UNIVERSITÀ DEGLI STUDI DI UDINE

in convenzione con Fondazione Edmund Mach

Corso di dottorato di ricerca in:

"Scienze e Biotecnologie Agrarie"

Ciclo XXXI

TESI DI DOTTORATO

Influence of the environment and viticultural practices on wine aroma. An untargeted metabolomics approach using comprehensive two-dimensional gas chromatography.

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Anno accademico 2017-2018

CONTENT

Riassunto

I composti volatili giocano un ruolo importante per l'accettabilità di ogni bevanda, ancor di più se si pensa alla valenza edonistica del vino. La presenza e la quantità di questi composti è influenzata da moltissimi fattori; da quelli ambientali a quelli umani, come la scelta della varietà da piantare in un determinato areale. L'insieme di queste variabili sarà sempre più determinante per l'ottenimento di un prodotto di qualità e che possa racchiudere in sè un pò di storia del territorio.

Senza la presunzione di essere esaustivi ho cercato di applicare una tecnica innovativa come la gascromatografia bidimensionale per studiare come cambia il profilo aromatico di alcune tipologie di vino modificando alcuni fattori climatico/agronomici.

Il primo lavoro riguarda lo spumante metodo classico prodotto in provincia di Trento. Fin dal lontano 1902 quando un giovane Giulio Ferrari, di ritorno dalla Francia, intravide le potenzialità del Trentino per la produzione di spumante la produzione di questa tipologia di vino ha preso sempre più piede finché nel 2007, dopo alcune tappe intermedie, viene creato il marchio Trentodoc che quest'anno comprende 51 case spumantistiche. Fin da subito questo prodotto è stato definito lo spumante di montagna per le sue caratteristiche organolettiche, sulla base dei risultati ottenuti da questo lavoro quest'asserzione può ritenersi un pò più oggettiva. Lo spumante Trentodoc si caratterizza infatti per una quantità più elevata di composti terpenici varietali e di composti prefermentativi.

La scelta dell'epoca di vendemmia è da sempre un annoso problema, come dimostra questo studio la composizione aromatica di vini da uve raccolte in date diverse può determinare delle caratteristiche sensoriali differenti. Lo studio, in collaborazione con un gruppo di ricerca australiano, ha dimostrato che, anche considerando 4 diverse zone, le uve raccolte nelle due date hanno dato origine a vini "analiticamente" distinguibili. L'analisi sensoriale condotta da un panel di esperti ha confermato queste differenze; i vini della prima epoca di vendemmia sono stati giudicati più freschi e con note da frutta rossa mentre quelli della seconda epoca presentavano sentori da frutta a bacca nera (mora, mirtillo).

Una delle varietà italiane a bacca bianca più versatili è sicuramente il Verdicchio che si presta alla spumantizzazione ma anche a lunghi invecchiamenti in legno. Possono le scelte agronomiche e l'areale di provenienza dare un'impronta al profilo dei composti volatili che aiuti a decidere che tipologia di vino sia più adatta? Quali sono i descrittori più importanti per ciascuna tipologia di prodotto? Ci sono dei composti volatili che caratterizzano il Verdicchio? Queste le domande a cui cerco di rispondere nei capitoli dedicati al Verdicchio.

Alcune recenti pubblicazioni hanno dimostrato l'importanza della popolazione microbica del vigneto nella definizione delle caratteristiche organolettiche di un vino proveniente da un certo territorio. Le scienze omiche hanno permesso un'esaustiva identificazione dei microorganismi presenti in un campione di mosto-vino e dei metaboliti ad essi correlati.Questo è stato fondamentale per l'esame accurato del contributo sia dell'ambiente viticolo che della fermentazione alcolica. Lo studio triennale del vigneto "Costalunga" in provincia di Verona ha dimostrato l'importanza di queste popolazioni microbiche per la produzione dell'Amarone.

L'utilizzo di un approccio metabolomico non mirato e di una tecnica separativa estremamente efficace come la gascromatografia bidimensionale ha permesso di evidenziare le modifiche del profilo aromatico nelle diverse situazioni.

Abstract

Volatile compounds play an important role in the acceptability of all drinks, especially when one thinks of the hedonistic value of wine. The presence and quantity of these compounds are influenced by many factors, both environmental and human, such as careful choice of the variety to be planted in a specific area. The combination of these variables will be increasingly decisive for obtaining a quality product that can encompass part of the history of the geographical area.

Without presuming to be exhaustive I have tried to apply innovative techniques by using two-dimensional gas chromatography coupled with time of flight mass spectrometry to study how the aromatic profile of some types of wine changes with modification of certain climatic/agronomic factors.

The first study, which kicked off this doctorate, concerns classic method sparkling wine produced in the province of Trento. From 1902, when the young Giulio Ferrari saw the potential of Trentino for producing sparkling wine on his return from France, production of this type of wine became increasingly popular. In 2007 the Trentodoc brand was created, after some intermediate stages, and now includes 51 sparkling wine producers. From the beginning, this product was defined as “mountain sparkling wine” due to its sensorial characteristics, and based on the results obtained in this work, the statement can be considered a little more objective. Trentodoc sparkling wine is indeed characterised by a larger quantity of varietal compounds such as teprenes and some prefermentative compounds.

Choosing when to harvest grapes has always been a yearly problem. As this study shows, the aromatic composition of wines from grapes harvested on different dates can lead to different sensory characteristics. This study, in collaboration with an Australian research group, showed that even considering four different areas, the grapes harvested on the two dates gave rise to wines that were "analytically" distinguishable. Sensory analysis conducted by a panel of experts confirmed these differences; wines from the first harvest period were judged to be fresher and with notes of red fruit, while those from the second period had hints of black fruit (blackberry and blueberry).

One of the most versatile Italian white berry varieties is certainly Verdicchio, which lends itself to sparkling wine but also to long ageing in wood. Can agronomic choices and the area of origin give rise to a volatile compounds profile that helps to decide which type of wine is most suitable? What are the most important descriptors for each type of product? Are there any volatile compounds that characterise Verdicchio and Trebbiano di Soave? These are the questions I try to answer in the chapters dedicated to Verdicchio.

Some recent publications have shown the importance of the microbial population of the vineyard in defining the sensorial characteristics of a wine coming from a certain geographical area. The omics sciences have allowed exhaustive identification of the microorganisms present in samples of must-wine and related metabolites. This is fundamental for accurate examination of the contribution of the viticultural environment to alcoholic fermentation. The three-year study at the "Costalunga" vineyard in the province of Verona has shown the importance of these microbial populations for the production of Amarone.

Use of a non-targeted metabolomic approach and an extremely effective separation technique, namely two-dimensional gas chromatography, made it possible to highlight the changes in the aromatic profile in different situations.

A mia mamma

Gli odori sono quasi un'immagine de' piaceri umani. Un odore assai grato lascia sempre un certo desiderio forse maggiore che qualunqu'altra sensazione. (Giacomo Leopardi, Zibaldone)

This is the smell I love. This and fresh-cut clover, the crushed sage as you ride after cattle, wood-smoke and the burning leaves of autumn. (Ernest Hemingway, For Whom the Bell Tolls)

Il est des parfums frais comme des chairs d'enfants, doux comme les hautbois, verts comme les prairies, - et d'autres, corrompus, riches et triomphants, ayant l'expansion des choses infinies, comme l'ambre, le musc, le benjoin et l'encens, qui chantent les transports de l'esprit et des sens. (Charles Baudelaire, Les Fleurs du mal)

Il Ciclo dei Mesi Torre dell'Aquila del Castello del Buonconsiglio di Trento; maestro Venceslao

Introduction and literature overview

Analysis of Aroma Compounds in Wine

This introduction has been adapted* and improved, including relevant parts, from:

Versini G., Dellacassa E., Carlin S., Fedrizzi B., Magno F. (2008). Analysis of aroma compounds in wine. In: Hyphenated techniques in grape and wine chemistry (edited by R. Flamini). Chichester Wiley: 173-225. *Hyphenated Techniques in Grape and Wine Chemistry Edited by Riccardo Flamini © 2008 John Wiley & Sons, Ltd. ISBN: 978-0-470-06187-9* © 2008 John Wiley & Sons, Ltd. ISBN: 978-0-470-06187-9

1.1. Aroma Compounds in Wines

The most important grape-derived aroma compounds are monoterpenes, norisoprenoids (various C_{13} megastigmane compounds), a number of benzenoid compounds called shikimates because of their origin from shikimic acid, and some polyfunctional sulphured compounds. In the winemaking process, these molecules are transferred to the wine both as free and bound forms. Monoterpenes[1], mostly present as alcohols, ethers and hydrocarbons, represent the most important category associated with basic floral aroma, beginning with linalool, recognised in Muscat grapes about 50 years ago (Cordonnier, 1956). Free and bound monoterpenes are synthesized in the berry during its ripening as diastereoisomeric and enantiomeric forms (Luan and Würst, 2002; Luan *et al.*, 2005). They are important markers of grape varieties (Rapp *et al.*, 1978). The aglycone profile remains a good marker even in aged wine (Versini *et al.*, 1997). Some monoterpenes are involved in chemical acid catalysed reactions that can reduce or change aroma contribution in the pH of must and wine: e.g. alcohols such as linalool (floral scenting) are partially transformed into α-terpineol and subsequently into 1,8-terpines; geraniol and nerol are transformed into linalool and α-terpineol etc. (Figure 1); some non-floral diols and a triol in part generate sensorially active substances such as hydrocarbons, and cyclic ethers such as neroloxide, roseoxide, anhydrofuranes and anhydropyranes (Strauss *et al.*, 1986). Recently, formation of the highly odorant wine-lactone from (*E*)-2,6-dimethyl- 6-hydroxyocta-2,7-dienoic acid was highlighted (Wintherhalter *et al.,* 1998). Most alcoholic compounds are present, sometimes in dominant amounts, as β-glycoconjugate forms, largely as monoglucosides like β-D-glucopyranoside and disaccharides such as 6-O-α-L-arabinofuranosyl-, 6-O-β-D-apiofuranosyl- and 6-O-α-L-ramnopyranosyl-β-D-glucopyranosides (Ribéreau-Gayon *et al.*, 1998). These can be converted into free forms, principally by hydrolysis in the case of α,*β*-unsaturated tertiary alcohols, or by enzymatic reaction by yeasts or supplied exogenous enzymes in other cases (Skouroumounis and Sefton, 2000). In these reactions, traces of potent flavourant ethyl ethers can arise (Strauss *et al.*, 1985). Other compounds are generated during fermentation, e.g. (*R*)-(+)-citronellol from geraniol and nerol, with enantiospecific reduction carried out by the yeast (Gramatica *et al.*, 1982; Di Stefano *et al.*, 1992).

[1] An extensive list of these compounds was reported by Strauss *et al.* (1986), later updated with *cis* and *trans* 8-hydroxylinalool (Rapp *et al.*, 1986) and the 6,7-dihydro form (Versini *et al.*, 1988), some menthanediols (Versini *et al.*, 1991), monoterpenoid acid (*E*)-2,6-dimethyl-6-hydroxyocta- 2,7-dienoic acid (Wintherhalter *et al.*, 1998) and ethers such as 2-*exo*-hydroxy-1,8-cineol (Bitteur *et al.*, 1990), wine-lactone (Guth, 1995) and 1,8-cineol (Fariña *et al.*, 2005). The structures are shown in Figure 2.

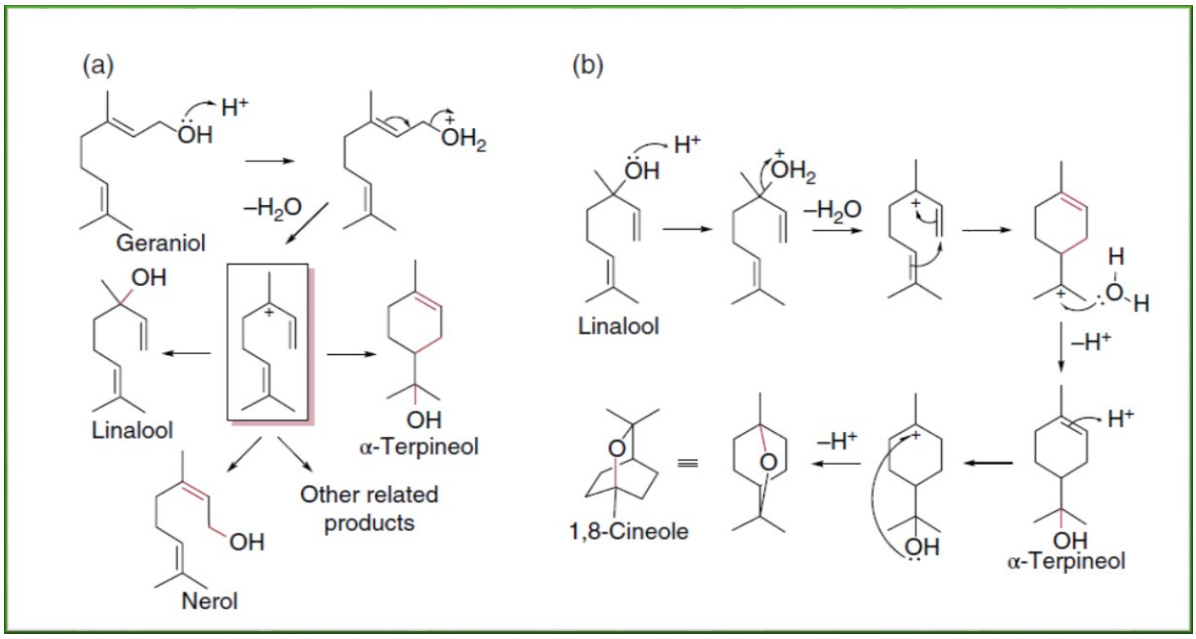

Figure 1 Acid-catalysed reactions of monterpenoids through carbocation intermediates, showing (a) hydrolysis of geraniol and subsequent rearrangement to form other monoterpenoids, including linalool, α-terpineol, and nerol, and (b) formation of 1,8-cineole from grape-derived linalool through hydrolysis and intramolecular cyclization *From Understanding WineChemistry By Andrew L. Waterhouse, Gavin L. Sacks and David W.Jeffrey.John Wiley and Sons,Chichester 2016. 470 pp., ISBN 978-1118627808*

The profiling and content of such compounds in grape products depends on the grape variety and how it interacts with berry ripeness and climatic and agronomic factors (Marais *et al.*, 1992).

During the last decade another group of terpenoids - the sesquiterpenes - have attracted the interest of wine researchers. This interest was beyond doubt stimulated by the discovery of the sesquiterpene ketone rotundone as the key aroma compound for the peppery character of high quality Syrah wines grown in cold climate regions of Australia (Siebert *et al*, 2008). Rotundone was found then also in autochthonous red wine from Italy such as Schioppettino and Vespolina, in Gruener Veltliner white wines (Mattivi *et al*., 2011) and in red varieties from La Rioja (Spain), as Graciano or Maturana Tinta (Cullerè *et al*., 2016).

Climate factors influence the profile of another group of chemical compounds – norisoprenoids – whose contribution is important in determining some specific ageing aromas due to the evolution of free and bound polyhydroxylate C_{13} megastigmane structures (Williams *et al.*, 1989 and 1992; Winterhalter *et al.*, 1990; Wintherhalter, 1992; Knapp *et al.*, 2002; Versini *et al.*, 2002). Of these, the most important flavouring compounds are reported in Figure 3, such as 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), vitispiranes, actinidols and β-damascenone, with kerosene, resinous/eucalyptus-like, woody and rose-like scents respectively. Their precursors seem to derive from carotenoid cleavage dioxygenase in relation to C_{13}-

norisoprenoid accumulation (Mathieu *et al.*, 2007). Some of these compounds are considered to be important contributors to the typical aroma evolution of certain white wines (*Chardonnay*, *Semillon* and *Sauvignon blanc*) and red wine varieties (*Shiraz*, *Grenache, Merlot*, *Cabernet Sauvignon*) (Williams *et al.,* 1989; Abbot *et al.*, 1991; Gunata *et al.,* 2002; Williams and Francis, 1996; Francis *et al.*, 1998) and of grapes (Flamini *et al.*, 2006).

Other aroma contributors can be shikimate compounds, i.e. benzenoid substances that result as free and bound forms (Williams *et al.*, 1983; Williams *et al.*, 1989; Winterhalter *et al.*, 1990). The most reported flavouring compounds are zingerone and zingerol, vanillin, methyl vanillate, and recently methyl salicylate, which is particularly abundant in a native Italian variety, *Verdicchio* or *Trebbiano di Soave*, where it has been found to have content ranging between 0.5 and 1 mg/L (expressed as aglycones). In this grape variety, a benzyl alcohol level ranging between 2 and 3 mg/L was also found (Versini *et al.*, 2005). The majority of volatiles in wines are however fermentation compounds, starting with so-called higher alcohols and continuing with important flavouring compounds such as acetates of higher alcohols and the ethyl ester of C_4-C_{10} fatty acids, providing fruity scents. When abundant, free fatty acids contribute to a 'goaty' flavour (Meilgaard, 1975). Some minor ethyl esters, such as the ethyl esters of 2-methyl and 3-methylbutyric acid may also play important role, due to their extremely low odor threshold. This group of molecules exhibit pleasant strawberry-liquorices-like odors. (Campo *et al*.,2007) Two hydroxyesters such as ethyl 2-hydroxy-3-methylbutyrate and ethyl 2-hydroxy-4-methylpentanoate and a new ethyl ester, ethyl cyclohexanoate, has revealed in wine by Campo et al 2006. These compounds could be important contributors to some of the specific fruity notes of aged wines. Other aroma compounds are C_6 alcohols such as 1-hexanol and *cis* and *trans* 3-hexen-1-ol, sometimes at relatively high levels, as in the case of *Müller-Thurgau* wines (Nicolini *et al.*, 1995), and others such as 2-phenylethanol and a significant acetate with a rose-like scent. The level of most such compounds is determined by the technology and procedures used in winemaking (vinification with white or red wines, skin contact, clarification, fermentation temperature, yeast strain and assimilable nitrogen level). In the latter case, a positive correlation has been proved between the nitrogen level in must and acetates, and a negative correlation with higher alcohols, 2-phenylethanol and methionol (Rapp and Versini, 1991). Extensive literature references are reported in the review papers of Schreier (1979) and Rapp (1988).

Organic sulphur compounds usually play a considerable role in the sensory characteristics of food and beverages, as they are frequently contributors to the nature of impact-scents. Examples of these products include truffle (Diaz *et al.*, 2003; Aprea *et al.*, 2007), garlic and onion (Yan

et al., 1993; Bocchini *et al.*, 2001), cooked meat (Hinrichsen and Pedersen, 1995; Andres *et al.*, 2002; Carrapiso *et al.*, 2002), coffee (Shimoda and Shibamoto, 1990; Semmelroch and Grosch, 1996), fruit juices (Winter *et al.*, 1976; Boelens and van Gement, 1993; Hinterholzer and Schieberle, 1998) and some distillates (Leppanen *et al.*, 1979; Cardoso *et al.*, 2004; Ledauphin *et al.*, 2006). As regards fermented drinks, several papers have studied beer (Burmeister *et al.*, 1992; Dercksen *et al.*, 1992) and wine (Schreier and Drawert, 1975; Ribereau-Gayon *et al.*, 1998; Flanzy, 1998), mainly to investigate and justify off-flavours resembling onion, garlic, cooked cabbage, rubber and putrefaction. In the last decade, the important positive contribution of some compounds with a box-tree and tropical fruit scent to aroma has been emphasised in wines obtained from different grape varieties, in particular using specific winemaking techniques (Darriet, 1993; Murat *et al.*, 2001; Tominaga *et al.*, 1998a; Fedrizzi *et al.*, 2007b). Sulphur compounds are formed through several pathways, involving both enzymatic and non-enzymatic processes. The former mainly concern yeast fermentation biochemistry and involve inorganic sulphates and sulphites, mostly sulphur-containing amino acids and oligopeptides (e.g. glutathione), as well as organic sulphur compounds such as cysteinyl or glutathionyl derivatives (Tominaga *et al.*, 1998b, Peyrot des Gachons *et al.*, 2002a; 2002b), while non-enzymatic mechanisms regard chemical, photochemical and thermal reactions during winemaking and storage (Mestres *et al.*, 2000). Usually considered as off-flavours, the sulphur compounds in wine are thiols, sulphides, thioesters and heterocyclic compounds, frequently divided into 'light' (b.p. < 90 °C) and 'heavy' (b.p. > 90 °C) compounds (Ribereau-Gayon *et al.*, 1998; Mestres *et al.*, 2000). Using methods including derivatization reaction and SPE/GC-MS, HS-SPME/GC-MS, is possible quantify the two poly-functional mercaptans 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate, with a tropical fruit-like scent typifying some wines such as *Sauvignon blanc*, and several others with a level depending on the variety and the technological procedure adopted (Fedrizzi *et al.*, 2007b; Fedrizzi *et al.*, 2007c, Larcher *et al*., 2015, Herbst-Johnstone *et al*., 2013), in particular so-called reductive vinification. 3-Mercaptohexan-1-ol and 4-methyl-4-mercaptopentan-2-one (the latter has a very sensitive box-tree like aroma typical of *Sauvignon blanc* wines at ppt levels or fractions), are mostly present in grapes as S-cysteine conjugates (Ribereau- Gayon *et al.*, 1998; Flanzy, 1998) and are freed to a minor extent (less than 5%), combined with production of 3-mercaptohexyl acetate by yeast metabolism (Swiegers and Pretorius, 2007; Swiegers *et al.*, 2007).

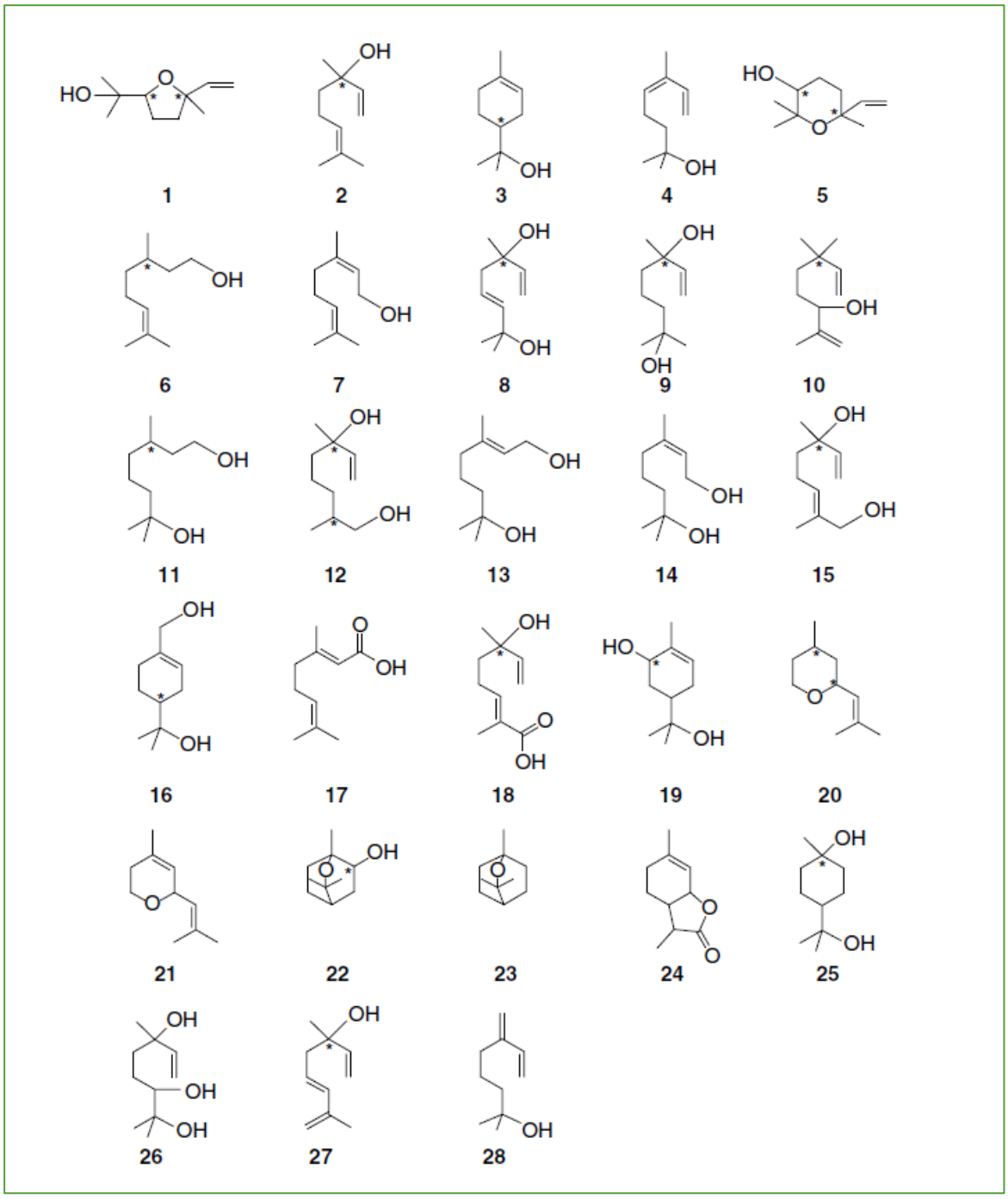

Figure 2: (1) *cis* and *trans* linalool oxide (5-ethenyltetrahydro-,,5-trimethyl-2-furanmethanol) (furanic form); (2) linalool (3,7-dimethyl-1,6-octadien-3-ol); (3) -terpineol (,,4-trimethyl-3-cycloexene-1-methanol); (4) (Z,E) ocimenol (2,6-dimethyl-5,7-octen-2-ol); (5) cis and trans linalool oxide (pyranic form); (6) citronellol (3,7-dimethyl-6-octen-1-ol); (7) nerol (Z), geraniol (E) (3,7-dimethyl- 2,6-octadien-1-ol); (8) Ho-diendiol I (3,7-dimethyl-1,5-octadiene-3,7-diol); (9) endiol (3,7-dimethyl-1-octene-3,7-diol); (10) Ho-diendiol II (3,7-dimethyl-1,7-octadiene-3,6-diol); (11) hydroxycitronellol (3,7-dimethyloctane-1,7-diol); (12) 8-hydroxydihydrolinalool (2,6-dimethyl-7-octene-1,6-diol); (13) 7-hydroxygeraniol (*E*-3,6-dimethyl-2-octene-1,7-diol); (14) 7-hydroxynerol (*Z*-3,6-dimethyl-2-octene-1, 7-diol); (15) *cis* and *trans* 8-hydroxy linalool (*E*- and *Z*-2,6-dimethyl-2,7-octadiene-1,6-diol); (16) p-menthenediol I (p-menth-1-ene-7,8-diol); (17) *E*-geranic acid (3,7-dimethyl-2,6-octadienoic acid); (18) *E*-2,6-dimethyl-6-hydroxyocta-2,7-dienoic acid; (19) *E*- and *Z*-sobrerol or pmenthenediol II (p-menth-1-ene-6,8-diol); (20) *cis* and *trans* rose oxide; (21) nerol oxide; (22) 2-*exo*-hydroxy-1,8-cineol; (23) 1,8-cineol; (24) wine lactone; (25) *cis* and *trans* 1,8-terpin; (26) triol (2,6-dimethyloctane-2,3,6-triol); (27) hotrienol [(5*E*)-3,5- dimethylocta-1,5,7-trien-3-ol]; (28) myrcenol (2-methyl-6-methylene-7-octen-2-ol)

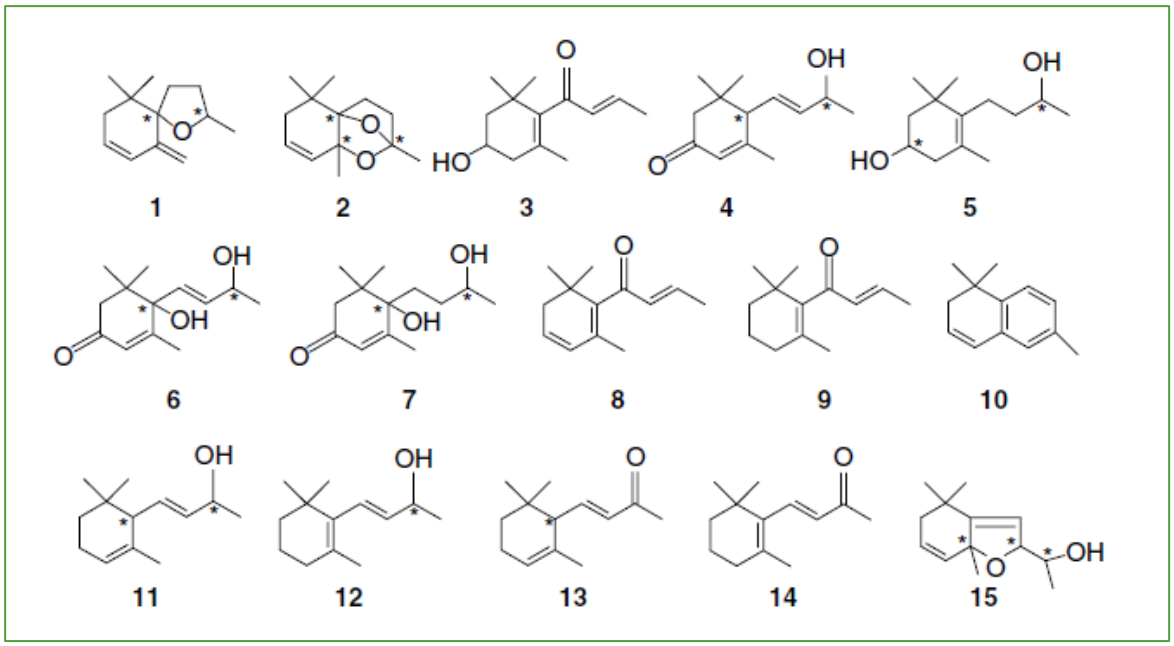

Figure 3 (1) vitispiranes (spiro [4.5]-2,10,10-trimethyl-6-methylene-1-oxa-7-decene); (2) riesling acetal (2,2,6-tetramethyl-7,11-dioxatricyclo[6.2.1.01_6]undec-4-ene); (3) 3-hydroxy-_-damascone; (4) 3-oxo-α-ionol; (5) 3-hydroxy-7,8-dihydro-β-ionol; (6) vomifoliol; (7) dihydrovomifoliol; (8) β-damascenone; (9)β-damascone; (10) TDN (1,1,6-trimethyl-1,2-dihydronaphthalene); (11) α-ionol; (12) β-ionol; (13) α-ionone; (14) β-ionone; (15) actinidols

1.2. Methods for Analysis of Aroma Compounds in Wines

In the last 15–20 years, several analytical methods have been reported for the extraction of aroma compounds from wine, aimed at replacing time-consuming (10–24 hours) continuous liquid-liquid methods. Liquid-liquid extraction is usually performed either with dichloromethane/pentane (2:1 v/v) (Drawert and Rapp, 1968) or Freon 11, sometimes in a Freon 11/dichloromethane 9:1 mixture (v/v) (Hardy, 1969; Rapp and Hastrich, 1978; Marais, 1986), both approaches being considered as relatively extensive and repeatable for volatile profiling of wines. Alternative methods are fast liquid-liquid discontinuous extraction with 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113 or 'Kaltron') (Ferreira *et al.*, 1993; Rapp *et al.*, 1994; Genovese *et al.*, 2005), and solid-phase extraction (SPE) with a polystyrenic resin like Amberlite XAD-2 (Gunata *et al.*, 1985; Versini *et al.*, 1988), or with a C18 reverse phase (Williams, 1982; Di Stefano, 1991). SPE approaches have the advantage that both 'free' forms and so-called glycosidically bound compounds can be isolated and separated in the same process. More recently, the highly crosslinked hydroxylated polystyrenic phase ISOLUTE®ENV+ has been marketed as a more active adsorbing material and applied to wine aroma analysis (Boido *et al.*, 2003, Vrhovsek et al., 2014), as well as the highly crosslinked ethylvinylbenzene-divinylbenzene copolymer LiChrolut EN (Genovese *et al.*, 2005).

Supercritical fluid extraction (SFE) has not been popular: methanol is sometimes added to CO_2 to achieve better recovery of more polar compounds (Blanch *et al.*, 1995). In the early 1990s, a new technique called solid-phase microextraction (SPME), was developed (Arthur and Pawliszyn, 1990). The key part of the SPME device is fused silica fibre coated with an adsorbent material such as polydimethylsiloxane (PDMS), polyacrylate (PA) and carbowax (CW), or mixed phases such as polydimethylsiloxane-divinylbenzene (PDMS-DVB), carboxenpolydimethylsiloxane (CAR-PDMS) and carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB). Sampling can take place either in the headspace (Vas *et al.*, 1998) or in the liquid phase (De la Calle *et al.*, 1996) of the samples. Headspace sampling in wine analysis is mainly useful for quantifying trace compounds with a particular affinity to the fibre phase, not easily measurable with other techniques. Exhaustive literature of materials used for the extraction-concentration of aroma compounds have been published by Ferreira *et al.* (1996), Eberler (2001), Cabredo-Pinillos *et al.* (2004) and Nongonierma *et al.* (2006). Analysis of volatile compounds is usually performed with gas chromatography (GC) coupled with either a flame ionization (FID) or mass spectrometry (MS) detector. In the next part of this chapter some of these techniques are compared, pointing out possible limitations and advantages in free and bound form aroma analysis, both in research and winemakers' laboratories. In one paragraph, recent quantification methods for some sulphur compound groups are discussed.

2.1. Sample Preparation

Solid-phase extraction with pre-packed polyhydroxylated styrene-divinylbenzene polymer

Solid phase extraction uses the difference of affinity between an analyte and interferents, present in a liquid matrix, for a solid phase (sorbent). This affinity allows the separation of the target analyte from the interferents. A typical solid phase extraction involves four steps:

1. the cartridge is equilibrated or conditioned with a solvent to wet the sorbent;
2. the loading solution containing the analyte is percolated through the solid phase. Ideally, the analyte and some impurities are retained on the sorbent;
3. the sorbent is then washed to remove impurities;
4. the analyte is collected during this elution step.

This technique allows to separate both free forms and precursors linked to sugars that can be analyzed after enzymatic or chemical hydrolysis. The first commercial materials were mostly

based on Silica C18, which unfortunately, do not have very good extraction ability from hydroalcoholic solutions. Much better results were obtained using polystyrene-divinylbenzene macro reticular copolymers suitable for quick SPE available at the end of the 90's. The first comprehensive method using a highly cross-linked polyhydroxylated styrene-divinylbenzene polymer ENV+® (Isolute, IST Ltd., Mid Glamorgan, UK) was proposed in 1997 (Carlin 1997) and it has been advantageously used in our laboratory since then. Other type of resins such as LiChrolut-EN resins was proposed in 2002 (Lopez et al., 2002, Cullere et al., 2004b; Escudero et al., 2007a; San Juan et al., 2012; Herrero et al., 2016)

Headspace solid-phase microextraction (HS-SPME)

Solid-phase microextraction (SPME) is a technique for the extraction of organic compounds from gaseous, aqueous and solid matrices such as many environmental samples. It is rapid and simple, which makes it ideal for automation and in situ measurements, and no harmful solvents are used. The principle of SPME is equilibration of the analytes between an organic polymeric phase coated on to a fused-silica fibre and the sample matrix. (Figure 1)

Solid phase microextraction (SPME) was developed by Arthur and Pawliszyn in 1990, as a solvent-free sample preparation method for aqueous sample matrices. In SPME a 1 or 2 cm long fused silica fibre, with different film thickness, coated with a sorbent, is either immersed in the sample or exposed to the headspace above the sample. During the sampling procedure, analytes partition between the fibre coating and the sample, resulting in enrichment of the analytes in the fibre coating. After sampling, the fibre is removed from the sample (ideally after equilibrium is reached) and the analytes are thermally desorbed in a conventional split/splitless GC injector at elevated temperature. Several parameters, such as the type of coating, extraction time and temperature, addition of salt (alteration of ionic strength), volume of the sample and volume of the headspace affect the extraction of analytes. Since the introduction of SPME, a range of different fibre coatings varying from PDMS to polar or mixed coatings has become commercially available. The main problem of the fiber is that wine vapors are extremely concentrated in ethanol, fusel alcohols and fatty acid ethyl esters. The amounts of these compounds present in the headspace exceed by far the extraction capacity of the fibers. This implies that the fiber becomes saturated, which brings as consequence that the amount of compound extracted becomes matrix-dependent. There are two suitable solutions: strong dilution and using good enough internal standards. (Siebert *et al*., 2005, Ferreira *et al*., 2015)

Recently, the effectiveness of three-phase fibre for aroma compounds analysis has been proved.

In particular, the CAR-PDMS-DVB phase (50/30μm×1cm) appears to be effective in overcoming the lack of selectivity of one-phase or two-phase fibre towards some compounds. Furthermore, the use of an autosampling system permits easier development of methods and wider applicability for quality control in the laboratory (Aprea, Biasioli, Carlin, Endrizzi, & Gasperi, 2009, Fedrizzi et al., 2012). SPME, immersed in the sample, was used for the analysis of rotundone in different wines, for example. (Caputi et al., 2011; F. Mattivi et al., 2011).

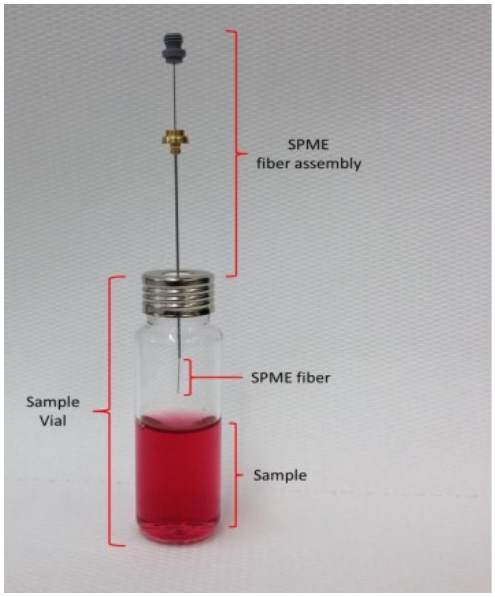

Figure 1 Headspace vial with fibre

2.2. GC-MS analysis

For unambiguous identification of compounds also present at trace level, the gas chromatography-mass spectrometry (GC-MS) approach is compulsory.

Once the components leave the GC column, they are ionized by the mass spectrometer using electron or chemical ionization sources. Using electron ionization source, the most common in volatile analysis, the first step is the production of gas phase ions of the compound, by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. (Figure 2) Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound. (Gross

2006) Working at 70 eV it is possible to obtain mass spectra comparable with the spectral libraries.

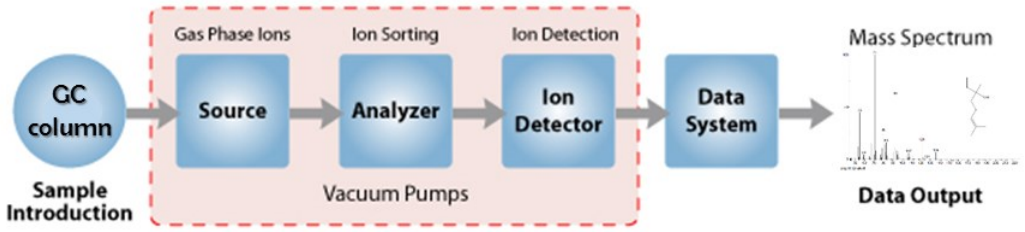

Figure 2 Components of a Mass Spectrometer

The final steps of the process involve ion detection and analysis, with compound peaks appearing as a function of their m/z ratios. Peak heights, meanwhile, are proportional to the quantity of the corresponding compound. A complex sample will produce several different peaks, and the final readout will be a mass spectrum. Using computer libraries of mass spectra for different compounds, it is possible identify and quantitate unknown compounds and analytes.

2.3 GC-MS/MS

The GC-MS/MS technique can offer enhanced sensitivity and selectivity by eliminating the coeluting gas chromatographic peak interference using the multiple reaction monitoring (MRM) mode. (Niessen 1999) This analysis technology may enhance signal-to-noise ratios to level that are orders of magnitude better than those obtained using SIM (single ion monitoring) mode of GC/MS

In this case the GC separation system is coupled with tandem mass spectrometry.Tandem quadrupole mass spectrometers are generally known as ‘triple quadrupoles.’ However, only two of the quadrupoles are involved in mass analysis: the third (middle) quadrupole transmits all the ions and is used to ‘activate,’ i.e., transfer energy, to the ions of interest. The multiple ion monitoring principle is that a particular m/z value of interest is selected from the mass spectrum in the first quadrupole and is directed into the collision cell that contains a neutral gas (argon) the ion is vibrationally excited by collision with the target gas, a process known as collision-induced dissociation. This generates fragment ions that are separated and recorded by the second quadrupole. (Figure 3). With this technique it is possible to be very selective and to quantify traces of volatile compounds in different matrices such as fruit and alcoholic beverages (Vrhovsek *et al*., 2014, Paolini *et al*., 2018)

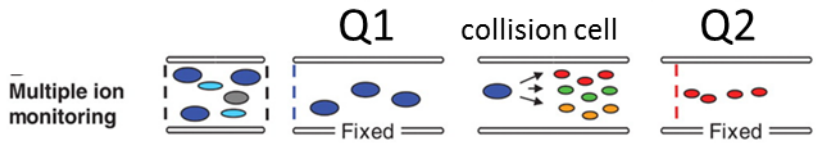

Figure 3 Scheme of mass spectrometry analysis in MRM mode (Adapted from Domon *et al*., 2006)

Other scan modes are available, including precursor ion scans, constant neutral loss and selected reaction monitoring.

2.4. GCXGC-ToF-MS comprehensive analysis

In the last ten years, another separation technique known as GCxGC time-of-flight mass spectrometry (GCxGC-TOF-MS) has been shown to greatly increase separation power and thus the detection of analytes. After the separation in the first column all the compounds before entering the second column compounds are modulated by hot and cold jets that trap and release the compounds as they leave the primary column.This is necessary to help maintain separation as secondary columns are much shorter than the primary. Knowing the timing of the pulses also allows the computer running the system to back calculate the retention time of the first column since analytes are only detected after they have passed through both. The preservation of first dimension separation depends on the number of modulations for first dimensional peak. To maintain well-resolved first dimension peaks, each first dimension peak should be sampled at least three or four times.

The development of this technique (Liu & Phillips, 1991) was followed by numerous reviews discussing the principles and experimental design of GC×GC (Ong & Marriott, 2002), (Dallüge, Beens, & Brinkman, 2003) (Cortes, Winniford, Luong, & Pursch, 2009). (Dymerski, 2018) These reviews have shown that GC×GC offers enhanced separation efficiency, reliability in terms of qualitative and quantitative analysis, the ability to detect low quantities and information on the overall sample and its components. In more recent years, there has been a shift towards the use of this technique in the analysis of real life samples including food and beverages, and samples from environmental, biological, and petrochemical studies (Adahchour, Beens, & Brinkman, 2008). A number of publications have appeared in the grape and wine field that have utilised HS-SPME and GC×GC as a technique. Rocha et al. (Rocha, Coelho, Zrostlíková, Delgadillo, & Coimbra, 2007) used GC×GC to analyse monoterpenes in grapes and identified 56 monoterpenes in the Fernão-Pires variety, of which 20 were reported for the

first time in grapes. This highlighted the advantage that structured chromatographic separation can provide in compound classification and compound identity confirmation. It has also shown promise in wine research, and was used for detailed investigation of the chemical composition of the uniquely South African wine cultivar Pinotage, proving able to tentatively identify over 200 compounds in different South African red wines (Vestner et al., 2011; Weldegergis et al., 2011). As can be seen in Robinson et al., using GCxGC-TOF-MS enabled them to identify over 360 compounds in the volatile headspace of Cabernet Sauvignon wines from western Australia (Robinson, Boss, Heymann, Solomon, & Trengove, 2011). GCxGC-TOF-MS was also used to identify twelve volatile compounds that can be used to differentiate and thus classify Cabernet Sauvignon, Merlot, Chardonnay, Sauvignon blanc and Pinot Noir varieties (Welke, Manfroi, Zanus, Lazzarotto & Alcaraz Zini, 2013Welke, Manfroi, Zanus, Lazarotto, & Zini, 2012; Welke, Zanus, Lazzarotto, Pulgati, & Zini, 2014)

It is anticipated that GC×GC will provide significant advantages in the identification of new and novel compounds which were previously unresolved using traditional one-dimensional chromatography. This would not have been possible with one dimensional GC due to the fact that many of the compounds co-elute on the primary, non-polar column, and are only able to be separated on the more polar secondary column.

Conventional 1-Dimensional GC is limited in its capacity to completely separate compounds in complex matrices, due largely to overlapping of similar compounds. Adding a second GC column allows a secondary phase of separation to take place prior to analyte detection. It is easy to see how, in general, GCxGC offers higher resolution, sensitivity and peak capacity compared to 1D-GC. To best exploit the second dimension separation space in GC×GC, the separation mechanisms of the two columns should be uncorrelated (independent), or in other words orthogonal. (Figure 4) An apolar×polar column combination is generally used (referred to as a normal column configuration), but the use of other column sets, such as polar×apolar (reversed column configuration), chiral×polar, or apolar×liquid crystalline, has also been reported.

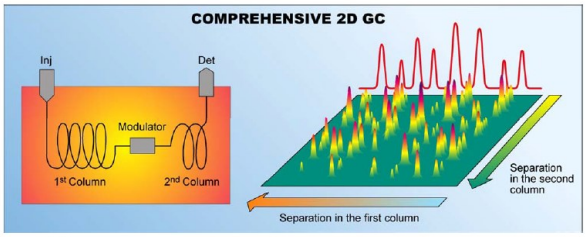

Figure 4 Diagram of a GCXGC oven and separation (adapted from Thermofisher Scientific)

As typical modulation periods in GC×GC vary from 3 to 8 seconds, the widths of first dimension peaks are supposed to be between 15 and 25 seconds. (Figure 5) It is apparent that not every first dimension peak fits this criterion. Narrower first dimension peaks therefore often undergo only 1 or 2 modulations, leading to convergence with neighbouring bands in the modulator. If too short a modulation period is used, peaks with high affinity to the second dimension phase might not elute within their modulation cycle, but in the next one. This phenomenon is called "wraparound". (Tadeusz Górecki Comprehensive two-dimensional gas chromatography (GCxGC) New Horizons and Challenges in Environmental Analysis and Monitoring, Publisher: 2003 CEEAM, Editors: Namiesik J, Chrzanowski W, Zmijewska P) Therefore, a compromise between sufficient sampling of first dimension peaks and avoiding wraparound must be reached when modulator settings are chosen.

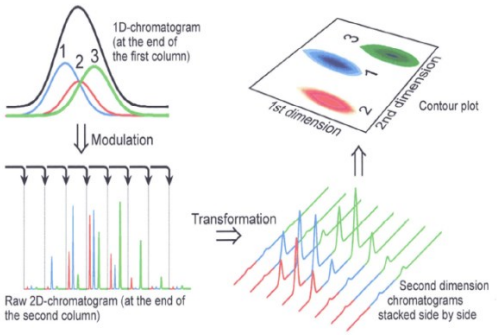

Figure 5 Generation and display of a GC×GC chromatogram (adapted and modified from Dallüge, Beens, & Brinkman, 2003).

Besides enhanced separation efficiency, GC×GC provides further advantages. Contour plots of GC×GC chromatograms often display structural retention patterns of related compounds that allow group type identification. For instance, peaks of homologous series typically form lines in GC×GC contour plots (Tranchida et al., 2008). Structural retention patterns are especially useful if no standard compounds and/or library reference spectra are available, or when mass spectra for different compounds are very similar (e.g. terpenes) (Rocha et al., 2007). Another advantage of GC×GC is improved sensitivity compared to one dimensional GC. The increased signal-to-noise ratios in GC×GC are a result of decreased peak width caused by re-focusing of analytes in the modulator and very fast second dimension analyses. Trapping of the effluent from the first dimension column in the modulator results in refocusing of the analytes by means of the combination of the stationary phase and cryotrapping prior to reinjection into the second dimension column. This focusing step 'resets' band broadening obtained in the first dimension column. Therefore, refocusing in the modulator leads to a substantial increase in signal-to-noise

ratios due to decreased peak width, and therefore to increased sensitivity for trace analyses. In addition, Chromatof software is able to perform data deconvolution, which is the process of computationally separating co-eluting components and creating a pure spectrum for each component (Du & Zeisel, 2013). Specifically, deconvolution calculates the contribution of each component for each observed m/z resulting from two or more components. The deconvolution of peaks makes it possible to obtain very clean mass spectra that facilitate identification with the spectral library (Figure 6), for example identification of the *cis* isomer of beta-damascenone (Figure 7)

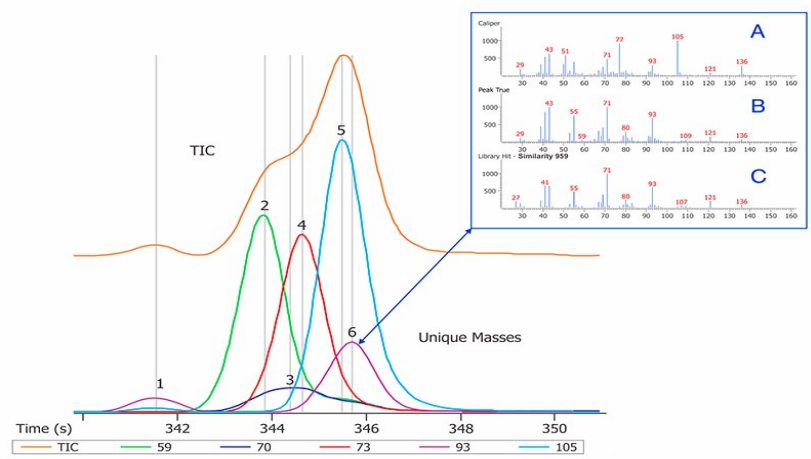

Figure 6 The deconvulation process also makes it possible to obtain "clean" mass spectra and higher library matching for compounds such as peak n° 6 (linalool) that are co-eluted with other compounds (adapted from Leco)

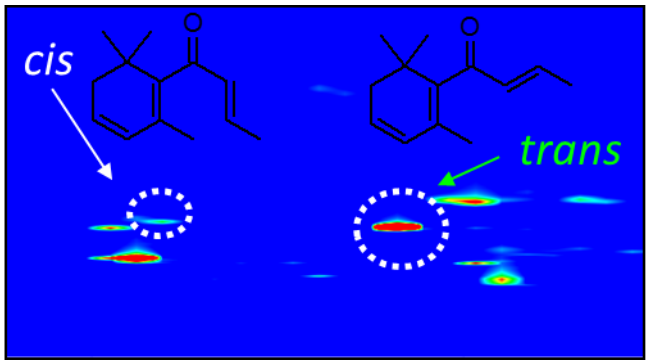

Figure 7 GCXGC makes it possible to identify compounds such as the *cis* isomer of β-damascenone

2.4. GC-O/MS analysis

GC-Olfactometry (GC-O) is a valuable method for the selection of odor components from a complex mixture. A trained human assessor is employed as a detector and can sniff the eluate in order to detect the presence of odor—active compounds via a specifically designed odor port (ODP) connected in parallel to conventional detectors (mass-spectrometry or flame-ionization).

Each separated compound, eluted by the GC, can be detected by a human assessor (odor present or not), who is able to measure the duration of the odor activity (start to end), to describe the quality of the odor perceived and to quantify its intensity. GC-O in combination with a mass spectrometer (GC-O/MS) not only enables the evaluation of odor compounds, but also their identification with mass spectral information.

GC-olfactometry (GC-O) refers to the use of human assessors as sensitive and selective detectors for odour-active compounds. The aim of this technique is to determine the odour activity of volatile compounds in a sample extract, and assign a relative importance to each compound. Methods can be classified into three types: detection frequency, dilution to threshold and direct intensity.(Delahunty, Eyres, & Dufour). Qualitative and quantitative evaluation of the odour is carried out for each analyte leaving the chromatographic column. This makes it possible to establish whether a given compound is sensorially active at a given concentration (i.e. whether it appears in the sample at a level higher than the threshold of sensory detection) and what its smell is, as well as determining the time of sensorial activity and the intensity of the odour. Determination of the analyte's odour is possible thanks to the presence of a special attachment, a so-called olfactometric port, connected in parallel to conventional detectors, such as a flame-ionization detector (FID) or mass spectrometer (MS) (Plutowska & Wardencki, 2008). The flow of the eluate is split in such a way that the analytes reach both detectors simultaneously, allowing both signals to be compared (Figure 8). Combination of an olfactometric detector with a mass spectrometer is particularly advantageous, as it makes identification of odour-active analytes possible. To avoid problems, since the mass spectrometer works in a vacuum while the olfactometric detector works under atmospheric pressure, it is necessary to install a restrictor (in the form of a narrow bore capillary) before the mass spectrometer to increase the pressure drop between the interface and the flow splitter, and to ensure careful selection of the carrier and auxiliary gas flows. It is known that only a few of the large number of volatiles occurring in a fragrant matrix contribute to its overall perceived odour (d'Acampora Zellner, Dugo, Dugo, & Mondello, 2008). Further, these molecules do not contribute equally to the overall flavour profile of a sample, hence a large GC peak area, generated by a chemical detector, does not necessarily correspond to high odour intensities, due to differences in intensity/concentration relationships. Consequently, the general interest of researchers has been directed at determining the contribution of individual constituents to the overall flavour of a product. In general, the sensory importance of an odour-active compound depends on its concentration in the matrix, and on its human nose limit of detection. Moreover, the unpredictable extent of interaction of flavour molecules with each

other and with other food constituents (lipids, protein, carbohydrates etc.) must also be considered. Some factors that should be considered to improve the value of GC-O analysis are the extraction method, GC instrumental conditions, including the design and operation of the odour port, methods of recording GC-O data and controlling the potential for human assessor bias using experimental design and a trained panel. Aroma extract dilution analysis (AEDA) is widely used for the screening of aroma-active compounds in gas chromatography–olfactometry (GC–O). During AEDA, stepwise dilutions of an original extract are performed and the diluted extracts are then evaluated by GC–O to provide flavour dilution (FD) factors (i.e. the maximum dilution of an extract at which the compound can be detected) (Schieberle & Grosch, 1987).

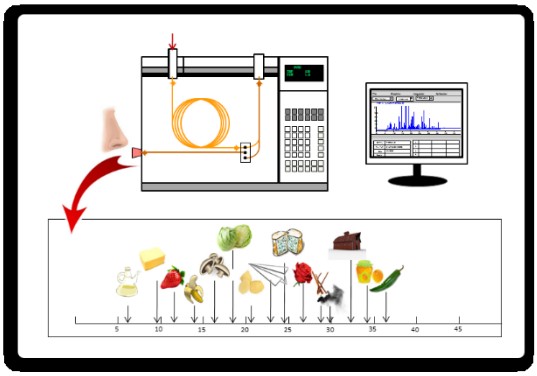

Figure 8 Diagram of a gas chromatograph equipped with olfactometric detector. (http://www.quimica.urv.es/)

Concluding remarks

In general, aroma enrichment using both a polyhydroxylated styrenedivinylbenzene/ ENV+ cartridge or polystyrene/XAD-2 resin is less time-consuming, requires less solvent vapour protected working space, is generally more healthy and consumes less organic solvent than traditional continuous liquid-liquid extraction methods. With reference to the most representative wine compounds included in different chemical categories in terms of functional groups and polarity, the analytical precision is similar for the three solid-phase methods, and comparable to that obtained using the rapid Kaltron liquid–liquid extraction method. However, this method does not permit the recovery of glycoside forms. These methods are also suitable for routine application in quality control laboratories. The use of either ENV+ – and highly sorbing phases as reported for LiChrolut EN – or a reverse phase cartridge provides a more exhaustive free-form profile, including most polar compounds. By performing a second step, both these approaches can achieve exhaustive and rapid separation of glycoside forms of aroma in wines, which are determined in the aglycone form after performing enzymatic hydrolysis.

For analysis of free compounds, headspace analysis with multiphase fibre is even more interesting and less time-consuming. This approach can replace most forms of free compound quantification using SPE techniques, considering also the possibility of sampling automation using a GC-MS system which can be coupled to statistical processing of fragment abundance (Kinton *et al.*, 2003; Cozzolino *et al.*, 2006). Moreover, HS-SPME/GC-MS is a very effective and efficient method for analysing specific compounds present in trace levels at ppt level, because they can be better and selectively enriched in the headspace. Since the probability of separating each component in a complex sample were relatively low when using a 1D separation process, depending on the complexity of the sample and the desired purity, the GCXGC technique allows high separation efficiency and enhanced sensitivity. It is also possible to extend the analysis to a broader range of volatile aroma compounds and find new compounds co-eluting in 1D GC, such as safranal, and *cis* beta damascenone. Moreover the identification of compounds is more reliable than with 1D GC-MS, since separation on two different phases, together with the deconvolution tool, produces clearer mass spectra. The use of GC-O also makes it possible to determine the most important compounds at sensorial level. A combination of all these analytical techniques makes it possible to carefully study the aromatic profiles of wines from different environment and viticultural practices, allowing the specific characteristics of each factor to be highlighted.

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Aim of the Ph.D project

Terroir can be defined as an ecosystem, in a given place, including many factors, like climatic conditions, cultivar and rootstock, geography and topography, as well as soil characteristics like mineral nutrition and water supply. (Reynolds A, 2010)

The term "terroir" involves a complex interplay of climate, soil, geology and viticulture, all of which influence the characteristics and quality of a wine from a given grape variety, rootstock and viticultural practice. Water availability is also important, and is based on climate (rainfall and humidity) and soil water-holding capacity. The soil structure reflects the geological history of a region and may have evolved over millions of years, influenced by faulting, weathering and bedrock mineralogy. The effect of region on the quality of wine is an old concept and there are now also numerous scientific publications on this topic. In 1993 Jackson and Lombard (Jackson & Lombard, 1993) published a review that described all these parameters well.(Figure 1)

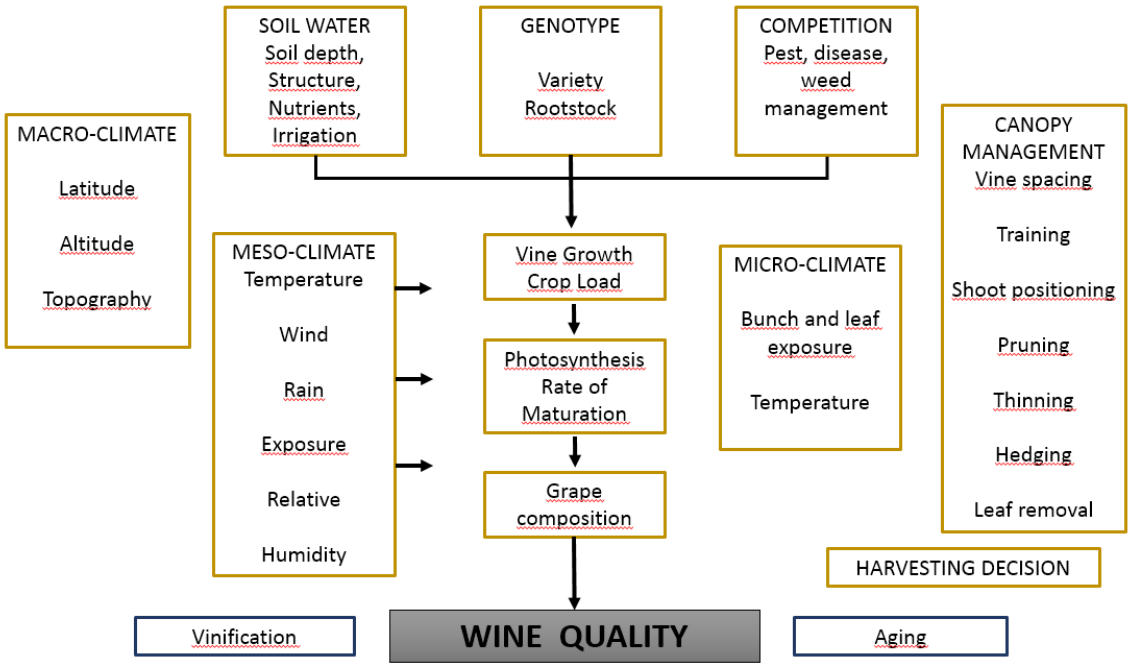

Figure 1 Environmental parameters influencing the characteristics and quality of a wine (modified from Jackson & Lombard, 1993)

The aim of this work is to study how certain environmental parameters affect the volatile profile of different wines:

- *Chapter 1* Macroclimate *sparkling wines*
- *Chapter 2* Harvesting decisions *Australian Shiraz wines*
- *Chapter 3* Mesoclimate, vintage, harvesting decisions *Verdicchio wines*
- *Chapter 4* Genotype peculiarity: *methyl salicylate glycosides in Verdicchio wines*
- *Chapter 5* Microbial differences in vineyards *Corvina Costalunga vineyard*

It is also expected that a very powerful separative technique such as the comprehensive two-dimensional gas chromatography will allow us to find some useful markers for the characterization of different climatic and human factors.

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Chapter 1: macroclimate

The effects of region on the volatile profiles of sparkling wine: a "comprehensive" view.

This chapter has been reprinted* from:

Regional features of northern Italian sparkling wines, identified using solid-phase micro extraction and comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry. Carlin S.; Vrhovsek, U.; Franceschi, P.; Lotti, C.; Bontempo, L.; Camin, F.; Toubiana, D.; Zottele, F.; Toller, G.; Fait, A.; et al. *Food Chem.* ***2016****, 208, 68–80.*

Preface to Chapter 1

1) Macroclimate

Climate plays a predominant role in grapevine growth. In 2013, Dal Santo stated that ~18% of genes in the grapevine can be impacted by environmental conditions and that climate has the biggest effect on gene expression at veraison (Dal Santo et al., 2013). In particular, phenylpropanoid-related genes involved in the formation of aroma compounds and polyphenols were strongly impacted by seasonally specific climate conditions. In this study the data suggest that veraison is a critical period, during which the seasonal climate has its greatest effect, whereas the microenvironment and agronomic practices have only a marginal impact. The direct influence of climate on berry quality has been demonstrated, particularly the cumulative effects of temperature and water availability (Jones & Davis, 2000; Savoi et al., 2016).

Studies on the role of climate on grape composition may be difficult to interpret because climate encompasses all environmental conditions related to sunlight, temperature, humidity and rainfall within a region, all of which play an important role in the growth and development of the vine and berry. In addition, levels of aroma and aroma precursor compounds vary in different climates and within climates. Low temperatures favour aroma production in the grapevine. This is clearly evident in the enhanced aromatic potential of cool climate white wines made from cultivars such as Gewürztraminer, Sauvignon Blanc or Riesling, where the major aroma compounds are isoprenoids, notably monoterpenes. For example, levels of lutein, β-carotene and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) have been found at higher concentrations in wines from warmer climates (South Africa) compared to cooler climates (Germany). (Marais, Wyk, & Rapp, 1992; Schneider, Razungles, Augier, & Baumes, 2001) On the other hand, the reverse relationship is observed, with higher levels of methoxypyrazines in Sauvignon blanc from cool climates (New Zealand) compared to warm climates (Australia) (Allen & Lacey, 1998). Untargeted metabolomic approaches using comprehensive GCXGC separation provide a promising tool for studying the volatile profile in complex systems, to better understand the impact of climate change on grapes and wine composition and quality. Well-designed future studies will be critical to ensure sustainable worldwide production of grapes and wines with desirable flavour attributes.

The purpose of this chapter is to focus on observing how two different areas could contribute to the final aroma of a complex product such as “classic method” sparkling wine. The two most

important areas for the production of such wine in northern Italy were selected: Trentodoc and Franciacorta. The cultivars allowed in "classic method" sparkling wine production are essentially Chardonnay and Pinot Noir. These premium quality wines are fermented twice, so the complexity of the final products is very high, considering the presence of varietal aroma but also fermentative compounds, ageing compounds from hydrolysis and sulphur compounds from the yeast.

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Food Chemistry 208 (2016) 68–80

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Regional features of northern Italian sparkling wines, identified using solid-phase micro extraction and comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry ☆

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ARTICLE INFO

Article history:
Received 24 November 2015
Received in revised form 18 March 2016
Accepted 29 March 2016
Available online 30 March 2016

Keywords:
GCxGC-TOF-MS
Sparkling wines
Volatile compounds
Untargeted metabolomics
Traceability
Correlation-based network analysis

ABSTRACT

We carried out comprehensive mapping of volatile compounds in 70 wines, from 48 wineries and 6 vintages, representative of the two main production areas for Italian sparkling wines, by HS-SPME-GCxGC-TOF-MS and multivariate analysis. The final scope was to describe the metabolomics space of these wines, and to verify whether the grape cultivar signature, the pedoclimatic influence of the production area, and the complex technology were measurable in the final product. The wine chromatograms provided a wealth of information, with 1695 compounds being found.
A large number of putative markers influenced by the cultivation area was observed. A subset of 196 biomarkers fully discriminated between the two types of sparkling wines investigated.
Among the new compounds, safranal and α-isophorone were observed. We showed how correlation-based network analysis could be used as a tool to detect the differences in compound behaviour based on external/environmental influences.

1. Introduction

A major development in analytical techniques and instruments has allowed progression from the first studies focusing on the analysis of major volatile compounds to the analysis of compounds present in very low concentrations (even at levels below ng L^{-1} but with very low odour thresholds). Wine aroma is one of the most important features of wine quality. The many different nuances that can be detected when we smell or drink wine have aroused the interest of winemakers and scientists researching the complexity of wine aroma. Due to the great complexity of the wine matrix, analysis of some minor but key aroma compounds may require either pre-concentration steps or analysis with GC-MS/MS or multidimensional gas chromatography, coupled with the most modern and powerful detectors, such as time-of-flight mass spectrometers (Weldegergis, Crouch, Gorecki, & de Villiers, 2011). The volatile profile of wines, obtained with one-dimensional gas chromatography using a mass spectrometric detector (1D-GC-MS), has been already used for differentiation and classification of wines according to their geographical origin (Green, Parr, Breitmeyer, Valentin, & Sherlock, 2011) or grape cultivar (Zhang et al., 2010), using different multivariate techniques. Comprehensive two-dimensional gas chromatography (GC × GC) has emerged as a powerful analytical technique that is an excellent choice for unravelling the composition of complex samples. This technique is based on the application of two GC columns coated with different stationary phases, connected in series through a modulator, allowing separation that is both comprehensive (the entire sample is subjected to both separation dimensions) and multidimensional (the sample undergoes two different separation processes and the separation accomplished in one dimension is not lost in the other dimension) (Beens, Janssen, Adahchour, & Brinkman, 2005). For this reason, GC × GC offers the best separation ability, with higher peak capacity, selectivity, structural chromatographic peak organisation and sensitivity enhancement as compared to 1D-GC. The

☆ Selected paper from the 9th Edition of the In Vino Analytica Scientia Symposium, 14–17 July 2015, Trentino Alto Adige, Italy.

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http://dx.doi.org/10.1016/j.foodchem.2016.03.112
0308-8146/

application of GC × GC to sparkling wine volatiles has recently been reviewed (Welke, Zanus, Lazzarotto, Pulgati, & Zini, 2014).

According to tradition, premium quality Italian sparkling wine is fermented twice. After the first alcoholic fermentation, it is subjected to a second slow fermentation in the bottle, through the addition of the 'liquor de tirage' (a suspension of yeasts and sugar). Thus its aroma is influenced by varietal components (monoterpenoids, sesquiterpenoids and C13-norisoprenoids), as well as by those produced during the double fermentation process (Francioli, Guerra, Lopez-Tamames, Guadayoi, & Caixach, 1999). In the north of Italy there are two areas that are particularly suitable for the production of sparkling wine; the province of Trento and a small area in Lombardy (Franciacorta), the two products being called Trentodoc and Franciacorta respectively. Along with the production method, the Trentino area also has some natural similarities with the Champagne region in relation to climate. Although the latitude is higher in France than in Italy, the difference between daytime and night-time temperatures typical of the Alpine environment compensates for this minor environmental difference and gives a high level of acidity to the base wine produced in Trentino, as happens with Champagne grapes.

Trentodoc is the name of the first Certified Brand of Origin for an Italian sparkling wine. "Trento" DOC (controlled designation of origin) is produced using the traditional method, according to which second fermentation in the bottle is followed by ageing of wines on the lees for at least 15 months for non-vintage wines, 24 months for vintage wines, and 36 months for 'riserva' wines before disgorging. The cultivars allowed, in order of decreasing importance, are Chardonnay, Pinot Noir, Pinot Blanc and Pinot Meunier. Trentodoc wines can be aged on the lees for up to 10 years, and these premium wines are expected to improve as the years go by, with excellent vintages and premium reserve released to market after 5–10 years. In 2012, 7 million bottles were produced and largely dispatched around the world.

Franciacorta is a sparkling wine from Lombardy with DOCG (controlled and guaranteed denomination of origin) status, produced from grapes grown within the confines of the Franciacorta area, on the hills in the Province of Brescia. It was awarded DOC status in 1967, the designation then also including still red and white wines. Since 1995 the DOCG classification has applied exclusively to sparkling wines from the area.

Established DOCG vineyards extend over 2200 hectares (5400 acres) and the distribution of the permitted grape cultivars includes, in decreasing order of importance, Chardonnay, 10% Pinot Noir and 5% Pinot Blanc. In 2012 14 million bottles of this fine wine were produced.

Taken together, Trentodoc and Franciacorta DOCG are considered to be the finest 'bubbly' in Italy. In 2013, the year of sampling, as many as 17 of these wines were awarded the "three glass" rating, which can be considered to be the most prestigious national wine award.

It is known that environmental factors (terrain morphology, agro-pedological characteristics, climate and viticultural practices), usually described with the French term "terroir", can influence grape and wine composition and quality (Welke et al., 2014). Volatile composition may be useful for characterisation and differentiation of varietal wines and in general for establishing criteria to improve the quality of wines. Aroma compounds can also contribute towards obtaining a geographical designation such as the designation of origin, which serves as a benchmark and guarantees product consistency, defining a product that is characteristic of a certain region (Robinson et al., 2012).

Moreover, several important questions arise for both producers and consumers: in particular, are the signature of the grape cultivar, the pedoclimatic influence of the production area and the complex technology evident in the final product? Fine wines produced in a restricted area with strict rules and complex technology are expected to have distinctive or common characteristics, so that wines with the same denomination can be recognised by consumers. In addition these products can retain the specific characteristics of individual wineries, which are the signature of the oenologist and the brand, of course associated with signs of ageing.

The aim of this work was to carry out comprehensive mapping of volatile compounds in a large and highly representative sample of both Trentodoc and Franciacorta sparkling wines, and to evaluate possible differences by applying HS-SPME followed by GCxGC-TOF-MS and multivariate analysis.

2. Material and methods

2.1. Samples, analytical reagents and supplies

All the samples used for the survey were commercial products normally present on the market in January 2013, which were purchased at the winery or at a local wine store. The first group of sparkling wines that we analysed included 47 samples of 2004–2009 vintage Trentodoc, produced in Trentino and including all the 37 independent Trentodoc wine producers. 21 samples were classified as young wines (less than 36 months on the lees) and 26 samples classified as old (more than 36 months on the lees).

The second group of sparkling wines included 23 samples of Franciacorta, produced by 11 different wineries, covering the same vintages as the Trentodoc wines. 6 samples were classified as young wines (less than 36 months on the lees) and 17 samples were classified as old wines (more than 36 months on the lees) (Table S4).

For both denominations, the sample included wines belonging to groups with all lengths of ageing on the lees, including the most prestigious reserve wines with the longest ageing on the lees, respectively Riserva Giulio Ferrari 2002 for Trentodoc and Cà del Bosco Anna Maria Clementi 2004 for Franciacorta. The wines were stored at 4 °C until analysis.

Pure reference compounds for analyte confirmation and n-alkanes (C9-C30) for linear retention index determination were from Sigma Aldrich and Supelco (Milan, Italy).

A stock solution of 122 compounds commonly present in grape/wine samples was prepared in ethanol at a concentration of 10 mg L^{-1} to verify the separation and peak identification ability of ChromaTOF software. A list of these compounds is given in Table (S1).

Safranal and α-isophorone pure standard from Sigma Aldrich were used to quantify these compounds in wine. The calibration curves were in the concentration range of 0.05–1 μg L^{-1}.

SPME fibre (50/30 divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) StableFlex) was purchased from Supelco (Bellefonte, PA). The fibre was conditioned according to the manufacturer's recommendations prior to first use. Sodium chloride (NaCl) of analytical grade was purchased from Sigma and was oven dried at 100 °C overnight before use.

2.2. Instrumentation

A Gerstel MultiPurpose Sampler autosampler (Gerstel GmbH & Co. KG Mülheim an der Ruhr Germany) with an agitator and SPME fibre was used to extract the volatiles from the sample vial headspace. The GC × GC system consisted of an Agilent 7890 A (Agilent Technologies, Santa Clara, CA) equipped with a Pegasus IV time-of-flight mass spectrometer (Leco Corporation, St. Joseph, MI). A VF-Wax column (100% polyethylene glycol; 30 m × 0.25 mm × 0.25 μm, Agilent J&W Scientific Inc., Folsom, CA) was used as first-dimension (1D) column, and a RTX-200MS-column (crossbond trifluoropropylmethylpolysiloxane) 1.50 m × 0.25 mm × 0.25 μm, Restek Bellefonte, USA) was used as a second-dimension (2D) column.

The GC system was equipped with a secondary column oven and non-moving quadjet dual-stage thermal modulator. The injector/transfer line was maintained at 250 °C. Oven temperature programme conditions were as follows: initial temperature of 40 °C for 4 min, programmed at 6 °C min^{-1} up to 250 °C, where it remained for 5 min. The secondary oven was kept 5 °C above the primary oven throughout the chromatographic run. The modulator was offset by +15 °C in relation to the secondary oven; the modulation time was 7 s and 1.4 s of hot pulse duration. Helium (99.9995% purity) was used as carrier gas at a constant flow of 1.2 mL min^{-1}. The MS parameters included electron ionisation at 70 eV with ion source temperature at 230 °C, detector voltage of 1317 V, mass range of m/z 35–450 and acquisition rate of 200 spectra s^{-1}.

2.3. Volatile extraction conditions

SPME extraction was carried out with slight modification of a previous method (Versini, Dellacassa, Carlin, Fedrizzi, & Magno, 2008): 5 mL of sparkling wine, sonicated for 2 min to remove the foam, were put into 20 mL glass headspace vials, 1.5 g of NaCl were added, the wine samples were spiked with 50 μL of alcoholic solution of 2-octanol at 2.13 mg L^{-1} and 50 μL of alcoholic solution of ethyl hexanoate d_{11} at 1.0 mg L^{-1} as internal standards. Samples were kept at 35 °C for 5 min and then extracted for 30 min at 35 °C. The headspace was sampled using 2-cm DVB/CAR/PDMS 50/30 μm fibre. The volatile and semi-volatile compounds were desorbed in the GC inlet at 250 °C for 4 min in splitless mode and the fibre was reconditioned for 7 min at 270 °C prior to each analysis. All samples were analysed with two different instruments: 1D-GC-MS and GCxGC TOF-MS, the details of 1D-GC-MS analysis are reported in the Supplementary material.

2.4. Data processing and statistical analysis

For GCxGC-MS data, LECO ChromaTOF Version 4.22 software was used for all acquisition control, data processing and Fisher ratio calculations. Automated peak detection and spectral deconvolution with a baseline offset of 0.8 and signal-to-noise of 100 were used during data treatment. With these settings it was possible to detect 1695 putative compounds. The shortlist considered in the statistical analysis was obtained by retaining only those present in at least 44 samples (969). The intensities were organised in a data matrix and the missing values in correspondence with undetected peaks were imputed by sampling from a uniform distribution of between 10 and 50 counts. To account for possible sample-to-sample variation, all intensities were normalized to the signal of 2-octanol (internal standard). All subsequent statistical analysis was performed in R (R Core Team, 2014).

Identification of wine volatile compounds was achieved by comparing the experimental linear temperature retention index (LTPRI) with retention indices reported in the literature for 1D-GC (VCF Volatile Compounds in Food 16.1 database); a description of this procedure has already been reported elsewhere (von Mühlen, Zini, Caramão, & Marriott, 2008). Retention data for a series of n-alkanes (C10–C30), in the same experimental conditions employed for chromatographic analysis of wine volatiles, were used for experimental LTPRI calculation. Mass spectrometric information for each chromatographic peak was compared to NIST 2.0, Wiley 8 and the FFNSC 2 mass spectral library (Chromaleont, Messina, Italy), with a library similarity match factor of 750.

2.5. Network analysis

Network analysis is a particularly effective technique for highlighting the coordinated relationship among metabolites. In a complex premium wine such as Trentodoc, a combination of factors determines the complexity of the products. These can be summarised as: 1) high quality, manually harvested grapes produced in a cool Alpine area; 2) complex production technology, including double fermentation; 3) long ageing on the lees, allowing the yeast to undergo autolysis, and 4) chemical evolution and aroma development associated with long storage (i.e., the so called "chemical age"). The untargeted approach with bidimensional chromatography and TOF detection was an ideal technique for providing an unbiased picture of the huge complexity of the aroma of these wines, while network analysis was able to reveal the subtle associations present in the dataset. For this paper these were explored in the annotated dataset, while in principle the analysis of networks could also be an interesting tool for exploring several hundred unknowns.

The coordinated behaviour of metabolites can be delineated using graph theory, where the nodes represent metabolites and the relationship between them is demonstrated as links. The generation of the graphs was based on correlation analysis of all metabolites in the young and aged wine datasets. Prior to correlation analysis, each metabolite was normalized using the internal standard, and this was followed by log transformation. Pairwise correlation analysis was conducted for each pair of metabolites within the respective datasets. Pearson correlation was used to estimate the correlation coefficients represented as links in the networks.

To reconstruct a network capturing coordinated changes in the metabolic profiles, first the corresponding p-value threshold of the Pearson product-moment correlation coefficient was determined, ensuring a q-value of 0.05. Secondly, an adequate correlation coefficient threshold was chosen by assessing four different network properties, i.e. average node degree, clustering coefficient, network density and diameter. For a full description of these network properties see Toubiana, Fernie, Nikoloski, & Fait, 2013. The correlation coefficient at which the network displayed robust behaviour across a range of p-values in all four properties was chosen as the threshold for network construction. Subsequently, the network was analysed for communities by employing two approaches: i) grouping the nodes according to the walktrap community algorithm (Pons & Latapy, 2005), ii) grouping nodes according to their compound class. Nodes in the network were attributed with the degree of connectivity reflected by their relative size, while links were attributed with the computed correlation coefficient reflected by their colour and relative width.

All computations for network visualization were generated in R (R Core Team, 2014), Cytoscape (Shannon et al., 2003) software, version 2.8.3 was used for network visualization. Network properties and communities were computed with the igraph R package.

2.6. Climate analysis

We researched a climatic difference between the Franciacorta and the Trentodoc production region. To account for the meteorological evolution over each growing season, we calculated the Heliothermal Index (Huglin, 1978) using the hourly measures of the air temperature at 7 Franciacorta sites ranging from 180 to 300 m asl (120 m of altitude range), and 39 Trentodoc sites from 158 up to 696 m asl (range = 538 m). In this context, we did not use the Heliothermal Index (HI) to identify suitable areas for the viticulture, but rather to check a consistent climatic difference of the calculated HI between the two production zones. To check this hypothesis, the HI values were scaled, centered and used to define a Bayesian hierarchical linear model with random intercepts: the scaled HI was associated with three main climatic drivers: the elevation of the meteorological stations above sea level (fixed effect), the production zone (fixed effect), and the year (from 2008 to 2015, random effect). This latent field was enforced as an independent

random noise. The R-INLA software was used (Rue, Martino, & Chopin, 2009) to estimate the posterior distribution of the parameters. The candidate model has been tested against every model built with the combinations of each fixed and random effect. To discriminate the significant effects we used the Watanabe-Akaike Information Criteria, WAIC (Watanabe, 2013).

3. Results and discussion

The chromatograms of the wines provided a wealth of information, with as many as 1695 compounds being found by ChromaTOF with automated peak find and spectral deconvolution. The high deconvolution and identification capacity of the software was also tested using a mixture of 122 compounds characteristic of grapes and wine, and using the automatic processing method it was possible to correctly annotate 111 of these compounds. The most evident errors were in the correct identification of some terpenes, which have an identical formula weight and very similar mass spectra (Table S1). Fig. S1 shows the colour plot chromatogram obtained with the standard. It is evident that there are a large number of compounds that are co-eluted in the first dimension and which obviously cannot be properly observed with conventional 1D-GC-MS.

In Fig. S2, showing the GC × GC Total Ion Chromatogram colour plot of a sparkling wine, only major peaks such as the ethyl ester of fatty acid, acetates and higher alcohols are evident, while the second plot, a partially extracted ion colour plot, shows several minor peaks belonging to different chemical classes such as monoterpenes, norisoprenoids, aldehydes.

Principal Component Analysis (PCA) on the autoscaled data matrix was performed to reduce the dimensionality and visualise the multidimensional dataset. The PCA scoreplot is presented in the left panel of Fig. 1. Each point in the plot represents the projection of an individual sample in the PC1 vs PC2 plane. The different colours identify samples belonging to Trentodoc and Franciacorta DOCG. The plot also shows that at this general level the two groups are partially separated, indicating that untargeted GCxGC analysis can be used to distinguish wines belonging to the two classes. This strongly suggests that wines belonging to each of the two denominations have a distinctive pattern of volatile metabolites. The next step was to explore the differences between the two classes.

To gain more insight into the chemical basis of this differentiation, 969 compounds were ranked according to their Fisher ratio and the first 162 were annotated (see "Data processing and statistical analysis").

The most important compounds in terms of separating the two classes were selected, using the univariate *t*-test on 969 putative compounds. False discovery rate correction was applied to account for multiple testing issues (Franceschi, Giordan, & Wehrens, 2013). By setting a cut-off q-value of 0.05 it was possible to retain a list of 196 biomarkers. PCA performed on the biomarkers is displayed in the right-hand plot in Fig. 1. As expected, the two classes are well separated in this scoreplot.

Table 1 gives the list of compounds that we annotated: for 54 of these compounds we obtained confirmation with pure standard, for 44 we found similar LTPRI values, while the others represent tentative identification.

The box plot in Fig. 2 shows some compounds belonging to the different chemical classes that will be discussed.

The variables that were most important for this separation between the two groups (Trentodoc vs Franciacorta) are given below, giving the average fold variation (mean max./mean min.) for the areas in brackets:

Monoterpenes such as *cis* rose oxide (3.0), linalool (4.3), nerol oxide (1.3), α-terpineol (1.8), 2,6 dimethyl 1,7 octadien 3-ol (2.2) and a *p*-menth-1-en-9-al (2.9) were higher in Trentodoc sparkling wines. We also found that the ethyl ethers of some monoterpenes such as linalyl, (4.7) neryl (1.4) geranyl (2.3) and α-terpenyl ethyl ether (1.8) were higher (Versini et al., 2008). These powerful flavouring compounds can develop during the reaction to convert glycoconjugated forms (Strauss, Wilson, Rapp, Guentert, & Williams, 1985) into the free form through hydrolysis or enzymatic reaction by the yeast. These data are in accordance with the results of traditional 1D-GC-MS data, where on average *cis* rose oxide was 0.26 μg L^{-1} in Trentodoc and 0.12 μg L^{-1} in Franciacorta, linalool 1.9 μg L^{-1} in Trentodoc and 0.6 μg L^{-1} in Franciacorta, geraniol 6.4 μg L^{-1} in Trentodoc and 5.0 μg L^{-1} in Franciacorta, geranyl ethyl ether 2.2 μg L^{-1} in Trentodoc and 0.7 μg L^{-1} in Franciacorta, α-terpineol 5.4 μg L^{-1} in Trentodoc and 3.2 μg L^{-1} in Franciacorta. The semi-quantitative data from 1D-GC-MS can be seen in Table S2.

Monoterpenes and sesquiterpenes are synthesized from isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) which are formed through the cytosolicmevalonatic-acid (MVA) pathway from three molecules of acetyl-CoA and through the plastidial 2-C-methylerythritol-4-phosphate (MEP) pathway from

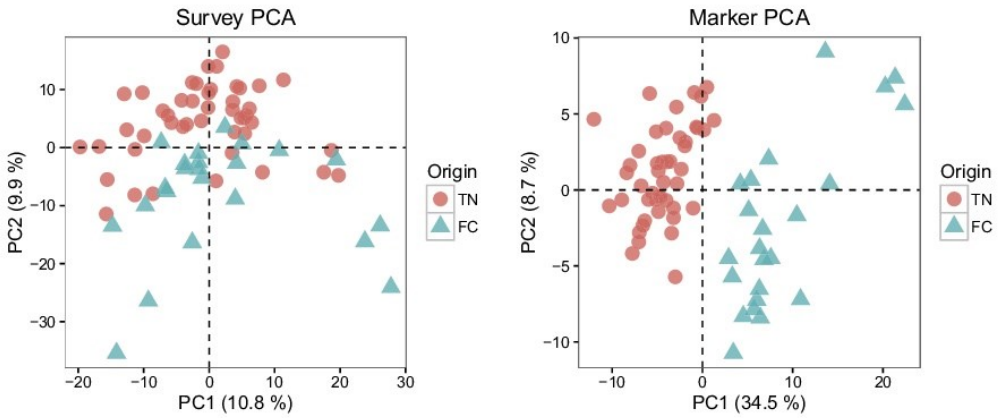

Fig. 1. Principal Component Analysis, projection of samples in the PC1 and PC2 plane. Different colours identify samples belonging to Trentodoc and Franciacorta DOCG. Panel A shows PCA with all compounds (969), panel B is based on 196 putative biomarkers selected after the univariate *t* test (cut-off value of 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
List of annotated compounds sorted by Fisher ratio descending, type of identification, class of compounds, CAS numbers, Fisher ratio, retention indices and average of areas for the 2 groups of sparkling wine.

Peak n°	Compound name	Ident.	Class of compounds	CAS number	FR	$LTPRI_{lit}$	$LTPRI_{exp}$	^{1}tR (s)	^{2}tR (s)	Trentodoc[a] n = 47	Franciacorta[a] n = 23
1	2-Methylbutyl acetate	A, B,C	Ester	624-41-9	174	1119	1111	386	2.79	27516	363,116
2	Gama-eudesmol	B, C	Terpene	1209-71-8	153	2175	2153	1605	1.83	3219	10,288
3	1-Hexanol	A, B,C	Alcohol	111-27-3	96	1357	1347	697	1.54	1,397,326	673,841
4	Benzofuran, 4,7-dimethyl-	C	Benzofuran	–	72	–	1684	1105	1.77	6812	16,082
5	2,6 dimethyl 1,7 octadien-3-ol	C	Terpene	–	71	–	1511	930	1.78	19,475	8756
6	1-Penten-3-ol	A, B,C	Alcohol	616-25-1	71	1147	1154	439	1.41	286,765	121,184
7	2-Penten-1-ol, (Z)-	A, B,C	Alcohol	1576-95-0	64	1317	1309	655	1.40	82,454	41,507
8	Propanoic acid, 3-ethoxy-, ethyl ester	B,C	Ester	763-69-9	58	1332	1336	673	2.18	55,801	125,675
9	3-Penten-2-one, 4-methyl-	C	Ketone	–	52	–	1526	943	2.58	48,966	116,852
11	2-Octen-4-one	C	Ketone	–	49	–	1470	867	2.55	43,798	75,922
12	3-Hexen-1-ol *trans*	A, B,C	Alcohol	928-97-2	46	1371	1353	709	1.49	825,607	262,549
13	Benzylidenemalonaldehyde	C	Benzenoid	–	42	–	1594	1006	1.73	32,002	57,299
14	Benzofuran, 4,7-Dimethyl-	C	Benzofuran	–	42	–	1698	1133	1.78	14,402	26,341
15	1-Undecyn-4-ol	C	Alcohol	–	39	–	1852	1308	1.87	23,145	9498
17	Riesling acetale	B,C,D	Norisoprenoid	Not found	38	1656	1638	1051	2.12	132,075	67,653
18	1,3-Dioxane, 2-methyl-	B,C	Acetal	626-68-6	38	1044	1056	318	1.84	27,946	82,207
21	1-Ethoxy-1-pentoxy-ethane	B,C	Acetal	13,442-89-2	34	1091	1100	363	2.92	66,161	156,627
23	2-Allylphenol	B,C	Benzenoid	–	31	–	1790	1227	1.88	4105	6819
26	Thiophene, 2-methyl-	B,C	S-compound	–	31	–	1477	884	1.73	83,727	158,982
28	3-Ethoxythiophene	C	S-compound	Not found	30	–	1466	857	1.65	417,742	1,143,002
29	Naphthalene, 1,2-dihydro-1,4,6-trimethyl-	C	Norisoprenoid	–	29	–	2010	1470	1.78	2345	3356
30	Lilial	B,C	Benzenoid	80-54-6	29	2037	2023	1488	2.07	5363	9342
31	1,3-Dioxolane, 4,5-dimethyl-2-pentadecyl-	C	Acetal	–	29	–	1146	421	2.60	378,979	758,803
32	Actinidol ethoxy 2	B,C,D	Norisoprenoid	Not found	29	1732	1733	1159	2.25	512,662	903,168
33	Propanedioic acid, diethyl ester	A, B,C	Ester	105-53-3	29	1580	1582	988	1.98	160,305	228,898
34	Pentanedioic acid, diethyl ester	A, B,C	Ester	818-38-2	29	1780	1786	1218	2.11	289,728	384,919
35	3-(Acetoxy)-4-methyl-2-pentanone	C	Ketone	–	29	–	1524	939	2.16	40,411	69,463
36	1,3-Dioxolane, 2,4,5-trimethyl-	B,C	Acetal	3299-32-9	28	967	970	232	2.01	121,835	356,007
37	1,3-Dioxane, 4-methyl-2-pentadecyl-	C	Acetal	–	28	–	1150	430	2.60	226,623	664,217
38	Actinidol ethoxy 1	B,C,D	Norisoprenoid	Not found	28	1706	1696	1127	2.27	744,480	1,334,068
39	Ethane, 1,1-diethoxy-	B,C	Acetal	105-57-7	28	889	897	165	1.91	659,482	1,349,646
40	3-Hexen-1-ol *cis*	A, B,C	Alcohol	928–96-1	27	1388	1385	737	1.47	1,665,105	873,645
41	1H-indene, 3-ethenyl-2,3-dihydro-1,1-dimethyl-	C	Norisoprenoid	–	26	–	2097	1551	1.88	4868	14,922
44	4-Decenoic acid, ethyl ester, (Z)-	B,C	Ester	7367-84-2	26	1699	1676	1089	2.49	11,390	28,878
45	Butane, 1,1-diethoxy-3-methyl-	B,C	Acetal	3842-03-3	26	1064	1061	327	2.93	11,451	26,965
47	Actinidol 1	C,D	Norisoprenoid	–	25	–	1905	1371	1.86	55,789	90,249
51	Succinic acid, Butyl ethyl ester	C	Ester	–	25	–	1848	1299	2.16	14,383	19,807
52	2(3H)-furanone, 5-ethoxydihydro-	B,C	Lactone	932-85-4	24	1728	1731	1155	1.87	28,769	47,413
53	Acetic acid, hexyl ester	A, B,C	Ester	142-92-7	24	1269	1261	592	2.76	16,946,133	3,162,138
54	Actinidol 2	C,D	Norisoprenoid	–	24	–	1936	1384	1.85	90,016	141,961
55	Dehydroaromadendrene	C	Terpene	–	24	–	2276	1695	1.73	14,961	34,198
56	2-Hexen-1-ol, (E)-	A, B,C	Alcohol	928-95-0	24	1410	1421	781	1.45	213,690	102,301
61	β-Bisabolol	A, B,C	Terpene	15,352-77-9	23	2189	2203	1649	1.74	27,620	12,921
62	Propyl octanoate	B,C	Ester	624-13-5	23	1514	1509	916	2.94	1,978,898	2,994,306
63	Propyl hexanoate	A, B,C	Ester	626-77-7	23	1306	1309	655	2.97	464,350	621,459
66	1,2,3-Trimethylindene	C	Norisoprenoid	–	23	–	1642	1060	1.94	13,577	26,587
67	2-Penten-1-ol, 2-methyl-5-(2-methyl-3-methylene-2-norbornyl)-	B,C	Terpene	77-42-9	23	1815	1795	1236	2.52	304,308	522,092
69	3-(Acetoxy)-4-methyl-2-pentanone	C	Ketone	–	23	–	1462	845	2.24	1,247,292	2,105,720
70	α-Terpineol	A, B,C	Terpene	98-55-5	21	1705	1693	1123	1.68	476,284	264,457
71	Diisobutyl succinate	C	Ester	–	20	–	2061	1501	2.18	22,142	8566
72	Naphthalene, 1,2,3,4-Tetrahydro-2,2-dimethyl-1-methylene-	C	Norisoprenoid	–	20	–	2126	1560	1.88	3638	9481
73	Cyclotetradecane	C	Hydrocarbon	–	20	–	2155	1609	1.78	100,876	346,584
76	Oxetane, 2-propyl-	C	Oxetane	–	19	–	850	147	1.72	66,915	124,310
78	p-Menth-1-en-9-al	B,C	Terpene	29,548-14-9	18	1637	1628	1032	2.29	41,366	14,238
80	Geranyl ethyl ether	B,C,D	Terpene	Not found	18	1532	1506	905	2.34	245,942	108,463
81	Alpha terpenyl ethyl ether	C,D	Terpene	–	18	–	1442	834	2.42	1,479,817	803,370
82	Ethyl 3-formylpropionate	C	Ester	–	18	–	1532	952	1.86	32,923	54,955
83	5-Ethoxy-3,4-dihydro-2h-pyrrole-2-carboxylic acid, ethyl ester	C	Ester	–	18	–	2204	1650	2.09	7933	14,989
84	Ethanone, 1-(2,3-dihydro-1,1-dimethyl-1H-inden-4-yl)-	B,C	Norisoprenoid	55,591-10-1	18	1739	1738	1168	1.87	17,762	47,296
85	Ethyl 2-(methylthio)-acetate	B,C	S-compound	4455-13-4	18	1439	1438	826	1.85	13,134	19,693
86	Naphthalene, 1,2-dihydro-1,5,8-trimethyl-	C	Norisoprenoid	–	17	–	1831	1267	1.82	319,848	513,509

Table 1 (*continued*)

Peak n°	Compound name	Ident.	Class of compounds	CAS number	FR	$LTPRI_{lit}$	$LTPRI_{exp}$	^{1}tR (s)	^{2}tR (s)	Trentodoc[a] n = 47	Franciacorta[a] n = 23
88	Ethyl hexadecanoate	A, B,C	Ester	628-97-7	17	2245	2262	1686	2.58	2,075,532	3,588,579
93	Octanoic acid, ethyl ester	A, B,C	Ester	106-32-1	17	1439	1432	809	2.88	123,214	57,368
94	3,6-Nonadien-5-one, 2,2,8,8-tetramethyl-	C	Ketone	–	17	–	1576	979	2.10	7926	19,631
95	1.5-Hexadiene, 2.5-dimethyl-3-methylene-	C	Terpene	–	17	–	1007	264	2.34	4336	6771
96	Dipropyl hydroxybutanedioate	C	Ester	–	16	–	2225	1677	1.75	10,285	20,742
97	4-Nonene, 2,3,3-trimethyl-, (E)-	C	Hydrocarbon, unsaturated	–	16	–	1300	637	2.54	7450	11,082
98	2,4-Pentanedione, 3-(2-propenyl)-	C	Ketone	–	16	–	1241	547	1.84	6368	9602
100	Nonanoic acid, ethyl ester	A, B,C	Ester	123-29-5	16	1528	1550	1384	2.19	5902	11,786
101	2-Propenoic acid, 3-phenyl-, ethyl ester	B,C	Ester	103-36-6	16	2132	2133	1571	1.72	41,383	94,471
103	4-(2,4,6-Trimethylphenyl)-butan-2-ol	C	Alcohol	–	16	–	2359	1796	1.53	6338	2989
104	Acetic acid, methyl ester	A, B,C	Ester	79-20-9	15	810	830	133	1.53	226,231	113,669
110	Butanoic acid, 2-pentenyl ester, (Z)-	C	Ester	–	15	–	1005	259	2.19	955,811	1,309,790
111	α-Methylene-gama-butyrolactone	C	Lactone	–	15	–	1570	970	1.72	10,394	18,331
112	Propanedioic acid, (1-methyl-2-propenyl)-, diethyl ester	C	Ester	–	15	–	1845	1294	2.11	5735	8431
115	β-Ionone	A, B,C	Norisoprenoid	14,901-07-6	15	1975	1995	1447	2.22	6052	8601
116	β-Damascenone	A, B,C	Norisoprenoid	23,726-93-4	15	1814	1807	1259	2.28	1,325,338	1,901,713
121	Naphthalene, 1,2,3,4-tetrahydro-1,8-dimethyl-	C	Norisoprenoid	–	14	–	1633	1042	2.01	4310	8098
122	Tridecanoic acid, 3-hydroxy-, ethyl ester	C	Ester	–	14	–	2091	1542	1.84	318,884	415,452
124	Ethanone, 1-[2,3-dihydro-2-(1-methylethenyl)-5-benzofuranyl]-, (R)-	C	Benzofuran	55,591-10-1	14	–	2091	1542	2.03	7389	38,150
125	(4-(1-Methylvinyl)-1-cyclohexenyl) methanol	C	Terpene	–	14	–	1638	1051	2.04	9982	1642
126	Propanoic acid, propyl ester	A, B,C	Ester	106-36-5	14	1056	1026	295	2.51	14,550	19,784
130	Butanedioic acid, diethyl ester	A, B,C	Ester	123-25-1	13	1687	1683	1103	2.05	707,935	440,185
133	Butanoic acid, ethyl ester	A, B,C	Ester	105-54-4	13	1040	1024	297	2.40	431,793	1,000,617
134	Z.Z farnesol	A, B,C	Terpene	4602-84-0	13	2272	2283	1713	2.16	16,841	33,160
136	α-Cyclocitral	B,C	Norisoprenoid	432-24-6	13	1420	1431	808	2.69	3929	2572
139	(E) ethyl undec-2-enoate	C	Ester	–	13	–	1200	498	2.48	15,650	25,221
141	2H-pyran-2-one, tetrahydro-6-methyl-	C	Lactone	–	13	–	1203	504	2.04	16,503	26,844
142	α-Ionone	A, B,C	Norisoprenoid	127-41-3	13	1818	1789	1114	1.94	3137	5612
143	Butanoic acid, 3-methyl-, ethyl ester	A, B,C	Ester	108-64-5	13	1055	1056	318	3.20	858,994	1,231,885
144	Butanedioic acid, hydroxy-, diethyl ester,	B,C	Ester	7554-12-3	13	2023	2019	1483	2.04	9900	25,920
147	Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-	C	Norisoprenoid	–	13	–	1437	823	2.39	31,805	130,366
148	2-Hexenoic acid, ethyl ester	B,C	Ester	1552-67-6	13	1343	1345	691	2.49	1,600,633	849,498
153	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	C	Terpene	–	12	–	1284	601	2.24	65,423	22,335
155	2H-pyran, tetrahydro-4-methyl-2-(2-methyl-1-propenyl)- (*trans* rose oxide)	A, B,C	Terpene	16,409-43-1	12	1383	1381	696	2.51	57,494	26,872
157	3-Hydroxy-2,2-dimethylhexyl ester of butanoic acid	C	Ester	–	12	–	1854	1311	2.09	27,133	46,725
158	α-Terpinene	A, B,C	Terpene	99-86-5	12	1189	1200	905	2.38	284,167	198,661
159	Thieno[2,3-b]thiophene,2-methyl-	C	S-compound	–	12	–	1939	1389	1.61	1812	6522
160	*trans*-4-Hydroxymethyl-2-methyl-1,3-dioxolane	C	Acetal	–	12	–	1623	1022	1.37	36,061	82,221
161	*cis*-Rose oxide	A, B,C	Terpene	4610-11-1	12	1337	1353	623	2.44	7356	2477
162	S-compound	B,C	S-compound	–	12	–	1070	345	1.75	7341	10,494
163	Trimethyl-tetrahydronaphthalene	C	Norisoprenoid	–	12	–	1424	790	2.41	23,017	52,183
164	2-Furanpropanoic acid, ethyl ester	C	Ester	–	12	–	1684	1105	1.83	51,381	82,997
165	Ethyl (4Z)-4-octenoate	C	Ester	–	12	–	1463	849	2.51	6058	9121
166	Cyclotetradecane	C	Hydrocarbon	–	12	–	2071	1515	1.75	11,768	32,654
168	2-Ethylhexyl 2-ethylhexanoate	C	Ester	–	12	–	1747	1186	3.51	41,908	77,620
172	1,3-Dioxolan-4-one, 2-(1,1-dimethylethyl)-5-(1-methylethyl)-, (2S-cis)-	C	Acetal	–	12	–	1891	1344	1.55	3685	6704
180	Nerolidol	A, B,C	Terpene	–	11	–	1951	1410	1.85	7885	4162
182	Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-	C	Norisoprenoid	–	11	–	1723	1141	2.04	5071	10,531
185	Benzene, 1-methyl-4-(1-methylethenyl)-	B,C	Terpene	1195-32-0	11	1447	1428	799	1.92	4153	2633
186	Butanedioic acid, hydroxy-, diethyl ester,	B,C	Ester	626-11-9	11	2036	2023	1488	1.60	5,101,450	8,821,596
187	2-Thiophenecarboxaldehyde	B,C	S-compound	98-03-3	11	1678	1684	1105	1.53	3070	4654
188	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	A, B,C	Terpene	99-85-4	11	1191	1162	457	2.53	12,681	5960

(*continued on next page*)

Table 1 (*continued*)

Peak n°	Compound name	Ident.	Class of compounds	CAS number	FR	$LTPRI_{lit}$	$LTPRI_{exp}$	^1tR (s)	^2tR (s)	Trentodoc[a] n = 47	Franciacorta[a] n = 23
189	3-Thiolannone tetrahydrothiophen-3-one	B,C	S-compound	1003-04-9	11	1568	1568	966	1.62	8217	12,844
191	1,3-cyclohexadiene, 1-methyl-4-(1-methylethyl)-	C	Terpene	–	11	–	1454	873	2.37	114,389	84,142
195	1,3-Dioxolane, 4,5-dimethyl-2-pentadecyl-	C	Acetal	–	11	–	1307	651	2.51	32,832	80,110
196	Hexyl octanoate	A, B,C	Ester	1117-55-1	11	1795	1802	1249	2.91	7785	3457
198	1-Butanol, 3 methyl, propanoate	B,C	Ester	105-68-0	11	1188	1171	475	3.02	649,265	782,984
199	Hydroxydihydroedulan	C	Norisoprenoid	–	11	–	1902	1366	1.84	47,801	16,392
202	Octanoic acid, 2-phenylethyl ester	C	Ester	–	11	–	2358	1794	1.97	48,288	60,190
203	2,6,6 -Trimethyl-2-cyclohexen-1-one	B,C	Norisoprenoid	Not found	11	1376	1398	763	2.47	135,241	206,458
208	Ethyl nicotinate	C	Ester	–	10	–	1805	1254	1.83	15,857	10,654
209	3-Hexenoic acid, ethyl ester, (E)-	B,C	Ester	26,553-46-8	10	1284	1298	633	2.35	183,046	136,895
210	Benzene, 1-methyl-4-(1-methylethenyl)-	A,B,C	Terpene	1195-32-0	10	1444	1474	876	2.02	377,461	611,358
213	Alpha calacorene	B,C	Terpene	21,391-99-1	10	1918	1899	1361	1.99	30,082	44,485
214	3-Heptanol, 3,5-dimethyl-	C	Alcohol	–	10	–	1332	664	2.0	14,215	31,093
218	unknown fatty acid, ethyl ester	C	Ester	–	10	–	2236	1861	2.53	51,450	75,171
219	2-Pentanone	A,B,C	Ketone	107-87-9	10	961	986	232	2.25	71,009	55,294
220	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, *trans*-	A, B,C	Terpene	1197-07-5	10	1843	1854	856	2.25	20,266	9931
222	Safranal	A,B,C	Norisoprenoid	116-26-7	10	1627	1645	1065	2.18	38,177	38,842
223	α-Isophorone	A,B,C	Norisoprenoid	78-59-1	10	1573	1591	1002	2.67	47,662	41,158
224	Octanal	A,B,C	Aldehyde	124-13-0	10	1296	1288	610	2.79	77,417	93,872
226	Vitispirane 1	B,C	Norisoprenoid	Not found	10	1524	1514	925	2.36	259,199	119,970
226	Vitispirane 2	B,C	Norisoprenoid	Not found	10	1524	1514	925	2.36	1,540,646	1,261,305
227	Naphthalene, 1,2-dihydro-1,1,6-trimethyl-	B,C	Norisoprenoid	30,364-38-6	<9	1739	1743	1177	1.92	1,259,295	1,551,632
231	Whiskey lactone isomers	B, C	Lactone	39,212-23-2	<9	1970	1946	1402	2.38	160,668	271,302
232	Ethanethioic acid, S-methyl ester	B, C	S-compound	1534-08-3	<9	1054	1047	300	1.39	25,689	13,891
251	Linalool	A,B,C	Terpene	22,564-99-4	<9	1554	1530	948	1.67	275,563	64,080
252	Linalyl ethyl ether	C,D	Terpene	–	<9	–	1331	660	2.58	88,212	18,811
233	Whiskey lactone isomers	B, C	Lactone	39,212-23-2	<9	1910	1884	1330	1.75	185,336	318,450
253	2-Undecanone	A,B,C	Ketone	112-12-9	<9	1604	1597	1011	2.90	1,208,853	1,849,381
254	Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-	B,C	Norisoprenoid	475-03-6	<9	1545	1536	957	2.30	17,257	34,754
238	Octanethioic acid, S-ethyl ester	C	S-compound	–	<9	–	1512	921	2.21	5,175,601	8,174,688
244	*cis*- Tetrahydroionone	C	Norisoprenoid	–	<9	–	1857	1317	2.43	4602	5688
263	Benzaldehyde	A,B,C	Aldehyde	100-52-7	<9	1525	1512	916	1.72	5,500,276	2,685,591
236	3-(methylthio)propyl acetate	B,C	S-compound	Not found	<9	1624	1636	1047	1.94	9249	5261
237	1-Propanol, 3-(methylthio)-	A,B,C	S-compound	505-10-2	<9	1727	1726	1146	1.38	434,396	344,688
239	Nerol oxide	B,C	Terpene	1786-08-9	<9	1471	1465	853	2.02	740,124	566,024
255	Naphthalene, 1,2,3,4-tetrahydro-1,4-dimethyl-	C	Norisoprenoid	–	<9	–	1740	1173	1.96	15,836	23,360
256	Disulfide, dimethyl	A,B,C	S-compound	624-92-0	<9	1080	1061	327	1.69	83,699	62,128
247	Neryl ethyl ether	C,D	Terpene	–	<9	–	1468	862	2.25	27,934	19,653
257	β-Pinene	A,B,C	Terpene	18,172-67-3	<9	1106	1123	412	2.59	45,618	50,132
240	2H-pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl- *trans*	A,B,C	Terpene	14,049-11-7	<9	1732	1748	1168	1.59	17,239	13,649
248	2-Decanone	A,B,C	Ketone	693-54-9	<9	1463	1477	885	2.91	178,285	135,055
258	Benzene, [(methylthio)methyl]-		S-compound	–	<9	–	1670	1092	1.69	34,818	23,944
250	2-Octanone	A,B,C	Ketone	111-13-7	<9	1297	1286	606	2.95	109,524	98,717
259	2H-pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl- *cis*	A,B,C	Terpene	14,049-11-7	<9	1756	1771	1195	1.55	6664	5774
260	4-(methylthio)-1-butanol	A,B,C	S-compound	20,582-85-8	<9	1812	1839	1281	1.42	22,539	21,764
261	4-Methyl-5-vinylthiazole	A,B,C	S-compound	1759-28-0	<9	1522	1512	921	1.64	24,361	21,900
243	Benzothiazole	A,B,C	S-compound	95-16-9	<9	1969	1944	1398	1.50	30,015	31,447
245	Norisoprenoid	C	Norisoprenoid	–	<9	–	1964	1434	1.83	18,306	18,091
262	Cyclohexanone, 2,2,6-trimethyl- (TCH)	B,C	Norisoprenoid	2408-37-9	<9	1320	1304	646	2.98	47,034	47,063
264	*2-Octanol, (S)- Internal standard*	A,B,C	*IS alcohol*	123-96-6	<9	1421	1423	786	1.72	713,166	723,034
265	*ethyl hexanoate d11 Internal standard*	A,B,C	*IS ester*	Not found	<9	–	1212	520	2.70	2,293,713	2,369,488

[a] Average of areas; Identification assignments: A comparing mass spectra and retention time with those of pure standard, B retention index mach on a similar phase column (VCF Volatile Compounds in Food 16.1, Schneider et al. J. Chromatogr. A 936 (2001) 145–157), C mass spectral database, D comparison with previous studies done at institute; FR = Fisher ratio.

pyruvate and glyceraldehyde-3-phosphate. Monoterpenes are formed from 2E-geranyl diphosphate (GPP) and sesquiterpenes are formed from 6E-farnesyl diphosphate (FPP) through the action of terpene synthases (TPS). Monoterpenes are important contributors to the aroma of white wines made from Muscat varieties and aromatic non-Muscat varieties with correlations between floral sensory attributes (Robinson et al., 2014).

The higher level of monoterpenes is probably due to a different microclimate caused by the mountains in Trentino. It is indeed well-known that low temperatures favour aroma production in grapevine berries, especially during ripening (Tonietto & Carbonneau, 2004). This is clearly shown in the enhanced aromatic potential of cool climate white wines (Duchêne & Schneider, 2005) made from cultivars such as Gewürztraminer, Sauvignon Blanc or

Riesling, where the main aroma compounds are isoprenoids, notably monoterpenes. Consequently, elevated temperatures potentially reduce the aromatic potential of grapes (Belancic et al., 1997). In a recent paper by Rienth et al. (2014) this matter was also supported from a transcriptional point of view. The Trentino region is predominantly mountainous or hilly and vines for the production of Trentodoc are located mainly on the slopes, reaching up to 800 m asl, while the Franciacorta area is characterised by plains and low hills. Furthermore, the production specifications for Franciacorta sparkling wine also state that all areas located at an altitude of over 550 m asl are excluded from production. From the climatic point of view, the Huglin Index values for the Franciacorta range from 2096 to 2770 (mean = 2507, sd = 175, range = 674) while for the Trentodoc the values of the index ranges from 1514 to 2848 (mean = 2284, sd = 245, range = 1334). (Fig. 4) If we consider only the subset of the Trentodoc stations that lies on the same altitude range of the Franciacorta territory, the distribution of the HI of the Trentodoc become similar to the Franciacorta district (range = 834, sd = 180). Anyway, the different altitude range in both production regions must be taken into account when describing the two territories and the larger variability of the HI index in the Trentodoc region reflects the variety of the alpine micro climates.

We used a standardized HI model to check a consistent climatic difference between the two production zones by using a Bayesian hierarchical model with an intercept varying randomly during 2008–2015. The effect of the elevation on the Huglin Index is well known and documented. However, adding the fixed effect of the production zone lowers significantly the WAIC from 484 to 436. The lower is the WAIC, the more preferable is the model: taking into consideration only the fixed effect of elevation or the production zone would exasperate the WAIC (769 and 957 respectively). So, the initial hypothesis of a consistent climatic difference between the two production zone as described with our model is supported by the meteorological data. Although the use of climatic indexes based on the temperature has proven effective, it could be interesting to take into account other climatic drivers like the shading effect of the mountains that dramatically changes the radiative energy budget during the development and ripening of the grapes.

It is also known that some authors have found increasing amounts of certain monoterpenes during second fermentation (Ganss, Kirsch, Winterhalter, Fischer, & Schmarr, 2011) and de novo synthesis of synthetic must by *S. cerevisiae* (Carrau et al., 2005), so we can also presume that some of the differences that we found may be the result of a "yeast" effect.

In the sesquiterpene class we found that gama-eudesmol (t.i.) would appear to be a good marker for sparkling wine from Franciacorta (3.2). This compound has been found by different authors (Alves, Nascimento, & Nogueira, 2005; Coelho, Rocha, Delgadillo, & Coimbra, 2006) in grapes and wine, but further study is necessary to better understand this higher level.

Trentodoc sparkling wines are also characterised by increasing levels of so-called "leaf C6-compounds": hexanol (2.1), *cis* and *trans* 3-hexenol (1.9–3.1) *trans* 2-hexenol (2.1) and hexyl acetate (5.4). These data are also confirmed by traditional 1D-GC-MS data, where on average hexanol was 1524 $\mu g\,L^{-1}$ in Trentodoc and 515 $\mu g\,L^{-1}$ in Franciacorta, *trans* 3-hexenol 2.6 $\mu g\,L^{-1}$ in Trentodoc and 0.9 $\mu g\,L^{-1}$ in Franciacorta, *cis* 3-hexenol 4.6 $\mu g\,L^{-1}$ in Trentodoc and 2.8 $\mu g\,L^{-1}$ in Franciacorta, hexyl acetate 13 $\mu g\,L^{-1}$ in Trentodoc and 3.9 $\mu g\,L^{-1}$ in Franciacorta. It has long been assumed that unsaturated fatty acids, linoleic and linolenic acids are the precursors of these compounds, and a pathway involving lipoxygenase and hydroperoxide lyase has been demonstrated (Tressl & Drawert, 1973). This reaction is related to the rupture of grape cells during the wine-making process and in particular this happens during the pressing and mashing of grapes (Tressl & Drawert, 1973) which are characterised by a grassy, herbaceous aroma. This group of C6 compounds were also considered likely candidates in relation to the formation of hexyl acetate, with a very fruity note that we found to be more present in Trentodoc wines, acting as precursors through alcohol acetyl transferase (AAT) activity during yeast fermentation. A direct relationship between hexanol concentration during fermentation and hexyl acetate production in wine via the action of yeast alcohol acetyl transferase was recently demonstrated (Dennis et al., 2012).

The C5 compounds 1-penten-3-ol (2.4) and 2-penten-1-ol (2.0) and 2-pentanone (1.3) were also much higher in Trentodoc samples. As compared to the well-known C6 volatile biosynthesis pathway, the synthesis of C5 compounds is less well-established. However, it seems that their synthesis in the tomato is catalysed in part by a 13-lipoxygenase (LOX), TomloxC, the same enzyme responsible for the synthesis of C6 volatiles (Shen et al., 2014). The hexanol content in wine may be due to several factors; the level of maturity of the grapes (Bindon, Varela, Kennedy, Holt, & Herderich, 2013) or different oenological practices such as the type of pressing and must sulfiting time (Nicolini, Versini, Amadei, & Marchio, 1996). In particular, differences in the content of C5 and C6 compounds in these wines could reflect the juice extraction methods: a lower presence of C5 and C6 compounds was expected in wines from Franciacorta since the whole cluster pressing technique is mandatory for the production of these wines, with the sole exception of the Franciacorta rosé wines.

Franciacorta sparkling wine is richer in diethyl esters, particularly diethyl glutarate (1.3), diethyl malate (1.7) and diethyl malonate (1.4). Data from 1D-GC-MS showed an average of 117 $\mu g\,L^{-1}$ of diethyl malate in Franciacorta and 87.4 $\mu g\,L^{-1}$ in Trentodoc; 7.3 $\mu g\,L^{-1}$ of diethyl glutarate in Franciacorta and 5.8 $\mu g\,L^{-1}$ in Trentodoc. As suggested by several authors, (Alexandre & Guilloux-Benatier, 2006), compounds coming from fermentation or caused by yeast autolysis, such as propanedioc acid diethylester (diethyl malonate), pentanedioic acid diethyl ester (diethyl glutarate) and butanedioic acid, hydroxy-, diethyl ester (diethyl malate) increase significantly with storage time, due to chemical esterification during the course of ageing (Camara, Alves, & Marques, 2006). This is in contrast with the ethyl esters of monoprotic acids, which decrease over time due to hydrolysis reactions.

Long ageing on the yeast lees, as takes place when producing this kind of wine, can have a very strong effect on the final quality. The sulphur compound content in wine, associated with yeast metabolism of nitrogen compounds in the media and their lysis, has been explored by different authors (Fedrizzi, Magno, Finato, & Versini, 2010; Moreira et al., 2002), showing a different evolution in the reductive and oxidative environment. We found that many of these sulphur compounds are higher in sparkling wines from Franciacorta, for example 3-ethoxy thiophene (2.7), ethyl 2-methylthioacetate (1.5), thieno[2,3-b] thiophene (3.6), 2-methylthiophene (1.9), 2-thiophenecarboxaldehyde (1.5) and tetrahydrothiophen-3-one (1.6). This is probably due to a slightly longer time on the yeast lees in the case of Franciacorta samples chosen for this study, the average time for our samples being 3.1 years for 48 Trentodoc wines and 4.0 years for 23 Franciacorta wines.

The content of 3-methylthiopropanol and 3-(methylthio)propyl acetate, which is produced by yeast from methionine via deamination, followed by decarboxylation (Ehrlich reaction), is similar in the two groups. In this case the compounds did not seem to be influenced by the contact time with the yeast, but rather by other winemaking techniques; the parameters that most increased 3-methylthiopropanol content were must turbidity, bisulfite addition and fermentation temperature (Karagiannis & Lanaridis, 1999).

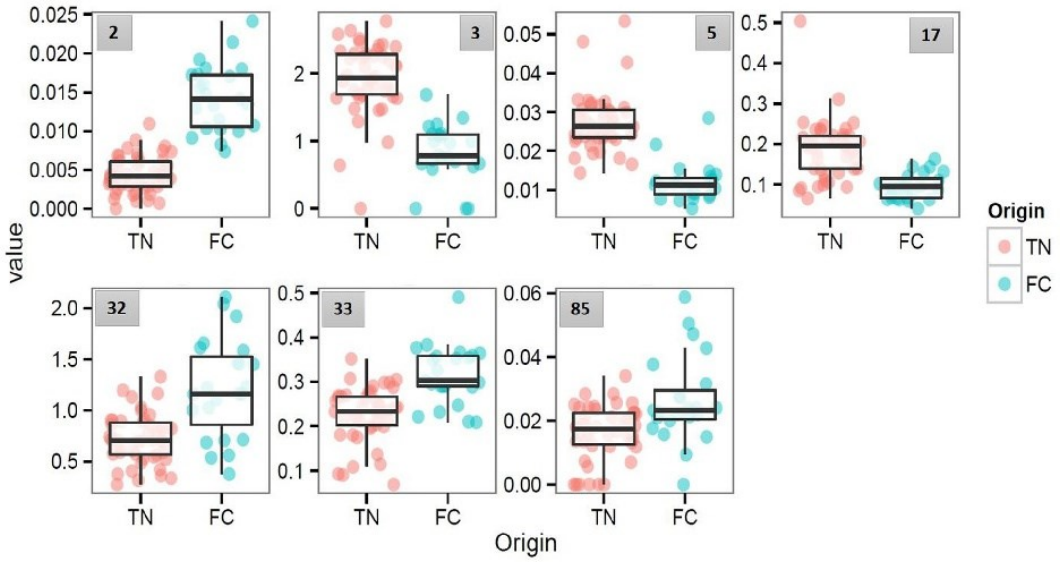

Fig. 2. Important compounds belonging to different chemical classes, numbers corresponding to peak n° of Table 1: 2 = gama eudesmol, 3 = 1-hexanol, 5 = 2,6 dimethyl 1,7 octadien 3-ol, 17 = riesling acetal, 32 = actinidiol ethoxy 2, 33 = diethyl malonate, 85 = ethyl 2-(methylthio)-acetate; value = area ratio.

Camara and Ferreira (Câmara, Marques, Alves, & Ferreira, 2003; da Silva Ferreira, Barbe, & Bertrand, 2002) observed that maturing of some wines such as Madeira and Port involves exposure to relatively high temperatures and oxygen, which affects the aroma and flavour composition, leading to the formation of the typical and characteristic bouquet of these wines. In the oxidative reactions occurring during the wine ageing process, the condensation reaction between glycerol and acetaldehyde in acid conditions (at wine pH) leads to the formation of heterocyclic acetals (1,3 dioxane – 1,3 dioxalane), while ethane 1,1-diethoxy is produced by the reaction between acetaldehyde and ethanol (Torrens, Riu-Aumatell, Vichi, Lopez-Tamames, & Buxaderas, 2010). In these samples we found some of this structure to be consistently higher in Franciacorta wines, such as ethane 1,1-diethoxy (2.0), 4-hydroxymethyl-2-methyl-1,3-dioxolane (2.3), 1,3 dioxane, 2-methyl (2.9) and 1,3-dioxolane 2,4,5-trimethyl (2.9). Since acetaldehyde can come from the yeast and varies depending on the strain, it is reasonable to surmise that in this case too, the longer time passed on the yeast by the Franciacorta samples may be the reason for the higher content of these compounds, in some cases possibly associated with exposure to slightly higher temperatures during storage. This was in agreement with the maximal concentration in these compounds found in the oldest reserve (vintage 2004).

The situation as regards the norisoprenoid content in the two datasets is not so clear. Although the majority of these compounds seem to have a slightly higher content in Franciacorta sparkling wines, some of these compounds, such as TDN and β-damascenone, had the same content and riesling acetale (2.0) and vitispiranes (2.2) were higher in Trentodoc wines. Data related to traditional approaches showed that vitispiranes were on average at 28.5 $\mu g\,L^{-1}$ in Trentodoc and 20.6 $\mu g\,L^{-1}$ in Franciacorta, β-damascenone 5.5 $\mu g\,L^{-1}$ in Trentodoc and 6.3 $\mu g\,L^{-1}$ in Franciacorta, TDN 10.2 $\mu g\,L^{-1}$ in Trentodoc and 11.2 $\mu g\,L^{-1}$ in Franciacorta and the sum of the two actinidol isomers was 4.2 $\mu g\,L^{-1}$ in Trentodoc and 5.8 $\mu g\,L^{-1}$ in Franciacorta.

Norisoprenoids can be formed by direct degradation of carotenoids such as β-carotene and neoxanthin, or they can be stored as glycoconjugates, which can then release their volatile aglycones during fermentation, via enzymatic and acid hydrolysis processes (Winterhalter & Schreier, 1994).

The norisoprenoids with important sensory properties that we identified in these sparkling wine were: TCH (2,2,6-trimethylcyclohexanone), β-damascenone, dihydro α-ionone, α-ionene (naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-), vitispiranes, actinidols, TDN (1,1,6-trimethyl-1,2-dihydronaphthalene), riesling acetal and some ethoxy derivates such as actinidols ethyl ether and vitispirane ethyl ether. Many other structures very similar to C13 norisoprenoids remained only tentatively identified. We observed that aged sparkling wines were particularly rich in several C13 norisoprenoids.

As reported by Marais, van Wyk, and Rapp (1992), the levels of some norisoprenoids such as TDN were significantly higher in grapes exposed to sunlight than in shaded grapes. However, the temperature effect on grape composition is complex and should not be dissociated from the different degree of sunlight exposure. It seems that light promotes the increase of carotenoids in unripe grapes, i.e. before veraison, as compared to shaded grapes. At maturity, grapes exposed to sunlight show a significant decrease in carotenoids as compared to grapes in shady conditions. Knowing that different light intensities can be associated with a higher or lower degree of shading of grape bunches by the canopy, it is reasonable to speculate about a potential effect of pruning techniques and leaf removal on carotenoid levels.

Two compounds that we found here for the first time in wine samples also belong to the class of norisoprenoids, but not C13: safranal with 10 carbon atoms and α-isophorone with 9 carbon atoms. Both are important constituents of the saffron aroma and their concentration was on average higher in the aged wines. β-isophorone, which we did not find here, was reported recently by Panighel et al., 2014) in some Italian grape varieties. α-isophorone is described as having a peppermint-like odour and was found in aged Riesling wine by Simpson & Miller in 1983. Safranal, the most powerful active aroma compound in saffron spice (Amanpour, Sonmezdag, Kelebek, & Selli, 2015), was reported by Rocha, Coelho, Zrostlíková, Delgadillo, and Coimbra (2007) in *Vitis vinifera* L. cv. 'Fernao-Pires' white grapes. Quantification of these

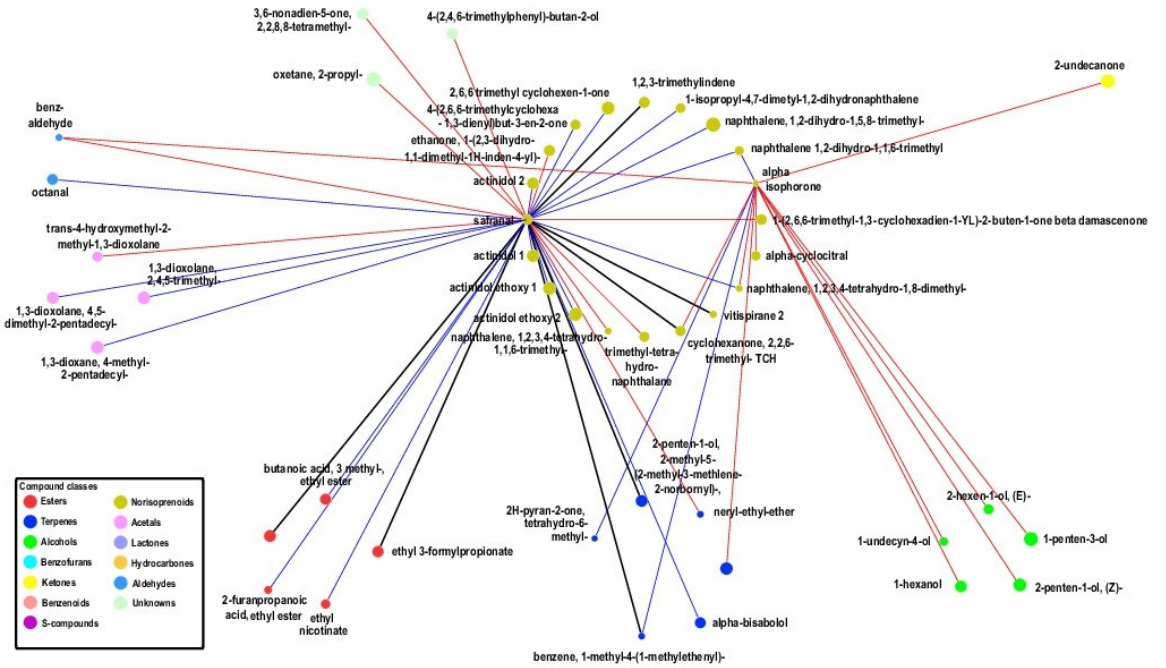

Fig. 3. Network analysis, symmetric difference plus intersect network of α-isophorone and safranal, compared in young and aged Trentodoc sparkling wines. Red edges correspond to the aged wine network, blue to the young wine network and the black line to intersections between them. Network visualization of metabolites is presented as nodes and their relations as links. The metabolites are colour-coded and clustered according to their chemical class. Pearson correlation was employed to compute all pairwise correlations between metabolites. Solely significant correlations were chosen for display. A significance level of $q < 0.05$ and an r-value of >0.4 were considered to be significant. The sizes of the nodes represent the relative degree of connectivity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

two compounds suggested that safranal was in a range between 0.12–0.6 μg L^{-1} and was similar in the two groups of wines, while α-isophorone was slightly higher in samples of Trentodoc (0.30 μg L^{-1} mean) than in Franciacorta (0.22 μg L^{-1}). Nevertheless, their olfactory impact in wine has still to be demonstrated.

3.1. Network analysis

In an attempt to understand common metabolic shifts in the young and aged wine datasets, we used correlation-based network analysis (CNA). The relationships for α-isophorone and safranal were scrutinised in particular.

In the generated networks (Fig. 3) each node represents a metabolite, and links joining two nodes represent the significant association between two metabolites - in our case a correlation (either positive = light blue link, or negative = light red). In order to be considered significant and appear as a link in the network, a correlation had to pass two threshold tests, i.e. a correlation coefficient greater than 0.4 in the young wine network and 0.5 in the aged wine network, and a significance level of q smaller than 0.05 in both networks. Nodes in the networks are colour-coded according to their compound classes. Furthermore, nodes were grouped into communities by applying the walktrap community algorithm (Pons & Latapy, 2005), identifying densely connected subgraphs based on short random walks. The degree of connectivity of nodes in the network is illustrated by the relative node sizes. To highlight the differences between the young and the aged wine networks, we generated difference, intersect and symmetric difference networks (set theory), combined them and analysed the topology of the networks, quantifying the different network properties, which are summarised in Table S3.

Network differences: young vs. aged wines

Based on the aforementioned threshold values for link detection, 899 significant connections were detected in the young wine network, while 1914 significant connections were recorded in the aged wine network (Table S3). Of all these connections, 472 were common to both networks (aged young intersect network), making 427 connections unique in the young wine network (young difference network) and 1442 in the aged wines network (aged difference network). The distribution of positive vs. negative correlations within the two networks was relatively similar. The symmetric difference network incorporates the connections unique for the networks analysed, which in our case amount to 1869 connections (427 + 1442).

We specifically analysed the network for links present on the nodes representing α- isophorone and safranal as interesting norisoprenoids, observed here in sparkling wines for the first time. Both compounds are reported to derive from complex degradation pathways of zeaxanthin (Sanchez & Winterhalter, 2013) and β-carotene, two of the main carotenoids in berries (Wehrens, Carvalho, Masuero, de Juan, & Martens, 2013; Winterhalter and Straubinger, 2000). This conversion produces carotenoid metabolites which could be progressively released during wine ageing and act as aroma precursors in wine. The connections of these nodes are highlighted in the network with dark blue (positive correlations) and dark red (negative correlations) and increased link width. In the aged wine network α-isophorone accounts for 11 and safranal for 17 connections, while in the young wine network the corresponding numbers are 3 and 25. To further emphasise the differences in these compounds within the network, we calculated the percentage of these connections in relation to the total number of connections in the respective networks. While in the aged wine

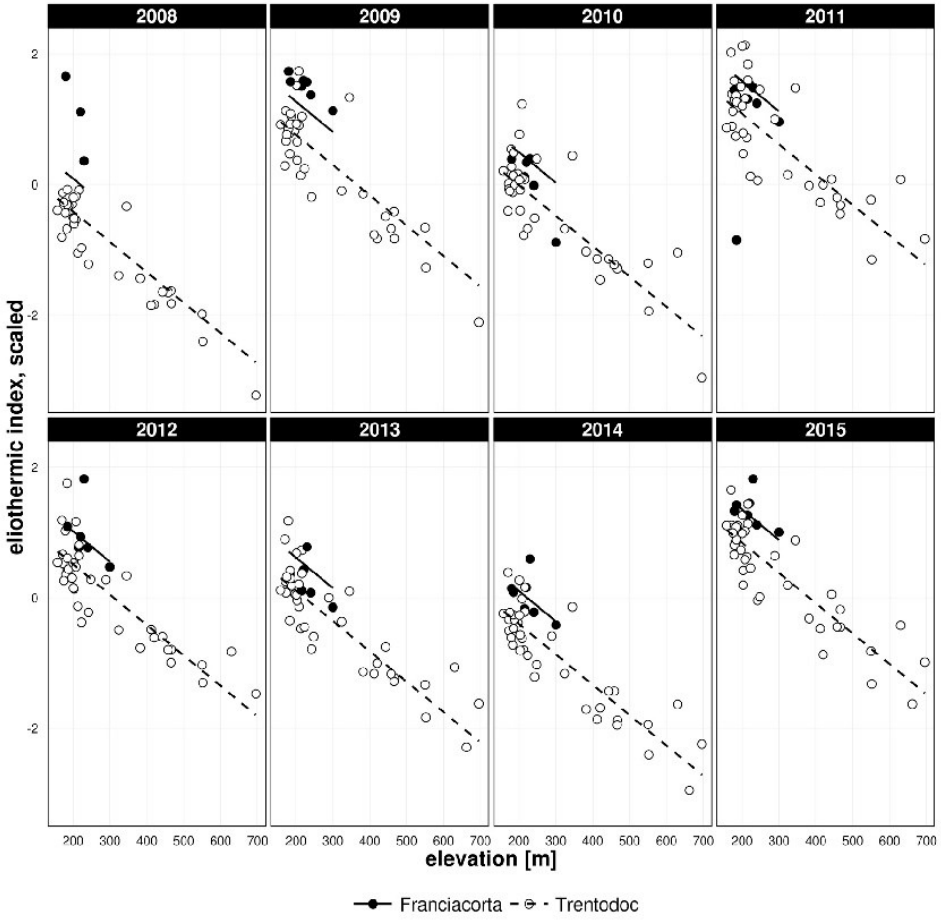

Fig. 4. Proposed model to describe the climatic differences between the Franciacorta and Trentodoc production areas. Huglin Index values have been rescaled and centred. Elevation above sea level and the production area are considered as fixed effects, while the year (from 2008 to 2015) is considered as a latent random noise. The lines show the prediction of the Bayesian hierarchical model (with random intercepts).

network α-isophorone made up 0.57% of all connections present, in the young wine network it made up only 0.33% of all connections. A more drastic change could be detected for safranal, accounting for 1.04% of all connections in the aged wine network, whereas it was 2.89% in the young wine network (Table S3). In the young wine network safranal connected to 13 other norisoprenoids, 5 esters, 3 acetals, 3 terpenes, and one aldehyde, while α-isophorone connected to 2 terpenes and one norisoprenoid. In the aged wine network, safranal connected to 7 other norisoprenoids, 3 terpenes, 2 esters, one aldehyde, one acetal, and 3 unknowns, while α-isophorone connected to 5 alcohols, 2 terpenes, 2 norisoprenoids, one ketone and one aldehyde. As shown in Fig. S5, several uncommon C9 and C10 carotenoid cleavage products were present in the network of young or aged wines. Some of them had a strong positive correlation with either safranal or isophorone, thus suggesting the presence of two groups of compounds with a biochemical origin similar to safranal or α-isophorone respectively. We should recall here that the correlations display the coordinated behaviour of the compounds throughout different conditions, in this case the differences between their respective backgrounds in young and aged Trentodoc sparkling wines (Fig. 3 and Figs. S3–S4). In the case of young sparkling wines, network analysis (Fig. S3, top), where the metabolites are colour-coded and clustered according to the chemical class, highlighted a strong positive correlation of safranal with the other C10 norisoprenoid α-cyclocitral and with the C9 norisoprenoid TCH, as well as with several C13-norisoprenoids. In particular, a large group of actinidiol derivatives were positively correlated to safranal, which also showed weak positive correlation with vitispirane 2 and negative correlation with 1,2,3,4-tetrahydro-1,8-dimethyl-naftalene. On the other hand, α-isophorone was instead positively and weakly correlated only to 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) within the class of C13 norisoprenoids. These correlations, even taking into consideration the fact that the connections shown are non-directed (so there is no cause and effect), clearly suggest a distinct biochemical origin of safranal and α-isophorone from the breakdown of carotenoids, but from different paths.

The network of aged sparkling wines was quite different, probably due to the importance of the chemical reactions occurring during ageing (Fig. S4). In this case several of the correlations observed in the network of young wines were lost, with the exception of those highlighted with black nodes in Fig. 3. The networks appearing in aged wines were better explored by clustering the metabolites according to the walktrap community algorithm. As shown in Fig. S4, at the bottom, a sub-network of compounds positively correlated to α-isophorone emerged, including one C10

norisoprenoid (α-ciclocitral), one monoterpenol and several alcohols formed from the degradation of unsaturated fatty acids. Their similarity over time during the course of wine ageing is probably highlighted here, not linked to common pathways or causality.

The symmetric difference plus intersect network of α-isophorone and safranal (Fig. 3) clearly summarises the different coordinated behaviour of these metabolites in young vs. aged Trentodoc sparkling wines (summarised in Table S3).

The example of network analysis directed at safranal and α-isophorone is suggested here as a useful tool for further exploring the rich dataset produced in untargeted GCxGC-MS experiments.

4. Conclusions

As we analysed large, representative samples of sparkling wines from six different vintages and 48 wineries, the behaviour described in our survey provides very general results on how the volatile compound profile differs in the two different Trentodoc and Franciacorta denominations, and how it evolves with wine ageing in Trentodoc. There is a relatively strong likelihood that these general observations will also be true for specific wines. This is exactly the reason why we preferred deductive reasoning in this experiment, following 70 different wines in search of general rules to be further investigated later. The GCxGC technique was successfully applied to the study of sparkling wines, allowing detailed investigation of the complex volatile flavour profile, making it possible to clearly separate the two types of sparkling wines. Improved separation, together with the use of deconvoluted mass spectra obtained using TOF-MS, made it possible to observe many new compounds that were probably co-eluting in 1 D. Many of the common wine flavour compounds were confirmed with the traditional technique, while a large number of newly identified or tentatively identified potential markers helping to place products in the production area were observed. Some varietal (terpenes) and pre-fermentative compounds (C5-C6) were significantly different in the two types of products, due to different micro-climates and levels of grape ripeness which resulted in higher values in Trentodoc wines, and analysis of the meteorological time series in the two production areas also supported this consideration. In the context of the considerable complexity of compounds deriving from first and second fermentation characterising these fine wines, it was possible to highlight the higher content of some ethyl esters of diprotic acids, sulphur compounds and cyclic acetal in samples of Franciacorta sparkling wine. CNA showed how it can be used as a tool to detect differences in compound behaviour based on external/environmental influences, particularly for safranal and α-isophorone.

Acknowledgements

This research was funded by the AGER, Agroalimentare e Ricerca, cooperative project between grant-making foundations, under the section "wine growing and producing", "New analytical methodologies for varietal and geographical traceability of oenological products" Project, contract no. 2011-0285. We thank Georg Weingart for his helpful suggestions in relation to the GCxGC equipment, and Andrea Angeli for sample preparation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.03.112.

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Supplementary Figure S1: Colour plot of 122 standards. For the peak names see Table S1

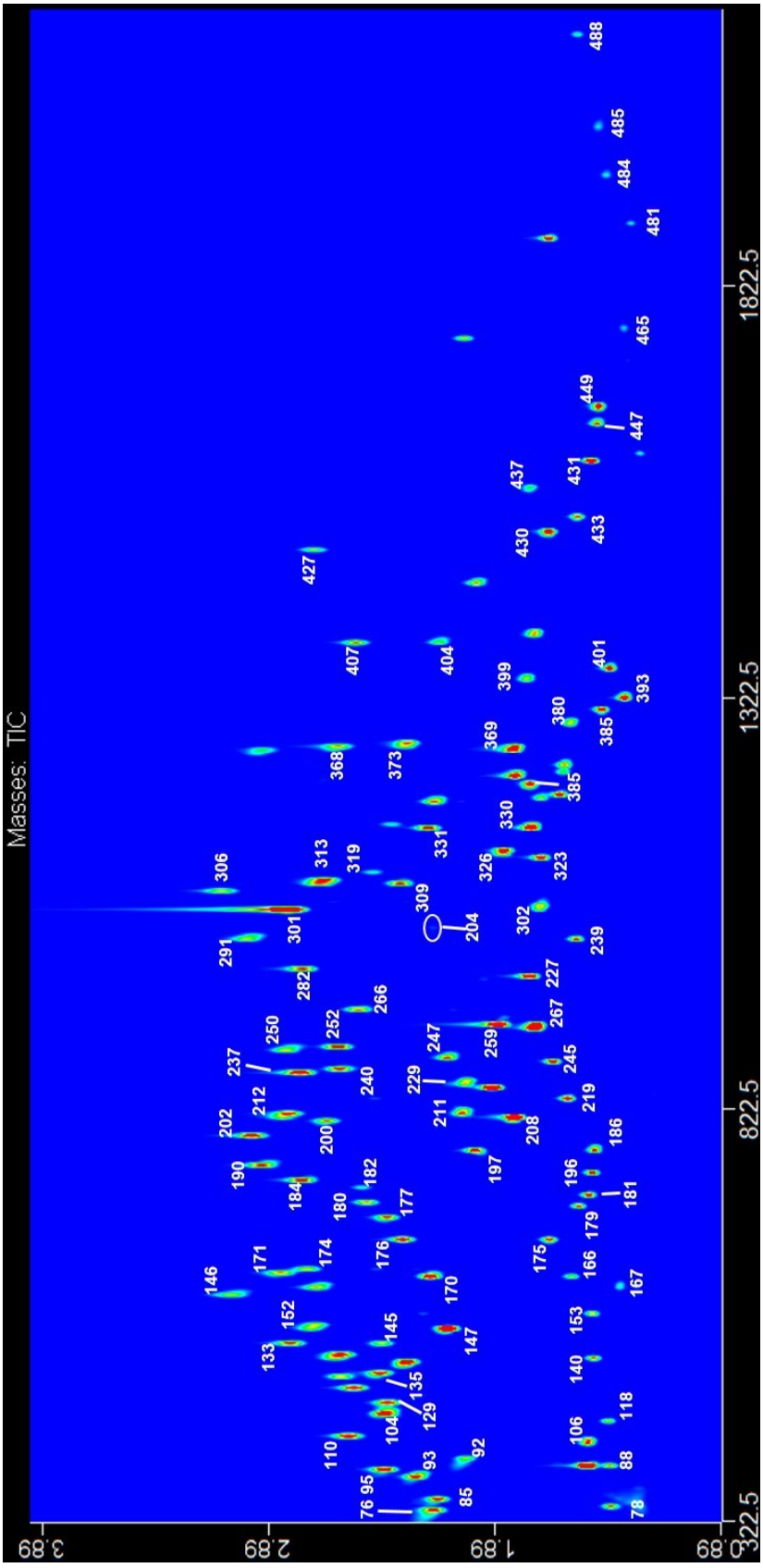


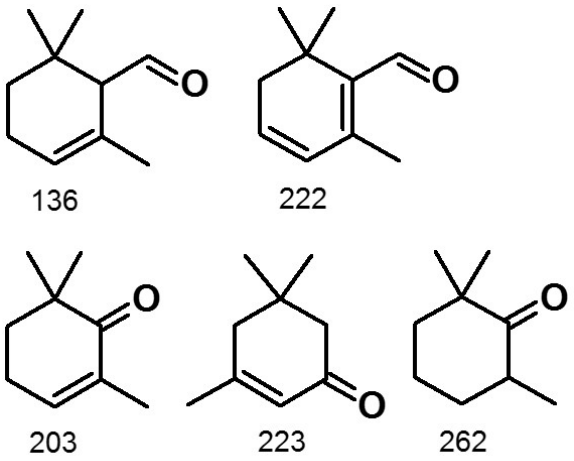
Table S1: List of 122 standard compounds identified with ChromaTof software using the automatic processing method; wrong identification is shown in italics

LTRI exp	Company	Peak Number	Compound	1st D (sec)	2nd D (s)	CAS	Similarity	UniqueMass
1065	Fluka	76	acetic acid, 2-methylpropyl ester	336	2.16	110-19-0	922	43
1070	Fluka	78	2-butanol	340	1.38	78-92-2	953	59
1072	Aldrich	85	butanoic acid, ethyl ester	349	2.15	105-54-4	943	71
1117	SAFC	92	2-butenal, 2-methyl-	372	2.12	1115-11-3	964	51
1106	Fluka	93	acetic acid, butyl ester	376	2.25	123-86-4	943	56
1110	Fluka	95	2-hexanone	385	2.39	591-78-6	941	58
1114	Fluka	97	1-propanol, 2-methyl-	390	1.39	78-83-1	950	74
1114	Aldrich	98	(R)-(-)-3-methyl-2-butanol	390	1.49	1572-93-6	966	73
1117	SAFC	101	2-butenal, 2-methyl-, (E)-	399	2.02	497-03-0	961	84
1121	Fluka	103	3-pentanol	408	1.48	584-02-1	853	59
1121	Fluka	104	α-pinene	408	2.49	127-91-3	914	93
1125	Fluka	106	(S)-(+)-2-pentanol	417	1.49	26184-62-3	979	45
1148	Fluka	110	***2-methylbutyl acetate (correct name 3-methylbutyl acetate)***	426	2.54	624-41-9	960	70
1156	Supelco	118	1-butanol	444	1.40	71-36-3	956	56
1160	Fluka	121	***α-pinene （correct name β-pinene)***	453	2.39	80-56-8	912	93
1167	SAFC	124	***2-pentenal, 2-methyl- (correct name 2-methylpropyl butanoate)***	466	2.37	623-36-9	955	98
1171	Aldrich	126	1,6-octadiene, 7-methyl-3-methylene-	475	2.22	123-35-3	899	69
1154	Fluka	129	acetic acid, pentyl ester	484	2.52	628-63-7	939	61
1200	SAFC	133	heptanal	498	2.58	111-71-7	945	70
1198	Fluka	134	***2-butanone (correct name 2-heptanone)***	502	2.07	78-93-3	917	72
1202	Fluka	135	hexanoic acid, methyl ester	502	2.40	106-70-7	955	74
1210	SAFC	140	1-butanol, 2-methyl-	520	1.46	137-32-6	954	56
1212	Fluka	142	1,8-cineole	525	2.58	470-82-6	951	111
1218	Aldrich	145	2-hexenal	538	2.40	6728-26-3	941	83
1218	Fluka	146	butanoic acid, butyl ester	538	2.80	109-21-7	962	71
1245	SAFC	147	furan, 2-pentyl-	556	2.11	3777-69-3	959	81
1247	Aldrich	152	hexanoic acid, ethyl ester	561	2.69	123-66-0	961	88
1253	Fluka	153	1-pentanol	574	1.47	71-41-0	964	55
1257	SAFC	159	***1,3,6-octatriene, 3,7-dimethyl-, (E)- (correct name α terpinolene)***	583	2.21	3779-61-1	909	93
1286	SAFC	167	acetic acid, hexyl ester	606	2.69	142-92-7	950	61
1292	SAFC	168	2-butanone, 3-hydroxy-	619	1.56	513-86-0	968	45
1292	SAFC	170	cyclohexene, 1-methyl-4-(1-methylethylidene)-	619	2.18	586-62-9	933	136
1294	Fluka	171	2-octanone	624	2.84	111-13-7	968	58
1296	Aldrich	174	octanal	628	2.73	124-13-0	959	84
1332	Fluka	175	2-heptanol	664	1.66	543-49-7	958	45
1332	SAFC	176	3-hexen-1-ol, acetate, (Z)-	664	2.30	3681-71-8	960	67
1345	Aldrich	177	5-hepten-2-one, 6-methyl-	691	2.37	110-93-0	924	108
1347	SAFC	178	propanoic acid, 2-hydroxy-, ethyl ester	696	1.58	97-64-3	979	45
1347	Supelco	179	1-hexanol	705	1.53	111-27-3	973	56
1353	Fluka	180	*cis* -rose oxide	709	2.46	876-17-5	941	139
1357	SAFC	181	3-hexen-1-ol, (E)-	718	1.48	544-12-7	953	67
1381	Fluka	182	***cis rose oxide (correct name trans rose oxide)***	727	2.48	876-17-5	917	139
1385	SAFC	184	acetic acid, heptyl ester	736	2.75	112-06-1	943	61
1389	Aldrich	186	3-hexen-1-ol, (Z)-	745	1.47	928-96-1	947	67
1393	Fluka	190	2-nonanone	754	2.93	821-55-6	966	58
1402	SAFC	196	2-hexen-1-ol, (E)-	772	1.46	928-95-0	948	57
1402	SAFC	197	2,4-hexadienal, (E,E)-	772	1.98	142-83-6	953	81
1402	SAFC	200	2-octenal, (E)-	772	2.58	2548-87-0	792	83
1424	SAFC	202	butanoic acid, hexyl ester	790	2.97	2639-63-6	925	71
1433	Aldrich	208	pyrazine, 2-methoxy-3-(1-methylethyl)-	813	1.81	25773-40-4	936	152
1435	Aldrich	211	*trans* -linalool oxide (furanoid)	817	2.04	5989-33-3	931	59
1435	Aldrich	212	octanoic acid, ethyl ester	817	2.81	106-32-1	937	88
1442	Fluka	219	1-heptanol	835	1.58	111-70-6	961	70
1460	Fluka	221	acetic acid	840	1.19	64-19-7	952	60
1465	Aldrich	227	2-furancarboxaldehyde	853	1.56	98-01-1	887	95
1465	Aldrich	229	*cis* -Linalool oxide (furanoid)	853	2.03	5989-33-3	921	59
1470	Aldrich	237	***1-hexanol, 2-ethyl-, acetate (correct name octyl acetate)***	867	2.76	103-09-3	950	61
1472	SAFC	239	2-octen-1-ol (E)	871	2.17	18409-17-1	863	53
1472	SAFC	240	citronellal	871	2.58	106-23-0	927	69
1475	Fluka	245	1-hexanol, 2-ethyl-	880	1.64	104-76-7	939	57
1475	SAFC	246	1,3,6-octatriene, 3,7-dimethyl-, (E)-	880	2.26	13877-91-3	810	79

continue

LTRI exp	Company	Peak Number	Compound	1st D (sec)	2nd D (s)	CAS	Similarity	UniqueMass
1477	SAFC	247	2,4-heptadienal, (E,E)-	885	2.11	4313-03-5	958	81
1481	Sigma-Aldr	250	decanal	894	2.82	112-31-2	966	57
1482	Aldrich	251	pyrazine, 2-methoxy-3-(1-methylpropyl)-	898	1.86	24168-70-5	895	124
1472	Aldrich	252	theaspirane A	898	2.59	36431-72-8	900	138
1512	Fluka	257	benzaldehyde	921	1.72	100-52-7	972	106
1514	SAFC	259	pyrazine, 2-methoxy-3-(2-methylpropyl)-	925	1.88	24683-00-9	945	124
1526	Aldrich	266	theaspirane B	943	2.50	36431-72-8	888	138
1532	Fluka	269	3,7-dimethyl-1,6-octadien-3-ol	952	1.67	0-00-0	863	71
1580	Aldrich	279	2-furancarboxaldehyde, 5-methyl-	984	1.74	620-02-0	963	110
1586	SAFC	282	acetic acid, nonyl ester	993	2.75	143-13-5	908	61
1591	Supelco	284	alpha isophorone	1002	2.67	78-59-1	915	82
1627	SAFC	289	2-octen-1-ol (Z)	1029	1.54	22104-78-5	925	57
1627	SAFC	291	hexanoic acid, hexyl ester	1029	3.00	6378-65-0	941	99
1640	Fluka	299	butanoic acid	1056	1.23	107-92-6	932	60
1645	Aldrich	301	decanoic acid, ethyl ester	1065	2.82	110-38-3	945	155
1647	Aldrich	302	benzeneacetaldehyde	1069	1.70	122-78-1	952	91
1645	SAFC	304	safranal	1065	2.18	116-26-7	860	107
1649	Sigma	305	β-caryophyllene	1074	2.61	87-44-5	877	93
1675	SAFC	306	octanoic acid, 3-methylbutyl ester	1087	3.10	2035-99-6	934	70
1680	Fluka	309	(E)-α-famesene	1096	2.32	18794-84-8	933	69
1682	Aldrich	313	***trans caryophillene (α-humulene)***	1101	2.65	6753-98-6	944	93
1684	Fluka	315	2-methylbutanoic acid	1105	1.25	116-53-0	878	74
1684	Aldrich	316	butanedioic acid, diethyl ester	1105	2.05	123-25-1	883	101
1687	Aldrich	319	2,6-octadienal, 3,7-dimethyl-	1110	2.43	106-26-3	940	69
1696	Fluka	323	α-terpineol	1128	1.69	98-55-5	940	136
1700	SAFC	326	phenylethyl acetate	1137	1.86	93-92-5	925	105
1736	Aldrich	330	acetic acid, phenylmethyl ester	1164	1.74	140-11-4	962	108
1736	Fluka	331	3,7-dimethyl-2,6-octadienyl acetate	1164	2.19	141-12-8	944	69
1783	SAFC	346	2,6-octadien-1-ol, 3,7-dimethyl-,	1195	2.16	105-87-3	944	69
1776	Aldrich	348	1-decanol	1200	1.69	112-30-1	954	83
1784	SAFC	352	β-citronellol	1204	1.61	106-22-9	968	95
1786	Fluka	356	methyl salicylate	1218	1.74	119-36-8	966	120
1790	Aldrich	359	benzeneacetic acid, ethyl ester	1227	1.81	101-97-3	946	164
1797	Aldrich	364	***2,6-octadien-1-ol, 3,7-dimethyl-, (E)- geraniol (correct name nerol)***	1240	1.59	106-24-1	922	69
1805	Bedoukian r	368	2-butanone, 4-(2,2,6-trimethylcyclohexyl)-, cis-	1254	2.85	60761-23-1	850	123
1807	SAFC	369	acetic acid, 2-phenylethyl ester	1258	1.82	103-45-7	936	104
1831	SAFC	373	β-damascenone	1267	2.28	23726-93-4	946	69
1843	SAFC	380	***2,6-octadien-1-ol, 3,7-dimethyl-, (Z)- nerol (correct name geraniol)***	1290	1.57	106-24-1	944	69
1848	Fluka	385	hexanoic acid	1299	1.27	142-62-1	942	60
1879	Sigma-Aldr	393	benzenemethanol	1321	1.33	100-51-6	960	108
1891	Fluka	399	3-buten-2-ol, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-	1344	1.76	25312-34-9	859	95
1897	Aldrich	401	benzeneethanol	1357	1.39	60-12-8	966	122
1907	SAFC	404	2-cyclopenten-1-one, 3-methyl-2-(2-pentenyl)-, (Z)-	1375	2.16	488-10-8	927	122
1939	Aldrich	407	3-buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, (E)-	1389	2.51	79-77-6	966	177
2017	Aldrich	423	phenol, 4-ethyl-2-methoxy-	1479	1.51	2785-89-9	967	137
2061	Aldrich	427	***ethyl pentadecanoate (correct name ethyl myristate)***	1501	2.70	41114-00-5	939	88
2071	Fluka	428	octanoic acid	1515	1.31	124-07-2	916	60
2078	Aldrich	430	2-methylaminobenzoic acid methyl ester	1524	1.66	85-91-6	964	165
2091	Fluka	433	1,8-terpin	1542	1.53	565-50-4	918	81
2137	Aldrich	437	2-propenoic acid, 3-phenyl-, ethyl ester	1578	1.74	103-36-6	965	131
2100	SAFC	440	phenol, 2-methoxy-4-(2-propenyl)-	1609	1.47	97-53-0	971	164
2158	Fluka	441	nonanoic acid	1614	1.32	0-00-0	899	60
2207	SAFC	447	acetophenone, 2'-amino-	1654	1.45	551-93-9	964	120
2225	Fluka	449	methyl anthranilate	1677	1.44	134-20-3	956	119
2283	Fluka	455	decanoic acid	1713	1.34	334-48-5	908	60
2345	SAFC	465	geranic acid	1771	1.33	459-80-3	888	69
2423	Fluka	473	benzoic acid	1852	1.21	1863-63-4	940	122
2615	SAFC	481	benzaldehyde, 4-hydroxy-3-methoxy-	1956	1.41	121-33-5	963	151
2557	Carlo Erba	484	2-furancarboxaldehyde, 5-(hydroxymethyl)-	2014	1.25	67-47-0	759	97
2623	SAFC	485	ethanone, 1-(4-hydroxy-3-methoxyphenyl)-	2014	1.44	498-02-2	958	151
2796	SAFC	488	2-butanone, 4-(4-hydroxy-3-methoxyphenyl)-	2127	1.53	122-48-5	926	137

Figure S5. Structures of C10 norisprenoids (136) α-cyclocitral and (222) safranal and C9 norisoprenoids, (203) 2,6,6 -trimethyl-2-cyclohexen-1-one; (223) α-isophorone and (262) cyclohexanone, 2,2,6-trimethyl- TCH, all found in sparkling wines, numbers of compounds corresponding to the peak numbers in Table 1



Chapter 2: Harvesting decision

Harvesting decisions for Shiraz wines.
It is possible to vary wine aroma?

This chapter has been reprinted* from:

Unravelling wine volatile evolution during Shiraz grape ripening by untargeted HS-SPME-GCXGC-TOFMS Šuklje K.[#], Carlin S.[#], Stanstrup J., Antalick G., Blackman J. W., Meeks C., Deloire A., Schmidtke L. M., Vrhovsek U. Food Chemistry, 2019, 277, 753-765

[#] *Equal contribution*

Preface to Chapter 2

2) Harvesting decisions

Deciding when to harvest grapes is probably one of the most important and challenging viticultural decisions for grape producers, due to the difficulty of assessing grape maturity in the vineyard and predicting wine quality. The yearly dilemma is whether to delay the harvest until desired quality parameters are reached, since once they have been picked grapes do not improve in terms of flavour, colour or sugar content. On the other hand, if the grapes are left hanging too long on the vine, the berries may shatter, get damaged by insects, or break down due to rot, with yields and quality negatively affected. The ripening of grapes involves many processes, including translocation, accumulation and metabolism of principal components within the berry. Many changes take place in grapes: after veraison the sugar concentration increases rapidly, sucrose, the predominant sugar transported in the phloem, is then cleaved and stored as D(+)-glucose and D(-)-fructose (Coombe, 1992; Davies & Robinson, 1996), L-(+)-tartaric acid and L-(-)-malic acid reach their highest levels near veraison and then decline during the ripening period (Sweetman, Deluc, Cramer, Ford, & Soole, 2009). After veraison, amino acid synthesis in the berry leads to an increase in arginine and proline in particular, coupled to a decline in ammonium (Stines et al., 2000), the synthesis of phenols decreases and condensed tannins are accumulated in the skin and seeds (Downey, Harvey, & Robinson, 2003), flavanols and anthocyanins (in red cultivars), and leuco-anthocyanins (in white cultivars) are also accumulated in the skins (Boss, Davies, & Robinson, 1996; Dokoozlian & Kliewer, 1996); the concentration of aroma precursors and volatile compounds also undergoes major changes (Razungles, Baumes, Dufour, Sznaper, & Bayonove, 1998), (Grose, Martin, Stuart, Albright, & McLachlan, 2016) (Figure 1). Several studies have monitored the effects of maturation on levels of flavour compounds and their precursors, including carotenoids and norisoprenoids, monoterpenes and methoxypyrazines. These studies show that carotenoids accumulating before veraison degrade rapidly after veraison. This rapid degradation occurs for β-carotene, lutein, and violaxanthin, while neoxanthin levels decrease steadily (Razungles, Babic, Sapis, & Bayonove, 1996). The subsequent accumulation of norisoprenoids is inversely proportional to the degradation of these carotenoids and positively correlated with sugar accumulation (Strauss et al. 1987). This relationship also exists for monoterpenes and is consistent with up-regulation of numerous genes involved in the early stages of terpene synthesis during maturation (Martin, Chiang, Lund, & Bohlmann, 2012).

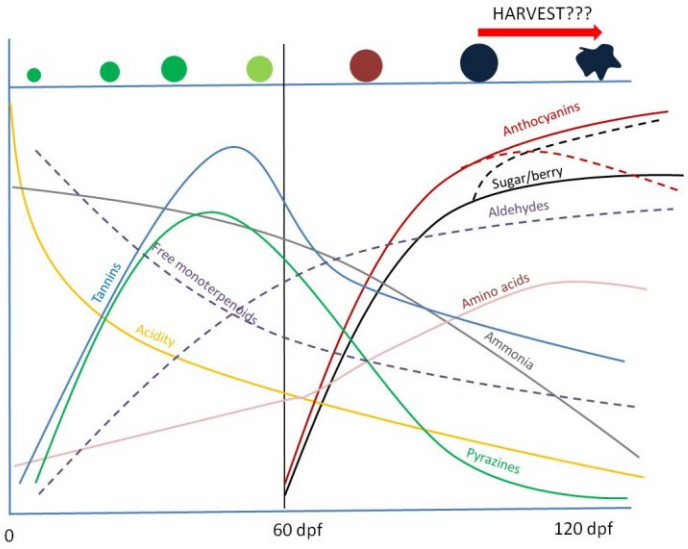


Figure 1: Evolution of metabolites during the vine green growth and ripening period**. (**Šuklje *et al*., 2016)

Temporal and spatial investigation of Shiraz wine volatile composition from four vineyards located in Riverina, Australia was performed. wines were from grapes harvested on two occasions, 10 to 12 days apart (harvest 1: H1 and harvest 2: H2 respectively), commencing 12 days after the berry sugar accumulation plateau. Chemical composition and sensory evaluation revealed a clear separation of wines based on the harvest date. Wines from grapes collected at H1 were perceived by a trained panel to be higher in red fruit notes, whereas wines from H2 were generally perceived as higher in dark fruit and plum characteristics. (Figure 2, Figure 3) These results indicate significant and coherent variations in wine volatiles in relation to grape harvesting date, irrespective of the vineyard site and management with a similar climate.

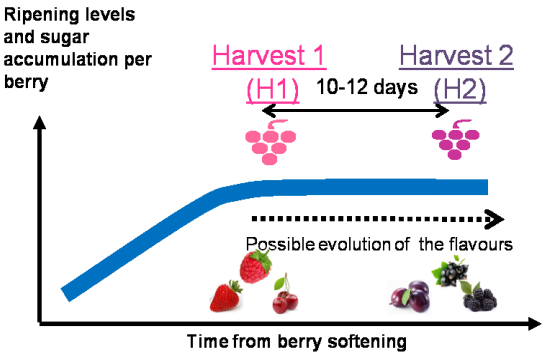


Figure 2 Sugar loading per berry as an indicator for predicting harvest dates for Shiraz, in relation to final wine style

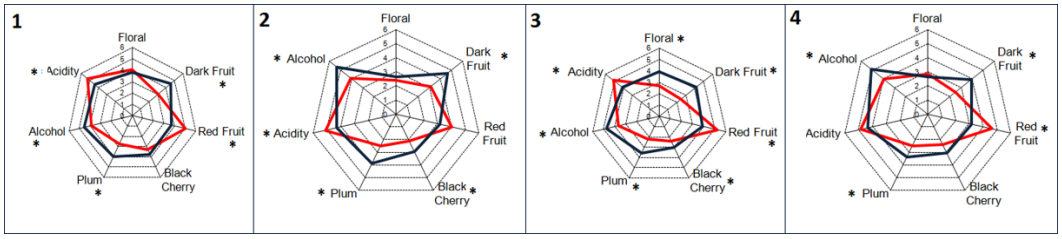


Figure 3 Descriptive wine sensory analysis. 1, 2, 3 and 4 refer to Vineyards 1, 2, 3 and 4 respectively. (**Red lines** for Harvest 1; **Black lines** for Harvest 2. Attributes significant at p≤0.05 are indicated with *.)

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Food Chemistry 277 (2019) 753–765

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Unravelling wine volatile evolution during Shiraz grape ripening by untargeted HS-SPME-GC × GC-TOFMS

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ARTICLE INFO

Keywords:
HS-SPME-GC × GC-TOFMS
Volatiles
Grape ripening
Maturity

ABSTRACT

The influence of grape maturity on wine volatome was investigated using HS-SPME-GC × GC-TOFMS. Shiraz wines were made from grapes harvested from four different vineyards from two berry maturity levels. A total of 1276 putative compounds were detected in at least one of the wine samples and 175 showed significant trends related to grape maturity. The first two dimensions of the Principal component analysis accounted for 57% of the variation and separated the samples according to the harvest date. Wines from the first harvest date were characterised by an abundance of lipoxygenase derived compounds, norisoprenoids and sulfur-containing compounds whereas a significant increase in some acetate esters was observed in wines produced from the more mature grapes.

This study demonstrated a common evolution of grape volatiles for Shiraz inside the same mesoclimate. During the late ripening stage of the grape, a direct nexus between sugar concentration and wine volatile evolution was not observed.

1. Introduction

Wine aromatic profile is one of the major determinants of wine quality. Hundreds of compounds, from a large number of different chemical classes, with concentrations ranging from ng/L to mg/L levels are present in wine. Even volatile compounds present in concentrations at below their perception threshold may contribute to the final wine aroma through synergistic effects with other compounds in wine (Pineau, Barbe, Van Leeuwen, & Dubourdieu, 2009). Traditional one-dimensional (1D) gas chromatography-mass spectrometry (GC–MS) has been widely used for targeted and untargeted analyses of several dozen (semi) volatile compounds. Employment of 1D GC–MS into the field of oenology has therefore brought important advances in understanding the complexity of both the grape matrix and wine. Additionally, gas chromatography coupled to olfactometric detection (GC-O) has assisted in identification of the distinct olfactory characteristic of individual wine volatiles. In 1D GC, volatiles elute along the retention time axis and often co-elute. Therefore the separation capacity has to greatly exceed the number of sample constituents (Mondello, Tranchida, Dugo, & Dugo, 2008). 1D GC-MS/MS has proven to be an extremely reliable and powerful tool for analyses and quantification of pre-selected targeted compounds. However, despite optimisation of chromatographic separation, selection of highly specific mass to charge ratios *(m/z)*, advancement of chromatogram deconvolutions techniques and multivariant data processing, the complexity of the grape/wine matrix results in a large number of compounds that are not able to be measured when a targeted approach is utilised. Two dimensional (2D) gas chromatography allows the identification of several hundred wine (semi) volatile compounds due to factors such as; better peak separation, higher peak capacity, sensitivity, selectivity and structural chromatographic peak organisation, and is therefore considered superior to conventional 1D GC–MS (Mondello et al., 2008). Due to the superior

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https://doi.org/10.1016/j.foodchem.2018.10.135
Received 1 March 2018; Received in revised form 20 September 2018; Accepted 29 October 2018
Available online 30 October 2018

separation power capability of GC × GC identification of previously unidentified wine compounds is possible (Carlin et al., 2016). Recently, GC × GC-TOFMS has been utilised to characterise volatile wine compounds from various treatments and regions (Carlin et al., 2016).

The timing of grape harvest is known to be crucial in the wine making process and a key determinant of wine style. Additionally, sequential harvests have been proposed as a tool to diversify wine styles (Bindon, Varela, Kennedy, Holt, & Herderich, 2013; Deloire, 2013). Cramer et al. (2014) reported changes in the transcript abundance of approximately 18,000 genes related to increased total soluble solids (TSS) content, with the majority of changes observed in the grape skins. Importantly, transcripts of several genes involved in isoprenoids and polypropanoids synthesis were significantly altered during grape maturation (Cramer et al., 2014). Other studies (Bindon et al., 2013; Pons, Lavigne, Eric, Darriet, & Dubourdieu, 2008) have emphasised marker compounds that are potentially linked to wine aromatic maturity. Pons et al. (2008) identified the contribution of γ-nonalactone to prune and jammy aromas in red wines. However, the aforementioned studies have focused on a few preselected compounds only.

This study aimed to quantitate changes in the wine volatome arising from sequential harvest dates in four vineyards within the same warm mesoclimate. Anecdotal observations had led the authors to speculate that a significant evolution of volatiles occurred during a late ripening stage of berry that was independent of sugar accumulation. Therefore, Shiraz wines were made using controlled triplicate fermentations from grapes harvested at two different berry maturity levels, based upon a sugar accumulation model. Comprehensive untargeted GC × GC-TOFMS analyses were conducted on the finished wines to reveal objective changes in wine volatome.

2. Materials and methods

2.1. Experimental vineyards

The investigation was carried out in four commercial Shiraz (*Vitis vinifera* L.) vineyards located in Griffith, Australia. The calculated Huglin index for the region in 2015 was 3140, inferring the region is classified as very warm. All vines, irrespective of vineyard, were own rooted, drip irrigated, mechanically pruned and trellised to open sprawling canopy. Clone, spacing and other basic vineyard characteristics are presented in Table 1. Inside each commercial vineyard, a smaller, 400 vine experimental plot across 8 rows was established. These sections were characterised by measuring mesoclimatic temperature, soil moisture profile and vine water status (data not shown). The average yield per vine and average number of bunches per vine were recorded on six vines at the first harvest date (H1), Table 1.

2.2. Harvest and wine making

Grapes were harvested sequentially on two occasions according to a well-established berry sugar evolution model (Deloire, 2013). Briefly, sugar accumulation per berry was monitored from veraison onwards. The first harvest date (H1) was predicted to be 12 days after the point of slowdown of sugar accumulation per berry, followed by the second harvest (H2), a further 12 days afterwards (Deloire, 2013). Harvest dates are presented in Table 1. At each harvest, 60 kg of grapes per replicate were randomly collected across the experimental site. Prior to transportation to the experimental winery grapes were sulfured with 40 mg/kg of sulfur dioxide (SO_2) in the form of dissolved potassium metabisulfite. At arrival to the winery, grapes were stored at +4 °C overnight. All biological replicates were kept separate during the grape processing. Grapes were destemmed, crushed and transferred to 100 L stainless steel tanks for fermentation. Acidity was adjusted with tartaric acid to pH 3.6. Grape must was inoculated with 300 mg/L *Saccharomyces cerevisiae* yeast EC118 (Lalvin) and fermentations were carried out at 25–26 °C. After the onset of fermentation, the yeast assimilable nitrogen (YAN) was adjusted to 220 mg/L for treatments that had an initial TSS level below 23.4° Brix using a combination of Fermaid K (Lallemand) and diammonium phosphate. Ferments that had a TSS in excess of 23.4° Brix were adjusted to 250 mg/L YAN. Malolactic fermentation was carried out by co-inoculation of Enoferm Alpha (*Oenococcus oeni*) (Lallemand) at a rate of 10 mg/L two days after the start of alcoholic fermentation. Wines were pressed off skins with a small hydraulic basket press up to a pressure of 1 bar when the residual sugar level had dropped below 0.5 g/L. Pressed wines were maintained at 22 °C until the completion of malolactic fermentation. Wines were then sulfured with 80 mg/L of SO_2 and pH was adjusted to 3.6. Wines were cold stabilised for 21 days at +0–2 °C and free SO_2 was re-adjusted to 30 mg/L prior to bottling in 0.75 L screw cap bottles.

Table 1
General vineyard parameters, yield per vine and harvest dates for the experimental sites.

	Vineyard 1	Vineyard 2	Vineyard 3	Vineyard 4
Plantation	1995	2008	1997	1997
Clone	Minato	BVRC12	SA1654	SA1654
Spacing (m)	2.5 × 3.7	2.5 × 3.7	2.5 × 3.7	2.5 × 3.7
Trellis system	Sprawling	Sprawling	Sprawling	Sprawling
Average Yield/vine (kg)	10.2 ± 2.2	18.5 ± 1.6	14.0 ± 1.8	17.7 ± 0.9
Plateau of sugar accumulation date	3.2.2015	5.2.2015	10.2.2015	10.2.2015
Days after plateau for H1	12	12	12	12
Days after plateau for H2	24	24	24	24

H1, Harvest 1, H2 harvest 2.

2.3. General analyses of grape maturity, yeast available nitrogen and basic wine parameters

Juice samples were collected after grape crushing for basic parameters of maturity. TSS expressed as °Brix was analyzed with a portable density meter (Anton Paar DMA 35N, Graz, Austria). Titratable acidity (TA) and pH were determined by sodium hydroxide titration to the end point pH 8.2 with an automatic titrator (Metrohm Fully Automated 59 Place Titrando System, Metrohm AG, Herisau, Switzerland). Ethanol was measured with an Anton Paar Alcolyser DMA 4500 density meter (Graz, Austria). Ammonia and α-amino acids (NOPA) were determined by commercially available enzymatic tests designed and developed for Arena discrete analyser (Thermo Fisher, Scoresby, Australia). YAN was calculated from ammonia and NOPA.

2.4. SPME-GC × GC-TOFMS

To a 20 mL headspace vial 1.5 g of sodium chloride was added, followed by 2.5 mL of wine spiked with 50 µL of 2-octanol and [$^2H_{11}$]-ethyl hexanoate at concentrations of 2 mg/L and 1 mg/L, respectively. Quality control samples (QC) consisted of equal proportion of each sample and were placed at the beginning of run (n = 5) and thereafter every 5th sample. GC × GC-TOFMS analysis of wines were performed using a GC Agilent 6890 N (Agilent Technologies Santa Clara, CA) coupled to a LECO Pegasus IV time-of-flight mass spectrometer (TOFMS) (Leco Corporation, St. Joseph, MI, USA) equipped with Gerstel MPS autosampler (GERSTEL GmbH & Co. KG), as described before with some modifications (Beckner Whitener et al., 2016; Carlin et al., 2016). Briefly, samples were incubated for 5 min at 35 °C and volatiles were extracted with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating 50/30 µm, and a 2-cm length SPME fibre (Supelco, Sigma Aldrich, Milan, Italy) for 20 min and desorbed for 3 min at 250 °C in splitless mode. The fibre was reconditioned between each sample for 7 min at 270 °C. Helium was used as a carrier gas at a flow rate of 1.2 mL/min. The oven was equipped with VF-WAXms 30 m × 0.25 mm, 0.25 µm film thickness (Agilent

Technologies) column in a first dimension (1D) and a Rxi 17 Sil MS 1.5 m × 0.15 mm, 0.15 µm film thickness (Restex Cooperation, Bellfonte, PA) column in the second dimension (2D). Oven temperature was held for 2 min at 40 °C and ramped up at the rate of 6 °C/min to 250 °C, held for 5 min, then returned to the initial conditions. The secondary oven temperature was held at 5 °C above the temperature of the primary oven throughout the chromatographic run. The modulator was offset by + 15 °C in relation to the secondary oven and modulation time was 7 s as described previously (Carlin et al., 2016). The ion source temperature was set at 230 °C and electron ionisation at 70 eV. Spectra were collected in a mass range of *m/z* 35–350 with an acquisition rate of 200 spectra/s and acquisition delay of 120 s.

2.5. GC × GC-TOFMS data alignment, processing and peak identification

ChromaTOF software version 4.32 was used to perform baseline correction, chromatogram deconvolution and peak alignment. The baseline offset was set to 0.8 and signal to noise (S/N) ratio was set at 100. A peak width of 42 s in the 1D was set and 0.1 s in 2D was established. Traditional, not adaptive integration was used. The required match (similarity) to combine peaks was set to 650. A library (NIST 2.0, Wiley 8 and FFNSC 2) search was conducted for molecular weights between 40 and 350 limited to report 5 library identifications. The mass threshold was set at 50 and the minimum similarity required to assign a compound name was set at 700. Under these conditions we were able to detect 1276 putative compounds. For identification of compounds with authentic standards, mix of 122 compounds was injected under identical GC × GC-TOFMS conditions as described previously (Carlin et al., 2016). Injected standards, calculated linear temperature retention index (LRI) and an unique mass is available in Carlin et al. 2016 (Supplementary material Fig. 1, Carlin et al., 2016). For the putative identification of compounds, a series of alkanes (C10-C30) was injected and the GC × GC-TOFMS was operated identically to the above described conditions. Retention indices were used to calculate the experimental LRI, which was compared to the literature (NIST 2.0 and Wiley 8 and FFNSC 2, VCF, Flavour net, ChemSpider). Mass spectra of the compounds that contributed significantly to the separation of samples according to the harvest date were compared to the mass spectra recorded in NIST 2.0 and Wiley 8 and FFNSC 2 mass spectral library (Chromaleont, Messina, Italy), with a library similarity match of 750. Based on the identification level following assignments were given; A comparing mass spectra and retention time with those of pure standard, B retention index match on a similar phase column, C mass spectral database. A typical two dimensional chromatogram is presented in Supplementary material Fig. 1.

2.6. Statistical analyses

Reported compounds to contribute significantly to sample separation in this study according to the harvest date were selected if the q-values for any of comparisons between any of the wines were below 0.05. Compounds identified as typical column bleed compounds were removed. In addition, variables/compounds observed to be from the same chromatographic peak (split peaks) after manual inspection were summed. The QC samples were used to assess the quality of the data, according to Want et al. (2010). The relative coefficient of variance (%CV) was calculated for each feature. The %CV for selected identified 175 compounds did not exceed 50% of variation, previously reported as the cut off value for compound selection (Beckner Whitener et al., 2016). All statistical analyses of wine volatiles were conducted in R v3.2.2 (RStudioTeam, 2012). For each putative compound, a linear model was fitted with harvest time (categorical), vineyard and interactions as fixed effects. The models were used to perform analyses of variance (ANOVA). As multiple significance testing of the variables was undertaken, a correction to the p-value associated with each variable was made using the Benjamini Hochberg procedure for false discovery, giving q (FDR corrected p) values (Benjamini, Krieger, & Yekutieli, 2006). A significance level of 5% was applied after this correction for all considerations. Post-hoc multiple comparison tests were performed to determine specific effects using the package multcomp v1.4–6 (Hothorn, Bretz, & Westfall, 2008). Unit variance scaling was used for PCA and heatmap generation. Values outside the range of 3 standard deviations were reassigned to 3 in the case of for the heatmaps. PCA was calculated using the pcaMethods package v1.60.0 (Stacklies, Redestig, Scholz, Walther, & Selbig, 2007) employing the NIPALS algorithm.

The Pearson correlation coefficient and Ward's minimum variance method were used for hierarchical clustering in the heatmap dendrograms. The ggplots package v3.0.1 (RStudioTeam, 2012) was used to draw heatmaps.

Basic juice and wine parameters were compared by one-way and two-way analyses of variance (ANOVA) using Statistica, Version 12 (StatSoft, Tulsa, OK, USA). The means were separated using Stats-Fisher's LSD test (different letters account for significant differences at $p \leq 0.05$). All quoted uncertainty is the standard deviation of three replicates of one treatment.

3. Results and discussion

3.1. Grape juice maturity parameters

Grapes were harvested according to the sugar loading model, 12 and 24 days after the slow-down of sugar accumulation into the berry for H1 and H2, respectively. A plateau of sugar accumulation was reached within a 7 day period across all four vineyards, causing a 7-day harvest gap for each nominal harvest time (Table 1). The average TSS juice concentration increase from H1 to H2 was 1.25 °Brix in vineyards 1, 2 and 3 whereas an increase of 2.2 °Brix was measured in Vineyard 4 (Table 2). It has been suggested, that during the late ripening period, phloem cessation occurs, resulting in reduced water and sugar flow into the berry (Rogiers, Greer, Hatfield, Orchard, & Keller, 2006). Further increase in TSS in late ripening stage is therefore primarily related to a concentration effect, due to the berry transpiration and xylem efflux (Rogiers et al., 2006). The observed slow-down of active sugar accumulation during the late ripening period supports this hypothesis, even though the decrease in berry fresh weight with ripening was not significant (Table 2). The small difference in grape juice TSS from Vineyard 1, did not result in a significant increase in wine ethanol content between two harvests, Table 2. Later harvest dates resulted in increased juice YAN and NOPA values at all sites except Vineyard 1. Minor changes related to grape maturity were noted for ammonia (Table 2). Contrasting trends in NOPA behaviour related to the grape maturity in Shiraz and Cabernet Sauvignon juice have been previously observed (Antalick et al., 2015).

3.2. Wine volatiles

Employing two-dimensional GC coupled to TOFMS in this investigation has enabled detection of 1276 putative compounds. This compares favourably with the untargeted GCMS approaches used to detect 253 peaks in Semillon (Schmidtke, Blackman, Clark, & Grant-Preece, 2013) or 99 compounds in Pinot Noir (Schueuermann, Khakimov, Engelsen, Bremer, & Silcock, 2016),

Chromatogram processing and univariate data analysing was used to determine the compounds that were different between harvest dates, and this narrowed the selection to 215 compounds. These compounds were further thoroughly checked for mass spectra similarities using the libraries (NIST 2.0, Wiley 8 and FFNSC 2), authentic standards and LRI match. Silicon containing compounds, likely originating form column bleeding or fibre were also removed from the list and split peaks were summed together. Finally, 175 compounds were identified to be relevant (see "Data processing and statistical analysis"). Criteria for

Table 2
Juice and wine basic composition.

	Harvest	Vineyard 1	Vineyard 2	Vineyard 3	Vineyard 4	H	V	H*V
TSS (°Brix)	H1	23.28 ± 0.2a	22.3 ± 0.10a	23.4 ± 0.18a	22.5 ± 0.0a	***	***	**
	H2	24.12 ± 0.1b	23.6 ± 0.10b	24.7 ± 0.52b	24.7 ± 0.18b			
Sugar/berry(mg)	H1	260.5 ± 3.7a	321.6 ± 13.5	282.9 ± 13.6	261.4 ± 12.4a	NS	***	NS
	H2	249.3 ± 15.2b	333.3 ± 3.5	305.9 ± 23.8	266.1 ± 11.4			
Berry fresh mass (g)	H1	1.11 ± 0.02	1.4 ± 0.06	1.27 ± 0.06	1.2 ± 0.04	NS	***	NS
	H2	1.04 ± 0.07	1.4 ± 0.04	1.20 ± 0.07	1.1 ± 0.06			
pH	H1	3.98 ± 0.02	3.93 ± 0.03b	3.71 ± 0.02b	3.66 ± 0.01b	***	***	***
	H2	4.01 ± 0.03	4.18 ± 0.01a	4.01 ± 0.01a	4.02 ± 0.01a			
TA (g/L)	H1	3.2 ± 0.01a	4.13 ± 0.11a	3.53 ± 0.11a	3.47 ± 0.06a	***	***	***
	H2	2.93 ± 0.06b	3.37 ± 0.06b	2.77 ± 0.01b	2.43 ± 0.06b			
YAN (mg N/L)	H1	127 ± 1.5	183 ± 4b	84 ± 3b	98 ± 1b	***	***	**
	H2	130 ± 3.1	192 ± 4a	104 ± 3 a	112 ± 6a			
NOPA (mg N/L)	H1	105 ± 0.6	142 ± 2.9b	70 ± 2.5b	76 ± 1.0b	***	***	***
	H2	109 ± 1.6	161 ± 4.0a	89 ± 2.6a	92 ± 4.6a			
Ammonia (mg N/L)	H1	25.3 ± 0.6b	49.0 ± 1.0a	18.0 ± 0.0	26.7 ± 0.6a	***	***	***
	H2	26.7 ± 0.6a	38.0 ± 0.0b	18.3 ± 0.6	24.0 ± 1.0b			
Ethanol (%)	H1	13.5 ± 0.11	12.6 ± 0.0b	13.3 ± 0.1b	12.4 ± 0.1b	***	***	***
	H2	13.6 ± 0.00	13 ± 0.1a	13.7 ± 0.1a	13.1 ± 0.1a			

H1, H2, refer to harvest 1, 2, respectively. TSS refers to total soluble solids, TA refers to titratable acidity, YAN refers to yeast assimilable nitrogen, NOPA refers to primary amino acids assimilable by yeast.
H refers to Harvest, V refers to Vineyard and H*V refers to interaction harvest*vineyard.
t test was performed on a raw data and means flowed by a different letter are different between 2 harvest dates for individual vineyard at $p \leq 0,05$ (Fisher's LSD test). All quoted uncertainty is the standard deviation of three replicates of one treatment.
Significance of two-way ANOVA for harvest date, vineyard and interaction harvest date × vineyard is indicated with *, where *** indicates $p \geq 0.001$. ** indicates $p \geq 0.01$ and NS indicates no significant differences.

considering compounds relevant were previous reports of these compounds in wines, compounds known to derive from fermentation and known grape metabolites (terpenes, norisoprenoids).

These compounds were also annotated as described in the Materials and methods section. 33 compounds were identified with the help of authentic standards, mass spectra and LRI comparison, 93 by mass spectra and LRI comparison and 37 compounds were tentatively identified only by comparison of mass spectra to the reference libraries. Only a few compounds remained unidentified due to a poor mass spectra match, even though it has been previously shown that the ChromaTOF software has high ability to correctly deconvolute and annotate compounds (Carlin et al., 2016).

In order to clarify the overall impact of harvest date on the wine volatile composition, a PCA and heatmap were generated (Figs. 1, 2). PCA accounted for 57% of the variability in data set and demonstrated that wines of different harvests can be clearly separated based on their volatile chemical composition, irrespective of the vineyard (Fig. 1). Samples from the different harvest dates (H1 vs H2) were separated along principal component (PC)1, irrespective of differences in TSS concentrations. PC2 accounted for 14.9% of the variation, with wines from vineyard 2 slightly separated from remaining samples at H1 and H2. This vineyard was also the highest yielding site and the abundance of some compounds, for example (*E*)-2-hexen-1-ol, some terpenoids and other compounds originating from the lipoxygenase (LOX) pathway were higher in these wines. Further on, slight separation of Vineyard 2 is also evident in Fig. 2, where H2 wines from Vineyard 2 showed fewer similarities to wines from other vineyards at H2. Clear grouping of treatments replicates were noticed and evolution of volatiles from H1 to H2 could be observed (Fig. 2). Irrespective of vineyard, LOX derived compounds, some terpenes and sulfur containing compounds decreased from H1 to H2, whereas an increase in some acetate esters at H2 was noticed (Fig. 2, Supplementary material Fig. 2).

Harvest date, sugar concentration and an interaction of both can influence wine volatile development in the earlier stages (up to 18 °Brix), of berry ripening, (Boss, Böttcher, & Davies, 2014). In particular, compounds that contribute negatively to the final wine flavour decrease with increasing TSS concentration and time, whereas aromas that are regarded as positive, such as fruity aromas, are more associated with TSS increase than time (Boss et al., 2014). The present study focused on the evolution of wine volatiles around physiological ripeness and commercial harvest dates, after rapid sugar accumulation into the berry. Despite small or no differences in final wine ethanol content amongst the wines, a clear differentiation of volatiles was noticed in wines from both harvest dates (Figs. 1, 2). Results suggest that volatile chemical evolution in the final wine is strongly related to the time after sugar plateau and can be predicted from this point onwards. It could be further hypothesised that evolution of volatiles after the plateau of sugar accumulation is not linked to grape sugar content at this stage of berry ripening. A similar conclusion was recently made by Böttcher, Boss, Harvey, Burbidge, and Davies (2017). This implies that sugar accumulation per berry may be able to be used as an indirect indicator of grape physiological maturity and consequently wine volatile evolution.

Compounds that contribute significantly to sample separation will be discussed according to their metabolic origin in the following paragraphs. Metabolites that significantly contribute to the separation of samples according to the harvest date are listed in Table 3. The metabolites are organised in a descending order according to the q value, which indicates the level of significance by which the compound abundance is affected by the later harvest date.

3.2.1. *Lipoxygenase derived compounds*

The concentration of LOX derived metabolites decreased in wines from H1 to H2 (Fig. 2). This was a consistent finding, despite different branching points in their formation being recognised. The only exceptions were the corresponding acetates formed by acetyl alcohol transferases (*ATF*) *1* and *2*. In particular, a significant decrease of (*Z*)-6-nonenol, 2-penten-1-ol,(*Z*), 4-hepten-1-ol and (*Z*)-3-hexenol was observed in H2 wines, irrespective of the vineyard (Fig. 2, Supplementary material Fig. 2). A decreasing trend of C5 and C6 compounds was recently reported in Cabernet Sauvignon grape skins in the late ripening period (Yang et al., 2015). This reduction coincided with a drastic decrease of alcohol dehydrogenase (ADH) activity and lesser decrease in activity of 13- and 9- hyperoxide lyase (HPL) (Yang et al., 2015). Similarly, Kalua and Boss (2009) hypothesised that activity of ADH and other enzymes involved in LOX pathway have greater impact on the variations in abundances of C6 alcohols than the substrate availability. These suggestions have been further substantiated by the finding in the

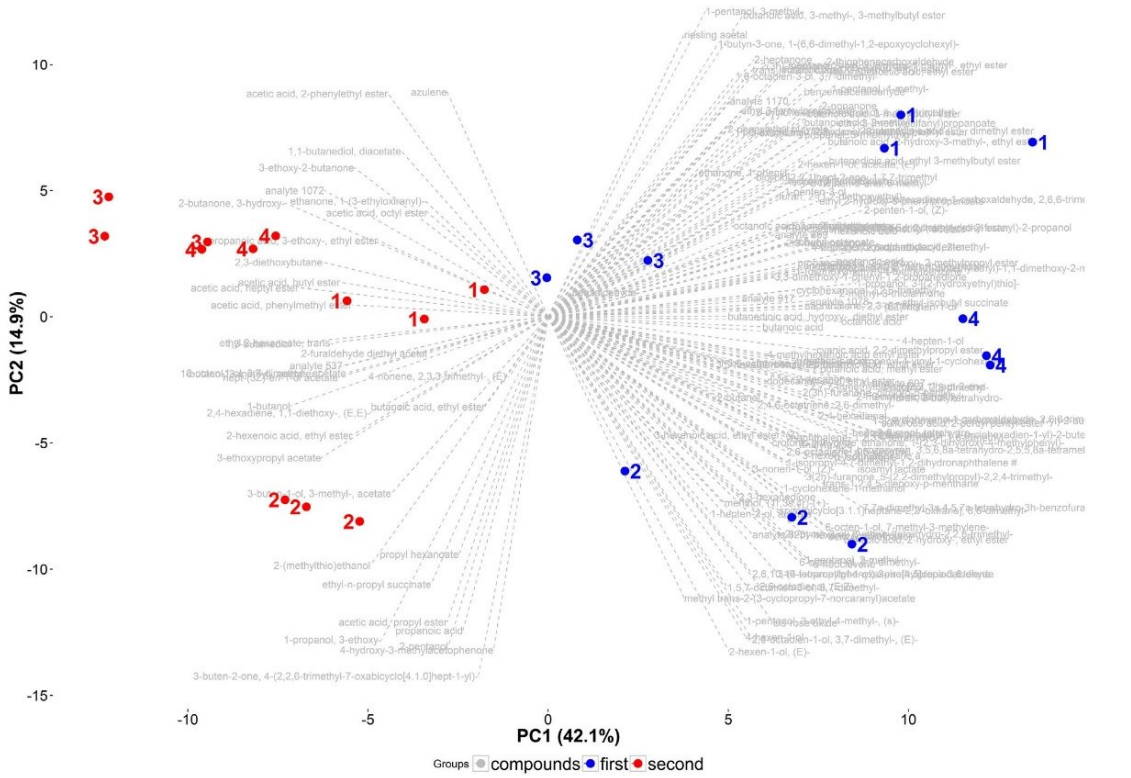


Fig. 1. Principle component analyses (PCA) after unit variance scaling. Blue colour identifies samples belonging to the first harvest (H1) and the samples from second harvest (H2) are marked in red. Numbers 1, 2, 3, 4 indicate vineyard 1, 2, 3, 4, respectively. PCA was conducted on 175 putative biomarkers selected after the two-stage Benjamini and Hochberg step-up false discovery rate at confidence interval of 5%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

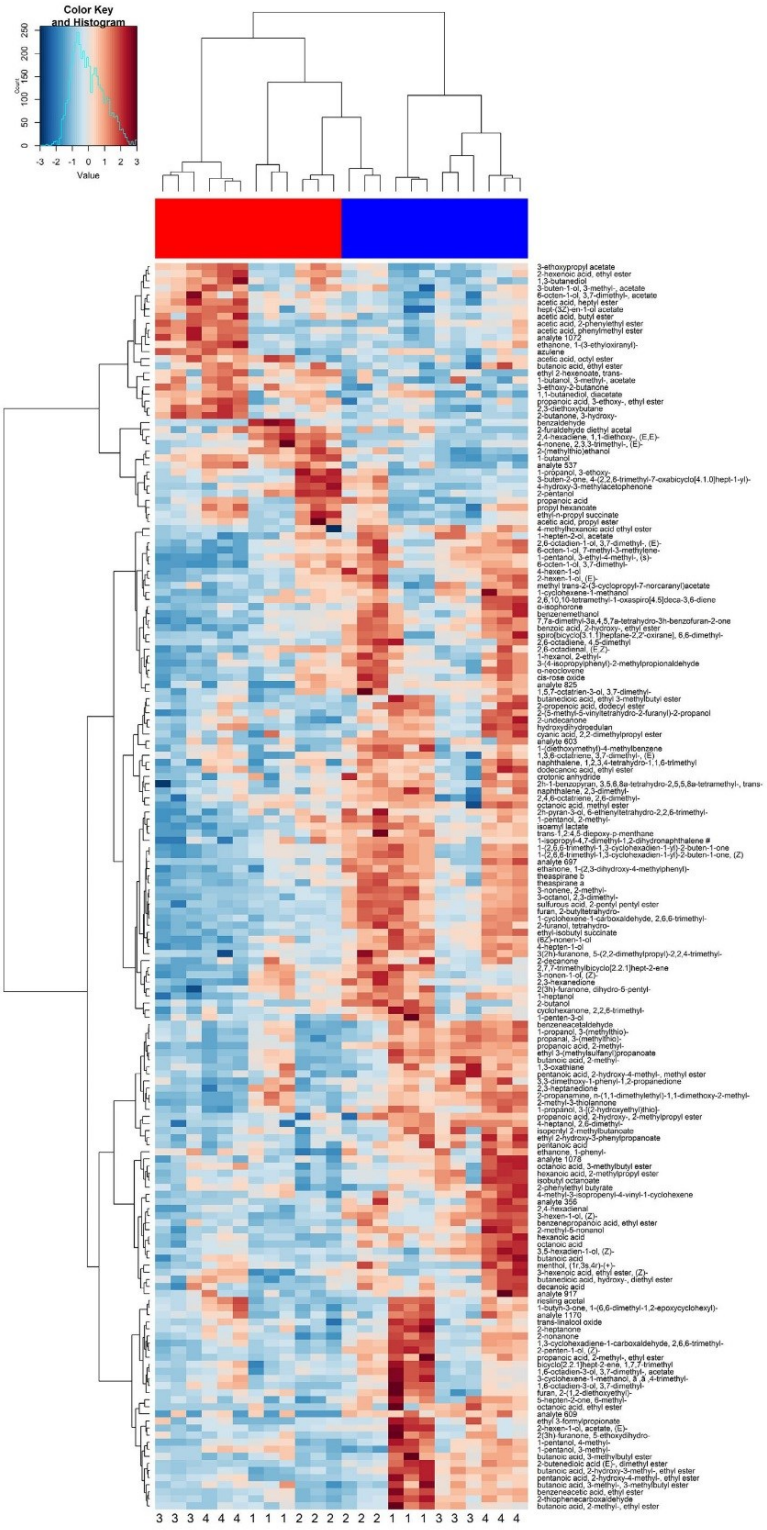
present study, that there was no significant differences in concentrations of linoleic and linolenic acid in grapes (data not presented), key substrates for production of green leaf volatiles between harvest dates.

The abundance of (*Z*)-3-hexenol in wines from all the vineyards decreased significantly from H1 to H2. This compound had the highest q value, and contributed the most to the separation of samples according to the harvest date. A decrease in abundance of (*Z*)-3-hexenol with ripening has been observed in previous studies across different vintages (Antalick et al., 2015; Kalua & Boss, 2009). Pedneault, Dorais, and Angers (2013) have also observed a decrease in LOX derived alcohols and aldehydes, in particular (*Z*)-3-hexenol and (*E*)-2-hexenol, with grape maturation. However, (*Z*)-3-hexenol in contrast to (*E*)-2-hexenol, seems to be only slightly altered during alcoholic fermentation (Herraiz, Herraiz, Reglero, Martin-Alvarez, & Cabezudo, 1990). Therefore (*Z*)-3-hexenol appears to be a valuable marker of grape maturity in Shiraz wines. While (*E*)-2-hexenol was also decreased in wines from the later harvest, the trend was less robust than observed for (*Z*)-3-hexenol. This is in accordance with previous work (Antalick et al., 2015). Despite a direct linear contribution of (*E*)-2-hexenol, hexanol and corresponding aldehydes to the formation of hexyl acetate (Dennis et al., 2012), no particular trend for this compound was observed in wines from H1 to H2. In contrast, abundances of (*E*)-2-hexenyl acetate decreased in all four vineyards between harvests suggesting its direct relationship with its precursor (E)-2-hexenol. Formation of acetates from corresponding alcohols is regulated by the activity of *ATF1* and *ATF2* and the availability of alcohols is not the limiting factor (Sumby, Grbin, & Jiranek, 2010). In addition, no correlation between acetates and corresponding higher alcohols was found in recent meta-study that reconsidered 104 publications that reported wine volatile data (Ilc, Werck-Reichhart, & Navrot, 2016). Similarly, in our study, acetates derived from C6 compounds decreased or showed no particular trend with delayed harvest.

3.2.2. Esters

Higher alcohol acetates (HAAs) that originate from yeast sugar and nitrogen metabolism exhibited an increasing trend with grape ripening (Fig. 2). The relative abundance of 1-butanol-3-methyl acetate (isoamyl acetate), acetic acid, propyl ester (propyl acetate), acetic acid heptyl ester (heptyl acetate), acetic acid octyl ester (octyl acetate), acetic acid, butyl ester (butyl acetate), 3-ethoxypropyl acetate and others increased from H1 to H2 (Fig. 2, Supplementary material Fig. 2). This is in agreement with previous work that reported an increase in HAAs during grape ripening (Bindon et al., 2013; Šuklje et al., 2016). The higher YAN levels in the H2 grape juices could have also influenced the acetates concentrations in these wines. Addition of amino acids in juice have resulted in significant increase in several yeast derived volatiles, such as esters, higher alcohols and even thiols on some occasions in particular varietals (Pinu et al., 2014). However, in the present study, higher alcohols exhibited inconsistent behaviours in relation to the harvest date. The relative abundance of 1-butanol increased from H1 to H2, whereas lower abundances of LOX derived alcohols were generally present in H2 wines, as discussed previously.

As a general trend, abundances of ethyl esters of fatty acids (EEFAs), branched fatty acids esters (EEBAs) and others decreased in wines from



(caption on next page)

Fig. 2. Heatmap conducted on 175 putative biomarkers selected after the two- stage Benjamini and Hochberg step-up false discovery rate at confidence interval of 5%. Compounds were subjected to unit variance scaling. Broad band of a red colour on the top of the heatmap indicates H1 and a broad band of a blue colour on the top of a heatmap indicates H2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

H1 to H2 (Fig. 2, Supplementary material Fig. 2). A similar pattern was observed for octanoic acid and the corresponding ethyl ester. Fatty acid concentrations directly influence production of corresponding EEFAs, whereas enzymatic activity is not a limiting factor (Saerens et al., 2008). However, the relative abundance of ethyl butyrate increased from H1 to H2, irrespective of the decreasing trend of corresponding butyric acid. The increase in ethyl butyrate abundance with Shiraz grape ripening has been reported previously (Antalick et al., 2015; Šuklje et al., 2016). An increase in ethyl butyrate concentrations with grape ripening was also reported in Cabernet sauvignon wines (Bindon et al., 2013). Production of ethyl butyrate and other EEFAs is regulated by several enzymes and it is difficult to determine which enzyme pathway is stimulated during ripening. Saerens et al. (2008) proposed an alternative manner of ethyl butyrate formation in wine through chemical synthesis.

The relative abundance of other minor isoamyl-, isobutyl-, methyl- and cinnamic acids esters, as observed for majority of EEFAs, decreased from H1 to H2 (Supplementary material Fig. 2). Contrary to a previous Cabernet Sauvignon study (Bindon et al., 2013), no direct relationship between the grape juice TSS and wine esters concentrations could be established in the present work.

3.2.3. Terpenoids

This study detected 28 monoterpenes and identified 2 sesquiterpenes that contributed significantly to sample separation between the two harvest dates. However, lack of consistency in the pattern of monoterpene behaviour was noted among the vineyards (Fig. 2, Supplementary material Fig. 2). An increase in grape monoterpene concentrations during rapid sugar accumulation (up to 20 °Brix) was reported (Marais, 1983), whereas Martin, Chiang, Lund, and Bohlmann (2012) found a decrease in monoterpene concentrations in late ripening. The same authors observed a significant decrease in the abundance of linalool and α-terpineol synthase from 82 to 96 days after anthesis. This appears to correspond to a study on Cabernet Sauvignon which reported a decrease in expression of genes involved in terpenoid metabolism after the berries reached 23 °Brix (Cramer et al., 2014). In wine, monoterpenes undergo acid catalysed rearrangements. Linalool and several derivatives such linalyl acetate as well as oxidation products (*E*)-and (*Z*)- linalool oxide and linalool oxide pyranosid, were identified in the wines. Linalool can be easily oxidised in wine conditions and result in formation of compounds with a higher detection thresholds compared to the 25 μg/L reported for linalool (Marais, 1983). Additionally, in wine conditions linalool can form α-terpineol and other terpenes such as nerol, limonene and terpinolene via protonation (Marais, 1983). According to hierarchical cluster analyses (Fig. 2), compounds with similar behaviour are grouped closely together. In our study, α-terpineol, linaly acetate, 2-bronene and (*E*)-linalool oxide were grouped jointly with linalool. This suggests linalool is probably a key substrate for formation of other monoterpenes in wine. Ilc et al. (2016) identified linalool as a key intermediate involved in the production through oxidation and acidic catalysis, of more than half the terpenes analysed in their study. However, in the present study, (*Z*)-linalool oxide and linalool oxide pyranoside were grouped apart of linalool (Fig. 2). A partial explanation to this finding could be the formation of furanoid linalool oxides through two pathways utilising 3,7-dimethyloct-1-ene-3,6,7-triol and linaly 6,7-epoxid, whereas pyranoid linalool oxides could be formed only via epoxide (Luan, Hampel, Mosandl, & Wüst, 2004). Other terpenes displayed diverse behaviour and were positioned dispersedly on the heatmap (Fig. 2). Terpenes are also reported to be released from aglycones in wines. Additionally, two sesquiterpenes, α-calacorene and α-neoclovene known to be synthetised from farnesyl diphosphate ions, contributed significantly to the separation of the samples with higher levels measured in H1 wines (Fig. 2, Supplementary material Fig. 1). The method utilised in this investigation was not able to identify rotundone, a potent sesquiterpene responsible for peppery aroma in Shiraz wines from cooler climates.

3.2.4. Norisoprenoids

Norisoprenoids are formed via the degradation of C40 carotenoids, either directly or released via chemical and enzymatic reactions in wine (Mendes-Pinto, Silva Ferreira, Caris-Veyrat, & Guedes de Pinho, 2005). With maturity, a trend of decreasing concentration of the majority of norisoprenoids with C9, C10, C11 and C13 backbones was noticed in the present study (Fig. 2, Supplementary material Fig. 2). Contradictory results on the evolution of norisoprenoids with ripening in grapes and wine have been reported. Marais, Van Wyk, and Rapp (1992) reported an increase in Riesling wines whilst Versini, Carlin, Dalla Serra, Nicolini, and Rapp (2001) observed no influence of grape ripeness on 1, 1, 6-Trimethyl-1, 2-dihydronaphthalene (TDN) concentrations in wine. Final concentrations in wines are also strongly influenced by wine pH and storage conditions (Mendes-Pinto et al., 2005). For example, the formation of C9 and C10 norisoprenoids, such as cyclohexanone, 2,2,6-trimethyl- (TCH) and β-cyclocitral, respectively, were suggested to occur via thermal degradation from β-carotene via 5,6-epoxy-β-ionone as key intermediate. A decrease in the relative abundance of TCH and β-cyclocitral in wines from H1 in comparison to H2 was observed. Safranal, another potent C10 norisoprenoid with a yet unknown contribution to the wine sensory profile (Carlin et al., 2016), was consistently found in lower abundances in wines from H2. Both isomers (*E*- and *Z*-) of β-damascenone were also identified. Traditional one-dimensional GC, enables detection of a prevalent (*E*)-β-damascenone isomer, whereas utilisation of two dimensional GC enabled the detection of both. In our experimental wines, (*Z*)- β-damascenone was found in lower abundances compared to the trans isomer, however both compounds exhibited a decreasing trend with increased grape maturity. (*E*)-β-damascenone was also reported as one of the key compounds contributing to the typicity of Shiraz and Sauvignon blanc wines (Mayr et al., 2014). Similarly, the relative abundance of both theaspirane isomers, A and B respectively, decreased from H1 to H2. Interestingly, hydroxydihydroedulan and edulan I abundances also decreased with grape maturity. These norisoprenoids were previously reported in Shiraz and Pinot Noir grapes and wines. The formation of hydroxydihydroedulan from the degradation of diastereoisomeric diols to vitispiranes at a ratio of 1:3 has been proposed (Waldmann & Winterhalter, 1992). As in the case for some monoterpenes, chemical rearrangement and release from glycosides might also contribute to the differences observed in the level of some norisoprenoids between H1 and H2.

3.2.5. Sulphur containing compounds

Volatile sulphur compounds (VSCs) have low detection thresholds in wine and are often associated with negative sensorial descriptors (Ugliano et al., 2011). In this study, 10 VSCs that contributed to the sample separation were identified. During grape ripening, all VSCs decreased with the exception of 2-(methylthio)ethanol, which was found to be increased significantly in H2 wines (Fig. 2, Supplementary material Fig. 2). This compound is characterised by French beans and meaty odours, the latter being a typical descriptor of (over)ripe Shiraz wines.

Coherent modifications of yeast sulphur metabolism from methionine were also noticed. Methionol, methional and ethyl 3-(methylsulfanyl) propanoate were significantly lower in wines from H2. Synthesis

Table 3
List of annotated compounds that were significantly different between harvest dates sorted descending according to the calculated q value, type of identification, CAS number, biochemical classification, q values for parameter H (harvest date), V (vineyard) and H:V interaction harvest date × vineyard and loadings for PC1 and PC2.

Analyte	Peak	Calculated LRI	Reported LRI	Identification	CAS	Class	H q value	V (q value)	H:V (q value)	PC1	PC2
1	3-hexen-1-ol, (*Z*)	1388	1384	A	928-96-1	GLV	**1,04E−10**	**2,77E−07**	**3,88E−04**	6.9567	4.9542
2	Propanoic acid, 2-methyl-/Isobutyric acid	1557	1567	B,C	79-31-2	Branched acid	**1,04E−10**	**6,53E−05**	**2,12E−03**	8.9866	−2.6221
3	2-Penten-1-ol, (*Z*)-	1311	1296	B,C	1576-95-0	GLV	**2,88E−10**	**5,57E−07**	**4,64E−06**	8.8101	−4.1334
4	2-Butenedioic acid (*E*)-, dimethyl ester/Dimethyl fumarate	1456	1530	B,C	624-49-7	Ester of fixed acids	**2,21E−09**	**5,44E−06**	**4,44E−05**	8.6666	−7.3426
5	Ethyl 3-(methylsulfanyl)propanoate	1557	1569	B,C	13327-56-5	VSC	**2,35E−08**	**3,65E−07**	**1,06E−03**	8.0245	−7.4701
6	Isoamyl lactate	1580	1583	B,C	19329-89-6	Ester of fixed acid	**3,71E−08**	**5,81E−06**	**1,93E−02**	8.6057	6.3664
7	1-Butanol	1146	1173	A	71-36-3	Higher alcohol	**4,44E−08**	**8,39E−04**	**8,16E−03**	−7.1616	−3.5335
8	4-Hepten-1-ol	1498	1502	B,C	20851-55-2	GLV	**5,22E−08**	**3,41E−05**	**5,00E−02**	9.8591	1.1189
9	Butanoic acid, 2-hydroxy-3-methyl-, ethyl ester/Ethyl 2-hydroxyisovalerate	1433	1430	B,C	2441-06-7	Ethyl ester of branched acid	**5,60E−08**	**9,98E−03**	**2,46E−05**	7.7616	−6.8307
10	2-Methyl-3-thiolanone	1535	1520	B,C	13679-85-1	VSC	**6,84E−08**	**2,39E−05**	**9,07E−07**	8.0625	−0.9397
11	3-Octanol, 2,3-dimethyl-	1436	NA	C	19781-10-3	Higher alcohol	**9,21E−08**	**6,80E−04**	2,26E−01	9.2603	2.8545
12	1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-/Safranal	1646	1648	A	116-26-7	Norisoprenoid	**1,09E−07**	**8,91E−07**	**5,86E−04**	8.6799	−4.6827
13	Ethyl-isobutyl succinate	1791	NA	C	NA	Ethyl ester of fatty acids	**2,86E−07**	**1,49E−02**	**9,79E−03**	9.8321	−0.6131
14	1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-/β- cyclocitral	1632	1631	B,C	432-25-7	Norisoprenoid	**3,25E−07**	**4,39E−04**	8,77E−02	9.2927	3.9546
15	Furan, 2-butyltetrahydro-	1845	NA	C	1004-29-1	Furan	**3,58E−07**	**3,93E−03**	1,30E−01	9.5735	3.1779
16	1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one/(*E*)-β-damascenone	1809	1823	A	23726-93-4	Norisoprenoid	**4,49E−07**	**1,41E−06**	**4,90E−02**	9.0653	4.2919
17	(*Z*)-β- damascenone	1751	NA	C	NA	Norisoprenoid	**5,35E−07**	**7,37E−07**	**4,30E−02**	8.8669	4.8755
18	1-Propanol, 3-(methylthio)-/Methionol	1705	1719	B,C	505-10-2	VSC	**5,65E−07**	**1,64E−05**	**1,23E−03**	7.2302	−7.3880
19	Analyte 697	1456	NA				**6,01E−07**	**1,07E−03**	**1,88E−03**	9.0224	2.4716
20	2,4-Hexadiene, 1,1-diethoxy-, (*E,E*)-	1451	NA	B,C	94088-28-5	GLV	**7,83E−07**	**5,57E−04**	**1,97E−03**	−4.8340	4.2976
21	3-Nonene, 2-methyl-	1256	NA	C	53966-53-3	Monoterpene	**7,75E−07**	**1,61E−03**	1,21E−01	9.0185	3.2697
22	Sulfurous acid, 2-pentyl pentyl ester	1786	NA	C	NA	VSC	**7,95E−07**	**2,37E−03**	3,43E−01	9.2566	4.2477
23	Pentanoic acid, 2-hydroxy-4-methyl-, ethyl ester	1542	1545	B,C	10348-47-7	Ethyl ester of branched acid	**8,43E−07**	**2,65E−04**	**1,05E−05**	6.7413	−10.0384
24	2-Nonanone	1394	1391	A	821-55-6	A-ketone	**8,77E−07**	**3,16E−06**	**1,14E−03**	7.6208	−8.6760
25	2-Thiophenecarboxaldehyde	1694	1689	B,C	98-03-3	VSC	**1,44E−06**	**1,24E−05**	**1,16E−02**	7.7512	−10.0255
26	2-Furanol, tetrahydro-	1394	NA	C	5371-52-8	Furan	**1,55E−06**	**7,63E−03**	3,97E−01	9.1456	4.7136
27	(6*Z*)-Nonen-1-ol	1705	1711	B,C	35854-86-5	GLV	**1,81E−06**	**1,47E−04**	2,31E−01	9.3664	−0.0526
28	Butanoic acid, 2-methyl-/ 2-Methylbutyric acid	1680	1671	B,C	116-53-0	Branched acid	**1,88E−06**	**8,96E−05**	**4,83E−02**	7.0647	−7.5195
29	Benzeneacetic acid, ethyl ester/Ethyl phenylacetate	1786	1781	A	101-97-3	Ethyl ester of branched acid	**1,97E−06**	**1,51E−05**	**2,58E−02**	7.9106	−9.5595
30	3,5-Hexadien-1-ol, (*Z*)-	1443	NA	C	2196-20-5	GLV	**2,32E−06**	**8,74E−07**	**5,19E−06**	4.5631	1.0856
31	7,7a-Dimethyl-3a,4,5,7a-tetrahydro-3H-benzofuran-2-one	1446	NA	C	NA	Monoterpene	**2,34E−06**	**2,40E−03**	5,46E−02	8.7310	7.4688
32	1-Oxaspiro[4.5]dec-6-ene, 2,6,10,10-tetramethyl-/Theaspirane B	1542	1543	A	43126-21-2	Norisoprenoid	**2,82E−06**	**8,04E−04**	1,35E−01	9.2321	4.7708
33	3-Ethoxypropyl acetate	1356	NA	C	NA	Higher alcohol acetate	**2,85E−06**	**2,37E−06**	6,51E−02	−6.2925	4.9247
34	2-Undecanone	1597	1596	B,C	112-12-9	A-ketone	**2,89E−06**	**5,35E−06**	**1,41E−02**	7.5370	−4.3077
35	2-Propanamine, n-(1,1-dimethylethyl)-1,1-dimethoxy-2-methyl-	1656	NA	C	62134-75-2	Amine	**3,77E-06**	**1,01E−03**	**6,43E−05**	7.7870	−1.9620
36	2-Butanone, 3-hydroxy-/Acetoin	1275	1265	B,C	531-86-0	A-hydroxyketone	**3,87E−06**	**1,81E−03**	**6,24E−04**	−7.2744	−4.9237
37	2,4-Hexadienal	1404	1409	B,C	142-83-6	GLV	**3,98E−06**	**1,27E−03**	1,12E−01	7.4469	3.2637
38	Octanoic acid	2051	2057	A	124-07-2	Fatty acid	**4,93E−06**	**2,68E−03**	3,81E−01	7.9104	−9.0184
39	1-Pentanol, 4-methyl-	1308	1317	B,C	626-89-1	Higher alcohol	**5,06E−06**	**2,72E−06**	1,85E−01	7.8621	9.0439
40	Propanal, 3-(methylthio)-/Methional	1453	1451	B,C	3268-49-3	VSC	**5,22E−06**	**3,11E−04**	**1,34E−02**	7.0387	−7.4181
41	2-(methylthio)ethanol	1534	1534	B,C	5271-38-5	VSC	**5,51E−06**	**4,75E−05**	4,49E−01	−4.8224	10.2463
42	4-hexen-1-ol	1433	1408	B,C	6126-50-7	GLV	**6,05E−06**	**4,96E−06**	**6,65E−04**	5.4432	12.5447
43	Benzoic acid, 2-hydroxy-, ethyl ester	1801	1819	B,C	118-60-5	Hydroxybenzoic acid ester	**7,16E−06**	**6,85E−03**	**1,37E−02**	8.2025	8.8521
44	2-Hexen-1-ol, (*E*)	1401	1402	A	928-95-0	GLV	**7,55E−06**	**7,55E−07**	**6,11E−04**	5.0205	−13.2612
45	Hexanoic acid	1842	1841	A	142-62-1	Fatty acid	**1,02E−05**	**7,53E−04**	4,35E−01	7.8912	−4.0527
46	2-Hexenoic acid, ethyl ester/Ethyl 2-hexenoate	1346	1329	B,C	1552-67-6	GLV	**1,13E−05**	**4,02E−05**	5,03E−02	−5.3841	4.1392
47	6-Octen-1-ol, 3,7-dimethyl-/β-citronellol	1755	1749	A	106-22-9	Monoterpene	**1,30E−05**	**1,17E−04**	**1,29E−03**	6.9755	10.1095
48	2-Methyl-5-nonanol	1583	NA	C	29843-62-7	Higher alcohol	**1,51E−05**	**8,34E−03**	6,78E−02	8.5536	2.6977
49	Hydroxydihydroedulan	1912	1902	B,C	NA	Norisoprenoid	**1,79E−05**	**1,36E−06**	**3,35E−02**	6.6678	−6.1205
50	2,3-Diethoxybutane	1178	NA	C	NA	Ether	**1,96E−05**	1,26E−01	**3,18E−03**	−6.1661	−2.4812

(*continued on next page*)

Table 3 (*continued*)

Analyte	Peak	Calculated LRI	Reported LRI	Identification	CAS	Class	H q value	V (q value)	H:V (q value)	PC1	PC2
51	6-Octen-1-ol, 7-methyl-3-methylene-/γ-geraniol	1786	1800	B,C	13066-51-8	Monoterpene	**1,94E−05**	**2,92E−02**	**1,05E−02**	7.6552	8.4405
52	Acetic acid, heptyl ester/Heptyl acetate	1366	1370	A	112-06-1	Higher alcohol acetate	**1,93E−05**	**1,15E−02**	7,18E−02	−6.8971	−1.7680
53	1-Pentanol, 2-methyl-/Isohexyl alcohol	1298	1320	B,C	105-30-6	Higher alcohol	**2,12E−05**	**1,95E−05**	4,48E−01	7.1853	9.8703
54	2-Hexen-1-ol, acetate, (*E*)-/ (*E*)-2-Hexenyl acetate	1339	1339	B,C	2497-18-9	GLV	**2,20E−05**	**1,95E−04**	**2,16E−04**	6.9968	−5.6354
55	Benzeneacetaldehyde	1646	1655	A	122-78-1	Aldehyde	**2,49E−05**	**1,37E−04**	7,33E−02	7.0673	−9.0631
56	Ethanone, 1-(2,3-dihydroxy-4-methylphenyl)-	1922	NA	C	69751-81-1	Phenolics	**2,89E−05**	**8,75E−03**	4,28E−01	8.7253	5.0851
57	2,7,7-Trimethylbicyclo[2.2.1]hept-2-ene	1532	NA	C	514-14-7	Monoterpene	**5,42E−05**	**3,37E−06**	5,47E−01	7.3524	3.4473
58	1-Pentanol, 3-ethyl-4-methyl-, (s)-	1501	1506	B,C	NA	Higher alcohol	**6,46E−05**	**6,18E−05**	**1,62E−04**	5.4873	12.3433
59	1-Heptanol	1496	1460	A	111-70-6	Higher alcohol/GLV	**7,39E−05**	**2,82E−03**	4,39E−01	8.2113	5.0116
60	2,3-Heptanedione	1149	1138	B,C	96-04-8	α-diketone	**8,67E−05**	**1,81E−03**	**9,67E−04**	6.5886	−2.7769
61	1-Propanol, 3-ethoxy-	1362	1377	B,C	111-35-3	Higher alcohol	**1,08E−04**	**2,98E−06**	**5,51E−02**	−4.6193	−12.7816
62	1,6-Octadien-3-ol, 3,7-dimethyl-, acetate/Linalyl acetate	1505	1538	B,C	115-95-7	Monoterpene	**1,27E−04**	**1,61E−03**	**7,50E−05**	6.6139	−3.2420
63	1-Propanol, 3-[(2-hydroxyethyl)thio]-/ 2-Ethanol 3-propanol sulfide	1755	NA	C	5323-60-4	VSC	**1,33E−04**	**1,10E−02**	**2,55E−02**	8.5697	−1.2101
64	Butanedioic acid, ethyl 3-methylbutyl ester	1897	1901	B,C	28024-16-0	Ester of fixed acid	**1,50E−04**	**3,70E−03**	**1,15E−02**	7.8329	−6.4752
65	Analyte 1072	1708	NA				**1,57E−04**	**1,67E−04**	**1,01E−02**	−6.2493	−5.6998
66	1,3-butanediol	1553	1556	B,C	107-88-0	Diol	**1,59E−04**	**4,92E−02**	1,33E−01	−7.1092	0.6919
67	Hexanoic acid, 2-methylpropyl ester/ Isobutyl hexanoate	1353	1350	B,C	105-79-3	Ester of fatty acid	**1,65E−04**	**2,31E−02**	1,36E−01	7.6088	−2.5705
68	4-Heptanol, 2,6-dimethyl-	1505	1506	B,C	108-82-7	GLV	**1,81E−04**	3,78E−01	**3,88E−02**	7.2629	−2.9258
69	Ethyl-*n*-propyl succinate	1751	1762	B,C	NA	Ester of fixed acid	**1,82E−04**	**2,99E−06**	**4,09E−02**	−3.1667	10.3817
70	α-Isophorone	1594	1591	A	78-59-1	Norisoprenoid	**1,86E−04**	**1,22E−02**	1,51E−01	7.9490	5.0194
71	2H-1-Benzopyran, 3,5,6,8a-tetrahydro-2,5,5,8a-tetramethyl-, trans-/Edulan I	1605	1602	B,C	41678-29-9	Norisoprenoid	**2,08E−04**	**5,56E−04**	4,80E−01	7.9418	5.0253
72	2,6-Octadien-1-ol, 3,7-dimethyl-, (*E*)-/ (*E*)- geraniol	1842	1824	A	106-24-1	Monoterpene	**2,11E−04**	**4,75E−04**	**2,81E−04**	5.6320	12.7049
73	α-Neoclovene	1801	NA	C	4545-68-0	Sesquiterpene	**2,24E−04**	**6,13E−04**	1,22E−01	7.3817	9.5718
74	Butanoic acid, 2-methyl-, ethyl ester/Ethyl 2-methylbutyrate	1048	1026	C,B	7452-79-1	Ethyl ester of branched acid	**2,97E−04**	**3,10E−03**	**2,81E−02**	6.4749	9.8044
75	Propanoic acid, 3-ethoxy-, ethyl ester	1339	1351	B,C	763-69-9	Ester of fatty acid	**3,07E−04**	**8,19E−03**	**1,60E−03**	−4.6591	−3.4192
76	Butanoic acid/Butyric acid	1635	1622	B,C	107-92-6	Fatty acid	**3,29E−04**	**4,44E−05**	**4,73E−02**	5.9143	−0.4859
77	Analyte 825	1528	NA				**3,45E−04**	**2,94E−03**	3,63E−01	5.6339	8.4394
78	Analyte 537	1304	NA				**3,81E−04**	1,96E−01	2,79E−01	−5.5996	1.9787
79	2-Pentanol	1133	1130	A	6032-29-7	Higher alcohol/GLV	**3,84E−04**	**3,45E−05**	**3,67E-04**	−2.7101	13.4481
80	Butanoic acid, 3-methylbutyl ester/Isoamyl butyrate	1218	1236	B,C	106-27-4	Ester of fatty acid	**4,31E−04**	**5,69E−03**	1,98E−01	7.2147	−7.8644
81	Acetic acid, 2-phenylethyl ester/Phenylethyl acetate	1805	1785	A	103-45-7	Higher alcohol acetate	**4,65E−04**	**2,04E−07**	**3,43E−03**	−4.5039	−9.6631
82	Propanoic acid, 2-methyl-, ethyl ester	983	959	B,C	105-37-3	Ethyl ester of branched acid	**4,74E−04**	**4,70E−02**	1,58E−01	8.1455	−3.3530
83	1-Oxaspiro[4.5]dec-6-ene, 2,6,10,10-tetramethyl-/Theaspirane A	1498	1507	A	36431-72-8	Norisoprenoid	**5,34E−04**	9,17E−02	3,18E−01	8.6498	5.5218
84	Ethyl 2-hydroxy-3-phenylpropanoate	2280	2273	B,C	NA	Ethyl ester of hydrocinnamic acid	**5,84E−04**	8,58E−02	6,59E−02	7.5042	−4.9456
85	Benzenepropanoic acid, ethyl ester/Ethyl hydrocinnamate	1864	1878	B,C	2021-28-5	Ethyl ester	**6,23E−04**	**2,05E−02**	1,81E−01	6.2046	1.1020
86	Trans-1,2:4,5-Diepoxy-p-Menthane	1485	NA	C	42569-59-5	Monoterpene	**6,97E−04**	**2,37E−02**	5,04E−01	7.6590	6.8848
87	2-(5-Methyl-5-vinyltetrahydro-2-furanyl)-2-propanol/(Z)-linalool oxide	1459	1441	A	5989-33-3	Monoterpene	**7,57E−04**	**4,58E−04**	4,97E−01	8.1559	−3.8406
88	Benzenemethanol	1861	1869	A	100-51-6	Phenolics	**8,34E−04**	**6,38E−03**	9,76E−02	7.8033	8.5194
89	Analyte 1078	1731	NA				**8,44E−04**	9,45E−02	1,05E−01	7.2237	−1.1014
90	Bicyclo[2.2.1]hept-2-ene, 1,7,7-trimethyl-/2-bronene	1491	1508	B,C	464-17-5	Monoterpene	**8,93E−04**	**3,64E−03**	**3,41E−05**	5.8560	−5.4526
91	4-Methyl-3-isopropenyl-4-vinyl-1-cyclohexene	1790	NA	C	NA	Monoterpene	**9,81E−04**	2,65E−01	**1,04E−02**	6.7628	1.3584
92	1-Cyclohexene-1-methanol/1-Hydroxymethylcyclohexene	1708	1732	B,C	4845-04-9	Monoterpene	**1,08E−03**	2,55E−01	**1,46E−02**	6.4774	6.5932
93	Pentanoic acid	1739	1736	B,C	109-52-4	Fatty acid	**1,16E−03**	2,87E−01	3,61E−01	8.0001	−2.4728
94	2,3-hexanedione	1136	1136	B,C	3848-24-6	A-diketone	**1,19E−03**	**6,59E−05**	**4,80E−02**	5.3261	7.8731
95	Hept-(3Z)-en-1-ol acetate	1408	1400	B,C	1576-78-9	Higher alcohol acetate	**1,23E−03**	**7,55E−04**	3,18E−01	−5.8476	1.6140
96	1-Penten-3-ol	1175	1130	B,C	616-25-1	GLV	**1,29E−03**	**4,39E−02**	6,89E−02	6.6115	−4.8522
97	2-Heptanone	1188	1185	B,C	110-43-0	A-ketone	**1,29E−03**	**3,51E−05**	**1,40E−03**	5.7930	−10.1342
98	3-Ethoxy-2-butanone	1094	NA	C	NA	A-ketone	**1,32E−03**	2,38E−01	3,57E−01	−5.3829	5.9128
99	Isobutyl octanoate	1549	1545	B,C	5461-06-3	Ester of fatty acid	**1,38E−03**	**1,08E−02**	2,36E−01	6.7323	−3.6792
100	Acetic acid, phenylmethyl ester/Phenylmethyl acetate	1733	1731	B,C	140-11-4	Higher alcohol acetate	**1,46E−03**	**1,06E−04**	**9,66E−03**	−5.4868	−1.2616

(*continued on next page*)

Table 3 (*continued*)

Analyte	Peak	Calculated LRI	Reported LRI	Identification	CAS	Class	H q value	V (q value)	H:V (q value)	PC1	PC2
101	1-Hexanol, 2-ethyl-	1490	1493	A	104-76-7	GLV	**1,51E−03**	**1,40E−03**	1,65E−01	7.1486	8.4592
102	Ethyl 3-formylpropionate	1546	NA	C	10138-10-0	Ester	**1,64E−03**	2,29E−01	**7,41E−03**	5.3603	8.1741
103	Analyte 356	1144	NA				**1,66E−03**	8,70E−02	1,97E−01	6.9425	4.5787
104	3-Hexenoic acid, ethyl ester, (*Z*)-/ Ethyl (Z)-3-hexenoate	1301	1295	B,C	64187-83-3	Ethyl esters of fatty acids/GLV	**1,67E−03**	**3,68E−07**	2,10E−01	2.9006	3.6527
105	2,6,10,10-Tetramethyl-1-oxaspiro[4.5]deca-3,6-diene	1546	NA	C	54344-61-5	Norisoprenoid	**1,70E−03**	**2,35E−03**	**1,66E−05**	5.6807	9.9339
106	1-Butyn-3-one, 1-(6,6-dimethyl-1,2-epoxycyclohexyl)-	1708	NA	C	NA	Norisoprenoid	**1,71E−03**	**9,12E−06**	**1,73E−04**	4.7530	−11.2624
107	(*Z*)-Rose oxide	1353	1353	B,C	16409-43	Monoterpene	**1,73E−03**	**1,43E−04**	1,56E−01	6.3019	12.1367
108	3-Cyclohexene-1-methanol, à,à,4-trimethyl-/α-terpineol	1694	1684	A	98-55-5	Monoterpene	**1,73E−03**	**1,47E−04**	**1,41E−04**	5.8554	−7.9508
109	4-Hydroxy-3-methylacetophenone	2197	2179	B,C	876-02-8	Monoterpene	**1,77E−03**	**1,83E−06**	6,53E−02	−1.4251	13.6418
110	2(3H)-Furanone, dihydro-5-pentyl-/γ- nonalactone	2036	2049	B,C	104-61-0	Lactone	**1,95E−03**	**5,56E−05**	5,59E−01	7.1657	3.4746
111	3(2H)-Furanone, 5-(2,2-dimethylpropyl)-2,2,4-trimethyl-	1747	NA	C	102307-37-9	Furan	**2,30E−03**	1,16E−01	7,78E−02	7.1711	6.4360
112	3,3-Dimethoxy-1-phenyl-1,2-propanedione	1362	NA	C	NA	Phenolics	**2,43E−03**	3,48E−01	1,00E−01	6.2374	−1.5237
113	Ethanone, 1-(3-ethyloxiranyl)-	1532	NA	C	17257-81-7	Ketone	**2,56E−03**	**4,49E−06**	**3,63E−02**	−3.4343	−5.6008
114	Cyclohexanone, 2,2,6-trimethyl-	1304	1308	B,C	2408-37-9	Norisoprenoid	**2,89E−03**	**9,12E−04**	8,14E−02	7.0109	−0.5727
115	2H-Pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl-/Linalool oxide pyranoside	1751	1721	B,C	14049-11-7	Monoterpene	**2,95E−03**	**4,09E−02**	3,97E−01	6.5540	8.7686
116	3-(4-Isopropylphenyl)-2-methylpropionaldehyde/Cyclamen aldehyde	1739	NA	C	103-95-7	Norisoprenoid	**3,09E−03**	**5,83E−04**	4,30E−01	6.4128	10.0655
117	Spiro[bicyclo[3.1.1]heptane-2,2′-oxirane], 6,6-dimethyl- /β-pinene oxide	1398	1384	B,C	6931-54-0	Monoterpene	**3,18E−03**	2,02E−01	**3,24E−02**	6.3688	7.3337
118	Analyte 609	1356					**3,39E−03**	3,92E−01	4,49E−01	6.2645	−3.3796
119	Ethyl 2-hexenoate, (*E*)-	1353	1345	B,C	27829-72-7	Ethyl ester of fatty acid/GLV	**3,71E−03**	4,41E−01	8,34E−02	−5.8735	1.1432
120	Propyl hexanoate	1311	1298	B,C	626-77-7	Ester of fatty acid	**3,75E−03**	**7,69E−06**	5,06E−01	−2.4199	8.7436
121	1,6-Octadien-3-ol, 3,7-dimethyl-/Linalool	1546	1553	A	78-70-6	Monoterpene	**3,86E−03**	**1,58E−03**	**1,56E−04**	5.2547	−9.5246
122	Pentanoic acid, 2-hydroxy-4-methyl-, methyl ester	1481	1510	B,C	40348-72-9	Methyl ester of branched acid	**4,00E−03**	**3,87E−02**	1,79E−01	5.3260	−7.3684
123	Riesling acetal	1639	1638	B,C	NA	Norisoprenoid	**4,20E−03**	**2,96E−06**	**2,26E−05**	3.8155	−11.6408
124	5-Hepten-2-one, 6-methyl-	1343	1347	A	110-93-0	Monoterpene	**4,26E−03**	2,92E−01	3,82E−01	7.2685	−5.3383
125	Octanoic acid, ethyl ester/Ethyl octanoate	1441	1438	A	106-32-1	Ethyl ester of fatty acid	**5,30E−03**	4,76E−01	5,25E−01	6.3919	−3.4892
126	Hotrienol	1604	1573	B,C	29957-43-5	Monoterpene	**6,41E−03**	**4,57E−03**	5,46E−01	4.9487	10.5093
127	2-Phenylethyl butyrate	1972	1958	B,C	103-52-6	Ester of fatty acid	**6,80E−03**	**3,24E−05**	**4,35E−02**	4.9543	−8.1298
128	2-propenoic acid, dodecyl ester	1916	1996	B,C	2156-97-0	Ester	**6,99E−03**	**9,24E−02**	2,20E−01	7.6271	−3.0440
129	Decanoic acid	2275	2288	A	334-48-5	Fatty acid	**7,03E−03**	2,12E−01	3,54E−01	7.0374	1.0987
130	6-Octen-1-ol, 3,7-dimethyl-, acetate/Citronellol acetate	1656	1671	B,C	150-84-5	Monoterpene	**7,35E−03**	1,91E−01	3,68E−01	−5.6030	1.6546
131	Analyte 603	1353	NA				**8,11E−03**	4,73E−01	4,07E−01	7.0782	−2.1159
132	Naphthalene, 2,3-dimethyl-/ guajen	1997	2008	B,C	581-40-8	Hydrocarbon	**9,28E−03**	7,54E−02	1,61E−01	7.2035	−0.5028
133	Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl	1446	1437	B,C	475-03-6	Norisoprenoid	**9,38E−03**	**1,02E−02**	5,15E−01	6.7926	4.4486
134	1-(Diethoxymethyl)-4-methylbenzene	1439	NA	C	NA	Phenolics	**9,64E−03**	2,75E−01	1,93E−01	6.8984	−1.8983
135	1-Butanol, 3-methyl-acetate/Isoamyl acetate	1131	1126	B,C	123-92-2	Higher alcohol acetate	**9,90E−03**	3,49E−01	4,54E−01	−6.5702	−2.2461
136	2-Butanol	1030	1033	A	78-92-2	Higher alcohol	**1,00E−02**	**9,04E−03**	5,16E−02	4.7531	3.6398
137	Acetic acid, butyl ester/Butyl acetate	1079	1066	A	123-86-4	Higher alcohol acetate	**1,01E−02**	7,93E−02	4,45E−01	−5.7754	−2.0446
138	1-Hepten-2-ol, acetate	1433	NA	B,C	1541-02-2	Higher alcohol acetate/GLV	**1,03E−02**	9,97E−02	2,30E−01	4.4589	7.9893
139	1,3-Oxathiane	1359	NA	C	646-12-8	VSC	**1,11E−02**	**1,70E−02**	2,44E−01	5.3219	−7.2096
140	Benzaldehyde	1532	1520	A	100-52-7	Phenolics	**1,12E−02**	**4,61E−07**	**7,44E−05**	0.7646	−0.3789
141	(*E*)-linalool oxide	1443	1453	A	5989-33-3	Monoterpene	**1,16E−02**	**3,72E−03**	**2,77E−02**	5.7285	−9.6598
142	Octanoic acid, methyl ester/Methyl octanoate	1394	1373	B,C	111-11-5	Methyl ester of fatty acid	**1,17E−02**	**1,45E−02**	2,07E−01	7.6353	1.9678
143	3-Nonen-1-ol, (*Z*)-	1687	1693	B,C	10340-23-5	GLV	**1,23E−02**	**7,37E−05**	3,25E−01	5.7680	6.6528
144	2(3H)-Furanone, 5-ethoxydihydro-/γ-Ethoxybutyrolactone	1733	1728	B,C	932-85-4	Lactone	**1,28E−02**	**1,10E−02**	1,72E−01	5.9233	−9.5397
145	Acetic acid, octyl ester/Octyl acetate	1485	1483	B,C	112-14-1	Higher alcohol acetate	**1,28E−02**	5,37E−02	8,54E−02	−3.3078	−4.5519
146	Butanoic acid, 3-methyl-, 3-methylbutyl ester/Isoamyl valerianate	1298	1285	B,C	659-70-1	Ester of fatty acid	**1,30E−02**	**7,92E−04**	3,31E−01	5.4333	−11.8774
147	Propanoic acid	1540	1535	B,C	79-09-4	Fatty acid	**1,40E−02**	**9,87E−07**	2,70E−01	−2.3233	12.2075
148	Butanedioic acid, hydroxy-, diethyl ester	2097	2060	B,C	7554-12-3	Ester of fixed acid	**1,40E−02**	**1,14E−03**	1,37E−01	4.9757	−0.9054
149	Isopentyl 2-methylbutanoate	1272	1273	B,C	27625-35-0	Ester of branched acid	**1,43E−02**	**9,09E−02**	2,84E−01	6.7225	−5.4496
150	Menthol, (1r,3s,4r)-(+)-	1639	NA	C	23283-97-8	Monoterpene	**1,51E−02**	**5,59E−05**	**3,26E−03**	4.9150	6.6623
151	Dodecanoic acid, ethyl ester/Ethyl laurate	1842	1833	B,C	106-33-2	Ethyl ester of fatty acid	**1,61E−02**	**2,63E−04**	3,81E−01	6.1430	1.8746

(*continued on next page*)

Table 3 (*continued*)

Analyte	Peak	Calculated LRI	Reported LRI	Identification	CAS	Class	H q value	V (q value)	H:V (q value)	PC1	PC2
152	Octanoic acid, 3-methylbutyl ester/Isoamyl octanoate	1653	1652	A	2035-99-6	Ester of fatty acid	**1,62E−02**	**5,85E−04**	3,37E−01	5.1319	−4.5444
153	4-Nonene, 2,3,3-trimethyl-, (*E*)-	1304	1300	B,C	63830-67-1	Monoterpene	**1,67E−02**	**2,23E−02**	1,69E−01	−0.9392	2.7775
154	Furan, 2-(1,2-diethoxyethyl)-	1597	NA	C	14133-54-1	Furane	**1,70E−02**	**4,07E−04**	**2,65E−02**	6.5143	−4.4974
155	Ethanone, 1-phenyl-	1649	1645	B,C	98-86-2	Ketone	**1,75E−02**	8,75E−02	1,83E−01	4.0924	−6.2813
156	2-decanone	1494	1496	B,C	693-54-9	Alpha-ketone	**1,76E−02**	1,36E−01	4,82E−01	6.9195	2.6022
157	Analyte 1170	1838					**1,82E−02**	**1,96E−03**	**1,25E−02**	5.0889	−8.9332
158	Azulene	1740	1710	B,C	275-51-4	Hydrocarbon	**1,83E−02**	**1,07E−03**	**3,54E−06**	−2.7761	−9.3502
159	2,6-Octadiene, 4,5-dimethyl	1298	NA	C	18476-57-8	Monoterpene	**1,86E−02**	3,62E−01	4,06E−01	6.6277	5.2609
160	4-Methylhexanoic acid ethyl ester	1301	NA	C	NA	Ethyl ester of branched acids	**1,87E−02**	5,62E−01	5,68E−01	6.0554	1.3024
161	Methyl *trans*-2-(3-cyclopropyl-7-norcaranyl)acetate	1705	NA	C	NA	Norisoprenoid	**2,16E−02**	**1,56E−02**	**4,84E−02**	3.7204	10.7776
162	Crotonic anhydride	1842	NA	C	623-68-7	Fatty acid	**2,21E−02**	1,11E−01	3,25E−01	6.2352	4.9774
163	Cyanic acid, 2,2-dimethylpropyl ester	1966	NA	C	1459-44-5	Ester	**2,39E−02**	2,66E−01	2,27E−01	7.4938	0.9718
164	Analyte 917	1587	NA				**2,46E−02**	6,31E−02	4,34E−01	5.3361	−1.5137
165	3-Buten-1-ol, 3-methyl-, acetate	1198	1190	B,C	5205-07-2	Higher alcohol acetate	**2,47E−02**	**4,62E−03**	3,39E−01	−4.3254	6.3472
166	1-Isopropyl-4,7-dimethyl-1,2-dihydronaphthalene #/α-calacorene	1907	1934	B,C	23267-57-4	Sesquiterpene	**2,63E−02**	**4,42E−02**	5,07E−01	6.6689	6.1080
167	2-Furaldehyde diethyl acetal/.Furan, 2-(diethoxymethyl)-	1450	1442	B,C	13529-27-6	Furan	**2,64E−02**	1,52E−01	5,08E−01	−3.2813	1.9093
168	2,6-Octadienal, (*E,Z*)-	1343	NA	C	76917-23-2	Monoterpenes	**2,65E−02**	**2,04E−04**	7,03E−02	5.9533	11.0216
169	Butanoic acid, ethyl ester/Ethyl butyrate	1040	1031	A	105-54-4	Ethyl ester of fatty acid	**2,73E−02**	**1,06E−02**	4,30E−01	−1.7491	−3.5581
170	1-Pentanol, 3-methyl-/3-Methylpentanol	1314	1316	B,C	589-35-5	Higher alcohol	**2,82E−02**	**2,49E−07**	**1,98E−02**	4.4344	−12.2371
171	1,3,6-Octatriene, 3,7-dimethyl-, (*E*)-/ (*E*)-β ocimene	1258	1267	B,C	3779-61-1	Monoterpene	**2,88E−02**	**2,60E−03**	4,86E−04	4.8255	1.6383
172	Propanoic acid, 2-hydroxy-, 2-methylpropyl ester/Isobutyl lactate	1453	1455	B,C	585-24-0	Ester of fixed acid	**2,92E−02**	4,96E−01	5,47E−01	6.9442	−2.1289
173	Acetic acid, propyl ester/Propyl acetate	993	969	B,C	109-60-4	Higher alcohol acetate	**3,05E−02**	**5,62E−04**	3,17E−01	−2.7395	12.0030
174	2,4,6-Octatriene, 2,6-dimethyl-(neoallo-ocimene)	1394	1394	B,C	673-84-7	Monoterpene	**3,08E−02**	**8,82E−07**	**3,07E−02**	5.9392	3.4549
175	1,1-Butanediol, diacetate	1443	NA	C	29949-17-5	Higher alcohol acetate	**3,13E−02**	4,64E−01	**3,26E−02**	−4.0032	−6.4066

Identification assignments: A comparing mass spectra and retention time with those of pure standard, B retention index match on a similar phase column, C mass spectral database. Q values in bold indicate significant differences. GLV refers to green leaf volatiles, VSC refers to volatile sulphur compounds.

of methional and its derivatives are strongly dependent on juice methionine and YAN concentrations (Moreira et al., 2002). Production of methional is an exclusively enzymatic reaction, catalysed by aminotransferase and α-ketoacids decarboxylase (Landaud, Helinck, & Bonnarme, 2008) and VCS formation is strongly influenced by fermentation media. Low YAN values during fermentation can stimulate the production of unwanted VSCs such as H_2S (Moreira, de Pinho, Santos, & Vasconcelos, 2011). Therefore, higher YAN values in juices from H2 may have potentially contributed to the lower VSC content in the corresponding wines.

4. Conclusion

In this study, GC × GC-TOFMS was utilised to determine the effect of harvest date on wine volatile compounds. This powerful approach allowed the detection of a vast array of compounds belonging to the classes of terpenoids, norisoprenoids, esters, acids, higher alcohols, sulphur compounds, ketones and others. Amongst the 1276 compounds detected, 175 significantly contributed to the separation of samples according to the harvest dates at signal to noise ratio above 100. A rational modification of volatile compound composition was able to be distinguished across the four vineyards located in the same mesoclimate. This was despite a temporal gap of a week between designated harvests and differences in various vineyard management strategies. Dozens of lipoxygenase derived compounds decreased with delayed harvest and a similar pattern was also observed for wine sulphur compounds. Abundances of some higher alcohol acetates increased. This study suggested that the plateau of sugar loading into the berry could be used as a reliable physiological indicator from which to determine potential harvest dates according to a specific wine aromatic composition. Further studies using other varieties from different climates and winemaking protocol would be valuable to further demonstrate the robustness of such a model.

Acknowledgements

The authors thanks to Australia's grape growers and winemakers for their financial support through their investment body Wine Australia. The research was also supported by the ADPP 2015 program funded by Autonomous Province of Trento (Italy). KS received a NWGIC travel bursary for her stay at FEM. The authors would also like to thank to the producers in Griffith for access to vineyards and grape donations.

Conflict of interest

The authors declare no competing financial interest nor ethical issues.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.10.135.

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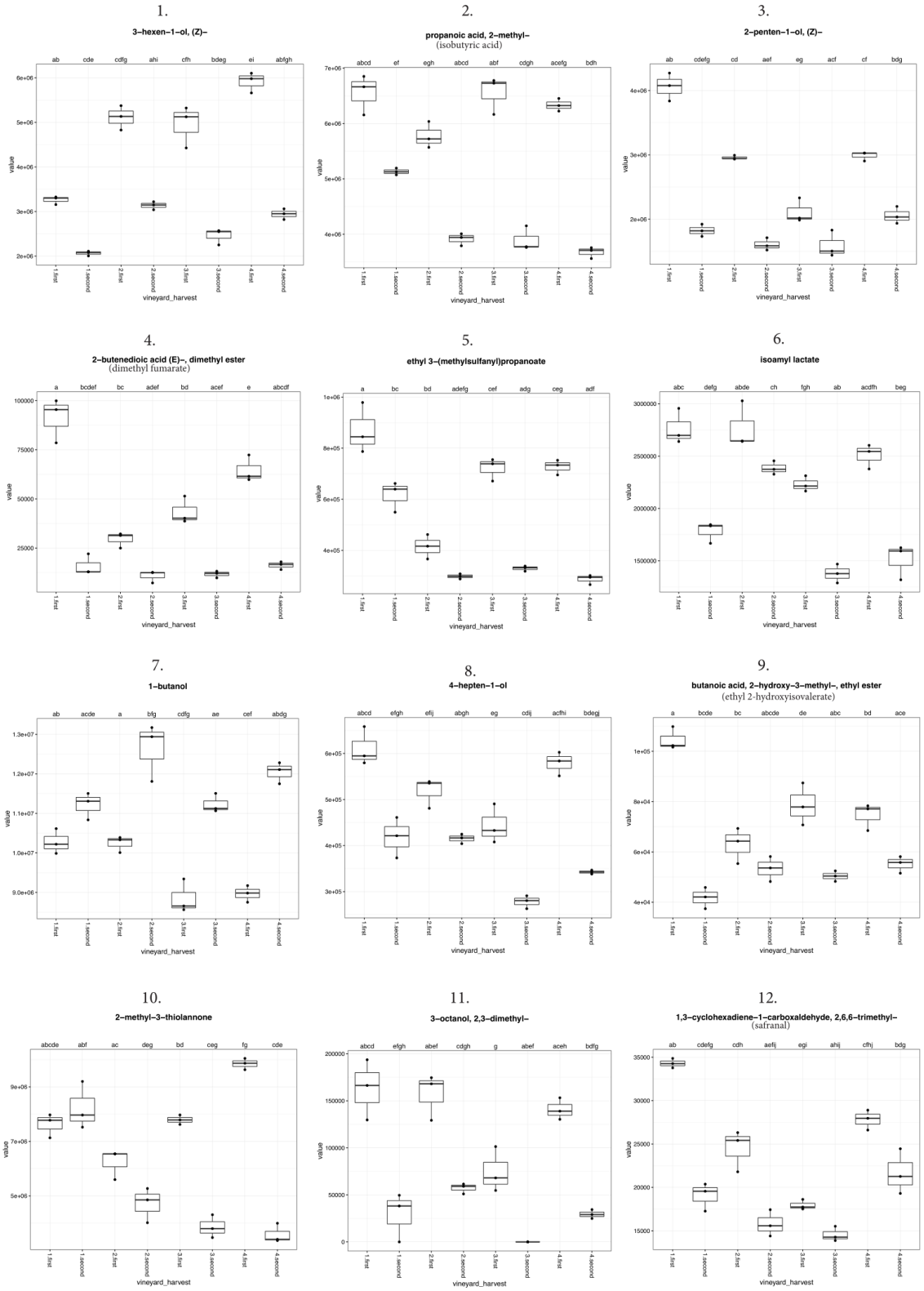
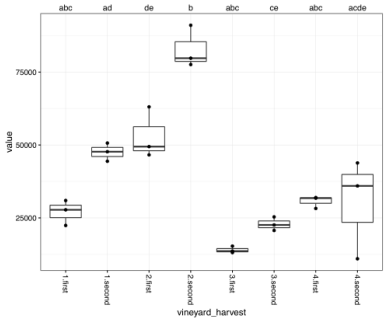
Figure S1. Boxplots of 175 compounds that ware significantly altered by the harvest date, presented in an order as listed in a Table 3. 1,2,3,4 refers to Vineyard 1,2,3,4, respectively, whereas first and second indicates H1 and H2, respectively.



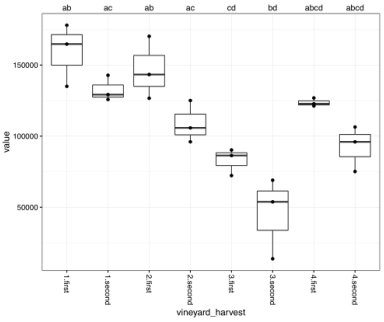
109.

4–hydroxy–3–methylacetophenone



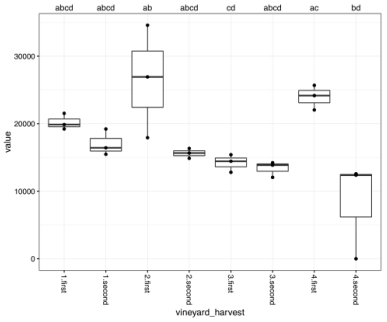
110.

2(3h)–furanone, dihydro–5–pentyl–
(γ- nonalactone)



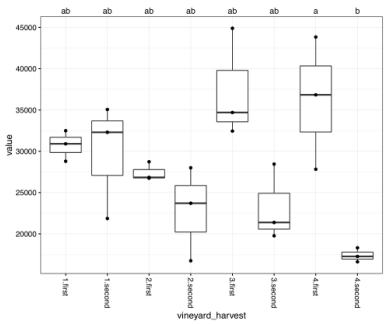
111.

3(2h)–furanone, 5–(2,2–dimethylpropyl)–2,2,4–trimethyl–



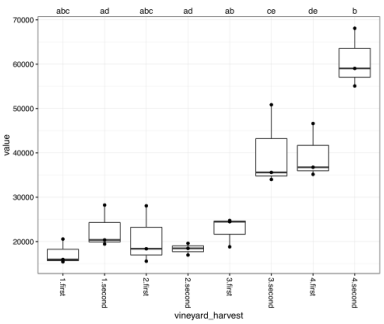
112.

3,3–dimethoxy–1–phenyl–1,2–propanedione



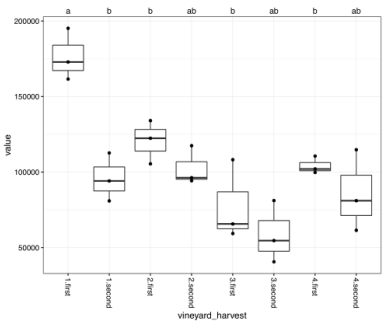
113.

ethanone, 1–(3–ethyloxiranyl)–



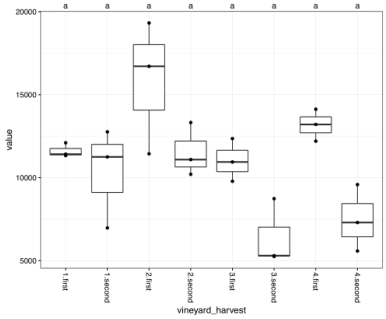
114.

cyclohexanone, 2,2,6–trimethyl–



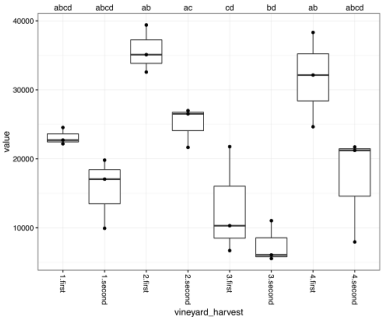
115.

2h–pyran–3–ol, 6–ethenyltetrahydro–2,2,6–trimethyl–
(linalool oxide pyranoside)



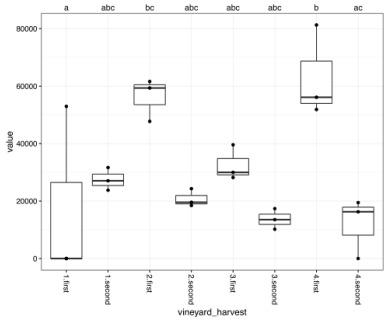
116.

3–(4–isopropylphenyl)–2–methylpropionaldehyde



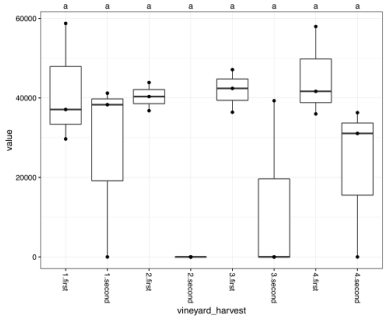
117.

spiro[bicyclo[3.1.1]heptane–2,2'–oxirane], 6,6–dimethyl–
(β-pinene oxide)



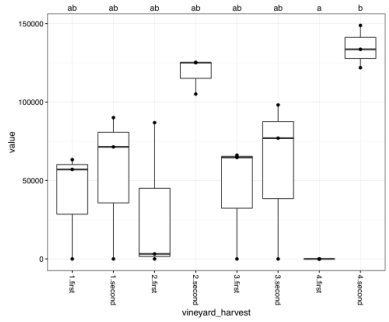
118.

analyte 609



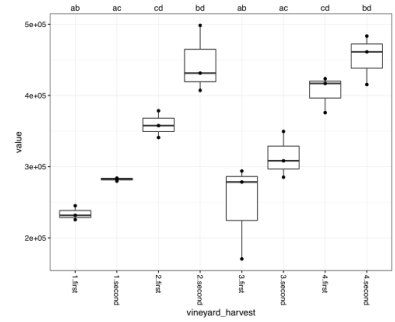
119.

ethyl 2–hexenoate, trans–



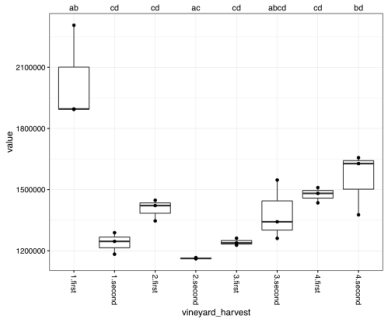
120.

propyl hexanoate



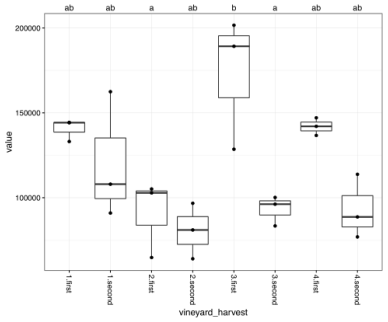
121.

1,6–octadien–3–ol, 3,7–dimethyl–
(linalool)



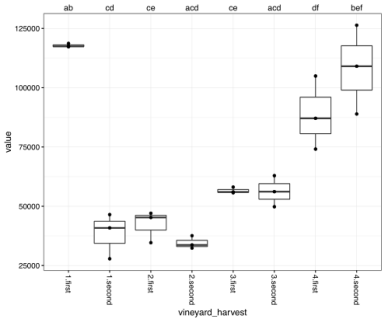
122.

pentanoic acid, 2–hydroxy–4–methyl–, methyl ester



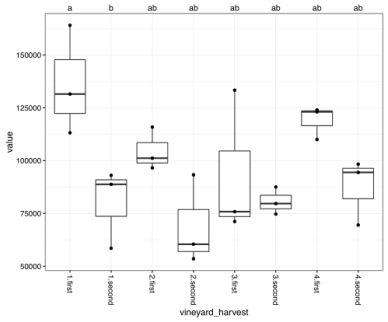
123.

riesling acetal



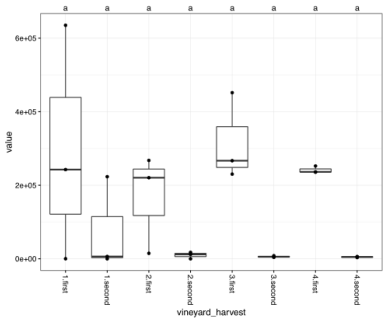
124.

5–hepten–2–one, 6–methyl–



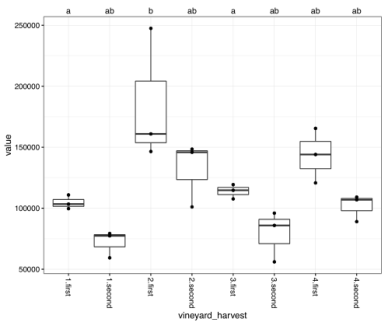
125.

octanoic acid, ethyl ester
ethyl octanoate)



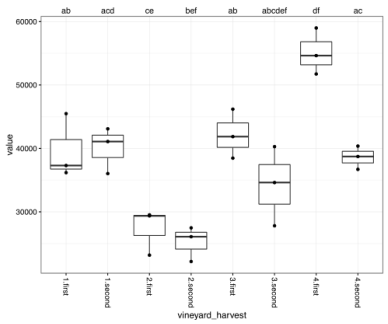
126.

1,5,7–octatrien–3–ol, 3,7–dimethyl–



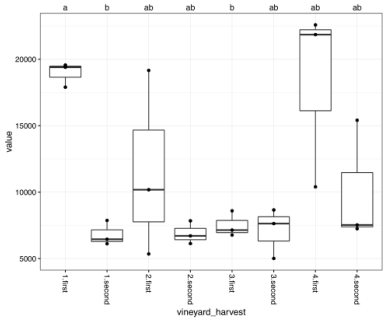
127.

2–phenylethyl butyrate



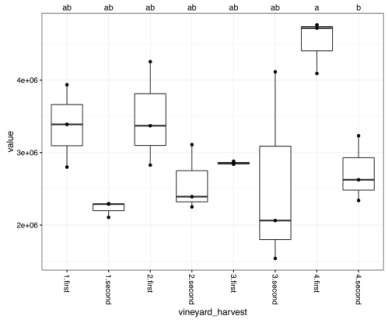
128.

2–propenoic acid, dodecyl ester



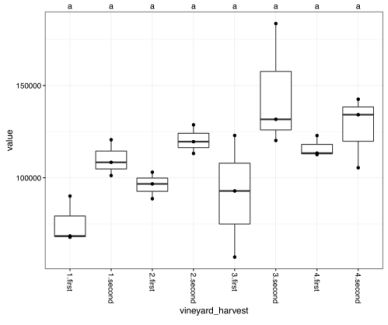
129.

decanoic acid



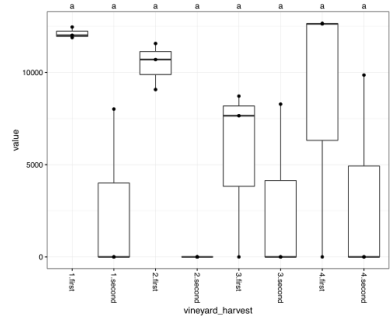
130.

6–octen–1–ol, 3,7–dimethyl–, acetate
(citronellol acetate)



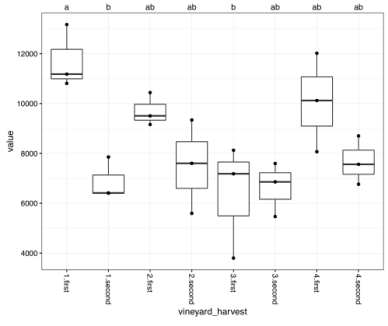
131.

analyte 603

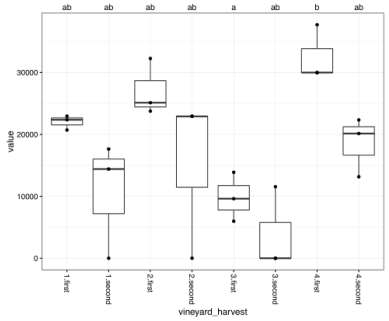


132.

naphthalene, 2,3–dimethyl–
(guajen)



133.



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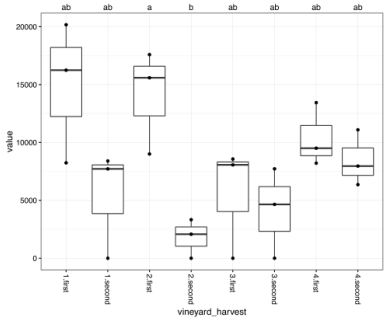
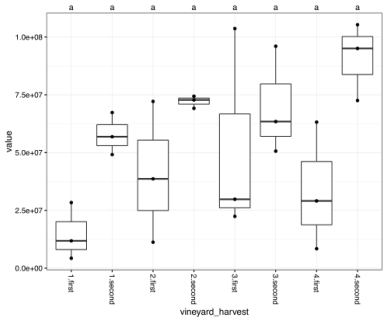
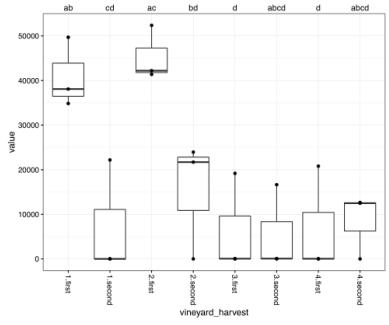


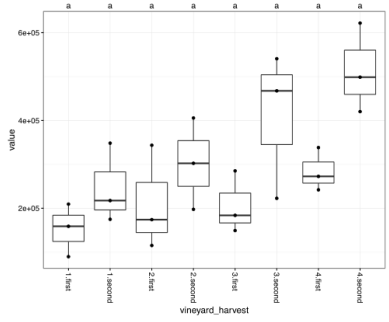
135.



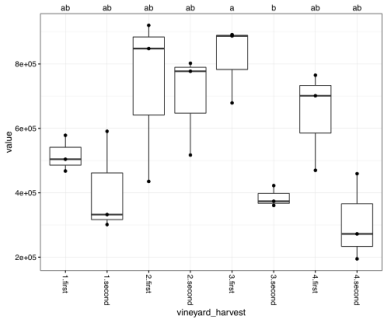
136.



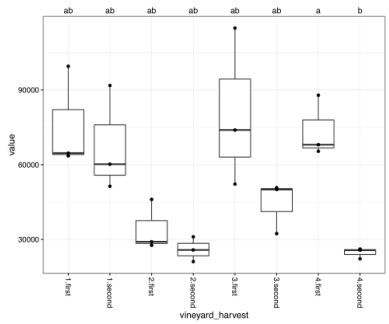
137.



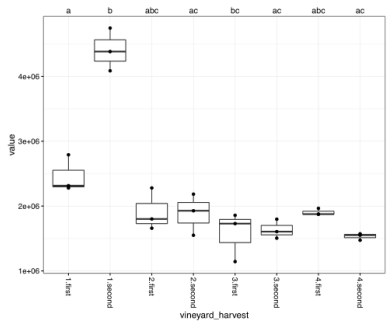
138.



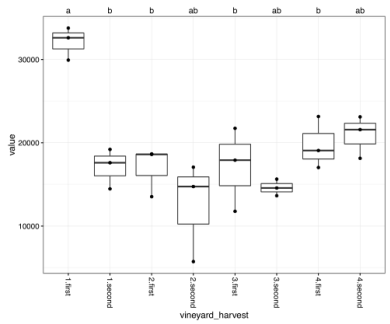
139.



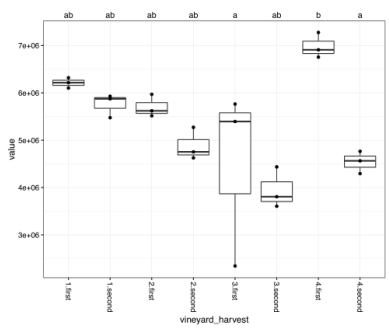
140.



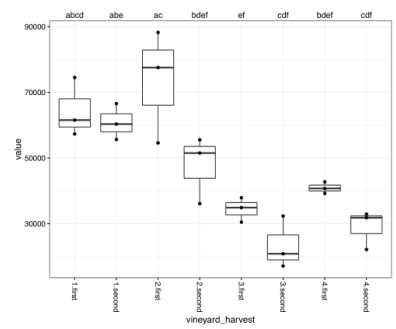
141.



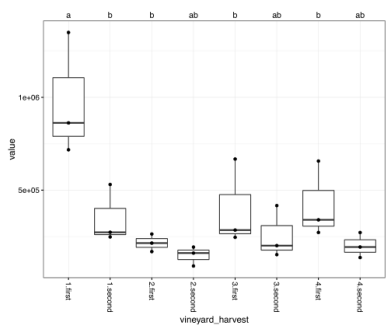
142.



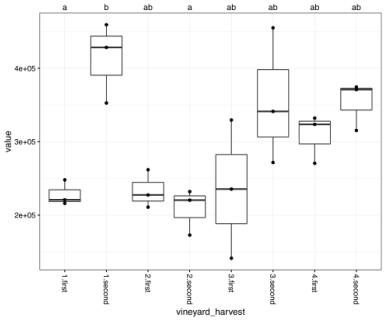
143.



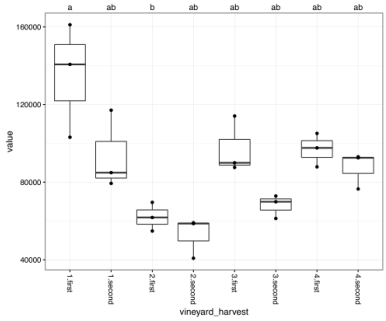
144.



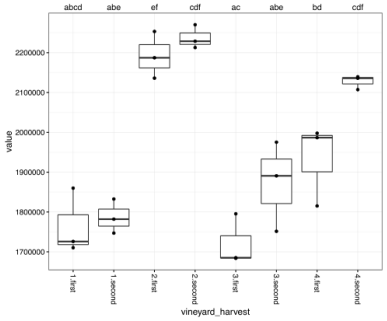
145.



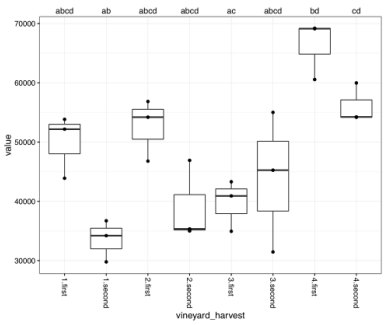
146.



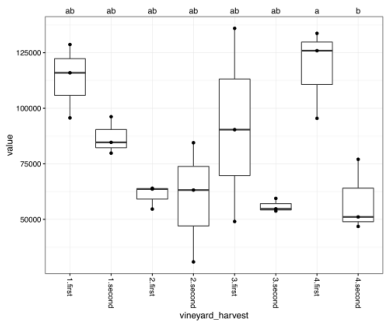
147.



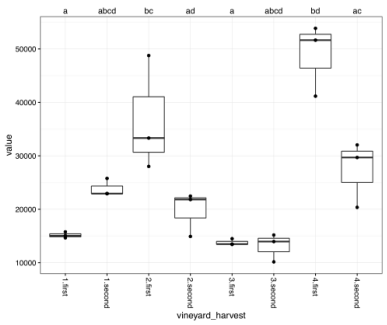
148



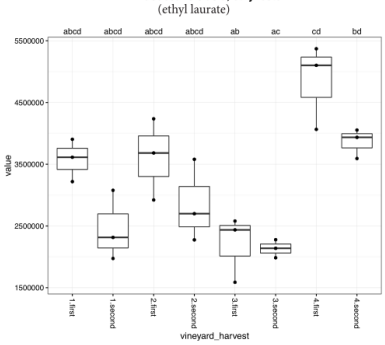
149.



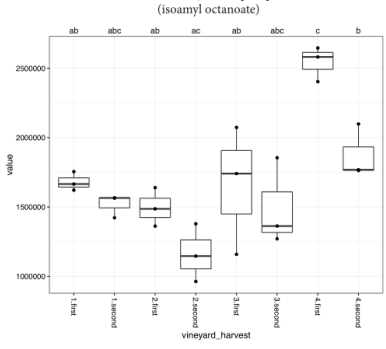
150.



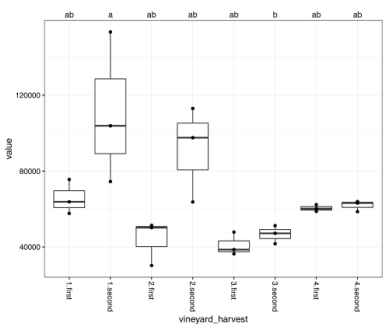
151.



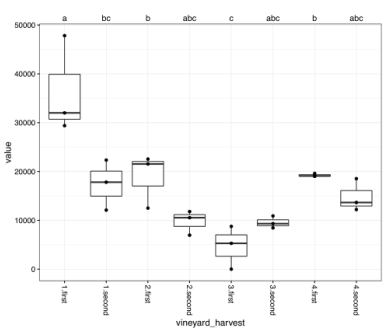
152.



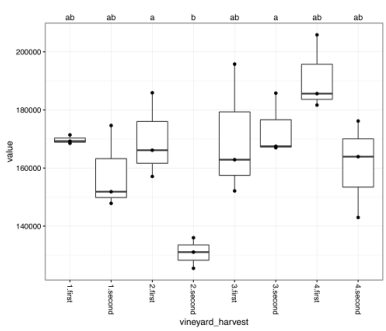
153.



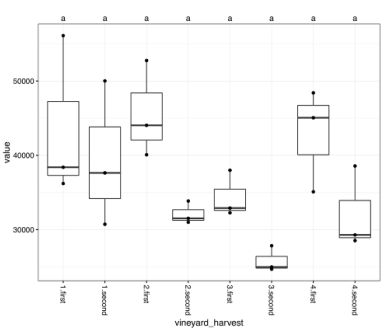
154.



155.

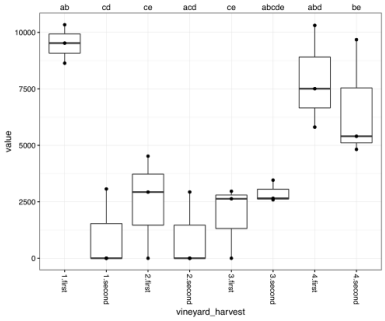


156.



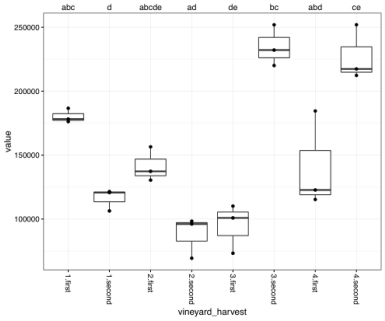
157.

analyte 1170



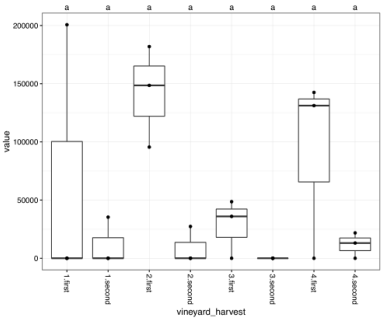
158.

azulene



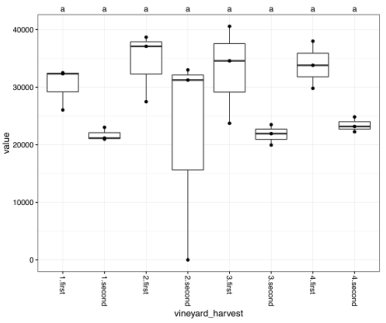
159.

2,6–octadiene, 4,5–dimethyl



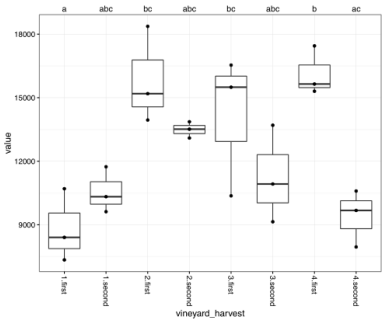
160.

4–methylhexanoic acid ethyl ester



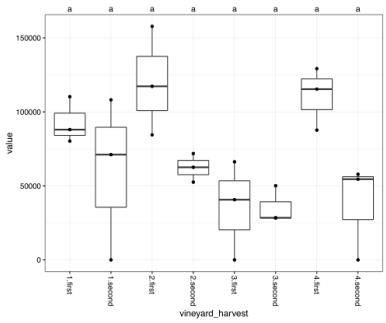
161.

methyl trans–2–(3–cyclopropyl–7–norcaranyl)acetate



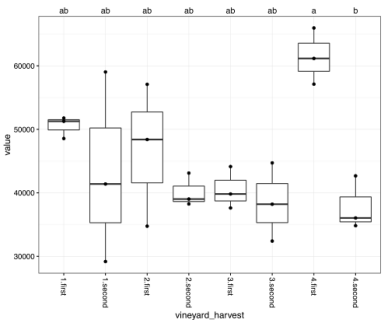
162.

crotonic anhydride



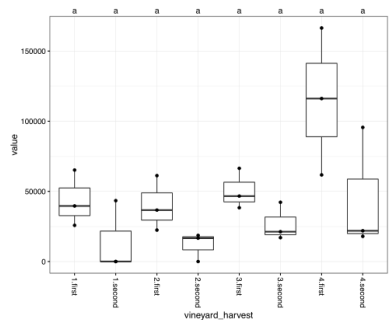
163.

cyanic acid, 2,2–dimethylpropyl ester



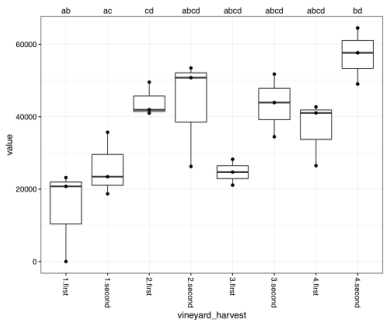
164.

analyte 917



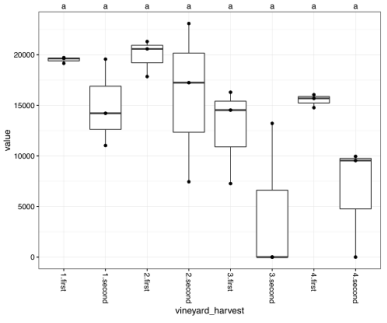
165.

3–buten–1–ol, 3–methyl–, acetate



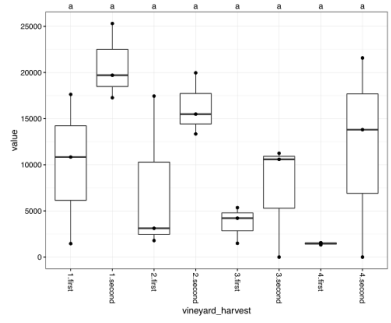
166.

1–isopropyl–4,7–dimethyl–1,2–dihydronaphthalene #
(α-calacorene)



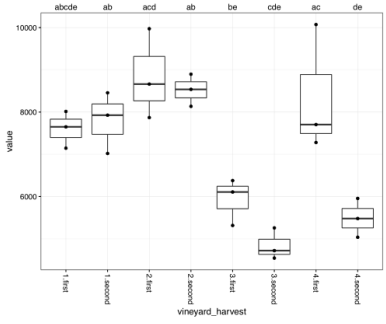
167.

2–furaldehyde diethyl acetal
(furan, 2-(diethoxymethyl))

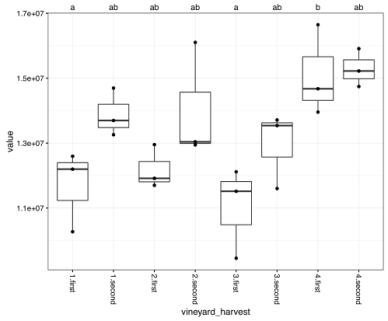


168.

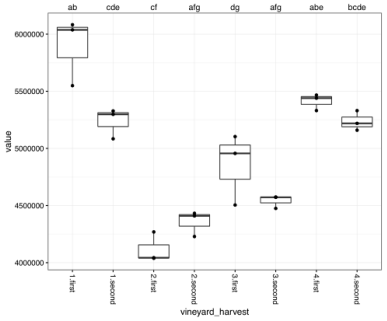
2,6–octadienal, (E,Z)–



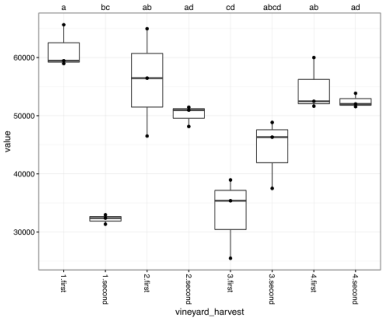
169.



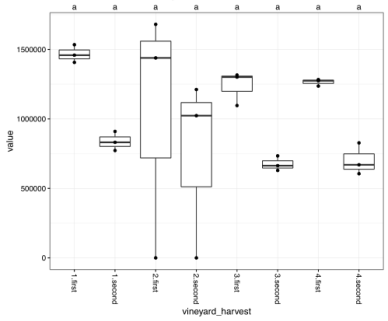
170.



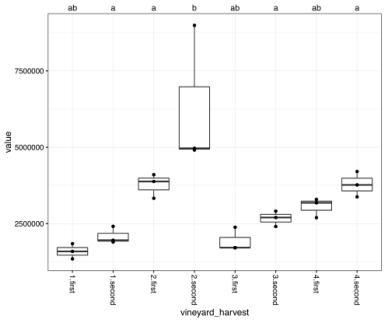
171.



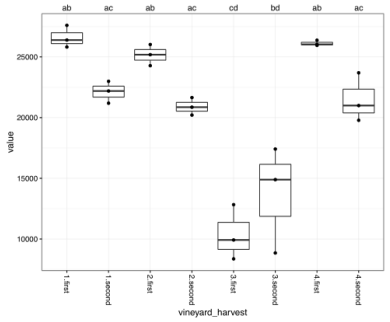
172.



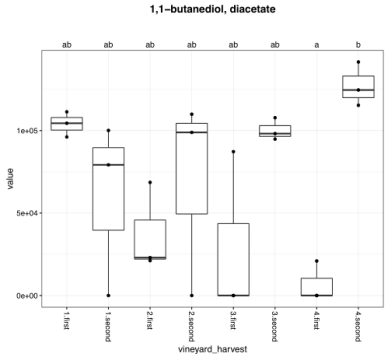
173.



174.

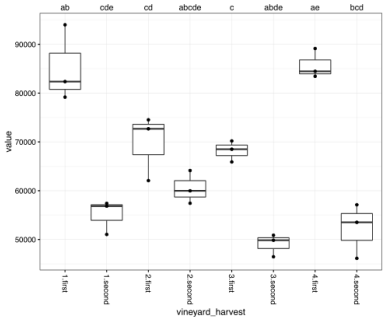


175.



13.

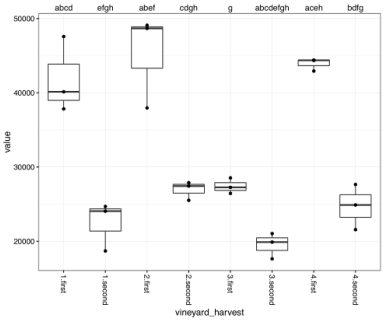
ethyl−isobutyl succinate



14.

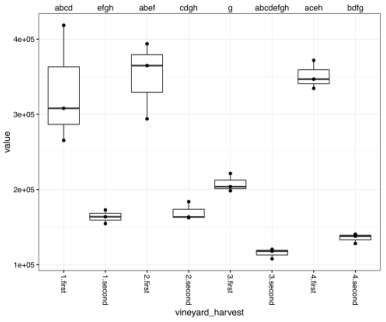
1−cyclohexene−1−carboxaldehyde, 2,6,6−trimethyl−

(β- cyclocitral)



15.

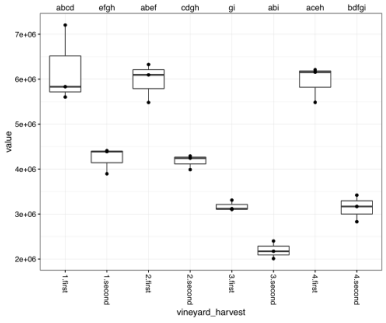
furan, 2−butyltetrahydro−



16.

1−(2,6,6−trimethyl−1,3−cyclohexadien−1−yl)−2−buten−1−one

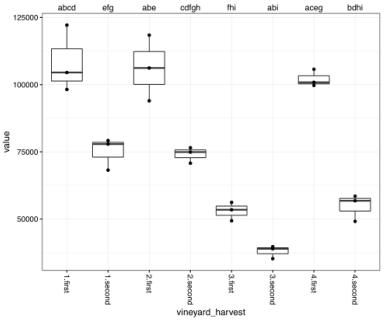
((*E*)-β- damascenone)



17.

1−(2,6,6−trimethyl−1,3−cyclohexadien−1−yl)−2−buten−1−one, (Z)

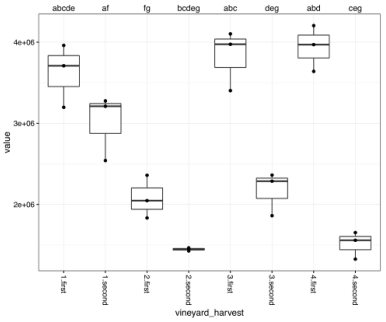
((*Z*)-β- damascenone)



18.

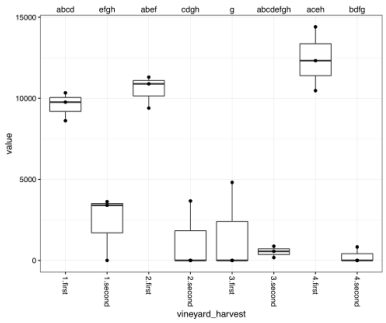
1−propanol, 3−(methylthio)−

Methionol



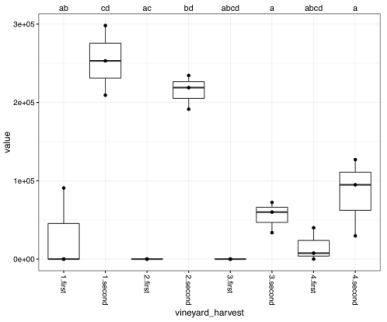
19.

analyte 697



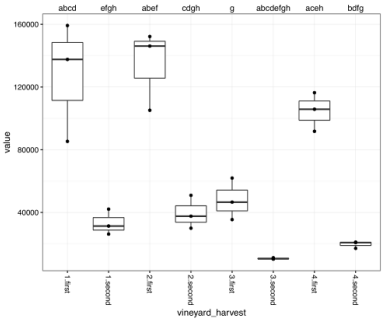
20.

2,4−hexadiene, 1,1−diethoxy−, (E,E)−



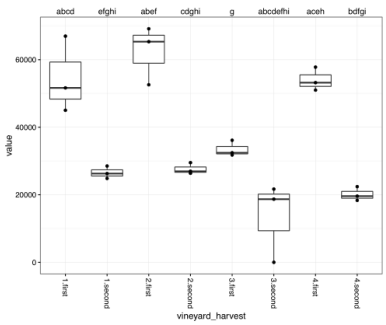
21.

3−nonene, 2−methyl−



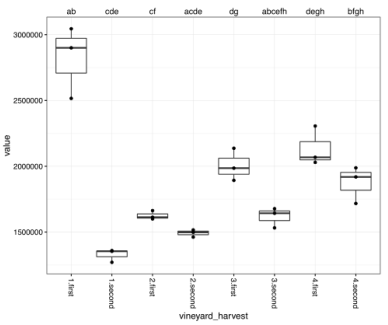
22.

sulfurous acid, 2−pentyl pentyl ester



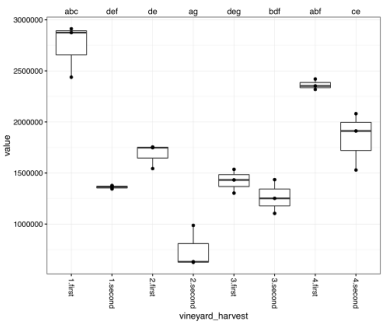
23.

pentanoic acid, 2−hydroxy−4−methyl−, ethyl ester



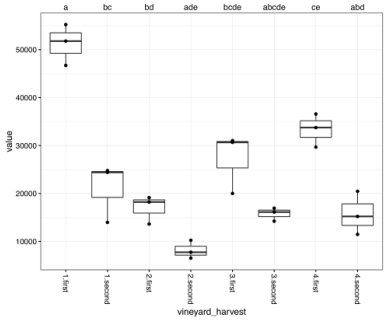
24.

2−nonanone



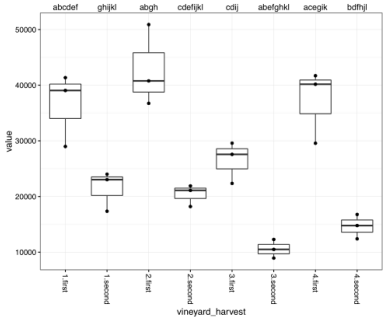
25.

2–thiophenecarboxaldehyde



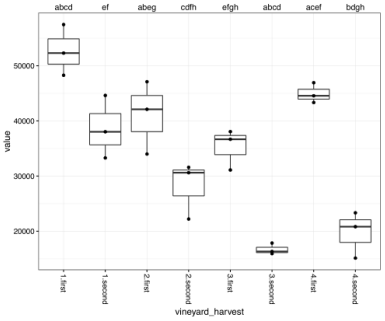
26.

2–furanol, tetrahydro–



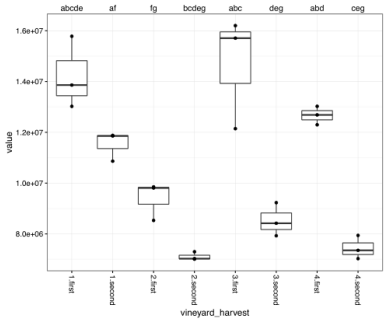
27.

(6Z)–nonen–1–ol



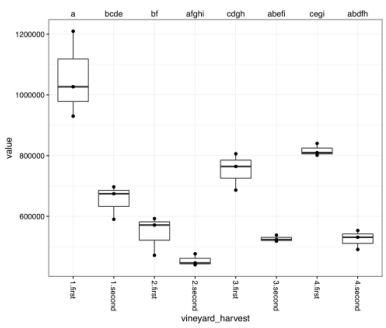
28.

butanoic acid, 2–methyl–
(2-methylbutyric acid)



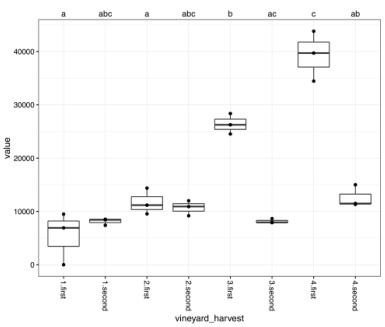
29.

benzeneacetic acid, ethyl ester
(ethyl phenylacetate)



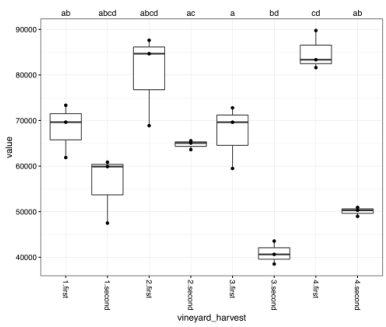
30.

3,5–hexadien–1–ol, (Z)–



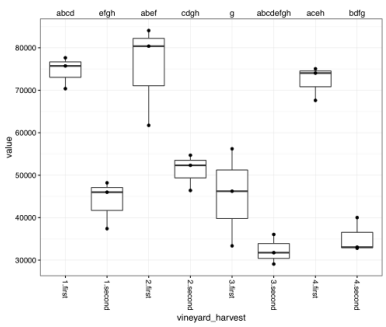
31.

7,7a–dimethyl–3a,4,5,7a–tetrahydro–3h–benzofuran–2–one



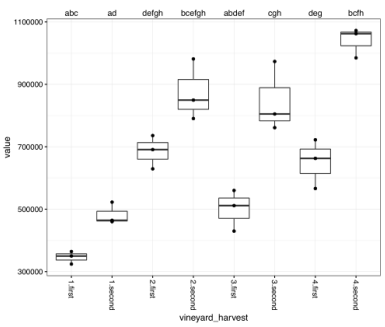
32.

theaspirane b



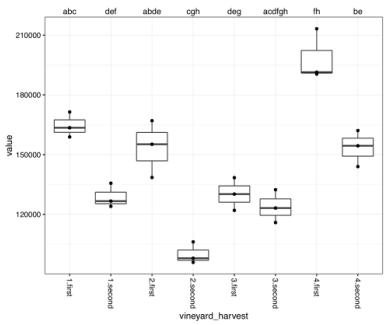
33.

3–ethoxypropyl acetate



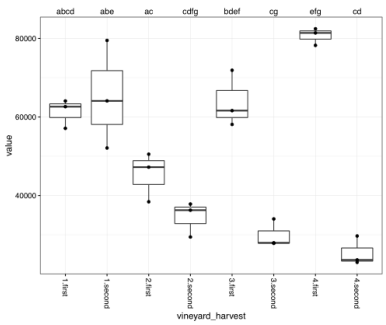
34.

2–undecanone



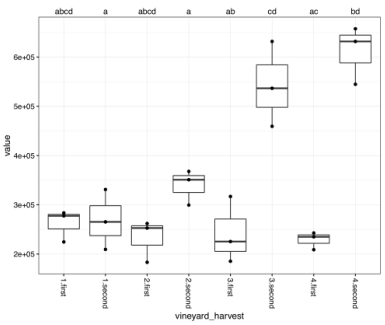
35.

2–propanamine, n–(1,1–dimethylethyl)–1,1–dimethoxy–2–methyl–



36.

2–butanone, 3–hydroxy–
(acetoin)



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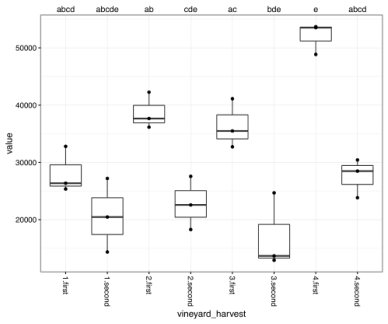

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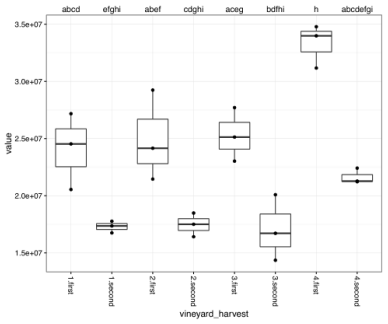

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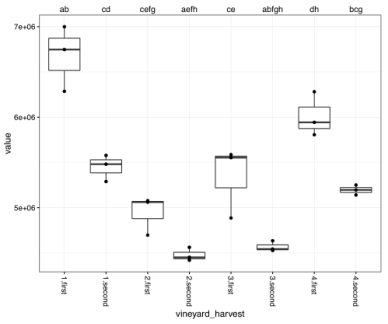

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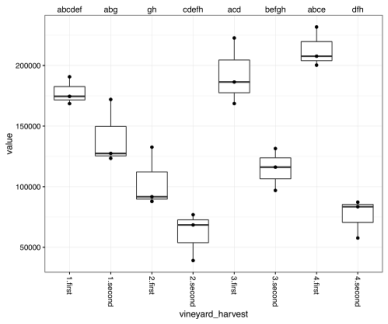

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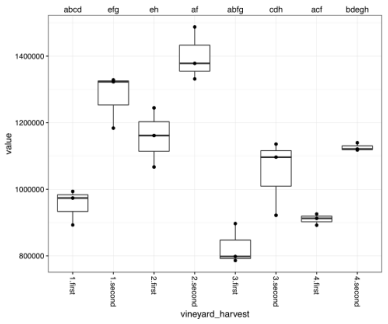

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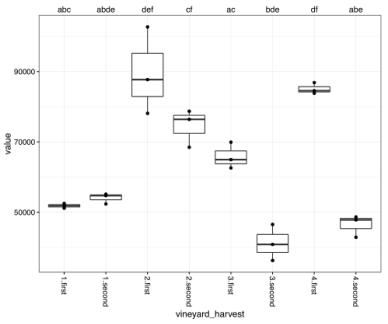

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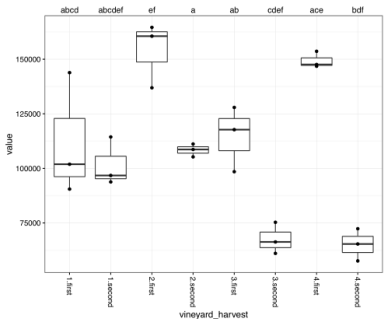

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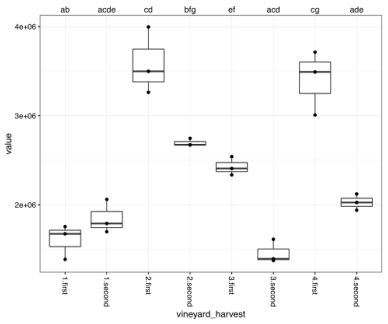

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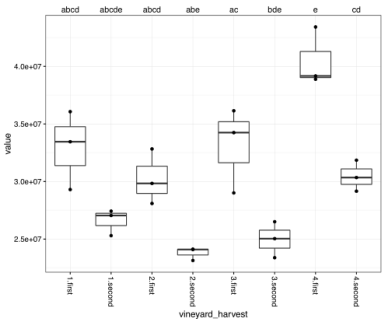

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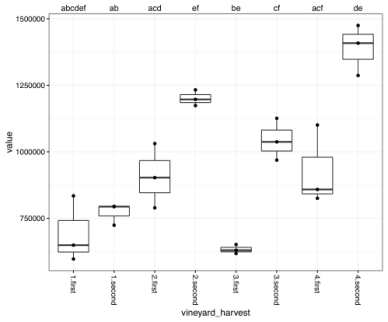

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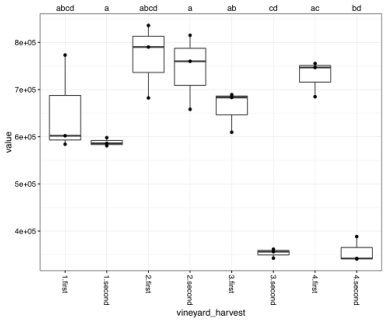

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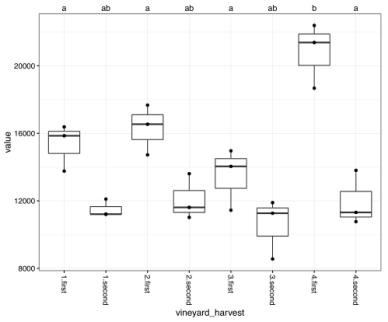

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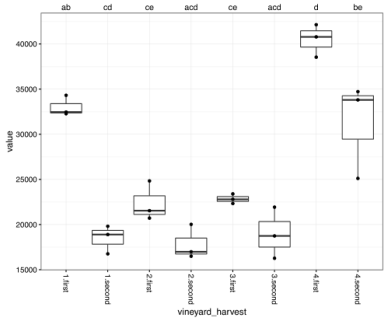

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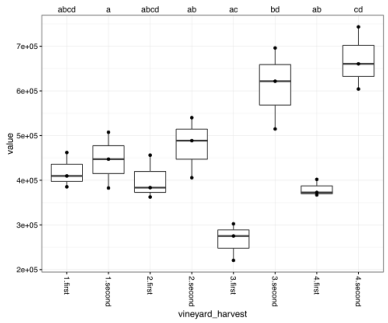

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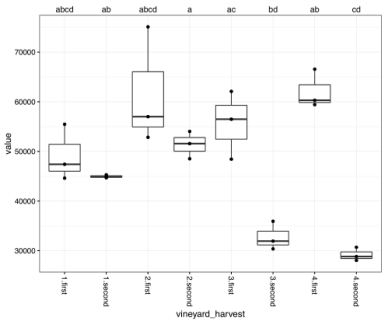

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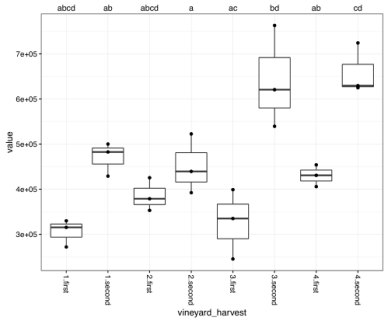

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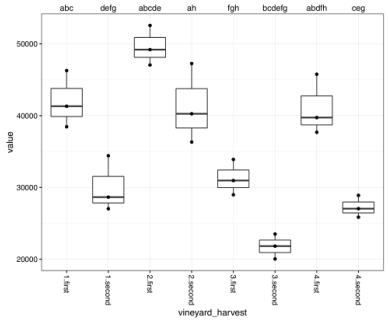

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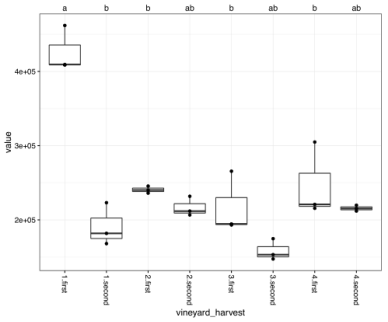

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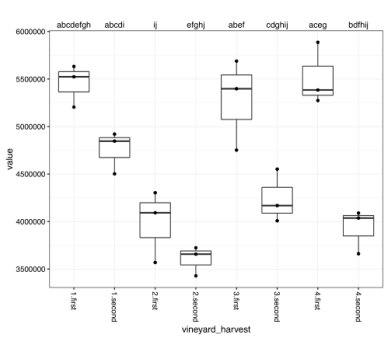

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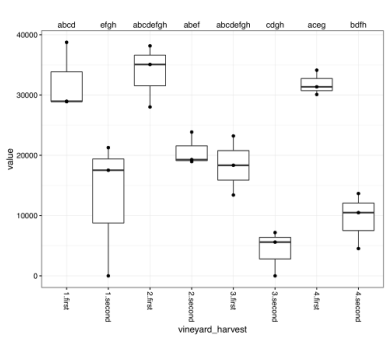

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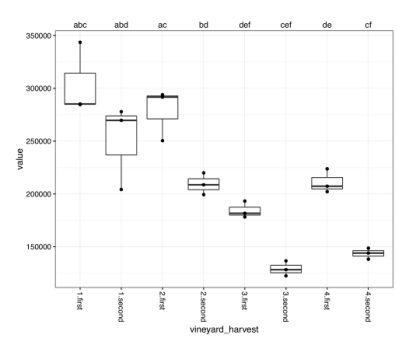

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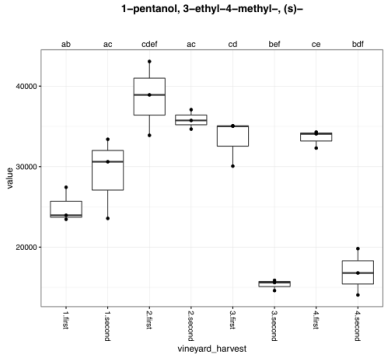

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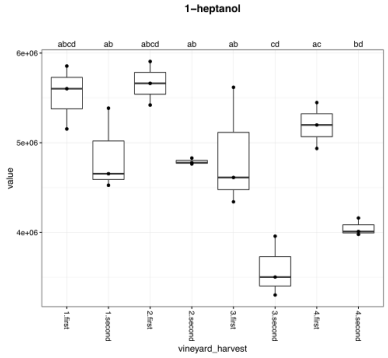

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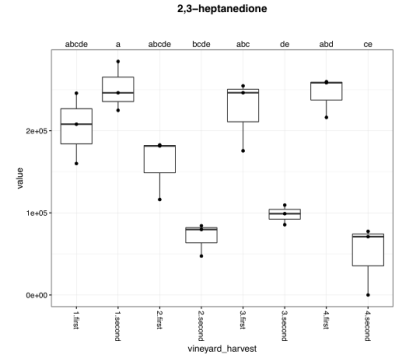

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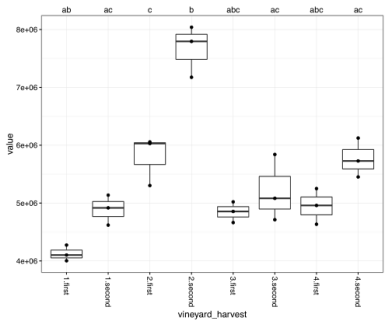

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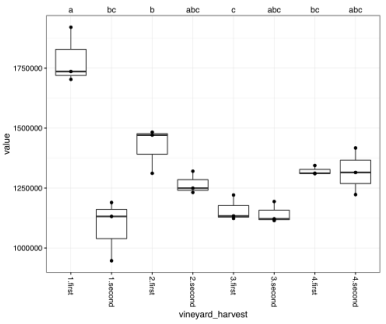

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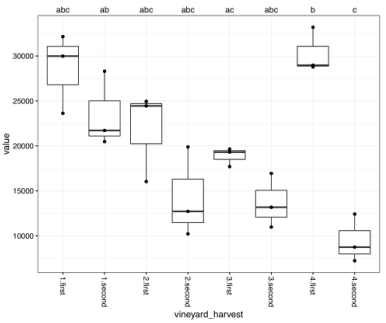

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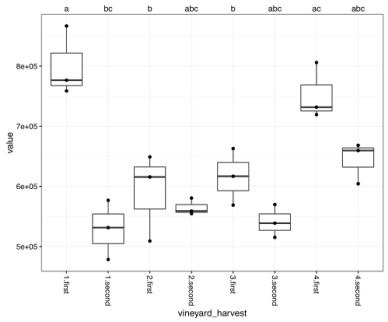

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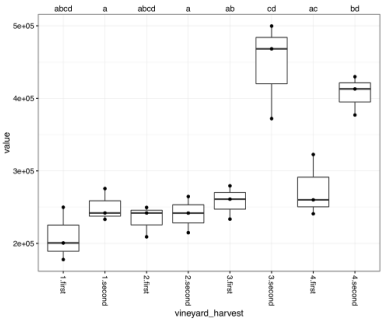

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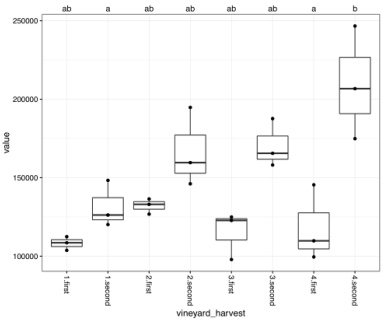

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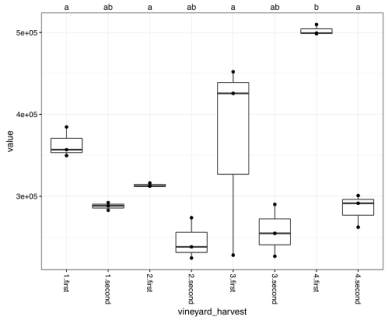

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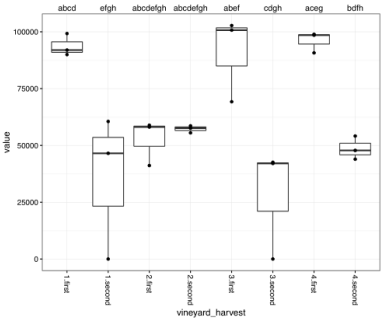

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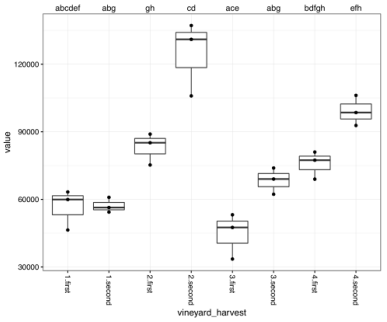

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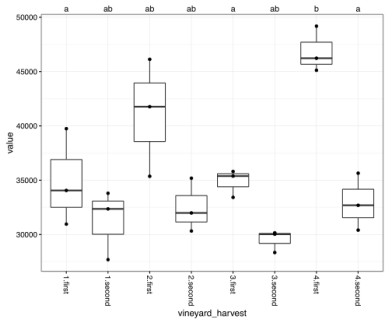

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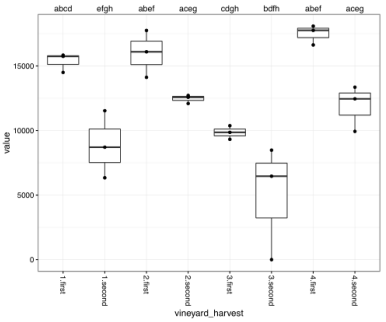

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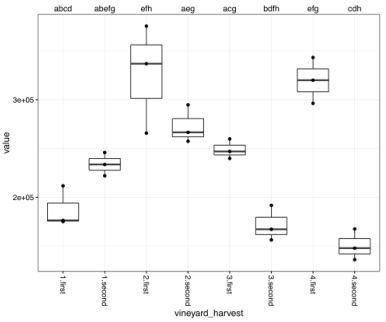

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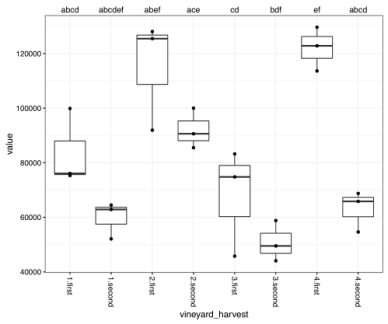

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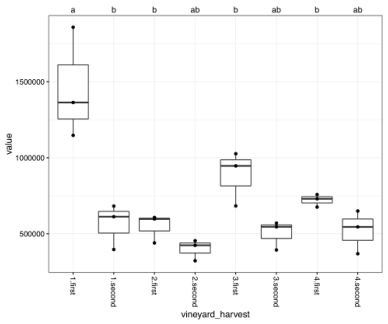

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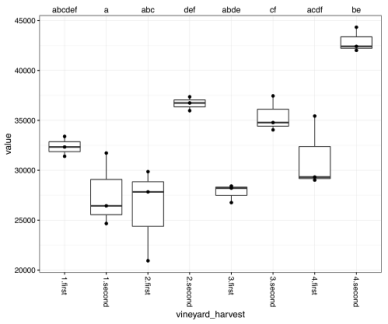

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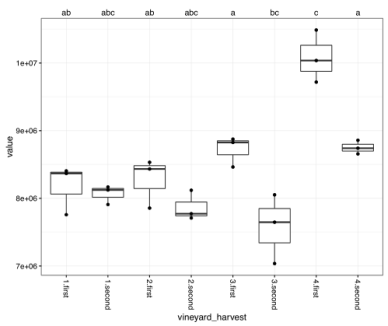

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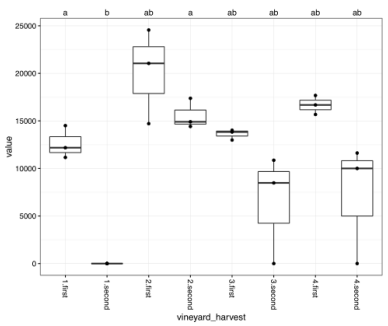

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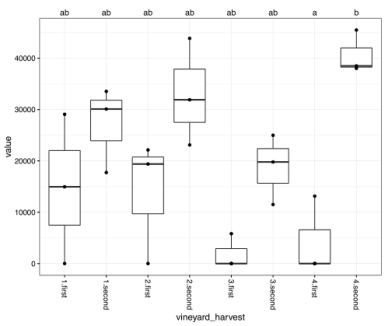

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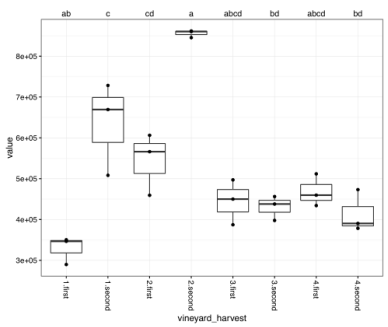

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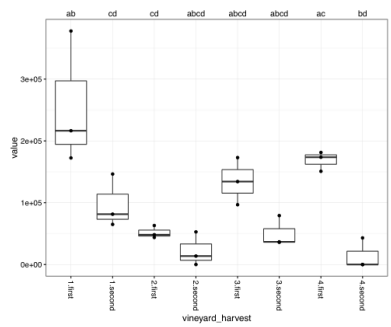

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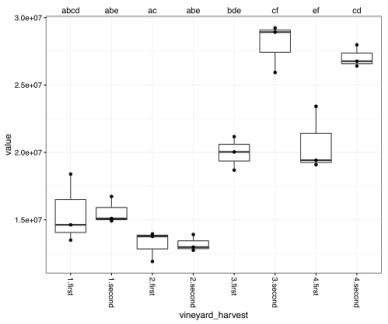

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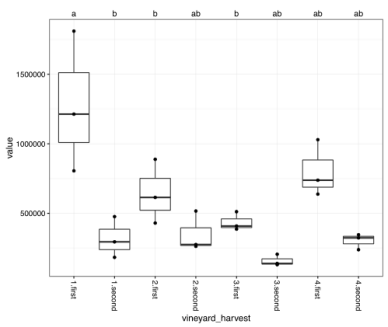

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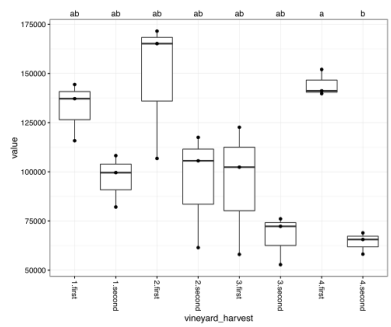

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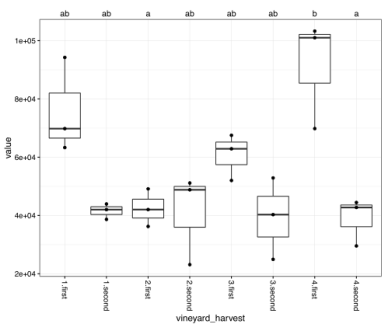

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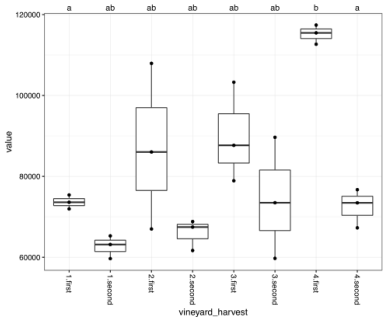

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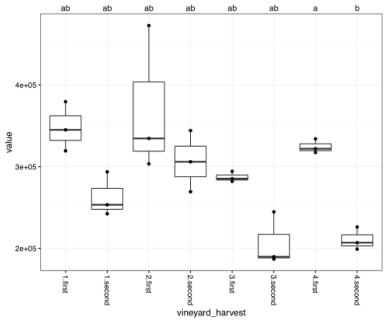

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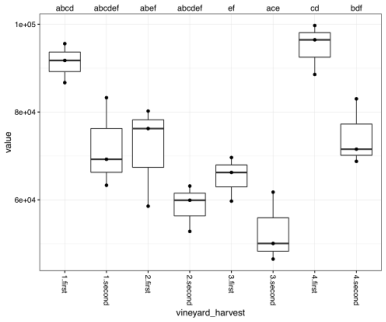

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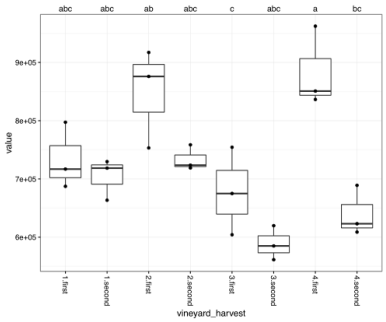

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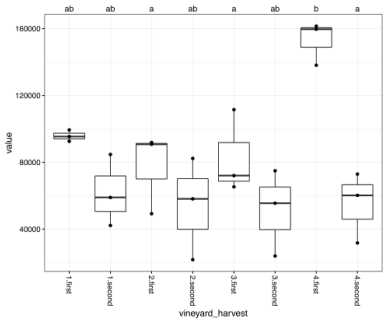

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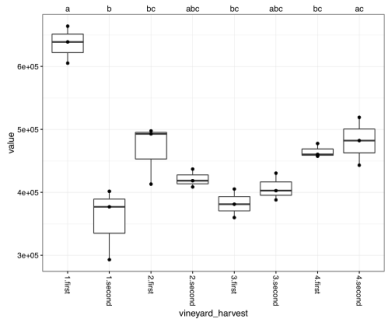

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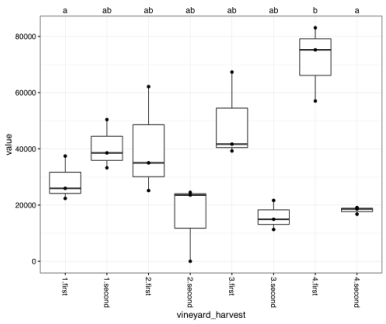

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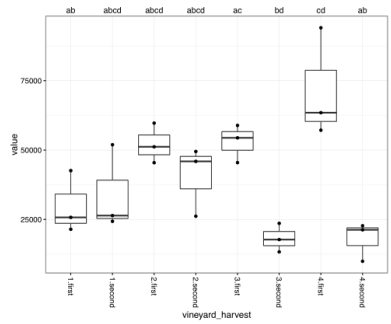

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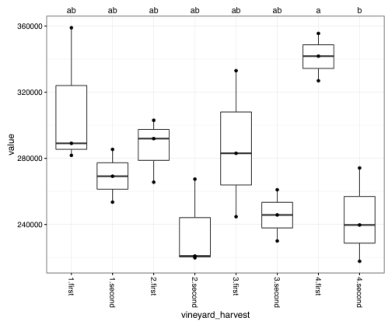

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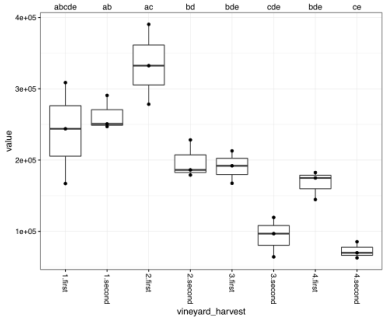

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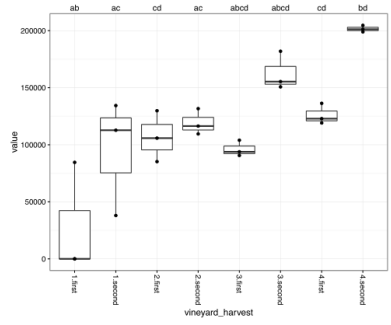

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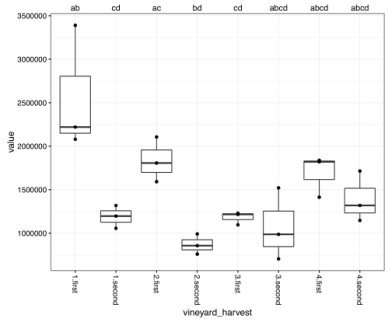

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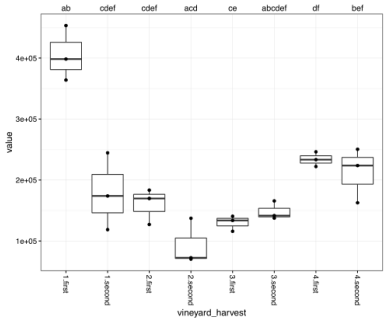
2–heptanone



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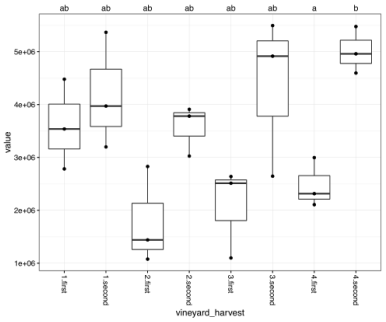
3–ethoxy–2–butanone



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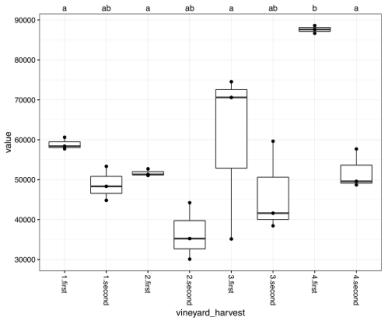
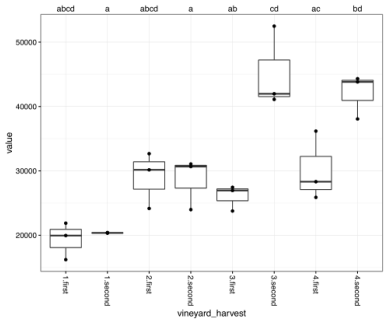
isobutyl octanoate



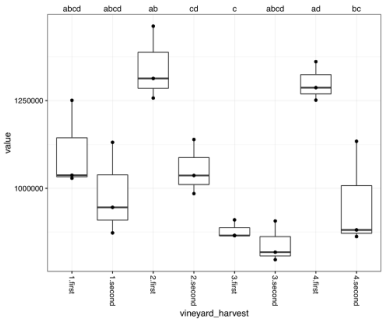
100.

acetic acid, phenylmethyl ester
(phenylmethyl acetate)



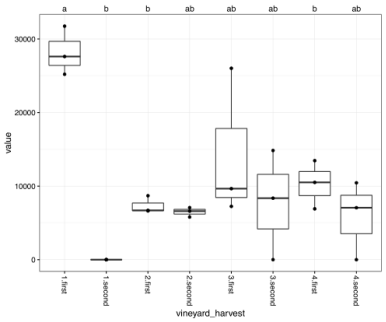
101.

1–hexanol, 2–ethyl–



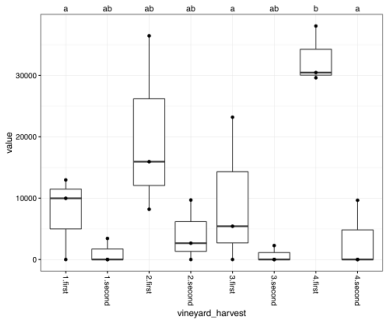
102.

ethyl 3–formylpropionate



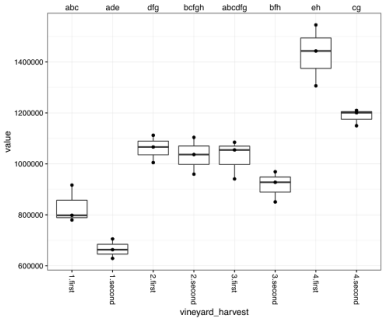
103.

analyte 356



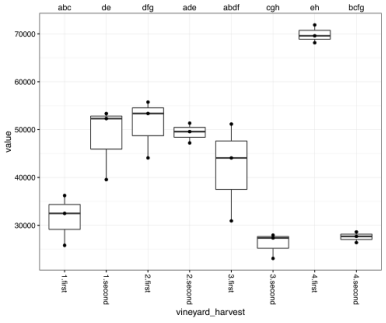
104.

3–hexenoic acid, ethyl ester, (Z)–
(ethyl (*Z*)-3-hexenoate)



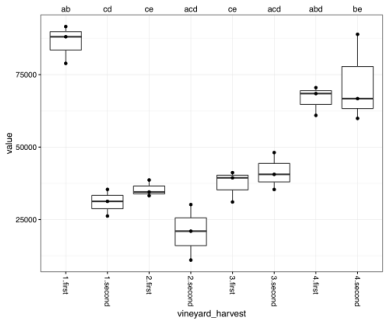
105.

2,6,10,10–tetramethyl–1–oxaspiro[4.5]deca–3,6–diene



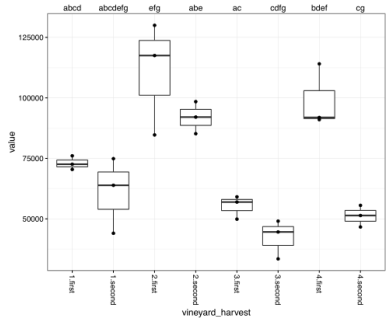
106.

1–butyn–3–one, 1–(6,6–dimethyl–1,2–epoxycyclohexyl)–



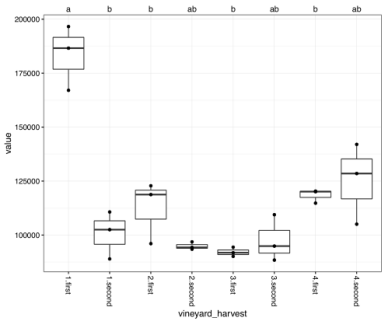
107.

cis–rose oxide



108.

3–cyclohexene–1–methanol, ä ,ä ,4–trimethyl–
(α-terpineol)



Verdicchio wines. Different environmental effects contribute to their complexity: a case study.

This chapter is ready for submission.

Preface to Chapter 3

Verdicchio is a white wine grape variety that has been cultivated for hundreds of years in the Marche region of central Italy. Verdicchio is used to produce all kinds of wines, dry, sweet and sparkling, some of which can easily be aged for ten or more years. For some experts it is also one of Italian native white grapes whose wines have the greatest affinity with oak ageing. (D'Agata I.). Verdicchio is one of the many Italian varieties named for its colour, in this case the green (*verde*) tinge of its berries. It is a versatile variety, used both for light, easy-drinking table wines, and for more complex wines worth ageing. It is commonly lauded by critics as being one of Italy's best white wine grape varieties, and is found in vineyards across the country. The use of Verdicchio has been documented in the Marche since the 14th Century, but it has been suggested that the variety could have originated in Veneto, where it is known as Trebbiano di Soave. Historians believe that Venetians migrated to the Marche area after the plague, bringing animals and plants, and it is thought that Verdicchio was among these. The variety adapted well to Marche's terroir, and nowadays, Verdicchio's spiritual home is in the hills along the Adriatic coast.

Some researchers (Vantini et al., 2003) have performed DNA profiling not just on Verdicchio and Trebbiano di Soave but also on Trebbiano di Lugana. They studied ten SSR microsatellite loci and confirmed the shared identity of Verdicchio and Trebbiano di Soave (the varieties have identical alleles at all ten loci). However, the researcher also found an abnormal allele of five hundred nucleotide bases in the VVMD36 locus that allows genetic differentiation between Trebbiano di Soave and Trebbiano di Lugana, which on the basis of these results can probably be more correctly viewed as biotypes. Apparently, Ghidoni and coworkers confirmed the synonymity of Verdicchio and Trebbiano di Lugana and established a new synonymity for Verdicchio and Trebbiano Valtenesi. (Ghidoni et al., 2010). Verdicchio is also a DNA match for Peverella, an old grape variety from Trentino which is also grown in Brazil - where many Trentino families emigrated in the late 19th century.

The replanted Trebbiano di Soave vines probably adapted to their new microclimate and the soil of the Marche, and this can explain some of the differences in aroma and flavour profiles found in wines made with Trebbiano di Soave or Verdicchio.

This study, funded by a winery in the Marche area, wished to extend knowledge of the aromatic profile of Verdicchio. Using this variety and carefully choosing the different areas, it is possible to obtain very different wines, some fresher and others more suitable for long ageing. The

research focused on identification of the most important sensorial compounds and on the characterisation of wines produced from:

- grapes in 3 different areas;
- different clones;
- vineyards with different levels of vigour (high and low);
- different harvest dates;
- old vintages;

The 2016 harvest and old vintages were analysed with different instrumental approaches, such as GCXGC, traditional GC-MS, also in MS-MS mode, and GC-O-MS. Unfortunately, for climatic reasons, it was not possible to analyse the 2017 harvest. The 2018 harvest will be analysed instead, but for reasons of timing it will not be possible to include the data in this work.

References not included in the paper:

D'Agata Ian Native Wine Grapes of Italy University of California Press, Berkeley, 2014 640 pp. ISBN 978-0-520-27226-2

Aromatic complexity in Verdicchio wines. A case study.

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Introduction

The aroma of a wine is influenced by the action of several different compounds on the sensory organs. These compounds are produced through metabolic pathways during the ripening and harvesting of grapes, and during the fermentation and storage of wine (Rapp & Mandery, 1986; Rapp & Versini, 1995). Of these factors, it is well known that the grape variety gives a characteristic aroma. This varietal aroma allows the classification and even the labelling of wines, so it is important to know the typical aromatic pattern of a wine variety in order to ensure its quality (Marais, 1983 altro)

Verdicchio is a white grape variety grown almost exclusively in the Marche region in central Italy. Genetic analysis has shown a very close relationship between Verdicchio, "Trebbiano di Soave", and "Trebbiano di Lugana" (Costacurta, Calò, & Crespan, 2003). The genetic origin of Verdicchio, and therefore of Trebbiano di Soave, is however still being debated. One

hypothesis, based on the documented migration of farmers from the province of Verona in northern Italy to the Marche, partially depopulated by the plague, in the 15th century, is now the most accredited. The settlers are thought to have brought their vines with them (Pollini, 2006), and thus it is surmised that they originally came from the Veneto region.

In a recent work (Ghidoni et al., 2010) it was reported that the three varieties are genetically identical, at least as far as the analysed part of the genome is concerned, but they could be traced to three different biotypes of the same variety, in relation to phenotypical events related to the environment. Indeed, the length of the separation would appear to justify some olfactory differences between Trebbiano di Soave and Verdicchio, over and beyond the demonstrated genetic identity (Vantini et al., 2003).

Verdicchio is not characterised by a large quantity of terpenes; indeed, in analysis carried out in the past, a very low content of the main monoterpene was observed. The floral terpene note could derive from the Ho-terpendiol I (about 20 μg L^{-1}), also present in bound forms, which could then be released over time ((Nicolini, Versini, Moser, Carlin, & Malossini, 2003)).

What was instead observed was the presence of a characterising compound: methyl salicylate, which in this variety can reach 45 μg L^{-1} in free forms and more than 500 μg L^{-1} in bound forms, as reported for Trebbiano di Soave and Verdicchio (Versini, Moser, & Carlin, 2005).

When added to wine, it is described as providing a floral note, slightly balsamic, tending to cinnamon-chestnut honey. A study involving Trebbiano di Lugana wines (genetically similar to Trebbiano di Soave and Verdicchio) highlighted that the grapes also contain sulphured aromatic compounds, in particular 3-mercaptohexanol and 3-mercapto hexyl acetate, which could impart tropical and citrus fruit scents (Mattivi et al., 2012).

In some cases this wine presents notes of anise, and one of the aims of this work was to understand if there is a compound that can determine this aroma. For this reason we used gas

chromatography–olfactometry (GC–O), a powerful tool for the study of chemical compounds responsible for wine aroma (van Ruth, 2001).

2. Materials and methods

Wine samples

For the preliminary survey, 4 types of wine obtained using Verdicchio grapes grown in different areas but in the same Italian region (Marche) by the Fazi Battaglia winery were used in the study. 11 vintages of "Masaccio" (2002-2015), 8 vintages of "San Sisto" (2001-2015), 3 vintages of "Titulus" (2013-2015) and 3 vintages of "Le Moie" (2008-2015) were analysed with SPME and GCXGC-ToF-MS. Masaccio is obtained from the highest and most south facing areas, San Sisto derives from colder areas with clayey soil, Le Moie is obtained in vineyards closer to the sea on sandy soils, and Titulus is produced with grapes grown in the 300 hectares of estate-vineyards, subdivided into 12 distinct vineyards with different exposure. From the 2016 harvest, wines from the 3 different Masaccio, San Sisto and Le Moie areas were analysed. In the case of San Sisto 3 different grape harvests were also sampled: the first (H1) on 23 September, the second (H2) after 1 week and the third during harvesting on 5 October (H3), with 3 different clones (VCR-3, VL-50, R-2), vines of high and low vigour, and 2 different vinification techniques, one with skin contact and the other known as "Archezia", using grapes infected with noble rot. The 2016 wines were extracted with the SPE technique and both free and bound forms were analysed after enzymatic hydrolysis. The most volatile compounds were analysed with the SPME technique, 3-methyl-2,4-nonanedione (3-MND) was quantified with SPME after derivatisation and the SPE extract was used for GC-O analysis.

SPE ENV+ extraction procedure. 50 mL of wine were extracted with solid phase extraction using ENV+ cartridges, 1 g (Biotage, Sweden). The cartridge was pre-conditioned with 15 mL of methanol followed by 20 mL of water. The aqueous extract was loaded onto the cartridge, which was then washed with 15 mL of water. The free aromatic compounds were eluted from the cartridge with 30 mL of dichloromethane and the bound aromatic compounds (i.e. glycosides) with 30 mL of methanol. The free volatiles was collected in a 100-mL flask and 60 mL of pentane were added to it followed by the addition of anhydrous Na_2SO_4 to remove water. Subsequently, the whole fraction was carefully concentrated prior to analysis up to 200 µL using a Vigreux column. Methanol was eliminated under vacuum and the residue solubilized in 5 mL of a citrate buffer pH 5, while the glycosidically bound fraction was hydrolysed with 400 uL of a commercial glycosidase rich enzyme (70 mg mL^{-1} Rapidase AR 2000 DSM) at 40°C for 12 h. The mixture with the aglycons was eluted through a ENV+ 1 g cartridge previously activated with 5 mL of methanol and 10 mL of milliQ water, and the free compounds were collected with 10 mL of dichloromethane. The relative amount of each volatile was expressed as µg/L of n-heptanol.

SPME with solution derivatisation for 3-MND determination

5 mL of wine were put in a 20 mL headspace vial with 20 uL of IS (4-methyl-3-penten-2one d^{11} 10 ug L^{-1}) and 400 uL of a o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) water solution (20 g L^{-1}) was added to the vial. The method was adapted from (Moreira, Meireles, Brandão, & De, 2013; Saison, De Schutter, Delvaux, & Delvaux, 2009) The PFBHA solution was prepared daily in ultrapure water at 20 g L^{-1}. The IS was prepared in ethanol at 1 g L^{-1} and further diluted at 10µg L^{-1}.

The vials were equilibrated for 20 min at room temperature, after that a 65um PDMS/DVB was inserted into the headspace for 40 min at 50°C.

Standard solutions were prepared in ethanol at 1 g L^{-1} and stored at 4°C. For the calibration curves, seven different concentrations of 3-MND, from 10 ng L^{-1} to 400 ng L^{-1}, were spiked in a white wine.

SPME GC-MS/MS method for 3-MND quantification

GC analysis was performed on a Trace GC Ultra gas chromatograph coupled with a XLS Tandem mass spectrometer. The system was equipped with a Pal autosampler (CTC Analytics AG, Zwingen, Switzerland). GC separation was performed on a 30 m VF Wax column with an internal diameter of 0.25 mm and a film thickness of 0.25 mm (Agilent). Supelco (Bellafonte, USA) PDMS/DVB 65 mm fibre was exposed in the sample for 40 min at 50°C and then desorbed into the GC liner, set at 250°C in splitless/surge mode for 3 min. Temperature programme: 80 °C held for 4 min after injection, 8°C/min up to 220°C, held for 1 min, 15°C/min up to 270°C, held for 5 min. Helium was used as the carrier gas at a constant flow rate of 1.2 mL min−1. The mass spectrometer was operated in electron impact (EI) ionisation mode at 70 eV. The filament current was 50 mA.

The temperature of the transfer line was 250°C and argon (99.9998% purity) was used as the collision gas, with collision cell pressure of 1.5 mTorr. Data acquisition and analysis were performed using the Xcalibur Workstation software supplied by the manufacturer. In order to determine the retention time and the characteristic mass fragments of 3-MND, full scan analysis (m/z 40–300) was performed. The retention times were 29.62 and 29.91 min for monosubstituted 3-MND and RT 35.83, 36.39 and 36.81 for disubstituted 3-MND (Fig 1S). For quantitative analysis, we selected the transitions m/z 168>71 (10 eV) for monosubstituded 3-MND and m/z 363>181 (10 eV) for disubstituted 3-MND. In addition, the transitions m/z 267> 86 (10 eV) and 307>181 (10eV) were also used as qualifiers for mono and disubstituted 3-MND respectively. For quantification, the chosen transitions were monitored in multiple

reaction monitoring (MRM) mode and the peak area ratios of 3-MND to 4-methyl-3-penten-2one d11 (internal standard) were calculated on the basis of the compound concentration. Quantification was performed using the internal standard (IS) method. For the calibration curves, seven different concentrations of 3-MND from 10 ng L−1 to 400 ng L−1 were spiked in a white wine. The repeatability of the method was checked by running 10 samples spiked with 2 different concentrations (20 ng L−1 and 100 ng L−1) and the cv% was < 13%.

SPME-GC×GC-TOF-MS

1.5 g of sodium chloride was added to a 20 mL headspace vial, followed by 1 mL of wine spiked with 50 µL of 2-octanol as internal standards (1 mg L^{-1}). Quality control samples (QC) consisting of equal proportions of each sample were taken at the beginning of the run (n=5), and then after every 10^{th} sample. GC×GC-TOF-MS analysis of wines was performed using a gas chromatograph (GC) Agilent 6890N (Agilent Technologies), coupled with a LECO Pegasus IV time-of-flight mass spectrometer (TOF-MS) (Leco Corporation, St. Joseph, MI, USA) equipped with a Gerstel MPS autosampler (GERSTEL GmbH & Co. KG). Samples were incubated for 5 min at 35 °C and volatiles were extracted with a DVB/CAR/PDMS coating of 50/30 µm and 2-cm long SPME fibre (Supelco Sigma-Aldrich, Milan, Italy) for 20 min, desorbed for 3 min at 250 °C in splitless mode. The fibre was reconditioned between each sample for 7 min at 270 °C. Helium was used as a carrier gas at a flow of 1.2 mL min^{-1}. The GC oven was equipped with a VF-Wax ms 30 m x 0.25 mm column with 0.25 µm film thickness (Agilent Technologies) for the first dimension (1D), while for the second dimension (2D) a Rxi 17 Sil MS 1.5 m x 0.15 mm column with 0.15 µm film thickness (Restex Cooperation, Bellefonte, PA, USA) was used. Oven temperature was held at 40 °C for 2 min and ramped at the rate of 6 °C/min to 250 °C, where it was held for 5 min before returning to the initial conditions. The secondary oven temperature was held at 5 °C above the temperature of the

primary oven throughout the chromatographic run. The modulator was offset by + 15 °C in relation to the secondary oven and modulation time was 7s. The ion source temperature was set at 230 °C and electron ionisation at 70 eV. Spectra were collected in a mass range of m/z 35-350 with an acquisition rate of 200 spectra/s and acquisition delay of 120 s.

SPE-GC-O-MS

One microlitre of SPE extract was analysed with a Trace GC Ultra chromatograph, coupled with a SQ EC single quad mass spectrometer (Thermo Scientific, Milan, Italy), equipped with a splitless injector maintained at 250 °C; the split vent was opened 0.5 min post injection. Compounds were analysed with a VF wax (Agilent J&W, Folsom, CA, USA) column (30 m × 0.25 mm i.d., 0.25 μm film thickness) in scan mode. The carrier gas was helium. The oven temperature was programmed to rise from 50 °C to 250 °C at 2.5 °C/min and finally kept at 250 °C for 10 min. To assess the olfactory potential of the extracts, the column was connected to a GC-O port Olfactory Port (GL Sciences Tokyo, Japan) maintained at 220 °C. The effluent was diluted with a large volume of air (20 $mLmin^{-1}$) prehumidified with an aqueous solution. For AEDA, the concentrated fraction was diluted stepwise (1:3) with pentane:dichloromethane. Complete aroma extract dilution analysis (AEDA) was performed by five trained panellists. The flavour dilution (FD) factor of each compound represented the maximum dilution at which the odorant could be perceived. The analysis was repeated in duplicate by each assessor.

Aroma identification

Aroma compound identification was achieved by comparison of odours and their retention indices (RI) with those of pure standards, when available.

Statistical data elaboration

Data from 1D-GC–MS and GC × GC-TOF-MS analyses were subjected to one-way analysis of variance (ANOVA), and average values were compared by Least Significant Difference (LSD) test at the level of $p < 0.05$. Data were further processed by multivariate techniques.

PLS-DA and heatmaps were generated using MetaboAnalyst v. 3.0 (http://www.metaboanalyst.ca), created at the University of Alberta, Canada (Xia, Sinelnikov, Han, & Wishart, 2015).

Results and discussion

The initial exploration was carried out using 4 different types of Verdicchio (Masaccio, San Sisto, Le Moie and Titulus) by analysing a series of vintages and using SPME with GCXGC-ToF-MS. Two-dimensional analysis made it possible to find many compounds characterising the different types of Verdicchio. Indeed, it is possible to notice clear separation in the PLS-DA (Partial least squares discriminant Analysis) plot (Figure 1). As shown in the graph, the first component alone can separate types of "Titulus", especially old vintages, from "Le Moie" and "San Sisto" wines. "San Sisto" and "Masaccio" are instead separated by the second component.

The volatile compounds determining this separation were whisky lactone, 5-methylfurfural, 2-furancarboxaldehyde, 2,4,5-trimethyl-1,3-dioxolane, benzofuran and 2-methylthiophene for the "San Sisto" samples. The first three compounds derive from the use of wood, while dioxolane is a compound correlated with ageing. "Le Moie" wines were characterised by a higher content of norisorenoids such as β-damascenone and safranal. "Titulus" wines were richer in other norisoprenoids like TDN and also in some sesquiterpenes such as alpha calacorene and monoterpenes such as alpha-terpineol and alpha terpinolene. Wines from the "Masaccio" areas were the richest in benzenacetaldehyde and linalool. "San Sisto" and "Masaccio" were also the richest in 4-methyl 1-pentanol and 2-acetyl furanone. It is also possible to observe the typical trend of TDN which increases over the years (Figure 2) It is known that this compound can derive from different precursors linked to sugar molecules and is formed by hydrolysis during ageing (Winterhalter & Schreier, 1994, Winterhalter 1991,

Versini et al., 2001) it is also known that pH, temperature and presence of oxygen are important parameters in the formation and degradation of this compound. (Silva Ferreira *et al*., 2004)

After analysing old vintages as a survey study, Verdicchio wines produced in 2016 were analysed in depth. In this case, several different tests were carried out in order to understand the aromatic profile of this variety. Results from SPE and SPME analyses were reported in Table 1.

The wines coming from the different Masaccio, San Sisto and Le Moie areas were again distinguishable (Figure 3 heatmap free). Moie wine was distinguished by a higher content of hexyl acetate, octanoic and decanoic ethyl ester and the corresponding acids, and by the highest amount of diendiol I, Le Moie is also the richest in the bound form. Masaccio was higher in norisoprenoids such as TDN and its precursor riesling acetale, methyl salicylate, homofuraneol and 2 (H)-pyran-2,6(3H)-dione. Some of these compounds will be better described in the GC-O section. Samples from San Sisto H1 were the richest in benzyl alcohol, methionol, 2-methyltetrahydrothiophen-3-one and 4-vinylguaiacol. It was also clear that overripe samples of San Sisto (H3) were richer in terpenes such as linalool, citronellol, limonene and alpha-terpineol, both in the free and in the bound forms (Figure 4). Late harvested "Archezia" obtained from grapes with noble rot and a withering stage was characterised by the presence of some marker compounds for Botritis infection, such as benzaldehyde, acetic acid, furfural and terpinen-4-ol. (Fedrizzi et al., 2011)

To better understand the key compounds in Verdicchio aroma, SPE extracts from the three different areas were injected into GC-O and sniffed by 6 expert panellists. 47 main odorants were found in the SPE extract (Table 2). What is perceived in the glass is a mixture of odour and taste molecules, which may combine to act in a suppressive or additive manner, or synergistically (Frijters & Schifferstein, 1994). In GC–O, by contrast, the odorants are delivered to the olfactory epithelia as single entities, which simplifies the recognition task.

Aroma extract dilution analysis (AEDA) was used to determine the relative odour potency of compounds present in the sample extract. Aroma compounds with a higher FD value (>3) were likely contribute considerably to overall Verdicchio type aroma. In particular, we observed that the most important compounds in terms of sensorial impact were ethyl 2-methylpropanoate, with notes of apple fruit, ethyl hexanoate with a bilberry-fruity note, β-damascenone with a baked apple note, 2-phenylethanol with a rose note, furaneol and homofuraneol with a cotton candy scent, vanillin with a characteristic sweet pastry note, phenylacetic acid, associated with honey-withered flower, one unknown compound with a toasted almond smell, isovaleric acid with a cheese odour, β-ionone with a violet note, and methional and methionol with a boiled potato odour. In some samples a passion fruit/tropical note was also found and the retention index was very close to 3-mercapto-hexanol. Moreover, in all samples a liquorice note was present, probably associated with the compound pantolactone, together with a clove/spice note given by 4-vinylguaiacol and a clear celery/vegetal scent, probably due to sotolone.

Furthermore, we focused our attention on the anise note noticeable in some vintages of Verdicchio. GC-O analysis made it possible to find the area where this note was sensed by the 6 expert panellists. In the literature, the compounds that can contribute to this note are *trans* and *cis* anethole, estragol (Zeller & Rychlik, 2007), ethyl hexanoate (San-Juan, Pet'ka, Cacho, Ferreira, & Escudero, 2010), R carvone (Pinar, Rauhut, Ruehl, & Buettner, 2017) and 3-methyl-2,4 nonanedione (Alexandre Pons, Lavigne, Eric, Darriet, & Dubourdieu, 2008). Except for ethyl hexanoate, which is eluted early, all the other compounds showed a RI on a polar column between 1687 and 1800. Analysis of the pure standards led to identification of the 3-methyl-2,4 nonanedione (3-MND) compound responsible for the anise note. Other researchers have reported an anise-like note in the same area that has been tentatively identified (Martí, Mestres, Sala, Busto, & Guasch, 2003; Peťka, Ferreira, González-Viñas, & Cacho, 2006). This compound was found in wine by French researchers (Alexandre Pons et al., 2008) and was

related to the plum note in prematurely aged red wines, and was also correlated with a loss of fruitiness (A. Pons, Lavigne, Darriet, & Dubourdieu, 2013). 3-MND is an intriguing compound that has recently emerged as the most potent known agonist for the human receptor OR1A1, with a submicromolar half-maximal effective concentration (Geithe, Noe, Kreissl, & Krautwurst, 2017). These results further emphasise the controversial role of this compound, whose presence at variable concentrations has previously been described as reminiscent of mint, anise, fruit kernels and prunes (Alexandre Pons et al., 2008) and has been associated with prematurely aged wines, oxidised soybean oil (Kao, Wu, Hammond, & White, 1998) and freshly brewed green tea (Guth & Grosch, 1993). The odour description of this compound in wine model solution is strong, depending on its concentration, as reported by Pons *et* al (Alexandre Pons et al., 2008). When the concentration is 100 ng L^{-1} it is perceived as minty, while around 1 µg L^{-1} it recalls anise, kernel and prune, and at 10 µg/L the perception is of anise.

In order to understand the contribution of this compound to the Verdicchio aroma, 3-MND was added to a "neutral" Verdicchio. The concentrations tested were 10 ngL^{-1}, 25 ngL^{-1}, 50 ng L^{-1}, 100 ng L^{-1} and 500 ng L^{-1}. Wine without additions was also include in the tasting.

The tasting was carried out by 10 trained people (8 males, 2 females), showing that the fruity note was masked only by the addition of 500 ng L^{-1} of 3-MND, as the anise note predominated in this wine. The samples preferred by the panellists were those with a 3-MND concentration of between 25 and 50 ngL^{-1}. No oxidative note was detected in any sample. In Verdicchio wines it instead seems capable of imparting a positive and characteristic note.

Unfortunately, a well-perceived smell on the nose (olfactometry) is very frequently not detected by chemical detectors (FID-MS). This is the case of some sulphur compounds and some carbonyl compounds such as 3-MND. In these cases it is necessary to use specific tests for the quantification of trace compounds. In order to quantify these compounds in our samples we

used a derivatisation method with headspace solid phase microextraction using GC- tandem mass spectrometry. The derivatisation reagent PFBHA reacts with the carbonyl group formed oximes, which showed relatively specific mass spectra and high sensitivity. This method has been extensively employed to identify and quantify aroma components in several matrices, including wine (Flamini, Vedova, Panighel, Perchiazzi, & Ongarato, 2005) and beverages (López-Vázquez, Orriols, Perelló, & De, 2012; Saison et al., 2009). Our method was modified from that of Moreira et al., 2013 and Saison et al., 2009, where the determination of carbonyl compounds in beer was reported. Table 1 shows the content of 3-MND in our samples.

Since Verdicchio is not an aromatic grape, one of the characteristics that has been shown to make this variety special in studies carried out in the past (Versini, Moser, & Carlin, 2005) is the presence of a large amount of methyl salicylate. Methyl salicylate was found in *V. riparia* grapes by Schreier & Paroschy, 1980 and in *V. vinifera* sp. (Cabaroglu et al., 1997) and in the Frontenac interspecific hybrid (Mansfield, Schirle-Keller, & Reineccius, 2011). The sensory impact of this compound in wine is not altogether clear. In the literature it is described as having an odour of wintergreen, mint and fresh green character (Mansfield et al., 2011). We found that in the free forms the concentration was in the range 25-40 μg L^{-1}, while in the bound forms a higher concentration of up to 0.5 mg L^{-1} was found after hydrolysis (Table 1). As the olfactory threshold of this compound is between 50-100 μg L^{-1}, it is possible that in some Verdicchio wines the balsamic note of methyl salicylate contributes to the fresh/anise scent. It is well known that wine odours are complex mixtures of many volatile chemicals, present in different concentrations. These chemicals can interact synergistically or additionally in the mixtures according to unpredictable rules and are the basis of the overall odour sensation (Brattoli et al., 2013). In a recent study (Parker et al., 2017), the sensory role of monoterpene glycosides during tasting through retronasal perception of odorant aglycones released in-mouth was also proposed. For this reason studies are underway to quantify the precursors of methyl salicylate

in wines, hence the total quantity, not only that deriving from hydrolysis of glycosylated compounds. In the literature it has been suggested that it can be bound as methyl salicylate 2-O-β-D-glucoside or as methyl salicylate 2-O-β-D-xylopyranosyl(1-6)–D-glucopyranoside (primeveroside), though in plants 9 different glycosides have been reported to date (Mao et al., 2014).

Conclusion

This work confirms that it is possible to obtain wines with very different characteristics from this variety of grapes, depending on the different location of the vineyard and winemaking techniques. Young wines are characterised by fruity, thiolic notes, while wines aged for longer are distinguished by their norisoprenoid content and by balsamic, anise and liquorice notes, which can be attributed to methyl salicylate released by precursors and the presence of 3-MND and pantolactone. With a new derivatisation MS-MS method it was possible to quantify 3-MND in the range of 10-50 ng L^{-1}. With the use of GC-O, 43 sensorially important compounds were found for this wine. Analysis of the compounds after hydrolysis confirmed that a high amount of methyl salicylate characterised this variety. Methyl salicylate has a balsamic note from wintergreen oil that is often perceived in Verdicchio tasting. In-depth studies are underway to understand which precursors are present in grapes and whether they are in any way dependent on agro-climatic variables.

FUNDING

This study was supported by Fazi Battaglia winery

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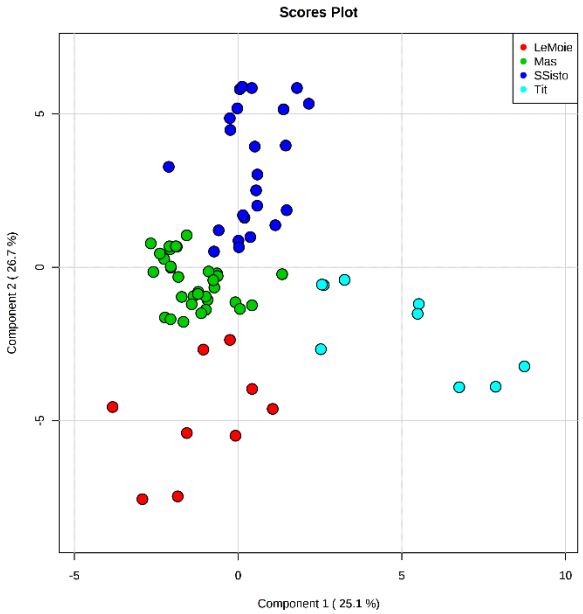
Figure 1 Partial least squares discriminant analysis (PLS-DA) graphs of Verdicchio grapes grown in different areas. 11 vintages of "Masaccio" (2002-2015), 8 vintages of "San Sisto" (2001-2015), 3 vintages of "Titulus" (2013-2015) and 3 vintages of "Le Moie" (2008-2015) Score scatter each point represents the vintages (3 replicas)



PLS-DA cross validation details:

Measure	1 comps	2 comps	3 comps	4 comps	5 comps
Accuracy	0.58667	0.8	0.86667	0.89333	0.89333
R2	0.53328	0.73823	0.80978	0.84497	0.88548
Q2	0.40976	0.63796	0.68923	0.70572	0.71244

Figure 2 increase of TDN content during ageing in Verdicchio wines produced in Masaccio area. 11 vintages (2002-2015)

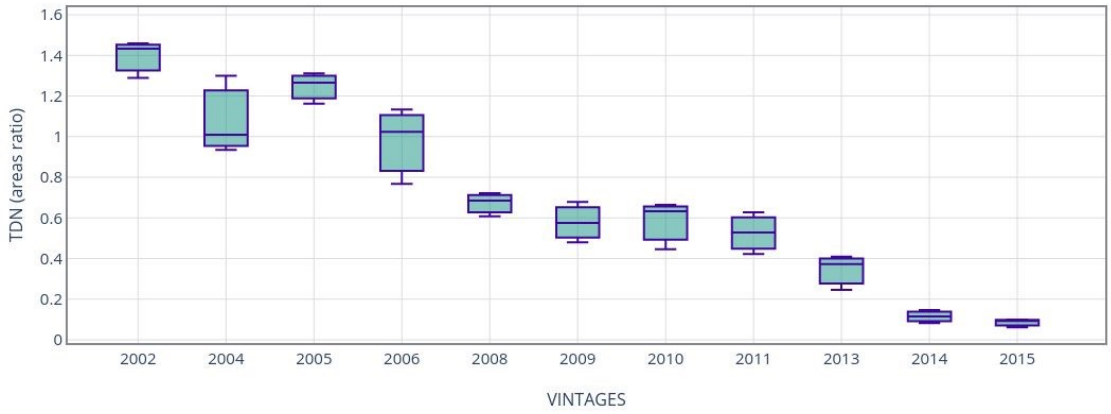


Figure 3: Hierarchical clustering analysis performed using volatile aroma compound profiles of 3 areas (Le Moie, Masaccio, San Sisto) and 3 harvest data (San Sisto). (Ward algorithm and Euclidean distance analysis using MetaboAnalyst v. 3.0) The heatmap was generated using 70 free compounds. The rows in the heatmap represent compounds and the columns indicate samples. The colours of heatmap cells indicate the abundance of compounds across different samples. The colour gradient, ranging from dark blue through white to dark red, represents low, middle, and high abundance of a compound.

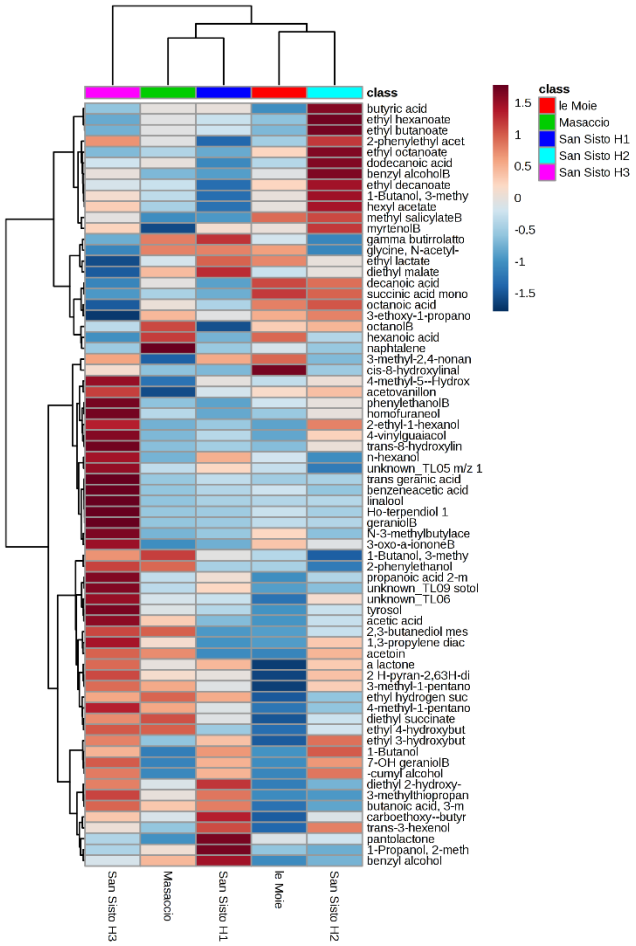


Table 1 Concentrations (as µg/L of internal standard n-heptanol) of free and bound compounds in 2016 Verdicchio wines. 3 different areas (Le Moie, Masaccio, San Sisto), 3 harvest time (San Sisto H1, H2, H3), low and high vigour, 3 different clones and 2 different vinification (late harvest and whitering process, skin maceration)

				Areas			*Harvest time*		*Vigour*		*Clones*			*vinification*	
	threshold ug/L	***RI exp***	***RI let***	le Moie	Masaccio	San Sisto H1	San Sisto H2	San Sisto H3	Low vigour	High vigour	VCR3	VL50	R2	Late harvest	Skin maceration
free ug/L as n-heptanol				**P019**	**P004**	**P003**	**P005**	**P016**	**P006**	**P007**	**P012V**	**P013**	**P014**	**P501**	**P015**
ethyl butanoate	20	*1030*	*1030*	316.6	365.3	354.0	502.3	313.2	296.1	306.5	327.6	352.3	287.0	188.1	345.3
1-propanol, 2-methyl	40000	*1087*	*1060*	2953	3563	5030	2794	3123	3023	2380	3862	3483	5195	8103	4975
1-butanol, 3-methyl-, acetate	30	*1122*	*1120*	1818	1646	1103	2637	1887	2795	3541	2311	2643	2554	823	1410
1-butanol	150000	*1135*	*1130*	253.2	210.1	640.5	723.9	597.3	592.2	493.9	217.2	196.3	202.1	169.4	659.1
limonene	10	*1197*	*1199*	17.0	3.8	2.7	2.3	2.1	1.0	1.6	31.8	22.3	3.7	4.5	1.7
1-butanol, 3-methyl-	30000	*1205*	*1221*	77381	92961	80576	66555	88127	94737	86592	95463	103955	110064	97078	92623
ethyl hexanoate	14	*1234*	*1236*	1149	1311	1255	1843	1090	906	969	1092	1231	1080	558	1013
hexyl acetate	1500	*1270*	*1272*	147.7	126.3	86.3	218.8	162.5	185.7	251.6	146.6	184.1	216.6	15.1	87.4
acetoin	150000	*1279*	*1268*	9.8	27.2	10.4	24.7	29.4	70.8	88.3	39.2	20.0	9.2	16.3	7.7
4-methyl-1-pentanol		*1312*	*1328*	20.9	37.2	31.3	26.7	44.4	44.0	42.8	35.5	49.4	37.4	24.6	29.7
3-methyl-1-pentanol	830	*1324*	*1341*	53.1	97.4	85.0	92.1	104.7	95.0	87.9	99.1	111.7	85.2	61.6	79.1
ethyl lactate	154000	*1340*	*1326*	3980	3036	4189	3219	1602	1027	956	2329	2874	3050	1988	3370
n-hexanol	8000	*1354*	*1356*	1362	1290	1455	1249	1581	1052	1162	1510	1579	1865	453	1757
trans -3-hexenol	400	*1363*	*1361*	50.7	67.3	103.2	97.0	80.8	63.1	65.5	71.1	67.1	67.3	14.3	73.0
3-ethoxy-1-propanol	100	*1371*	*1389*	572.9	562.0	487.2	610.5	251.4	173.9	164.9	551.4	455.3	583.4	94.4	746.1
cis 3-hexenol		*1386*	*1379*	45.5	31.9	35.6	33.7	23.4	40.0	53.2	58.7	53.0	86.2	12.4	116.1
trans -2-hexen-1-ol		*1407*	*1410*	9.0	10.6	22.0	10.4	9.3	6.6	10.1	11.1	12.6	12.6	12.5	10.3
ethyl octanoate	20	*1437*	*1435*	1871	1679	1554	2299	1591	1320	1329	1418	1547	1472	636.4	1186
acetic acid	200000	*1453*	*1430*	253.0	403.0	290.0	337.8	554.4	212.8	138.4	676.1	612.9	814.5	1145.0	392.8
2-ethyl-1-hexanol		*1491*	*1485*	38.2	39.6	42.0	57.3	63.7	58.8	51.5	43.5	31.1	33.3	36.7	67.0
benzaldehyde		*1506*	*1508*	15.9	9.7	29.2	25.5	19.1	9.6	9.6	14.7	11.1	11.3	40.6	13.3
ethyl 3-hydroxybutanoate		*1511*	*1499*	221.3	315.6	423.4	479.5	467.9	439.0	411.9	284.1	310.4	304.7	186.8	535.0
2-methyltetrahydrothiophen-3-one		*1514*	*1538*	16.1	18.1	27.7	27.3	23.8	35.5	38.5	14.0	18.3	17.7	11.9	11.7
2,3-butanediol meso	100000	*1530*	*1525*	1397	2464	1372	1762	2542	1514	2010	2573	2495	2812	3056	1412
propanoic acid		*1540*	*1546*	56.1	63.8	70.6	71.7	75.6	82.8	64.8	95.4	74.0	78.3	88.6	69.1
linalool	25	*1551*	*1542*	11.8	7.4	10.7	12.1	56.4	14.4	16.2	7.3	7.7	8.2	5.1	33.1
1-octanol		*1554*	*1552*	17.5	20.0	25.9	13.0	13.6	10.6	9.5	17.2	11.4	13.6	15.1	23.6
propanoic acid 2-methyl-		*1567*	*1583*	366.1	462.8	510.0	447.5	714.1	442.7	378.2	940.1	522.8	622.9	1537.6	493.7
cis -4-hydroxymethyl-2-methyl-1,3-dioxolane		*1602*	*1623*	2.1	4.8	1.9	3.1	8.3	10.2	8.5	12.2	3.6	3.3	24.9	2.6
gamma butirrolattone	35000	*1611*	*1650*	339.7	406.3	434.8	276.5	298.5	168.7	149.4	443.1	484.5	328.6	565.3	395.1
butyric acid	173	*1623*	*1600*	970.6	1114	1119	1349	1033	761.6	835.7	1140	1016	907.0	667.9	1035
phenylacetaldehyde		*1626*	*1612*	4.7	3.6	3.7	3.2	4.3	3.4	4.0	4.7	4.6	4.8	44.7	4.0
ethyl decanoate		*1641*	*1637*	1392.2	1234.0	872.5	1811.9	1263.4	1459.2	1173.3	1280.0	1339.4	1168.5	644.4	965.0
butanoic acid, 3-methyl-	33	*1667*	*1642*	544.9	696.4	741.5	585.2	754.3	735.3	691.0	859.3	645.5	781.8	1089.2	953.2
diethyl succinate	200000	*1672*	*1667*	1962	4378	3295	3161	4062	3473	3467	3201	2876	2374	3218	2502
alpha terpineol		*1686*	*1684*	10.3	7.9	6.4	5.1	19.9	6.6	6.7	9.1	8.5	10.4	4.5	18.6
3-methylthiopropanol	1000	*1705*	*1725*	453.4	682.9	866.8	477.5	928.7	1161	1112	518.3	568.8	668.7	1315.3	1045.3
naphtalene		*1715*	*1728*	9.0	29.5	4.7	4.6	4.9	3.9	4.7	29.4	24.9	28.5	24.7	5.3
1,3-propylene diacetate (t.i.)		*1730*	-	611.7	749.7	603.0	776.2	925.3	981.9	1111.4	681.9	523.2	776.7	762.0	896.9
α-cumyl alcohol		*1750*	*1779*	24.2	24.4	66.5	76.5	75.8	71.3	74.6	26.8	25.5	24.2	25.5	71.8
methyl salicylate	100	*1754*	*1759*	30.5	44.8	41.7	35.3	33.5	26.5	25.3	37.9	24.9	21.1	19.2	23.2
methyl-4-hydroxybutanoate (t.i.)		*1758*	-	3.1	5.4	3.3	4.4	6.0	4.3	3.9	11.0	6.4	6.4	42.7	15.4
ethyl 4-hydroxybutanoate		*1799*	*1827*	1167	2880	1757	2006	2908	1665	1301	7072	4033	3254	10540	3778
2-phenylethyl acetate	250	*1802*	*1803*	209.8	250.8	126.6	372.3	323.2	719.5	837.7	392.9	430.0	430.9	186.5	147.3
4-(methylthio)-1-butanol		*1833*	*1812*	8.2	7.9	11.9	12.2	12.0	9.0	8.6	6.8	6.4	6.7	4.5	12.5
hexanoic acid	420	*1840*	*1830*	4420	4645	2823	3163	2556	1957	2481	3027	3595	3980	1876	2277
N-(3-methylbutyl)acetamide		*1855*	*1855*	128.7	23.2	42.2	34.2	293.3	15.5	17.5	42.4	31.4	42.4	2119.8	359.8
benzyl alcohol		*1863*	*1876*	168.9	311.3	407.3	199.5	254.5	285.2	285.4	234.3	158.8	165.8	41.6	485.3
2-phenylethanol	50000	*1895*	*1893*	24108	33267	24159	19319	34585	35058	38909	37031	39963	43355	52908	28076
Ho-terpendiol 1		*1943*	*1957*	27.6	8.6	10.9	13.4	152.1	11.2	11.0	10.8	9.6	7.0	18.8	49.6
trans -2-hexenoic_acid		*1960*	*1971*	29.4	38.2	33.5	28.5	42.9	45.4	46.5	80.2	53.9	78.1	10.0	105.1
2 (H)-pyran-2,6(3H)-dione (t.i.)		*1979*	-	566.2	926.6	898.3	976.8	1124.3	1008.5	930.5	1002.1	811.5	852.1	360.9	776.1
pantolactone		*2013*	*2033*	56.6	45.6	78.6	55.4	53.0	40.4	45.8	77.4	55.9	79.1	89.0	85.1
diethyl malate		*2029*	*2070*	2027	2520	3141	2197	1139	1081	1122	1374	1809	1691	1196	1823
octanoic acid	500	*2053*	*2043*	7782	6848	6291	8071	5010	4572	5254	4901	6336	6071	2123	4502
homofuraneol	40	*2075*	*2090*	11.7	14.6	7.2	20.2	48.2	76.7	29.0	92.5	40.6	21.9	33.9	8.7
unknown		*2094*	-	263.4	262.6	279.4	232.6	324.0	300.5	299.5	440.2	331.4	448.7	235.0	287.0
unknown		*2100*	-	102.2	279.2	263.6	319.5	543.2	632.1	526.2	434.2	372.8	165.4	670.1	168.4
diethyl 2-hydroxy-3-methylsuccinate (t.i.)		*2137*	-	49.6	83.9	128.1	63.9	114.4	83.2	129.8	97.3	69.1	89.2	92.7	250.3
4-vinylguaiacol		*2175*	*2175*	66.4	78.5	108.1	160.7	278.5	162.8	149.0	95.3	140.5	95.9	142.6	113.7
carboethoxy-γ-butyrolactone		*2211*	*2241*	673.3	996.9	1362.4	1005.8	1113.8	887.1	1026.3	589.9	743.7	770.7	519.2	1106.0
unknown		*2180*	-	102.5	132.5	161.2	116.4	236.3	206.8	196.9	145.4	149.3	179.2	251.4	176.8
decanoic acid	1000	*2269*	*2257*	3885	3010	2368	3724	2188	2719	3038	2458	2957	2744	1211	2113
succinic acid monoethylester		*2267*	*2256*	50.5	35.4	32.4	48.4	30.8	40.0	39.8	31.5	38.9	33.6	15.7	29.9
4-methyl-5-(β-hydroxyethyl)thiazole		*2285*	*2300*	208.1	168.7	219.3	221.9	281.9	152.6	227.3	547.9	137.2	383.7	99.1	65.1
unknown		*2354*	-	168.7	435.0	509.6	485.5	584.4	459.5	530.1	853.3	551.2	367.0	1107.3	282.8
ethyl hydrogen succinate		*2373*	*2367*	18934	41368	37520	27026	37685	34261	33732	41644	41629	31707	40166	26664
dodecanoic acid		*2472*	*2465*	317.8	350.5	253.6	502.2	332.1	505.8	501.7	276.1	243.2	271.0	154.7	238.5
benzeneacetic acid	2500	*2539*	*2534*	41.5	30.6	36.5	30.6	100.1	30.8	33.0	36.7	31.3	57.9	233.0	112.2
acetovanillon		*2607*	*2626*	117.6	93.2	113.1	121.1	131.9	143.2	140.1	122.1	107.1	102.2	199.7	223.7
tyrosol		*2990*	*2985*	2922	4892	4267	4560	8809	9986	9654	6338	5078	6669	6965	4624
C13-norisoprenoids SPME															
β-damascenone	0.05	*1801*	*1820*	4.9	3.1	1.6	2.8	3.3	5.2	5.3	3.4	3.2	3.6	3.3	2.6
VTP (sum of isomers)	800	*1524*	*1514*	2.5	2.9	3.9	2.9	0.5	2.8	2.5	2.3	2.4	1.8	3.7	3.6
riesling acetale		*1656*	*1638*	2.5	2.7	2.5	2.5	2.6	3.0	2.9	2.7	2.6	2.7	2.3	2.5
TDN	2	*1739*	*1743*	1.1	3.3	1.5	2.2	1.5	4.0	3.1	3.4	1.8	2.6	1.3	3.1
SPME with derivatization															
3-methyl-2,4-nonanedione ng/L	0.016	*1732*	*1723*	77	16	67	35	68	24	28	30	29	38	60	54
				Areas			*Harvest time*		*Vigour*		*Clones*			*vinification*	
bound ug/L as n-heptanol		***RI exp***	***RI let***	le Moie	Masaccio	San Sisto H1	San Sisto H2	San Sisto H3	Low vigour	High vigour	VCR3	VL50	R2	Late harvest	Skin maceration
n-hexanol		*1354*	*1356*	54.8	44.0	39.4	44.8	59.5	34.3	30.9	33.2	38.5	28.4	285.5	73.3
n-octanol		*1554*	*1552*	478.1	566.2	275.3	496.2	408.7	418.6	433.7	494.5	530.1	655.1	458.2	249.5
trans linalool oxide furan		*1444*	*1429*	3.14	1.80	1.86	2.03	8.41	2.34	2.38	2.46	2.40	2.31	3.84	6.85
cis linalool oxide furan		*1463*	*1454*	3.98	3.04	3.02	3.33	4.34	3.85	3.96	3.95	4.35	3.94	4.29	5.75
linalool		*1546*	*1545*	1.11	0.46	0.31	0.67	7.63	0.77	0.85	0.61	0.48	0.46	0.75	4.26
tricyclo octane (t.i.)		*1679*	-	0.54	0.33	0.26	0.42	0.62	0.60	0.52	0.71	0.57	0.50	1.53	1.37
alpha terpineol		*1686*	*1698*	6.04	5.48	6.07	6.38	8.39	7.41	8.24	8.70	7.35	8.83	7.49	21.86
trans linalool oxide pyran		*1727*	*1716*	7.31	6.02	6.12	8.41	12.73	8.18	9.23	6.84	7.90	7.32	10.58	11.84
cis linalool oxide pyran		*1755*	*1781*	5.05	3.94	3.99	5.16	4.97	2.88	3.60	3.63	4.13	4.17	3.69	3.44
citronellol		*1762*	*1772*	1.89	1.69	2.87	3.75	2.61	2.31	5.00	1.28	0.89	2.72	1.89	2.83
nerol		*1790*	*1819*	4.51	4.37	4.58	4.91	17.83	5.96	5.57	5.39	4.47	4.75	4.43	10.63
geraniol		*1847*	*1831*	12.98	9.76	10.32	12.05	37.14	11.89	12.39	10.57	10.46	10.75	8.81	25.07
exo-2-hydroxycineole		*1849*	*1861*	19.67	19.31	21.40	24.80	21.08	23.04	25.53	22.21	22.85	24.44	37.88	50.07
terpendiol I		*1943*	*1957*	7.67	8.52	6.48	8.92	7.81	8.14	8.53	8.27	8.87	10.07	7.02	5.03
trans -8-hydroxylinalool		*2264*	*2277*	35.95	36.17	39.18	49.07	81.57	57.11	65.36	41.82	50.08	46.43	62.86	82.47
7-OH geraniol (t.i.)		*2299*	-	18.86	16.15	63.77	68.83	78.97	71.90	61.97	15.75	16.83	17.45	17.18	81.44
cis -8-hydroxylinalool		*2302*	*2315*	51.37	14.53	13.00	15.75	26.09	12.88	14.68	16.51	18.93	15.41	28.54	30.12
trans geranic acid		*2301*	*2289*	6.77	6.35	8.43	7.33	26.26	10.48	8.70	7.39	8.72	8.40	5.97	31.12
myrtenol		*2500*	-	171.4	136.6	173.1	197.0	176.1	161.1	170.3	142.7	159.2	187.5	272.5	353.1
3-hydroxy-β-damascone		*2512*	*2558*	32.26	31.77	21.98	27.24	31.76	33.85	35.37	23.09	24.45	28.36	26.26	38.77
3-oxo-α-ionone		*2603*	*2582*	67.01	52.57	55.31	62.53	79.18	102.4	90.44	85.88	73.10	91.79	116.1	150.4
dihydro-β-ionone		*2642*	-	17.69	11.89	13.64	13.17	17.44	19.75	16.59	16.30	15.19	16.68	22.36	45.40
3-hydroxy-5,6-epoxy-β-ionone		*2696*	-	6.27	5.64	3.22	4.32	11.32	29.28	26.15	45.00	13.17	25.59	11.18	141.12
3-hydroxy-7,8-dihydro-β-ionol		*2739*	*2722*	23.53	21.18	22.27	24.21	27.05	27.76	25.49	21.46	24.46	27.94	38.66	40.41
4-oxo-β-isodamascol (t.i.)		*2856*	-	13.87	11.42	10.97	12.94	14.77	13.26	12.18	9.01	12.04	16.24	29.87	20.89
benzenoids															
benzyl alcohol		*1863*	*1876*	1294.3	985.4	882.2	2156.9	1332.2	1302.6	855.9	1346.0	1188.5	1106.6	1082.4	1199.2
phenylethanol		*1895*	*1893*	246.6	229.1	218.0	255.1	330.0	296.4	302.8	255.4	261.9	308.9	492.3	435.9
2-methoxy benzyl alcohol		*2138*	*2082*	7.66	6.62	7.95	8.88	7.96	9.62	9.91	6.63	7.53	8.70	9.67	10.38
α-cumyl alcohol		*1750*	*1779*	4.96	5.12	15.94	15.58	12.87	17.47	18.03	4.36	5.60	5.61	4.39	15.21
vanillic alcohol		*2778*	*2787*	16.06	9.29	10.89	7.97	8.65	11.12	10.40	8.94	14.71	15.64	21.32	17.94
methyl salicylate		*1754*	*1759*	477.5	337.3	344.7	491.2	410.1	276.8	248.9	296.9	399.2	358.6	255.4	219.7

Figure 4 Hierarchical clustering analysis performed using volatile aroma compound profiles of 3 areas (Le Moie, Masaccio, San Sisto) and 3 harvest data (San Sisto). (Ward algorithm and Euclidean distance analysis using MetaboAnalyst v. 3.0) The heatmap was generated using 30 most significant bound compounds after enzymatic hydrolysis (the highest Fisher ratios). The rows in the heatmap represent compounds and the columns indicate samples. The colours of heatmap cells indicate the abundance of compounds across different samples. The colour gradient, ranging from dark blue through white to dark red, represents low, middle, and high abundance of a compound.

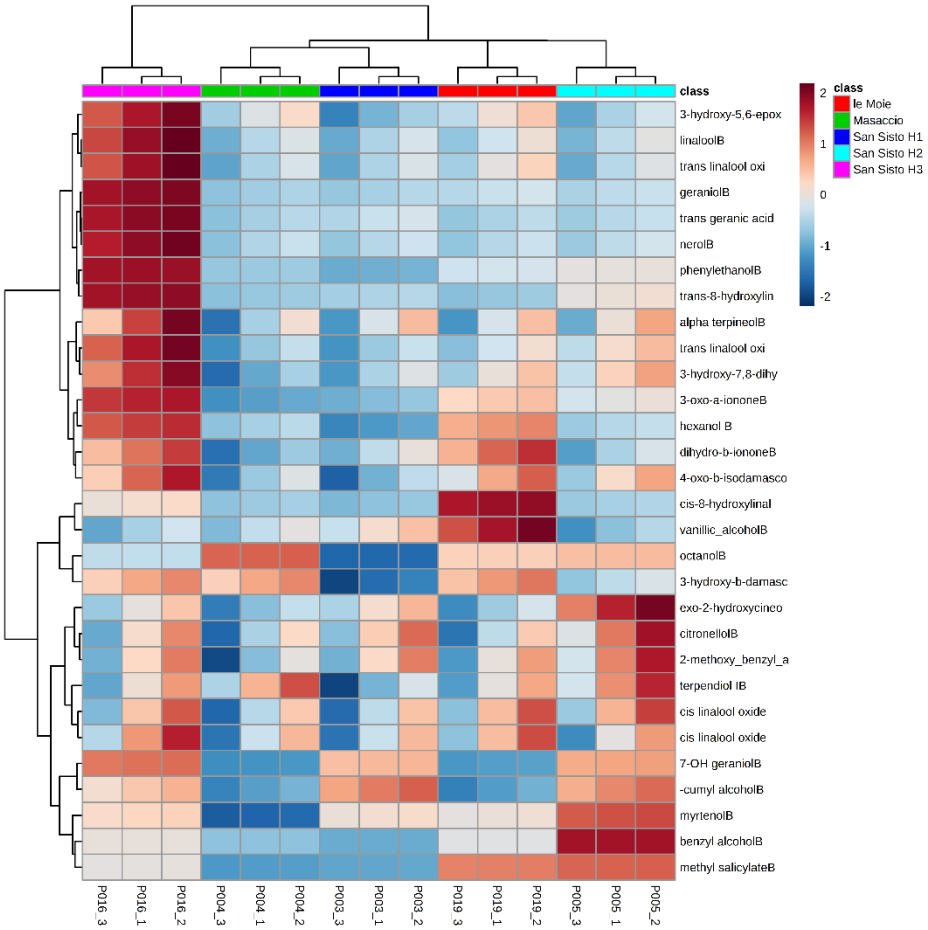


Table 2: Odoriferous zones perceived by GC–AEDA (each GC–O analysis was performed by six experienced judges)

RI wax^{exp}	RI ^{lit}	FD	Descriptor	Impact compound	CAS
1006	958	243	Apple	ethyl 2-methylpropanoate	97-62-1
1016	938	27	Kiwi	ethyl propanoate	105-37-3
1035	1041	81	Bilberry	ethyl 3-methylbutanoate	108-64-5
1024	1110	81	Banana	isoamyl acetate	123-92-2
1094	-	81	Toasted almonds	unknown	-
1080	1033	9	Apple/pineapple	ethyl butanoate	105-54-4
1201	1118	3	Herbaceous (fusels)	1-butanol	71-36-3
1229	-	3	Balsamic	unknown	-
1179	1174	27	Harsh, stale	isoamyl alcohol	123-51-3
1225	1232	243	Bilberry/fruity	ethyl hexanoate	106-32-1
1264	1269	9	Fruity	hexyl acetate	142-92-7
1304	1302	27	Mushroom	1-octen-3-one	4312-99-6
1348	1345	3	Yeasty-creamy	ethyl lactate	97-64-3
1376	1328	3	Fruity	4-methyl-pentanol	626-89-1
1437	1434	27	Fruity/apple	ethyl octanoate	106-32-1
1443	1454	27	Boiled potato	methional	3268-49-3
1466	1447	3	Winegar	acetic acid	79-09-4
1523	1532	9	Fruity	butanoic acid, 3-hydroxy-, ethyl ester	5405-41-4
1586	1570	27	Cheese, dog	isobutyric acid	503-74-2
1629	1635	3	Creamy, oily	γ butyrolactone	96-48-0
1646	1620	27	Cheese	butyric acid	107-92-6
1686	1671	81	Taleggio cheese	isovaleric acid	503-74-2
1685	1693	3	Fruity/sweaty	ethyl 9-decenoate	67233-91-4
1730	1730	27	Boiled potato	3-methylthio-1-propanol	505-10-2
1732	1747	3	Aniseed	3-methyl-2,4-nonanedione	113486-29-6
1740	1730	9	Not defined	methyl salicylate	119-36-8
1815	1838	243	Baked apple	β-damascenone	23726-93-4
1815	1810	9	Flowery/rose	phenethyl acetate/ethyl-4-hydroxybutanoate	103-45-7
1845	1875	3	Tropical/unpleasant	1-hexanol, 3-mercapto	51755-83-0
1864	1855	3	Pungent/chemical	hexanoic acid	142-62
1881	1875	9	Floral	benzenmethanol	100-51-6
1919	1909	243	Rose	2-phenylethanol	60-12-8
1962	1951	3	Sweet/green	2,6-dimethyl-3,7-octadiene-2,6-diol	13741-21-4
1956	1939	81	Coconut	whiskey lactone I	39212-23-2
1960	1920	81	Violet	β-ionone	14901-07-6
2003	-	27	Licorice/vegetal	2H-pyran-2,6(3H)-dione (t.i.)	5926-95-4
2045	2047	243	Cotton candy	furaneol	3658-77-3
2050	2025	9	Acetic/bad	octanoic acid	124-07-2
2093	2080	243	Cotton candy	homofuraneol	27538-09-6
2044	-	9	Mushroom	unknown	-
2098	2123	27	Peach/sweet	γ-decalactone	706-14-9
2137	2168	9	Smoke-roast	ethyl 5-oxotetrahydro-2-furancarboxylate	1126-51-8
2196	2186	27	Leather/weiss beer	4-vinylguaiacol	7786-61-0
2200	2202	243	Celery like/sweet	sotolone	5579-78-2
2559	2556	243	Pastries, white chocolate	vanillin	121-33-5
2571	2555	27	Withered flower/honey	phenylacetic acid	103-82-2
2631	2640	27	Wood /clove	acetovanillone	498-02-2

Chapter 4: Genotype peculiarity

Methyl salicylate glycosides
Evidence of a variety characteristic.

This chapter is ready for submission.

Preface to Chapter 4:

As mentioned in the introduction, volatile aroma compounds frequently occur in plant tissues as non volatile glycosidically-bound precursors, the so-called "aroma glycosides". Glycosides are ubiquitous in the plant kingdom and found in all major plant organs, including fruit, leaf, seed, flower, bark and root. Glycosides are plant secondary metabolites consisting of a non-sugar component, called an aglycone, attached to one or more sugars. These compounds may contribute to aroma only after acidic or enzymatic cleavage of the glycone; in winemaking this reaction takes place during fermentation and storage. Several factors are important in determining the eventual concentrations of free aroma compounds arising from glycosidic precursors, including the amounts of precursors present in juice or must, enzymatic activity during fermentation, and pH/temperature during storage. Glycosides of volatile aglycones include aliphatic alcohols (e.g., C6 compounds), shikimate derivatives (e.g., methyl salicylate, benzyl alcohol, phenols and vanillin) and mevalonate/deoxyxylulose phosphate (DXP) derivatives (e.g. monoterpenoids and C13-norisoprenoids) The aglycone is always attached directly to β-D-glucose, yielding a monosaccharide (i.e. an O-β-D-glucoside, but the glucose can be further substituted by other sugars (α-L-arabinofuranose, α-L-rhamnopyranose, β-D-xylopyranose, β-D-apiofuranose and β-D-glucose) (Figure 1) to give the corresponding disaccharides. (Waterhouse, Sacks, & Jeffery, 2016)

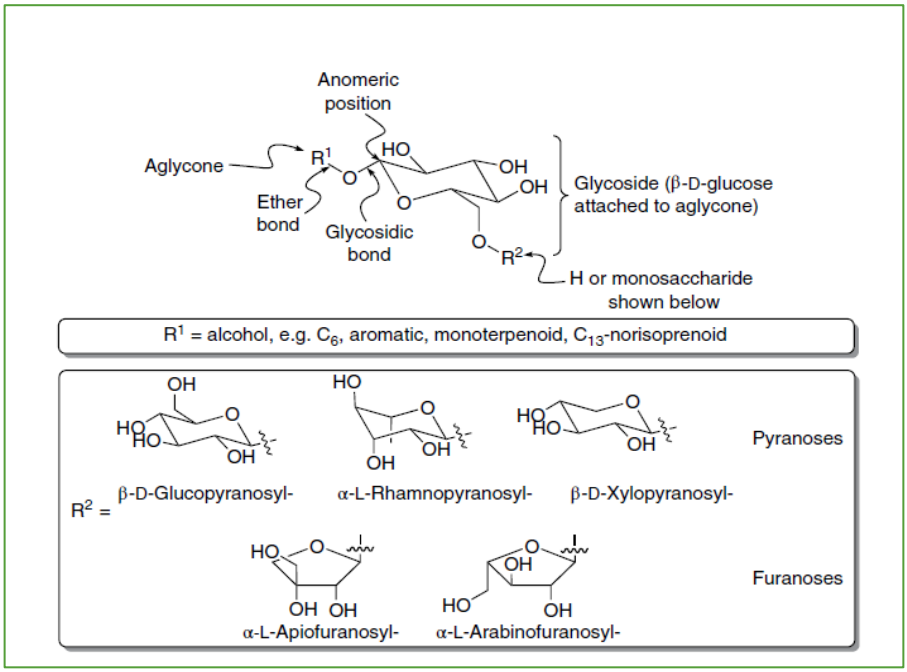


Figure 1 Structure of a generic glycoside, with the volatile compound (aglycone) (R^1) attached to the anomeric hydroxyl of D glucose (β-anomer shown). Different sugars (R^2) can be attached to the 6-position hydroxyl of glucose. *From Understanding WineChemistry By Andrew L. Waterhouse, Gavin L. Sacks and David W.Jeffrey.John Wiley and Sons,Chichester 2016. 470 pp., ISBN 978-1118627808*

The mechanisms underpinning the glycosylation of volatiles in grape berries have not been fully elucidated, but their formation putatively involves the reaction of an alcohol with a sugar. Currently, there is limited understanding regarding the biosynthesis, structures and composition of glycosides in plants and foods, particularly the glycosidic precursors of volatile aroma compounds. These limitations are primarily due to a lack of analytical methods that directly analyse glycosides. Most current techniques require derivatisation or hydrolytic cleavage of the glycone, followed by analysis of free volatiles and/or the released sugar moiety (Hjelmeland, Zweigenbaum, & Ebeler, 2015)

This research focused on methyl salicylate glycoside precursors.

Methyl salicylate (MeSA) (Figure 2) is an organic ester naturally produced by many species of plants, particularly wintergreens. It is synthesised from salicylic acid, a phytohormone that contributes to plant pathogen defence. MeSA is synthesised by members of a family of O-methyltransferases. The compound was first extracted and isolated from the plant species *Gaultheria procumbens* in 1843. It is also synthetically produced and is used as a fragrance in foods and beverages and in liniments. From the sensorial point of view, methyl salicylate has a balsamic-sweet odour that is known as "Wintergreen oil". This oil is made by steam processing of warmed, water-soaked wintergreen (*Gaultheria procumbens*) leaves. Is also used for acute joint and muscular pain, both in human and animals, and as a flavouring agent in chewing gum and mints in small concentrations, and added as an antiseptic to mouthwash solutions. This compound is very interesting due both to its healthy and sensorial qualities. Many studies, especially in China, have highlighted the healing properties of this compound, without the side effects that may be caused by aspirin, a compound with a very similar structure, (acetylsalicylic acid).

methyl salicylate

Figure 2 chemical structure

Role of methyl salicylate in plants

Plants respond to pathogen attack by activating both local and systemic defences that restrict pathogen growth and spread. In the infected leaf, these defences often involve a hypersensitive response, in which necrotic lesions form at the infection site(s) (Dempsey, Shah, & Klessig, 1999); uninoculated tissues subsequently develop systemic acquired resistance (SAR), a state of heightened defence throughout the plant. SAR is similar to acquired immunity in animals, in that it is systemic and long-lasting; it also resembles innate immunity, as it provides broad-spectrum resistance to secondary infection. (Durrant & Dong, 2004). A paper published in 2007

in Science with the title: Methyl Salicylate Is a Critical Mobile Signal for Plant Systemic Acquired Resistance argues that this compound is a mobile SAR signal. This hypothesis is supported by gas chromatography–mass spectrometry analysis, which revealed increased methyl salicylate levels in primary infection. (Park, Kaimoyo, Kumar, Mosher, & Klessig, 2007)

Methyl salicylate in wine

We found that in some Italian varieties methyl salicylate in "bound" forms is present in high amounts.

It can be assumed that during ageing this compound hydrolyses from precursors and gives the balsamic note that appears in some aged Verdicchio wines.

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Methyl salicylate glycosides in some Italian varietal wines

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Glycosides are plant secondary metabolites consisting of a non-sugar component, called an *aglycone*, attached to one or more sugars. Glycosides are ubiquitous in the plant kingdom and they are present in all plant organs, such as fruit, flowers, roots, seed and bark (Winterhalter & Skouroumounis, 1997). Most aglycones are non polar and glycosylation increases their water solubility and facilitates the transport, accumulation and storage of these compounds, together with the detoxification of some of them (Anna K. Hjelmeland & Ebeler, 2015; Sarry & Günata, 2004). The aglycone part can be an aliphatic alcohol (C6 compounds), a shikimate derivate (benzyl alcohol, phenols or methyl salicylate) or terpenoid (monoterpenoid or norisoprenoid). All these compounds can be a reserve of odour compounds after hydrolysis (I. L. Francis, Sefton, & Williams, 1992).

The first volatile aroma glycosides were found in the rose in 1969 (M. J. O. Francis & Allcock, 1969). In 1974 Cordonnier and Bayonove suggested that these compounds could be present in

the grape, but it was only in 1982 that these aroma precursors were identified in the grape by William et al. (P.J. Williams, Strauss, Wilson, & Massy-Westropp, 1982) Some researchers then observed that glycosides in terpenic grapes and wines were an important reserve of potential wine flavour. They also reported that 90% of monoterpenes were found in the glycosylated or bound form in Muscat of Alexandria grapes (S. K. Park, Morrison, Adams, & Noble, 1991). Glycosides are made up of an aglycone that is linked to one or more sugar moieties, which are glycones.

Hydrolysis of this "bound" compound happens during fermentation or storage. The most important factor that increases this reaction is enzymatic activity during fermentation and pH/ temperature during storage, and of course the amount of precursors in the grapes. It is well-known that the monoterpenes released during winemaking and ageing by glycosides are an important factor in the fruity and floral flavour of "aromatic" wines such as Muscat and Gewürztraminer (Gunata, Bayonove, Baumes, & Cordonnier, 1985). In other wines the glycosides of C13-norisoprenoids are more important, with tea and grassy notes in Chardonnay and Semillon wines (Patrick J. Williams, Sefton, & Francis, 1992), but also kerosene-like characteristics in aged Riesling wine. Monoterpene glycosides produce volatile aroma compounds directly after hydrolysis, while norisoprenoids glycosides may produce odourless products after hydrolysis that require other chemical reactions to produce volatile aroma compounds (Sefton, Skouroumounis, Elsey, & Taylor, 2011; Winterhalter & Skouroumounis, 1997) Glycosides in wine originate from the grape berry during ripening and appear to be well correlated with the concentration of their corresponding aglycones. A recent work demonstrated that grapevine exposure to bushfire smoke can lead to accumulation of volatile phenols in glycoconjugate forms and therefore to release of the free form during winemaking (Hayasaka, Dungey, Baldock, Kennison, & Wilkinson, 2010). The aglycone is always attached directly to β-D-glucose, but the glucose can be further substituted by other sugars such α–L-

arabinofuranose, α–L-rhamnopyranose, β-D-xylopyranose, β-D-apiofuranose and β-D-glucose to give the corresponding disaccharides. A rhamnosyl-glucoside is commonly called rutinoside, glucosyl-glucoside, gentiobioside or sophoroside, depending on 2-O rather than 6-O linkage (Waterhouse et al., 2016). Due to its potential role in the aroma characteristics of wine, quantification of this precursor could be useful for winemakers to determine, for instance, the optimal maturity of grapes and the most suitable winemaking processes to best enhance them (Schneider, Charrier, Moutounet, & Baumes, 2004).

One of the aglycones also found in grapes and in wine is methyl salicylate, the so-called methyl salicylate glycoside that has aroused widespread concern. Structurally, a methyl group is attached to the C(1) position of the mother nucleus, while the C(2) position is connected to it by a O-glycosidic bond. Some of these glycosides are expected to substitute aspirin due to their long-term effects and fewer side effects. To date nine different methyl salicylate glycosides from plants have been reported (Mao et al., 2014). All of them have one glucopyranosyl unit connected directly to methyl salicylic acid. These methyl salicylate glycosides are mainly spread over the genera *Gaultheria, Camellia, Polygala, Filipendula* and *Passiflora.* Some of these plants have also been used in traditional medicine.

Methyl salicylate was found in *V. Riparia* grapes by (Schreier & Paroschy, 1980) and in *V. vinifera* sp. (Cabaroglu et al., 1997; Versini, Moser, & Carlin, 2005a) and the Frontenac interspecific hybrid (Mansfield, Schirle-Keller, & Reineccius, 2011). We found that the methyl salicylate glycoside content in Verdicchio, Trebbiano di Soave and Trebbiano di Lugana wines was very high in comparison to other varieties (up to 0.5 mg/L). All these varieties are genetically similar (Vantini et al., 2003). This is a confirmed by studies done in the past, in which the existence of two glycosides was highlighted: a methyl salicylate 2-O-β-D-glucoside and a methyl salicylate 2-O-β-D-xylopyranosyl (1-6) β-D-glucopyranoside (MeSA-primeveroside or Gaultherin), while the possible presence of other glycosides was hypothesised

(Versini, Moser, & Carlin, 2005b). As the olfactory threshold of this compound is between 50 and 100 μg/L, it is possible that methyl salicylate contributes to the balsamic scent in Verdicchio wines. For this reason the aim of this work was to identify and quantify precursors of methyl salicylate in wines in their native glycosidic forms (Table 1).

Materials and methods

Methyl salicylate 2-O-β-D-glucoside (MeSAG) and Methyl salicylate 2-O-β-D-xylopyranosyl (1-6) β-D-glucopyranoside (MeSA-primeveroside or Gaultherin) were from Sigma Aldrich and iChemical Technology (Shangai) respectively. Plants, dried leaves and fruit: *G. procumbens, G. yunnanensis (Franch.) Rehder, V. cornuta V. tricolor, P. Ginseng, C. Sinensis* and *P. Edulis* were from local stores. All solvents for GC and HPLC analysis (MS grade) were purchased from Sigma-Aldrich (Milan, Italy)

Forty different white wine varieties bought in a local store present in our experimental cellar were analysed, including Verdicchio, Trebbiano di Soave, Trebbiano di Lugana, Pevarella and international and local varieties (Table 2).

SPE ENV+ extraction procedure. 10 mL of wine, added to 25 uL of n-heptanol (200 mg/L) as internal standard, were extracted with solid phase extraction using ENV+ cartridges, 1 g (Biotage, Sweden). The cartridge was pre-conditioned with 15 mL of methanol followed by 20 mL of water. The aqueous extract was loaded onto the cartridge, which was then washed with 5 mL of water. The free aromatic compounds were eluted from the cartridge with 10 mL of dichloromethane and 20 mL of pentane were added to it followed by the addition of anhydrous Na_2SO_4 to remove water. Subsequently, the whole fraction was carefully concentrated prior to analysis up to 200 μL using a Vigreux column. The bound aromatic compounds (i.e. glycosides) were eluted with 10 mL of methanol. Methanol was eliminated under vacuum and the residue

solubilised in 1 mL of methanol/water 1/9, filtered through a 0.22 µm PTFE filter into a 2 mL amber vial.

GC–MS analysis of free methyl salicylate. Analysis of free volatiles was performed using a Trace GC Ultra gas chromatograph coupled to a Quantum XLS mass spectrometer (Thermo Scientific, Milan, Italy) mounted with a PAL combi-xt autosampler (CTC, Zwingen, Switzerland). A Rxi®-5Sil MS capillary column (30 m x 0.25 mm x 0.25 µm, Agilent Technologies) was used. One microlitre of sample was injected in splitless mode with a GC inlet temperature of 250 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL/min. The oven temperature was programmed as follows (i) initial temperature 50 °C held for 2 min, (ii) linearly raised by 8 °C/min to 150 °C, and (iii) in the final step the temperature was ramped at 20°C/min to 280°C, maintained for 1 min (total run-time was 22 min). The mass spectrometer was operated in positive electron ionisation mode at 70 eV and all spectra were recorded in full scan with a mass range of 40–350 Da; transfer line and source temperatures were set at 250 °C. Electron ionisation was applied at 70 eV with an emission current of 50 µA. Thermo Excalibur software (1.1.1.03, Thermo Scientific) was used for all acquisition control and data processing. A calibration curve was generated, spiking the model wine solution with 5-500 µg/L of methyl salicylate.

UPLC-Q-TOF-HDMS analysis of glycosides. An Acquity UPLC system interfaced with a Waters Synapt HDMS-QTOF mass spectrometer with electrospray ionisation system (ESI) (Waters Corporation, Manchester, UK) was used to perform LC–HDMS analysis of glycosylated precursors. All samples were analysed on a reversed phase (RP) ACQUITY UPLC 1.8 m 2.1 × 150 mm HSS T3 column (Waters) protected with an Acquity UPLC® BEH HSS T3 1.8 m, 2.1 × 5 mm precolumn (Waters), at 40 ◦C and with a mobile phase flow rate of 0.28 mL/min. Water was used as weak eluting solvent (A) and methanol as strong eluting solvent (B); formic acid0.1% v/v was used as an additive in both eluents. The multistep linear

gradient used was as follows: 0–1 min, 100% A isocratic; 1–3 min, 100–90%A; 3–18 min, 90–60%A; 18–21 min, 60–0%A; 21–25.5 min, 0% A isocratic; 25.5–25.6 min, 0–100% A; 25.6–28 min 100% isocratic. The injection volume was 2 µL. Mass spectrometric data were collected in positive ESI mode over a mass range of 50–2000 m/z, with a scan duration of 0.3 s in centroid mode (Arapitsas et al., 2016). For quantification sodiate masses were used (337.09 for methyl salicylate 2-O-β-D-glucoside, m/z 469.13 for the 3 isomers MeSA primeveroside, Canthoside A and MeSA-vicianoside (violutoside), m/z 499.14 for MeSA-gentiobioside and m/z 483.15 for MeSA- rutinoside. The calibration curves were prepared in solvent using 15 levels of MeSA 2-O-β-D-glucoside and MeSA-primeveroside with concentrations between 1 µg/L e 10 mg/L.

Isolation

500 g of *Gaultheria procumbens L*. dry leaves (A. Minardi & Sons Italy) were placed twice in 2 L of hot milliQ water (90°C) and infused for 4 hours (room temperature), then centrifuged and filtered through a 0.44 µm filter.

Flash chromatography with Isolute ENV+ and preparative HPLC for the isolation of the single glycosides

For chromatographic separation a Shimadzu SCL-10 AVP preparative HPLC system with a Shimadzu SPD-10 AVP UV-VIS detector, 8A pumps and Software Class VP (Shimadzu Corp., Kyoto, Japan) were used. For flash chromatography 30 g of ENV+ resin were used (IST Ltd, United Kingdom), conditioned with 300 mL of methanol and 500 mL of milliQ water. 500 mL of water extract were loaded each run and elution was carried out using 15 mL/min of water as solvent A and methanol as solvent B. The linear gradient went from 0 to 100% of solvent B in 120 min. The fraction with the peaks of interest was collection. Separation of the fraction was done using a *Develosil ® 100DIOL-5 300 x 20,0 mm* (CPS ANALITICA, Milan). The mobile

phase was acetonitrile as solvent A and methanol 3% milliQ water (Solvent B). Run temperature was set to 65°C, 0-15 min 0% B, 15-30min linear gradient, 0 to 15% of B; 30-40 min 15%-100%B; 40-50 min 100% B. The injection volume was 2 mL. Each peak of interest was fractionated and injected into a HPLC-DAD Alliance 2695 (Waters, Milford, MA, USA) with a Discovery HS-C18 25 cm x 10 mm, 10 µm (SUPELCO, USA) column and a UV-VIS 2996 (Waters) detector at 280 nm. The injection volume was 100µL. Mobile phase milliQ water (Solvent A) and methanol (Solvent B). Run temperature was set to 40°C, 0-2 min 5-20%B; 2-20 min 32.5%; B wash with 100% B for 2 min. Peaks of interest were fractionated with a Waters Fraction Collector 3 (Waters, Milford, MA, USA).

Discussion

The methanolic extract of Verdicchio wine after SPE was injected into a UHPLC coupled to a high resolution mass spectrometer. In the chromatogram it was possible to see the methyl salicylate monoglycoside peak (MeSAG) (m/z+ 337,0900 (M+Na)), confirmed by the standard. This compound is the structural foundation of all glycosides, a methyl salicylate group linked with the –OH of a glucopyranosyl unit. MeSAG was also the most widely distributed compound in all the plants that we injected. We also found 3 diglycoside isomers (m/z+ 469,1322 (M+Na) (Figure 1), which have the structural unit of MeSAG connected to another monosaccharide that could be apiose/xylose/arabinose. In Figure 2 (chromatogram of hexose isomers) there is one diglycoside (m/z+ 499,1428 (M+Na)) that can be attributed to MeSA-lactoside or MeSA-gentiobioside, and in Figure 3 there is one diglycoside with m/z+ 483,1479 (M+Na), which corresponds to MeSA-rutinoside. We did not find any MeSA-triglycosides.

In order to understand which glycosides were present in wine, we tried to extract different plants that were particularly rich in such glycosides, according to the literature (Mao et al., 2014). Of these, the plant *Gaultheria* is perhaps the richest source. Indeed, wintergreen oil (98% methyl salicylate) is obtained from this species by steam distillation of leaves of the plant, following

maceration in warm water in order to promote enzymatic action and hydrolyse the methyl salicylate from glycosides. To date, five glycosides have been found in this plant: MeSAG, MeSA-primeveroside, MeSA-lactoside and two triglycosides. In the methanolic extract of *Gaultheria procumbens* we saw three peaks with mass m/z+ 469,1322 (M+Na), the largest corresponding to MeSA-primeveroside, also confirmed with the pure standard, whereas the other two isomers should be MeSA-vicianoside (violutoside) and Canthoside A. In order to identify the 2 isomers, the methanolic extracts of two *Viola* species (*V. cornuta* and *V. tricolor*) that are the richest in MeSA-vicianoside (Violutoside) according to the literature (Mao et al., 2014) were analysed, and a major peak with mass m/z+ 469,1322 (M+Na), corresponding to MeSA-vicianoside (Violutoside), was found. In the wine chromatogram (Fig. 1) is therefore possible to deduce that the first isomer with mass m/z+ 469,13 (M+Na) and a retention time of 13.59 is Canthoside A, while the second with RT 13.86 should be MeSA-vicianoside (Violutoside), and the last with RT 14.35 should be MeSA-primeveroside. It is also evident that the most abundant peak in wine is MeSA-vicianoside (Violutoside). Methanolic extract from green tea leaf *(C. Sinensis*) confirmed the presence of monoglycoside and MeSA-primeveroside, as reported in some papers (D. Wang, Yoshimura, Kubota, & Kobayashi, 2000; Dongmei Wang, Kubota, Kobayashi, & Juan, 2001; Dongmei Wang, Kurasawa, Yamaguchi, Kubota, & Kobayashi, 2001). In the same way, we found the presence of MeSA-rutinoside in the methanolic extract of *Passiflora edulis* (Chassagne, Crouzet, Bayonove, & Baumes, 1997). We also tried to identify the 2 diglycoside (m/z+ 499,1428 (M+Na) isomers. Unfortunately, the diglycoside glucosyl-glucoside (MeSA-gentiobioside) present in the *Ginseng Panax* plant according to the literature (Mao et al., 2014) was not found in our ginseng extract. Several studies have shown that MeSA-lactoside was isolated from *Gaultheria yunnanensis (Franch.) Rehder* (Yang et al., 2016; Zhang et al., 2015), so we tried to inject an extract of this plant in order to identify which isomer corresponds to MeSA-lactoside. The injection excluded the

presence of this compound in wine, and therefore the one present in wine is the other MeSA-gentiobioside isomer. Isolation and subsequent characterisation with NMR will help us to confirm the correct identification of these compounds.

In order to understand if these glyconjugated compounds are characteristic of Verdicchio and homologous varieties (Trebbiano di Soave and Lugana) we analysed 40 white wines (Table 2). We found that in the free forms the concentration of MeSA in "Trebbiano di Soave/Lugana and Verdicchio" varieties was in the range 14-110 μg/L, while the sum of the 6 glycoside precursors varied from 33-300 μg/L (Table 2), while in "other" white wines the free form of MeSA was from 1 to 21 μg /L and lower than 15 μg /L as the sum of glycosides. In Verdicchio/Trebbiano di Soave and Lugana wines the most abundant glycoside precursor was MeSAG, followed by MeSa-vicianoside (Violutoside) (Figure 4).

The sensory impact of these compounds in wine is not particularly clear. In the literature they are described as having an odour of wintergreen, mint and fresh green character (Mansfield et al., 2011) and the threshold is around 50-100 ug/L. Some preliminary tests made by adding β-glycosidase enzymes during vinification are underway to measure how much methyl salicylate is released by the precursors. In tasting of old Verdicchio vintages a balsamic note often appears and could derive from release of this compound by the precursors.

Conclusions

Methyl salicylate is a very intriguing molecule, both in sensorial terms and due to the role it plays in defending the plant and in relation to human health. This work confirmed the presence of this compound in bound form as a characteristic of Verdicchio, Trebbiano di Soave and Lugana. The different glycosylated forms were analysed and it was found that monoglycoside and violutoside are the forms most present in wine. The compound released by precursors during ageing can be considered to be responsible for the balsamic aroma perceived in old

Verdicchio vintages. Only after isolation and NMR analysis of compounds from the dry leaves of *Gaultheria procumbens* will be possible to correctly identify some of these molecules.

Table 1. Chemical name, common name, molecular formula and monoisotopic masses of different glycosides of MeSA found in wines.

Chemical name	Common name	Molecular formula	Monoisotopic Mass	MM+Na
methyl salicylate (benzoic acid, 2-hydroxy-, methyl ester)	MeSA	C8H8O3	152.0473	175.0371
methyl salicylate 2-O-β-D-glucoside	MeSAG	C14H18O8	314.1002	337.09
methyl salicylate 2-O-β-D-xylopyranosyl (1-6) β-D-glucopyranoside	MeSA-primeveroside or gaultherin	C19H26O12	446.1424	469.1322
methyl salicylate 2-O-α-L-arabinopyranosyl(1-6)-β-D-glucopyranoside	MeSA-vicianoside or violutoside	C19H26O12	446.1424	469.1322
methyl salicylate 2-O-β-D-apiofuranosyl(1-6)-β-D-glucopyranoside	MeSA-canthoside A	C19H26O12	446.1424	469.1322
methyl salicylate 2-O-β-D-galactopyranosyl(1-4)-β-D-glucopyranoside	MeSA-lactoside	C20H28O13	476.1529	499.1428
methyl salicylate 2-O-β-D-glucopyranosyl(1-6)-O-β-D-glucopyranoside	MeAS-gentiobioside	C20H28O13	476.1529	499.1428
methyl salicylate 2-O-α-L-rhamnopyranosyl(1-6)-β-D-glucopyranoside	MeSA-rutinoside	C20H28O12	460.158	483.1478

Table 2 Content of different MeSA glycosides and free methyl salicylate in different wine varieties.

		Glycosides							*Free*
Variety	**vintage**	**monoglycosides**	**diglycosides**					***Sum of all***	
		MeSAG	MeSA-primeveroside	MeSA-violutoside[a]	MeSA-canthoside A[a]	MeSA-gentiobioside[a]	MeSA-rutinoside[a]		**MeSA**
Trebbiano di Soave*	2017	28,82	0,05	11,5	NF	NF	0,92	**41,3**	68,6
Trebbiano di Soave*	2017	27,17	0,07	10,5	NF	NF	0,80	**38,5**	44,5
Trebbiano di Soave*	2016	105,5	8,08	59,0	8,36	12,0	3,46	**196,4**	45,4
Trebbiano di Lugana*	2016	43,75	1,28	14,7	NF	2,77	2,91	**65,4**	33,9
Trebbiano di Lugana*	2017	108,9	12,0	45,8	10,3	15,6	11,3	**203,8**	110,7
Trebbiano di Lugana*	2016	117,1	14,58	84,6	4,69	18,3	4,87	**244,2**	31,7
Trebbiano di Lugana*	2016	129,9	15,73	100,7	3,36	29,0	16,4	**295,1**	23,9
Verdicchio*	2015	47,28	1,65	13,6	0,015	3,17	5,91	**71,6**	14,0
Verdicchio*	2014	32,32	0,97	24,8	1,82	5,02	0,04	**65,0**	5,46
Verdicchio*	2013	30,07	0,67	35,3	NF	20,3	NF	**86,3**	18,2
Verdicchio*	2016	22,94	0,02	9,07	NF	NF	1,22	**33,3**	30,4
Verdicchio*	2016	27,68	0,96	15,05	5,96	0,94	2,94	**53,5**	17,7
Verdicchio*	2016	36,91	1,84	20,09	NF	9,23	3,97	**72,0**	20,7
Verdicchio*	2016	23,29	0,39	14,75	NF	1,14	1,12	**40,7**	18,7
Verdicchio*	2016	42,93	2,40	35,75	2,93	0,64	0,01	**84,7**	14,8
Verdicchio*	2016	22,82	NF	10,87	NF	NF	1,47	**35,2**	16,7
Verdicchio*	2008	99,9	16,0	50,3	0	28,6	1,77	**196,6**	66,8
Verdicchio*	2010	94,6	5,63	29,9	1,45	15,3	21,5	**168,4**	32,7
Peverella *	2013	0,039	NF	6,34	NF	NF	NF	**6,38**	56,5
Psarades [§]	2017	NF	NF	NF	NF	NF	NF	NF	4,47
Baiano [§]	2017	NF	NF	NF	NF	NF	NF	NF	3,93
Chardonnay [§]	2016	4,86	NF	2,81	NF	NF	NF	**7,7**	7,39
Ribolla gialla [§]	2017	1,53	NF	0,418	NF	NF	NF	**2,0**	2,07
Bianca [§]	2016	2,60	2,50	1,37	NF	NF	NF	**16,5**	12,0
Riesling renano[§]	2016	0,069	0,02	0,15	NF	NF	NF	**0,2**	2,95
Catarratto[§]	2016	5,10	NF	2,51	NF	NF	NF	**7,6**	7,69
Helios[§]	2016	0,707	NF	8,95	NF	NF	NF	**9,7**	2,08
Gewürztraminer[§]	2009	NF	NF	NF	NF	NF	NF	NF	3,06
Grüner Veltliner[§]	2009	NF	NF	NF	NF	NF	NF	NF	2,10
Müller Thurgau[§]	2016	NF	8,5	0,057	NF	NF	NF	**8,5**	5,24
Tocai friulano[§]	2012	NF	NF	NF	NF	NF	NF	NF	2,99
Chardonnay [§]	2016	NF	NF	NF	NF	NF	NF	**0,0**	3,44
Pinot gris[§]	2016	NF	NF	NF	NF	NF	NF	**1,3**	3,07
Goldtraminer[§]	2016	NF	NF	NF	NF	NF	NF	**NF**	5,28
Goldtraminer[§]	2009	NF	NF	NF	NF	NF	NF	NF	1,95
Soave[§]	2016	17,5	2,43	18,0	1,87	3,36	0,82	**44,0**	7,45
Soave [§]	2016	42,0	4,59	38,6	1,21	1,72	0,45	**88,6**	21,0
Muscaris[§]	2017	0,415	NF	0,94	NF	NF	NF	**1,36**	9,43
Solaris[§]	2017	NF	NF	NF	NF	NF	NF	NF	3,29

* Verdicchio and genetically similar; [§] others Variety; concentration expressed in µg/L ([a] as MeSA primaveroside)

Figure 4: Content of MeSA-monoglycosides, MeSA-diglycoside and methyl salicylate (MeSA) in free form (value in µg/L) in "Verdicchio, Trebbiano di Soave and Lugana wines" compared to other Italian and international varieties (see Table 2)

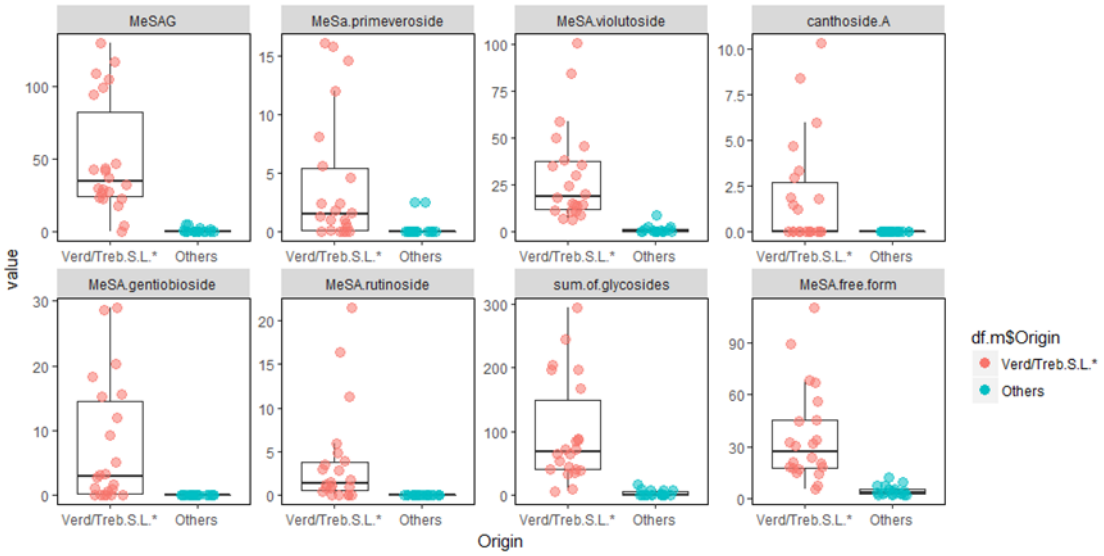


Figure 1: Extracted chromatogram of m/z 469.13 with the 3 diglycoside isomers found in Verdicchio wine

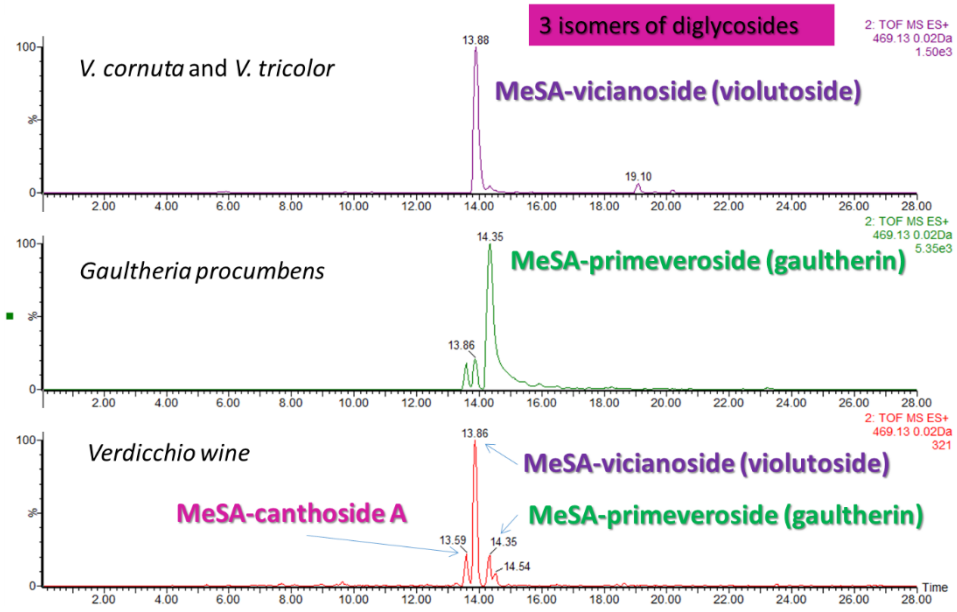


Figure 2: Extracted chromatogram of m/z 483.14 with rutinoside diglycoside found in Verdicchio wine

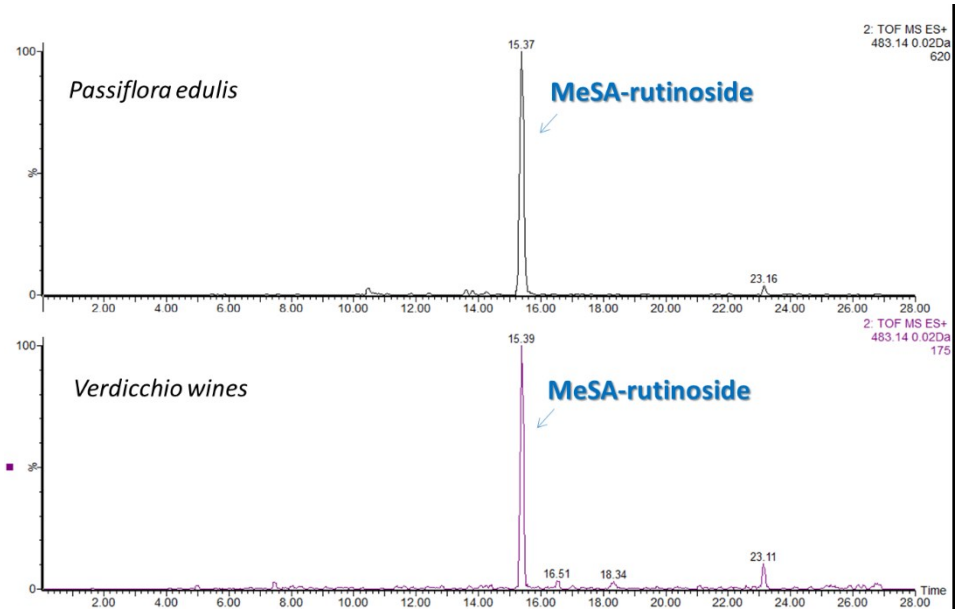
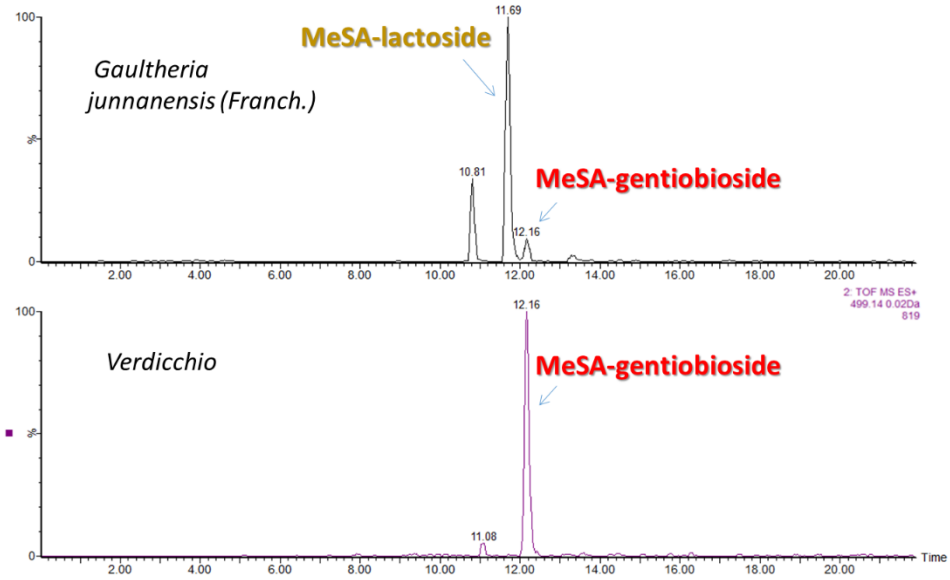


Figure 3 Extracted chromatogram of m/z 499.14 with the 2 diglycoside isomers, MeSA-gentiobioside is the only one in Verdicchio wine



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Chapter 5: Microbial differences in vineyards

The Costalunga vineyard, microbiota and volatile metabolome in *Corvina* grapes and wines

This chapter has been reprinted* from:

Core Microbiota and Metabolome of Vitis vinifera L. cv. Corvina Grapes and Musts. Stefanini I.[#], Carlin S.[#], Tocci N., Albanese D., Donati C., Franceschi P., Paris M., Zenato A., Tempesta S., Bronzato A., Vrhovsek U., Mattivi F., Cavalieri D. (2017) Frontiers in Microbiology. 8:457. doi: 10.3389/fmicb.2017.00457

[#] *Equal contribution*

Preface to Chapter 5:

The aim of this work was to study the microbiota and volatile metabolome of a newly planted vineyard to understand the possibility of carrying out the fermentation process partially spontaneously, identifying a set of micro-organisms characteristic of two parts of the vineyard. The project consisted of defining the role of vineyard exposure and distance from the forest on the microbial communities of grapes, paying particular attention to the role of withering in defining or enhancing the characteristics of vineyard microbiota. The object of study was the "Costalunga" vineyard, located in Valpolicella, (Verona, Italy), an area suited for the production of *Corvina*, *Corvinone* and *Rondinella* grapes, important autochthonous varieties used to make Amarone. The hill is characterised by two slopes, the first ("Quarry") oriented towards the Verona plain and closed to the east by a small quarry, the second ("Lake") open to the west towards Lake Garda. The vineyard ecosystem is marked by microclimatic variables such as exposure, hours of sunshine, ventilation and maximum and minimum temperatures, playing a fundamental role in the growth of the vineyard and the maturation of grapes, and contributing to the interaction between vines, grassy areas between the rows and insects. The vineyard is surrounded by a wooded belt and a farm estate with hedges located in the middle, representing possible areas for sheltering wildlife, which suggests a particular contribution to the microbiome.

In the year before planting, the area was subjected to agricultural reclamation which involved modifying the surface area, making it less steep and creating ridges for optimal draining of surface water, while building work facilitated the construction of the road network within the vineyard. Mixing of the first 50 cm of ground soil cancelled the "soil" factor characterising the typical terroir of the vineyard.

The first part of the study, the subject of the publication, focused on studying the composition and changes in the fungal population and metabolites present in the grapes and wines of cv *Corvina* cultivated in this vineyard.

The second part of the study, which has not yet been published, instead investigated differences at sensorial level between wines coming from the two sides of the vineyard (Lake and Quarry). The grapes from the two parts of the vineyard were harvested, then subjected to a withering period and vinified separately following the same protocol. The samples were analysed with the SPE technique, followed by GCXGC-ToFMS and GC-MS/O analysis (materials and methods are reported in detail in Chapter 3). The SPE extracts were injected into GC-MS/O

and the samples were sniffed by five trained panellists (2 females and 3 males) to determine aroma-impact compounds. We applied AEDA (aroma extraction dilution analysis) to determine the relative odour potency of compounds present in the extract as the factor dilution (FD). The extract was diluted with a series of 1:3 dilutions, and each dilution was sniffed until no significant odour was detected. Several injections were required to reach a dilution of the aroma extract in which odorous regions were no longer detected. The factor dilution (FD value) is the last dilution at which an odour active compound is detected.

Most of the substances were present in both wines but they differed mainly in terms of the dilution factor value. The “Lake” sample was distinguished by floral notes and notes of celery and honey that are not present in the “Quarry” wine, whereas this sample was richer in cotton candy, but also camphor and other notes recalling wood. All the 46 compounds are reported in Table 1 with the FD value.

Table 1. Main odourants resulting for the two "Lake" and "Quarry" wines from GC-O analysis.

Impact compound	CAS	RI^{exp}	RI^{lit}	Descriptor	FD[a]	
					Lake	Quarry
ethyl 2-methylpropanoate	97-62-1	1006	958	Apple	3	9
ethyl propanoate	105-37-3	1016	938	Kiwifruit	27	81
ethyl butanoate	105-54-4	1076	1034	Ripe kiwifruit	27	81
ethyl 2-methylbutanoate	7452-79-1	1080	1033	Red fruits	81	81
isoamilic alcohol	123-51-3	1220	1208	Harsh, stale	81	-
unknown	-	1222	-	Toasted almonds	9	9
1-butanol	71-36-3	1150	1140	Herbaceous (fusels)	3	9
1-octen-3-ol	3391-86-4	1326	1447	Mushroom	1	3
1-hexanol	111-27-3	1338	1353	Hay, grass	-	3
unknown	-	1365	-	Cinnamon	-	1
vitispirane	65416-59-3	1449	1513	Camphor	-	1
methional	3268-49-3	1443	1454	Boiled potato	243	81
benzaldehyde	100-52-7	1490	1520	Glue, chemical	-	1
acetic acid	79-09-4	1466	1447	Winegar	-	1
linalool	78-70-6	1583	1545	Citrus, lemon	-	3
isobutyric acid	79-31-2	1586	1570	Stink, cheesy	9	81
butyric acid	107-92-6	1635	1620	Cheese	9	3
2-furanmethanol	98-00-0	1644	1654	Burnt plastic	-	?
acetophenone	98-86-2	1659	1636	Rotten flowers, honey	-	?
isovaleric acid	503-74-2	1668	1671	Cheese	243	243
alpha-terpineol	98-55-5	1700	1691	Terpene resin	-	1
3-methylthio-1-propanol	505-10-2	1732	1730	Cauliflower, potato	81	27
3-methyl-2,4-nonanedione	113486-29-6	1732	1747	Aniseed	243	3
unknown	-	1789	-	Grassy	81	-
β-damascenone	23696-85-7	1815	1838	Baked apple, apple strudel	-	3
unknown	-	1820	-	Tomato	-	1
phenethyl acetate/ethyl-4-hydroxybut.	101-97-3	1815	1810	Floral	9	9
benzenmethanol	100-51-6	1881	1875	Floral	9	-
unknown	-	1900	-	Grassy	243	-
2-phenylethanol	60-12-8	1919	1909	Rose	243	243
unknown	-	1941	-	Vanilla	-	9
whiskey lactone	39212-23-2	1956	1939	Coconut	27	81
furaneol	3658-77-3	2045	2047	Cotton candy	243	243
γ-decalactone	706-14-9	2058	2134	Peach, apricot	1	3
unknown	-	2065	-	Violet	9	-
unknown	-	2098	-	Rotten flowers, honey	3	81
unknown	-	2139	-	Floral	243	-
4-ethyl phenol	123-07-9	2158	2174	Horse, leather	-	1
γ–carboethoxy-γ-butyrolactone	1126-51-8	2180	2168	Burnt, smoky	3	?
4-vinyl guaiacol	7786-61-0	2196	2186	Cloves	-	1
sotolone	28664-35-9	2200	2202	Celery	27	-
unknown	-	2235	-	confectioner's sugar,	1	243
unknown	-	2220		Whiskey, wood	-	27
unknown	-	2403	-	Rotten flowers	-	1
vanillin	121-33-5	2530	2566	Vanilla	27	-
phenylacetic acid	103-82-2	2571	2555	Honey, floral	243	81

[a] FD values: indicates the last dilution at which an odor compound is percepted. (n = 5 assessors). Identification based on retention index (VFWax column).

frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 March 2017
doi: 10.3389/fmicb.2017.00457

Core Microbiota and Metabolome of *Vitis vinifera* L. cv. Corvina Grapes and Musts

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 15 January 2017
Accepted: 06 March 2017
Published: 21 March 2017

Citation:
Stefanini I, Carlin S, Tocci N, Albanese D, Donati C, Franceschi P, Paris M, Zenato A, Tempesta S, Bronzato A, Vrhovsek U, Mattivi F and Cavalieri D (2017) Core Microbiota and Metabolome of Vitis vinifera L. cv. Corvina Grapes and Musts. Front. Microbiol. 8:457. doi: 10.3389/fmicb.2017.00457

The composition and changes of the fungal population and of the metabolites present in grapes and in ferments of *Vitis vinifera* L. cv. Corvina, one of the major components of the Amarone musts, were dissected aiming at the identification of constant characteristics possibly influenced by the productive process. The fungal populations and metabolomic profiles were analyzed in three different vintages. 454-pyrosequencing on the ribosomal ITS1 region has been used to identify the fungal population present in Corvina grapes and fresh must. Samples were also subjected to metabolomics analysis measuring both free volatile compounds and glycosylated aroma precursors through an untargeted approach with comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry. Albeit strongly dependent on the climate, both the mycobiota and metabolome of Corvina grapes and fresh musts show some characteristics recursive in different vintages. Such persistent characteristics are likely determined by the method adopted to produce Amarone or other dry wines made from partially dried grapes. In particular, the harsh conditions imposed by the prolonged withering appear to contribute to the shaping of the fungal populations. The fungal genera and metabolites present in different vintages in *V. vinifera* L. cv. Corvina grapes and fresh musts represent core components of the peculiar technique of production of Amarone. Their identification allows the in-depth understanding and improved control of the process of production of this economically and culturally relevant wine.

Keywords: *Vitis vinifera* L. cv. Corvina, volatile compounds, untargeted metabolomics, metataxonomics, fungal populations, grapes, musts

INTRODUCTION

Amarone is a dry wine produced exclusively in the Italian region of Valpolicella (Verona) by the combination of *Vitis vinifera* L. cv. Corvina and *V. vinifera* L. cv. Rondinella withered red grapes (45–95% Corvina, 5–30% Rondinella, Paronetto and Dellaglio, 2011). Since 2010 Amarone is a DOCG (Denominazione di Origine Controllata e Garantita, "Controlled and Guaranteed Denomination of Origin") wine, a category reserved for the highest quality wines from Italy, thus its production is subjected to a strict regulation (reviewed by Paronetto and Dellaglio, 2011). Corvina

grapes, composing the main part of the Amarone must, have been shown to hold a fundamental role in conferring the organoleptic characteristics to the wine (Di Carli et al., 2011; Fedrizzi et al., 2011; Toffali et al., 2011). As an additional step characterizing the Amarone production, grapes are subjected to a long withering period. Grapes harvested at ripening are stored in well-aired warehouses until they lose up to 40% of their initial mass (Williams et al., 1989). During this process, which can last up to 2–3 months due to the relatively low environmental temperatures of the late autumn/early winter, grapes are dried and increase their sugar content up to about 30% (Consonni et al., 2011). Several metabolites relevant for the aromatic bouquet of wine have been shown to evolve in this phase of the process (Consonni et al., 2011; Fedrizzi et al., 2011; Paronetto and Dellaglio, 2011). The withering process is also known to favor the growth of fungi of the genus *Botrytis*, the "noble rot" whose relevance in determining the microbial and chemical characteristics of withered wines has been suggested (Fedrizzi et al., 2011; Consonni et al., 2011; Bokulich et al., 2012). In a recent study, Salvetti and co-workers have shown that the microbial populations present after the grape withering is widely defined by drying parameters such as temperature, relative humidity, and ventilation (Salvetti et al., 2016). Nevertheless, other factors have been shown to severely affect the microbial populations present on grape skins and thus in the early phases of must fermentation. Among these, the environmental changes associated to the vintage, climate, geography, and cultivar (Bokulich et al., 2014). Aiming at the disclosure of the influence of the productive process on the final product regardless to other environmental parameters, we studied the changes in microbial populations and in the metabolome of grapes of Corvina during and after the withering period. As a case-study, we analyzed grapes and musts sampled from the warehouse of Cantina Zenato, located in Valpolicella, the Italian viticultural zone where the grape varieties used for Amarone production (Corvina and Rondinella) are typically produced. To assess whether the influence of the productive process was constant across different years, we compared the fungal populations and metabolome among vintages characterized by both standard (the 2013 and 2015 vintages) and extreme climates (the 2014 vintage, characterized by abnormal, and abundant rainfalls). As a consequence of these peculiar atmospheric conditions, the wine-makers adopted extraordinary approaches in the vineyard (with repeated treatments with antifungals), in deciding the day of grape harvesting (which has to be done in absence of precipitations) and in the following steps of the wine-making process. Considering this, the inclusion in our study of this vintage, unfortunate by the wine-maker economic viewpoint, gave us the opportunity to observe the natural behavior of fungal communities in an extremely wet year, representing an interesting "case study."

MATERIALS AND METHODS

Sampling

Samples were collected from the warehouse of Cantina Zenato, located in Valpolicella, the Italian viticultural zone located in the province of Verona (Italy), where red wine is typically made from three grape varieties: Corvina (*V. vinifera* L. cv. Corvina), Rondinella, and Molinara. Grapes and musts were sampled during three vintages (2013–2015). The time of harvesting was decided by the wine-makers according to the evaluated grade of grape maturation. After the harvesting in the vineyard "Costalunga" (also located in the Valpolicella region, Sant'Ambrogio, Verona, Italy), grapes were subjected to withering in a dedicated warehouse located a few kilometers far from the vineyards (<5 Km). The warehouse was equipped with automatic systems able to control and modify the internal temperature and humidity. The duration of grape withering was defined according to the regional rules for Amarone production (reviewed in Paronetto and Dellaglio, 2011), which defines the time in which the grapes for Amarone vinification can be mashed. In light of this, the withering period varied in the 3 years of study (71 days in 2013, 107 days in 2014, 77 days in 2015). Grapes were then mashed in the warehouse used for the withering and fermentations were carried out in stainless steel tanks. Grapes were collected at two time-points during the withering period. The first grape sampling (T0) occurred a few hours after the harvesting, once the grapes were transferred from the vineyard to the warehouse. The second sampling occurred after 7–8 weeks since the start of the withering (58th, 49th, and 63rd day after harvesting in the 2013, 2014, and 2015 vintages, respectively). For each time-point of grapes sampling, six biological replicates were collected in the 2013 vintage and eight in the 2014 vintage (a bunch for each replicate). Grapes were mashed at the end of the withering period, namely at the 71st, 107th, and 77th day after harvesting in the 2013, 2014, and 2015 vintages, respectively (**Table 1**). Musts samples were collected as soon as the grapes were mashed. To ensure sampling representativity musts were mixed with sterile tools before sampling. In addition, for each sampling equal amounts were sampled from the bottom, from the middle and from the top of the tank, then mixed again and analyzed as an unique sample. Two must samples were collected in 2013 and 2015 vintages, eight in 2014. Samples were collected in sterile tubes then stored at −80°C until DNA extraction. A summary of samples details is shown in **Table 1**.

DNA Extraction

Aiming at the quantification of the total amounts of microbial populations, microbial DNA was extracted from both grape and must samples collected in all the studied vintages. DNAs extracted from the 2013 and 2014 vintages samples were also used for meta-taxonomic analyses. Extraction of DNA was carried out from 4 grapes or 2 ml thawed must. While grapes were directly subjected to microbial DNA extraction, must samples were subjected to a prior treatment to remove substances (i.e., polyphenols) which could interfere with the DNA extraction. Musts were centrifuged 30 min at 14,000 g and at 4°C, and the pellet was dissolved in 2 ml TE buffer. Must was centrifuged again for 15 min at 14,000 g at 4°C, and the pellet was dissolved in 300 μl TE buffer. Extraction of DNA was then carried out with the FastDNA Spin Kit for Soil (MP biomedicals) following the manufacturer's instructions.

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Quantification of Total Fungi and Bacteria

To monitor the absolute amounts of microbes present in the samples, we carried out quantitative Real Time PCR (qRT-PCR) as previously described (Stefanini et al., 2016). Total bacterial and fungal DNAs extracted from all the matrices collected in all the studied vintages were quantified by using universal primers specific for either the V1–V3 region of 16S rRNA gene for bacteria (Baker et al., 2003), or the ITS1 region for fungi (Findley et al., 2013). Real-time PCR was performed with a LightCycler(R) 480 (Roche) using optical grade 96-well plates. The PCR reaction was performed in a total volume of 12.5 μl using the KAPA SYBR(R) Fast qPCR Kit (KAPABiosystems) as previously described (Stefanini et al., 2016). Wilcoxon-Mann-Whitney test was carried out to compare the total amount of either fungi or bacteria in different groups of samples.

Meta-Taxonomic 454-Pyrosequencing and Data Analysis

Meta-taxonomics analysis was carried out on samples collected in the 2013 and 2014 vintages. Library preparation, sequencing, and initial raw sequences processing were carried out by the Sequencing Platform at Fondazione E. Mach. Fungal genera identification was carried out by mean of 454-pyrosequencing of the ITS1 region. To avoid biases in abundance detection due to preferential amplifications of sequences showing different lengths (ITS1-5.8S-ITS2), we sequenced the ITS1 region which is not subjected to the wide length polymorphism affecting the entire ITS region (Op De Beeck et al., 2014). For each sample, fungal ITS1 rDNA region was amplified using a specific fusion primer set coupled with forward primer 18SF (5′-GTA AAAGTCGTAACAAGGTTTC-3′) and reverse primer 5.8S1R (5′-GTTCAAAGAYTCGATGATTCAC-3′; Findley et al., 2013) containing adaptors, key sequence and barcode (Multiple IDentifier) sequences as described by the 454 Sequencing System Guidelines for Amplicon Experimental Design (Roche, Switzerland). The forward primer sequences were made up of: the "LIB-L" primer A sequence specific for "Lib-L" chemistry and "One-Way Reads" sequencing methods (Roche, Branford, CT), the key sequence TCAG, the bar code MID (Multiple IDentifier) sequence specific for any sample and the forward primer sequence. The reverse primer contained the "Lib-L" primer B sequence, the key sequence TCAG and the reverse primer sequence. For each sample, a PCR mix of 25 μl was prepared containing 1X PCR buffer, 1.25 U of FastStart High Fidelity polymerase blend (Roche) and dNTPs from the FastStart High Fidelity PCR system (Roche), 0.4 μM of each primer (PRIMM, Milano) and 10 ng of gDNA. Thermal cycling consisted of initial denaturation at 94°C for 3 min followed by 25 cycles (35 for fungal ITS) of denaturation at 94°C for 15 s, annealing at 60°C (58°C for fungal ITS) for 45 s, and extension at 72°C for 1 min, with a final extension of 8 min at 72°C. Products of PCR were analyzed through gel electrophoresis and cleaned using the AMPure XP beads kit (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions. Products of the different samples were quantified via quantitative PCR using the Library quantification kit—Roche 454 titanium (KAPA Biosystems, Boston, MA) and pooled in an equimolar way in a final amplicon library. 454-pyrosequencing was carried out on the GS FLX + system using XL + chemistry, following the manufacturer's recommendations. Pyrosequencing produced a total of 31,4559 reads of ITS1 region (for 28 sequenced samples). The sequences were assigned to samples according to sample-specific barcodes. This allowed us to collect FASTA formatted files containing an average of 11234.25 ± 3132.04 (*SD*) sequences per sample. Sequences were then checked for the following criteria: (i) no more than one mismatch/deletion/insertion both in the bar code and in the primer, (ii) length of at least 150 nucleotides (barcodes and primers excluded) and (iii) no more than two undetermined bases (denoted by N). Data were submitted to the European Nucleotide Archive with accession number PRJEB15229 (http://www.ebi.ac.uk/ena/data/view/PRJEB15229). The correspondence between submitted data IDs and samples is shown in **Table 1**.

Raw data files generated by the Roche 454 sequencer were de-multiplexed using Roche's sfffile software. Reads were pre-processed using the micca pipeline v0.1 (Albanese et al., 2015). Forward and reverse primers trimming and quality filtering were performed using micca-preproc (parameters -f GTTTCCGTAGGTGAACCTGC -r TCCTCCGCTTATTGATATGC -O 16 -l 150 -q 18), truncating reads shorter than 150 bp. *De novo* sequence clustering, chimera filtering and taxonomy assignment were performed using micca-otu-denovo (parameters –s 0.97 –c): OTUs were assigned by clustering the sequences with a threshold of 97% pairwise identity, and their representative sequences were classified using blast against the "unite" database (Kõljalg et al., 2013) (release 09/02/2014). For ITS data, multiple sequence alignment (MSA) and phylogenetic tree inference were performed using the online version of T-Coffe (Notredame et al., 2000). The taxonomies of all the representative sequences identified in the samples were further checked by manually blasting each sequence through the National Center for Biotechnology Information nucleotide collection database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Blasting results are shown in Supplementary Table 1. Sampling heterogeneity was reduced by rarefaction (2,500 sequences per sample). Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates were computed using the phyloseq r package (McMurdie and Holmes, 2013). Two-sided, unpaired Welch *t*-statistics were computed using the function mt() in the phyloseq library (McMurdie and Holmes, 2013), and the *p*-values were adjusted for multiple comparison controlling the familywise Type I error rate (minP procedure) (Westfall and Young, 1993). PERMANOVA (Permutational multivariate analysis of variance) was performed using the adonis() function of the *vegan* R package with 999 permutations. Correlations among fungal species and chemicals were evaluated with the psych r package (Revelle, 2015). False discovery rate (FDR)-adjusted *P*-values were computed using the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995).

Metabolomics Measurement and Analysis

Metabolomics analysis was carried out for all the samples collected in the 2014 and 2015 vintages. Free and bound (glycosylated aroma precursors) volatile organic compounds (VOCs) were extracted following the SPE method reported in Vrhovsek et al. (2014). Extracts were injected using a Gerstel MultiPurpose Sampler autosampler (Gerstel GmbH & Co. KG Mülheim an der Ruhr Germany) into a comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC × GC-MS) system consisting of an Agilent 7890 A (Agilent Technologies, Santa Clara, CA) equipped with a Pegasus IV time-of-flight mass spectrometer (Leco Corporation, St. Joseph, MI). A VF-Wax column (100% polyethylene glycol; 30 m × 0.25 mm × 0.25 μm, Agilent J&W Scientific Inc., Folsom, CA) was used as first-dimension (1D) column, and a Rxi-17Sil MS-column (Restek Bellefonte, USA) (mid polarity phase) 1.50 m × 0.15 mm × 0.15 μm, (Restek Bellefonte, USA) was used as a second-dimension (2D) column. The GC system was equipped with a secondary column oven and non-moving quadjet dual-stage thermal modulator. The injector/transfer line was maintained at 250°C. Oven temperature programme conditions were as follows: initial temperature of 40°C for 4 min, programmed at 6°Cmin^{-1} at 250°C, hold for 5 min. The secondary oven was kept 5°C above the primary oven throughout the chromatographic run. The modulator was offset by +15°C in relation to the secondary oven; the modulation time was 7 and 1.4 s of hot pulse duration. Helium (99.9995% purity) was used as carrier gas at a constant flow of 1.2 mL min-1 The MS parameters included electron ionization at 70 eV with ion source temperature at 230°C, detector voltage of 1317 V, mass range of m/z 35–450 and acquisition rate of 200 spectra s^{-1}. For GC × GC-MS data a LECO ChromaTOF (Version 4.22) software was used for acquisition control, and data processing. The identification of volatile compounds was done using NIST 2.0, Wiley 8, and the FFNSC 2 mass spectral library (Chromaleont, Messina, Italy), with a library similarity match factor of 750. Raw metabolomic data were submitted to the MetaboLights database with accession number MTBLS392. Free and glycosylated aroma precursors volatile organic compounds were annotated by comparing their mass spectra to NIST 2.0, Wiley 8, and FFNSC 2 libraries, with a similarity match factor of 750. Compounds with a similarity match factor lower than 750 were classified as "unknown". In the 2014 vintage samples, 93 glycosylated aroma precursors (89 tentatively identified + 4 unknown) and 728 free compounds (664 tentatively identified + 64 unknown) were measured. In the must samples collected in the 2015 vintage 947 free volatile compounds were measured. The response of internal standard 1-heptanol was used for normalization and to make a relative estimation of the identified compounds as commonly accepted in the analysis of aroma compounds. Wilcoxon-Mann-Withney test was carried out to evaluate the significance in the differences abundance of each chemical among either sample types (grapes or musts) or vintages. Pearson coefficients were calculated between chemical compound content and fungal genera relative abundances. All the calculated *p*-values were adjusted for multiple comparison (FDR) (Westfall and Young, 1993).

RESULTS

Climate and Withering Effects on the Mycobiota Diversity

To compare the populations of grapes and musts sampled in different vintages, we first sought to compare their general characteristics. Aiming at this, the total amounts of fungi and bacteria were quantified in *Vitis vinifera* L. cv Corvina grapes and must samples collected in two vintages, 2013 and 2014, by mean of quantitative Real Time PCR (qRT-PCR) (**Figure 1A**). Grapes were sampled at two timepoints: T0, when grapes were transferred in the warehouse, and T1, after 7–8 weeks of withering (see **Table 1** for details). Must samples were collected right after grape mashing at the end of the withering period, whose length varied in different vintages (**Table 1**). The slow withering process imposed environmental stresses to the grapes microbiota, potentially selecting the tolerant bacteria, and fungi, we thus expected to find significant variations in the total amounts of bacteria and fungi. Noteworthy, despite widely changing during the process, neither the total amount of fungi nor the total amount of bacteria significantly changed during the initial withering (grape_T0 versus grape_T1) in any of the studied vintages (**Figure 1A**). In addition, in the first sampled vintage (2013), the amounts of total bacteria and fungi did not differ between grape and fresh must samples. Contrarily, the total amount of bacteria decreased from the beginning to the end of the 2014 withering, being it significantly lower at each sampled time-point (Wilcoxon-Mann-Whitney FDR < 0.05, **Figure 1A** and Supplementary Table 2). To note, the fungal total amount significantly changed only between grapes sampled at the second withering time-point and fresh musts in 2014 vintage (Wilcoxon-Mann-Whitney FDR < 0.05, **Figure 1A**). To further explore the differences in microbial populations present in Corvina grapes and musts in the two vintages, we carried out 454-pyrosequencing meta-taxonomics analysis based on fungal ITS1 region sequencing aiming at the identification of the fungal genera present in the samples. We were able to describe at the genus level the fungal populations present in the samples (OTUs relative abundances are shown in **Figure 1B**). Differences between fungal populations were observable by comparing the genera relative abundances (**Figure 1B**), highlighting wide differences between samples of the different vintages and similarities between grape time-points of the same vintage, while slight differences were observable between grapes and fresh musts in both vintages. We went further and quantified the changes by comparing alpha (within sample) and beta (between samples) diversities. As observed when comparing the total amounts of fungi and bacteria, the observed number of OTUs did not differ between grapes sampled at the two time-points in any of the studied vintages (**Figure 2A**). In addition, both time-points of grapes sampled in 2013 bore a lower number of fungal genera than the 2014 must samples (Wilcoxon-Mann-Whitney FDR = 0.028 for both comparisons, Supplementary Table 2), but no significant differences were found between different sample types (grapes and musts) of the same vintage (**Figure 2A**). The same situation was observable by comparing Chao1 and Shannon indexes of alpha diversity (Supplementary Figure 1). On the

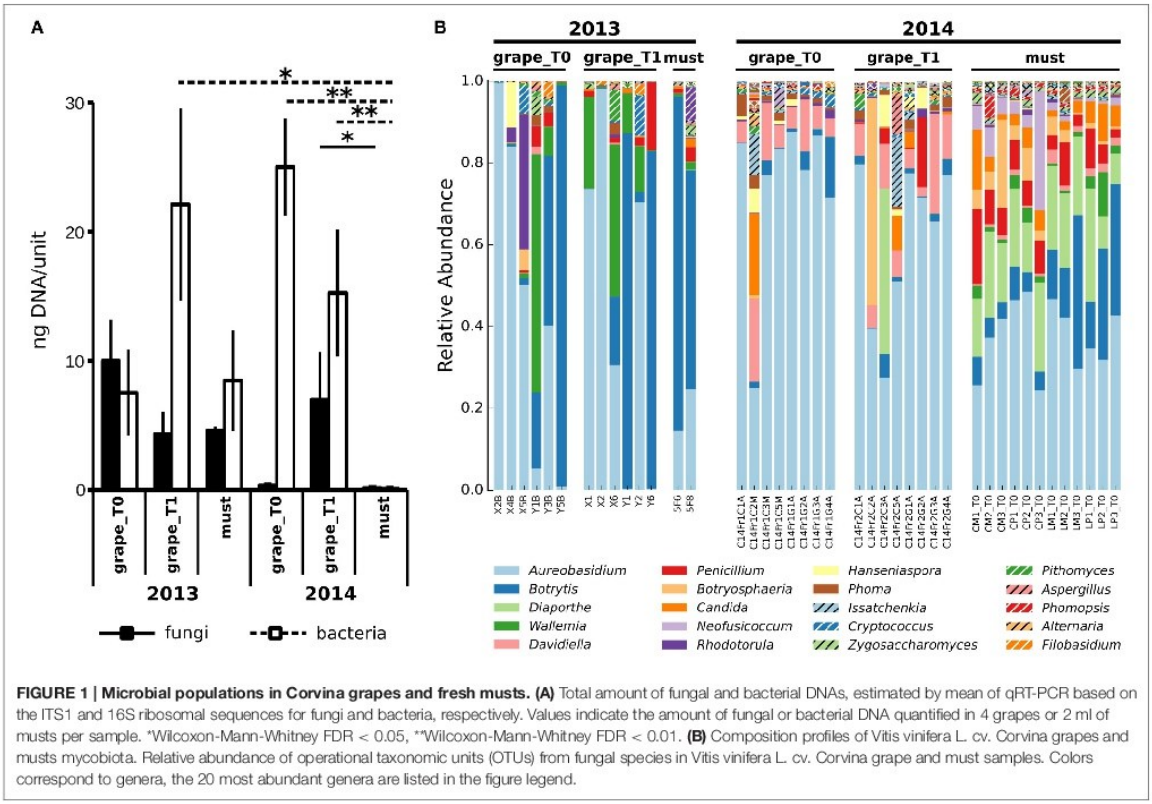

FIGURE 1 | Microbial populations in Corvina grapes and fresh musts. (A) Total amount of fungal and bacterial DNAs, estimated by mean of qRT-PCR based on the ITS1 and 16S ribosomal sequences for fungi and bacteria, respectively. Values indicate the amount of fungal or bacterial DNA quantified in 4 grapes or 2 ml of musts per sample. *Wilcoxon-Mann-Whitney FDR < 0.05, **Wilcoxon-Mann-Whitney FDR < 0.01. **(B)** Composition profiles of Vitis vinifera L. cv. Corvina grapes and musts mycobiota. Relative abundance of operational taxonomic units (OTUs) from fungal species in Vitis vinifera L. cv. Corvina grape and must samples. Colors correspond to genera, the 20 most abundant genera are listed in the figure legend.

other hand, the beta diversity analysis (in terms of unweighted UniFrac distance) showed that the samples were significantly grouped according to the vintage (PERMANOVA corrected for nested variables, FDR = 0.001) but also to the sample type (i.e., grapes vs. musts, PERMANOVA corrected for nested variables, FDR = 0.001) (**Figure 2B**). No significant differences were found between grapes at the beginning of the withering period (T0) and T1 in the same vintage (Supplementary Table 2). In addition, while neither grapes collected at T0 nor grapes collected at T1 in 2013 showed significant differences compared to the must of the same year, both grapes timepoints significantly differed from musts in 2014 (PERMANOVA FDR < 0.05, Supplementary Table 2). The same results were observed by using two other widely used beta diversity measures, namely the Bray-Curtis and weighted UniFrac beta dissimilarities (Supplementary Table 2 and Supplementary Figure 2).

The Corvina Mycobiota: Effects of the Climate and Constant Genera

By comparing fungal populations of grape and must samples during different vintages, we could identify the fungal genera whose presence was modified by the withering process, irrespective of the seasonal changes. As observed with other parameters (see previous section), the grape samples collected at T0 and at T1 were substantially similar even when comparing the fungal genera relative abundances (Welch *t*-test FDR > 0.05, Supplementary Figure 3). To note, despite the wide similarity among grapes at T0 and T1, the effect of the vintage on fungal genera relative abundances was not identical (Supplementary Figure 4). Indeed, the relative abundances of Sporobolomyces, Periconia, Peniophora, Leptosphaerulina, Issatchenkia, Bathalimia, Diaporthe, and Botrytis differed in T0 grapes harvested in 2014 and in 2013 (Welch *t*-test FDR < 0.05, Supplementary Figure 4) and did not differ in abundances in T1 samples of the two vintages. Grapes sampled at T1 were shown by several parameters to be indistinguishable from samples collected at T0 in the same vintage, suggesting that the withering affects fungal populations after prolonged periods. To identify the changes triggered by the withering on genera fungal abundances, we thus compared grapes sampled at T1 and fresh musts. Several genera were identified to be more abundant in grape than in must samples, without abundance differences between the vintages (**Figure 3**). The fungal genera *Pithomyces*, *Phoma*, *Leptospherulina*, *Cryptococcus,* and *Alternaria* were significantly more abundant in grape than in must samples (Welch *t*-test FDR < 0.05, **Figure 3**). *Vice-versa*, must samples showed a higher amount of the genera

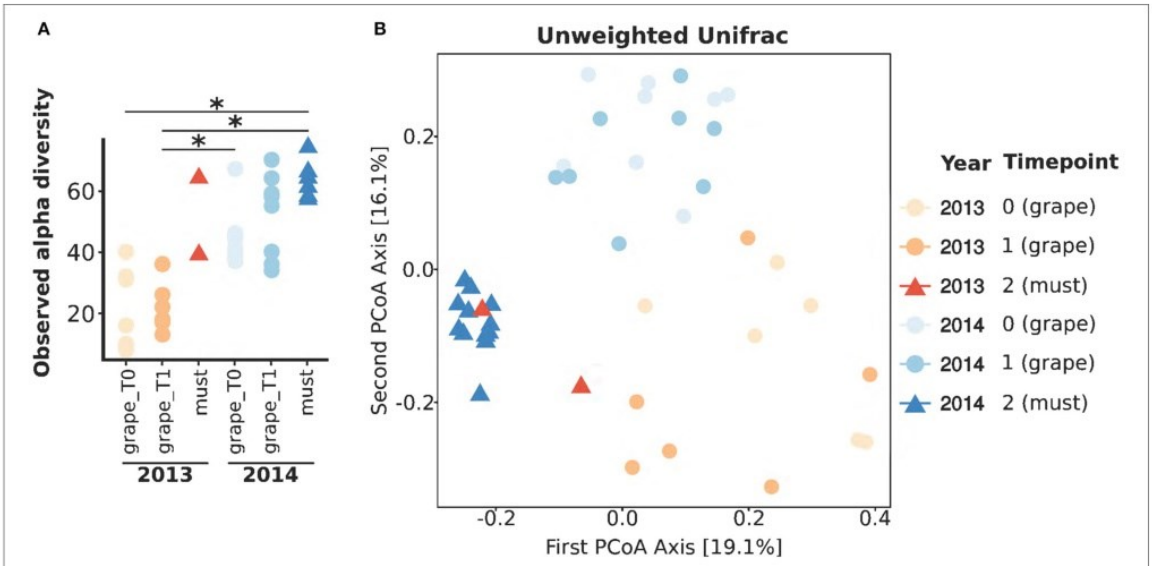

FIGURE 2 | Fungal population diversities in Corvina grapes and fresh musts. (A) Observed OTUs of fungal populations present in grape and must samples. *Wilcoxon-Mann-Whitney FDR < 0.05. **(B)** First two coordinates if the Principal Coordinates Analysis (PCoA) carried out on samples distances calculated with the unweighted UniFrac metric.

Zygosaccharomyces, *Metschnikowia*, *Diplodia*, *Cytospora*, and *Candida*, compared to grape samples (FDR < 0.05, **Figure 3**). In particular, *Diplodia* was found exclusively in must samples (Supplementary Figure 5). Considering the known problems in taxonomic assignments of species belonging to the Candida and Metschnikowia genera, we further evaluated our classification by clustering the representative sequences found in our samples with the Candida spp. and Metschnikowia spp. sequences deposited in the UNITE database (Supplementary Figure 6). Also other fungal genera were differentially represented in the two matrices, but the differences were strongly influenced by seasonal changes (Supplementary Figure 4). As an example, the *Davidiella* (*Cladosporium*, Bensch et al., 2012) and *Nigrospora* genera were significantly more abundant in all the samples collected in the 2014 vintage (Supplementary Figure 4), and were estimated to be more abundant in grape samples compared to must (**Figure 3**). Other examples of this situation are shown by the fungal genera *Phomopsis*, *Neofusicoccum*, *Diaporthe*, and *Diaporthe_unidentified*, which were significantly more abundant in must samples, but whose presence was significantly higher in the 2014 vintage. To assess whether the genera enriched in grapes and musts were mutually exclusive (the presence of one corresponds to the absence of another one or vice-versa), we calculated the Pearson coefficient between every possible couple of genera enriched in grapes and must samples. As expected, strong positive correlations were found among fungal genera that were enriched in the same matrix (grape or must) (Supplementary Figure 7). When observing the correlations between genera more abundant in musts and those more abundant in grapes, we found negative correlations among: (i) *Zygosaccharomyces* and *Metschnikowia* with genera more abundant in grapes, (ii) *Candida* (must) and *Leptosphaerulina* (grape) and (iii) *Cytospora* (must) and *Alternaria* and *Pithomyces* (grape) (Supplementary Figure 7).

The Corvina Metabolome: Relations with the Climate

To further describe the process of Amarone production from the microbial viewpoint, we explored the metabolomic profiles of *V. vinifera* L. cv. Corvina grapes and musts in different vintages by mean of untargeted analysis (Versini et al., 2008). We assessed the contribution of grape withering in modifying the wine metabolome by comparing free and glycosylated precursor compounds in grapes and musts (before and after the withering) collected in the same vintage (2014). In addition, we evaluated which part of the chemical profiles were conserved in different vintages by comparing the free compounds (the most relevant for the wine flavor) in musts collected in two different vintages (2014 and 2015) (Michlmayr et al., 2012). Because grapes sampled at T1 were shown to be indistinguishable from samples collected at T0 in the same vintage, and aiming at the minimization of the matrix effect on metabolite measurement (grapes at drier at T1 than at T0, then more similar to musts) we carried out the following analyses by using the latter only and comparing them to must samples. To evaluate the effect of the withering process, we compared the relative amounts of free and glycosylated precursor compounds in grapes (before withering) and fresh musts (after withering) sampled in the 2014 vintage. Among the free compounds, 11 were significantly more abundant in must than in grape samples and 170 were showing an opposite

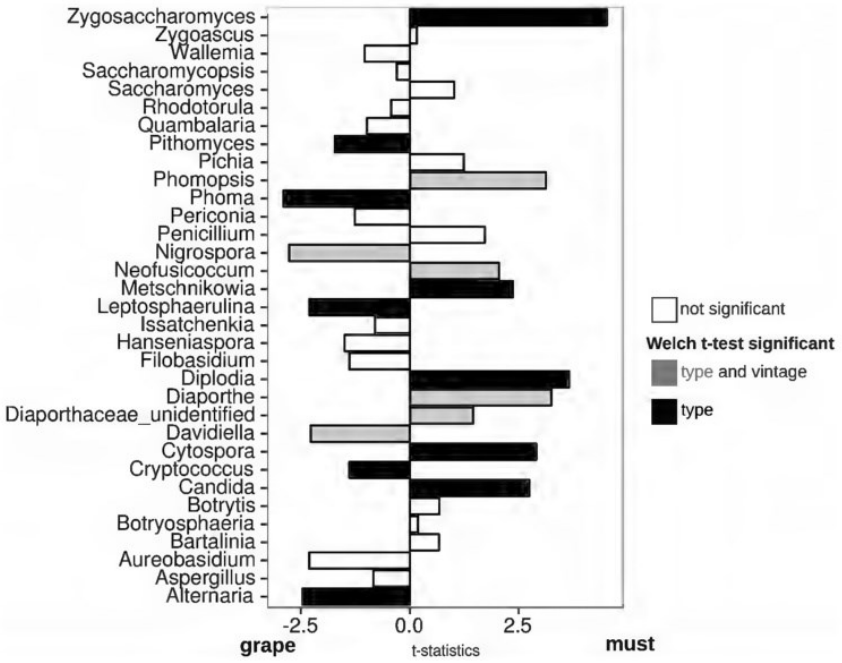

FIGURE 3 | Welch's t-statistics comparing the relative abundances of fungal genera present in grapes and must samples. Welch's *t*-test was carried out to compare the relative abundances of fungal genera in grape and must samples. Black = FDR < 0.05 among sample matrices and FDR > 0.05 among vintages, Gray = FDR < 0.05 among matrices and FDR < 0.05 among vintages, white = FDR > 0.05.

trend (Supplementary Table 3). In terms of glycosylated aroma precursors, none was more abundant in grape than in must samples, while, vice-versa, 67 of them were significantly more abundant in musts (Supplementary Table 4). A similar increase in glycosylated precursor compounds was previously observed to occur during grape maturation (Ryona and Sacks, 2013), thus suggesting that the processes begun in grape ripening proceed also during withering as previously proposed (Di Carli et al., 2011). Beyond that, the relative amount profiles suggest a very effective extraction of the highly polar and water-soluble bound precursors from the skins into the juices.

We then focused on the free compounds consistently present in 2014 and 2015, since they directly influence the aroma characteristics. It is worth mentioning that even if the total number of measured compounds is different in the 2 years (947 vs. 664 tentatively identified, respectively, **Figure 4A**), a large part of the ones measured in 2014 (219 of 664) was also present in 2015 (**Figure 4A**). Interestingly, out of the 219 common volatiles, 187 show significant differences in relative amounts in the 2 years (Wilcoxon-Mann-Withney FDR < 0.05, Supplementary Table 5, **Figure 4B**), and only 31 do not significantly change (**Figure 4B** and Supplementary Table 5). The latter ones, which can be considered "core" compound, hold the potential to be central in the definition of the Amarone organoleptic characteristics.

Relations between Mycobiota and Metabolome

The identification of correlations between fungal genera abundance and quantified volatile compounds further contributes to the understanding of the changes to which Corvina grapes are subjected during the withering process. To assess if the presence of metabolites characterizing the Amarone fermentation process could be associated to a specific fungal activity, we searched for significant correlations (FDR < 0.05) among the fungal genera constantly present in the 2013 and 2014 vintages (**Figure 3**) and the 32 "core" Amarone compounds (**Figure 4B**). We carried out this analysis focusing on the "core" mycobiota and metabolome of Corvina grapes processed for Amarone production, i.e., those fungal species and compounds present in different vintages, because the identification of eventual correlations among them might have a relevant biotechnological potential. Among the fungal genera enriched in grape samples, *Phoma* showed significant positive correlations with a free compound, (3E)-3-hexenoic acid (**Figure 4C**). The fungal genera more abundant in musts were found to be negatively correlated with free compounds, with the only exception of *Diplodia*, which was found to be positively correlated with 1-pentanol and 2,6-dimethoxy phenol (**Figure 4C**). In addition, *Cytospora*, *Metschnikowia,* and *Candida* were found to be negatively correlated with several

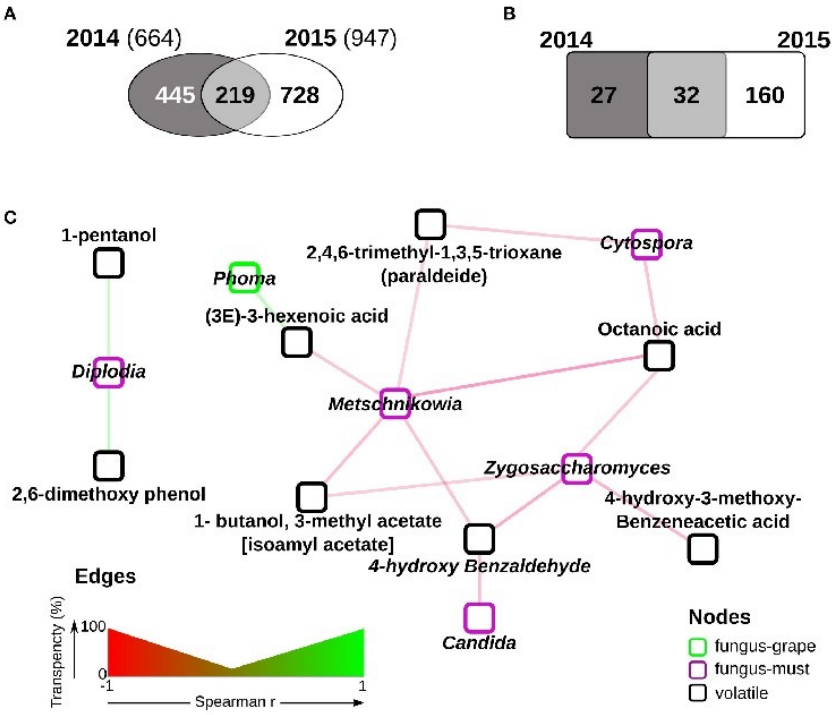

FIGURE 4 | The Corvina volatolome. (A) comparison of free compounds present in must samples of the two vintages. 219 compounds were found in both the 2014 and 2015 samples; **(B)** comparison of the relative amounts of the 219 free compounds measured in both vintages. Free compounds were considered significantly enriched in either the 2014 or the 2015 samples when Wilcoxon-Mann-Whitney FDR < 0.05; **(C)** correlations among free compounds and fungal genera found in Corvina samples. Significant Pearson correlations (FDR < 0.05) were calculated among the free compounds and the fungal genera measured in must samples at similar relative amounts in the two vintages. The network was drawn with Cytoscape 3.4 (Shannon et al., 2013) with the significant correlations only.

free compounds known to have a relevant role in wine aroma (**Figure 4C** and Supplementary Table 6) (Zalar et al., 2005; Gomes et al., 2013).

DISCUSSION

The Microbiota and Metabolome of *Vitis vinifera* L. cv. Corvina Grapes and Musts Are Influenced by the Climate and the Process

The composition of the fungal microbiota present on grape skins and maintained in early phases of must fermentation has been shown to be strongly influenced by environmental factors (Bokulich et al., 2014). Nevertheless, the withering period to which Corvina grapes are subjected for Amarone production also influences the microbial populations (Salvetti et al., 2016). Here, we show that in *V. vinifera* L. cv. Corvina both the climate and the duration of withering contribute in defining the microbial populations present on grapes both at the beginning and at the end of withering period. Several parameters indicate that the fungal populations present on grapes changes only after a 7–8 weeks withering. After this period, fungal genera, despite not changing in numerosity, change in relative abundances, suggesting a selection of genera able to survive to the harsh environmental conditions imposed by the long lasting withering.

In occurrence of heavy and frequent rainfalls, wine-makers repeatedly treat vineyards with antifungal products to avoid colonization by pathogenic fungi. For this reason, the number of fungal genera present in grape and must samples could be expected to be lower in these vintages than in standard ones. Contrarily, our study revealed that samples collected during the wet vintage (2014) show a higher number of fungal genera compared to the "dry" vintage (2013), not corresponding to an increase of total amount of either bacteria or fungi. Similarly, the total amount of fungi quantified in recently harvested-grapes was higher (even if not significantly) in 2014 than in 2013. Despite the microbial quantification could be biased by the amplification of the DNA of dead cells (Nocker and Camper, 2006; Carini et al., 2016), since we observed a decrease of the total amount of fungal and bacterial DNA during the withering process we are confident that DNA does not accumulate in these samples. The fact that the total amount of fungi only slightly increased but the number of genera widely increased in wet-vintage samples

compared to dry-vintage samples is ascribable to the effect of antifungal treatments in the vineyard: the killing of sensitive fungi may favor the overgrowth of otherwise not competitive fungi. The increase in number of not-sensitive fungi compensates the decrease of the sensitive ones, finally resulting in a similar total amount of fungi. The relative abundance of some fungal genera was drastically influenced by the vintage. Indeed, the relative abundance of fungal genera present prevalently either in musts (*Phomonas*, *Neofusicoccum*, *Diaporthe*, Diaporthaceae unidentified, *Davidiella*, *Botrytis,* and *Botrysphaeria*) or in grapes (*Nigrospora*, *Davidiella,* and *Wallemia*) were significantly different in the studied vintages. To note, two genera of the Botryosphaeriaceae family and known *Vitis* pathogens, *Neofusicoccum* and *Botryosphaeria*, were more abundant in 2014 than in 2013 must samples, indicating that the adverse environmental conditions could have favored their persistence on grapes even after a prolonged withering period. On the other hand, the genus *Botrytis*, found to be more abundant in 2013 than in 2014 must samples, is an example of the ability of the process to modify the environmental fungal populations. This genus is known to grow better in presence of high levels of humidity, thus potentially in the wet 2014 grapes (Paronetto and Dellaglio, 2011). Nevertheless, it has to be considered that the humidity of the warehouse used for withering was controlled and modified by an automatic system, potentially compensating for the vintage-specific environmental differences. This could explain the higher abundance of this genus in 2013 musts, composed by grapes subjected to a shorter period of withering than the 2014 grapes. Even the metabolome of Corvina is affected by the climate, since we found compounds significantly enriched in either the wet or the dry vintages. In addition, the wet-vintage samples were enriched in a higher number of compounds compared to the dry-vintage, recapitulating what observed for fungal populations, which showed a lower biodiversity in dry-vintages compared to wet-vintages. As a whole, these results indicate that either the environmental characteristics or the actions adopted to reduce the impact of environmental changes (i.e., repeated antifungal treatments and prolonged withering in presence of heavy rainfalls) can impose severe stresses resulting in a simplified microbiota adapted to this harsh environment. As a consequence of the reduced fungal biodiversity, it is likely that the ability to efficiently metabolize present compounds is reduced, finally impacting on the volatile component of the metabolome released by the microbial component (volatile compounds). As observed from the comparison of different vintages, other environmental conditions, imposed by the withering, can shape the grape mycobiota: while the initial withering does not affect the grape mycobiota, the harsh conditions imposed by a prolonged withering contribute to the shaping of the fungal populations.

Conserved Microbial and Chemical Characteristics of Amarone Fermentation

Despite the several differences observed among samples and associated to the vintage, both grapes and fresh musts of *V. vinifera* L. cv. Corvina showed fungal genera which were constantly present in different years. As expected, the genera characterizing the fungal mycobiota of Corvina grapes are those usually found in the environment, confirming the environmental origin of the microbial populations inhabiting the grape skin (Barata et al., 2011). Among the fungal genera whose relative abundances remain unchanged during different vintages, Leptosphaerulina, *Pithomyces,* and *Phoma* are commonly associated to plants and soil, while *Alternaria* and *Cryptococcus* are commonly found associated to fruits and vegetables. Even more important, several fungal genera among those constantly present in must samples of different vintages, like *Zygosaccharomyces*, *Metschnikowia,* and *Candida*, are known to hold relevant roles (either useful or detrimental) in the fermentation for the production of volatile aroma compounds (Loureiro and Malfeito-Ferreira, 2003; Sipiczki, 2006; Oro et al., 2014; Whitener et al., 2015, 2016; Ciani et al., 2016). Interestingly, several negative correlations were found among fungal genera more abundant in grapes and genera more abundant in musts. Such negative correlations could indicate either the presence of competition among genera or the inability of the two genera to exploit the same environment. To note, in a recent study Salvetti et al. found some fungal genera at the end of the *V. vinifera* L. cv. Corvina grapes withering (i.e., *Aspergillus* and *Penicillium*) which we did not find in either grapes or fresh musts in the studied vintages (Salvetti et al., 2016). These known airborne molds mainly grow in humid and warm environments. The use of temperature and humidity controller systems in the warehouse used for withering the grapes could have prevented the growth of these molds.

Interestingly, we found that *Diplodia* (found exclusively in must samples) was positively correlated with compounds usually detected in traces in grapes and fresh fruits, 1- pentanol and 2,6-dimethoxyphenol (the latter also called syringol) (Escudero et al., 2007). These compounds increase during grapes withering, confirming previous observations for compounds of the same class (Bellincontro et al., 2016). We also found that octanoic acid (caprylic acid) had a negative correlation with fungal genera increasing in relative abundance during the withering process, indicating a decrease of the compound during the withering. Considering that this compound is known to inhibit *Saccharomyces cerevisiae* fermentation (Stevens and Hofmeyr, 1993), by subjecting the grapes to a prolonged withering process the protocol for Amarone production could favor the growth of this yeast in the following fermentation.

CONCLUDING REMARKS

As all the other fermentative processes, also Amarone production is strongly affected by the environmental conditions during grapes maturation, thus by the climate. Our findings highlight that, despite some changes that could be ascribed to the vintage, both microbial populations and chemical profile of *V. vinifera* L. cv. Corvina grapes and musts show characteristics that are maintained over different vintages. These characteristics, either defined by the process or by chemico-physical parameters (i.e., of the Corvina cultivar, of the soil), contributes to the typicity of Amarone. The disclosure of these persistent microbial and chemical features helps in understanding the details of this production technology and will thus give pivotal insights to further optimize the process.

AUTHOR CONTRIBUTIONS

DC, IS, and FM conceived and designed the study. IS, MP, and NT handled the samples and carried out DNA extractions, qRT-PCR, and PCR amplification before library preparation. AZ, ST, and AB were responsible for the processes conducted in the winery. SC, UV, NT, and FM extracted and analyzed the metabolites. PF supervised and carried out statistical analyses. CD and DA supervised and carried out metataxonomics analyses. All the authors discussed the results and contributed at writing the manuscript.

FUNDING

This study was supported by Cantina Zenato.

ACKNOWLEDGMENTS

The authors would thank Fabio Bellai, Fausto Bianchera, and Massimo Guidetti for their contribution to grape and fermentation management at Cantina Zenato and the Sequencing Platform of Fondazione Edmund Mach, in particular Massimo Pindo, Simone Larger, Erika Stefani and Daniela Niccolini, for carrying out the 454-pyrosequencing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00457/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Concluding remarks

GC×GC has been shown to be a very promising technique for analysis of wine volatiles, since it allows the separation and identification of a large number of compounds. Improved separation, together with the use of deconvoluted mass spectra obtained using GCXGC-ToF-MS, has made it possible to observe many new compounds that were probably co-eluting in 1 D. Many of the common wine flavour compounds have been confirmed with the traditional technique, while a large number of newly identified or tentatively identified potential markers that help to place products in the production area have been observed. Some norisoprenoids such as ***safranal***, ***alpha-isophorone*** and the ***cis β-damascenone*** isomer have been seen here for the first time.
The GCXGC technique should act as an overview, allowing us to find important ***markers*** that can then be better quantified with targeted methods or smelled with GC-O to evaluate their sensorial characteristics.

Different types of wine were chosen to include the largest number of volatile compounds.

- The GCxGC technique was successfully applied to the study of sparkling wines, allowing detailed investigation of the complex volatile flavour profile, making it possible to clearly separate two types of sparkling wines produced with grapes harvest in different climatic areas. Some varietal (terpenes) and pre-fermentative compounds (C5-C6) were significantly different in the two types of products
- More than 1.200 compounds were detected by SPME-GC×GC-TOFMS in Shiraz wines. 240 of the compounds were found at significantly different levels between the four vineyards in at least one of the harvests. Separation of samples was noticed based on the harvest date according to the principal component analyses. Derivates of lipoxygenase pathway, i.e. C5, C6 and C9 compounds were typically found in higher levels in wines from H1, irrespective of vineyard site. These compounds are known to contribute to fresh fruit aromas. These results indicate significant and coherent modulations of wine volatiles in relation to grape harvest date irrespective of vineyards site and management with similar climate.
- Verdicchio wines could be very different depending on the different location of the vineyard and winemaking techniques. Young wines are characterised by fruity, thiolic notes, while wines aged for longer are distinguished by their norisoprenoid content and by balsamic, anise and liquorice notes, which can be attributed to methyl salicylate released by precursors and the presence of 3-MND With a new derivatisation MS-MS method it was possible to quantify 3-MND in the range of 10-50 ng/L. The use of GC-O allowed to found 43 sensorially important compounds. Analysis of the compounds after hydrolysis confirmed that a high amount of methyl salicylate characterised this variety.

- ❖ Six different methyl salicylate glycosides were identified in wine. All of them were higher in Verdicchio and Trebbiano (Soave-Lugana) compare to others white wines varieties. The compound released by precursors during ageing can be considered to be responsible for the balsamic aroma perceived in old Verdicchio vintages.
- ❖ Amarone production is strongly affected by the environmental conditions during grapes maturation, thus by the climate. Despite some changes that could be ascribed to the vintage, both microbial populations and chemical profile of Corvina grapes and musts show characteristics that are maintained over different vintages.

The metabolomics approach applying GCxGC presented in this work and the results obtained may also have practical importance. Such detailed profiles of volatile compounds obtained by GC × GC-TOF-MS may serve experts, producers to better define the regional or varietal typicity of different wines, and in this way reinforce their identity and their link with the territory From the agronomic point of view, the understanding of changes in the aromatic profile depending on the harvesting time can allow a more rational decision of the date, also on the basis of the sensorial characteristics that we wish to bring out in the wine.

As known from the literature, the vineyard and surrounding environment can significantly influence the aromatic profile of wine. Climate change will make it necessary to think very carefully where the variety should be planted in order to give maximum expression to the final product.

***In brief**:*

The key areas examined are the ***terroir*** for Trentodoc, where some varietal compounds linking it to the local area are highlighted, the possibility of diversifying the aromatic expression of Shiraz wines according to ***harvesting time***, the versatility of the Verdicchio variety in relation to ***agronomic/human*** choices and ***aromatic peculiarities*** and finally the influence of the ***microbiota*** present in the vineyard for the production of Amarone.

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Ringraziamenti

Se l'inglese è la lingua scientifica, l'italiano resta quella degli affetti e quindi in questa lingua mi accingo a ringraziare le persone che mi hanno supportarto in questo mio "tardivo" desiderio.

Desidero ringraziare innanzitutto la Dr.ssa Urska Vrhovsek, per avermi permesso di intraprendere questo percorso e per la fiducia ed il supporto che mi ha sempre dimostrato.

Ringrazio il Prof. Fulvio Mattivi per avermi reso partecipe di alcuni suoi progetti, alcuni dei quali discussi in questa tesi.

Grazie a Katja ed Irene per la proficua collaborazione.

Ringrazio di cuore i miei colleghi: Cesare, per non avermi mai negato il suo valido supporto tecnico e Domenico per la preziosa collaborazione in tante occasioni. Grazie ad Andrea, Alessio, Domen, Marynka, Panagiotis e Daniele per aver reso il laboratorio una grande famiglia! Grazie a Mar per il suo aiuto con R e per le sue lezioni di catalano, grazie a Lais per i continui foraggiamenti di biscotti di un famoso mulino italiano…

Ringrazio Adriano per avermi su-sopportato anche quando non avevo molto tempo da dedicargli e le mie figlie Agata ed Ester alle quali, in quest'ultimo anno ,ho sicuramente negato un po' di tempo.

*Un grande **GRAZIE** alla mia mamma, per esserci sempre stata quando ne avevo bisogno e anche al papà che ne ha subito le conseguenze.*

Grazie ad Anita per i suoi preziosi consigli e per l'incoraggiamento.

Grazie alle mie amiche di "Udin" Mara, Annalisa e Nadia che tante volte mi hanno dato ospitalità!