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It is often perceived that late maturity of grape gives a more complex aroma profile to Pinot noir wine, however, there is little understanding of the basic flavor chemistry of grape maturity on wine aroma. The aroma contributing compounds in Pinot noir were first identified by aroma extract dilution analysis (AEDA). Based on the AEDA results, the most important aroma compounds for Pinot noir include acids, alcohols, ethyl esters as well as β-damascenone, vanillin, eugenol, nonalactone, whiskey lactone, trans-geraniol. Those important aroma compounds were investigated in wines made from early, middle and late maturity grapes by the stir bar sorptive extraction- gas chromatography/mass spectrometry (SBSE-GC/MS) method. Quantitative analysis showed that the Pinot noir wine made from late harvest grapes contained more monoterpenes, more C13-norisoprenoids, more γ-nonalactone, guaiacol, and 4-ethylguaiacol, which contributed to more cherry, berry, more complex aroma characters; while wine produced with early harvest grapes have more short chain esters. The development of those aroma compounds in grapes was further investigated. The free aroma compounds were directed extracted from grape juice with the stir bar sorptive extraction and analyzed with gas chromatography-mass spectrometry, the glycoside bound aroma precursors were isolated with a reversed phase C18 column and hydrolyzed with glycosidic enzymes. The released aglycones were analyzed with SBSE-GC-MS. It was found that free monoterpenes and C13-norisoprenoids decreased during grape development, while free benzenoid

alcohols increased. However, the bound C13-norisoprenoids dramatically increased during grape maturation. Since the glycoside bound aroma precursors had much higher concentrations than the free form, these precursors will be hydrolyzed during wine making process, and contribute to more cherry, berry, and more complex aroma to the finished wine.

Development of Volatile Compounds in Pinot noir Grapes and Their Contributions to Wine Aroma

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Yu Fang, Author

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DEVELOPMENT OF VOLATILE COMPOUNDS IN PINOT NOIR GRAPES AND THEIR CONTRIBUTIONS TO WINE AROMA

Chapter 1. General Introduction (literature review)

1.1 Analysis techniques for wine aroma

Since the appearance of wine thousands of years ago, wine lovers have been eager to unlock the secret of wine flavors. In the 19th century, analytical methods focused on the determination of major wine components such as ethanol, organic acids, and sugars. The development of chromatographic techniques in the early 1900s and particularly the development of gas chromatography in the early 1950s ushered in a new area of discovery for analytical chemists. Currently, more than 680 volatile compounds have been identified in wines [1]. These volatile organic compounds in wine were believed to be responsible for wine bouquet. However, recent research found that many of them do not actually contribute to wine aroma because of their high sensory thresholds. On the other hand, some of the odor-active compounds, which may be present at very low concentrations (sometimes lower than μg/L) but have low sensory thresholds, determine the aroma character. Therefore, more and more researchers are beginning to focus on looking at odor-active aroma compounds instead of simply all volatile compounds. New analytical techniques that can model the complex relationships between aroma compounds and sensory properties have been developed.

1.1.1 Extraction

Having pigment and sugar residues, wine samples are difficult to directly analyze by gas chromatography (GC), so making an aroma extract is necessary for aroma analysis. Numerous methods to isolate volatiles have been developed, but each one alters to some extent the overall volatile composition obtained from wine. Moreover, since aroma compounds generally have low concentrations in wine, a pre-concentration step is reqired prior to analysis.

1.1.1.1 Solvent extraction

Liquid-liquid extraction is one of the most commonly used sample preparation techniques for the analysis of wine volatiles. Pentane/ether (1:1), dichloromethane, and Freon 11 are generally used as solvents for extraction [2-4]. Continuous extractions typically are employed to improve sensitivity for low analyte concentrations, due to the continuous re-circulation of fresh organic solvent. However, continuous extractions require heating the extracting solvent to its boiling point, so thermal degradation and chemical reactions can be a major problem during this process.

Generally, distillation is required after liquid-liquid analysis for separating out the sugars, pigments and other non-volatiles. Therefore, selecting an appropriate distillation technique is critical for aroma analysis. During a successful distillation, odor-active compounds should not be discriminated, the condition applied should not alter the structure of key aroma compounds, and non-volatile compounds should be completely removed. A compact and versatile distillation unit, called solvent assisted flavor evaporation (SAFE), was developed recently, which results in higher yields compared to previously used techniques,

such as high vacuum transfer [5]. After distillation, the extract is dried and concentrated prior to chromatographic analysis.

Solid phase separation, such as with silica gel and C18 pre-packed cartridges, also have been used for purification and fractionation of solvent extracts of wine [3, 6]. The advantages of this technique are easy operation and the extracts are often more concentrated compared to distillation.

Though solvent extraction techniques have been widely used, long preparation time, the costs for solvent disposal, as well as safety and environmental concerns, are prompting researchers to search for other methods that minimize or eliminate the use of organic solvents.

1.1.1.2 Static and dynamic headspace extraction

Headspace samplings in the static or dynamic mode are solvent-free techniques widely used to analyze the volatile fraction of liquid and solid matrices. Static headspace extraction is a simple technique, and mainly depends on the equilibrium between the sample and the gas phase in the sample vial, which is characterized by a partition coefficient representing the ratio of analyte concentrations in sample and gas phase. The dynamic headspace extraction technique is based on flushing the sample with an inert gas, and then transferring the volatiles onto a trap of adsorptive polymers, such as Tenax or Porapak Q. Tenax is most often used for analysis of wine samples, due to its low affinity for water and ethanol [7].

These methods are directly relatable to the vapor that can enter the human nose, and therefore to the perceived aroma. Their efficiency is affected by analysis time, sample size and number of volatile components [8]. However, there can be a disadvantage to these methods, since it may not be possible to process sufficient

vapor to ensure that extremely small quantities of particular compounds are detected [9]. These compounds may have extremely low sensory thresholds, so they may be potentially important contributors to aroma. Therefore, only limited information can be provided by these traditional headspace sampling methods [10].

1.1.1.3 Solid phase micro-extraction (SPME)

As an alternative to traditional pre-concentration methods, solid-phase microextraction (SPME) lies between static headspace and dynamic headspace techniques, and offers a simple and quick extraction method. The typical SPME fiber is housed within a small diameter stainless steel tubing and coated with different materials that can absorb and thermally release organic volatiles. Extracted by the SPME fiber, the volatile compounds are directly concentrated and can be immediately injected onto a GC column for analysis. Currently, SPME has been successfully used to investigate volatile compounds from the headspace of various samples [11-16].

Headspace SPME extraction efficiency is based on the equilibrium of analytes among the three phases: the coated fiber, the headspace and the sample solution. Depending on how fast the analytes transfer to the headspace from the sample solution and then get adsorbed by the fiber, the length of extraction time and temperature can be critical for SPME extraction efficiency. Generally, longer extraction time and high temperature benefited the equilibrium and increased the responses of less volatile analytes. However, because the SPME fiber only has a limited number of adsorption sites, and higher molecular weight compounds can displace lower molecular weight compounds as a consequence of competition for active sites on the fiber [17], quantification can only be achieved under non-equilibrium conditions using shorter extraction times, particularly for complex

matrices [18-20]. Other factors, such as salt addition and sample stirring, has also been found to be important to fiber extraction efficiency [21, 22].

Some limitations have been observed when SPME is used for the analysis of mixtures of volatile compounds. For instance, since SPME fibers are not uniformly sensitive to all compounds, the adsorption selectivity of the fiber and its discrimination between compounds can be a drawback for quantification in complex matrices, such as wine [23]. It is also reported that the decomposition or reaction of analytes in the fiber cause some problems during sample preparation and GC injection, such as oxidation of dimethyl sulfide to dimethyl sulfoxide [12] and generation of dimethyl disulfide from methanethiol [16].

1.1.1.4 Stir bar sorptive extraction (SBSE)

In 1999, Baltussen et al. described a new extraction technique, known as the stir bar sorptive extraction (SBSE) method [24], which is based on the partition coefficient between poly(dimethylsiloxane) (PDMS) and water. In SBSE, a magnetic stirring bar encapsulated in a glass jacket and coated with PDMS, is added to liquid samples to promote the transport of analytes into the polymer coating. After a predetermined extraction period, the analytes can be thermally desorbed in the GC injector or solvent extracted for HPLC analysis.

Though the fundamental aspects of SBSE for liquid phase sampling are similar to the principles of in-sample solid phase microextraction (IS-SPME), it has been found that SBSE has much higher recoveries than IS-SPME, because the 24 μL of PDMS is used in SBSE (0.5mm of phase thickness) compared to only 0.5μL with SPME (100μm of fiber thickness) [25, 26]. Moreover, compared to SPME, a 500-fold increase in sensitivity can be attained using SBSE with extraction times between 30 to 60 min [24].

Recently, this method has been widely used to detect the volatile and semivolatile compounds in water, tea, coffee bean, beer and wine [25, 27-32]. Applied to wine, the SBSE technique was found to be orders of magnitude more sensitive than modern conventional methodology, allowing for lower detection and quantification levels. Moreover, SBSE often gave better signal to noise ratios in scan mode than other methods in selective ion monitoring (SIM) mode, and thus improved confirmation of identity. With the help of characteristic mass spectra, Hayasaka et al [33] unambiguously identified all agrochemicals at concentrations of 10 μg/L in wine, and further detected 100 constituents in a Cabernet Sauvignon sample. Thus, it is now possible to analyze complex samples such as wine by scan mode, with better confirmation of identity, and without sacrificing sensitivity, where previously SIM methodology had to be used.

Like SPME, the SBSE extraction efficiency is affected by many factors, such as temperature, salting out effect, addition of methanol/ethanol, volume of samples, equilibration time, and so on. For optimization of SBSE condition, numerous studies have been done [34, 35].

1.1.2 Identification and quantification

Developments in chromatography have revolutionized the field of flavor chemistry by allowing a large number of individual aroma compounds to be separated, identified and quantified in complex mixtures. Gas chromatography (GC) coupled with mass spectrometry (MS) is commonly used to analyze the volatile compounds in wines. GC-MS is also proving increasingly useful for quantification of volatiles, where internal standards are generally used to monitor analyte recoveries and to reduce variability associated with sample preparation and injection. However, for odor-active compounds, special detection techniques are

required to link them to aroma properties, and to separate them from any interfering volatiles.

1.1.2.1 Gas chromatograph – olfactometry (GC/O)

The combination of olfactometric practices with gas chromatography, known as GC/O techniques, has been developed to detect aroma compounds using the human nose. Odor-active compounds can be perceived by sniffing the GC effluent and the associated aroma properties are described at the same time. Recently, more comprehensive approaches to research into wine aroma have been taken, with increasing use of GC/O methods.

Two techniques, charm analysis [36, 37] and aroma extract dilution analysis (AEDA) [38, 39], obtain information about the odor-active compounds in the wines with dilution experiments. In both procedures, the extracts containing aroma compounds are diluted stepwise with solvent and each dilution then analyzed by GC/O. In the case of AEDA, the result is expressed as flavor dilution (FD) factors, which is the ratio of the concentration of the odorants in the initial extract to its concentration in the most dilute extract in which the odor is still detectable by GC/O. Charm analysis constructs chromatographic peaks, the areas of which are proportional to the amount of the chemical in the extract. The primary difference between the two methods is that charm analysis measures the dilution value over the entire time the compounds elute, whereas AEDA simply determines the maximum dilution value detected [40]. The AEDA technique has been further developed using static headspace injection [41], which could evaluate more of the highly volatile odorants lost during solvent extraction.

Another GC/O technique applied in wine aroma analysis is OSME, which uses non-diluted aroma extracts [42, 43]. In this method, the odor intensities

perceived in replicates by several assessors are averaged, yielding a consensus aromagram. Considering Stevens' law of psychophysics, OSME measures the response to odorants on a scale of time-intensity, so the results could reflect more real aroma intensity in complex matrix. However, the results were not significantly affected when Stevens' law was not taken into account.

Using GC/O techniques, most potent compounds of significance to wine aroma have been identified. It also indictes that differences between wines are mainly dependent on the amount of odorants, or specifically relative proportion of compounds in the sample, rather than the presence or absence of specific compounds [44].

1.1.2.2 Quantification and calculation of odor active value (OAV)

Due to the complexity of the volatile fraction of wine and the large differences in concentration, volatility and reactivity of its odorants, it is not possible to quantify the odorants precisely by using conventional methods [40]. Stable isotope dilution assays (SIDA) have provided greatly improved confidence in the analytical data, which use stable isotopes of the analytes as internal standards. However, since stable isotopically-labeled internal standards are not commonly commercially available, these standards must often be synthesized. Recently, Diez et al. [45] used SBSE-GC/MS for quantification of phenols in wine. They reported that the detection limit of phenols in wine could be as low as a few ppb after optimization of extraction conditions, where 15 ml of 1:4 diluted wines were extracted for 60 min at 900 rpm agitation without salt addition. The results also showed that methods using SBSE have good repeatability, high recovery, and low analytical sensitivity, and the matrix effect on the stir bar could be minimized using internal standards.

After careful quantification, the odor active values (OAVs) of volatile compounds are generally calculated by dividing the concentration of the odorant in the sample by the detection threshold concentration for that compound. The OAVs are useful measures to indicate the relative importance of individual compounds to sample aroma.

However, it should be noted that aroma threshold determinations are themselves subject to a degree of uncertainty, and threshold values in the published literature have been determined using widely different methods with differing degrees of rigor and in diverse matrices, including air, water, model systems, and different wines [44]. Therefore, the reference threshold value used for calculation should be carefully chosen. Moreover, since the interactions among volatiles are not taken into account when calculating OAVs, OAVs themselves cannot be used as the only standard for predicting the aroma mixture. Further reconstitution and omission experiments should be carried out, as explained in the following section.

1.1.3 Reconsitution and omission studies

To fully understand wine aroma, the last step is to determine the importance of the odor-active compounds in wines by reconstitution and omission experiments [40]. In reconstitution experiments, synthetic blends of odorants are prepared based on the obtained analytical data, and their aromas are compared with those of the originals. Oppositely, odorants are removed from the matrix in omission studies to detect their individual effect on overall aroma. Though these experiments have been increasingly carried out in the last decades, only limited successful studies have been reported due to the given difficulties of undertaking such technically demanding experiments [44].

An excellent example is a study conducted by Grosch [40], which

investigated the aroma of Gewurztraminer wine. After identification and quantification, the OAVs were calculated. Reconstitution results showed that an aroma model containing only compounds with OAV ≥10 was not satisfactory, while the aroma matched very well to that of the original wine when the model was completed by including the odorants with OAVs of 1 to 9. Further omission studies indicated that acetaldehyde (OAV=4), β-damascenone (OAV=17) and geraniol (OAV=7) had only a small effect on wine aroma.

In a majority of studies, it has been found that compounds with OAV < 1 do not appear to be crucial to wine aroma, and the presence of one or two specific compounds will have a major impact on that particular wine variety [46-48]. To fully understand the secrets of wine aroma, more and more of these types of studies are essential.

1.2 Wine aroma compounds and formation

The aroma of wine is directly associated with the grape growing and chemistry of the entire winemaking process. According to origin, the aroma compounds found in wines could be divided to three main types: (1) primary aromas, compounds already present in the grapes and persisting through vinification; (2) secondary aromas, generated primarily during fermentation, which are qualitatively and quantitatively the largest amount of the volatile compounds present in the wine; and (3) tertiary aromas, generated during maturation or aging processes, which are subsequent to vinification. Since the wine aroma is determined by the grape variety, certain primary aromas characterize a wine.

In the following sections, the major aroma compounds in wine and their formation are summarized based on chemical classes.

1.2.1 Alcohols

Except for ethanol, many fusel alcohols have been identified in wine, which generally have a characteristic pungent odor, such as 2-methylpropanol, 3-methylbutanol, 1-butanol, and so on. At low concentrations, these compounds add to the desirable aspects of wine aroma, though they become negative quality factors at high levels. Several GC/O studies have found that 3-methylbutanol is one of most potent aroma compounds [49, 50]. However, recent sensory analysis of white wine made with Devin grapes shows that the fusel aroma note is rather weak, and only in the retronasal perception it reaches 50% [51]. These findings may be attributed to good solubility of this alcohol in wine and to the fact that this compound is a fixed constituent of wine aroma and forms part of the general concept of wine aroma. Something similar happens to the 2-methylpropanol as well as other compounds that are considered to be generic contributors to wine aroma [48, 50, 52].

These fusel alcohols are secondary yeast metabolites, and their biosynthesis in wine yeast is shown in Figure 1.1 [53]. The use of different yeast strains during fermentation contributes considerably to variations in fusel alcohol profiles and concentrations in wine [54]. Moreover, the concentration of amino acid, ethanol concentration, fermentation temperature, the pH and composition of grape must, aeration, level of solids, grape variety, maturity and skin contact time also affect the concentration of fusel alcohol in final wines [55].

The C6 alcohols, such as 1-hexanol, *trans*- and *cis*- 3-hexenol, have been reported as green odorants in wines. Using the wine models, Herraiz et al. [56] studied the change of these compounds during alcoholic fermentation. The results showed that the presence of 1-hexanol in wine arises from the 1-hexanol present in

the must as well as from reduction of hexanal, *trans*-2-hexenal, *trans*-2-hexenol, and *cis*-2-hexenol. *cis*-3-Hexenol and *trans*-3-hexenol come from grape must, and are stable during alcoholic fermentation.

In grape must, levels of C6 alcohols and aldehydes depend on the grape variety [57], the ripeness rate of grapes [58], treatment of the must [59], and time and temperature affecting the contact with skins [60]. Consequently, data found for these compounds in the final wine could be helpful for characterizing the corresponding grape variety and for studying the technological treatment applied to the initial must.

1-Octen-3-ol, having a remarkable mushroom-like odor, is reported to be present in numerous wines [43, 61]. This compound as well as 1-octanol are formed during ripening as a result of attack by gray mold, and if present in a high concentration, may be considered a defect [62]. Its presence in wine is due to the action of *Botrytis cinerea* on grapes. Some research has shown that pesticide residues in grape must and malolactic fermentation can significantly affect the concentration of these compounds in wines [63, 64].

Benzyl alcohol and 2-phenylethanol are two common aromatic alcohols found in wines, which give strong floral and rosy odors [43, 50]. Both compounds are generated by the shikimate pathway, which is a common aromatic biosynthesis pathway. The proposed pathways for these two compounds are shown in Figure 1.2 and 1.3 respectively [65, 66]. In 1999, Antonelli and coworkers studied the effect of yeast on wine volatiles, and found that the concentration of 2-phenylethanol in wines significantly depended on the yeast strain used [67]. Moreover, it has been reported that the grape skins can produce these compounds by cell immobilization [68], which indicated that those compounds are present as precursor forms in grape skin.

1.2.2 Acids and Aldehydes

Though numerous acids have been identified in wines, only some of them may have recognizable odors, which are variedly described as cheesy, green, fruity or animal [69]. In a Mourvedre wine, all these acids up to octanoic acid were identified, but in this aroma complex only three acids, butanoic, 3-methyl-butanoic and hexanoic acids, were included in total GC peak area assessment, consisting of 0.78% of the total [70]. In an investigation of 13 young Spanish white wines, Aldave et al. [71] only reported quantitative information on octanoic acid, averaging 1.3mg/L in wines made where sulfur dioxide had not been used, and 2.6 mg/L in wines made with sulfur dioxide.

It should be noted that these acids generally do not impart important odors to wine aroma, especially when headspace SPME technique was applied in the analysis [72]. Except for their high sensory thresholds, another reason is that these acids will be rather soluble in water and will transition slowly into the headspace. Therefore, the sampling technique should be taking into account when examining the results of wine aroma analysis.

Most acids are generally related to yeast lipid metabolism during fermentation. Since these acids are necessary for the further generation of ester compounds, their concentration in wines will not only directly affect wine quality, but also affect ester concentration in samples, which will further influence wine aroma.

Ribereu-Gayon et al. [73] listed 18 aldehydes (mostly alkyls) in wine, but stated that, with the exception of acetaldehyde present at around 0.1g/L, these aldehydes are only present in trace amounts. In wines, acetaldehyde is a fermentation product, and can combine with sulfur dioxide. Other aldehydes present in grapes will be largely oxidized to the corresponding alcohols under the

conditions of vinification. Therefore, aldehydes are generally not considered to be important aroma contributors.

The "leaf aldehydes" (hexanal, *trans*-2-hexenal, and *cis*-3-hexenal) are reported present in Mourvedre grapes and wines [70]. Their presence is due to the crushing of grapes, prior to vinification, when enzymatic oxidation of linolenic acid can occur. However, it is also stated that this wine aroma is a result of the use of unripe grapes [73]. During fermentation, these aldehydes can be transformed into the corresponding alcohols, which have a similar "grassy" aroma at low concentration.

Several aromatic aldehydes have shown wine aroma importance. Vanillin and cinnamaic aldehyde are often recognized as vanilla-like, floral odorants. Having a bitter almond aroma, benzaldehyde is a potential defect in wines, but characteristic of some grapes, such as Gamay [73]. Developed during aging in oak barrels, these aldehydes increase in concentration in aged wines due to oxidation. Their changes are likely to be influenced by the amount of sulfur dioxide present, irreversibly reducing oxygen content and other factors.

1.2.3 Esters

In wine, esters of all kinds are regarded as especially important to wine aroma. They are usually generated during fermentation, and some of them arise from the aging process due to alcohol-acid rearrangements.

Ethyl fatty acid esters and acetates are the most abundant esters in wines, which comprise about 30% of all the volatile compounds detected in red wines [47, 74]. It is generally recognized that the lower aliphatic ethyl esters show fruity notes of different kinds, such as apple, tropical tree fruit, banana, etc., whereas the higher homologues tend towards soapy, oily, and candle-like characteristics.

These esters are formed from acyl-SCoA by yeast during fermentation, which can be dramatically affected by many factors, such as fermentation strains, fermentation temperature and oxygen availability [73]. For example, lower temperatures favor the formation of "fruity" esters, which are especially significant in young white wines, and contribute to their "fruity" character. It has been also discovered that branched fatty acid ethyl esters are influenced by nitrogen levels during fermentation [75], because the nitrogen composition of grape musts affects the growth and metabolism of yeast, thus the fermentation rate, and the completion of fermentation [76]. Checked in Muscat wines, aged 1-5 years, the branched fatty acid ethyl esters increased along with aging, while straight-chain ethyl esters decreased [77]. In this study, researchers investigated three hypothetical pathways suggested in the literature, and the results showed that the acid-ester equilibrium was the most effective in generating the branched fatty acid ethyl esters from their corresponding acids during wine aging. Therefore, as explained above, the acid level will be critical for ester generation.

Similar fruity characteristics are also associated with other esters, such as ethyl benzoate, ethyl phenyl acetate and hexyl hexanoate. Even with low concentration (only a few ppm in wine [74]), these esters are still considered as potent, and hence important, aroma compounds due to their low sensory thresholds (< 50 ppb). However, none of these esters themselves appears to offer a number of other fruity characteristics found in many wines, such as cherry, blackcurrant, gooseberry, or plum.

In 1995, ethyl and methyl anthranilate, ethyl cinnamate, and ethyl dihydroxycinnamate were identified in Pinot noir [78]. Described as cherry, blackcurrant, and stone fruit, these compounds were suspected to influence the characteristic flavor quality in Pinot noir wines of Burgundy according to GC-O

results. However, later quantification showed that amounts of these esters were below the sensory thresholds [79], so their contributions to Pinot noir aroma is still unclear. In other kinds of wines, these compounds have also been identified as potent and/or important aroma contributors [49, 69].

1.2.4 Terpenes

The large family of terpene compounds is very widespread in the plant kingdom. Within this family, odor-active compounds are mainly monoterpenes (with 10 carbon atoms) and sesquiterpenes, formed from two and three isoprene units, respectively. In grapes and wines, monoterpenes, which could exist as hydrocarbons, alcohols, aldehydes, ketones or esters, have been found to be responsible for the floral aroma. The main monoterpene compounds found in grape juice and wines are summarized in Figure 1.4 by Maricas and Mateo [80]. Since wines gain these compounds directly from grapes, monoterpenes express the typical sensory characteristics of the wine bouquet, and they can therefore be used analytically for its variety.

Terpene compounds belong to the secondary plant constituents, of which the biosynthesis begins with acetyl-coenzyme A (CoA). Figure 1.5 shows the mechanism of biosynthesis of monoterpenes in plants [81]. Three types of categories of monoterpenes exist in grapes with some interrelationships between the categories: free form aroma, free odorless polyols, and glycosidically conjugated form precursors. They are largely present in the skins of grapes and among the three forms, glycoside precursors are most abundant [82]. Their content in grapes varies with different varieties (0-1 mg/L) [83]. However, no satisfactory explanation has been agreed upon to account for why certain grape varieties consistently produce more monoterpenes than others do. Strauss et al.

suggested four pathways for metabolism of linalool in grapes [84]. Muscat varieties contain a relatively high concentration of free linalool, and also readily utilizes all four pathways. In Chardonnay, where the terpene content close to zero, it is likely that only one or two pathways are utilized.

During winemaking, terpene glycosides can be hydrolyzed by the action of glycosidase enzymes, which are produced by the grapes, yeast and bacteria. Therefore, increasing glucosidase enzyme activity is a way for enhancing the terpenoid aroma in wines. Generally, enzymatic hydrolysis of monoterpenes involves two steps. In the first step, an α-L-rhamnosidase and an α-L-arabinofuranosidase or a β-apiofuranosidase (depending on the structure of the aglycone moiety) cleave 1,6-glycosidic linkages. In the following step, the monoterpenes are liberated from monoterpenyl β-D-glucosides by the action of a β-glucosidase [54]. To improve wine aroma, many enzymes from yeast and bacteria are screened based on the desired enzyme properties [85-87]. The glycoconjugated aroma compounds are often investigated by enzyme hydrolysis because they can produce more "natural" aromas [82, 88].

Besides enzymatic hydrolysis, acidic hydrolysis can be used to release the monoterpenes from their precursors in grapes. It should noted that acid hydrolysis induces molecular rearrangement of the monoterpenols, such as transformation of linalool to α-terpineol, hydroxyl linalool, geraniol, and nerol, as shown in Figure 1.6 [89]. These various ways to liberate terpenes simulate the reactions taking place during aging of wines, and the different terpenic alcohols are produced in similar quantitative ratios. It has been confirmed that the progressive release of aroma with long periods of mild acid hydrolysis is reflected in the increase in intensity of the same aroma attributes in wines undergoing natural aging or mild heating [90]. Therefore, more and more mild acid hydrolysis reactions are used to

analyze the content of terpene glycosides [88, 91, 92].

1.2.5 Ketones

Some simple aliphatic ketones in wines are formed during fermentation, but only a few of them are considered to contribute to wine aroma. Diacetyl (2,3-butadione) may reach high enough concentration levels to produce a sweet, buttery or butterscotch odor, though it can be regarded in "spoiled" wines as an off-flavor. Acetoin (3-hydroxybutan-2-one) has a similar slightly milky odor, and may be perceptibly present in wines.

The complex ketones, β-damascenone and α,β-ionones are found as important aroma compounds in wine with highly desirable flavor properties and have low odor thresholds (respectively 2 ng/L and 7 ng/L) [93]. β-Damascenone has a narcotic scent reminiscent of exotic flowers with a heavy fruity undertone and is described as apple, rose and honey, while α,β-ionone has a distinct aroma of violets. These compounds are C13-norisoprenoid compounds, and arise from the enzymatic oxidation and cleavage of carotenoid during the crushing of the grapes [73]. There may also be an increase in the amount because of "in-bottle" aging. Oak aging may also release some α- and β- ionone.

Like the monoterpenes, the norisoprenoids occur in grapes and wines predominately as glycosidically bound precursors, which will be released by enzyme and acid during winemaking. In a study investigating the precursors of C13-norisoprenoids in Riesling wine, it has been found that β-damascenone arises from different conjugated glycosides [93]. It is also reported that those precursors developed in the fruit with sugar accumulation. Based on their positive correlation, Strauss et al. [94] suggested that between changes in the juice °Brix readings and changes in precursor concentrations, grape maturity is implicated as a causative

factor in the ultimate bottle aging of Riesling wines.

1.2.6 Phenols

Phenolic compounds are responsible for all the differences between red and white wines, especially the color and flavor of red wines. In particular, sensory analyses of wines, obtained from Cabernet franc grapes grown in different Loire Valley locations, pointed out that intensity variables (color, taste, and flavor), mellowness and balance are affected by complex wine phenolic compositions [95]. Therefore, the quality of red wines depends to a large extent on their phenolic composition, including both grape constituents and products formed during winemaking.

Though phenolic and polyphenolic compounds found in grapes, musts and wines, are widely studied, the volatile phenols directly related to wine aroma were only paid attention to in recent years [96]. Volatile phenols are normally known for their contribution to off-flavor such as "band-aid" or "barnyard", but recently it was reported that they can contribute positively to the aroma of some wines [97]. Among these phenols, vinyl-4-phenol, vinyl-4-guaiacol, ethyl-4-phenol and ethyl-4-guaiacol are regarded as being especially important in an olfactory defect known as "phenol" character [96]. In addition, several volatile phenols have also been described as having a "smoky" or "tarry" character, including 2-methoxy-guaiacol and 2-ethyl-cresol, among others.

Trace amounts of these compounds are present in grape musts, but they are predominantly produced either during fermentation or generally released during aging. Vinylphenols are formed by enzymic decarboxylation by the yeast during fermentation from two cinnamic acids present, while the presence of ethylphenols arises not during fermentation but rather during the aging process [98, 99]. In red

wines, ethylphenols could be associated with spoilage by *Brettanomyces* [100]. Red wines have a much higher level of tannin than do white wines as they are extracted from the skins of grapes during red wine fermentation. These compounds were primarily degraded to weakly smelling intermediates (4-vinyl phenol and 4-vinyl guaiacol), and then further enzymatically degraded by *Brettanomyces* to the strong smelling 4-ethyl phenol and 4-ethyl guaiacol respectively. Therefore, formation of these compounds is suspected to associate with anthocyanins in grapes and red wines.

The use of oak barrels, after toasting, during aging is the main factor in determining the presence of the other phenols identified in wine, in particular eugenol in large amounts and some cresols in very small amounts. It has also been reported that these compounds could be extracted from oak barrel, and toasting of the oak barrels could lead to thermal degradation of lignin and the subsequent production of the volatile phenols [101, 102]. Data has been presented relating the degree of toasting to the extractability of the various phenols [96].

1.2.7 Lactones

Lactones can be present in wine via a number of pathways. The simple lactones like γ-butyrolactone, which has an aromatic odor, can arise in the fermentation, by the lactonization of γ-hydroxybutanoic acid. The acid itself is formed by the deamination and decarboxylation of free glutamic acid or from protein present [103]. However, this compound has a very high threshold, thus contributes little to wine aroma.

Widely distributed in fruit, lactones may also come from the grapes, as is the case in Riesling, where they contribute to the varietal aroma. For example, sotolon, which is involved in the toasty aroma characteristic of wines, is produced by

Botrytis cinerea present on the grape skins [96]. Sotolon also can result from a condensation reaction between α-keto butyric acid and ethanal, which is not catalyzed by enzymes [73]. Another compound, 3a,4,5,7a-tetrahydro-3,6-dimethylbenzofuran-2(3H)-one, known as wine lactone, has been identified as an important odorant of Scheurebe and Gewürztraminer wines [41]. The 3*S*,3a*S*,7a*R* isomer has a coconut, woody, and sweet aroma with an odor threshold of 0.02 pg/L air [83]. Winterhalter et al. [104] postulated that a monoterpenoid precursor is acid converted to wine lactone at typical wine pH (pH 3.2).

Some lactones present in wine arise during aging processes. One of the most important is β-methyl-γ-octalactone, commonly known as oak or whiskey lactone. There are two isomers of oak lactone. Both isomers have a woody, oaky, coconut-like aroma; however, the aroma threshold for the *cis* isomer has been observed at 92 ppb, compared to 460 ppb for the *trans* isomer. Though the exact mechanisms and the origin of the methyl-octalactone precursors in wood and their hydrolysis are still unknown, it has been proposed that the ratio of *cis* to *trans* forms of oak lactone can be used to differentiate between wines fermented in American and French/European oak [105]. Chatonnet [106] observed that these compounds were influenced by the wood treatment before making barrel.

1.2.8 Thiols

The volatile thiols have been found to be one of the most potent groups of aroma compounds in wine. They usually contribute positive aroma at low concentration, while imparting negative aroma at high concentration. Due to their extremely low perception thresholds (3-60ng/L), 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) are found as strong odorants in wine, which have box tree (4MMP), passionfruit,

grapefruit, gooseberry, and guava aroma (3MH and 3MHA) respectively [107]. These sulfur-containing compounds have been identified in wines (Sauvignon Blanc, Colombard, Riesling, Semillon, Merlot and Cabernet Sauvignon) in varying concentrations and can potentially impact aroma [108, 109]. Furfurylthiol is also a potent aroma sulfur-containing compound in wine, which presents a roasted coffee aroma with a perception threshold of 0.4 ng/L [110].

The volatile thiols are almost non-existent in the grape juice and only develop during fermentation. It has been shown that production of furfurylthiol is linked to the production of the HS^- anion, which is not produced when ammonium sulfate is added in sufficient quantities in a fermentation [111]. However, there is evidence showing that 4MMP and 3MH do exist in the grapes but in the form of non-volatile, cysteine bound conjugates and that yeast is responsible for the cleavage of the thiol from the precursors [112].

1.3 Wine Sulfur Off-flavor Compounds and Formation

In addition to aroma compounds that provide positive notes, the study of off-flavor compounds in wines has also received more attention in recent years, especially of sulfur volatiles. To gain a better understanding of the mechanism for sulfur volatiles in wines, many studies have been done to investigate their origin, formation, and reaction during grape growing and winemaking.

1.3.1 Analytical Method for Sulfur Volatiles in Wines

Like aroma compounds, sulfur off-flavor compounds are generally present in trace amounts in wine, therefore a pre-concentration step is required before chromatographic analysis [18]. Solvent extraction [113, 114] and static headspace

techniques [115, 116] have been widely used for volatile extraction, but time consumption and lack of sensitivity are the two major downfalls to limit their application for sulfur analysis in wine. In addition, some sulfur compounds are extremely volatile and chemically reactive so it is often impossible to use traditional techniques to enrich them.

As an alternative to traditional pre-concentration methods, solid-phase microextraction (SPME) has been successfully used to extract volatile compounds, including sulfur compounds, from the headspace of various samples [11-16]. SPME technique has been previously used to analyze volatile sulfur compounds in wines [117-120], but quantification has not been successful due to the challenges involved with the reactive nature of sulfur compounds as well as competitive adsorption within the SPME fiber [17]. A SPME extraction coupled with stable isotope dilution assay was successfully developed to analyze ethanethiol and diethyl disulfide in Syrah wine [121, 122].

Due to low concentrations in food, sulfur compounds are typically analyzed by gas chromatography (GC) with sulfur-specific detection, including flame photometric detection (FPD) [115, 116], sulfur chemiluminescent detection (SCD) [123] and atomic emission detection (AED). Recently, pulsed flame photometric detection (PFPD) has proven to be very sensitive for sulfur compounds, and it has been widely used to analyze trace sulfur compounds [16, 124-126]. This technique uses a pulsed flame, rather than a continuous flame as with traditional FPD, to achieve the generation of flame chemiluminescence [127]. With PFPD, light emissions due to hydrocarbons and flame background can be ignored during each pulse of the flame by electronically gating the emission, allowing for only the sulfur portion of the spectrum to be integrated, thereby greatly increasing the selectivity and sensitivity for this detector.

However, it is still very difficult to exactly quantify most sulfur volatiles, especially to hydrogen sulfide and methanethiol, due to their high volatility and reactivity. It becomes increasing important to develop a quick and reliable analytical method to quantify volatile sulfur compounds in wine.

1.3.2 Aroma Properties of Sulfur Volatiles

Volatile sulfur compounds are known to have very powerful and characteristic odors, and these compounds can contribute to pleasant or unpleasant aromas of a wine according to their nature and concentration [128]. Usually, when volatile sulfur compounds are present at very low concentrations, they contribute a positive impression to the wine aroma [129]. However, when present at higher concentrations, they are responsible for "reduced", "rotten egg", or "sulfury" off-flavors [130]. The sensory thresholds in various mediums along with aroma descriptions of common volatile sulfur compounds found in wines have been summarized (Table 1.1) [52, 128, 131]. For most volatile sulfurs, their sensory threshold is extremely low, so they are easy to become a defect in wines.

Recently, Tsai [131] investigated the odor suppression of four important sulfur volatiles in Oregon Pinot noir wines. It was found that ethanethiol (EtSH) affects wine aromas more when both methanethiol (MeSH) and ethanethiol (EtSH) are present in base wine. Additionally, MeSH governed wine off-odors more than EtSH under the influence of sub-threshold levels of two disulfides, dimethyl disulfide (DMDS) and diethyl disulfide (DEDS) respectively. Mercaptans can significantly affect aroma quality of Oregon Pinot noir wine at very low concentrations (in ppb level), and they have a stronger effect than disulfides. Regarding the impact caused by these four sulfur compounds on the base wine aroma, the base wine lost its fruity and floral character and increased overall

intensity, overall stinky, nose burn and sulfur-related odors when concentrations of the four volatile sulfur compounds in base wine increased.

1.3.3 Formation of Sulfur Volatiles in wines

A variety of biochemical as well as chemical mechanisms are involved in the formation of sulfur compounds in wine, but many of these mechanisms are still poorly defined [128, 132]. The development of these sulfur compounds by yeasts includes the degradation of sulfur-containing amino acids, the degradation of sulfur-containing pesticides, and the metabolism of grape derived sulfur-containing precursors [133, 134].

Probably the best-studied sulfur volatile in wine is hydrogen sulfide (H_2S), since it is associated with the most common problems in winemaking. Hydrogen sulfide can be formed metabolically by yeast either from inorganic sulfur compounds (sulfates and sulfites) or from organic sulfur compounds (cysteine and glutathione) [132, 135]. Under two synthetic juice conditions, Spiropoulos et al. [133] investigated hydrogen sulfide production by 29 strains of *Saccharomyces cerevisiae* and the sulfate reduction sequence (SRS) pathway is suggested. When nitrogen is limited, the SRS pathway will be activated and sulfides will accumulate due to the lack of precursors. Surplus sulfide is then liberated from the cell as H_2S [136]. Moreover, H_2S is a highly reactive compound, which can take part in a variety of reactions to generate other sulfur volatiles that impact wine aroma [137]. For example, mercaptans can be formed by the reaction of H_2S with ethanol or acetaldehyde [132].

The mercaptans, including MeSH and EtSH, are mainly produced as by-products of yeast metabolism of methionine, and can be formed during fermentation in association with H_2S [132]. Thioacetic acid esters of these

mercaptans are also observed to form during fermentation, and these compounds can slowly hydrolyze to the parent mercaptan in the later aging [138]. It should be noted that thioacetates might not contribute to the off-odors in wine since they are believed to have relatively high sensory thresholds, but hydrolysis to mercaptans can create aroma defects [132].

The formation of dimethyl sulfide (DMS) is not clear yet. It has been observed that DMS formation during formation was linked to cysteine, cystine or glutathione metabolism in yeast, and its formation during wine maturation is related to the cleavage of S-methyl-L-methionine to homoserine and DMS [132]. The formation of polysulfides is believed to involve oxidation of the mercaptans. On the other hand, yeast can also reduce disulfides to mercaptans.

In addition to yeast, it also has been reported that lactic acid bacteria isolated from wine (*Oenococcus Oeni* strain) are able to metabolize methionine to form sulfur volatiles including MeSH, DMDS, and 3-(methylthio)propanol, commonly known as methionol [116]. Overall, since many pathways and factors are involved, the formation of sulfur volatiles in wine is very complex and is still not well understood.

1.3.4 Effects of Vinification on Sulfur Volatiles in Wine

Since sulfur volatiles, especially H_2S and mercaptans, are generally related to wine off-flavor, ways to control their amounts in wine has become a hot topic among winemakers. However, this is a significant challenge, since many factors such as deficiencies of nutrients (amino acids and vitamins), yeast strains, metal ions, redox potential, and fermentation temperature, can all influence the formation of volatile sulfur compounds [139].

Several studies [140, 141] indicate that the presence of elemental sulfur from

the vineyard can cause H_2S formation during fermentation. However, this claim has been recently disputed [142, 143].

Nitrogen deficiency in grape must has been widely accepted as one of the major reasons to cause volatile sulfur production [128]. Vos and Gray [144] suggested that the yeast breaks down extracellular proteins in order to scavenge α-amino groups, leaving behind the sulfide residues of the sulfur-containing amino acids when musts are deficient in nitrogenous components. Later evidence effectively argues against this mechanism, and has shown that yeast reduce sulfites under these deficient conditions, resulting in increased levels of H_2S [145, 146]. Recently, more research [134, 147] has been done which indicates that the formation of sulfur volatiles associated with the yeast metabolism of nitrogen compounds is much more complex. The addition of different amino acids to grape musts generates different sulfur compounds in wines [147]. Moreover, it has been reported that H_2S production was even significantly higher if the concentration of yeast assimilable nitrogen content (YANC) was increased when pantothenic acid was deficient [134].

The effects of other vinification parameters on volatile sulfur production in wine have been reported, such as temperature, light exploration, and bisulfite addition [113, 148-150]. However, it is very difficult to compare these results. One of major reasons is that the parameters examined are evaluated with different yeast strains and must turbidity. Therefore, there are still many disagreements in this field on how to effectively control the production of volatile sulfur compounds in wine and the subsequent possibility of off-aroma formation.

Table 1.1 Sensory thresholds in various mediums and aroma description of common volatile sulfur compounds [52, 128, 131].

Compound	Threshold value (ppb)			Aroma description
	Wine		Ethanol water**	
Hydrogen sulfide	0.001-150		0.8	Rotten egg, decaying seaweed, rubbery
	40-100*			
Methanethiol	1.72-1.82	(red)	0.3	Rotten cabbage, cooked cabbage, burnt rubber, pungent, putrefaction
Ethanethiol	1.1	(white)	0.1	Onion, rubber, fecal, burnt match, earthy, durian
	0.19-0.23	(red)		
Carbon disulfide	30	(white)		Rubber, choking repulsive, cabbage, sulfidy
Dimethyl sulfide	10-160		5-10	Cabbage, asparagus, cooked corn, truffles, vegetal, molasses, black olive
	25	(white)		
	60	(red)		
Diethyl sulfide	0.92-18		6	Garlic, onion, cooked vegetables, rubbery, fecal
	0.92	(white)		
Dimethyl disulfide	20-45		2.5	Cabbage, cooked cabbage, onion-like
	29	(white)		
	11.2-23.6	(red)		
Diethyl disulfide	4.3-40		20	Garlic, onion, burnt rubber
	4.3	(white)		
	1.4-2.2	(red)		
Dimethyl trisulfide				Beany
Methyl thioacetate				Sulfurous, rotten vegetables, cheesy, onion, burnt
Ethyl thioacetate				Sulfurous, cheesy, onion, burnt
Methionol	1200-4500			Raw potato, soup-like, meat-like
Methional			50	Onion, meat, mashed potato, soup, bouillon
Benzothiazole	24		50	Rubber
	50-350			
2-mercaptoethanol	130 -10000		1000 - 10000	"Boxer", poultry, farmyard, alliaceous
4-methylthiol-1-butanol	100		80-1000	Chive, garlic, onion, earthy, alliaceous

* Flavor threshold

**The percentage of ethanol in water is 12% v/v.

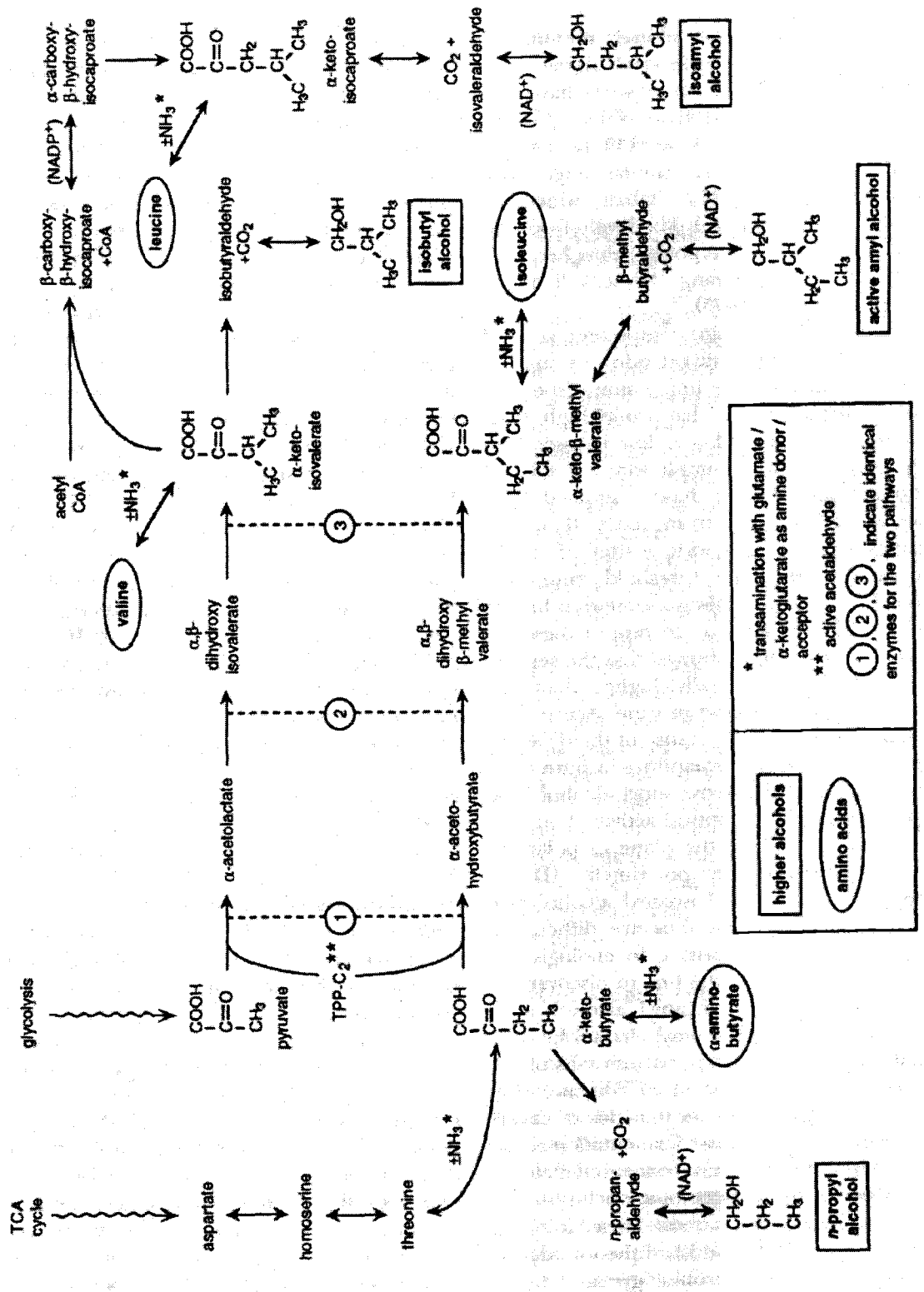

Figure 1.1 Pathway for formation of higher alcohols from glucose [53]

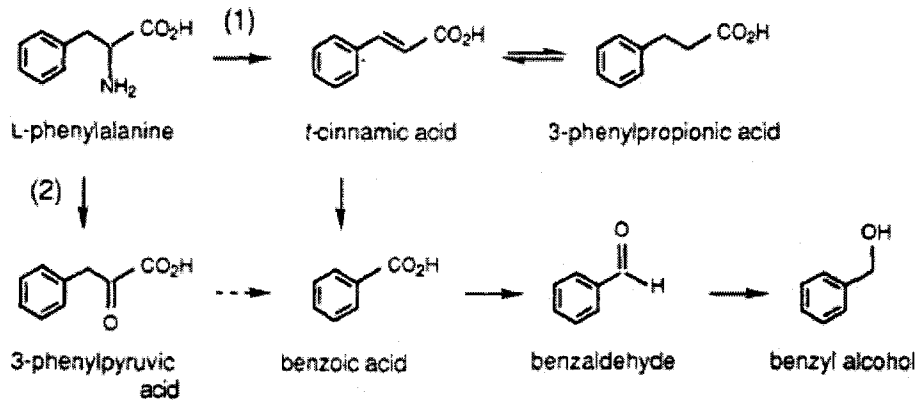

Figure 1.2. the Proposed production pathway of benzaldehyde and benzyl alcohol by stain K2606: (1) phenylalanine ammonia-lyase; (2) transaminase or L-Amino acid oxidase [65]

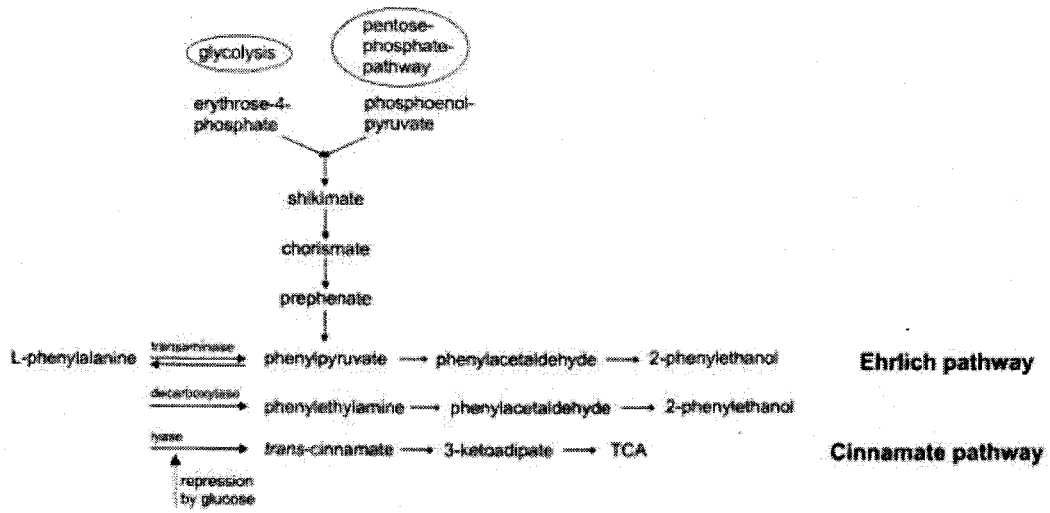

Figure 1.3. The Proposed production pathway of 2-phenylethanol [66]

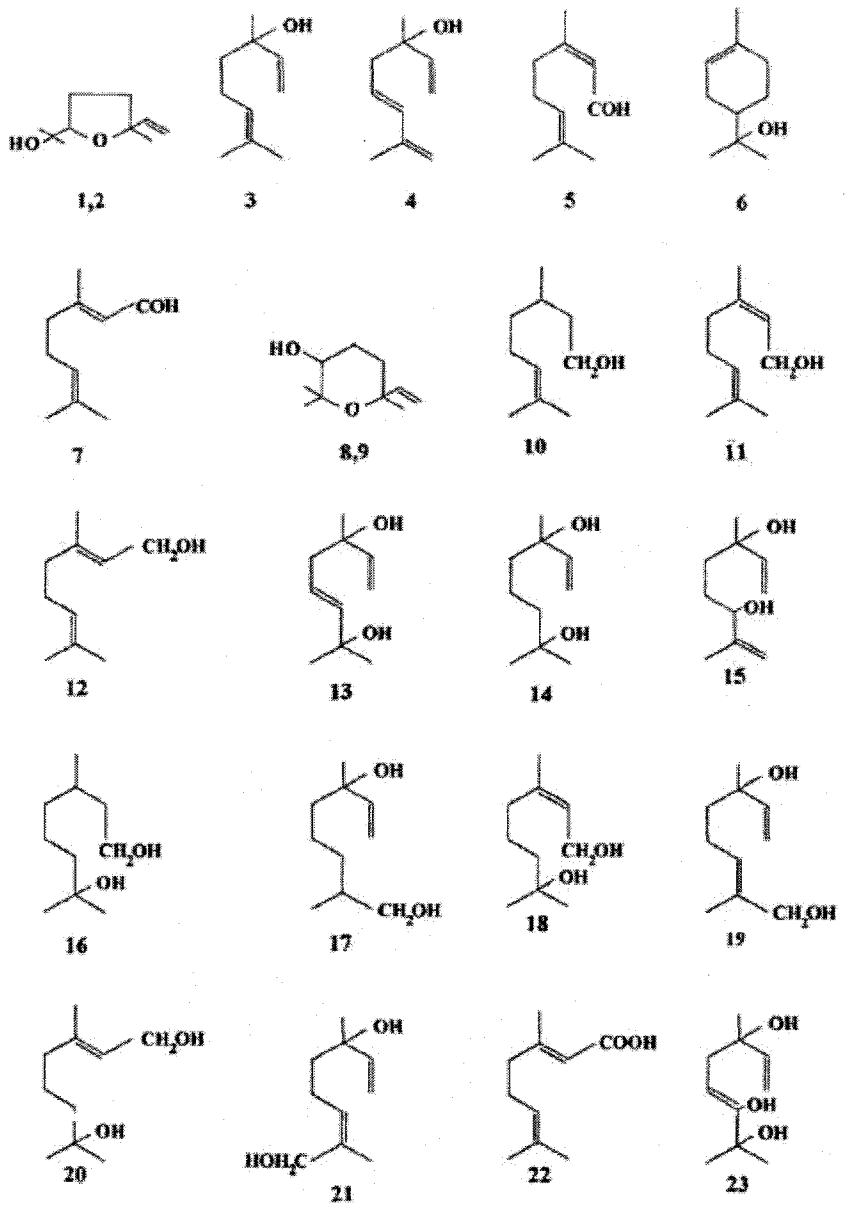

Figure 1.4 Main monoterpene compounds in grape juice and wines [80]
1,2: *trans*- or *cis*- Furan linalool oxide; 3: linalool; 4: hotrienol; 5: neral; 6: α-terpineol; 7: geranial; 8,9: *trans*- or *cis*- pyran linalool oxide; 10: citrobellol; 11: nerol; 12: geraniol; 13: diol I; 14: endiol; 15: diol II; 16: hydroxyl-cityronellol; 17: 8-hydroxy-dihydroxy-linalool; 18: hydroxyl-nerol; 19: *trans*-8-hydroxy-lianlool; 20: hydroxyl-geraniol; 21: *cis*-8-hydroxy-linalool; 22: geranic acid; 23: triol

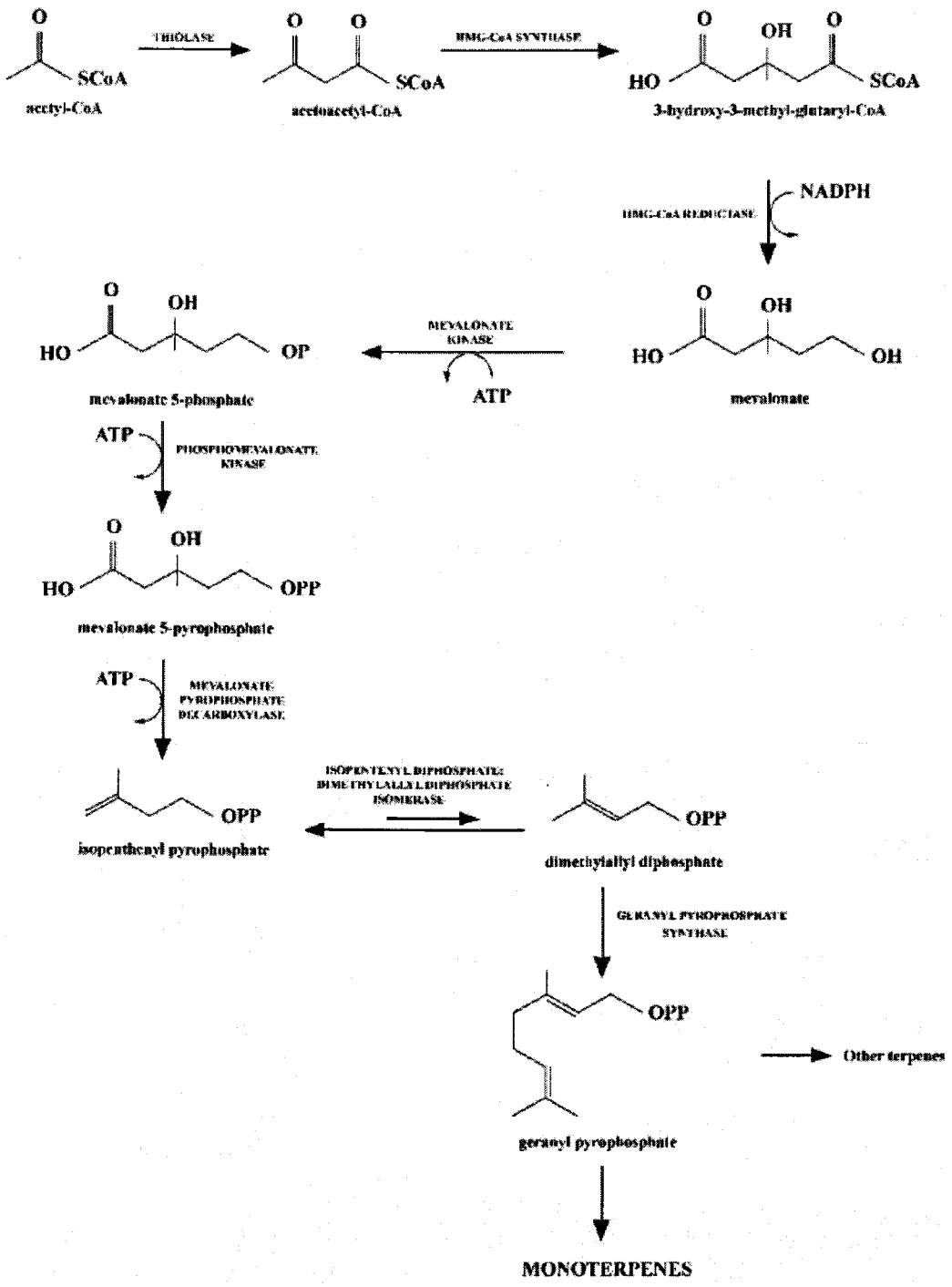

Figure 1.5 The mechanism of biosynthesis of monoterpenes in plant [81]

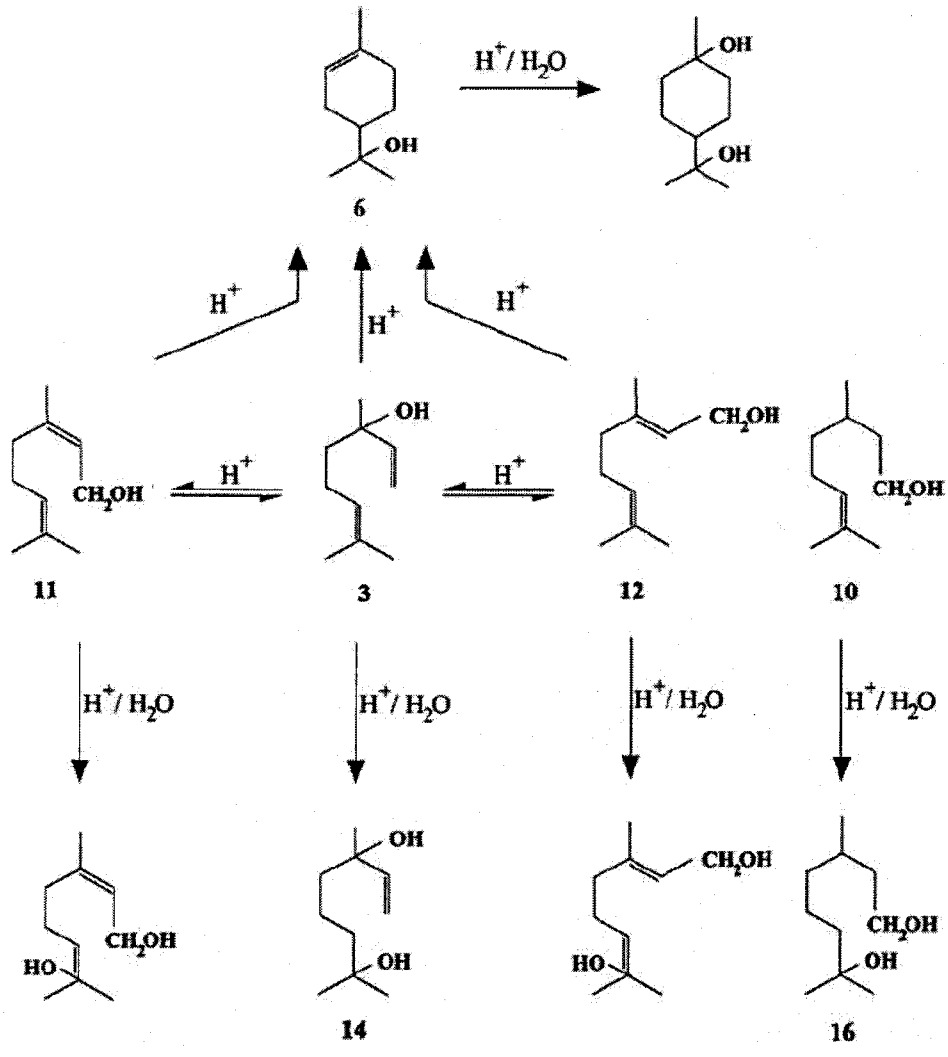

Figure 1.6 Acid catalyzed rearrangement of monoterpenes [89]

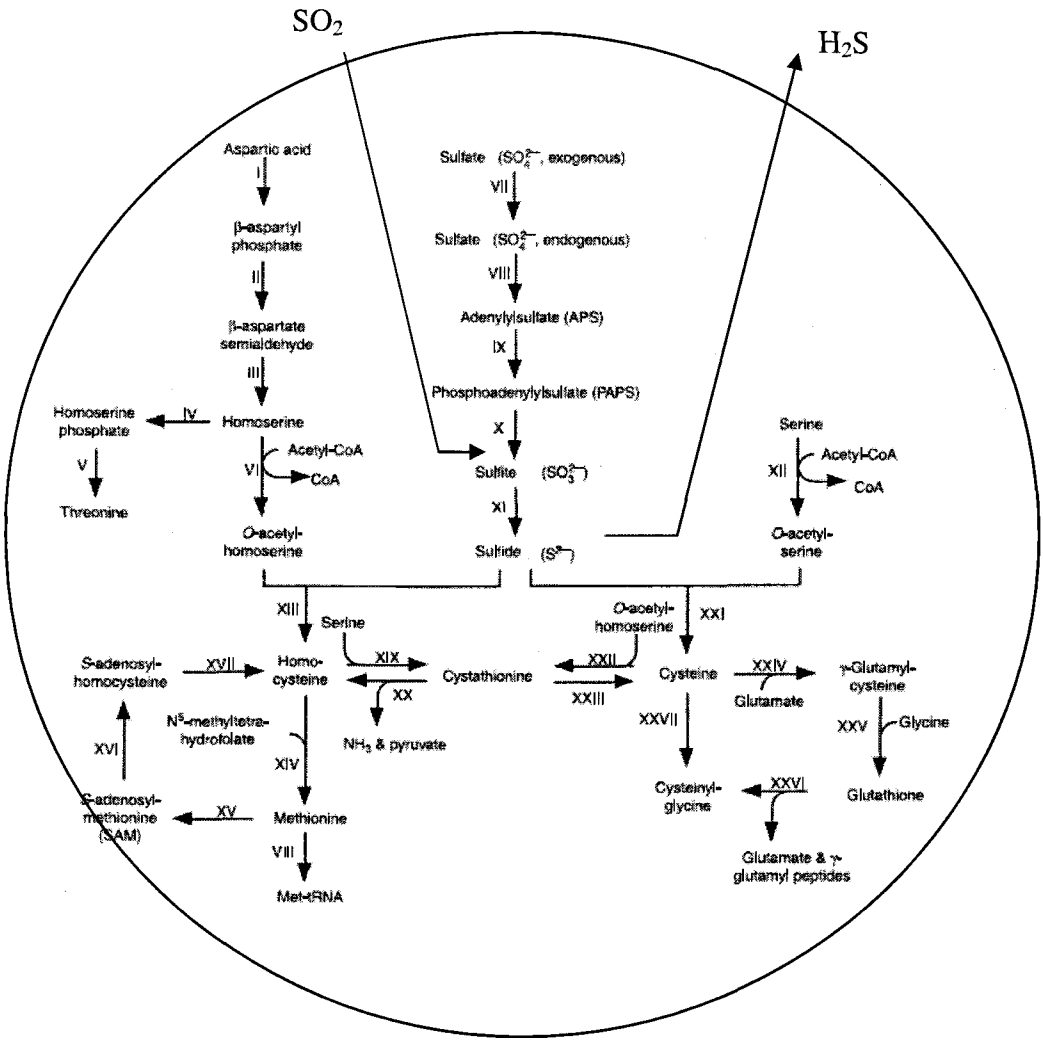

Figure 1.7 A schematic representation of the sulfur metabolism of wine yeast based on Spiropoulos et al [133] and Wang et al [134].

(I) Aspartate kinase; (II) aspartate semi-aldehyde dehydrogenase; (III) homoserine dehydrogenase; (IV) homoserine kinase; (V) threonine synthase; (VI) homoserine O-transacetylase; (VII) sulfate permeases; (VIII) ATP sulfurylase; (IX) APS kinase; (X) PAPS reductase; (XI) sulfite reductase; (XII) serine acetyltransferase; (XIII) O-acetylhomoserine and O-acetylserine sulfydrylase; (XIV) homocysteine methyltransferase; (XV) S-adenosylmethionine synthetase; (XVI) S-adensylmethionine demethylase; (XVII) adenosylhomocysteinase; (XVIII) methionyl-tRNA synthetase; (XIX) b-cystathionine synthase; (XX) b-cystathionase; (XXI) cysteine synthase; (XXII) c-cystathionine synthase; (XXIII) c-cystathionase; (XXIV) c-glutamylcysteine synthetase; (XXV) glutathione synthetase; (XXVI) c-glutamyltranspeptidase; (XXVII) cysteinylglycine dipeptidase

Chapter 2. Aroma Compounds in Oregon Pinot noir Wine Determined by Aroma Extract Dilution Analysis (AEDA)

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2.1 Abstract:

The aroma profiles of Oregon Pinot noir wines were investigated with aroma extract dilution analysis (AEDA). The wines were extracted with pentane-diethyl ether, the aromas were distilled using solvent-assisted flavor evaporation (SAFE), and separated into acid/water-soluble and neutral/basic fractions. In the acid/water-soluble fraction, 2-phenylethanol and 3-methyl-1-butanol showed the highest AEDA values, followed by 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, 2-methylpropanol, hexanol, trans-3-hexenol, cis-3-hexenol, benzyl alcohol, methionol, 3-ethylthio-1-propanol, linalool, and geraniol (all with FD≥ 64). In neutral/basic fractions, ethyl 2-methylpropanoate, ethyl butanoate, isoamyl acetate, ethyl hexanoate, and benzaldehyde had very high AEDA values (all with FD≥ 64), followed by ethyl 3-methylbutanoate, isoamyl 2-methylpropanoate, ethyl octanoate, ethyl decanoate, benzyl acetate, phenylethyl formate, phenylethyl acetate, ethyl dihydrocinanamate, ethyl anthranilate, methional, citronellal, whiskey lactone , and γ-nanalactone (all with FD≥ 16). Overall, the results indicated that there is no single compound that characterizes the aroma of Pinot noir, and the characteristic aroma comes from a blend of numerous compounds.

2.2 Keywords:

Pinot noir, aroma, GC-O, AEDA, wine

2.3 Introduction

Pinot noir wine is one of the oldest wines, originating from the Burgundy region of France. From 1970s, production of this aromatic wine increased in areas of Oregon, California, Australia, and New Zealand. As Pinot noir wine became more popular in the United States over the past decade, its characteristic and distinct flavor began to receive more scrutiny from consumer. Pinot noir wine is known to exhibit distinct red fruit aromas evoking particularly the odors of small-stone fruits (plum and cherry), and of strawberry, raspberry, black currant, and blackberry [151].

Wine aroma has been meticulously studied over the last few decades, and several comprehensive reviews [152-155] reported more than 800 compounds as volatile constituents in wine. However, most of these compounds are not odor-active [41, 156], and they do not contribute to wine aroma. A number of gas chromatographic/olfactometric (GC-O) techniques, including Charm Analysis [36], aroma extract dilution analysis (AEDA) [38] and OSME [40], has been used to determine odor-active compounds in wine.

Many studies have been performed to identify which odorants are responsible for the characteristic bouquet of white wines. Using Charm Analysis, researchers have found ethyl hexanoate, ethyl butanoate, and ethyl 2-methylbutanoate to be the most potent odorants of Chardonnay and white Riesling wines [157, 158]. In addition to these ethyl esters, Moio et al. demonstrated vanillin, 2,3-butanedione, guaiacol, 4-vinylguaiacol, and ethyl cinnamate as further potent odorants of the variety Chardonnay due to their high Charm values. β-Damascenone and 2-phenylethanol were also identified as key odorants in Chardonnay-Semillon wines based on their high FD factors[159]. Ethyl 2-

methylbutanoate, ethyl methylpropanoate, 2-phenylethanol, 3-methylbutanol, 3-hydroxy-4,5-dimethyl-2(5H)-furanone, 3-ethylphenol, and wine lactone (3a,4,5,7a-tetrahydro-3,6-dimethylbenzofuran-2(3H)-one) were identified to be important to the aroma of Scheurebe and Gewürztraminer wines [41].

Some odorants in red wines have also been identified within the past ten years [47, 160-162]. Ferreira et al. [156] quantified odorants in 52 young red wines made from Grenache, Tempranillo, Cabernet sauvignon, and Merlot grapes. Thirty-three odorants were detected in these wines at concentrations higher than their corresponding odor thresholds. The most important odorants include ethyl octanoate, β-damascenone, ethyl hexanoate, 2-methylpropanoic and 3-methylbutanoic acids, 3-methylbutyl acetate, 3-methylbutanol and 2-phenylethanol, 2,3-butanedione, ethyl butanoate, β-ionone, 3-methylthio-1-propanol, ethyl cinnamate, ethyl dihydrocinnamate, γ -nonalactone, eugenol, cis-3-hexenol, geraniol, guaiacol, 3-isobutyl-2-methoxypyrazine, 4-ethylguaiacol, acetoin and whisky lactone.

Aznar et al. [47] analyzed aged red wine from Rioja and found that the most important aroma compounds were 4-ethylguaiacol, whisky lactone, 4-ethylphenol, β-damascenone, 3-methylbutanoic and hexanoic acids, eugenol, ethyl 2-methylbutanoate, ethyl 2-methylpropanoate, ethyl cinnamate, furaneol, phenylacetic acid and *trans*-2-hexenal. More recently, Ferreira et al. [163] found that 3-hydroxy-4,5-dimethyl-2(5H)-furanone might be a key odorant of the aged Port wine. In addition, Boido et al. [164] identified C13-norisoprenoid, including 3-hydroxy-β-damascenone, 3-oxo-α-ionol, vomifoliol, 4-oxo-β-ionol, 3-oxo-7,8-dihydro-α-ionol, 4-oxo-7,8-dihydro-β-ionol, grasshopper ketone, and 7,8-dihydrovomifoliol in Tannat, a typical red wine from Uruguay.

There have been only a few studies investigated on the aroma of Pinot noir

wines. Ethyl and methyl vanillate, acetovanillone, and 3-methylthio-1-propanol, along with 3-methylbutanoic, hexanoic, octanoic, and decanoic acids, 2-pheylethanol and benzyl alcohol, have been identified to be important in Pinot noir [2, 165-167]. Additionally, ethyl anthranilate, ethyl cinnamate, ethyl 2,3-dihydrocinnamate, and methyl anthranilate have also been suspected to contribute the typical aroma of Pinot noir wine [78]. Despite of those studies, Pinot noir wine aroma is still not well understood due to its complexity. As Oregon State becomes one of the major producers of Pinot noir wine, it is important to characterize Pinot noir wine aroma from this region.

2.4 Materials and Methods:

2.4.1 Wines:

Vintage 2000 Pinot noir wines were produced from Oregon State University viticulture trials with grapes grown at Benton-Lane vineyard in the Oregon Southern Willamette Valley appellation. Pinot noir clone FPMS 2A vines were grafted onto 7-year-old Teleki 5C rootstocks. These vines were treated with different nitrogen fertilization and with/without irrigation to simulate various vineyard practices in Oregon. After harvest, grapes from each treatment were collected, crushed, stemmed and fermented separately (1 g/L Lavin RC 212 Bourgorouge yeast). The wines were settled and racked off the primary yeast, followed by malo-lactic fermentation with Lalvin malo-lactic bacteria. The wines were cold stabilized, bottled at nine months of age, and stored in the pilot winery at 18°C. Based on preliminary sensory evaluation, two wines with distinct variation of "typical Oregon Pinot noir aroma" were selected for AEDA analysis and labeled as Wine A and Wine B [168].

2.4.2 Wine aroma extraction and distillation:

One liter of each Pinot noir wine was extracted with freshly distilled diethyl ether: pentane (1:1 v/v) three times in a separatory funnel (extracts totaled 750 ml). Distillation of these extracts was performed with solvent assisted flavor evaporation (SAFE) (Glasbläserei Bahr, Manching, Germany) to remove the nonvolatile constituents at 50°C under 29 in Hg vacuum[5]. After distillation, the receiving part of SAFE in the system was carefully rinsed with 10ml of distilled diethyl ether, and combined with the distillates in the volatile-receiving flask. Finally, the distillates were dried over anhydrous sodium sulfate and concentrated to 10 ml under nitrogen.

2.4.3 Wine aroma fractionation:

To facilitate the GC analysis, aroma extracts were separated into acidic/water-soluble and neutral fractions [169]. Distilled water (10 ml) was added to the concentrated extract. The aqueous phase was adjusted to pH 11 with 1 N sodium carbonate solution, then separated in a separatory funnel and retained. The organic phase was further washed with 10 ml of diluted sodium hydroxide solution (pH=11) three times, and the washings were combined with the aqueous phase. The organic phase was dried over sodium sulfate, filtered, concentrated to 200 µl, and labeled as "neutral fraction" for GC/O analysis. The aqueous solution was adjusted to pH 1.7 with 1 N H_2SO_4, then 10 g NaCl was added, and the solution was extracted three times with 50 ml of diethyl ether: pentane (1:1 v/v). These extracts were combined, dried with anhydrous sodium sulfate, filtered, concentrated to 500 µl, and labeled as "acidic/water-soluble fraction" for further GC/O analysis.

2.4.4 Gas Chromatography-Olfactometry Analysis (GC-O):

The analysis was performed using a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and an olfactometer. Samples were analyzed on a Stabilwax column (30 m length, 0.32 mm ID, 1 μm film thickness, Restek Corp., Bellefonte, PA) and on a DB-5 column (30 m length, 0.32 mm ID, 1 μm film thickness, J&W Scientific, Folsom, CA). Two micro-liters of samples were injected into the GC in split-less mode. The column carrier gas was nitrogen at constant pressure (15 psi, 2 ml/min flow-rate measured at 25°C). The column effluent was split 1:1 (by volume) into the FID and a heated sniffing port with a fused silica outlet splitter (Alltech Associates, Inc., Deerfield, IL). The oven temperature was programmed for a 2 min hold at 40°C, and then increased at a rate of 4°C/min, to 230°C, finishing with a 10 min hold. Injector and detector temperatures were 250°C. Retention indices (RI) were estimated in accordance with a modified Kovats method [170].

2.4.5 AEDA:

The fractionated extracts were stepwise diluted with diethyl ether: pentane (1:1 v/v) and analyzed by AEDA. The acidic/water-soluble fractions were performed on a Stabilwax column (same as above), and the neutral/basic fractions were performed on both Stabilwax and DB-5 columns (same as above). Two panelists, one male and one female, who had been trained more than six months for GC-O analysis, performed GC-O on original and diluted extracts. Flavor dilution (FD) factors for the odor active compounds in each fraction were determined.

2.4.6 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis:

Capillary GC-MS identification was carried out using an Agilent GC 5973 N GC-MSD system. Both a DB-Wax fused silica column (30 m ×0.25 mm i.d., 0.5 μm film thickness, J&W Scientific) and a DB-5 column (30 m × 0.32 mm i.d., 1 μm film thickness, J&W Scientific). The oven temperature and injector were identical to that use d in GC-O analysis as described previously. Helium was used as the column carrier gas at a constant flow rate of 2 ml/min. The electron impact (EI) energy was 70 eV, and the ion source temperature was set at 230°C. System software control and data management/analysis were performed through Enhanced ChemStation Software, GCA v. C.00.01.08 (Agilent Technologies Inc.). Mass spectra of unknown compounds were compared with those in the Wiley 275.L (G1035) Database (Agilent Technologies Inc.), and confirmed by their retention indices.

2.5 Results and Discussion

Table 1 lists the aroma compounds and their FD values in acidic/water-soluble fraction. Thirty-seven odorants were identified by MS and retention indices from standards (RI) / literatures (RIL) in this fraction, while five were tentatively identified by aroma descriptor and RI/RIL. Among them, twelve were acids, twenty-five were alcohols, four were ketones, and one was sulfide.

Based on AEDA values, the potential important acids were 2-methylpropanoic, butanoic, 2-methylbutanoic and 3-methylbutanoic acids (FD≥ 64). These acids impart strong sweaty odors in wines. Moreover, propanoic, hexanoic, and octanoic acid, were also found at high FD factors in this fraction (FD≥ 16), which was consistent with the previous report [2].

3-Methylbutanol and 2-methylpropanol, which give nail polish-like odors, had extremely high FD (FD≥ 4096) values in both samples. These fusel alcohols normally arise from sugar catabolism, as well as from decarboxylation and deamination of amino acids [83]. They have already been reported in Pinot noir wines [43, 171].

trans-3-Hexenol and *cis*-3-hexenol, which give green odors, showed potential importance in Pinot noir aroma (FD≥ 16), while 1-hexanol had low FD values (FD≤ 16) in both samples. A number of investigators mentioned these C_6 green odorants are present in grapes and wines [172, 173]. The biosynthesis and composition of C_6 compounds are dependent on several enzyme activity in grapes during their biosynthesis [174]. In this study, *trans*-3-hexenol has higher FD values than *cis*-3-hexenol in both analyzed samples. Previous quantitative results in Pinot noir wines by Girard et al also showed that the amount of *trans* form is higher than *cis* form in Pinot noir wines [171].

2-Phenylethanol (rosy) was found as a key characteristic aroma compound in Pinot noir wine based on its high AEDA value (FD= 8192). It is suggested to be important, previously based on GC-Osme study [43]. Benzyl alcohol, linalool, and geraniol, which contribute floral, dried fruity aroma, were also significant odorants in this fraction (FD≥ 64). Guaiacol, α-terpineol, 4-ethylguaiacol, and eugenol also showed to be important (FD≥16). In addition, *m*-cresol, isoeugenol and 4-vinylphenol were detected by AEDA with low FD factors (FD≤ 16). Most of these compounds belong to the secondary plant constituents and are synthesized from acetyl-coenzyme A (CoA) [175]. They are not formed by yeast metabolism during fermentation, but rather from either the degradation of free odorless poly-ols or hydrolysis of glycosidically conjugated forms during wine aging [82]. Although present in only a small amount, many of them may play significant roles in

contributing to floral and cherry flavors to wine due to their high AEDA values.

Two sulfur containing compounds, 3-methylthio-1-propanol and 3-ethylthio-1-propanol, were found to have very high AEDA values in the acidic/water-soluble fraction (FD≥ 64). Both compounds gave cooked potato odors, which negatively correlated to pleasant descriptors [4]. 3-Methylthio-1-propanol can be formed from photo-degradation of methionine precursor, a common amino acid in wine [176]. Several researchers have demonstrated that 3-methylthio-1-propanol is a potent aroma compound in wine [43, 46], and its concentration in Pinot noir wines from British Columbia varied from 400 to 2070 μg/L. However, to our knowledge, this is the first time that 3-ethylthio-1-propanol has been detected in Pinot noir wines, though it has already been found in Port wine [113] and Muscat wine [177]. In addition, 3-mercaptohexanol, which is one of the most powerful sulfurous odorants of Grenache Rose wines [178], was also found to be important in this fraction (FD≥ 16).

The AEDA results of the neutral fraction on both polar (Stabilwax) and nonpolar (DB-5) GC columns were summarized in Table 2 and Table 3. On the Stabilwax column, forty-one compounds were identified on the stabilwas column. The majority consisted of esters, ketones and aldehydes.

Esters, being the most common odorants in the neutral fraction, mostly result from reaction of acids with alcohols during wine aging. Many ethyl esters, including ethyl methylpropanoate, ethyl butanoate, ethyl 3-methylbutanoate, and ethyl hexanoate, showed high importance in both wine samples (FD≥ 64). High levels of these esters explained the strong perception of tropical fruit aroma. Ethyl acetate is the most abundant ester in wine and results from the process of acidification. It has an ether-like odor reminiscent of pineapple and is responsible for a tart-like odor in wine [179]. However, because its threshold is quite high

(~20 ppm), its contribution to wine aroma may be limited (FD≤ 16).

3-Methylbutyl acetate, 3-methylbutyl 2-methylpropanoate, ethyl decanoate, benzyl acetate, phenylethyl formate, and phenylethyl acetate were also important esters in Pinot noir wine (FD≥ 16). These compounds are often associated with fruity, banana and blackcurrant aromas. In addition, one sulfur containing ester, ethyl 3-(methylthio)propanoate, was found in both wines with AEDA values greater than 16, and it was described as a cooked rice odor by panelists. This compounds has been reported in Port wine [113] and some white wines [180]. Moreover, ethyl anthranilate and ethyl 2,3-dihydrocinnamate were identified by GC-O and confirmed by GC-MS in this fraction, and showed potential importance, based on AEDA values (FD≥ 16). Ethyl cinnamate and methyl anthranilate was tentatively identified by aroma descriptor and RI, and had low importance (FD< 16) in this fraction. These esters were reported as minor constituents in Burgundy Pinot noir wines, contributing fruity, cherry, and cinnamon-like odors [78].

Several ketones were identified in the neutral/basic fraction on the polar column to have high AEDA values. Whisky lactone, a woody odorant, was found to have high AEDA values in the wines (FD> 16). It has been reported that this compound constitutes a key difference between young and aged Rioja wine [47]. γ-Nonalactone, associated with coconut odor, could also be important in this fraction (FD≥16). Additionally, 4-sulfunyl-4-methylpentane-2-one, which contributes a typical black currant odor to Scheurebe wines [83], was detected by panelists (FD<16). This odorant is thought to be released from the bound precursor in grapes by cysteine β-lyase [83].

With regard to the C13-norisoprenoids, found in both wines, β-damascenone was identified. This compound mainly comes from degradation of carotenoids in grapes [181]. Additionally, other precursors to β-damascenone

present in wine have been reported, including 3-hydro-7,8-didehydro-β-ionol and 3,5-hydroxy-6,7-megastigmadien-9-ol [182]. Many researchers have reported that β-damascenone is important in wines due to its low threshold level (~0.009ppb in water). However, only moderate AEDA values (8 and 16) were obtained in this fraction.

Several aldehydes were identified in the neutral/basic fractions. Among them, benzaldehyde could be very important to Pinot noir aroma (FD≥ 64). Benzaldehyde could be generated from benzyl alcohol [183] and phenylalanine [184]. In addition, 3-methylthio-1-propanal was found to be important (FD≥ 16).

Most of these aroma compounds were also detected on the non-polar column (DB-5). The potentially important aroma compounds identified on the DB-5 column included 3-methylbutyl acetate, ethyl hexanoate, ethyl 3-(methylthio)propanoate, ethyl octanoate, whisky lactone, ethyl dihydrocinnamate, methyl and ethyl vanillate, and ethyl cinnamate (FD> 16).

Preliminary sensory comparison of these two wines showed that wine A was more fruity, cherry, and earthy/musty, while wine B was more spicy, vegetative and floral [168]. The AEDA analysis showed that there were not significant differences in the FD factors for most of the fatty acids. However, Wine B had much higher AEDA values for propanoic and octanoic acids (goaty, earthy odors). Wine B also had much higher AEDA values for C_6 compounds than wine A, most likely a direct correlation to the stronger vegetable and green odor perception in wine B [168]. In the neutral/basic fractions, wine A had more esters than wine B. Since esters are associated with fruity notes, the AEDA results were consistent with descriptive analysis that Wine A was fruitier. Overall, it was determined that both wines contained very similar compounds, and their aroma difference probably came from the different proportions of those odorants.

2.6 Conclusions

The present work has characterized the aromatic profile of two typical Pinot noir wines from Oregon. Based on the FD factors, 2-phenylethanol and 3-methylbutanol showed most significance in contributing to overall aroma in both wines. In addition, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, 2-methylpropanol, hexanol, *trans*-3-hexenol, *cis*-3-hexenol, benzyl alcohol, 3-methylthio-1-propanol, 3-ethylthio-1-propanol, ethyl 2-methylpropanoate, ethyl butanoate, 3-methylbutyl acetate, ethyl hexanoate, whisky lactone, benzaldehyde, 3-methylthio-1-propanal and γ-nonalactone demonstrated their contribution toward aroma of Pinot noir wine.

The results indicated that characteristic Pinot noir aroma is a complex formulation of aroma compounds, and different proportions of these compounds give rise to different perceived odors. Because the flavor dilution (FD) factor is the ratio of an odorant's concentration in an initial GC/O extract to its concentration in the most dilute extract that still allows detection, it is simply a relative measure [185]. Even though AEDA is an effective screening method for potent odor-active compounds in wine, the data alone does not allow making precise conclusions about the specific role of each constituent in the overall wine aroma. The next step of our research will be quantitative analysis and calculation of odor activity values (OAVs), followed by reconstitution and omission sensory tests, a strategy that has been successfully applied to wine by Guth et al [41, 46, 186] and Ferreira et al [178].

2.7 Acknowledgement

We would like to thank the Oregon Wine Advisory Board for funding this project. Thanks also to Barney Watson for wine making in this study.

Table 2.1. Potent odorants in acidic/water-soluble fraction detected by AEDA in stabilwax column

Compounds	RI	RI (Reference)	Basis of identification	Descriptor	FD factors	
					Wine A [a]	Wine B [a]
Unknown	1039			Cabbage	8	4
1-Propanol	1060	1037[187]	MS[a], A[b], RI[c]	Fruity	8	8
2-Methylpropanol	1097	1108[69]	MS, A, RI	Nail polish	256	256
1-Butanol	1161	1143[188]	MS, A, RI	Fruity	4	4
1-Penten-3-ol	1177	1158[188]	MS, A, RI	Fruity	2	4
3-Methyl-1-butanol	1214	1209[43]	MS, A, RI	Nail polish	8192	8192
1-Octen-3-one	1275	1298[189]	MS, A, RI	Mushroom	16	64
3-Hydroxy-2-pentanone	1338	1338[179]	MS, A, RIL[d]	Sweet bread, toasty	8	8
Unknown	1354			Earthy	64	16
1-Hexanol	1367	1355[188]	MS, A, RI	Grape juice	4	16
trans-3-Hexenol	1381	1365[188]	MS, A, RIL	Green	32	256
cis-3-Hexenol	1390	1386[188]	MS, A, RI	Fruity green	32	128
Dimethyl trisulfide*	1404	1367[189]	A, RI	Cabbage	16	32
Acetic acid	1467	1450[187]	MS, A, RI	Vinegar	4	8
1-Octen-3-ol	1467	1453[188]	MS, A, RI	Mushroom	8	64
Propanoic acid	1548	1510[190]	MS, A, RI	Pungent	16	128
Linalool	1569	1557[43]	MS, A, RI	Caramel, apple-sweet	64	64
2-Methylpropanoic acid	1588	1584[47]	MS, A, RIL	Rancid	128	256
2(3H)-Dihydro-furanone**	1628		MS, A	Caramel	8	4
Butanoic acid	1652	1644[47]	MS, A, RI	Sweaty	64	128
2-Methylbutanoic acid	1682	1693[191]	MS, A, RIL	Rancid, sweaty	64	256
3-Methylbutanoic acid	1691	1686[47]	MS, A, RI	Rancid, sweaty	512	256
α-Terpineol	1720	1708[43]	MS, A, RI	Floral	16	32
3-Methylthio-1-propanol	1746	1738[47]	MS, A, RI	Cooked vegetable	128	128
Citronellol*	1760	1786[69]	A, RI	Fruity rosy	4	1
3-Ethylthio-1-propanol**	1802		MS, A	Cooked potato	64	32
β-Damascenone	1828	1832[47]	MS, A, RIL	Sweet, tea, floral	8	32
Hexanoic acid	1857	1850[43]	MS, A, RI	Sweaty	32	32
Geraniol	1863	1875[47]	MS, A, RI	Floral	256	128
3-Sulfanylhexan-1-ol*	1869	1863[47]	A, RIL	Sulfur, unpleasant	16	32
Guaiacol	1872	1875[47]	MS, A, RI	Sweet pungent, chemical, phenolic	32	8
Benzyl alcohol	1898	1884[43]	MS, A, RI	Floral, dried fruit	128	256
2-Phenylethanol	1946	1933[43]	MS, A, RI	Rose	8192	8192
trans-2-Hexenoic acid	1983	1969[192]	MS, A, RI	Leaf	4	8
Whisky lactone	1993	1977[47]	MS, A, RIL	Floral sweet	32	16
4-Ethylguaiacol*	2055	2048[47]	A, RIL	Phenolic, spicy	4	4
Octanoic acid	2083	2083[47]	MS, A, RI	Goaty rancid cheese	8	32
m-Cresol	2129	2114[47]	MS, A, RIL	Animal	4	1
Eugenol	2215	2186[47]	MS, A, RI	Smoky	16	64
p-Cresol	2231	2195[47]	MS, A, RIL	Phenolic, smoky	32	32
Isoeugenol	2319	2309[193]	MS, A, RIL	Woody, toasted	8	16
9-Decenoic acid	2369		MS, A, RI	Oily, fatty, cooked meaty	4	2
4-Vinylphenol*	2411	2427[194]	A, RIL	Caramel	2	1
Benzoic acid**	2446		MS, A	Floral	16	4
Benzeneacetic acid	2556	2571[47]	MS, A, RIL	Musty, fruity, sour	2	4

*: tentatively identified by aroma and retention index

**: tentatively identified by mass spectra and aroma

a. MS: compounds were identified by the MS spectra; b. A: compounds were identified by the aroma descriptors; c, RI: compounds were identified by compared with pure compound standard, d. RIL: compounds were identified by compared with retention index from literatures.

Table 2.2 Potent odorants in neutral fraction detected by AEDA in Stabilwax column

Compounds	RI	RI (reference)	Basis of identification	Descriptor	FD factors	
					Wine A	Wine B
Ethyl acetate	914	904[191]	MS[a], A[b], RI[c]	Sweet fruity	16	8
Ethyl propanoate	955	925[195]	MS, A, RI	Fruity	8	4
Ethyl 2-methylpropanoate	971	955[196]	MS, A, RIL[d]	Sweet, apple fruity	128	64
2-Methylpropyl acetate	1029	989[197]	MS, A, RI	Floral	16	8
Ethyl butanoate	1048	1028[187]	MS, A, RI	Fruity peach	512	128
Ethyl 2-methylbutanoate	1062	1053[191]	MS, A, RI	Honey sweet	8	1
Ethyl 3-methylbutanoate	1076	1070[47]	MS, A, RI	Ester fruity	64	32
3-Methylbutyl acetate	1114	1096[171]	MS, A, RI	Fruity banana	256	128
Ethyl pentanoate	1146	1158[47]	MS, A, RI	Mint, fruity	4	2
3-Methylbutyl 2-methylpropanoate	1214		MS, A, RI	Ester fruity floral	32	16
Ethyl hexanoate	1242	1244[47]	MS, A, RI	Fruity, wine	256	128
Hexyl acetate	1254	1251[171]	MS, A, RI	Sweet floral	16	4
Unknown	1270			Smoky	4	8
4-Sulfanyl-4-methylpentan-2-one*	1373	1380[198]	A, RIL	Wet woody, box tree	4	8
Ethyl octanoate	1438	1436[198]	MS, A, RI	Cooked fruity, pleasant	64	16
3-Methylthio-1-propanal	1462	1469[47]	MS, A, RI	Cooked vegetable	16	64
Furfural	1474	1474[47]	MS, A, RI	Toasty	8	4
Citronellal	1508	1485[199]	MS, A, RI	Green lemon fruity	16	32
Benzaldehyde	1557	1523[171]	MS, A, RI	Nutty cherry	64	128
Ethyl 3-(methylthio) propanoate	1580		MS, A	Cooked rice, green	32	64
Ethyl decanoate	1644	1641[171]	MS, A, RI	Fruity	32	64
Ethyl 9-decanoate	1701	1694[171]	MS, A, RIL	Grape, leaf	16	2
Benzyl acetate	1739	1738[200]	MS, A, RI	Floral herbal	32	64
Phenylethyl formate**	1750		MS, A	Fresh grassy rose	32	16
Phenylethyl acetate	1804	1831[171]	MS, A, RIL	Floral, honey	64	16
β-Damascenone	1827	1832[47]	MS, A, RIL	Fruity, green apple	8	16
Ethyl dihydrocinnamate	1909	1885[78]	MS, A, RIL	Floral, sweet juice	64	32
Whisky lactone	1993	1977[47]	MS, A, RIL	Floral, wine-like	32	16
γ-Nonalactone	2064	2063[194]	MS, A, RI	Sweet, coconut, cream	16	64
Ethyl cinnamate*	2129	2133[78]	A, RIL	Fruity floral cherry	8	4
Methyl anthranilate*	2254	2245[78]	A, RIL	Tea, fruity	4	8
Ethyl anthranilate	2291	2280[78]	MS, A, RIL	Sweet fruity	16	16
Unknown	2445			Black pepper	2	16
Vanillin	2567	2581[47]	MS, A, RIL	Vanilla	2	1
Methyl vanillate	2585	2598[43]	MS, A, RIL	Tea, green	8	2

*: tentatively identified by aroma and retention index

**: tentatively identified by mass spectra and aroma

a. MS: compounds were identified by the MS spectra; b. A: compounds were identified by the aroma descriptors; c, RI: compounds were identified by compared with pure compound standard, d. RIL: compounds were identified by compared with retention index from literatures

Table 2.3. Potent odorants in neutral fraction detected by AEDA in DB-5 column

Compounds	RI	RI (reference)	Basis of identification	Descriptor	FD factors	
					Wine A	Wine B
Ethyl acetate	612	610[191]	MS[a], A[b], RI[c]	Sweet, tart	16	8
Ethyl propanoate	704	704[195]	MS, A, RI	Fruity	8	4
Ethyl 2-methylpropanoate	756	756[196]	MS, A, RIL[d]	Fruity sweet	64	16
2-Methylpropyl acetate	788	768[201]	MS, A, RI	Sweet, floral	16	4
Ethyl butanoate	804	804[196]	MS, A, RI	Sweet, fruity	256	64
Furfural	840	837[202]	MS, A, RI	Toasty	8	2
Ethyl 2-methylbutanoate	849	853[47]	MS, A, RI	Sweet honey	8	1
Ethyl 3-methylbutanoate	858	856[47]	MS, A, RI	Fruity, sweet apple	64	32
3-Methylbutyl acetate	869	876[201]	MS, A, RI	Banana	256	128
Ethyl pentanoate	904	900[201]	MS, A, RI	Mint, green fruity	4	2
3-Methylthio-1-propanal	910	897[191]	MS, A, RI	Cooked vegetable	16	64
4-sulfanyl-4-methylpentan-2-one*	941	944[198]	A, RIL	Moldy, woody	4	8
Benzaldehyde	968	962[201]	MS, A, RI	Cherry	32	64
Ethyl hexanoate	1000	999[201]	MS, A, RI	Sweet fruity	128	128
Unknown	1080			Smoky	4	8
Ethyl 3-(methylthio)propanoate	1098	1098[201]	MS, A, RIL	Vegetable, cooked rice	32	32
Unknown	1105			Barbecue sauce	8	2
Citronellal	1160	1161[199]	MS, A, RI	Green lemon	8	8
Benzyl acetate	1164	1164[201]	MS, A, RI	Floral	32	32
Ethyl octanoate	1196	1194[201]	MS, A, RI	Green fruity floral	64	16
Phenylethyl acetate	1260	1255[201]	MS, A, RIL	Floral, honey	32	16
Whisky lactone	1310	1289[47]	MS, A, RIL	Green floral	32	8
Methyl anthranilate	1354	1354[78]	MS, A, RIL	Green peach	4	16
Ethyl 2,3-dihydrocinnamate	1360	1359[78]	MS, A, RIL	Fruity	32	32
γ-Nonalactone	1370	1372[161]	MS, A, RI	Coconut, cream	8	32
β-Damascenone	1378	1392[191]	MS, A, RIL	Green apple	8	8
Ethyl decanoate	1380	1392[201]	MS, A, RI	Fruity, sweet	64	64
Vanillin	1398	1406[203]	MS, A, RIL	Vanillin floral		
Ethyl anthranilate*	1419	1425[78]	A, RIL	Floral fruity	4	8
Ethyl cinnamate	1480	1475[78]	MS, A, RIL	Fruity cinnamon	16	4
Methyl vanillate**	1496		MS, A	Green tea	8	2
Ethyl vanillate	1574	1579[47]	MS, A, RIL	Floral tea	32	8
Unknown	1618			Vegetable	4	8
Unknown	1765			Vegetable grassy	4	2
Unknown	1985			Smoky floral rancid	4	32
Unknown	2151			Green rose floral sweet	16	8

*: tentatively identified by aroma and retention index

**: tentatively identified by mass spectra and aroma

a. MS: compounds were identified by the MS spectra; b. A: compounds were identified by the aroma descriptors; c, RI: compounds were identified by compared with pure compound standard, d. RIL: compounds were identified by compared with retention index from literatures

Chapter 3. Effect of Grape Maturity on Aroma Compounds in Pinot Noir Wines Determined by Stir Bar Sorptive Extraction – Gas Chromatography-Mass Spectrometry

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3.1 Abstract:

Effect of grape maturity on aroma compounds in Pinot noir wine was investigated using stir bar sorptive extraction-gas chromatograph-mass spectrometry (SBSE-GC-MS). Calibration curves of aroma compounds were built using five internal standards in a synthetic wine matrix. High correlation coefficients (>0.95) and standard deviations (<10%) were obtained for all aroma compounds of interest. Two vintages of Pinot noir wines, with three different grape maturities each, were analyzed by this method. Statistical analysis showed that both grape maturity and growing year significantly affected the aroma composition of the final wine. Analysis of wine samples from the same vintage indicated that grape maturity could affect aroma compounds in different ways, based on their biochemical formation in the wines. For most fermentation related short-chain fatty acid esters, there were no obvious trends for their concentrations with grape maturity, however, it was observed that the concentrations of ethyl 2-methylpropanoate, ethyl 3-methylbutanoate consistently decreased with grape maturity. The decreasing trend also observed for other important characteristic esters for Pinot noir, including ethyl cinnamate, ethyl dihydroxycinnamate, and ethyl anthranilate, with the exception of ethyl vanillate, which increased with grape maturity. Most of the grape-derived aroma compounds including C_{13} norisoprenoids, monoterpenes, guaiacol and 4-ethylguaiacol had increasing trends in wine with grape maturation. However, linalool showed a decreasing trend with grape maturation.

3.2 Keywords:

Stir-bar sorption extraction (SBSE), aroma, Pinot noir wine, grape maturity

3.3 Introduction

Aroma composition is one of the most important attributes of wine quality. By nature, aroma compounds are volatile. However, most of the volatile compounds in wine may not contribute to the wine aroma because they have too high sensory thresholds to be odor-active. On the other hand, odor-active compounds, which may be present at very low concentrations (sometimes lower than 1 μg/L) but have low sensory thresholds, determine the aroma character [204]. Due to the extremely low concentrations of most aroma compounds, a pre-concentration step is usually carried out prior to their analysis by GC-MS.

Traditionally, liquid-liquid extraction followed by concentration has been the most widely used technique for aroma isolation. Solid-liquid extraction has gained popularity for aroma extraction [83] due to its simplicity and sensitivity. The most widely used technique is headspace solid-phase microextraction (HS-SPME) or in-sample immersion solid-phase microextraction (IS-SPME). More recently, a stir bar-sorptive extraction (SBSE) technique has been developed [24], and the commercial instrument has become available. The fundamental aspects of SBSE for liquid phase sampling are similar to the principles of IS-SPME, which is based on the partition coefficient between the solid phase and the liquid. However, it has been found that SBSE has much higher recoveries than IS-SPME due to its much larger volume of polymeric coating [25, 26]. This SBSE has been applied to analyze aroma and volatile phenolic compounds in wines recently [33, 45], and the technique was found to be very sensitive and reproducible, allowing for lower detection and quantification.

Pinot noir is one of the oldest wine cultivars. It originated in the Burgundy region of France, and has become popular in the United States, especially in

Oregon. It exhibits distinct red fruity aromas evoking particularly the odors of small-stone fruits (plum and cherry). However, the grape requires a long, cool growing season to develop the right flavor attributes, and it is difficult to turn the grapes into good wine [205]. It has been noticed that the wine from late harvested grapes has a different flavor profile from the early harvested grapes. A preliminary sensory evaluation with 7 panelists showed that the late stage wines had more complex aroma with more floral, more dried fruity and more oak-like aroma, while the early stage wines showed the highest fresh fruity aroma. However, it is not clear the chemical basis for this difference.

Both the volatile and aroma compounds in Pinot noir wine have been studied [165, 167]. Using the gas chromatography-olfactometry technique, it was found that the most important odor-active compounds in Oregon Pinot noir include 2-methylpropanol, 3-methylbutanol, hexanol, *trans*-3-hexenol, *cis*-3-hexenol, 3-ethylthio-1-propanol, ethyl 2-methylpropanoate, ethyl butanoate, 3-methylbutyl acetate, ethyl hexanoate, ethyl lactate, ethyl octanoate, ethyl dodecanoate, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, hexanoic acid, octanoic acid, decanoic acid, tridecanoic acids, benzaldehyde, linalool, methionol, 2-phenylethyl acetate, benzyl alcohol, 2-phenyl ethanol, γ-octalactone, γ-nonalactone, ethyl and methyl vanillate, acetovanillone, and whisky lactone [2, 43, 50]. In addition, ethyl and methyl anthranilate, ethyl cinnamate, and ethyl dihydroxycinnamate were identified in Burgundy Pinot noir, and they were suspected to influence the characteristic flavor quality of this wine [78]. However, later quantification of these 4 compounds showed that the concentrations were below the sensory thresholds [79], so their contributions to Pinot noir aroma are still unclear.

Quantification of important aroma compounds in Pinot noir wines had been

attempted [79, 171, 206]. However, the limited studies only reported the relative concentration by semi-quantification method or the concentrations of a few aroma compounds due to a lack of a suitable method for quantification. The objective of this study is to develop a sensitive SBSE-GC-MS technique to quantify the important aroma compounds in Pinot noir wine, and employ this technique to study the impact of grape maturity on the aroma composition of Pinot Noir wines.

3.4 Material and Methods

3.4.1 Chemicals.

All aroma standards listed in Table 1 were purchased from Sigma-Aldrich (St. Louis, MO). The ethanol was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY), and tartaric acid was from Mallinckrodt Inc. (Paris, KY).

A synthetic wine solution was made by dissolving 3.5 g L-tartaric acid in 1 L of 12% ethanol solution, and adjusting the pH to 3.5 with 1 M NaOH [207]. Standard stock solutions (1000 ppm) were prepared in ethanol first and then diluted to the proper concentrations of working standards in synthetic wine. An internal standard solution was made by dissolving 46 ppm of hexyl formate, 48 ppm of octyl propanoate, 7 ppm of trans-carveol, 9 ppm tran-2-nonenal, and 9 ppm of linanyl 3-methylbutanoate in ethanol, and stored at -15°C.

3.4.2 Wine samples.

Vintage 2003 and 2004 Pinot noir wines were produced from grapes grown at the Oregon State University experimental vineyard planted in 1984 as described previously [208]. During each growing seasons, fruits were harvested when the

grape sugar reached around 21 °Brix, and were labeled as "early stage of maturity". On the following two weeks, fruits were collected in each week, and were labeled as "middle stage of maturity" and "late stage of maturity". Harvested grapes were crushed, destemmed and fermented separately (1g/L Lavin RC 212 Bourgorouge yeast). New wines were settled and racked off the primary yeast, followed by malo-lactic fermentation. The wines were cold stabilized, bottled at nine months of age, and stored in the pilot winery at 15~20°C. Each wine was manufactured in triplicate in different fermentors, and 3 bottles of each wine from different fermentors were combined for analysis.

3.4.3 Aroma extraction and analysis.

Wine sample (10 mL) was diluted with 10 mL of water into a 40 mL vial, in which 6 g of sodium chloride had been added, and 20 μL of internal standard solution was added into the vial. A stir bar coated with Poly(dimethylsiloxane) (PDMS) phase (2 cm length, 100 mm thickness, Gerstel Inc., Baltimore, MD) was used to extract the aroma compounds from the wine sample. The Twister bar was constantly stirred for 12 hours at a speed of 1000 rpm. After sampling, the twister bar was rinsed with distilled water, dried with a Kimwipe (Kimberly-Clark Professional Inc, Roswell, GA) tissue paper, and placed into the glass sample holder of the TDS tray (Gerstel, Inc.).

The analyses were performed using a TDU autosampler (Gerstel, Inc., Baltimore, MD) mounted on an Agilent GC-MS system (Agilent 5973 GC-MS, Agilent Technologies, Little Falls, DE). The analytes were thermally desorbed at the TDU-2 in splitless mode, ramping from 35°C to 300°C at a rate of 700°C/min, and held at the final temperature for 3 min. The desorbed analytes were cryofocused (-60°C) in a programmed temperature vaporizing (PTV) injector (CIS

4, Gerstel, Inc.) with liquid nitrogen. After desorption, the PTV was heated from -60°C to 250°C at a rate of 10°C/sec and held at 250°C for 3 min. The solvent vent injection mode was employed with a venting flow of 20 mL/min at 20 psi venting pressure for 0.01min. A RTX-1 capillary GC column (60m, 0.25mm I.D., 0.5μm film thickness, Resteck Inc., Bellefonte, PA) was employed to separate the analytes. Helium at a constant flow of 1.8 mL/min was used as the carrier gas. The oven temperature was initially set at 50°C for 2 min, raised to 210°C at a rate of 2°C/min, then to 250°C at a rate of 10°C/min, and held at 250°C for 15 min. An Agilent 5973 MSD was used for identification. The electron impact (EI) energy was 70eV, and the ion source temperature was set at 230°C. Enhanced ChemStation Software (GCA v. D.00.01.08, Agilent Technologies Inc.) was used for data acquisition and analysis.

3.4.5 Standard Calibration curve.

The stock solutions were prepared by dissolving around 10000 ppm each target compound individually into ethanol solution. Before analysis, a certain amount of stock solution was added to synthetic wine to make the first level mixed standard solution (**Table 1**) and diluted at 4:1(v/v), 3:2 (v/v), 2:3 (v/v) and 1:4 (v/v) ratio with synthetic wine to give a range of concentrations. The standard solutions were analyzed using the same procedure as described above. To avoid wine matrix interferences, selective ion-monitoring (SIM) mass spectrometry was used to analyze the aroma compounds. The selected target ions for qualification and quantification ions were listed in table 1. The calibration curve for individual target compounds was built up by plotting the selected ion abundance ratio of target compounds with their respective internal standard against the concentration

ratio. For each calibration curve, the regression coefficients were calculated using the Chemistation data analysis software, and RSDs were calculated based on triplicate analysis of the combined wine samples.

3.4.6 Quantification of aroma compounds in Pinot noir wine.

Aroma compounds in 6 wines from 3 different maturity grapes in 2 years were quantified. A 10 mL of wine sample along with 20 μL of internal standard solution were added into a 40 mL vial with 10 mL of water and 6 g of sodium chloride. The SBSE and GC-MS conditions were the same as described previously. The concentration of aroma compounds were calculated based on their calibration curves. Triplicate analysis was performed on all samples, and the average values are reported.

3.4.7 Statistic Analysis.

The effect of grape maturity on the aroma composition of Pinot noir wine was investigated using multivariate analysis of variance (MANOVA). In the MANOVA model, year, maturity, and the two-way interaction (year×maturity) were studied.

3.5 Results and discussion

3.5.1 Quantification of aroma compounds in wines.

Both AEDA and Osme techniques have been used to characterize the aroma profile of typical Oregon Pinot noir wines [43, 50]. The results indicate that Pinot noir aroma is a complex formulation of many aroma compounds, and there is

no single compound responsible for the characteristic aroma of Pinot noir wine. Different proportions of these compounds give rise to different perceived odors. Ultimately, concentration of these aroma compounds and their balance in the wine matrix will affect the quality of Pinot noir wines. Based on the results of previous GC-olfactometry identification of aroma compounds in Pinot noir [42, 78, 79, 165], 29 key aroma compounds were selected for quantification, which included 9 alcohols, 17 esters, 2 ketones, and 1 lactone. Acids and higher alcohols are formed primarily during fermentation, so they were not quantified although they are important to wine aroma [50].

Calibration curves of selected aroma compounds were constructed individually using pure aroma compounds and internal standards in synthetic wine. Due to the wide range of concentration and different chemical and physical properties of the aroma compounds, five internal standards, including one alcohol, one aldehyde, and three esters, were used to quantify all aroma compounds. The chromatographic conditions were selected to give good resolution for the aroma compounds, and the quantifying ions were carefully selected to eliminate any interfering ions from coeluted compounds and give good sensitivity. The correlation coefficients for most of the aroma compounds were greater than 0.99 (**Table 1**). The method is reproducible with a relative standard error (RSD) less than 10% for most of the aroma compounds quantified (**Table 1**).

Ester was the major class of aroma compounds analyzed in this study. Ethyl esters of butanoate, hexanoate, octanoate, and decanoate were all quantified, and high concentrations were found for all these esters. Branch-chained esters such as ethyl 2-methylpropanoate, ethyl 3-methylbutanoate, 3-methylbutyl acetate, and 2-methylbutyl acetate also had high concentration (ranging from 0.1 to 1 ppm), which were consistent with the literature [103]. Because sensory thresholds of

these esters are at ppb levels, they should contribute to characteristic fruity aromas of the wines.

Several aromatic esters were also studied, which are typically described as floral, cherry, stone-fruit, and dry-plum. Ethyl phenylacetate, 2-phenylethyl acetate and ethyl 3-phenylpropanoate have been identified as important to wine aroma [33, 72]. Ethyl dihydoxycinnamate, ethyl cinnamte, and ethyl anthranilate were pointed out to be important in Pinot noir wines of Burgundy [78]. The threshold of ethyl cinnamate in water has been determined to be 16 ppb [79], and the concentration of this compound in the Pinot noir wine was lower than the sensory threshold. Therefore, its contribution to the wine aroma is probably limited, which is consistent with the quantification results by stable isotope dilution assay [79]. There is no sensory threshold data for ethyl dihydroxycinnamate and ethyl anthranilate, thus their aroma contributions are not clear.

Among the aroma compounds quantified, phenylethanol, which gives rosy and honey aromas, showed the highest concentration (24 to 38 ppm). This compound has been reported as a key characteristic aroma compound in Pinot noir wines [2, 50]. Benzene alcohol, which was described as floral, also was present at ppm level in the wine samples.

Guaiacol, typically described as smoky, spicy, and medicine-like, was found to be from 70 to 200 ppb in the wine samples. Compared to its sensory threshold (20 ppb in dry white wine) [209], this compounds may contribute to the wine aroma. Eugenol and 4-ethylguaiacol, which were also described as smoky and spicy, were detected at ppb level in the wines. Though generally considered to be faults at high concentration, these phenoic compounds can contribute attractive elements of aroma to a wine's bouquet, and this positive effect may vary based on

the grape variety [97].

It had been widely reported that monoterpenes are responsible for the characteristic floral aroma in grapes and wines. In this study, four monoterpenes, linalool, geraniol, nerol, and citronellol, were quantified. The results showed that all of them were present at ppb levels in the wines. Since the sensory thresholds of these compounds are generally very low, they may play significant roles in contributing floral and cherry flavors to Pinot noir wine.

β-Damascenone, which has a scent reminiscent of exotic flowers with a heavy fruity undertone, is variably described as apple, rose and honey. It had concentrations from 5 to 10 ppb in the wines. β-Ionone has a distinct berry and violet-like aroma, which had a high concentration from 300 ppb to 1 ppm. The high concentration and low sensory threshold of β-ionone make it a very important aroma compound for Pinot noir wine. γ-Nonalactone, which usually described as coconut and peach, was also detected at ppb levels in wine samples.

3.5.2 Effect of grape maturity on wine aroma composition.

MANOVA analysis was performed on all quantified aroma compounds. As shown in **Table 3**, both grape harvest maturity and producing year could affect the aroma composition of Pinot noir wine ($p<0.05$), and these effects were independent to each other ($p=0.16$). To further investigate the effects of grape maturity on wine aroma, the aroma composition of wine samples in the same year were compared.

Aroma compounds in wines could be divided into three groups based on their biological origins. Primary aromas are the compounds already present in the grapes and persisting through vinification. They were mainly the C13 norisoprenoids and terpenens. Secondary aromas are those compounds primarily

generated during fermentation, which are qualitatively and quantitatively the largest amount of volatile compounds present in the wine. This group of compounds includes alcohols formed from fermentation as well as esters from esterification. Tertiary aromas are the compounds generated during wine maturation or aging processes. Although many new compounds can be formed or generated during wine maturation, the most widely studied tertiary compounds include guaiacol and 4-ethylguaiacol that are primarily related to oak barrel aging. Generally, primary compounds characterize the aroma of the wine.

Among the alcohols studied, the concentration of geraniol, nerol and citronellol increased with the grape maturity (**Figure 1**), which partially explained why the late stage wine presents more floral-aroma than early one. However, the linalool concentration decreased slightly. Terpene compounds belong to the secondary plant constituents. Generally, 90% of the terpenes were present as nonvolatile glycosides that can be hydrolyzed (enzymatically or chemically) to the free form during fermentation and aging [83]. Except for hydrolysis, acid-catalyzed rearrangements during wine processing and aging also can result in changes in concentration and formation of new compounds that were not present in the original grapes and young wines [210, 211]. For this reason, the decrease of linalool may due to the transformation of linalool to geraniol and nerol during wine producing. Moreover, it had been reported that geraniol and nerol could be further changed to citronellol through enzymatic reactions [212, 213], and the latter one has a much lower sensory threshold than other two [73].

Low levels of guaiacol and 4-ethylguaiacol were also detected, and their concentrations dramatically increased along with grape maturation (**Figure 2**). Most of the guaiacol and 4-ethyl guaiacol in red wines are related to oak barrel aging. It has been reported that toasting of the oak barrels leads to thermal

degradation of lignin and produces the volatile phenols, which are extracted into the wine [101, 102]. In addition, they could be associated with spoilage by *Brettanomyces* [100] in red wine. Tannins in red wine can be degraded to 4-vinylphenol and 4-vinylguaiacol, and *Brettanomyces* can convert them to 4-ethylphenol and 4-ethylguaiacol respectively. However, phenolic compounds were also detected in non-oak aged alcoholic beverages [214, 215], which indicated another pathway. Since the experimental wines were not aged in oak barrels, guaiacol and 4-ethylguaiacol were probably formed from lignin degradation. As more tannins are formed during grape maturation, more guaiacol and 4-ethylguaiacol can be generated.

β-Damascenone and β-ionone, two C13 norisoprenoids, are thought to arise from carotenoid degradation during grape ripening [83]. Predominantly occurring in grapes as glycosidically bound precursors, those compounds could be released in wine by enzyme and acid hydrolysis. For both years, the late maturity wines had much higher concentrations than the early stages (**Figure 3**), which could contribute a more berry-like aroma in the late maturity wine sample.

The result also showed that the late stage wines had higher concentration of γ-nonalactone (**Figure 3**). Lactones are widely distributed in the fruit of plants, although some of them could originate from aging in oak barrels.

It is widely known that esters are especially important to wine flavor. Esters are usually considered secondary aromas, and they are formed from acyl-SCoA by yeast during fermentation. Ester formation can be affected by many factors such as yeast strain, fermentation temperature, and oxygen availability and nitrogen level during fermentation [75, 103].

Two acetate esters and six fatty acid esters, including 2-methylbutyl acetate, 3-methylbutyl acetate, ethyl 2-methylpropanoate, ethyl butanoate, ethyl 3-

methylbutanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate were analyzed in this study. Though those esters had high concentrations in wines, there was no obvious correlation with grape maturity for most of the esters. However, it was observed that the concentrations of ethyl 2-methylpropanoate, and ethyl 3-methylbutanoate consistently decreased with grape maturity.

The decreasing trend with grape maturity was also observed for other important characteristic esters for Pinot noir, including ethyl anthranilate, ethyl cinnamate, ethyl dihydroxycinnamate, ethyl phenylacetate, phenethyl acetate, ethyl 3-phenylpropinoate, and methyl vanillate (**Figure 4**). The opposite trend was observed for ethyl vanillate. The decreasing of total esters might partially explain why the late stage of wines showed less fruity aroma. Further research need to be done to understand the formation mechanism of those compounds during grape maturity and the wine making process.

In conclusion, a rapid method using SBSE-GC/MS was developed to quantify the aroma compounds in wine. The correlation coefficient and RSD of calibration curves showed that this method could be used to accurately analyze most key aroma compounds in Pinot noir wines. Moreover, this method was applied to investigate how grape maturity affects the aroma compounds in wine. The results demonstrated that grape maturity could significantly affect the aroma composition of the wine aroma. The concentration of most grape-derived aroma compounds increased along with grape maturity, except for linalool. Different trends were observed for other compounds.

Table 3.1. Standard curve and quantification of aroma compounds in wine (n=6)

Compounds	Quantity ion	Qualify ion	First Level Concentration (ppb)	Equation*: $C_{TC} = A \times \frac{C_{IS}}{R_{IS}} \times R_{TC}$	Coefficient	RSD (%)
trans-Carveol (IS)	109	84				
Guaiacol	109	81,124	45	A = 7.86	0.968	7.49
Linalool	71	93	54	A = 0.33	0.997	3.60
Nerol	69	41,93	43	A = 0.32	0.995	5.26
Geraniol	69	41	50	A = 0.33	0.998	2.87
Eugenol	164	149	52	A = 0.63	0.996	4.07
Benzyl alcohol	108	79	8950	A = 99.4	0.999	3.11
Phenylethanol	122	91	7510	A = 40.7	0.999	2.51
Citronellol	69	81	44	A = 0.36	0.968	9.70
4-Ethylguaiacol	152	137	45	A = 7.71	0.999	5.65
Hexyl formate (IS)	56	69				
Ethyl 2-methylpropanoate	71	116	184	A = 10.5	0.999	7.21
Ethyl butyrate	71	88	194	A = 11.6	0.999	5.42
3-Methylbutyl acetate	70	87	196	A = 1.66	0.999	3.95
2-Methylbutyl acetate	70	43,55	190	A = 1.65	0.999	3.22
Ethyl 3-methylbutanoate	88	57	264	A = 1.60	0.998	4.73
Octyl propinoate (IS)	112	75				
Ethyl hexanoate	88	99	202	A = 0.32	0.990	6.56
Ethyl octanoate	88	101,127	261	A = 0.16	0.983	4.68
Ethyl decanoate	88	101,155	246	A = 0.23	0.981	7.05
2-Nonenal (IS)	70	55,83				
β-Damascenone	121	69	53	A = 0.26	0.997	3.26
β-Ionone	177		503	A = 108	0.987	3.68
γ-Nonalactone	85		49	A = 1.13	0.997	3.98
Linalyl isobutyrate (IS)	93	121				
Ethyl phenylacetate	164	91	53	A = 1.55	0.998	7.99
Ethyl dihydroxycinnamate	178	104	45	A = 0.92	0.996	8.98
Ethyl anthranilate	165	119	61	A = 1.02	0.983	9.94
Ethyl cinnamate	131	103	71	A = 0.31	0.996	5.70
Methyl vanillate	151	182	52	A = 23.1	0.987	4.70
Ethyl vanillate	196	151	41	A = 1.00	0.983	5.88
Phenylethyl acetate	104	91	78	A = 0.51	0.999	5.57
Ethyl 3-phenylpropanoate	104	91	53	A = 0.42	0.997	8.46

* C_{TC}: Concentration of target compound; C_{IS}: Concentration of internal standard; R_{TC}: MS Response of target compound; R_{IS}: MS Response of internal standard

Table 3.2. The concentration (ppb) of potential aroma compounds in Pinot noir wine samples (n=3)

Compounds \ Wine Sample	2003			2004		
	early stage	middle stage	late stage	early stage	middle stage	late stage
Alcohols						
Guaiacol	84 ± 6	106 ± 8	182 ± 6	75 ± 7	118 ± 11	144 ± 13
Linalool	12.6 ± 0.3	11.7 ± 0.3	10.5 ± 0.3	14.8 ± 0.5	11.8 ± 1.1	8.9 ± 0.1
Nerol	2.45 ± 0.16	2.88 ± 0.34	4.55 ± 0.17	9.44 ± 0.15	9.69 ± 0.67	11.53 ± 0.09
Geraniol	5.8 ± 0.1	9.8 ± 0.6	13.2 ± 0.3	15.1 ± 0.4	20.2 ± 1.0	24.9 ± 0.1
Eugenol	4.24 ± 0.12	3.74 ± 0.20	3.37 ± 0.08	3.18 ± 0.13	3.19 ± 0.29	2.90 ± 0.03
Benzylalcohol (ppm)	1.14 ± 0.03	1.18 ± 0.06	1.61 ±0.05	1.84 ± 0.05	1.92 ± 0.04	2.06 ± 0.06
Phenylethanol (ppm)	38.3 ± 0.7	38.4 ± 1.2	38.0 ± 0.3	24.6 ± 1	24.6 ± 0.8	24.3 ± 0.5
Citronellol	6.42 ± 0.22	7.28 ± 0.34	9.66 ± 0.64	3.80 ± 0.27	7.10 ± 2.89	9.17 ± 0.51
4-Ethylguaiacol	ND	2.01 ± 0.16	3.59 ± 0.25	ND	2.13 ± 0.05	5.77 ± 0.32
Ketones						
β-Damascenone	6.39 ± 0.16	6.94 ± 0.37	9.68 ± 0.11	4.61 ± 0.16	5.28 ± 0.16	6.04 ± 0.24
β-Ionone	701 ± 24	757 ± 40	1,004 ± 29	367 ± 12	366 ± 12	475 ± 20
γ-Nonalactone	13.3 ± 0.1	14.1 ± 0.2	18.5 ± 1.3	10.6 ± 0.5	10.9 ± 0.3	15.5 ± 1.1
Esters						
Ethyl 2-methylpropanoate	789 ± 35	563 ± 48	442 ± 38	370 ± 24	215 ± 23	135 ± 6
Ethyl butyrate	218 ± 2	207 ± 12	221 ± 29	149 ± 3	152 ± 8	117 ± 6
3-Methylbutyl acetate	476 ± 23	562 ± 24	493 ± 41	274 ± 4	295 ± 11	246 ± 2
2-methylbutyl acetate	117 ± 5	133 ± 5	116 ± 6	72 ± 1	73 ± 3	55 ± 0
Ethyl 3-methylbutanoate	81.5 ± 4.6	57.6 ± 4.0	49.2 ± 2.9	24.6 ± 0.8	15.2 ± 0.5	9.2 ± 0.3
Ethyl hexanoate	234 ± 7	304 ± 20	296 ± 28	242 ± 11	184 ± 18	245 ± 14
Ethyl octanoate	291 ± 14	244 ± 10	253 ± 6	196 ± 11	187 ± 14	196 ± 7
Ethyl decanoate	103 ± 6	111 ± 6	146 ± 19	81 ± 6	81 ± 6	96 ± 3
Ethyl phenylacetate	6.27 ± 0.41	3.93 ± 0.26	3.92 ± 0.24	2.24 ± 0.14	1.66 ± 0.16	1.28 ± 0.16
Ethyl dihydroxycinnamate	1.24 ± 0.09	0.91 ± 0.09	0.54 ± 0.04	0.84 ± 0.05	0.49 ± 0.04	0.36 ± 0.04
Ethyl anthranilate	0.82 ± 0.07	0.62 ± 0.04	0.66 ± 0.06	0.34 ± 0.02	0.19 ± 0.02	0.16 ± 0.04
Ethyl cinnamate	1.03 ± 0.03	0.93 ± 0.02	0.67 ± 0.06	2.21 ± 0.03	1.49 ± 0.15	1.40 ± 0.12
Methyl vanillate	44.3 ± 1.3	33.7 ± 1.2	34.4 ± 0.7	39.9 ± 1.6	32.9 ± 2.3	27.6 ± 2.4
Ethyl vanillate	13.3 ± 0.5	13.4 ± 0.5	17.8 ± 0.9	10.7 ± 0.2	14.0 ± 1.6	17.8 ± 1.5
Phenylethyl acetate	25.7 ± 1.6	24.3 ± 0.7	19.7 ± 1.4	12.1 ± 0.1	11.1 ± 0.9	8.0 ± 0.7
Ethyl 3-phenylpropanoate	1.55 ± 0.09	1.14 ± 0.11	0.69 ± 0.05	0.96 ± 0.11	0.64 ± 0.05	0.39 ± 0.03

ND: not detected by this method

Table 3.3. The statistics results of Multivariate Tests [a] for effects of year and grape maturity on wine aroma

Effect	Wilks' Lambda	F value	Hypothesis df	Error df	Sig.
YEAR	.000	647.660	12	1	.031
MATURITY	.000	62.291	24	2	.016
YEAR * MATURITY	.000	5.710	24	2	.160

a Model: YEAR+MATURITY+YEAR * MATURITY

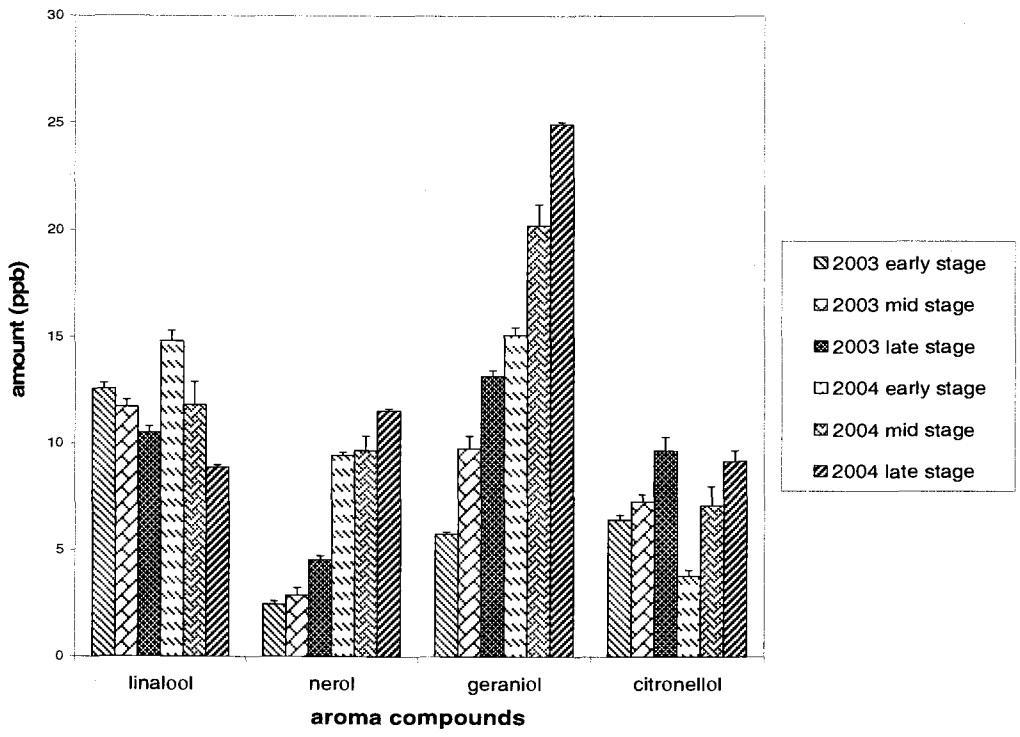

Figure 3.1. The changes of linallol, nerol, geraniol and citronellol content in wine samples with different grape maturity

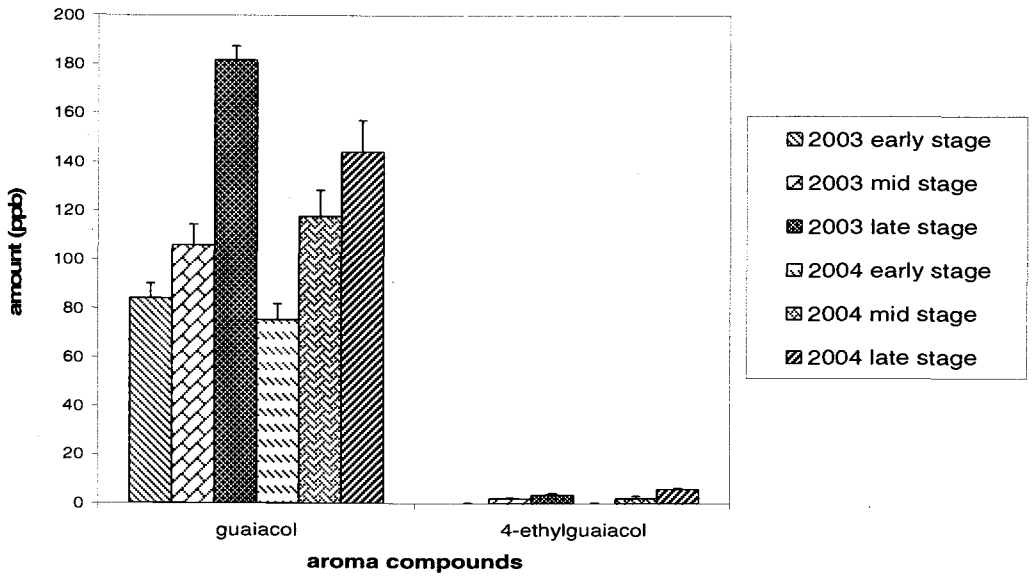

Figure 3.2. The changes of guaiacol and 4-ethylguaiacol content in wine samples with different grape maturity

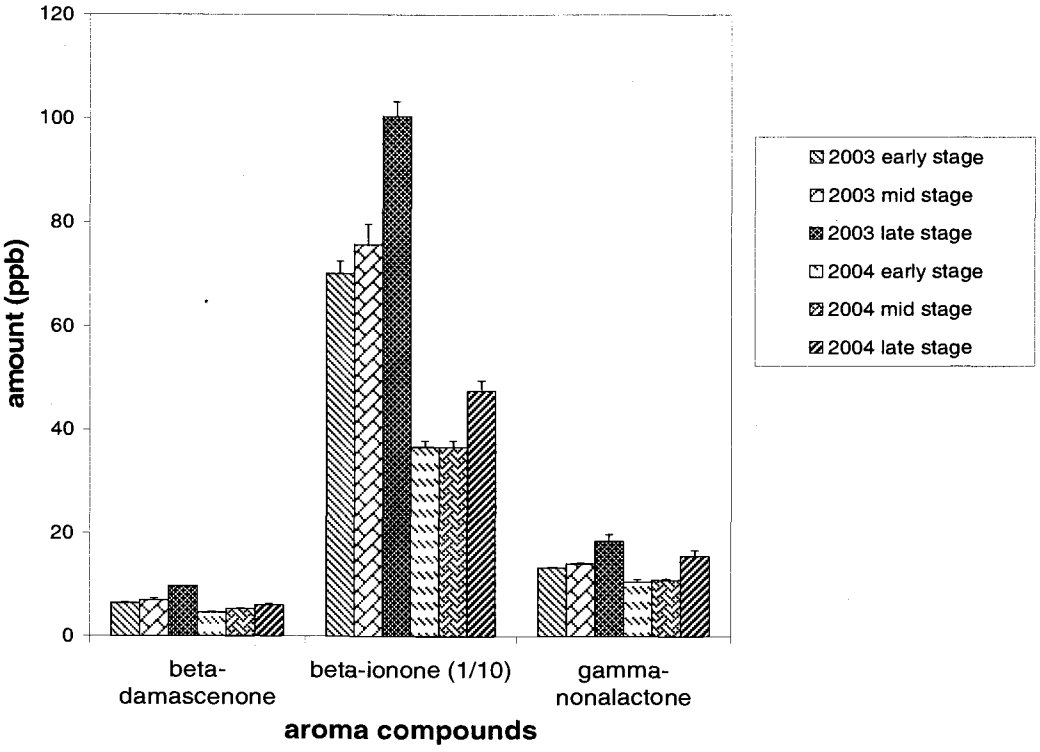

Figure 3.3. The changes of β-damascenone, β-ionone, and γ-nonalactone content in wine samples with different grape maturity

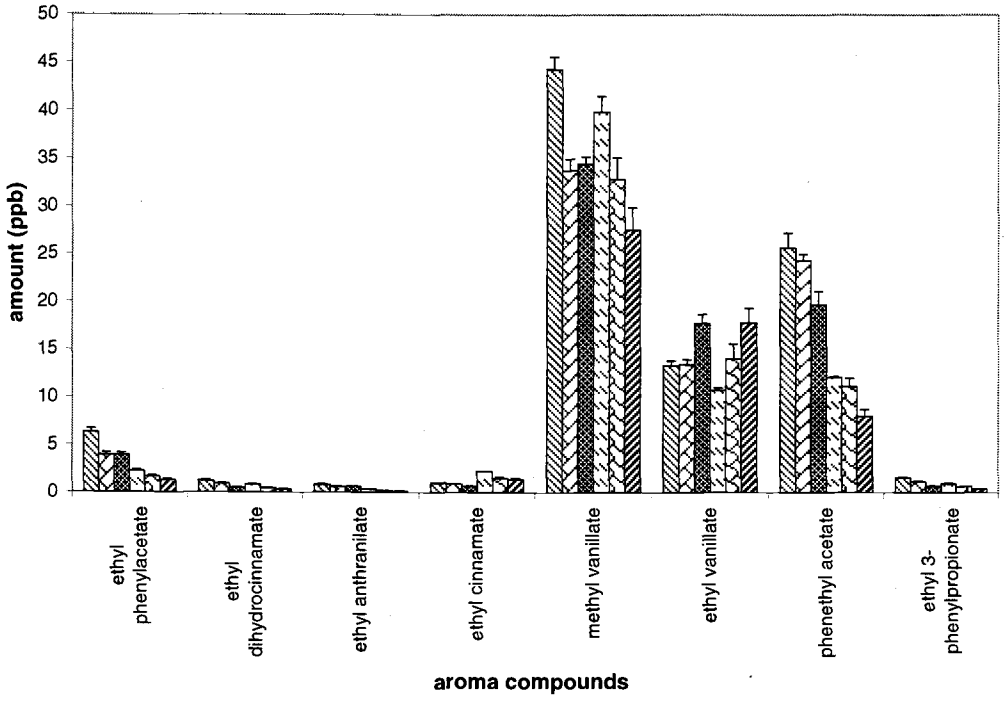

Figure 3.4. The changes of some minor esters content in wine samples with different grape maturity

Chapter 4. Preliminary Study of Aroma Compounds in Pinot noir Grapes and Their Development by Putgr-trap Technique

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To be submitted

4.1 Abstract:

The potential aroma compounds in Pinot noir grapes were identified by solvent extraction/gas chromatography-olfactometry (GC-O). Selected aroma compounds were further analyzed to investigate their changes during grape berry development, which will further help in understanding their relative importance in wine flavor and wine quality. Two years of Oregon Pinot noir grape samples were collected during the growing season, and analyzed individually using purge-trap/gas chromatography/mass spectrometry (PT/GC/MS). The results indicated that different aroma compounds followed different paths during grape development, which is not the same as sugar and acid development. Generally, the flavor compounds have very low concentration prior to veraison. At the beginning of veraison, many green odor-active flavor components, such as hexanal, *trans*-2-hexanal, hexanol and *trans*-2-hexenol, developed very rapidly and approached their maximum level. During further grape maturation, their amounts began to decrease. However, 2-methylbutanal, 3-methylbutanal, isoamyl alcohol and isobutyl alcohol continued increasing through harvest. The fruity and floral aroma compounds, geraniol and benzaldehyde, remained at low concentration and showed little change during grape development.

4.2 Key words:

Development, aroma compounds, Pinot noir grape, Purge-trap extraction,

4.3 Introduction:

Great Pinot noir wine creates a lasting impression on the palate and in the

memory. Its aroma can be intense with a ripe-grape, vaguely pepper, mint or black cherry aroma [205]. Ripe tomato, mushroom, and barnyard are also common descriptors for identifying Pinot Noir. Pinot noir is also one of most difficult wines to make due to the variability of the grapes. Little research has been done about the aroma of Pinot noir wine [2, 43, 50]. However, little research has been done about flavor and flavor precursors in the grape itself.

During the growing season, berry growth shows a double-sigmoid pattern and can be divided into three relatively distinct phases or stages [216]. In stage I, the grape shows the first rapid increase in size of the seeds and flesh, lasting four to six weeks. The berry is green and hard, and accumulates organic acids but little sugar. Stage II is the lag phase during which the embryo develops rapidly, while the berry grows only insignificantly. As to stage III, which is the ripening period, its onset is termed 'veraison' and is marked by berry softening and rapid color change from green to red or purple in dark-skinned varieties and to more or less yellow in white varieties. This last phase is characterized by a further increase in berry volume, which is initially very rapid but slows down progressively towards fruit maturity. It has been reported that the production of aroma compounds is influenced by viticulture conditions and practice during stage III [217].

The objectives of this research project are to understand the aroma compounds formed during stage III berry development in Pinot noir fruit. In this experiment, potentially important aroma compounds were identified and their changes during berry development were investigated. The information generated from this study could supplement other grape composition studies and correlate grape composition with wine quality.

4.4 Materials and Methods:

4.4.1 Grape material:

Pinot noir grapes were grown at the Oregon State University experimental vineyard located in Alpine (Woodhall vineyard, maturity/C block, pommard clone), planted in 1984. During the growing seasons of 2002 and 2003, twenty clusters were collected from the week prior to veraison up until harvest and frozen at –20°F. Berries were destemmed while still frozen, and then placed in a glass jar and kept at –10°F prior to analysis.

4.4.2 Solvent extraction/gas chromatography-olfactometry (GC-O):

A 500g Pinot noir grape sample was blended and continuously extracted with 300ml diethyl ether/pentane (1:1) for 8 hours. The crude extracts obtained from grape were distilled using solvent assisted flavor evaporation (SAFE), dried over anhydrous sodium sulfate, and then concentrated to 100ul under nitrogen. The aroma extracts were injected onto a DB-FFAP GC column (30m length, 0.32mm I.D., and 0.5um film thickness) for analysis. The carrier gas used was helium at 2ml/min. The initial oven temperature program was 40°C (for 2min), then it increased at 4°C/min to 220°C, and was held at this temperature for 10 min. The column effluent was split 1:1 via a fused silica outlet splitter to both an Agilent 5973 Mass Selective detector (MSD) and a sniffing port, where the port effluent was mixed with humidified air. Two panelists were used to select the most important aroma compounds. The electron impact (EI) energy was 70eV, and the ion source temperature was set at 230°C. System software control and data management/ analysis were performed through Enhanced ChemStation Software,

GCA v. C.00.01.08 (Agilent Technologies Inc.). Mass spectra of unknown compounds were compared with MS of purified chemicals and those in the Wiley 275.L (G1035) Database (Agilent Technologies Inc.).

4.4.3 Purge-trap-gas chromatography/mass spectra (PT-GC/MS) analysis:

One hundred grams of grape berries were blended after thawing for an hour at room temperature. 3-Heptanone (0.05ppm) was added to the berry as internal standard and well mixed. Ten grams of blended fruit were transferred to a frit tube, and then the volatiles were purged by nitrogen at a flow of 40 ml/min for 40min at 50°C (Tekmar ALS 2016 and LSC 2000 purge-and-trap equipment). A Tenax trap (#12-0083-003, Tekmar Co.) absorbed the volatiles, and was then dry purged with nitrogen for 3min. Volatiles were thermally desorbed (250°C for 2 min) and transferred with helium carrier gas directly to the GC injection port by a 1.5m×1.6 mm id transfer line. The GC/MS conditions were identical as solvent extraction/GC-O analysis. The amount of target compounds were calculated based on their total ion peak area compared with the peak area of the internal standard. Triplicate analysis was performed on all samples and the average and standard deviation are reported.

4.5 Results and Discussion:

4.5.1 Identification of aroma compounds in grapes:

The free aroma compounds in ripe Pinot noir grape berries are shown in Table 1, which were identified by solvent extraction/GC-O analysis. About 19 alcohols,

3 aldehydes, 5 acids, 3 ketones and 1 ester were identified as potential aroma compounds. In this study, the floral aroma of grape berries was attributed mainly to five alcohols: 2-butanol, propanol, geraniol, benzyl alcohol, and phenylethyl alcohol. Most of them were found as characteristic aromas in the Pinot noir wine. At the same time, 1-butanol, isoamyl alcohol, 1-hexanol, *trans*-3-hexenol, *cis*-3-hexenol, acetic acid, butanoic acid, 3-methyl-butanoic acid, hexanoic acid, and *trans*-2-hexenoic acid were also identified [50]. The most important volatiles representing green and vegetal aromas were 1-hexanal and *trans*-2-hexenal. These aldehydes, considered important aroma precursors, can be transformed into the corresponding alcohols during wine fermentation, which have a similar "grassy" aroma at low concentrations [96].

4.5.2 Development of important aroma compounds in grape

The grape berry samples were collected during the growing seasons of 2002 and 2003, and the growing degree-days are summarized in Figure 1 (A). Based upon the data, 2003 was a warmer year so overall berry development in 2003 occurred about one week earlier than in 2002, which is illustrated in Figure 1 (B).

By using a quick dynamic headspace PT-GC/MS technique, the changes of several important aroma compounds in grapes have been investigated. The results are shown in Figure 2-6. Hexanal and *trans*-2-hexenal (Figure 2), which contribute a green, unripe odor, showed a sharp increase after veraison, and then decreased after the middle of September. This trend is similar for both the 2002 and 2003 vintages. However, the 2003 vintage showed a much sharper decrease compared with 2002. Hexanol and *trans*-2-hexenol also showed similar trends, but the magnitude of this change was less (Figure 3).

2-Methylbutanal and 3-methylbutanal also contribute to green aroma. These two compounds continue to increase significantly even after berry maturation (Figure 4). For the 2003 vintage, their concentrations were higher than 2002. Both 2-methylbutanal and 3-methylbutanal are flavor precursors and can be oxidized to 2-methylbutanoic acid and 3-methylbutanoic acids during wine making. Isoamyl and isobutyl alcohols followed similar trends over the two vintages (Figure 5).

Geraniol, an important floral aroma compound, kept in low concentration and showed little change during development (Figure 6). However, benzylaldehyde, which contributes a cherry-like aroma, showed a quite different development pattern in 2002 than in 2003. Benzylaldehyde showed little increase in 2002 while its concentration continued to increase in 2003.

However, some important aroma compounds, such as phenylethyl alcohol, benzyl alcohol and β-ionone, could not be quantified in our studies, though they were identified by solvent extraction/GCO analysis. These compounds usually have extremely low sensory thresholds (ppb level), so they were easy to detect by panelists even at very low concentration. In PT-GC/MS analysis, the signals for these compounds were too low to be quantified.

In conclusion, two years' data indicated that flavor development in grapes does not follow the same path of sugar and acid development. Most flavor compounds have very low concentration prior to veraison. Beginning at veraison, many green odor-active flavor compounds, such as hexanal, *trans*-2-hexanal, hexanol and *trans*-2-hexenol, developed very rapidly and showed a sharp peak during this time, while during grape maturation, most aldehydes decreased sharply. However, 2-methylbutanal, 3-methylbutanal, along with isoamyl alcohol and

isobutyl alcohol, continued to increase through harvest. One important aroma compound, geraniol, stayed at a low concentration and showed little change during grape development. Although some important compounds, like benzyl alcohol and phenylethyl alcohol, were identified in grapes, their concentrations were too low to be quantified. Further studies about those compounds and their glycoside precursors should be done to better understand their formation.

4.6 Acknowledgement:

We would like to thank the Oregon Wine Advisory Board for funding this project. Thanks also to the OSU department of Horticulture for providing grape samples in this study. Specifically, thanks to Jose Pastor for assistance in sample collection and berry weight analysis.

Table 4.1. Important Aroma Compounds in Ripe Pinot noir Grape

Compound	Descriptor	Retention index (RI)	Intensity
Phenylethyl alcohol	Rosy	1948	very strong
Ethyl acetate	Pungent sweet fruity stimulate	877	strong
n-Hexanal	green vegetable	1101	strong
1-Butanol	green herb	1157	strong
3-Methylbutanol	herb pungent	1221	strong
trans-2-Hexenal	green grape	1245	strong
1-Hexanol	cooked fruity, green	1371	strong
Acetic acid	Sour	1467	strong
Geraniol	floral, green fruity	1871	strong
Benzyl alcohol	floral	1912	strong
2-Butanol	floral	1066	moderate
Propanol	sweet floral	1074	moderate
cis-3-Hexenyl butyrate	fruity,	1118	moderate
2-Hexanol	green herb	1332	moderate
trans-3-Hexen-1-ol	fresh grass	1381	moderate
cis-3-Hexenol	heated grass, green	1403	moderate
Nonanal	sweet rubber	1416	moderate
2-Hexen-1-ol	green grape	1426	moderate
Butanoic acid	sweaty	1649	moderate
2-Methyl-butyric acid	sweaty	1689	moderate
Nerol	vegetable, sweet fruity	1822	moderate
Hexanoic acid	sweaty	1865	moderate
B-lonon	fruity	1959	moderate
trans-2-Hexenoic acid	sweaty	1990	moderate
Benzenepropanol	fruity strawberry	2076	moderate
2-propanol	sweet fruity	937	weak
2-pentenone	sweet fruity	1051	weak
3-Methyl-2-butanol	sweet	1131	weak
3-hydroxy-2-butanone	sweet caramel fruity	1313	weak
2-hepten-1-ol	sweet	1529	weak
Octanol	herb tea	1599	weak
3.7-dimethyl-6-octen-1-ol*	fresh vegetable	1785	weak

* This compound was tentatively identified by MS

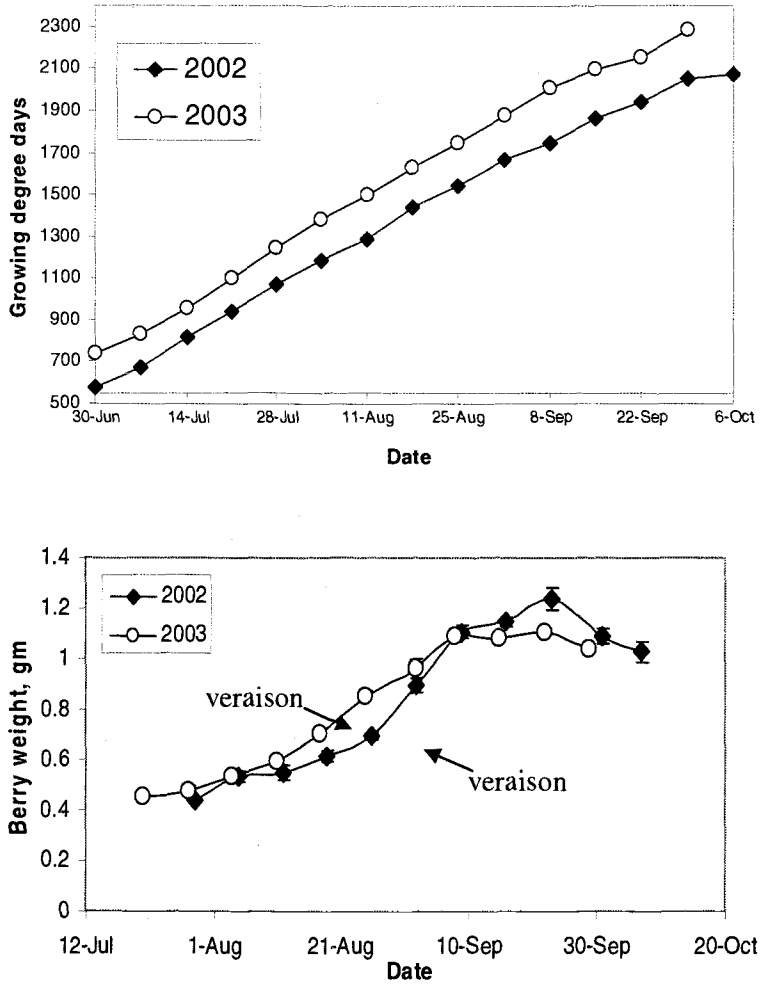

Figure 4.1. Cumulative growing degree days and berry weight change during the period of berry growth for the 2002-2003 growing seasons (error bars indicating ±SEM, N=5)

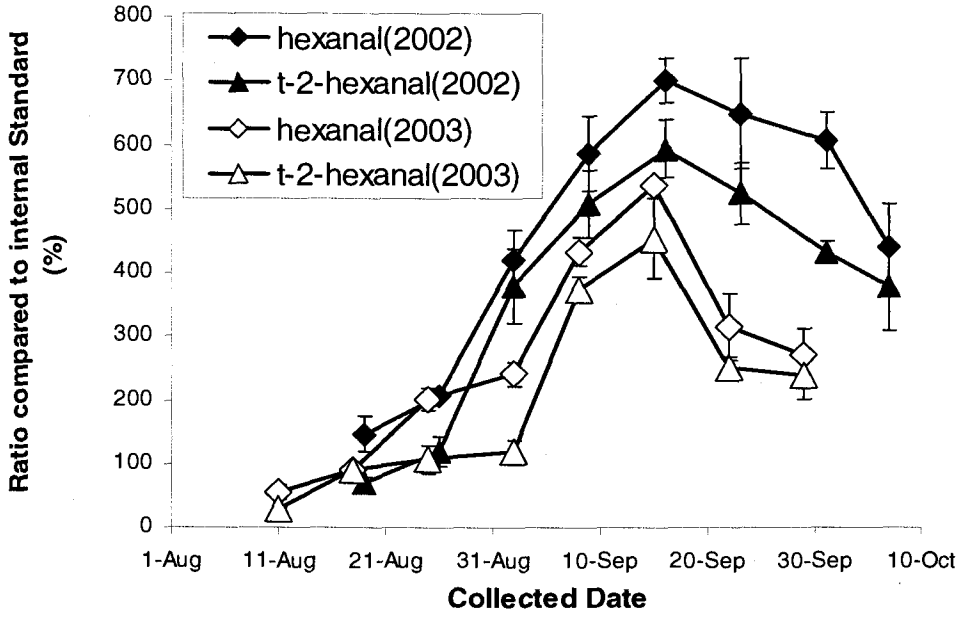

Figure 4.2. Development of hexanal and *trans*-2-hexenal in Pinot noir grapes in 2002 and 2003, with error bars indicating ±SEM (N=3), detected by PT-GC/MS

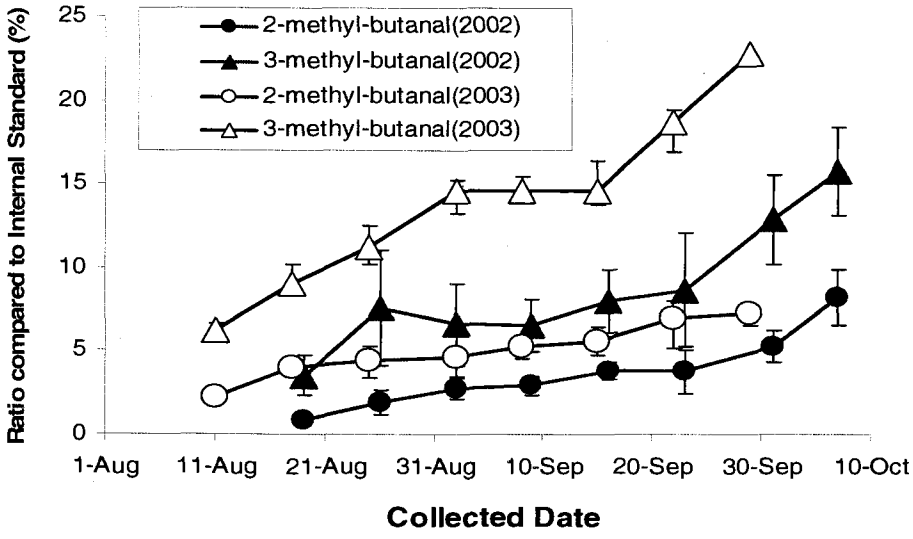

Figure 4.3. Development of 2-methyl-butanal and 3-methyl-butanal in Pinot noir grapes in 2002 and 2003, with error bars indicating ±SEM (N=3), detected by PT-GC/MS

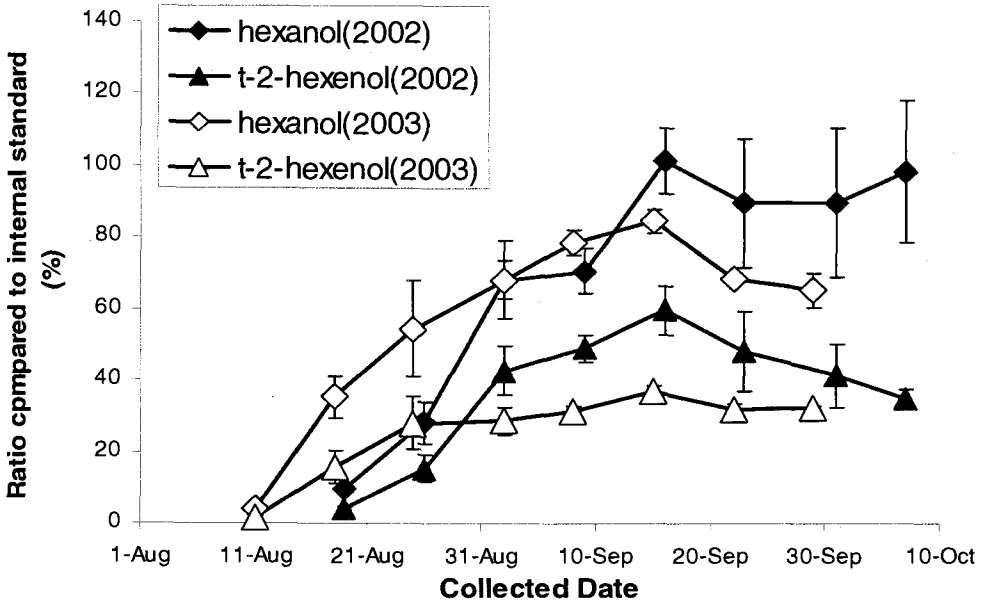

Figure 4.4. Development of hexanol and *trans*-2-hexenol in Pinot noir grapes in 2002 and 2003, with error bars indicating ±SEM (N=3), detected by PT-GC/MS

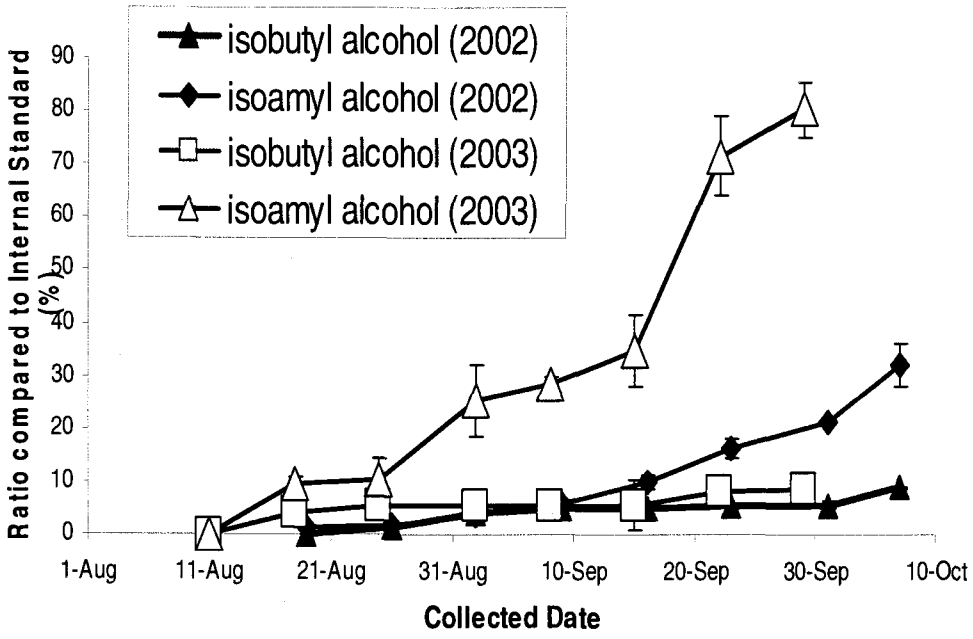

Figure 4.5. Development of isobutyl alcohol and isoamyl alcohol in Pinot noir grapes in 2002 and 2003, with error bars indicating ±SEM (N=3), detected by PT-GC/MS

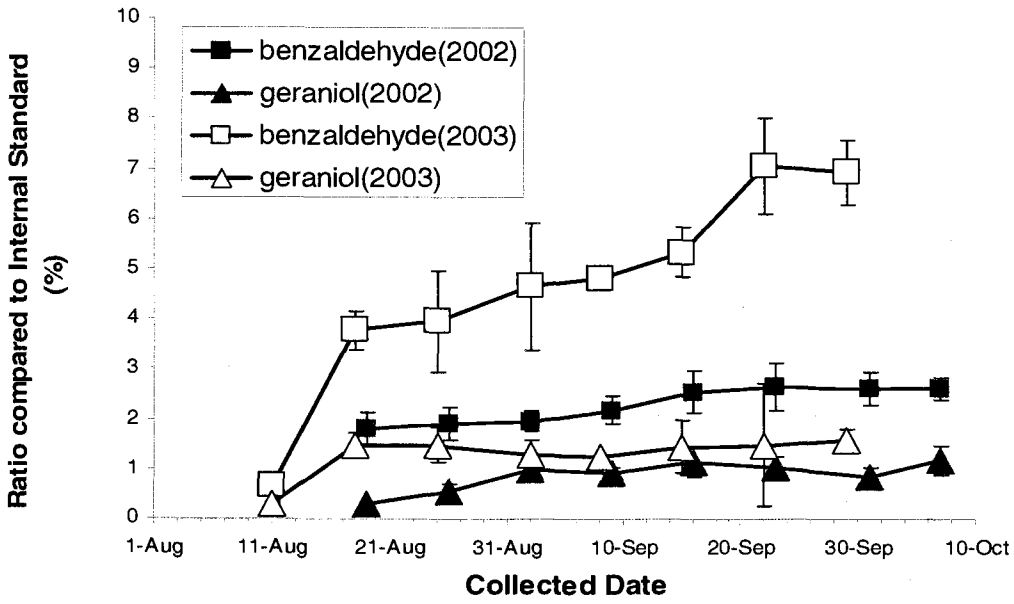

Figure 4.6. Development of benzaldehyde and geraniol in Pinot noir grapes in 2002 and 2003, with error bars indicating ±SEM (N=3), detected by PT-GC/MS

Chapter 5. The Development of Free Wine Aroma Compounds in Pinot noir Grapes Determined by Stir Bar Sorptive Extraction – Gas Chromatography-Mass Spectrometry

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5.1 Abstract:

A quick quantification method for volatile compounds in grape juice was developed using a stir bar sorptive extraction-gas chromatography-mass spectrometry (SBSE-GC-MS) technique. In this method, calibration curves for individual target aroma compounds were built up based on three internal standards. The regression coefficient and RSD showed that it is a reliable method for quantifying volatiles in grape juices. This method was applied to investigate the development of volatiles in Pinot noir grapes sampled during three growing seasons. The results showed that different compounds undergo different progressions during grape development. Green C6 alcohols and aldehydes sharply increased in the early stage, and decreased in the late stage. Major monoterpenes (geraniol, nerol and citronellol) increased during grape development, and this increase stopped or diminished in the late stage, while other monoterpenes decreased. Both β-ionone and vanillin only showed an increase in the very early stage, and diminished during grape maturity, while floral alcohols dramatically increased over the whole season. Trace amounts of β-damasonone and γ-nonalactone were detected in grape juices, and there was no or little change for these compounds. Since glycoside precursors in grapes are important to wine aroma, further studies involving hydrolysis should be done in the future to understand the correlation between grape composition and wine.

5.2 Key words:

Grape development, aroma compounds, Pinot noir grape, stir bar sorptive extraction (SESB)

5.3 Introduction:

During the growing and ripening processes, grape berries undergo both physical and chemical changes. These changes include expansion (and later shrinkage) of the berry volume, structural changes in the skin, pulp, and vascular tissues, switches in metabolic pathways, rapid accumulation of sugars, decrease in acidity, and increase in pH [218]. Ingredients other than water, sugars, acids, and nitrogen make up only a small proportion of the berry weight, but make up a very large proportion of what constitutes fruit and wine quality, such as color and flavor [73].

In wines, the development of flavor is of great importance to the consumer. Flavor is derived from a range of compounds, differing in chemical functionality. These compounds originate from primary or secondary metabolic pathways in grapes berries, which can be formed by many routes, such as mevalonic acid, shikimate, polyketide, and carotenoid breakdown pathway [96]. Therefore, the wine makers obviously are concerned with the composition of the berries at harvest and the variations near harvest that can affect the wine's composition and quality.

Hundreds of volatiles belonging to many different classes of chemicals have been reported in grapes, and some of them have been identified as important aroma compounds in grapes and wines. Varieties differ greatly in the type and amount of volatiles they produce, and these differences are responsible for the characteristic varietal aroma and flavor. There also is substantial fruit-to-fruit variation within a variety due to differences in fruit location, growth temperature and sunlight, nutrition, harvest date (maturity), and post-harvest handling [217, 219]. However, due to the lack of reliable and sensitive analytical methods, it is

still not clear how these volatiles change during grape berry ripening.

A new extraction technique, stir bar sportive extraction (SBSE), was investigated for aqueous samples [24]. Coated with 24μL of poly(dimethylsiloxane) (PDMS), the stir bar can extract most volatile and semi-volatile compounds based on the partition coefficient between PDMS and water. The SBSE technique has been proven to have lower detection and quantification levels compared with other modern conventional methodology [220-222]. With this technique, the analysis of trace amounts of aroma compounds in grapes, which have great importance in grape and wine aroma, becomes possible.

Pinot noir originated in the Burgundy region of France, and has become popular in the United States, especially in Oregon. Recently, a preliminary sensory evaluation along with instrumental analysis showed that grape maturity (harvest date) significantly affected some key aroma compounds in wine [223]. The late stage of wines contains higher concentration of C13-norisoprenoids and monoterpenes than the early stage. However, it is still unknown if this difference comes from the grape aroma volatiles or their glycoside precursors.

In this experiment, we developed a fast quantify method using SBSE-GC/MS, and investigated the important aroma compounds development during Pinot noir grape ripening. The information generated from this study will supplement flavor and flavor precursor development of other components (anthocyanins, sugar and acid), and further help understanding the correlation of grape composition and wine quality.

5.4 Materials and Methods:

5.4.1 Chemicals:

All chemical standards and internal standards were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide was bought from J.T. Baker (Philipsburg, NJ), citric acid was from Staley Manufacturing Company, (Decatur, IL), and sodium chloride was from VWR International (West Chester, PA).

5.4.2 Standard solution and internal standard solution preparation:

The citric buffer solution (0.2 M) was prepared to dissolve 42g of citric acid into 1L of Milli-Q water (Continental Water System, Millipore Corporation, Billerica, MA), which was adjusted the pH value to 3.1 by sodium hydroxide. Standard stock solutions (about 1000 ppm) were prepared in ethanol individually and stored at -15°C. Before analysis, the standards were diluted to the proper concentrations of working standards in the citric buffer solution.

An internal standard solution was made by dissolving 1.93ppm of octyl propanoate, 0.55ppm of trans-carveol, and 0.94ppm tran-2-nonenal in ethanol, and was stored at -15°C.

5.4.3 Grape sampling and juice preparation:

Pinot noir grapes were grown at the Oregon State University experimental vineyard located in Alpine, OR. During the growing seasons of 2002, 2003 and 2004, eight grape samples were collected yearly as described previously [224]. While sampling, ten clusters were randomly picked in the vineyard and were immediately frozen at -29°C. Berries were destemmed while still frozen, and then

placed in a glass jar and kept at -23°C. Prior to analysis, the juice was prepared in the following procedures. About 200g of grape berries were thawed at 4°C overnight and then ground using a commercial blender (Waring Products Division, New Hartford, CT). After settling for 5 min, skins and seeds were separated from the juice using cheesecloth, and then the grape juice was immediately analyzed.

5.4.4 Extraction of volatiles in grape juice by SBSE:

The 10ml fresh-made grape juice and 10 mL of 0.2 M citrate buffer (pH=3.1) as well as 6 g of sodium chloride were mixed in a 40ml vial, and 20 μL of internal standard solution was also added. A pre-cleaned twister bar coated with PDMS phase (2 cm × 100 mm, Gerstel Inc., Baltimore, MD) was used to extract the volatile compounds from grape juices. The twister bar was constantly stirred for 3 hours at a speed of 1000 rpm. After extraction, the twister bar was rinsed with Milli-Q water, dried with Kimwipe (Kimberly-Clark Professional Inc, Roswell, GA) tissue paper, and placed into the glass sample holder of the TDS autosampler tray (Gerstel Inc.).

5.4.5 Gas chromatography-mass spectra (GC-MS) analysis:

The extracted samples were analyzed using a thermal desorption unit (TDU) autosampler (Gerstel Inc.) mounted on an Agilent GC-MS system (Agilent 6890 GC coupled with Agilent 5973 MS, Agilent Technologies, Little Falls, DE). The analytes were thermally desorbed in the TDU in splitless mode, ramping from 35°C to 300°C at a rate of 700°C/min, and held at the final temperature for 3 min. The desorbed analytes were cryofocused (-80°C) in a programmed temperature vaporizing (PTV) injector (CIS 4, Gerstel Inc.) with liquid nitrogen. The solvent

vent injection mode was employed with a venting flow of 25 mL/min at 10 psi venting pressure for 0.01min. After SBSE desorption, the PTV was heated from -60°C to 250°C at a rate of 10°C/sec and kept at 250°C. A ZB-FFAP capillary GC column (30m, 0.32mm ID, 0.25μm film thickness; Phenomenex, Torrance, CA) was employed to separate the analytes. The column carrier gas was helium at 2 mL/min. The oven temperature programmed initially 40°C (for 2min), then increased at 6°C/min to 180°C, further increased at 4°C/min to 240°C, and held at this temperature for 20min. The electron impact (EI) energy was 70eV, and the ion source temperature was set at 230°C. Enhanced ChemStation software (GCA v.D.00.01.08, Agilent Technologies Inc.) was used for data acquisition and analysis.

5.5.6 Calibration and quantification of aroma compounds in grape juice:

The stock solutions were prepared by dissolving ca 10,000 ppm of each target compound individually into ethanol solution. Before analysis, certain amounts of stock solutions were added in synthetic wine to make the first level mixed standard solution (Table1) and diluted at 4:1(v/v), 3:2 (v/v), 2:3 (v/v) and 1:4 (v/v) ratio with synthetic wine to give a range of concentrations.

After adding 6g of sodium chloride and 20 μL of internal standard solution, the mixture as well as its 4:1(v/v), 3:2 (v/v), 2:3 (v/v), and 1:4 (v/v) dilutions in synthetic wine were then extracted by SBSE for 3 hours. The SBSE extracts were than analyzed using the same procedure as described above. To avoid the interferences between coeluting compounds, the MS analysis was carried out in the single ion-monitoring (SIM) mode. The selected target ions for quantification are listed in Table 1. The calibration curves for individual target compounds were built up by plotting the selected ion abundance ratio of target compounds with their respective internal standard against the concentration ratio, which was force

to pass through the origin (0,0). The calibration equation and their regression coefficients were calculated using the ChemStation data analysis software.

The amounts of target compounds in each sample vial were calculated based on the calibration curves individually, and then converted to the concentrations in original grape juices. Triplicate analysis was performed on all samples, and the average values are reported.

5.5 Results and Discussion:

Since the SBSE technique efficiency is based on the equilibrium of analytes between PMDS solid phase and sample solution, the extraction of analytes was influenced by numerous factors [24]. Several researches have been done to optimize the SBSE extraction [225, 226]. During grape development, the pH value of grape juice varied in a wide range, which definitely affected the extraction efficiency of the stir bar. Therefore, for quantification, 10mL of buffer solution (pH=3.1) were mixed with grape juice to eliminate the sample matrix effect. Moreover, 6g of sodium chloride were also added to improve the sensitivity.
A total of 28 aroma compounds were investigated in the grapes, including 16 alcohols, 3 ketones, 7 aldehydes and 2 esters. Three different internal standards were used to quantify those aroma compounds based on their chemical properties. Table 1 lists the characteristics of calibration curves for those aroma compounds using SBSE-GC/MS methods. The correlation coefficients (R^2) for most compounds were greater than 0.99 (Table 1), and the relative standard deviation (RSD) calculated in later analyses of grape juices were less than 15% for most quantified compounds (data not shown).

Target compounds were selected based on the results of the previous GC/O studies of Oregon Pinot noir grapes and wines [50, 224]. Except for acids, short

carbon-chain alcohols, and ethyl acetate, all aroma compounds having moderate odor-activity in grapes were quantified in our study. In addition, monoterpenes (linalool, linalool oxide, nerol, citronellol and α-terpineol), C13-norisoprenoid (β-damascenone) and other aroma compounds (1-octal-3-ol, γ-nonalactone, vanillin, methyl and ethyl vanillate) were also included, since they showed some important aroma impacts in final wines.

Table 2, 3 and 4 shows the concentrations of aroma compounds in different stages of Pinot noir grapes during 2002, 2003 and 2004. To further investigate the development of individual compounds, the results were plotted based on different vintage.

In grapes, C6 alcohols (1-hexanol, *trans*-2-hexenol, *trans*-3-hexenol, and *cis*-3-hexenol) and aldehydes (1-hexanal and *trans*-2-hexenal) are well known as green and vegetable odorants [173]. The developments of these compounds are shown in Figure 1.

In all three years, *trans*-2-hexenol was the most abundant compound among these green odorants, which showed a sharp increase after verasion and decreased in the middle of September. 1-Hexanol, 1-hexanal and *trans*-2-hexenal showed the similar trend during grape development, which is consistent with the previous study using purge-trap extraction [224]. It has been reported that the chemical reduction of hexanal, *trans*-2-hexenal, *trans*-2-hexenol is responsible for the 1-hexanol present in the must [56]. Therefore, wines from late harvest grapes should contain less 1-hexanol, which partially explains why they generally have less green and un-ripe aromas.

For *cis*- and *trans*-3-hexenol, they generally come directly from grape must, and stay stable during fermentation [56]. In all three vintages, a sharp peak of *cis*-3-hexenol after verasion was observed, and it indicates that this compound might

be converted to other comopounds during grape development. Only small amount of *trans*-3-hexenol (< 10 μg/L juice) was found in all samples, and it steadily increased. However, in AEDA analysis of Pinot noir wines, the *trans* form was found to have higher FD values than the *cis* form [50], which may due to the transformation of these two isomers occurring during wine storage [59].

Monoterpene compounds belong to the secondary plant constituents, of which the biosynthesis begins with acetyl-coenzyme A (CoA). During wine making, these compounds cannot be generated by yeast itself. However, acid rearrangement of the monoterpenols can occur and change their composition, such as transformation of linalool into α-terpineol, hydroxyl linalool, geraniol, and nerol [89]. Therefore, total terpene levels in grapes should be considered when evaluating their impact on wine aroma. Figure 2 shows the development of free form monoterpenes in three vintages. In Pinot noir, geraniol is among the most important monoterpenes, since it has a higher concentration compared to others, and it has also been shown to have an important impact on both grape and wine aroma [224]. The results showed that geraniol as well as nerol and citronellol increased in the early stage, and stopped or diminished in the late stage. Similar trends were also observed with free terpenols in Muscat de Frontignan [227]. Only trace amounts of linalool, linalool oxide, and α-terpineol were detected in grape juices (<2 ppb), and they decreased along with grape development.

However, it is difficult to explain the concentration of monoterpenes in wine increasing along with grape maturity. This is because the free volatile form is not the only form of terpenes in grape musts. Around 90% of terpenes are present in grapes as glycosides, which could be hydrolyzed by enzymes and acid [82]. Therefore, the bound form of monoterpenes should also be studied to fully understand their effects on wine.

Large amounts of benzyl alcohol, phenylethyl alcohol and phenol were found in grape juices. As the grapes developed, their concentration dramatically increased (Figure 3). These compounds contribute to the typical floral aroma in grapes and wines, especially phenylethyl alcohol. Phenylethyl alcohol in wine is generated through the shikimate pathway, which is significantly dependent on the yeast strain used [67], thus there is no direct correlation between the amount present in grapes and that in wines.

1-Octen-3-ol, having a remarkable mushroom-like odor, is reported to be present in numerous wines [43, 61]. In Pinot noir grapes, the results showed that there is no change in this compound during the growing season. It has been reported that this compound is formed during ripening as a result of attack by gray mold, and if present in a high concentration, may be considered a defect [62].

Figure 4 shows the development of three ketones in grapes. β-Damascenone and β-ionone are C13-norisoprenoid compounds, and have been found as important aroma compounds in wine with highly desirable flavor properties [33, 93]. The results showed that there was a trace amount of β-damascenone in grape juices, and it barely changed throughout the whole season, which is consistent with literature [94]. However, it is interesting to note that there is quite a large amount of free form β-ionone, which is consistent with the previous GC/O analysis. During grape development, β-ionone sharply increased in the very early stage, and then diminished during maturity.

Small amounts of vanillin and γ-nonalactone were found in the grapes. Vanillin decreased during grape maturity, though a slight increase was observed in the very early stage, while γ-nonalactone didn't change along with the grape development (Figure 4). Like monoterpenes as well as norisoprenoids, these

compounds occur in grapes and wines predominately as glycosidically bound precursors, and arise from the enzymatic hydrolysis and acid cleavage during the crushing of the grapes [73]. To investigate their effect on wines, it is necessary to study their glycoside precursors. Other volatile compounds were also quantified in our study, such as long carbon chain aldehydes, and methyl and ethyl vanillates. There were no obvious trends for these compounds, though they may contribute to grape and wine aroma.

Most winemakers have experienced that warmer climates produce less flavor and aroma constituents in grapes, but it has not been confirmed. Herrick and Nagel [228] found the phenol content of Riesling wines from Alsace (13mg/L) was much lower than those from eastern Washington State and California (123 mg/L). Later, Ewart et al. [229], comparing diferent vineyard sites in south Australia, found in the cool site that total volatile terpenes increased more slowly but were at higher concentrations in the warm site. In our study, some compounds do increase along with harvest year, since 2004 and 2003 were much hotter than 2002. However, the difference among different vintages was not clear enough to make any conclusion.

In summary, free wine aroma compounds can be analyzed using SBSE/GC-MS technique. The regression coefficient and RSD showed that it is a reliable method for analyzing volatiles in grape juices. Analysis of grape samples during three growing seasons showed that different compounds undergo different routes during grape development. Moreover, since glycoside precursors in grapes are important to wine aroma, hydrolysis studies should be done to better understand the correlation between grape composition and wine.

5.6 Acknowledgement:

We would like to thank the Oregon Wine Advisory Board for funding this project. Thanks also to the OSU Department of Horticulture for providing grape samples in this study. Specifically, thanks to Jose Pastor for assistance in sample collection.

Table 5.1. Standard curve and quantification of aroma compounds in grape juice

Compounds	Quantity ion	First Level Concentration (ppb)	Equation*: $C_{TC} = A \times \frac{C_{IS}}{R_{IS}} \times R_{TC}$	Coefficient
trans-Carveol (IS)	109			
Linalool	71	29	A = 1.104	0.998
Nerol	69	24	A = 0.791	0.997
Geraniol	69	27	A = 0.677	0.997
Eugenol	164	28	A = 3.299	0.998
Citronellol	81	15	A = 3.700	0.989
Linalool oxide#	94			
α-Terpineol	93	15	A = 14.18	0.992
Phenol	94	5160	A = 138.0	0.993
1-Hexanol	69	55	A = 7.375	0.993
Benzyl alcohol	108	7160	A = 148.1	0.996
Phenylethyl alcohol	122	6010	A = 77.83	0.999
trans-3-Hexenol	82	1450	A = 52.27	0.994
cis-3-Hexenol	82	1550	A = 49.77	0.994
trans-2-Hexenol	57	2030	A = 104.4	0.994
1-Octen-3-ol	57	7	A = 0.881	0.996
3-Methylbutanol	70	11870	A = 173.7	0.979
2-Nonenal (IS)	70			
β-Damascenone	121	14	A = 0.442	0.998
β-Ionone	177	54	A = 72.93	0.988
γ-Nonalactone	85	13	A = 0.568	0.991
Vanillin	151	596	A = 33.43	0.983
Hexanal	82	32	A = 26.96	0.981
trans-2-Hexenal	83	46	A = 15.49	0.960
Heptanal	70	18	A = 3.538	0.943
Octanal	84	17	A = 3.056	0.996
Nonanal	98	7	A = 3.129	0.974
Decanal	112	4	A = 5.236	0.932
Octyl propinoate (IS)	112			
Methyl vanillate	151	52	A = 1.190	0.987
Ethyl vanillate	196	67	A = 0.043	0.977

* C_{TC}: Concentration of target compound; C_{IS}: Concentration of internal standard; R_{TC}: MS Response of target compound; R_{IS}: MS Response of internal standard

Linalool oxide was calculated based on the calibration curve of linalool

Table 5.2. The concentration (μg/L juice) of free volatile compounds in Pinot noir grapes during 2002

	8/19/2002	8/26/2002	9/2/2002	9/9/2002	9/23/2002	10/1/2002	10/7/2002
Alcohol							
Linalool	1.27±0.02	0.87±0.01	0.75±0.01	0.58±0.01	0.57±0.08	0.36±0.02	0.30±0.01
Nerol	1.17±0.01	1.02±0.02	1.84±0.02	2.51±0.02	3.62±0.02	3.74±0.01	2.50±0.01
Geraniol	2.26±0.01	3.01±0.01	7.66±0.01	8.94±0.01	10.78±0.01	8.35±0.01	7.54±0.01
Eugenol	0.28±0.02	0.24±0.01	0.51±0.01	1.15±0.01	1.22±0.00	1.01±0.01	1.54±0.01
Citronellol	0.72±0.01	0.95±0.01	1.76±0.00	1.59±0.01	1.77±0.01	1.06±0.01	1.17±0.01
Linalool oxide	0.86±0.03	0.42±0.02	0.11±0.02	0.06±0.03	0.06±0.02	0.09±0.05	0.06±0.03
α-Terpineol	0.45±0.01	0.31±0.01	0.22±0.01	0.17±0.01	0.13±0.01	0.12±0.01	0.12±0.01
Phenol	45.9±1.8	38.8±2.8	38.9±0.9	27.4±2.9	33.5±2.3	34.6±0.5	36.0±4.4
1-Hexanol	24.9±0.1	32.9±0.1	59.9±0.1	83.2±0.1	78.0±0.1	61.7±0.1	51.1±0.1
Benzyl alcohol	141±1	91±6	254±3	525±3	1,367±6	1,742±8	1,824±7
Phenylethyl alcohol	52±5	52±2	119±2	212±3	305±4	335±5	565±7
trans-3-Hexenol	2.49±0.21	3.63±0.23	4.14±0.67	3.47±0.45	2.33±0.14	8.13±0.80	5.65±0.37
cis-3-Hexenol	190.1±1.0	413.4±0.7	421.8±0.6	241.6±0.7	52.4±0.5	25.5±0.6	25.8±0.5
trans-2-Hexenol	102±2	297±1	344±1	375±1	326±1	288±1	235±1
1-Octen-3-ol	10.69±0.01	5.71±0.01	5.43±0.01	4.42±0.01	4.56±0.01	4.65±0.01	5.08±0.01
3-Methylbutanol	ND*	ND	88±10	130±5	300±12	375±11	573±5
Ketone & Aldehyde							
β-Damascenone	0.33±0.01	0.47±0.02	0.91±0.01	0.27±0.01	0.12±0.02	0.07±0.01	0.07±0.01
β-Ionone	46.1±0.1	46.7±0.1	55.6±0.1	46.6±0.1	29.2±0.1	23.4±0.1	24.1±0.1
γ-Nonalactone	0.20±0.01	0.25±0.01	0.18±0.01	0.35±0.01	0.20±0.01	0.22±0.01	0.23±0.01
Vanillin	11.58±1.31	10.60±0.68	9.60±0.19	6.64±0.08	6.20±0.46	5.84±0.53	6.02±0.21
Hexanal	16.9±0.1	37.2±0.1	69.2±0.1	78.1±0.1	98.1±0.1	86.8±0.1	79.2±0.1
trans-2-Hexenal	4.5±0.1	10.1±0.0	42.5±0.0	45.3±0.0	50.3±0.0	34.5±0.0	33.5±0.0
Heptanal	1.56±0.01	1.65±0.01	1.85±0.00	1.38±0.01	1.37±0.01	1.09±0.02	1.08±0.01
Octanal	1.45±0.01	1.40±0.01	1.40±0.02	0.56±0.00	0.42±0.01	0.44±0.02	0.31±0.01
Nonanal	1.94±0.01	1.93±0.00	1.69±0.00	1.13±0.00	1.00±0.01	0.87±0.00	0.61±0.01
Decanal	2.48±0.00	1.97±0.00	1.31±0.00	0.55±0.00	0.50±0.01	0.39±0.01	0.25±0.00
Esters							
Methyl vanillate	13.8±0.1	14.1±0.0	27.9±0.1	75.0±0.1	30.6±0.1	23.3±0.1	23.5±0.0
Ethyl vanillate	0.78±0.09	1.01±0.06	0.84±0.15	0.78±0.63	0.34±0.03	0.18±0.01	0.51±0.07

* ND: not detected

Table 5.3. The concentration (μg/L juice) of free volatile compounds in Pinot noir grapes during 2003

	8/11/2003	8/18/2003	8/25/2003	9/2/2003	9/8/2003	9/15/2003	9/22/2003	9/29/2003
Alcohol								
Linalool	0.80±0.03	0.77±0.01	0.71±0.03	0.53±0.02	0.54±0.02	0.53±0.02	0.54±0.01	0.49±0.02
Nerol	0.78±0.02	0.74±0.01	1.31±0.02	2.99±0.01	3.37±0.01	3.15±0.01	2.84±0.01	2.73±0.01
Geraniol	1.67±0.01	4.70±0.01	7.98±0.01	9.50±0.01	13.51±0.00	11.48±0.01	8.51±0.01	7.47±0.01
Eugenol	0.20±0.02	0.21±0.02	0.39±0.01	0.74±0.01	1.55±0.01	0.99±0.01	0.82±0.01	0.91±0.02
Citronellol	0.58±0.01	1.23±0.01	1.48±0.01	1.54±0.01	2.15±0.01	1.59±0.01	1.10±0.01	1.05±0.01
Linalool oxide	0.66±0.04	0.23±0.02	0.09±0.02	0.06±0.02	0.06±0.03	0.05±0.01	0.06±0.02	0.08±0.01
α-Terpineol	0.35±0.01	0.24±0.01	0.20±0.01	0.19±0.01	0.19±0.01	0.17±0.01	0.18±0.01	0.16±0.01
Phenol	30.7±2.5	32.5±2.2	32.8±0.8	30.3±3.5	34.0±2.3	38.7±1.9	39.1±1.3	39.8±1.2
1-Hexanol	19.1±0.1	73.9±0.1	107.3±0.1	137.1±0.1	65.6±0.1	36.7±0.1	27.2±0.1	23.5±0.1
Benzyl alcohol	61±4	79±5	249±6	766±6	1,074±3	1,382±3	1,436±8	1,379±3
Phenylethyl alcohol	24±3	35±2	127±2	264±6	318±3	325±4	361±5	496±1
trans-3-Hexenol	3.16±0.17	2.38±0.55	3.94±0.28	4.14±0.35	5.39±0.57	5.78±0.15	6.30±0.36	6.92±0.80
cis-3-Hexenol	160.4±1.0	469.0±1.3	389.7±0.2	143.4±1.0	81.3±0.7	42.6±0.7	37.5±0.9	35.1±0.3
trans-2-Hexenol	221±2	463±2	554±0	552±1	541±1	432±1	316±1	294±1
1-Octen-3-ol	6.88±0.01	3.64±0.01	3.70±0.01	2.62±0.01	2.98±0.01	3.02±0.01	3.41±0.01	3.84±0.01
3-Methylbutanol	41±19	45±17	72±9	182±14	202±5	254±4	425±6	512±4
Ketone & Aldehyde								
β-Damascenone	0.07±0.01	0.20±0.01	0.16±0.01	0.12±0.01	0.13±0.01	0.12±0.02	0.07±0.02	0.06±0.01
β-Ionone	28.7±0.1	44.0±0.1	37.7±0.1	36.7±0.1	32.5±0.1	30.1±0.1	23.2±0.1	24.2±0.1
γ-Nonalactone	0.29±0.01	0.21±0.01	0.11±0.01	0.17±0.01	0.13±0.01	0.13±0.01	0.27±0.01	0.33±0.01
Vanillin	10.12±0.54	6.66±0.19	5.11±0.63	4.77±0.79	4.13±0.31	4.17±0.58	3.83±0.69	3.91±0.52
Hexanal	3.6±0.1	9.4±0.1	13.3±0.1	26.2±0.1	58.2±0.1	65.6±0.1	53.5±0.1	53.7±0.1
trans-2-Hexenal	1.0±0.1	3.2±0.1	7.5±0.1	16.9±0.1	41.3±0.1	42.7±0.1	43.4±0.1	34.8±0.1
Heptanal	0.36±0.02	0.38±0.07	0.36±0.01	0.54±0.01	0.60±0.08	0.50±0.01	0.57±0.01	0.25±0.01
Octanal	0.34±0.02	0.31±0.01	0.28±0.03	0.27±0.01	0.26±0.01	0.22±0.01	0.58±0.01	0.35±0.01
Nonanal	0.73±0.01	0.69±0.01	0.47±0.01	0.49±0.01	0.58±0.01	0.46±0.01	0.58±0.01	0.53±0.01
Decanal	0.66±0.01	0.50±0.01	0.39±0.01	0.33±0.01	0.39±0.01	0.32±0.01	0.34±0.01	0.35±0.01
Esters								
Methyl vanillate	21.5±0.1	16.1±0.1	63.5±0.1	168.3±0.0	182.1±0.1	153.0±0.1	144.9±0.1	147.1±0.1
Ethyl vanillate	ND*	0.03±0.76	0.33±0.10	0.66±0.02	1.01±0.04	0.87±0.08	2.37±0.13	8.43±0.06

* ND: not detected

Table 5.4. The concentration (μg/L juice) of free volatile compounds in Pinot noir grapes during 2004

	7/28/2004	8/2/2004	8/9/2004	8/18/2004	8/27/2004	9/7/2004	9/14/2004	9/20/2004
Alcohol								
Linalool	1.76±0.01	1.24±0.01	1.10±0.01	1.09±0.01	0.74±0.02	0.55±0.02	0.33±0.01	0.39±0.01
Nerol	1.36±0.01	1.26±0.01	1.05±0.02	1.63±0.02	3.71±0.02	3.91±0.01	3.67±0.02	3.57±0.02
Geraniol	4.30±0.01	2.36±0.01	3.35±0.01	7.94±0.01	15.79±0.01	15.03±0.01	13.07±0.02	13.07±0.01
Eugenol	0.32±0.02	0.21±0.02	0.22±0.01	0.42±0.01	0.86±0.01	1.05±0.02	0.82±0.02	1.21±0.01
Citronellol	0.46±0.01	0.38±0.02	0.64±0.01	1.56±0.01	2.20±0.01	2.24±0.01	1.72±0.01	1.94±0.01
Linalool oxide	1.02±0.01	0.77±0.01	0.45±0.02	0.31±0.02	0.10±0.03	0.08±0.04	0.10±0.02	0.08±0.05
α-Terpineol	0.63±0.01	0.46±0.01	0.44±0.01	0.38±0.01	0.22±0.01	0.19±0.01	0.11±0.01	0.11±0.01
Phenol	37.6±2.7	37.4±2.3	33.5±2.7	31.9±3.3	29.5±3.3	30.5±2.7	35.4±3.6	40.0±3.2
1-Hexanol	34.5±0.1	30.3±0.1	49.3±0.1	105.0±0.1	86.3±0.1	91.7±0.1	90.2±0.1	85.4±0.1
Benzyl alcohol	203±8	196±9	218±7	225±3	697±5	1,563±11	1,607±10	1,658±8
Phenylethyl alcohol	71±6	54±6	76±7	64±5	245±3	280±6	299±3	309±3
trans-3-Hexenol	3.00±0.13	9.01±0.64	10.15±1.51	12.02±0.51	9.33±1.01	6.89±1.26	7.16±0.64	7.36±0.73
cis-3-Hexenol	201.5±1.4	170.0±1.1	284.6±0.5	485.1±0.6	177.2±1.2	75.0±1.7	39.5±0.9	35.4±0.3
trans-2-Hexenol	333±1	341±2	578±1	693±1	753±1	777±1	590±1	481±1
1-Octen-3-ol	7.89±0.01	7.05±0.01	5.08±0.01	4.93±0.01	5.07±0.01	5.74±0.01	5.87±0.01	6.03±0.01
3-Methylbutanol	90±14	91±20	102±22	163±30	194±11	270±13	277±11	314±4
Ketone & Aldehyde								
β-Damascenone	0.05±0.01	0.05±0.01	0.13±0.01	0.11±0.01	0.12±0.01	0.07±0.01	0.04±0.01	0.05±0.02
β-Ionone	20.9±0.1	20.4±0.1	29.5±0.1	24.0±0.1	21.4±0.1	19.1±0.1	14.1±0.1	14.5±0.1
γ-Nonalactone	0.08±0.01	0.11±0.01	0.12±0.01	0.07±0.01	0.09±0.01	0.07±0.01	0.06±0.01	0.08±0.01
Vanillin	4.38±0.48	5.80±0.64	5.32±0.25	2.86±0.43	2.80±0.37	2.13±0.64	1.41±0.19	2.03±0.40
Hexanal	9.7±0.1	13.1±0.1	7.0±0.1	11.1±0.1	20.7±0.1	35.5±0.1	45.1±0.1	38.3±0.1
trans-2-Hexenal	3.3±0.1	5.8±0.1	6.4±0.1	8.1±0.1	16.2±0.1	26.6±0.1	35.7±0.1	35.6±0.1
Heptanal	0.21±0.01	0.23±0.01	0.51±0.06	0.44±0.02	0.39±0.03	0.41±0.07	0.36±0.01	0.27±0.01
Octanal	0.36±0.02	0.43±0.01	0.51±0.04	0.20±0.01	0.28±0.03	0.31±0.02	0.20±0.05	0.22±0.02
Nonanal	0.70±0.01	0.75±0.01	0.77±0.01	0.39±0.01	0.48±0.01	0.51±0.01	0.42±0.01	0.41±0.01
Decanal	0.81±0.01	0.82±0.01	0.98±0.01	0.36±0.01	0.44±0.01	0.54±0.01	0.30±0.01	0.28±0.01
Esters								
Methyl vanillate	27.8±0.1	17.7±0.1	23.1±0.1	33.9±0.1	75.2±0.1	128.4±0.1	104.5±0.1	102.5±0.1
Ethyl vanillate	ND*	ND	ND	ND	ND	0.19±0.02	0.16±0.01	0.27±0.04

* ND: not detected

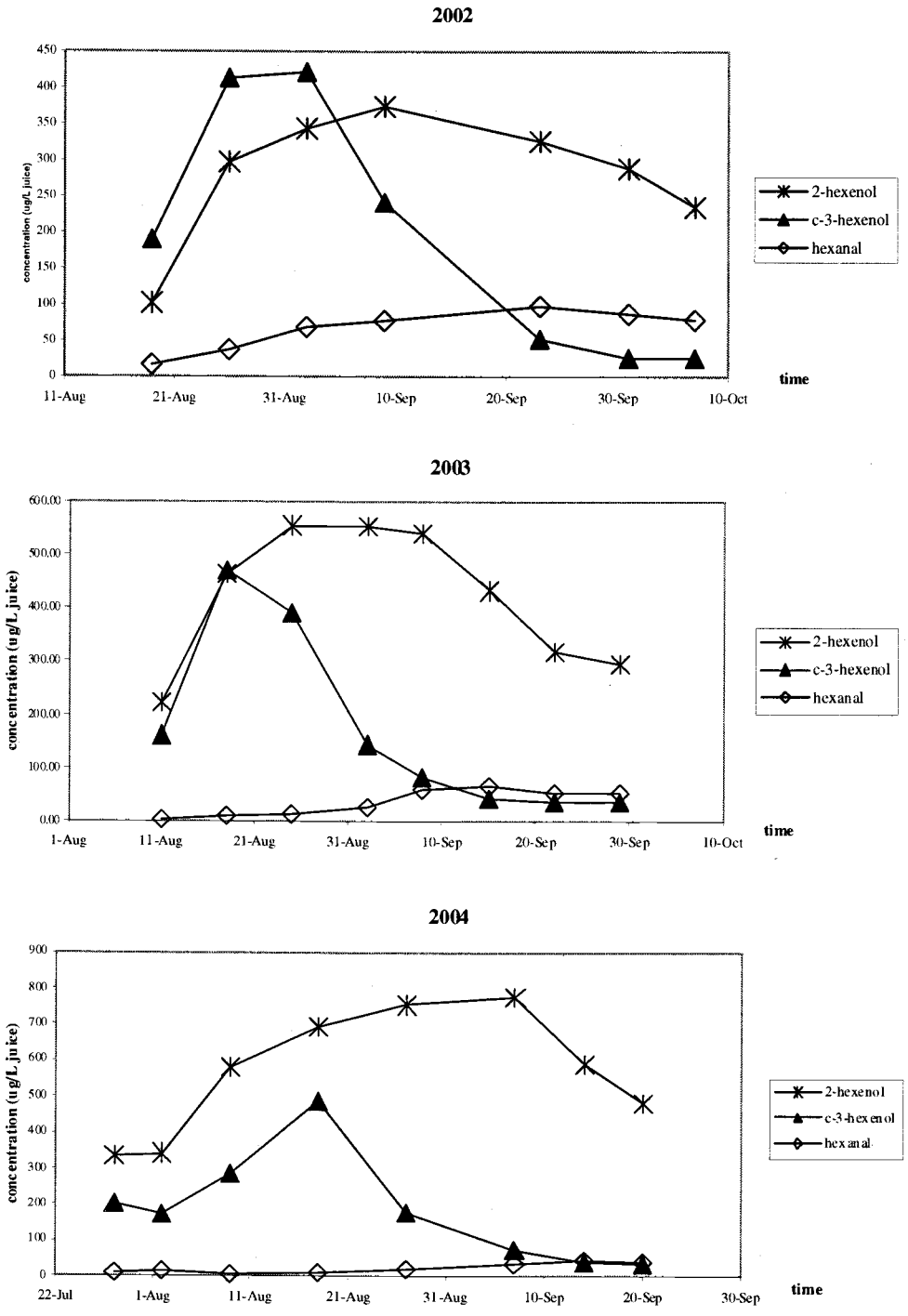

Figure 5.1. The development of free form of green aroma compounds in grapes during 2002, 2003, and 2004

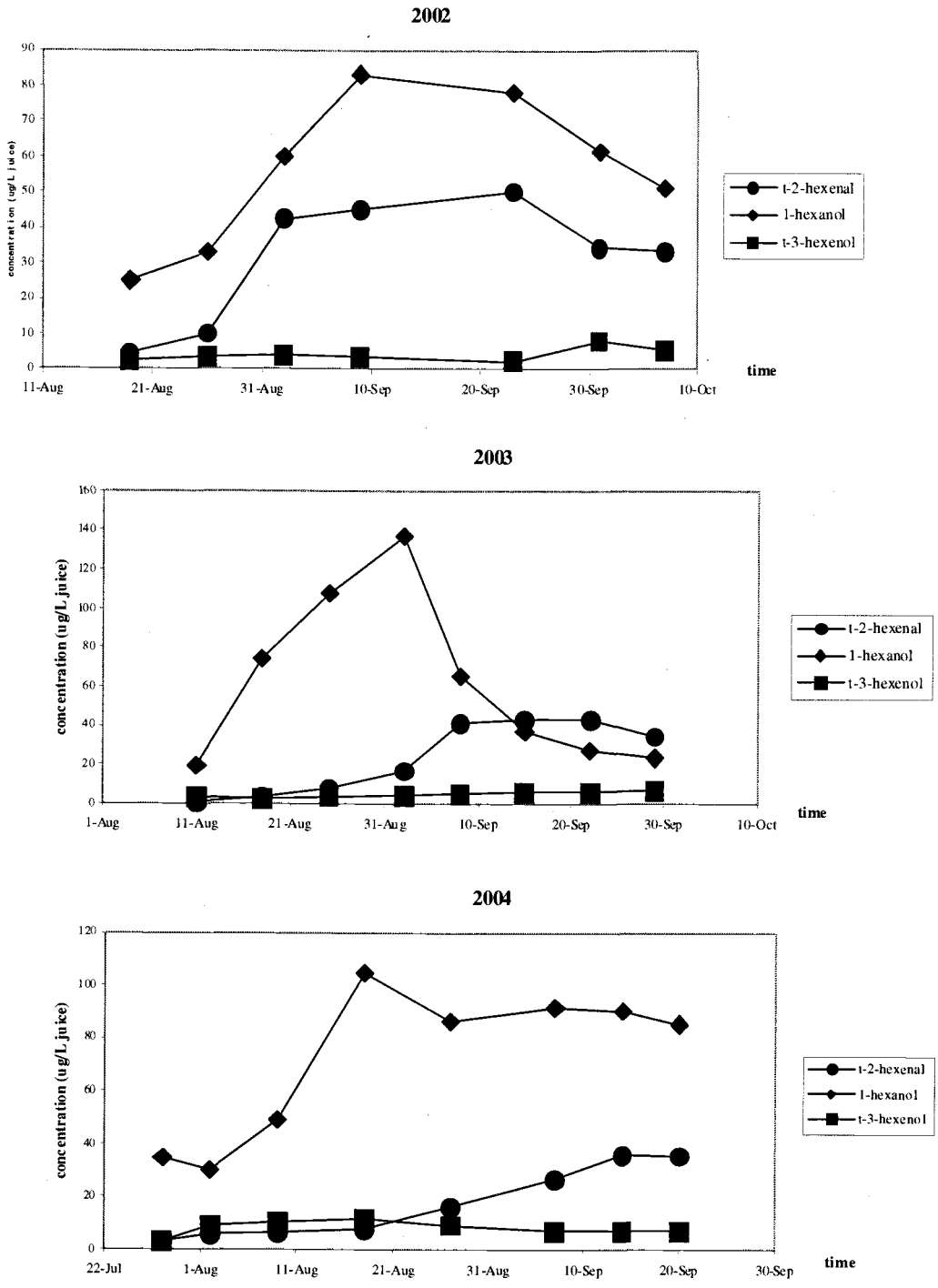

Figure 5.1 (cont.). The development of free form of green aroma compounds in grapes during 2002, 2003, and 2004

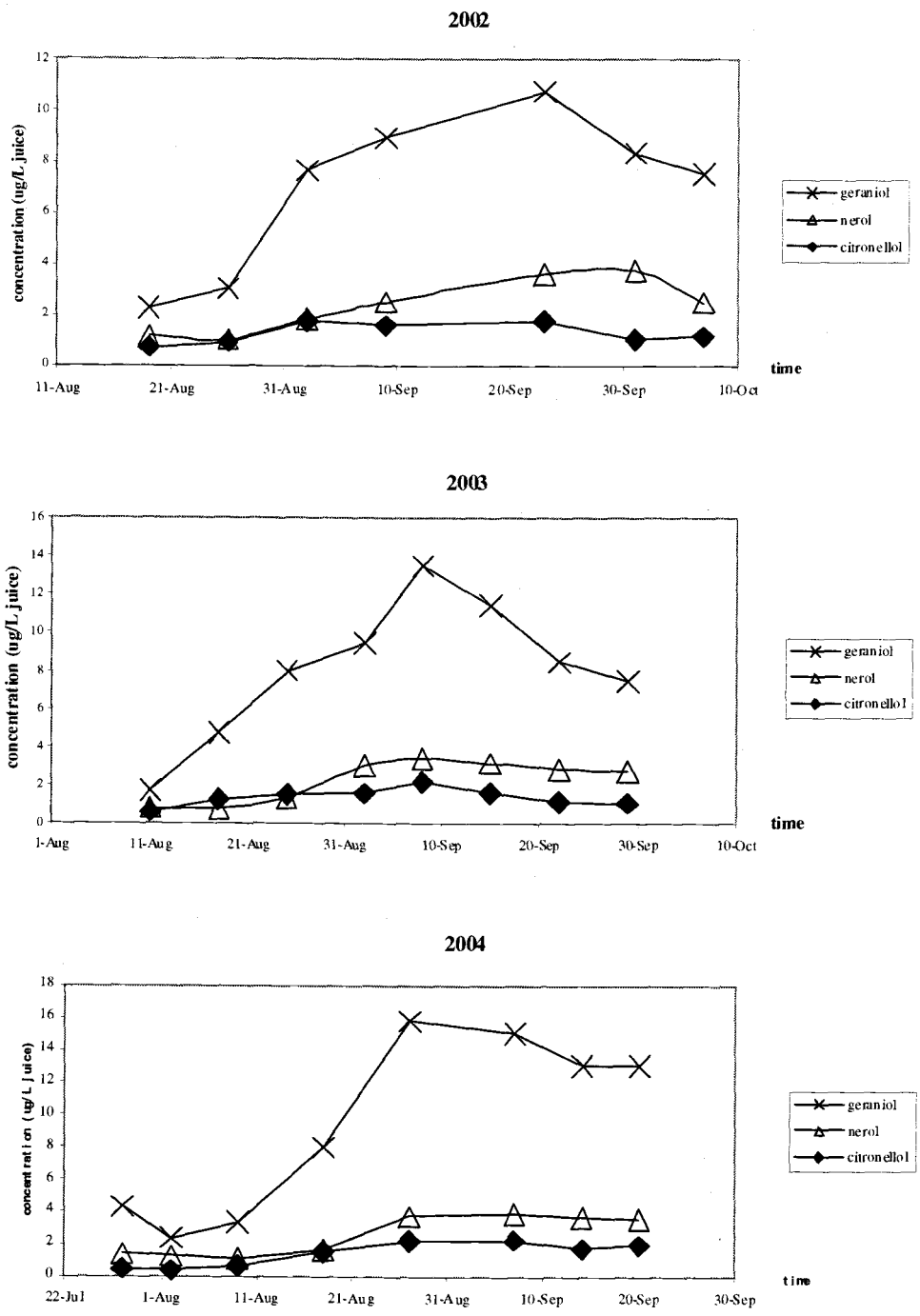

Figure 5.2 The development of free form of monoterpenes in grapes during 2002, 2003, and 2004

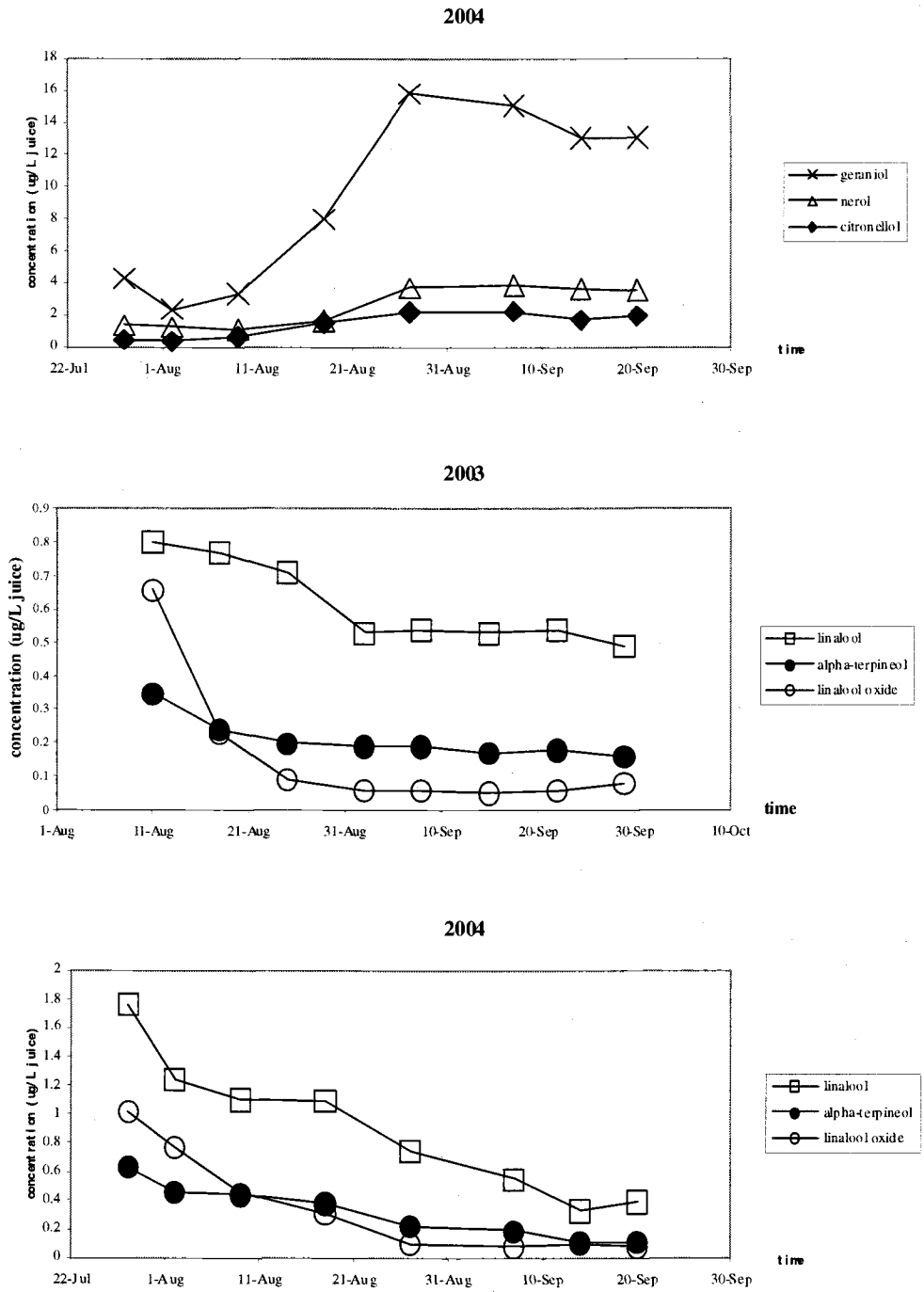

Figure 5.2 (Cont.) The development of free form of monoterpenes in grapes during 2002, 2003, and 2004

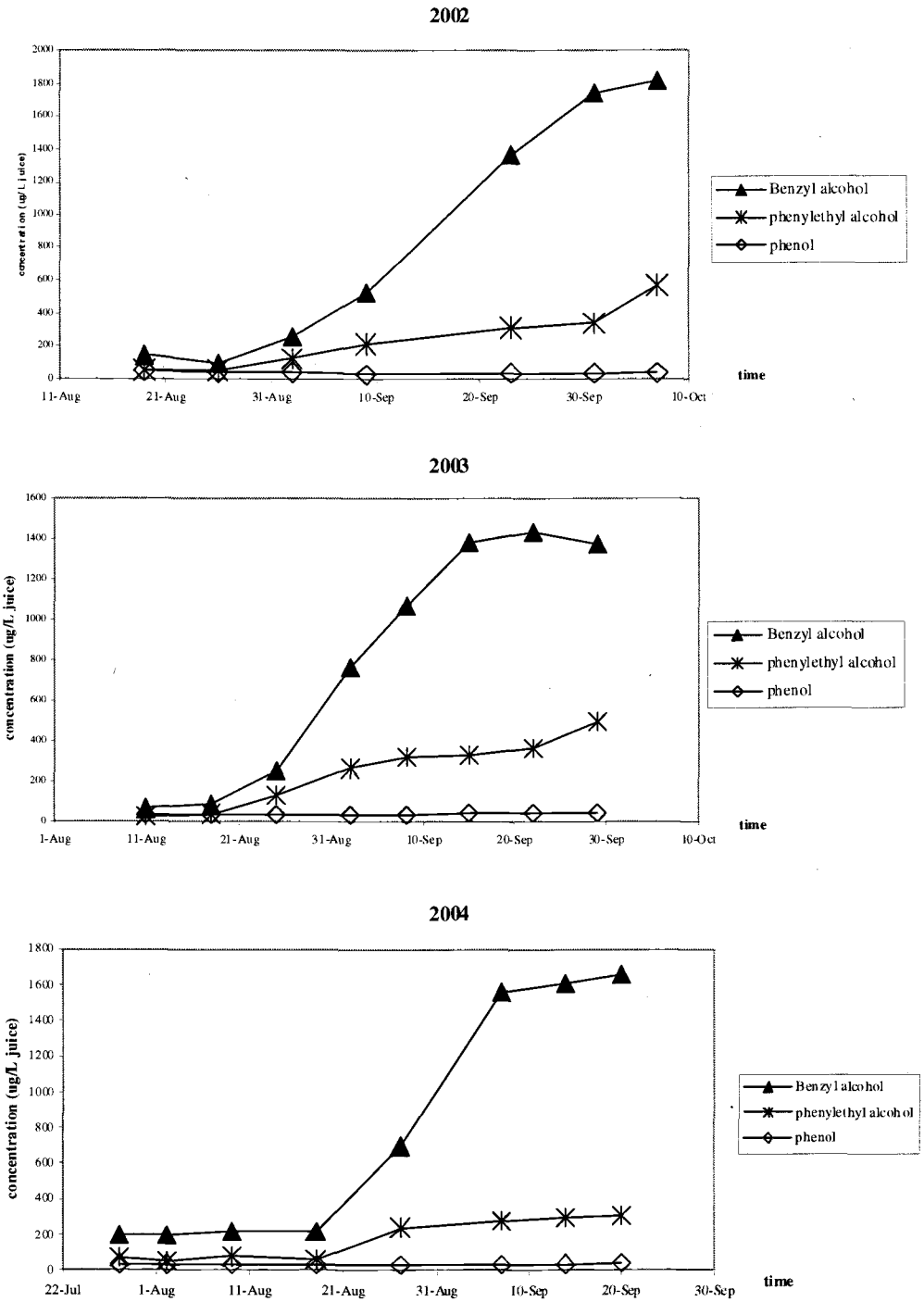

Figure 5.3. The development of free form of phenol, benzyl alcohol and phenylethyl alcohol in grapes during 2002, 2003, and 2004

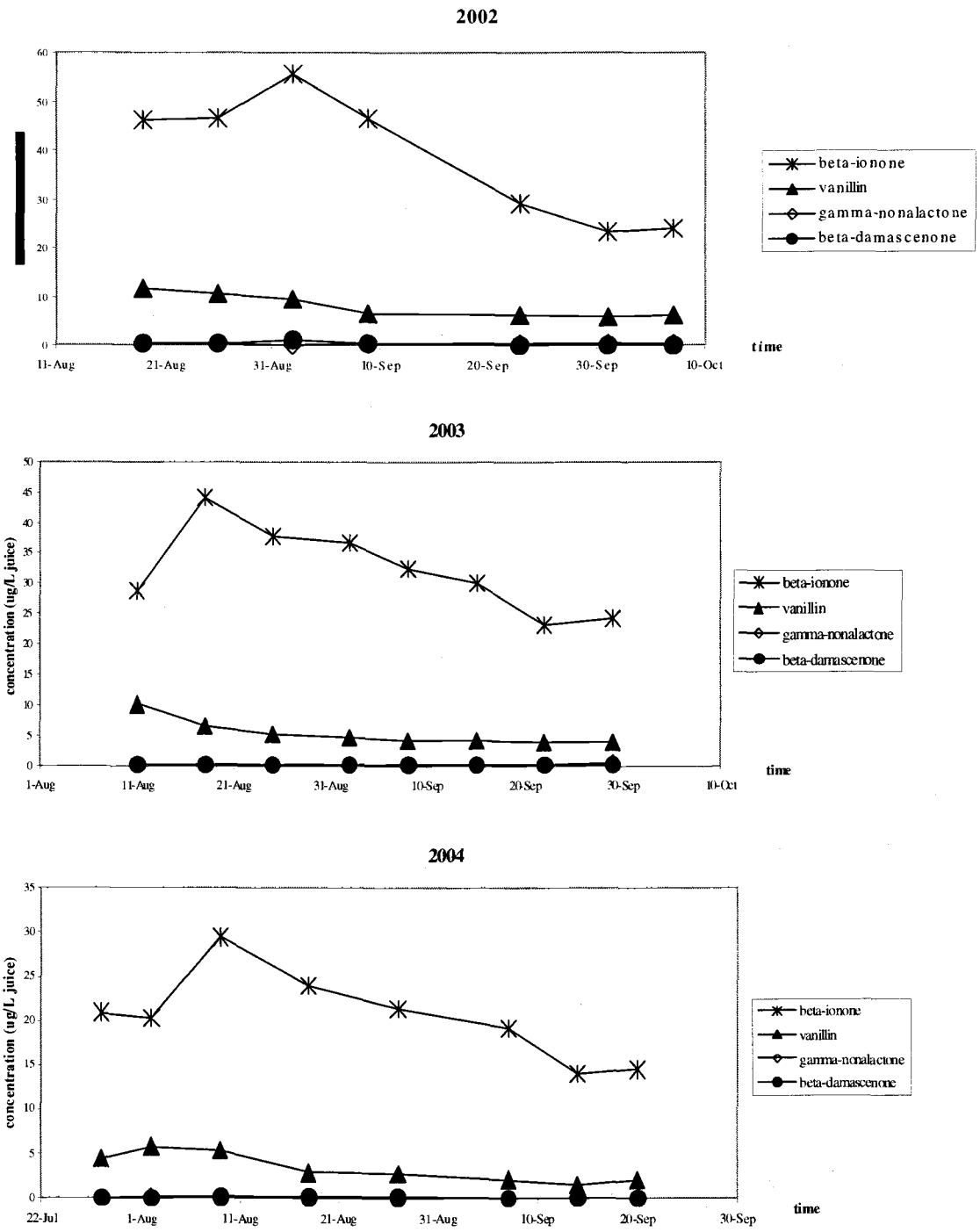

Figure 5.4. The development of free form of β-damascenone, β-ionone, γ-nonalactone, and vanillin in grapes during 2002, 2003, and 2004

Chapter 6. Analysis of Glycoside Bound Aroma Precursors in Pinot noir Grapes by Enzyme-Acid Hydrolysis Followed by Stir Bar Sorptive Extraction-Gas Chromatography-Mass Spectometry

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6.1 Abstract:

Pinot noir grape berry samples were collected during the growing seasons of 2002, 2003, and 2004. The development of bound aroma compounds in Pinot noir grape juice was investigated using stir bar sorptive extraction-gas chromatography-mass spectrometry (SBSE-GC-MS) after enzymatic and acidic hydrolysis. The results showed that the amount of C13-norisoprenoids released from bound precursors was more than ten times the amount of the free form in juices, and these compounds dramatically increased during grape maturation. Vanillin and methyl vanillate as well as γ-nonalactone also showed similar trends. Benzenoid compounds (phenol, benzyl alcohol, and phenylethyl alcohol) decreased in the very early stage, and increased during later stages of maturation. However, bound monoterpenes decreased during grape development, though this decrease could be stopped or reversed in the late stage, which is not consistent with studies in other grape cultivars.

6.2 Key words:

Grape development, aroma precursor, Pinot noir grape, stir bar sorptive extraction (SBSE)

6.3 Introduction:

Quality wines have different flavor properties, which often depend on varietal characteristics. Their typical flavor is mainly due to aroma compounds that are present in the grapes, whether they are in free volatile form or in bound form [73]. Therefore, for making high quality wine, it is very important to use grapes with specific desirable characteristics.

During ripening, grape berry quality generally reaches a peak and then declines as they become overripe. It is at this peak, or optimum stage, of maturity that winemakers aim to harvest the fruit. However, it is still a challenge to determine precisely when the optimum is reached. Although the final judgment is the

subjective assessment of tasting the end product, this is too late, too slow and costly. Tasting fruit to evaluate maturity may work only within very confined limits, mostly because sweetness, acidity, and astringency can be tasted, while many flavor and aroma compounds are locked up as non-volatile glycosides and are only released during the winemaking process [53]. Therefore, the grape aroma in both free and bound form is critical to both the grape grower and the wine maker.

A preliminary sensory evaluation of different stages of wine showed that wines from the late harvest grapes had more complex aroma with more floral, more dried fruit and more oak-like aroma, while the early stage wines showed the highest fresh fruity aroma. Instrumental analysis has shown that grape maturity (harvest date) significantly affects some key aroma compounds in wine [223]. However, the relationship between grape development and wine aroma is still unclear.

The development of free form aroma compounds in Oregon Pinot noir were investigated by stir bar sorptive extraction- gas chromatography- mass spectrometry (SBSE-GC-MS) [230]. In the current experiment, the bound aroma compounds were released from their precursors in grape juice by a quick enzyme-acid hydrolysis. These hydrolyzed aroma compounds were then quantified by SBSE-GC-MS. Our objective is to investigate the development of glycoside bound aroma compounds during grape growing, which will further help to understand the relationship between grape maturity and wine aroma.

6.4 Materials and Methods:

6.4.1 Chemicals and Materials:

All chemical standards and internal standards were purchased from Sigma-Aldrich (St. Louis, MO), and organic solvents (pentane, dichloromethane, methanol) were obtained from VWR scientific (West Chester, PA). Sodium hydroxide was bought from J.T.Baker (Philipsburg, NJ), citric acid from Staley Manufacturing Company, (Decatur, IL), and sodium chloride from VWR International (West Chester, PA).

A citrate buffer solution (0.2 M) was prepared by dissolving 42g of citric acid

in 1L of Milli-Q water (Continental Water System, Millipore Corporation, Billerica, MA), which was adjusted to pH 3.1 by sodium hydroxide. Standard stock solutions (about 1000 ppm) were prepared in ethanol individually and stored at -15°C. Before analysis, certain amounts of stock solutions were added to buffer solution to make the first level mixed standard solution and diluted at 4:1(v/v), 3:2 (v/v), 2:3 (v/v) and 1:4 (v/v) ratios with buffer solution to give a range of concentrations.

An internal standard solution was made by dissolving 1.93ppm of octyl propanoate, 0.55ppm of *trans*-carveol, and 0.94ppm *trans*-2-nonenal in ethanol, and stored at -15°C.

The Macer8^{TM} FJ enzyme solution was provided by Biocatalysts Limited (Wales, UK), which contained a balanced mix of pectinases and pectin lyase.

6.4.2 Grape sampling and juice preparation:

Pinot noir grapes were grown at the Oregon State University experimental vineyard located in Alpine, OR. During the growing seasons of 2002, 2003 and 2004, eight grape berry samples were collected yearly as described previously [224, 230]. While sampling, ten clusters were randomly picked in the vineyard and were immediately frozen at –29°C. Berries were destemmed while frozen, and then kept in a glass jar at –23°C. Prior to analysis, the juice was prepared as follows. About 200g of grape berries were thawed at 4°C overnight and then ground using a commercial blender (Waring Products Division, New Hartford, CT). After settling for 5 min, skins and seeds were separated from the juice using cheese cloth, and then the grape juice was filtered under vacuum through filter paper (VWR Scientific, West Chester, PA).

6.4.3 Isolation of glycosides and enzyme hydrolysis:

Isolation of the glycoside compounds from the grape juice, obtained as described above, was achieved using BAKERBOND^{TM} SPE Octadecyl (C18) disposable extraction columns (J.T.Baker, Philipsburg, NJ) as reported previously [231]. Each C18 column was pre-conditioned with 10ml of methanol, then with

10ml of Milli-Q water (Continental Water System, Millipore Corporation, Billerica, MA). Five mL of filtered grape juice was loaded onto the C18 cartridge. The cartridge was washed with 10ml of Milli-Q water and then with 6ml of pentane/dichloromethane (2:1, v/v). The glycoside extracts were finally eluted from the cartridge with 6ml of methanol into a 40mL vial, and concentrated to dryness at 45°C under vacuum. Twenty mL of 0.2 M citrate buffer solution and 100 µl of Macer8™ FJ enzyme solution were added into the glycoside extracts obtained from each C18 cartridge. The mixture incubated at 45°C for 24 hours.

6.4.4 Analysis of hydrolyzed volatiles by SBSE-GC-MS:

After enzyme hydrolysis, the mixture was cooled at room temperature, and 6 g of sodium chloride as well as 20 µL of internal standard solution were added to the vial. A pre-cleaned Twister™ stir bar (2 cm × 100 mm, Gerstel Inc., Baltimore, MD) was put into the hydrolyzed mixture. The stir bar was constantly stirred in the sample for 3 hours at a speed of 1000 rpm. After extraction, the stir bar was rinsed with Milli-Q water, dried with Kimwipe (Kimberly-Clark Professional Inc, Roswell, GA) tissue paper, and placed into the glass sample holder of the thermal desorption system (TDS) autosampler tray (Gerstel, Inc., Baltimore, MD).

The analytes were thermally desorbed in the thermal desorption unit (TDU; Gerstel Inc.) in splitless mode. The TDU temperature ramped from 35°C to 300°C at a rate of 700°C/min, and hold at the final temperature for 3 min. An Agilent GC-MS system (Agilent 6890 GC coupled with an Agilent 5973 MS; Agilent Technologies, Little Falls, DE) was used for analysis extracts. To cryfocus the desorbed analytes, a programmed temperature vaporizing (PTV) injector (CIS 4, Gerstel Inc.) with liquid nitrogen was used with the solvent vent injection mode. The PTV temperature was programmed from -60°C to 250°C at a rate of 10°C/sec and kept at 250°C. The venting flow was 25 mL/min, the venting pressure was 10 psi, and the venting valve was closed after 0.01min. A ZB-FFAP capillary GC column (30m, 0.32mm ID, 0.25µm film thickness; Phenomenex, Torrance, CA) was employed to separate the analytes. The column carrier gas was helium at 2 mL/min. The initial oven

temperature was set as 40°C for 2min, then increased at 6°C/min to 180°C, further increased at 4°C/min to 240°C, and held at this temperature for 20min. The electron impact (EI) energy was 70eV, and the ion source temperature was set at 230°C. Enhanced ChemStation software (GCA v.D.00.01.08, Agilent Technologies Inc.) was used for data acquisition and analysis.

6.4.5 Calibration and quantification of volatiles:

The calibration curves were built up as in previous work [130]. Adding 6g of sodium chloride and 20 μL of internal standard solution, the standard mixture as well as 4:1(v/v), 3:2 (v/v), 2:3 (v/v), and 1:4 (v/v) dilutions in buffer solution were analyzed using SBSE-GC-MS as described above. The MS analysis was carried out in the single ion-monitoring (SIM) mode to avoid the interferences between coeluting compounds. Then the calibration curve for individual target compounds was ploted the selected ion MS responce ratio of target compounds with their respective internal standard against the concentration ratio. The calibration equation and their regression coefficients (R^2) were calculated using the ChemStation data analysis software, and all calibaration curves were forced to pass through the origin (0,0).

The amounts of target compounds in the hydrolyzed solution were calculated based on the calibration curves individually, and then converted to the concentrations in original grape juices. Triplicate analysis was performed on all samples.

6.5 Results and Discussion:

Most grape aroma compounds are present in the grape either as free volatiles, which may contribute directly to odor, or as non-volatile bound sugar conjugates. The bound sugar conjugates, or glycosides, are nonvolatile and, for the most part, represent aroma precursors. They can undergo acid or enzyme hydrolysis, releasing free volatiles and potentially enhancing aroma [80]. Research by Francis and co-workers compared the effect of hydrolysis conditions on the aroma compounds released from grape glycosides [90, 232]. Based on sensory descriptive analysis,

they found that hydrolysis catalyzed by only enzymes had no detectable effect on aroma, whereas acid-catalyzed hydrolysis produced sensory properties similar to those of bottle aged wines. Therefore, in this study, enzyme combined with mild acid (pH=3.1) hydrolysis was investigated as a way to release the bound aroma compounds from glycoside extracts in grape juice.

Grape glycoside precursors generally released a wide range of compounds, which represent, in part, the potential aroma of a grape variety. These compounds include monoterpenes, C13-norisoprenoids and volatile phenols, which were the main target compounds in this experiment. The quantification method for these target compounds in grape juice was previously developed using SBSE-GC-MS for the analysis of free form aroma compounds. Since the same citrate buffer solution was used in this experiment as that for making the calibration curves, the method is also suitable to quantify compounds in the hydrolyzed solution.

A total of 19 aroma compounds were quantified in the hydrolyzed solution. Tables 1, 2 and 3 presented the concentration of those compounds that are released from bound precursors in Pinot noir grape juices from the 2002, 2003, and 2004 growing seasons.

Throughout the development, grapes produce pigments. The most important class of pigment, in terms of wine quality, is the carotenoids. They are accumulated early on in berry development to protect berry tissues from oxidative stress, and appear to be converted to potent wine aroma components after veraison. The degradation of carotenoids can form C13-norisoprenoids, two of which have been identified as key aroma compounds in wine, β-damascenone ('rose' or 'exotic fruit' aroma) and β-ionone ('violet' or 'raspberry' odor) [50, 232, 233]. 3-Hydroxy-β-damascenone was also considered as an important bound aroma compound in grapes [234]. However, this compound has not been reported in wines, probably since it can be converted to β-damascenone during winemaking.

Figure 1 shows the development of bound β-damascenone, β-ionone, and γ-nonalactone during 2002, 2003, and 2004. Compared to free form [230], about 10~100 times more β-damascenone was hydrolyzed from bound precursors in the

same grape juices, and its amount was dramatically increased along with grape development, even when the grapes were sampled close to harvest time. These results are consistent with literature [94]. Similar trends were also found for β-ionone and γ-nonalactone. However, the concentration of bound β-ionone is less than its free form in juice. This indicated that unlike other C13-norisoprenoids, the glycoside precursor of β-ionone is not the major form present in grapes.

In our previous study, it was found that wines made with late stage grapes contained more β-damascenone, β-ionone and γ-nonalactone than those made with the early stage ones [223]. Our results confirmed that this difference is mainly dependent on the increase of bound aroma precursors during grape maturation. Therefore, late harvested Pinot noir grapes can produce wine with more "exotic fruit", "raspberry", and "coconut" aroma, which is associated with these three compounds.

Vanillin was generally considered an oak-related odorant, and can be generated due to hydrolysis during storage [96]. The results showed that the bound precursor of vanillin increased during the grape development. Therefore, grapes of different maturity may generate different levels of vanillin, even under the same wine aging process. A similar trend was found for methyl vanillate.

Monoterpenes belong to the secondary plant constituents, of which the biosynthesis begins with acetyl-coenzyme A (CoA) [81]. They are largely present in the skins of grapes and glycoside precursors are most abundant form [82], which varies with different varieties of grapes (0-1 mg/L) [83]. About 10 μg/L juice of geraniol was hydrolyzed from glycoside precursors, which is the most abundant monoterpene released from hydrolysis.

It has been reported that the content of various monoterpenes change during bottle storage or during the maturation of wine by acid catalyzed reaction [89]. During the maturation of wine, linalool can be transformed in an aqueous acid medium to α-terpineol by cyclization, to hydroxyl-linalool through hydration in the seventh position and to geraniol and nerol by a nucleophilic 1,3-transition. Since an average wine pH (3.1) was used in the hydrolysis procedure, these acid

rearrangement reactions may occur.

Figure 2 shows the changes of six kinds of monoterpenes during three years of grape development. All monoterpenes decreased in the early stage of grape development, while they showed no or little changes in the late stage. A slight increase of nerol was observed when the grape matured. However, these results disagreed with most found in the literature [227, 235].

One reason may be that the results are presented as the concentration in grape juice, rather than the concentration in grape berries. Berry growth is mostly due to water increase, where the juice yield before veraison is much lower than that close to harvest. Therefore, in the early stage, berry volume increases so quickly that aroma precursors in juices become diluted. In the later stage, berry volume development slows down, and the aroma precursors begin to increase, so the concentrations of these compounds increase or stay at the same level.

The thing may similarly happen to the benzenoid compounds (phenol, benzyl alcohol, and phenylethyl alcohol). Phenylethyl alcohol decreased in the very early stage of grape growing, and then increased in the late stage (Figure 3). However, this compound can be generated from yeast during fermentation [67], so the initial concentration in grapes is not as critical for wine makers. Similar trends were also found for phenol and benzyl alcohol.

Moreover, enzymatic hydrolysis of monoterpenes involves two steps [82]. In the first step, an α-L-rhamnosidase and an α-L-arabinofuranosidase or a β-apiofuranosidase (depending on the structure of the aglycone moiety) cleave 1,6-glycosidic linkages. In the following step, the monoterpenes are liberated from monoterpenyl β-D-glucosides by the action of a β-glucosidase [54]. Therefore, the hydrolysis efficiency of enzymes is mainly based on the desired enzyme properties [85-87]. Besides enzymatic hydrolysis, acidic hydrolysis can also be used to release the monoterpenes from their precursors in grapes. It has been confirmed that the progressive release of aroma with long periods of mild acid hydrolysis is reflected in the increase in intensity of the same aroma attributes in wines undergoing natural aging or mild heating [90]. However, it should noted that long periods (more than

two weeks) of mild acid hydrolysis reactions are generally used in most research [88, 91, 92]. In this experiment, short time and non-specific enzymes were used for hydrolysis, so bound monoterpenes may not be completely released from glycoside precursors. Therefore, complete hydrolysis using other conditions is suggested to confirm these results.

In conclusion, the developments of bound aroma compounds in Pinot noir grapes were investigated in this study. Most precursors of bound aroma compounds increased along with grape maturity, except those of bound monoterpenes. These results as well as those from the previous free form study helped to explain why the late stage wines contained more grape-derived aroma compounds than early stage wines, and can help further guide winemakers to select the optimum harvest time for Pinot noir grapes. However, further study is suggested to confirm the findings in this experiment.

6.6 Acknowledgement:

We would like to thank the Oregon Wine Advisory Board for funding this project. Thanks also to the OSU department of Horticulture for providing grape samples in this study. Specifically, thanks to Jose Pastor for assistance in sample collection.

Table 6.1. The concentration (μg/L juice) of bound aroma compounds in grapes during 2002

	8/19/2002	8/26/2002	9/2/2002	9/9/2002	9/23/2002	10/1/2002	10/7/2002
Alcohols							
Linalool	7.41±0.04	6.40±0.06	4.81±0.04	3.47±0.03	3.47±0.05	3.15±0.03	3.42±0.01
Nerol	3.53±0.05	3.05±0.08	2.96±0.03	1.81±0.02	2.02±0.02	2.07±0.01	2.15±0.01
Geraniol	21.04±0.04	18.51±0.06	12.97±0.02	7.70±0.01	7.09±0.04	6.22±0.01	6.50±0.01
Eugenol	14.56±0.02	11.14±0.06	7.68±0.02	6.50±0.01	4.74±0.05	3.26±0.02	3.56±0.01
Methoxyeugenol	16.45±0.01	16.06±0.05	11.40±0.01	10.85±0.01	10.59±0.06	11.59±0.03	11.07±0.01
Citronellol	7.90±0.02	8.27±0.04	3.47±0.01	1.86±0.01	1.29±0.05	1.18±0.03	1.01±0.09
Linalool oxide	4.23±0.03	2.54±0.03	2.15±0.02	1.78±0.03	1.85±0.05	1.70±0.04	1.99±0.01
α-Terpineol	9.71±0.01	7.44±0.01	5.27±0.01	4.57±0.01	5.99±0.01	4.70±0.01	4.95±0.01
Phenol	92±3	62±24	52±5	76±9	75±13	98±14	131±3
Benzyl alcohol	2,679±3	1,653±30	1,409±18	1,351±8	1,802±15	1,952±15	2,065±11
Phenylethyl alcohol	414±2	304±11	236±13	242±17	243±16	261±11	333±5
1-Octen-3-ol	1.55±0.04	1.12±0.04	0.75±0.01	0.59±0.01	1.04±0.01	1.33±0.02	1.74±0.01
Ketones & Aldehydes							
β-Damascenone	1.15±0.04	1.91±0.04	8.19±0.01	11.65±0.02	15.44±0.02	16.12±0.01	17.22±0.02
3-Hydroxy-beta-damascenone	< 0.1	0.18±0.05	0.17±0.01	0.19±0.04	0.19±0.05	0.18±0.01	0.22±0.02
β-ionone	7.27±0.03	8.80±0.24	12.02±0.25	14.92±0.01	15.11±0.24	20.08±0.12	24.55±0.05
γ-Nonalactone	1.18±0.05	1.18±0.04	1.14±0.02	1.14±0.01	1.75±0.06	2.23±0.01	2.91±0.01
Vanillin	27.5±1.4	27.4±0.9	34.5±4.2	35.7±0.5	38.7±1.9	45.2±2.7	52.2±0.3
Esters							
Methyl vanillate	61.1±0.1	47.1±0.1	80.5±0.0	107.2±0.1	163.7±0.1	159.3±0.2	150.4±0.1
Ethyl vanillate	ND*	ND	ND	ND	0.16±0.06	0.27±0.08	0.57±0.10

* ND: not detected

Table 6.2. The concentration (μg/L juice) of bound aroma compounds in grapes during 2003

	8/11/2003	8/18/2003	8/25/2003	9/2/2003	9/8/2003	9/15/2003	9/22/2003	9/29/2003
Alcohols								
Linalool	5.31±0.03	6.06±0.02	3.68±0.13	3.24±0.00	3.22±0.05	3.51±0.06	3.09±0.02	3.73±0.03
Nerol	3.21±0.09	3.30±0.01	2.15±0.07	1.87±0.03	2.06±0.02	1.89±0.05	2.00±0.03	2.74±0.03
Geraniol	17.97±0.05	19.95±0.01	8.86±0.15	7.46±0.03	8.02±0.02	7.74±0.06	6.85±0.03	8.42±0.04
Eugenol	9.10±0.02	8.22±0.01	4.48±0.14	3.87±0.08	2.94±0.01	2.84±0.04	3.02±0.03	3.45±0.01
Methoxyeugenol	11.06±0.02	10.19±0.01	9.49±0.06	9.22±0.18	9.06±0.02	9.64±0.09	10.09±0.04	11.74±0.02
Citronellol	4.74±0.04	5.61±0.01	1.64±0.13	1.62±0.09	1.40±0.01	1.19±0.13	1.03±0.02	1.76±0.12
Linalool oxide	2.41±0.04	2.00±0.01	1.45±0.10	1.23±0.04	1.36±0.01	1.29±0.05	1.26±0.03	1.22±0.03
α-Terpineol	6.18±0.01	5.16±0.01	3.55±0.01	3.17±0.01	3.54±0.01	3.35±0.01	3.38±0.01	3.42±0.01
Phenol	64±23	74±7	59±16	61±43	78±15	86±11	103±9	110±9
Benzyl alcohol	1,476±29	1,390±9	1,024±24	1,020±2	1,127±16	1,366±3	1,593±11	1,604±6
Phenylethyl alcohol	324±18	313±1	202±44	195±7	168±9	161±23	201±17	242±4
1-Octen-3-ol	1.34±0.02	1.20±0.01	0.80±0.03	0.85±0.04	0.95±0.02	1.28±0.06	1.31±0.01	2.47±0.01
Ketones & Aldehydes								
β-Damascenone	1.41±0.05	3.28±0.01	12.43±0.04	12.92±0.02	13.92±0.01	14.44±0.02	16.35±0.02	17.71±0.02
3-Hydroxy-beta-damascenone	0.16±0.05	0.21±0.01	0.53±0.14	0.57±0.30	0.64±0.09	0.89±0.08	0.99±0.07	1.39±0.06
β-ionone	16.04±0.11	17.37±0.28	18.52±0.03	19.27±0.49	19.55±0.13	21.78±0.13	21.75±0.04	24.32±0.06
γ-Nonalactone	1.06±0.01	0.82±0.05	1.00±0.04	1.02±0.02	1.29±0.02	2.02±0.04	2.49±0.02	2.63±0.03
Vanillin	39.4±1.2	35.0±1.4	37.9±4.5	36.1±1.0	40.6±1.7	48.2±3.3	57.7±2.3	61.2±4.4
Esters								
Methyl vanillate	32.8±0.2	45.0±0.2	82.4±0.3	85.1±0.5	109.6±0.2	118.9±0.3	115.8±0.0	114.1±0.2
Ethyl vanillate	ND*	ND	ND	ND	ND	0.07±0.02	0.16±0.05	0.82±0.13

* ND: not detected

Table 6.3. The concentration (μg/L juice) of bound aroma compounds in grapes during 2004

	7/28/2004	8/2/2004	8/9/2004	8/18/2004	8/27/2004	9/7/2004	9/14/2004	9/20/2004
Alcohols								
Linalool	13.26±0.02	11.95±0.01	10.70±0.01	8.55±0.05	4.39±0.03	3.17±0.03	3.11±0.02	3.77±0.01
Nerol	4.66±0.02	4.05±0.02	3.81±0.03	3.67±0.07	1.88±0.03	1.54±0.02	1.79±0.02	1.89±0.03
Geraniol	36.99±0.03	33.66±0.03	30.32±0.01	25.44±0.05	9.79±0.02	6.51±0.02	6.65±0.01	7.53±0.02
Eugenol	9.72±0.01	7.90±0.02	6.14±0.01	5.76±0.02	2.95±0.02	2.30±0.03	2.77±0.01	3.26±0.03
Methoxyeugenol	10.74±0.02	9.59±0.04	8.74±0.00	7.13±0.02	6.05±0.01	4.66±0.04	5.93±0.01	6.54±0.05
Citronellol	12.15±0.02	11.57±0.01	11.42±0.01	7.35±0.04	1.81±0.01	1.16±0.02	1.42±0.03	1.36±0.02
Linalool oxide	6.16±0.03	4.86±0.04	4.48±0.02	3.52±0.02	2.42±0.02	1.99±0.03	1.91±0.02	2.12±0.03
α-Terpineol	13.36±0.01	11.04±0.01	12.75±0.01	8.00±0.01	5.34±0.01	4.41±0.01	4.24±0.01	4.52±0.01
Phenol	85±1	83±4	90±7	66±11	69±10	72±16	99±4	92±5
Benzyl alcohol	3,024±14	2,456±11	2,238±11	1,964±7	1,779±1	1,774±9	2,150±1	2,281±6
Phenylethyl alcohol	445±6	387±7	399±4	320±1	192±5	138±14	161±3	194±6
1-Octen-3-ol	1.43±0.01	1.35±0.05	1.28±0.02	0.76±0.01	0.58±0.01	0.65±0.01	0.96±0.01	1.14±0.02
Ketones & Aldehydes								
β-Damascenone	1.75±0.02	1.76±0.04	3.42±0.03	6.10±0.02	12.00±0.02	12.04±0.01	12.11±0.02	13.10±0.03
3-Hydroxy-beta-damascenone	0.12±0.01	0.11±0.04	0.13±0.07	0.18±0.01	0.28±0.14	0.33±0.10	0.38±0.18	0.44±0.16
β-ionone	10.11±0.04	11.00±0.22	13.43±0.26	13.32±0.11	14.13±0.55	14.28±0.10	15.43±0.07	16.96±0.10
γ-Nonalactone	1.26±0.06	1.39±0.03	1.51±0.03	1.40±0.02	1.36±0.01	1.50±0.04	1.62±0.03	2.14±0.06
Vanillin	30.9±3.0	34.0±1.2	35.5±2.4	35.2±1.0	37.1±1.3	39.7±1.8	40.4±1.9	48.8±2.3
Esters								
Methyl vanillate	70.8±0.4	54.6±0.2	61.5±0.2	92.5±0.3	91.4±0.1	106.0±0.2	111.1±0.1	102.7±0.1
Ethyl vanillate	ND	ND	ND	ND	ND	ND	ND	0.12±0.02

* ND: not detected

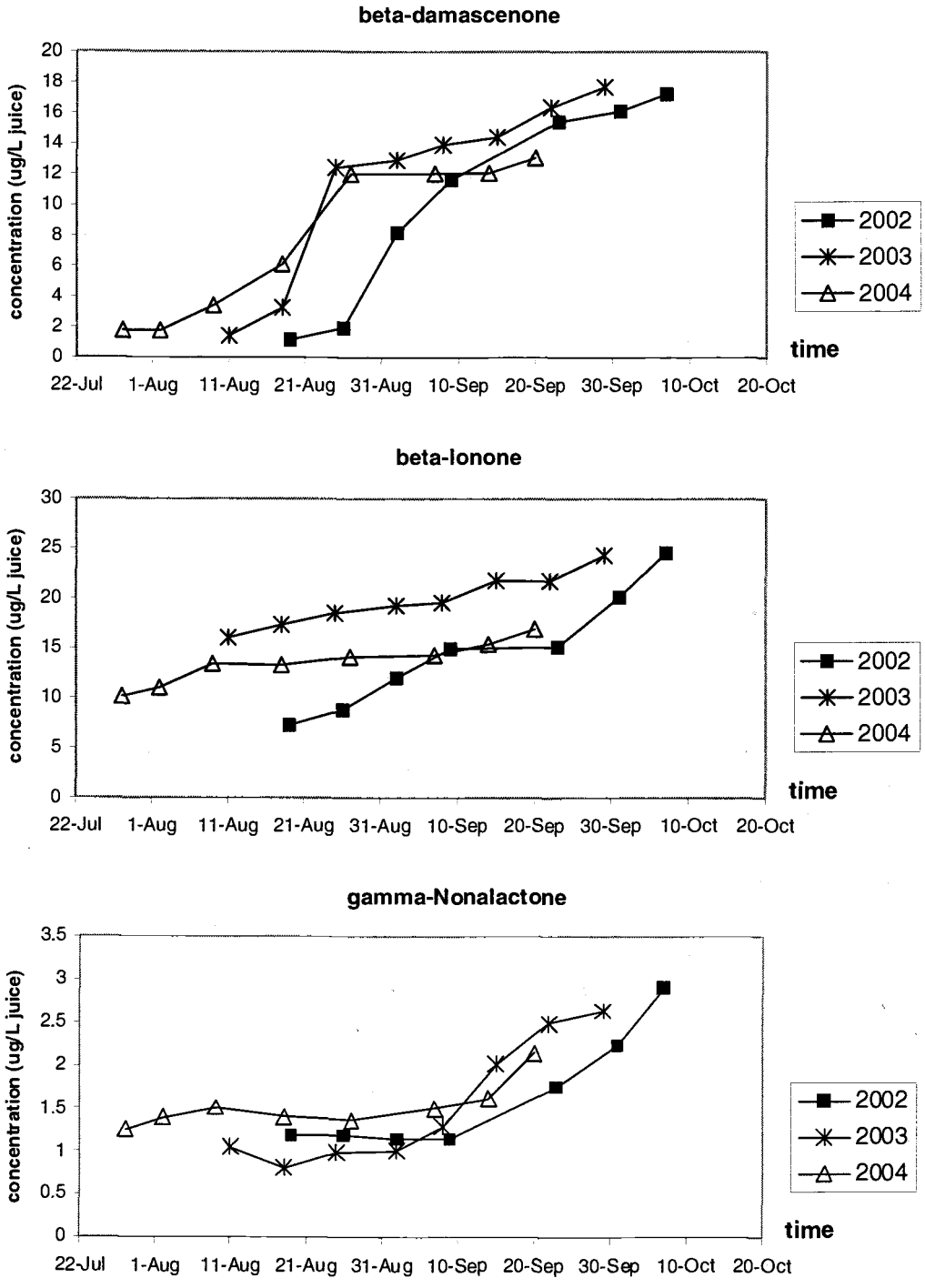

Figure 6.1. The development of bound β-damascenone, β-ionone, γ-nonalactone in Pinot noir grapes during 2002, 2003, and 2004

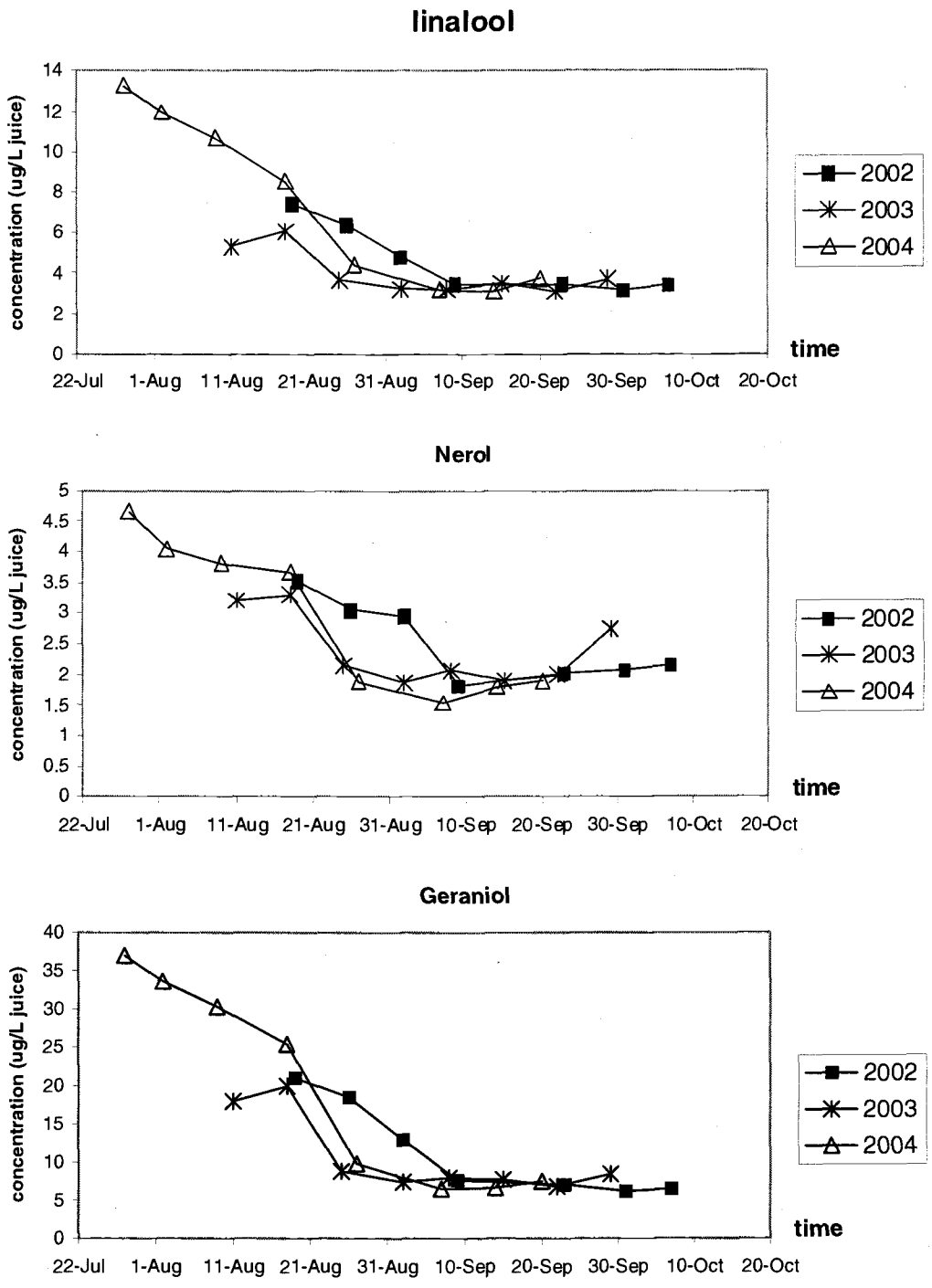

Figure 6.2. The development of bound monoterpenes in Pinot noir grapes during 2002, 2003, and 2004

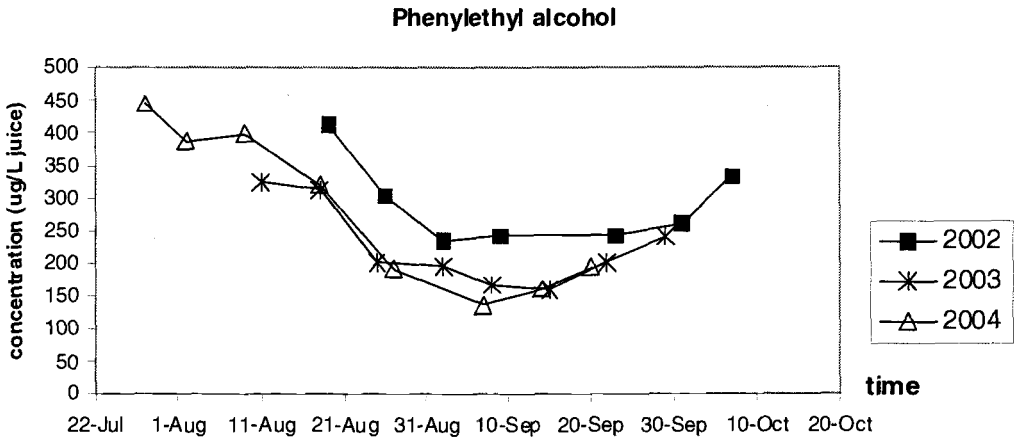

Figure 6.3. The development of phenylethyl alcohol in Pinot noir grapes during 2002, 2003, and 2004

Chapter 7. Sensitive quantification of sulfur compounds in wine by headspace solid-phase microextraction technique

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7.1 Abstract

A sensitive solid-phase microextraction and gas chromatography-pulsed flame photometric detection technique was developed to quantify volatile sulfur compounds in wine. Eleven sulfur compounds, including hydrogen sulfide, methanethiol, ethanethiol, dimethyl sulfide, diethyl sulfide, methyl thioacetate, dimethyl disulfide, ethyl thioacetate, diethyl disulfide, dimethyl trisulfide and methionol, can be quantified simultaneously by employing three internal standards. Calibration curves were established in a synthetic wine, and linear correlation coefficients (R^2) were greater than 0.99 for all target compounds. The quantification limits for most volatile sulfur compounds were 0.5 ppb or lower, except for methionol which had a detection limit of 60 ppb. The recovery was studied in synthetic wine as well as Pinot noir, Cabernet Sauvignon, Pinot Grigio, and Chardonnay wines. Although the sulfur compounds behaved differently depending on the wine matrix, recoveries of greater than 80% were achieved for all sulfur compounds. This technique was applied to analyze volatile sulfur compounds in several commercial wine samples; methionol concentrations were found at the ppm level, while the concentrations for hydrogen sulfide, methanethiol, and methyl thioacetate were at ppb levels. Only trace amounts of disulfides and trisulfides were detected, and ethanethiol was not detected.

7.2 Keywords:

Volatile sulfur compound, quantification, wine off-flavor, SPME, pulsed flame photometric detection

7.3. Introduction

Volatile sulfur compounds are known to have very powerful and characteristic odors, and these compounds can contribute to pleasant or unpleasant aromas of a wine according to their nature and concentration [128]. Usually when volatile sulfur compounds are present at very low concentrations, they contribute a

positive impression to the wine aroma [129]. However, when present at higher concentrations, they are responsible for "reduced", "rotten egg", or "sulfury" off-flavors [130]. Balancing the two can be a significant challenge to winemakers, since many factors such as deficiencies of nutrients (amino acids and vitamins), yeast strains, metal ions, redox potential, and fermentation temperature, can all influence the formation of volatile sulfur compounds [139]. The mechanisms that form these compounds are still poorly understood, which is partially because there is no sensitive, reliable analytical method available to measure them. For this reason, it has become increasingly important to develop a quick and reliable analytical method to quantify volatile sulfur compounds in wine.

Sulfur compounds are present in trace amounts in wine, therefore a pre-concentration step is required before chromatographic analysis [18]. Solvent extraction [113, 114] and static headspace extraction [115, 116] have been widely used for volatile extraction, but time consumption and lack of sensitivity are the two major downfalls to limit their application for sulfur analysis in wine. In addition, some sulfur compounds are extremely volatile and chemically reactive so it is impossible to use traditional technique to enrich them.

As an alternative to traditional pre-concentration methods, solid-phase microextraction (SPME) has been successfully used to extract volatile compounds, including sulfur compounds, from the headspace of various samples [11-16]. SPME technique has been previously used to analyze volatile sulfur compounds in wines [117-120], but quantification has not been successful due to the challenges involved with sulfur compounds as well as competitive adsorption [17]. A SPME extraction coupled with stable isotope dilution assay was successfully developed to analyze ethanethiol and diethyl disulfide in Sarah wine [121, 122], but this technique is time-consuming. Moreover, not all important volatile sulfur compounds, such as hydrogen sulfide and methanethiol, could be quantified by this method.

Due to low concentrations in food, sulfur compounds are typically analyzed by gas chromatography (GC) with sulfur-specific detection, including flame photometric detection (FPD) [115, 116], chemiluminescent detection (SCD) [123]

and atomic emission detection (AED). Recently, pulsed flame photometric detection (PFPD) has proven to be very sensitive for sulfur compounds [16, 124-126]. This technique uses a pulsed flame, rather than a continuous flame as with traditional FPD, to achieve the generation of flame chemiluminescence [127]. With PFPD, light emissions due to hydrocarbons and flame background can be ignored during each pulse of the flame by electronically gating the emission, allowing for only the sulfur portion of the spectrum to be integrated, thereby greatly increasing the selectivity and sensitivity for this detector.

In this study, a quick, sensitive method was developed to quantify the trace amounts of volatile sulfur compounds in wines by SPME and GC-PFPD. Parameters for SPME extraction were optimized to increase sensitivity, and highly reactive sulfur compounds were stabilized during the analysis. The technique was used to measure the concentrations of volatile sulfur compounds in several commercial wines.

7.4. Experimental

7.4.1. Chemicals

Sodium sulfide, methanethiol (MeSH), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and isopropyl disulfide (IsoProDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanethiol (EtSH), diethyl sulfide (DES), methyl thioacetate (MeSOAc), ethyl thioacetate (EtSOAc), 3-methylthiopropanol (methionol), and 4-methylthiobutanol were obtained from Johnson Mattey Catalog Company Inc. (Ward Hill, MA, USA). Ethyl methyl sulfide (EMS), dimethyl sulfide (DMS), diethyl disulfide (DEDS) were supplied by TCI America (Portland, OR, USA). Methanol and L-tartaric acid were obtained from J.T. Baker (Phillipsburg, NJ, USA), and the ethanol was from AAPER Alcohol and Chemical Co. (Shelbyville, KY, USA).

7.4.2. SPME extraction condition

An automatic headspace sampling system (CombiPAL autosampler equipped

with a SPME adapter, from CTC Analytics, Zwingen, Switerland) with an 85μm Carboxen-PDMS StableFlex SPME fiber (SUPELCO, Bellefonte, PA, USA) was used for extraction of sulfur compounds. Five milliliters of samples were placed in 20 ml autosampler vials. The vials were tightly capped with Teflon-faced silicone septa, and placed in an automatic headspace sampling system. The SPME conditions were set as following: samples were equilibrated at 30°C for 30 min with 500 rpm agitation; and extracted for 15 min with 250 rpm agitation (on for 8s, off for 2s) at the same temperature.

7.4.3. Detection of volatile sulfur compound by GC-PFPD

The analyses were made on a Varian CP-3800 gas chromatography equipped with a PFPD detector (Varian, Walnut Creek, CA, USA) operating in sulfur mode. After extraction, the SPME fiber was directly injected into the GC injection port with the splitless mode at 300°C and kept for 7 min. The separation was performed using a DB-FFAP capillary column (30 m × 0.32 mm I.D., 1 μm film thickness, from Agilent, Palo Alto, CA, USA). The oven temperature was programmed as follows: 35°C (initial hold 3 min), ramp at 10°C/min to 150°C (hold for 5 min), and then ramp at 20°C/min to 220°C (final hold 3 min). The carrier gas was nitrogen with a constant flow rate of 2 mL/min. The temperature of the detector was 300°C, and the detector was supplied with 14 mL/min hydrogen, 17 mL/min air 1, and 10 mL/min air 2. The detector voltage was 500 V, the gate delay for sulfur compounds was 6 ms, and the gate width is 20 ms. All sulfur compounds were identified by comparing their retention times with those of the pure standards. The sulfur responses of specific compounds were calculated by the square root of peak area.

7.4.4. Quantification of volatile sulfur compounds

7.4.4.1. Synthetic wine

The synthetic wine was made according to Mestres et al. [117] where 3.5 g L-tartaric acid was dissolved into 1 L of 12% ethanol solution, and the pH was adjusted to 3.5 with 1 M NaOH.

7.4.4.2. Sulfur standards and internal standard preparation

Hydrogen sulfide (H_2S) was generated by adding sodium sulfide solution into synthetic wine. Different concentrations of sodium sulfide solutions were made by dissolving the salt in distilled water (pH = 7). The solutions were stored at 4°C. Before analysis, the sodium sulfide solutions were directly added into sample vials containing synthetic wines (pH = 3.5). The concentrations of H_2S were calculated based on the amounts of sodium sulfide added into the synthetic wines. The MeSH standard was prepared by bubbling pure MeSH gas directly into cooled methanol (-15°C). Its concentration was calculated by weight. Standard solutions of 2000 ppm (w/w) of DMS, DMDS, DMTS, EtSH, DES, DEDS, MeSOAc, EtSOAc and methionol were individually prepared in cooled methanol (-15°C) and stored at -15°C. Dilutions were made with cooled methanol at the same temperature.

An internal standard solution was made by dissolving 500 ppb (w/w) of EMS, 2 ppb (w/w) of IsoProDS, and 100 ppm (w/w) of 4-methylthiobutanol in methanol with 1% of acetaldehyde, and stored at -15°C.

7.4.4.3. Suppression the interference of SO_2 with acetaldehyde:

To eliminate the interference of SO_2 on the sulfur analysis, acetaldehyde was added into the wine to suppress the interference of SO_2. The impact of acetaldehyde on the extraction of volatile sulfur compounds was investigated. Five milliliters of wine samples with and without 200 ppm of acetaldehyde were prepared. The samples were equilibrated at 30°C for 30 min with 500 rpm agitation, and the sulfur compounds were extracted with SPME fiber for 15 min with 250 rpm agitation and analyzed by GC-PFPD.

7.4.4.4. Investigation of SPME fiber selectivity to sulfur compounds

The target sulfur compounds were dissolved in methanol (each compound at a concentration of 3.4 ppm) and 0.5 µL of sample was directly injected into GC-PFPD (split ratio 1:10) to determine the detector sulfur responses for different compounds. Another mixture of target compounds (each at 136 ppb in synthetic wine) was put into a 20 mL vial, and the sample was equilibrated at 30°C for 45 min

with stirring. The headspace (10 μL) was directly injected into GC-PFPD with the splitless mode. Moreover, a mixture of sulfur compounds (1.36 ppb of each in synthetic wine) was analyzed by the SPME technique (pre-equilibrated for 30 min and extracted for 15 min at 30°C). The GC-PFPD conditions were the same as described previously. The response of MeSH was assigned to be 1, and was used as a reference against which other sulfur compounds were calibrated. The ratio of sulfur responses of static headspace injection with those of solvent injection represented the volatility of sulfur compounds in synthetic wine under experimental condition. The selectivity of SPME fiber was calculated by comparing sulfur responses in SPME analysis with those in static headspace.

7.4.4.5. Calibration of standard curves:

Five milliliters of synthetic wine containing different concentrations of sulfur standards and 100 μL of internal standard solutions were placed in 20 ml autosampler vials. The vials were tightly capped with Teflon-faced silicone septa, and placed in an automatic headspace sampling system. The SPME conditions and GC-PFPD conditions were set as described previously. The standard curve for individual sulfur compounds was built up by plotting the sulfur response ratio of target compound and its internal standard against the concentration ratio.

7.4.4.6. Calculation of recovery rates:

The recovery rates of sulfur compounds were evaluated in synthetic wine as well as in Pinot noir, Cabernet Sauvignon, Pinot Grigio, and Chardonnay wines. Known amounts of sulfur compounds were added to these wines separately. The concentrations of the sulfur compounds in these wines before and after the sulfur addition were quantified by the procedure described previously. The recovery rate was calculated by the following equation:

$$\text{Recovery rate} = \frac{\textit{Detected amount after addition} - \textit{Detected amount before addtion}}{\textit{Added amount}} \times 100\%$$

7.4.5. Wine analysis:

Seven different commercial white wine samples (5 varieties) and seven red wine samples (3 varieties) from California, Oregon and Canada were obtained from market place. All wine samples were stored at 4°C before analysis. Five milliliters of wine sample and 100 µL of internal standard solution were placed in 20 mL autosampler vials. The vials were tightly capped with Teflon-faced silicone septa. The sample vials were placed in the automatic headspace sampling system and the same SPME fiber as that used in the calibration curve was used. The SPME and GC-PFPD conditions were set as mentioned above. Triplicate analysis was performed on all samples.

7.5. Results and discussion

7.5.1. SPME extraction of volatile sulfur compounds in wine

High reactivity and low concentration are two of the biggest challenges for volatile sulfur analysis in wine. A lot of work has been done to evaluate different SPME fibers for sulfur extraction, and the results show that the fiber coated with a bi-layer of Carboxen and PDMS (polydimethylsiloxane) has high sensitivity for volatile sulfur compounds [117, 119, 236]. This fiber can extract highly volatile compounds such as H_2S and DMS, which cannot be easily recovered by solvent extraction or purge-trap methods.

However, some limitations have been observed with this fiber concerning the decomposition or reaction of analytes during sample preparation and GC injection, such as oxidation of DMS to dimethyl sulfoxide [12] and generation of DMDS from MeSH [16]. We found that the artifact formation of MeSH was also related to sample matrix. MeSH is even unstable in methanol and can be easily oxidized to DMDS. This oxidation was much more severe in phosphate buffer than in water. Therefore, the stability of target sulfur compounds was a major concern in our study.

In order to stabilize sulfur compounds during analysis, it was found that pre-treatment of the instrument was required. In this experiment, the GC injection port

was deactivated with BSTFA (bis(trimethylsilyl)-trifluoroacetamide), and the sample vials were flushed with inert gas. Since MeSH is not stable and the commercial MeSH solution contained detected amount of DMDS, MeSH gas was used to prepare the standard solution. All the sulfur standards were freshly prepared and dissolved in a synthetic wine matrix containing 0.35% tartaric acid and 12% ethanol. In addition, the extraction temperature was kept low. When sulfur standards were checked individually only single peak was detected (Figure 1), which indicated that artifact formation was prevented under the experimental conditions.

Headspace SPME extraction efficiency is based on the equilibrium of analytes among the three phases: the coated fiber, the headspace and the sample solution. Depending on how fast the analytes go to the headspace and are adsorbed by the fiber, the length of extraction time and temperature will be critical for SPME extraction efficiency. Generally, longer extraction time and high temperature benefited the equilibrium and increased the responses of less volatile analytes. However, because the Carboxen-PDMS fiber only has a limited number of adsorption sites, and higher molecular weight compounds (less volatile) can displace lower molecular weight compounds as a consequence of competition for active sites on the fiber [17], the quantification can only be achieved under non-equilibrium conditions using short extraction time, particularly for complex matrices [18-20]. In addition, it was noticed that more water was adsorbed by SPME fiber at above 40°C, causing baseline shift in the chromatogram. Therefore, a short extraction time (15min) and a low temperature (30°C) were chosen in our study.

7.5.2. Quantification of volatile sulfur compounds

Quantification of volatile sulfur compounds thus far has had minimum success due to the difficulties involved in the analysis. Sulfur dioxide can be added to wine as an antioxidant and anti-microbial agent. Commercial wines can contain up to 50 ppm free SO_2 or more. The high PFPD response for SO_2 interferes with the detection of other volatile sulfur compounds, which occur in wine at significantly lower concentrations. Since SO_2 reacts with carbonyl compounds, acetaldehyde

(200 ppm) was added to the wines to eliminate the interference of SO_2. As shown in Figure 2 (A), the addition of acetaldehyde can efficiently eliminate free SO_2. Moreover, addition of acetaldehyde had no effect on the measurement of other volatile sulfur compounds (Figure 2 B).

It is well known that SPME fibers have different selectivity for different compounds. The selectivity of Carboxen-PDMS fiber towards different volatile sulfur compounds in wine was investigated. As shown in Table 1, the fiber selectively extracted much more disulfides and trisulfides than DMS, EtSH and MeSH, which resulted in much higher detection sensitivity for disulfides and trisulfides. Therefore, trace amount of contaminating disulfides and trisulfides in other sulfur standards can generate very large signal. Since the concentrations for disulfides and trisulfides were very low in the experimental wine samples, the high purity of sulfur standards was critical for successfully quantification. Since the selectivity was very different among different sulfur compounds, it would be inaccurate to quantify all sulfur compounds based on only one internal standard. In this study, multiple internal standards were used to quantify different types of sulfur compounds.

To build up the calibration curves, different concentration of target compounds as well as internal standards were spiked in synthetic wine, and analyzed by SPME-GC-PFPD (Figure 3). MeSH, EtSH, H_2S, DMS, DES, MeSOAc, and EtSOAc were calculated with EMS as the internal standard. For most of these sulfur compounds, linear responses were obtained up to a quantification limit of 0.5 ppb with the correlation coefficient (R^2) greater than 0.99 (Figure 4 A & B) and the relative standard deviations (RSD) were less than 10%. For H_2S, a quantification limit of 1 ppb and a relative standard deviation of 15% were achieved even though it is extremely volatile. IsoProDS has a similar response to that of poly-sulfides, so it was used to quantify DMDS, DEDS and DMTS (Figure 4 C). For these compounds, the quantification limits could go as low as 0.01 ppb (R^2 of the linear relationship > 0.99, RSD < 10%). Methionol was calculated based on 4-(methylthio)butanol as the internal standard (Figure 4 D), and the detection limit was 60 ppb (R^2 of the linear

relationship = 0.98). Although methionol responses varied a lot based on the time after the sample was prepared, its RSD value could be reduced to below 20% by internal standard correction.

To investigate the influence of the wine matrix on the recovery of volatile sulfur compounds, known amounts of target compounds (1.74 ppb of H_2S, 2.69 ppb of MeSH, 3.16 ppb of EtSH, 3.16 ppb of DMS, 0.63 ppb of DES, 1.58 ppb of MeSOAc, 0.79 ppb of EtSOAc, 63.3 ppt of DMDS, 63.3 ppt of DEDS, 63.3 ppt of DMTS, and 0.32 ppm of methionol) were added to five different types of wines. The concentrations were measured before and after the spiking of sulfur compounds. Table 2 shows the recovery rates of target compounds in synthetic wine, Pinot noir, Cabernet Sauvignon, Pinot Grigio, and Chardonnay. The recovery rates in the synthetic wine were all close to 100%. For real wine samples, the matrix did show a different effect on the recovery. However, most recovery rates fit into the range of 80%~120%, which is within the analytical error. Thus, this method is reliable to quantify the amount of sulfur compounds in different wines.

7.5.3. Sulfur analysis of commercial wines

Several red and white wines purchased in the market were analyzed by this method, and the results were shown in Table 3 and Table 4. For these commercial wines, no sulfur off-flavor problem was detected by a preliminary sensory evaluation. EtSH and DEDS were not detected in either white or red wines. Concentrations of H_2S and MeSH in all tested wines were found to be ranging from 0.48 ppb to 9.26 ppb. Although previous research reported that the concentration of MeSH as low as 1.5 ppb could cause the occurrence of off-flavors in wine [123], the MeSH in our study did not cause any sulfur off-flavor problems even at concentration as high as 4.88 ppb, which may be due to its different threshold in different wines. Only a trace amount of disulfide and trisulfide were found in some wine samples. The results for methionol showed that its concentration was generally lower in white wine than in red wine.

7.6. Conclusion

A sensitive SPME-GC-PFPD technique was developed to analyze volatile sulfur compounds in wines. This method can be applied for detection and quantification of H_2S, MeSH, EtSH, DMS, DES, MeSOAc, DMDS, EtSOAc, DEDS, DMTS, and methionol in both red and white wines. The quantification limits can be as low as 0.5 ppb for most volatile sulfur compounds, and 0.01 ppb for disulfide and trisulfide, which are well below sensory detection limits. The development of this method makes it possible to reliably study the sulfur aroma compounds in wine.

7.7. Acknowledgement

The authors thank for the financial support of USDA-CSREE grant and Oregon Wine Board grant.

Table 7.1. Volatility of sulfur compounds in synthetic wine and selectivity of SPME Carboxen-PDMS fiber (presented based on MeSH as 1) (n=3)

	Volatility in Synthetic wine	Selectivity of SPME fiber
MeSH	1.00	1.00
EtSH	0.93	0.93
DMS	0.61	1.14
DES	0.65	4.32
MeSOAc	0.19	5.21
DMDS	0.65	6.36
EtSOAc	0.31	7.39
DEDS	0.79	13.96
DMTS	0.49	14.84
Methionol	0.18	-[a]

[a] the selectivity of methionol cannot be detected based on this experiment.

Table 7.2. Recovery rates of sulfur compounds in different wine matrices (presented as 100%, n=3)

	Synthetic Wine	**Pinot noir**	**Cabernet Sauvignon**	**Pinot Grigio**	**Chardonnay**
H_2S	100	89	99	80	98
MeSH	99	83	93	117	117
EtSH	101	104	110	117	125
DMS	101	111	116	94	86
DES	100	98	106	108	96
MeSOAc	98	121	103	87	85
DMDS	100	107	104	108	96
EtSOAc	101	117	93	98	81
DEDS	98	84	95	110	117
DMTS	101	90	96	109	114
Methionol	101	82	106	90	120

Table 7.3. The concentration of volatile sulfur compounds in commercial white wine samples (n=3)

Wine sample / Sulfur compound	Wine A Pinot Grigio from California	Wine B Pinot Grigio from Canada	Wine C Pinot Gris, from Oregon	Wine D Pinot Blanc from Oregon	Wine E Chardonnay from California	Wine F Chardonnay from California	Wine G Chardonnay From Oregon
H_2S (ppb)	4.60 ± 1.20	1.66 ± 0.49	7.89 ± 1.32	9.03 ± 1.60	1.45 ± 0.58	2.14 ± 0.43	3.59 ± 0.39
MeSH (ppb)	4.88 ± 0.37	1.09 ± 0.32	4.28 ± 0.77	2.94 ± 0.29	1.02 ± 0.40	0.48 ± 0.11	1.64 ± 0.14
EtSH (ppb)	nd	nd	nd	nd	nd	nd	nd
DMS (ppb)	17.00 ± 1.03	35.37 ± 2.15	18.08 ± 0.84	12.05 ± 0.25	27.38 ± 1.13	52.60 ± 1.54	31.57 ± 1.20
DES (ppb)	nd	nd	nd	0.27 ± 0.05	nd	nd	nd
MeSOAc (ppb)	1.68 ± 0.11	0.32 ± 0.00	1.55 ± 0.29	3.50 ± 0.82	2.18 ± 0.10	1.42 ± 0.06	1.60 ± 0.06
EtSOAc (ppb)	0.17 ± 0.00	1.00 ± 0.19	20 ± 6	22 ± 6	0.51 ± 0.03	0.58 ± 0.04	11 ± 0
DMDS (ppt)	19 ± 1	70 ± 10	0.34 ± 0.02	0.64 ± 0.20	65 ± 7	24 ± 2	nd
DEDS (ppt)	nd	nd	nd	nd	nd	nd	nd
DMTS (ppt)	18 ± 2	55 ± 6	nd	nd	111 ± 29	35 ± 6	11 ± 1
Methionol (ppm)	0.41 ± 0.14	0.22 ± 0.06	0.75 ± 0.02	0.83 ± 0.04	0.43 ± 0.11	0.47 ± 0.13	0.67 ± 0.10

nd: not detected

Table 7.4. The concentration of volatile sulfur compounds in commercial red wine samples (n=3)

Wine sample / Sulfur compound	Wine H Gamay noir from Oregon	Wine I Cabernet Sauv. from California	Wine J Cabernet Sauv. from California	Wine K Pinot noir from Oregon	Wine L Pinot noir from Oregon	Wine M Pinot noir from Oregon	Wine N Pinot noir from California
H_2S (ppb)	2.68 ± 0.12	5.41 ± 1.74	7.64 ± 2.69	2.11 ± 0.41	4.70 ± 1.62	2.60 ± 0.71	9.26 ± 2.36
MeSH (ppb)	0.95 ± 0.01	1.26 ± 0.08	2.41 ± 0.24	1.56 ± 0.20	2.17 ± 0.35	1.19 ± 0.03	2.92 ± 0.29
EtSH (ppb)	nd	nd	nd	nd	nd	nd	nd
DMS (ppb)	9.34 ± 0.86	45.54 ± 0.60	67.53 ± 4.97	26.41 ± 4.03	13.58 ± 0.48	14.44 ± 0.08	11.90 ± 0.14
DES (ppb)	0.28 ± 0.04	nd	0.49 ± 0.06	nd	nd	0.34 ± 0.03	0.35 ± 0.07
MeSOAc (ppb)	2.74 ± 0.08	7.51 ± 0.07	6.83 ± 0.46	1.59 ± 0.15	1.50 ± 0.03	9.21 ± 0.28	4.10 ± 0.10
EtSOAc (ppb)	nd	0.70 ± 0.01	0.99 ± 0.06	10 ± 1	0.35 ± 0.01	13 ± 1	0.46 ± 0.04
DMDS (ppt)	0.17 ± 0.00	13 ± 1	13 ± 2	nd	31 ± 9	1.23 ± 0.04	36 ± 7
DEDS (ppt)	nd	nd	nd	nd	nd	nd	nd
DMTS (ppt)	nd	nd	nd	nd	nd	nd	21 ± 6
Methionol (ppm)	1.06 ± 0.03	1.73 ± 0.35	2.06 ± 0.24	1.13 ± 0.26	1.50 ± 0.15	1.97 ± 0.32	1.83 ± 0.41

nd: not detected

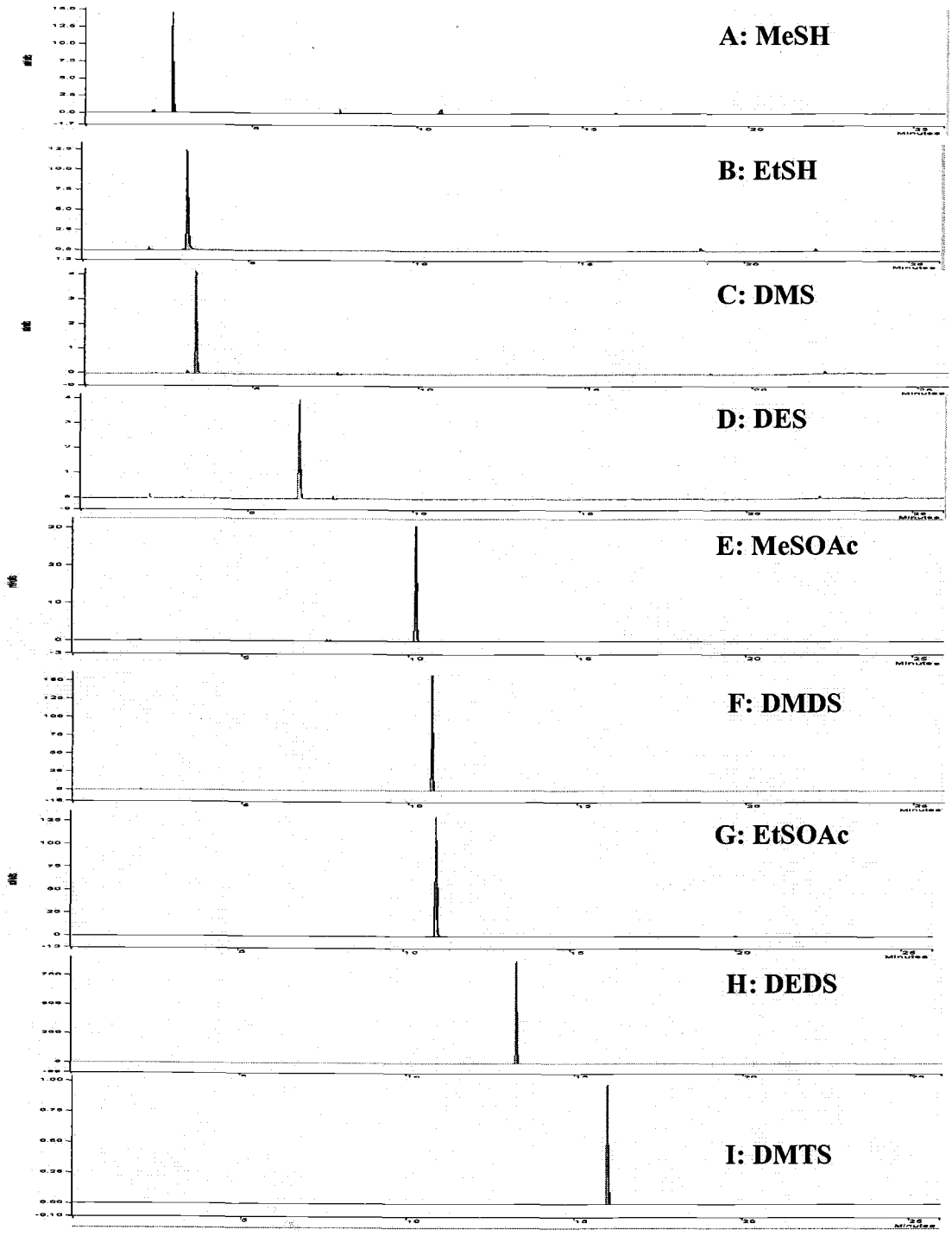
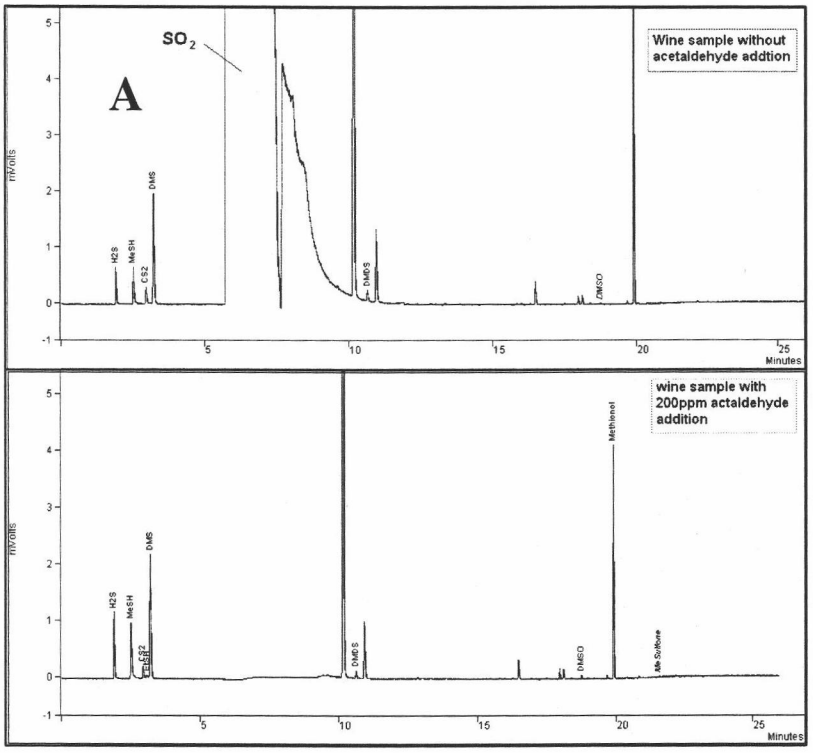


Fig 7.1. The artifacts determination of sulfur compounds under SPME extraction condition in this study



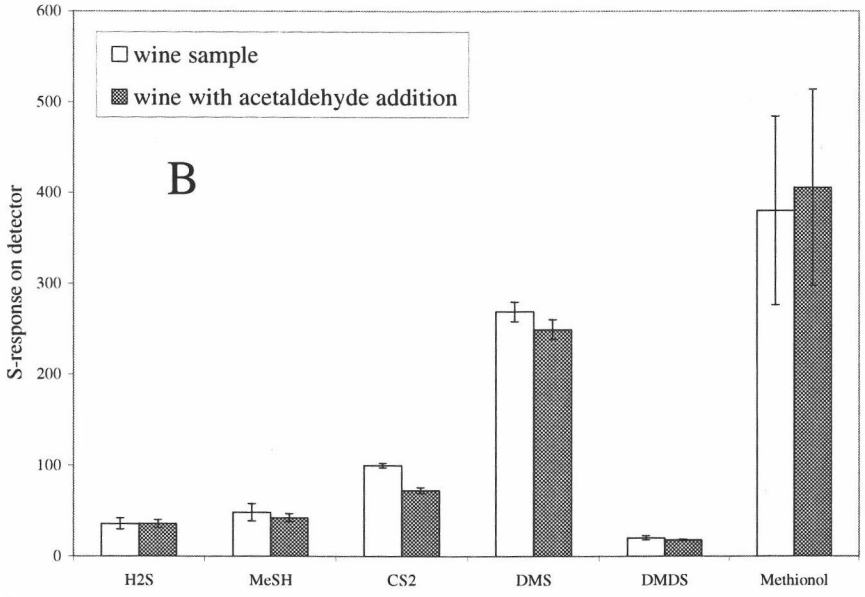


Fig 7.2. (A) Chromatogram showing the effect of acetaldehyde addition on SO_2; (B) The effects of acetaldehyde addition on the extraction of volatile sulfur compounds (n=3)

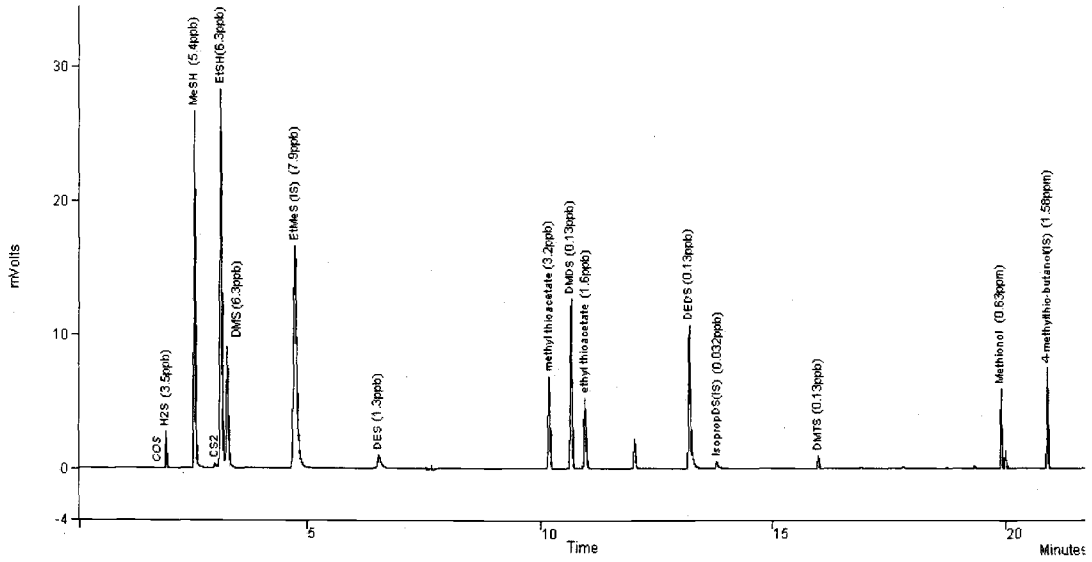


Fig 7.3. Chromatogram of volatile sulfur compounds and internal standards in synthetic wine by SPME-GC-PFPD

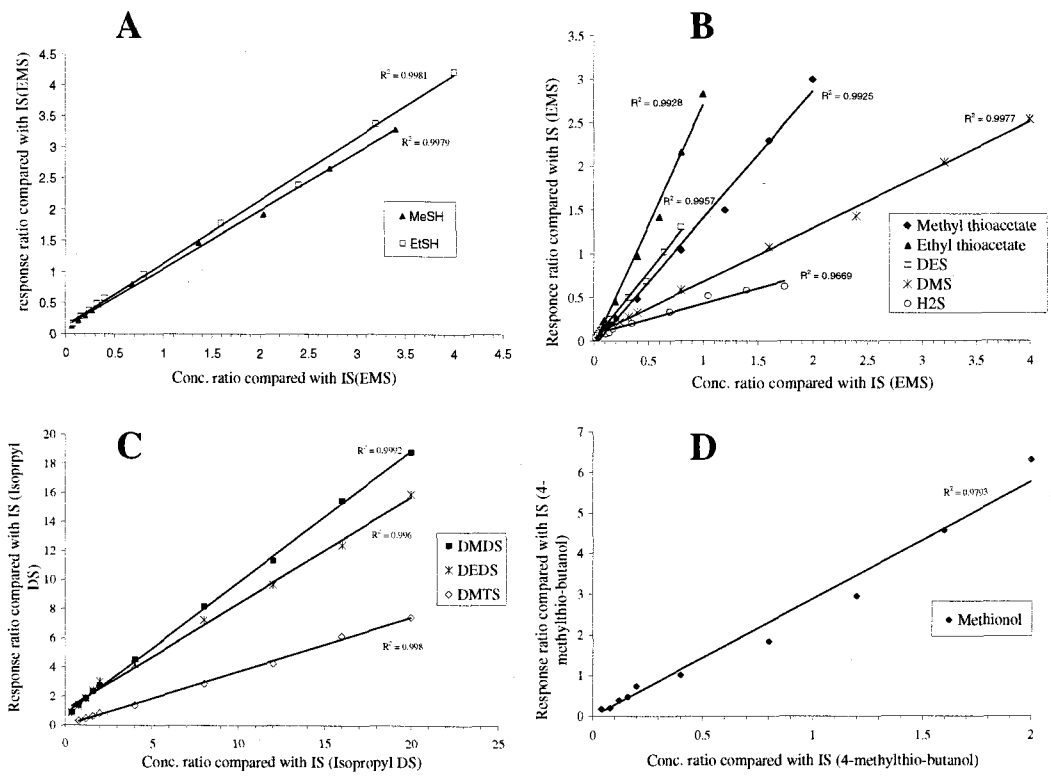


Fig 7.4. Calibration curves for (A) MeSH and EtSH; (B) H_2S, DMS, DES, MeSOAC and EtSOAc; (C) DMS, DES and DMTS; (D) methionol.

Chapter 8. Sulfur Compound Analysis of Oregon Pinot noir Wines as Affected by Irrigation, Tillage and Nitrogen Supplementation in the Vineyard

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To be submitted

8.1 Abstract:

The effects of nitrogen fertilization, tilling and irrigation on contents of volatile sulfur compounds in Pinot noir wines were studied using the solid phase microextraction and gas chromatography/ pulse flame photometric detection (HS-SPME-GC/PFPD). Wines were made from two field blocks of twelve combinations of irrigation (dry or irrigated), tillage (tilled or not tilled) and fertilization (none, foliar nitrogen supplementation or soil applied nitrogen) from three vintages (1999, 2000 and 2001) of *Vitis vinifera cv.* Pinot noir. After quantification by HS-SPME-GC/PFPD, the concentrations of volatile sulfur compounds were statistically analyzed. Multivariate analysis of variance (MANOVA) results showed that year, irrigation, and nitrogen had significant effects on concentrations of these target sulfur compounds ($p<0.01$). Results performed by analysis of variance (ANOVA) and Principal Components Analysis (PCA) showed that nitrogen fertilization had a major impact on levels of hydrogen sulfide (H_2S) and methanethiol (MeSH). Foliar nitrogen supplementation or soil nitrogen application significantly increased contents of H_2S ($p<0.01$) and MeSH ($p<0.01$) in Pinot noir wines. Dimethyl sulfide (DMS), methionol, methyl thioacetate (MeSOAc), and ethyl thioacetate (EtSOAc) were mainly affected by vintage.

8.2 Keywords:

Quantification, sulfur volatiles, wine, vineyard practices, SPME-GC/PFPD

8.3 Introduction

Volatile sulfur compounds are known to have very powerful and characteristic odors. When presented at supra-threshold concentrations (at ppb levels), these compounds are responsible for off-flavors such as reduced, sulfury, rotten egg...etc. [130]. Since many factors such as deficiencies of nutrients (amino acids and vitamins) and sulfite residues are associated with the formation of volatile

sulfur compounds [139], optimizations of vine health and fruit quality are among the top topics of grape growing and winemaking.

The mechanisms of forming these compounds in wine are still not fully understood. Most studies indicate that the sulfur amino acids of grape juice, especially methionine, seem to act as precursors of some sulfur compounds. The evidence showed that yeast breaks down the extra-cellular proteins and leaves sulfide residues of the sulfur-containing amino acids behind when deficiency of nitrogenous components occurs in must. Although several studies also showed that the presence of elemental sulfur from vineyard sprays can also cause hydrogen sulfide (H_2S) formation during fermentation, this claim has been recently challenged by Thomas [143]. H_2S can also act as a precursor for other volatile sulfur compounds (i.e. mercaptans) that also impart off-odor to wine.

The effects of different yeast strains and must turbidity on H_2S production have already been widely studied. Karagiannis and Moreira also examined the effect of vinification parameters (addition of sulfite or sulfur-containing amino acid to the must, and fermentation temperature) on the development of volatile sulfur compounds in wines and grape musts [147, 148].

However, little information is available on the formation of highly volatile sulfur compounds other than H_2S in wine. One of the major reasons is that there is no sensitive, reliable analytical method available to measure these highly volatile sulfur compounds due to their extremely low concentration in wine and high reactivity [128]. Recently, a quick, sensitive method was developed to quantify trace amounts of volatile sulfur compounds in wines by the SPME and GC-PFPD [238]. With this method, the quantified volatile sulfur compounds could go as low as 0.5 ppb, which below their sensory detection thresholds [131].

In Oregon, grapevines are subject to low soil water availability, accompanied by high levels of solar irradiance, temperature, and air vapor pressure deficits during the summer. Under these conditions, photosynthesis is greatly reduced, particularly toward the end of the growing season. The inability of vines to photosynthesize prior to harvest results in a shortage of carbohydrates, and is further responsible for a

reduction of nitrogenous compounds in grapes at harvest. If assimilable nitrogen levels at harvest are too low, fermentations may be slow and sluggish, producing wines with residual sugar or off-flavors at undesirable levels. Commercial Oregon must samples at harvest have been found to contain low assimilable nitrogen compared to the minimum requirements of healthy fermentation. To improve fruit quality, various vineyard practices such as additional nitrogen supplementation, irrigation, and tillage have been applied.

Using the newly developed SPME and GC-PFPD methods, this study integrates the volatile sulfur content of wines with various viticulture strategies that may improve nitrogen availability to the vine, particularly during ripening. By examining three consecutive vintages (1999, 2000 and 2001), we aim to determine the effect of vineyard practices used for nitrogen management on sulfur volatiles in Pinot noir wine.

8.4 Materials and Methods

8.4.1 Vine treatments and Wine preparation:

Vintage 1999, 2000 and 2001 Pinot noir wines were produced from Oregon State University viticulture trials with grapes grown at Benton-Lane vineyard in the Oregon Southern Willamette Valley appellation. Pinot noir clone FPMS 2A vines were grafted onto 7-year-old Teleki 5C rootstocks. There were 24 wine samples: 12 treatment combinations and 2 field replications (Table 1). The treatments included nitrogen supplement (three levels: none, foliar applied, and soil applied), irrigation (two levels: dry and irrigated) and tillage (two levels: alternate in-row tilling and not tilled). The irrigation treatment involved water applied at the rate of 0.5gal/hour for four hours daily for a total of 200 hours during ripening. Tilling was done in early spring to encourage nitrogen utilization and reduce nutrient and water competition. Fertilizer was applied to either soil or foliar: soil nitrogen was applied manually one time in May at the rate of 39 Kg urea/ha. Foliar N was split into two applications of 1.5 kg/ha applied by spraying on the leaves.

After harvest, grapes from each treatment were collected, crushed, stemmed, treated with 50 mg/L sulfur dioxide, and fermented separately (inoculated 1 g/L Lavin RC 212 Bourgorouge yeast). The musts were punched down twice daily during fermentation and pressed after seven days of fermentation. After wines were settled and racked off the primary yeast lees, 0.025g/gallon OSU 1-step (Lalvin) malolactic bacteria was used to induce secondary malolactic fermentation. The new wines were cold stabilized, racked, bottled with the addition of 25 mg/L of sulfur dioxide, aged for nine months, and stored in the experimental winery at 18°C.

8.4.2 Quantification of Volatile Sulfur Compounds in Wines:

The quantification of volatile sulfur compounds in wines was performed by a previously published method [238]. In general, five milliliters of wine samples and 100 µl of internal standard solution, which included 500 ppb (w/w) of EMS, 2 ppb (w/w) of IsoProDS, and 100 ppm (w/w) of 4-methylthiobutanol, were placed in 20 ml pre-flushed autosampler vials. The sulfur volatiles were equilibrated for 15min at 30°C, and extracted at the same temperature for 30min with agitation by an 85µm Carboxen™-PDMS StableFlex™ SPME fiber (SUPELCO, Bellefonte, PA, USA). After extraction, the SPME fiber was injected directly into GC injection port with the splitless mode at 300°C. The GC/PFPD analyses were made on a Varian CP-3800 gas chromatography equipped with a pulsed flame photometric detector (PFPD) (Varian, Walnut Creek, CA, USA) operating in sulfur mode. The separations were performed using a DB-FFAP capillary column (30m×0.32 mm I.D., 1 µm film thickness, Agilent, Palo Alto, CA, USA).

The purified chemicals, H_2S, methanethiol (MeSH), ethanethiol (EtSH), dimethyl sulfide (DMS), diethyl sulfide (DES), dimethyl disulfide (DMDS), diethyl disulfide (DEDS), dimethyl trisulfide (DMTS), methyl thioacetate (MeSOAc), ethyl thioacetate (EtSOAc), and methionol were used to built up calibration curves as presented in the previous publication [238]. The sulfur responses of target compounds were calculated by the square root of peak area. Triplicate analysis was

performed on all samples, and amounts of sulfur compounds in wines were determined by comparing their own standard curves.

8.4.3 Statistical analysis:

A complete randomized block design was used. The four treatments, vintage (year), irrigation (irrigate), tillage (till) and nitrogen were fixed effects and replication (rep) was considered as a block effect. The data were first analyzed by multivariate analysis of variance (MANOVA) to examine whether significant differences were found on concentrations of eight volatile sulfur compounds (H_2S, MsSH, DMS, DMDS, DMTS, MeSOAc, EtSOAc, and methionol) in wine samples with different treatments. Year, irrigate, till, nitrogen, and rep were considered as main effects. All 2-way, one 3-way (irrigate×till×nitrogen) and one 4-way (year×irrigate×till×nitrogen) interaction were included in the MANOVA model. The level of significance (α) was 0.05. The MAVONA results (Wilk's λ) showed that rep and the interactions containing rep were not statistically different from various levels of treatments ($p>0.05$). Therefore, year, irrigation, till, nitrogen and all their interactions were included in the four-way ANOVA model on the eight volatile sulfur compounds individually. To understand the paired mean differences, mean concentrations of volatile sulfur compounds in different treatments were compared by multiple comparisons adjusted by Tukey-HSD method. Principal components analysis (PCA) was also performed on the mean data with a varimax rotation. The minimum of 0.7 for the correlation of original sulfur compounds with the new factor generated was used as a selection criterion. All statistical analyses were performed using SPSS 13.0 for windows (SPSS Inc., Chicago, IL).

8.5 results and discussion

8.5.1 Quantification of volatile sulfur compounds

In all wine samples, ethanethiol and diethyl disulfide were not detected. Diethyl sulfide was detected in only a few wine samples, and its concentrations were very low (not shown). Combined results from the present study with the previous

results in commercial wines , show that highly volatile sulfur compounds containing an ethyl group are not major products during normal wine making. A previous sulfur survey in Oregon wines conducted in this laboratory also showed that trace amounts of ethanethiol could generate off-flavor problems . Except for its low detection threshold, its absence in most normal wines is probably the other reason that subjects can immediately recognize its sensory characteristics in Pinot noir wine.

The concentrations of other target sulfur compounds in wine samples are presented in Tables 2 to 4. H_2S levels in wines ranged from 0.08 to 7.27 ppb, methanethiol concentrations from 0.79 to 4.87 ppb, and dimethyl sulfide concentrations from 7.15 to 23.35 ppb. The most abundant sulfide compound in wine samples was methionol, which ranged from 1.07 to 3.35 ppm. Only less than 1ppb of DMDS and DMTS were found in all these wines. Concentrations of these sulfur compounds in experimental Pinot noir wines were below their detection thresholds in wine [128].

Sensory descriptive analyses of the three vintage Pinot noir wines was also performed by a trained panel . Neither off-flavor nor sulfur-related aroma difference was found in all these wines, which is consistent with the instrumental analysis performed. Therefore, it is impossible to relate sulfur volatiles to these vineyard practices only based on the sensory evaluation results.

With the new developed quantification method, the concentration of target compounds can be precisely determined as low as the ppb level (RSD< 15%), which provides high sensitivity. Moreover, it required less amount of wine sample and less analysis time than the traditional method, thus it could be possible to use in online analysis in wineries.

8.5.2 Statistical analyses

MANOVA results showed that vintage year, irrigation and nitrogen were significantly different from the various levels of treatments ($p<0.05$) (Table 5). Significant two-way interactions were year×nitrogen and irrigation×nitrogen ($p<0.05$). The results indicated that the concentrations of eight volatile sulfur

compounds in wine samples were dependent on three main factors, but also on a combination of vintage year and nitrogen, and a combination of irrigation and nitrogen.

The four-way ANOVA results on each target compound showed that vintage year greatly influenced concentrations of the volatile sulfur compounds in wine samples ($p< 0.05$) except for MeSH. Table 6 contains the concentration means of volatile sulfur compounds over three years. Vintages bearing different superscripts are significantly different at $p<0.05$ by ANOVA and Tukey's HSD. Multiple comparison results showed that the 1999 wine samples contained higher H_2S, DMS, DMDS, DMTS and methionol, but lower MeSOAc and EtSOAc. However, the reason is not clear yet.

Tables 7, 8 and 9 contain the concentration means of target compounds in wine samples by different viticulture treatment. Treatments bearing different superscripts are significantly different at $p<0.05$ by ANOVA and Tukey's HSD. There is no significant difference between viticulture treatments on DMMS, EtSOAC, DMTS, and methionol. Multiple comparison results showed that the wine samples without nitrogen supplementation have significantly lower concentrations of H_2S and MeSH. Moreover, irrigation significantly increased the amount of MeSOAc, and tillage significantly increased the amount of DMS.

In ANOVA, one interaction factor (irrigate×nitrogen) showed a significant impact on concentration of H_2S and MeSH in wines. In Figure 1, the combination of irrigation and soil nitrogen supplement had the highest amount of both H_2S and MeSH, and followed by the combination of irrigation and Foil nitrogen supplement. It indicated that the effects of nitrogen supplement on these two compounds are influenced by irrigation treatment.

In PCA, there were four principal components (PCs) extracted and they accounted for 87.1% of total data variance. Figures 2A and 2B showed loading scores of the 36 treatment combinations after PC1 and PC4 (2A) and PC2 and PC3 (2B) were plotted. In Figure 2A, wine samples appear to be separated into three groups based on vintage year. Compared to 1999 and 2000 vintages, most 2001

wines have more sulfite esters (MeSOAc and EtSOAc) and less dimethyl sulfide. The vintage 2000 wines have a higher content of methionol than 1999 and 2001 vintages.

H_2S and its off-flavor have been studied the most in wine. It can further react with wine components and generate mercaptans, which are more difficult to eliminate in winemaking . Therefore, the concentrations of H_2S and MeSH in wines are generally correlated with each other. In the present study, they are represented by PC2 (explained variance 18.9%; Figure 2B). Concentrations of both compounds were different among various nitrogen treatments on PC 2.

It was well known that nitrogen deficiency in grape must is one of major reasons to form H_2S off-flavor, but formation of H_2S is much more complex [133]. Sea et al. measured the production of H_2S during wine fermentation during the two seasons, and reported poor correlation between H_2S and nitrogen concentrations in must during wine fermentation . Recently, researchers reported that H_2S production was even significantly higher when the concentration of yeast assimilable nitrogen content (YANC) was increased if pantothenic acid was deficient [134]. Results in the present study also showed that the concentrations of H_2S and MeSH were significantly higher when nitrogen supplementation was applied in the vineyard. Previous analysis showed that YANC of the 12 vineyard treatments is not significantly different within the three vintage years , which indicated that there is no direct correlation between concentrations of these sulfur volatiles and YANC.

Overall, in this study, a new quantification method was applied to investigate the effects of various vineyard practices on contents of volatile sulfur compounds in Pinot noir wines, even when the levels of these compounds are below their sensory detection thresholds. The data showed that these sulfur volatiles could be affected by year, nitrogen supplement, and irrigation. Nitrogen supplementation can increase the H_2S and MeSH levels in wines. However, this effect was not significant, and no sulfur off-flavor was detected in these wine samples.

8.6 Acknowledgment

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Table 8.1. The experimental design for the grape treatments

	Tilled		Not tilled	
	Irrigated	Dry	Irrigated	Dry
Zero nitrogen	I T 0N	D T 0N	I NT 0N	D NT 0N
Foil nitrogen	I T FN	D T FN	I NT FN	D NT FN
Soil nitrogen	I T SN	D T SN	I NT SN	D NT SN

Table 8.2. The concentration of sulfur compounds in 1999 wines samples

Treatment	H2S (ppb)	MeSH (ppb)	DMS (ppb)	MeSOAc (ppb)	DMDS (ppt)	EtSOAc (ppb)	DMTS (ppt)	Methionol (ppm)
I NT 0N	0.98	0.79	18.78	3.60	27.14	Nd	106.80	2.42
I NT 0N	1.57	1.85	18.92	4.93	30.54	0.35	157.08	1.47
I T 0N	2.84	1.96	19.41	3.32	23.42	Nd	95.86	2.45
I T 0N	0.25	1.54	14.53	3.78	24.01	Nd	88.09	2.08
D NT 0N	2.00	1.65	17.11	1.89	26.66	Nd	127.77	2.15
D NT 0N	1.31	1.92	20.89	3.18	28.98	Nd	127.64	1.83
D T 0N	2.95	0.90	23.35	4.30	58.38	Nd	256.11	2.15
D T 0N	1.26	2.29	19.40	5.71	32.67	0.35	122.85	1.91
I NT FN	2.62	1.97	18.38	4.20	26.07	Nd	128.70	2.15
I NT FN	2.87	1.78	20.24	3.56	52.70	Nd	192.65	1.66
I T FN	3.35	2.28	22.04	3.58	49.58	Nd	265.18	2.04
I T FN	5.66	2.17	19.80	5.91	32.45	0.28	121.94	2.90
D NT FN	3.38	2.49	16.16	4.41	42.60	Nd	153.64	2.59
D NT FN	4.61	2.67	22.06	1.35	88.99	Nd	298.73	2.14
D T FN	2.22	1.98	22.73	2.70	30.57	Nd	128.22	2.24
D T FN	3.73	3.34	19.80	2.26	44.68	Nd	186.28	2.12
I NT SN	5.39	2.32	19.32	4.75	35.63	0.27	216.81	2.68
I NT SN	2.31	1.86	17.89	3.48	59.30	Nd	191.59	2.00
I T SN	3.29	2.61	15.15	4.91	38.01	Nd	121.93	2.01
I T SN	7.14	4.87	20.52	14.21	29.36	0.82	160.05	2.07
D NT SN	3.13	1.90	21.39	2.84	27.52	Nd	133.75	2.71
D NT SN	1.41	2.01	17.82	2.56	47.20	Nd	217.66	3.08
D T SN	1.98	2.60	16.29	2.17	39.77	Nd	162.63	3.06
D T SN	1.48	2.21	12.65	1.94	36.28	Nd	225.98	2.58

Table 8.3. The concentration of sulfur compounds in 2000 wines samples

Treatment	H2S (ppb)	MeSH (ppb)	DMS (ppb)	MeSOAc (ppb)	DMDS (ppt)	EtSOAc (ppb)	DMTS (ppt)	Methionol (ppm)
I NT 0N	1.62	1.63	10.81	7.54	30.00	0.65	125.72	2.40
I NT 0N	2.27	1.43	14.19	4.64	36.83	0.42	222.61	2.44
I T 0N	1.27	1.48	13.49	8.06	45.52	0.59	253.66	1.97
I T 0N	1.63	1.37	9.47	6.93	28.84	0.58	163.45	2.31
D NT 0N	1.82	1.43	11.80	5.71	25.38	0.53	162.50	3.02
D NT 0N	1.52	1.46	14.30	5.08	36.45	0.42	221.45	2.59
D T 0N	1.74	1.51	11.47	5.59	27.06	0.44	186.97	2.50
D T 0N	2.98	1.84	17.20	2.54	26.62	0.17	123.30	2.54
I NT FN	3.67	1.82	12.05	6.94	17.86	0.62	98.09	2.34
I NT FN	3.87	1.99	11.93	8.15	28.92	0.77	208.07	1.72
I T FN	2.66	1.71	12.11	6.56	27.90	0.60	184.02	2.42
I T FN	2.78	1.94	19.21	6.62	47.61	0.44	303.80	2.70
D NT FN	1.84	1.90	15.47	2.69	43.13	0.26	334.74	1.56
D NT FN	2.31	1.61	11.53	5.75	19.22	0.47	137.38	2.82
D T FN	3.79	1.76	15.41	4.20	21.55	0.40	160.90	2.32
D T FN	3.43	1.92	13.66	6.15	34.27	0.49	225.34	2.20
I NT SN	5.47	2.80	13.80	6.69	15.61	0.57	46.59	2.37
I NT SN	4.79	1.92	15.84	6.00	31.79	0.49	88.91	2.86
I T SN	7.27	2.62	13.47	7.23	16.18	0.59	72.87	2.47
I T SN	2.87	1.78	15.94	5.04	45.27	0.45	202.72	2.09
D NT SN	3.95	1.88	12.32	4.54	21.18	0.40	144.31	3.35
D NT SN	2.74	1.67	12.67	5.12	23.45	0.56	162.23	2.02
D T SN	2.99	1.61	12.75	3.66	25.67	0.47	150.09	2.02
D T SN	4.71	2.42	16.09	3.65	26.45	0.44	138.89	3.22

Table 8.4. The concentration of sulfur compounds in 2001 wines samples

Treatment	H2S (ppb)	MeSH (ppb)	DMS (ppb)	MeSOAc (ppb)	DMDS (ppt)	EtSOAc (ppb)	DMTS (ppt)	Methionol (ppm)
I NT 0N	0.28	1.17	7.19	7.80	19.08	0.61	57.03	2.39
I NT 0N	0.52	1.26	10.03	6.86	30.77	0.77	56.52	1.33
I T 0N	0.60	1.71	11.68	9.72	20.96	0.79	70.43	1.28
I T 0N	0.08	1.65	9.65	10.20	30.22	1.09	80.62	2.55
D NT 0N	1.14	1.47	10.10	9.15	25.18	0.94	56.84	2.91
D NT 0N	0.64	1.48	11.37	11.22	30.93	1.08	104.96	1.37
D T 0N	1.30	2.17	16.80	5.52	45.78	0.45	196.06	1.21
D T 0N	1.23	2.10	11.35	12.67	19.32	1.48	37.79	2.14
I NT FN	1.53	2.45	11.24	12.95	33.08	1.44	90.23	2.75
I NT FN	1.67	1.71	11.72	9.48	26.23	0.78	70.66	1.67
I T FN	3.73	3.00	16.95	10.08	63.21	0.78	192.67	1.61
I T FN	0.70	1.63	10.95	6.87	26.07	0.51	59.03	1.99
D NT FN	1.10	1.75	8.75	5.22	27.67	0.26	68.62	1.97
D NT FN	1.42	3.18	9.75	12.12	58.87	0.84	218.59	2.18
D T FN	3.44	2.91	13.64	17.06	44.60	1.81	135.20	3.09
D T FN	0.97	2.18	15.45	10.73	46.69	0.69	158.71	2.05
I NT SN	1.53	1.58	10.09	5.99	25.05	0.38	86.90	2.12
I NT SN	1.11	2.56	11.67	9.19	33.16	0.60	123.39	1.26
I T SN	4.55	1.45	12.82	6.61	31.10	0.24	115.55	1.95
I T SN	3.08	3.71	15.70	11.72	55.08	1.02	287.01	1.09
D NT SN	0.78	2.05	12.05	9.46	31.87	0.80	109.35	1.11
D NT SN	1.99	2.08	11.12	13.54	35.03	1.07	133.77	3.08
D T SN	0.95	1.67	13.53	4.16	37.44	0.53	98.02	1.29
D T SN	1.43	1.72	13.69	6.43	31.99	0.42	130.13	2.68

Table 8.5. The MANOVA results using SPSS 13.0 (α=0.05)

Effect	Value (Wilks' λ)	F	p value
YEAR	0.002	26.186	**0.000**
IRRIGATE	0.152	6.253	**0.006**
TILL	0.706	0.469	0.850
NITROGEN	0.042	4.352	**0.002**
REP	0.489	1.174	0.405
YEAR * IRRIGATE	0.134	1.945	0.088
YEAR * TILL	0.398	0.657	0.798
YEAR * NITROGEN	0.012	2.506	**0.005**
YEAR * REP	0.192	1.439	0.227
IRRIGATE * TILL	0.447	1.393	0.315
IRRIGATE * NITROGEN	0.094	2.538	**0.030**
IRRIGATE * REP	0.780	0.317	0.940
TILL * NITROGEN	0.231	1.214	0.343
TILL * REP	0.254	3.303	**0.047**
NITROGEN * REP	0.174	1.573	0.177
YEAR * IRRIGATE * TILL	0.232	1.212	0.345
YEAR * IRRIGATE * NITROGEN	0.105	0.916	0.597
YEAR * IRRIGATE * REP	0.182	1.514	0.197
YEAR * TILL * NITROGEN	0.071	1.140	0.351
YEAR * TILL * REP	0.282	0.993	0.502
YEAR * NITROGEN * REP	0.033	1.661	0.073
IRRIGATE * TILL * NITROGEN	0.149	1.786	0.118
IRRIGATE * TILL * REP	0.523	1.024	0.481
IRRIGATE * NITROGEN * REP	0.131	1.986	0.081
TILL * NITROGEN * REP	0.325	0.849	0.626
YEAR * IRRIGATE * TILL * NITROGEN	0.029	1.746	0.055

Table 8.6. The means of sulfur volatile compound concentrations in Pinot noir wine by three vintage years (n=24)

	1999	2000	2001
H_2S (ppb)	2.82 ^{b}	3.08 ^{b}	1.49 ^{a}
MeSH (ppb)	2.17	1.81	2.03
DMS (ppb)	18.94 ^{b}	13.62 ^{a}	11.97 ^{a}
MeSOAc (ppb)	3.98 ^{a}	5.63 ^{b}	9.36 ^{c}
DMDS (ppt)	39 ^{b}	29 ^{a}	35 ^{ab}
EtSOAc (ppb)	0.09 ^{a}	0.49 ^{b}	0.81 ^{c}
DMTS (ppt)	166 ^{b}	172 ^{b}	114 ^{a}
Methionol (ppm)	2.27 ^{ab}	2.43 ^{b}	1.96 ^{a}

Vintages bearing different superscripts are significantly different at $p<0.05$ by ANOVA and Tukey's HSD.

Table 8.7. The means of sulfur volatile compound concentrations in Pinot noir wine by different nitrogen supplements (n=24)

	Zero Nitrogen	Foliar Nitrogen	Soil Nitrogen
H_2S (ppb)	1.41 [a]	2.81 [b]	3.18 [b]
MeSH (ppb)	1.59 [a]	2.17 [b]	2.25 [b]
DMS (ppb)	14.30	15.46	14.77
MeSOAc (ppb)	6.08	6.65	6.25
DMDS (ppt)	30	39	33
EtSOAc (ppb)	0.42	0.49	0.48
DMTS (ppt)	133	172	147
Methionol (ppm)	2.14	2.22	2.30

Treatments bearing different superscripts are significantly different at $p<0.05$ by ANOVA and Tukey's HSD.

Table 8.8. The means of sulfur volatile compound concentrations in Pinot noir wine by with or without irrigation treatment (n=36)

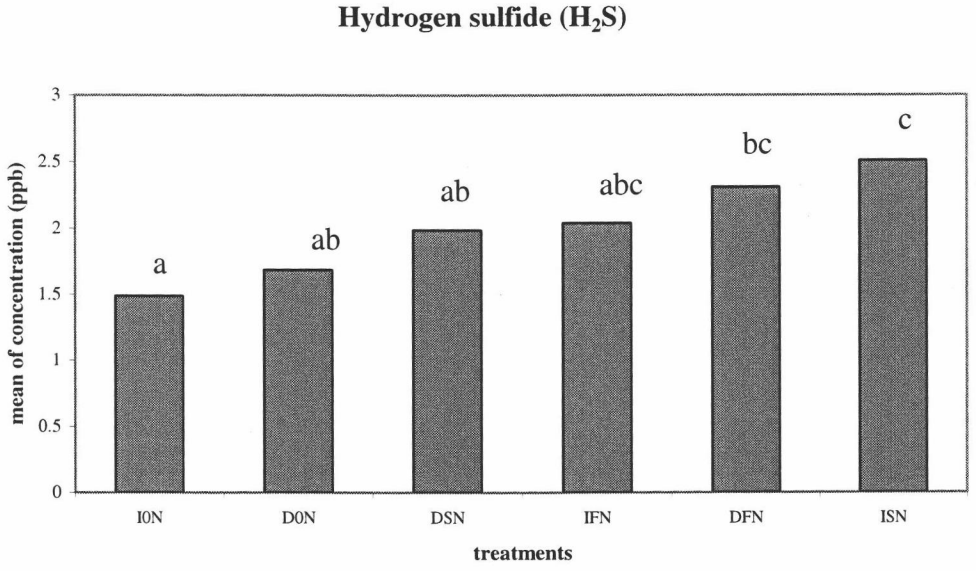
	With irrigation	Without irrigation (Dryness)
H_2S (ppb)	2.72	2.21
MeSH (ppb)	2.13	1.88
DMS (ppb)	14.64	15.05
MeSOAc (ppb)	6.89 [b]	5.76 [a]
DMDS (ppt)	35	33
EtSOAc (ppb)	0.49	0.44
DMTS (ppt)	142	159
Methionol (ppm)	2.11	2.33

Treatments bearing different superscripts are significantly different at $p<0.05$ by ANOVA and Tukey's HSD.

Table 8.9. The means of sulfur volatile compound concentrations in Pinot noir wine by with or without tillage treatment (n=36)

	With tillage	Without tillage
H_2S (ppb)	2.68	2.26
MeSH (ppb)	2.13	1.88
DMS (ppb)	15.50 [b]	14.19 [a]
MeSOAc (ppb)	6.47	6.18
DMDS (ppt)	35	33
EtSOAc (ppb)	0.47	0.45
DMTS (ppt)	157	144
Methionol (ppm)	2.20	2.24

Treatments bearing different superscripts are significantly different at $p<0.05$ by ANOVA and Tukey's HSD.



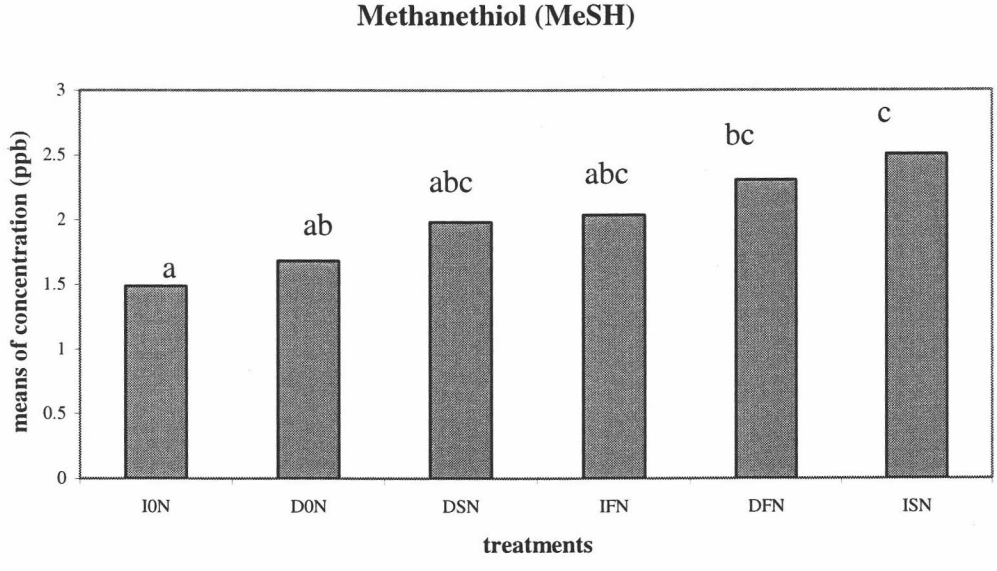


Figure 8.1. The concentration means of hydrogen sulfide (H_2S) and methanethiol (MeSH) by different irrigation and nitrogen treatment combination

Treatments bearing different superscripts are significant different at $p<0.05$ by ANOVA and Tukey's HSD.

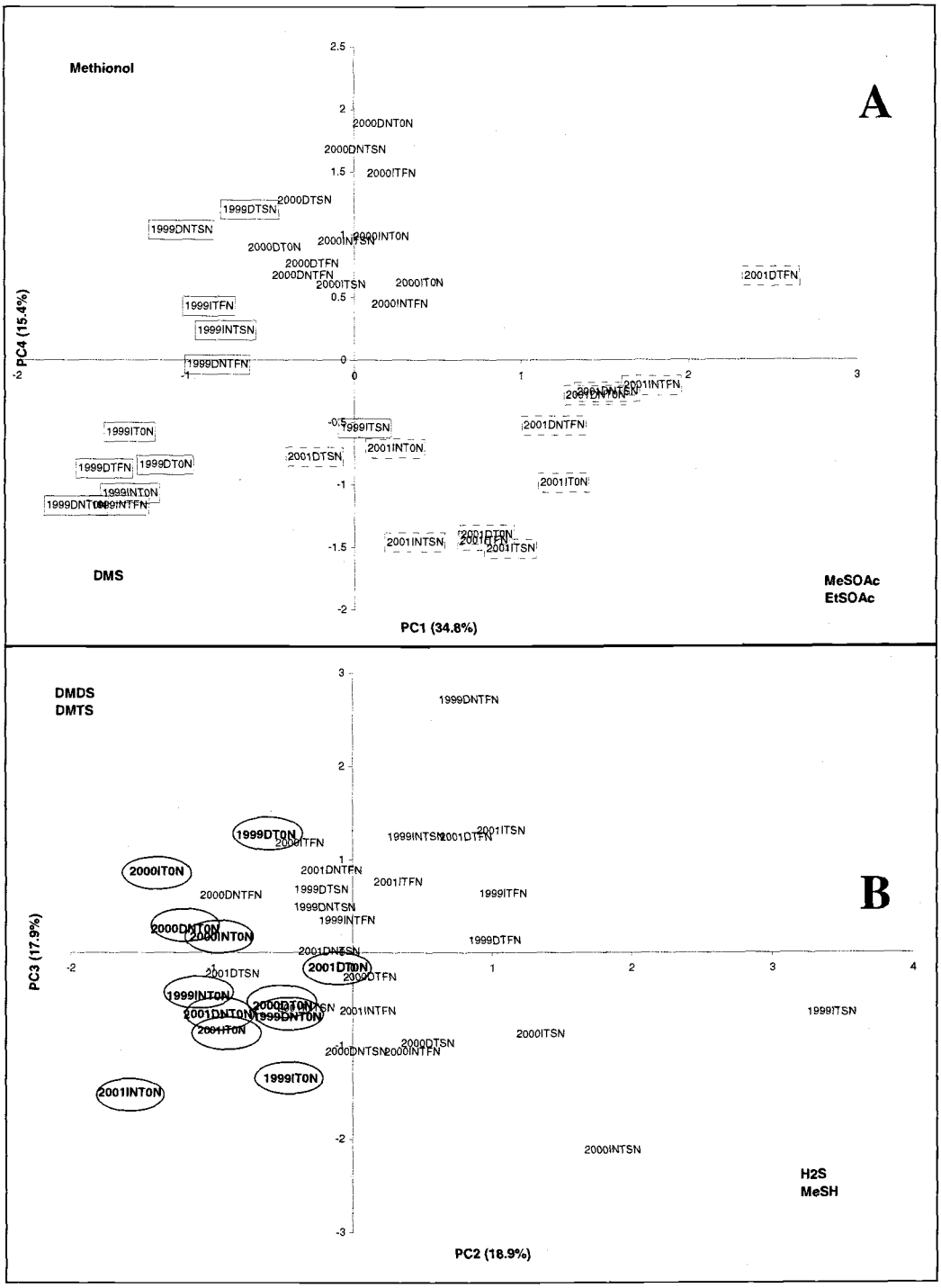


Figure 8.2. Principal components score plot from the sulfur analysis of Pinot noir wine

▭ : The wine samples in 1999; ▭ : The wine samples in 2001;
⬭ : The wine samples without nitrogen supplement

Chapter 9. General Summary

The aroma profiles of Oregon Pinot noir wines were investigated with aroma extract dilution analysis (AEDA). The wines were extracted with pentane-diethyl ether, the aromas were distilled using solvent-assisted flavor evaporation (SAFE), and separated into acid/water-soluble and neutral/basic fractions.

In the acid/water-soluble fraction, 2-phenylethanol and 3-methyl-1-butanol showed the highest AEDA values, followed by 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, 2-methylpropanol, hexanol, trans-3-hexenol, cis-3-hexenol, benzyl alcohol, methionol, 3-ethylthio-1-propanol, linalool, and geraniol (all with FD≥ 64).

In neutral/basic fractions, ethyl 2-methylpropanoate, ethyl butanoate, isoamyl acetate, ethyl hexanoate, and benzaldehyde had very high AEDA values (all with FD≥ 64), followed by ethyl 3-methylbutanoate, isoamyl 2-methylpropanoate, ethyl octanoate, ethyl decanoate, benzyl acetate, phenylethyl formate, phenylethyl acetate, ethyl dihydrocinanamate, ethyl anthranilate, methional, citronellal, whiskey lactone , and γ-nanalactone (all with FD≥ 16).

Therefore, no single compound characterized the aroma of Pinot noir, and the characteristic aroma comes from a blend of numerous compounds.

A method using stir bar sorptive extraction-gas chromatograph-mass spectrometry (SBSE-GC-MS) was developed to quantify these aroma compounds. Calibration curves of aroma compounds were built using five internal standards in a synthetic wine matrix. A high correlation coefficient (>0.95) and RSD (<10%) were obtained for all aroma compounds of interest.

Two vintages of Pinot noir wines, with three different grape maturities each, were analyzed by this method to investigate the effect of grape maturity on aroma compounds in Pinot noir wine. Statistical analysis showed that both grape maturity and growing year significantly affected the aroma composition of the final wine. Analysis of wine samples from the same vintage indicated that grape maturity could

affect aroma compounds in different ways, based on their biochemical formation in the wines.

For most fermentation related short-chain fatty acid esters, there were no obvious trends for their concentrations with grape maturity, however, it was observed that the concentrations of ethyl 2-methylpropanoate, and ethyl 3-methylbutanoate consistently decreased with grape maturity. The decreasing trend was also observed for other important characteristic esters for Pinot noir, including ethyl cinnamate, ethyl dihydroxycinnamate, and ethyl anthranilate, with the exception of ethyl vanillate, which increased with grape maturity. Most of the grape-derived aroma compounds including C_{13} norisoprenoids, monoterpenes, guaiacol and 4-ethylguaiacol had increasing trends in wine with grape maturation. However, linalool showed a decreasing trend with grape maturation.

The potential aroma compounds in Pinot noir grapes were also identified by solvent extraction/gas chromatography-olfactometry (GC-O). To investigate the relationship of grape and wine aroma, both free form and bound form of aroma compounds in Pinot noir grapes were quantified using three internal standards. The results showed that different compounds show different trends during grape development.

Free forms of green C6 alcohols and aldehydes sharply increased in the early stage, and decreased in the late stage. For most monoterpenes, free volatiles decreased during grape maturation, and bound precursors slightly increased or stayed at a similar level. Free forms of both β-inone and vanillin only showed an increase in very early stage, and diminished during grape maturity, while bound precursors increased during the whole season. Either free or bound floral alcohols dramatically increased during grape development. For β-damasonone and γ-nonalactone, their precursor significantly increased to a large amount, while only trace amount of free forms were observed in grape juice.

Moreover, a sensitive solid-phase microextraction and gas chromatography-pulsed flame photometric detection technique was developed to quantify volatile sulfur compounds in wine. Eleven sulfur compounds, including hydrogen sulfide,

methanethiol, ethanethiol, dimethyl sulfide, diethyl sulfide, methyl thioacetate, dimethyl disulfide, ethyl thioacetate, diethyl disulfide, dimethyl trisulfide and methionol, can be quantified simultaneously by employing three internal standards. Calibration curves were established in a synthetic wine, and linear correlation coefficients (R2) were greater than 0.99 for all target compounds. The quantification limits for most volatile sulfur compounds were 0.5 ppb or lower, except for methionol, which had a detection limit of 60 ppb.

The effects of nitrogen fertilization, tilling and irrigation on content of volatile sulfur compounds in Pinot noir wines were studied using this method, and the concentrations were analyzed by MANOVA, ANOVA, and PCA technique. The results showed that year, irrigation, and nitrogen had significant effects on concentrations of these target sulfur compounds ($p<0.01$ in MANOVA). Further ANOVA and PCA analysis showed that nitrogen fertilization had a major impact on levels of hydrogen sulfide (H2S) and methanethiol (MeSH).

Bibliography:

1. Marrse, H. and C.A. Visscher, *Volatile compounds in food, qualitative and quantitative data*. 1994, Zeist, The Netherlands: TNO-CIVO, Food Analysis Institute.

2. Miranda-Lopez, R., et al., *Identification of additional odor-active compounds in Pinot noir wines*. American Journal of Enology and Viticulture, 1992. **43**(1): p. 90-92.

3. Lee, S.-J. and A.C. Nobel, *Characterization of odor-active compounds in CalifornianChardonnay wines using GC-olfactometry and GC-Mass Spectrometry*. Journal of Agricultural and Food Chemistry, 2003. **51**(27): p. 8036-44.

4. Aznar, M., et al., *Prediction of aged red wine aroma properties from aroma chemical composition. Partial least squares regression models.* Journal of Agricultural and Food Chemistry, 2003. **51**(9): p. 2700-07.

5. Engel, W., W. Bahr, and P. Schieberle, *Solvent assisted flavour evaporation - a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices.* European Food Research and Technology, 1999. **209**: p. 237-41.

6. Soleas, G.J., et al., *A derivatized gas chromatographic- mass spectrometric method for the analysis of both isomers of resveratrol in juice and wine.* American Journal of Enology and Viticulture, 1995. **46**(3): p. 346-52.

7. Salinas, M.R., G.L. Alonso, and F.J. Esteban-Infantes, *Adsorption-Thermal Desorption-Gas Chromatography Applied to the Determination of Wine Aromas.* Journal of Agricultural and Food Chemistry, 1994. **42**(6): p. 1328-31.

8. Qian, M. and G.A. Reineccius, *Quantification of Aroma Compounds in Parmigiano Reggiano Cheese by a Dynamic Headspace Gas Chromatography-Mass Spectrometry Technique and Calculation of Odor Activity Value.* Journal of Dairy Science, 2003. **86**: p. 760-76.

9. Takeoka, G.R. and G.H. Full, *Analysis of volatile constituents of friut*, in *Modern methods of plant analysis, Vol 19: plant volatile analysis*, H.F. Linskers and J.F. Jackson, Editors. 1997, Springer-Verlag: New York.

10. Noble, A.C., R.A. Flath, and R.R. Forrey, *Wine Headspace Analysis. Reproducibility and Application to Varietal Classification.* Journal of Agricultural and Food Chemistry, 1980. **28**(2): p. 346-53.

11. Pelusio, F., et al., *Headspace Solid-Phase Microextraction Analysis of Volatile Organic Sulfur Compounds in Black and White Truffle Aroma.* Journal of Agricultural and Food Chemistry, 1995. **43**(8): p. 2138-43.

12. Haberhauer-Troyer, C., E. Rosenberg, and M. Grasserbauer, *Evaluation of solid-phase microextraction for sampling of volatile organic sulfur compounds in air for subsequent gas chromatographic analysis with atomic emission detection.* Journal of Chromatography, A, 1999. **848**(1 + 2): p. 305-15.

13. Blanc, P., et al., *Development of new techniques in the field of quantitative and isotopic analyses of aromatic and sulfur-aromatic compounds in water samples : Potential for petroleum and environmental geochemistry.* Abstracts of Papers, 221st ACS National Meeting, San Diego, CA, United States, April 1-5, 2001, 2001: p. GEOC-020.

14. Fan, X., et al., *Volatile sulfur compounds in irradiated precooked turkey breast analyzed with pulsed flame photometric detection.* Journal of agricultural and food chemistry, 2002. **50**(15): p. 4257-61.

15. Lestremau, F., et al., *Evaluation of Solid-Phase Microextraction for Time-Weighted Average Sampling of Volatile Sulfur Compounds at ppb Concentrations.* Analytical Chemistry, 2003. **75**(11): p. 2626-32.

16. Qian, M.C. and H. Burbank, *Volatile sulfur compounds in Cheddar cheese determined by headspace SPME-PFPD.* Abstracts of Papers, 228th ACS National Meeting, Philadelphia, PA, United States, August 22-26, 2004, 2004: p. AGFD-153.

17. Murray, R.A., *Limitations to the use of solid-phase microextraction for quantitation of mixtures of volatile organic sulfur compounds.* Analytical Chemistry, 2001. **73**(7): p. 1646-49.

18. Lestremau, F., et al., *Development of a quantification method for the analysis of malodorous sulphur compounds in gaseous industrial effluents by solid-phase microextraction and gas chromatography-pulsed flame photometric detection.* Journal of Chromatography, A, 2003. **999**(1-2): p. 71-80.

19. Nielsen, A.T. and S. Jonsson, *Q uantification of volatile sulfur compounds in complex gaseous matrices by solid-phase microextraction.* Journal of Chromatography A, 2002. **963**: p. 57-64.

20. Tuduri, L., V. Desauziers, and J.L. Fanlo, *Dynamic versus static sampling for the quantitative analysis of volatile organic compounds in air with*

polydimethylsiloxane–Carboxen solid-phase microextraction fibers. Journal of Chromatography A, 2002. **963**: p. 49-56.

21. Arthur, C.L., et al., *Automation and optimization of solid-phase microextraction.* Analytical Chemistry, 1992. **64**(17): p. 1960-6.

22. Demyttenaere, J.C.R., et al., *Flavor analysis of Greek white wine by solid-phase microextraction-capillary gas chromatography-mass spectrometry.* Journal of Chromatography, A, 2003. **985**(1-2): p. 233-46.

23. Tuduri, L., V. Desauziers, and J.L. Fanlo, *Determination of absolute amount extracted by solid-phase microextraction: Different approaches under examination.* Journal of Microcolumn Separations, 2000. **12**(10): p. 550-57.

24. Baltussen, E., et al., *Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: theory and principles.* Journal of Microcolumn Separations, 1999. **11**(10): p. 737-47.

25. Bicchi, C., et al., *Headspace Sorptive Extraction (HSSE), Stir Bar Sorptive Extraction (SBSE), and Solid Phase Microextraction (SPME) Applied to the Analysis of Roasted Arabica Coffee and Coffee Brew.* Journal of Agricultural and Food Chemistry, 2002. **50**(3): p. 449-59.

26. Baltussen, E., C.A. Cramers, and P.J.F. Sandra, *Sorptive sample preparation - a review.* Analytical and Bioanalytical Chemistry, 2002. **373**(1-2): p. 3-22.

27. Benanou, D., F. Acobas, and M.R. de Roubin, *Trace analysis of malodorous compounds in drinking water by Twister stir-bar sorptive extraction (SBSE) and GC/MS.* LCGC North America, 2003(Suppl.): p. 24.

28. Benanou, D., et al., *Analysis of off-flavors in the aquatic environment by stir bar sorptive extraction-thermal desorption-capillary GC/MS/olfactometry.* Analytical and Bioanalytical Chemistry, 2003. **376**(1): p. 69-77.

29. Bicchi, C., et al., *SBSE-GC-ECD/FPD in the Analysis of Pesticide Residues in Passiflora alata Dryander Herbal Teas.* Journal of Agricultural and Food Chemistry, 2003. **51**(1): p. 27-33.

30. Bonnlaender, B., et al., *Analysis of enantiomeric linalool ratio in green and roasted coffee.* Colloque Scientifique International sur le Cafe, 2005. **20**: p. 346-50.

31. De Villiers, A., et al., *Stir bar sorptive extraction-liquid desorption applied to the analysis of hop-derived bitter acids in beer by micellar electrokinetic chromatography.* Electrophoresis, 2004. **25**(4-5): p. 664-69.

32. Alves, R.F., A.M.D. Nascimento, and J.M.F. Nogueira, *Characterization of the aroma profile of Madeira wine by sorptive extraction techniques.* Analytica Chimica Acta, 2005. **546**(1): p. 11-21.

33. Hayasaka, Y., et al., *Application of stir bar sorptive extraction for wine analysis.* Analytical and Bioanalytical Chemistry, 2003. **375**(7): p. 948-55.

34. Leon, V.M., et al., *Analysis of 35 priority semivolatile compounds in water by stir bar sorptive extraction-thermal desorption-gas chromatography-mass spectrometry I. Method optimisation.* Journal of Chromatography, A, 2003. **999**(1-2): p. 91-101.

35. Guerrero, E.D., et al., *Optimisation of stir bar sorptive extraction applied to the determination of volatile compounds in vinegars.* Journal of Chromatography, A, 2006. **1104**(1-2): p. 47-53.

36. Acree, T.E., J. Barnard, and D.G. Cunningham, *A procedure for sensory analysis of gas chromatographic effluents.* Food Chemistry, 1984. **14**: p. 273-86.

37. Acree, T.E., *GC/olfactometry.* Analytic Chemistry, 1997. **69**(New Features): p. 170A-75A.

38. Schieberle, P. and W. Grosch, *Identifizierung von Aromastoffen aus der Krume von Roggenbrot - Vergleich mit den Aromastoffen der Kruste.* Z Lebensm Unters Forsch, 1984. **178**: p. 479.

39. Grosch, W., *Detection of potent odorants in foods by aroma extract dillution analysis.* Trends Food Science and Technology, 1993. **4**: p. 68-73.

40. Grosch, W., *Evalution of the Key Odorants of Foods by Dilution Experiments, Aroma Models and Omission.* Chemical Sense, 2001. **26**: p. 533-45.

41. Guth, H., *Identification of chracter impact odorants of different white wine varieties.* Journal of Agricultural and Food Chemistry, 1997. **45**: p. 3022-26.

42. McDaniel, M.R., et al. *Pinot noir aroma: a sensory/gas chromatographic approach.* in *Charalambous, G. (ed.), Flavours and Off-flavours.* 1990. Amsterdam: Elsevier Science.

43. Miranda-Lopez, R., et al., *Odor analysis of Pinot noir wines from grapes of different maturities by a gas chromatography-olfactometry technique (Osme).* Journal of Food Science, 1992. **57**(4): p. 985-93, 1019.

44. Francis, I.L. and J.L. Newton, *Determining wine aroma from compositional data.* Australian Journal of Grape and Wine Research, 2005. **11**(2): p. 114-26.

45. Diez, J., et al., *Optimisation of stir bar sorptive extraction for the analysis of volatile phenols in wines.* Journal of Chromatography, A, 2004. **1025**(2): p. 263-67.

46. Guth, H., *Quantitation and sensory studies of charater impact odorants of different white wine varieties.* Journal of Agricultural and Food Chemistry, 1997. **45**(8): p. 3027-32.

47. Aznar, M., et al., *Identification and quantification of impact odorants of aged red wines from Rioja. GC-olfactometry, quantitative GC-MS, and odor evaluation of HPLC fractions.* Journal of Agricultural and Food Chemistry, 2001. **49**(6): p. 2924-29.

48. Escudero, A., et al., *Characterization of the aroma of a wine from Maccabeo. key role played by compounds with low odor activity values.* Journal of Agricultural and Food Chemistry, 2004. **52**: p. 3516-24.

49. Lopez, R., et al., *Impact odorants of different young white wines form Canary Islands.* Journal of Agricultural and Food Chemistry, 2003. **51**(3419-3425).

50. Fang, Y. and M. Qian, *Aroma compounds in Oregon Pinot Noir wine determined by aroma extract dilution analysis (AEDA).* Flavour and Fragrance Journal, 2005. **20**(1): p. 22-29.

51. Petka, J., et al., *Sensory and Chemical Characterization of the Aroma of a White Wine Made with Devý´n Grapes.* Journal of Agricultural and Food Chemistry, 2006. **54**: p. 900-15.

52. Ferrari, G., *Influence of must nitrogen composition on wine and spirit quality and relation to aroma composition and defects. A review.* Journal International des Sciences de la Vigne et du Vin, 2002. **36**(1): p. 1-10.

53. Boulton, R.B., et al., *Principles and Practics of Winemaking.* 1996, New York: Chapman & Hall.

54. Giudici, P., P. Romano, and C. Zambonelli, *A biometric study of higher alcohol production in Saccharomyces cerevisiae.* Canadian Journal of Microbiology, 1990. **36**: p. 61-64.

55. Fleet, G.H. and G.M. Heard, *Yeast: Growth during fermentation*, in *Wine Microbiology and biotechnology*, G.H. Fleet, Editor. 1993, Harwood Academic Publishers: Chur, Switzerland.

56. Herraiz, T., et al., *Changes in the composition of alcohols and aldehydes of C6 chain length during the alcoholic fermentation of grape must.* Journal of Agricultural and Food Chemistry, 1990. **38**: p. 969-72.

57. Martin-Alvarez, P.J., et al., *Applications of Chemometrics to the Characterization of Spanish Wines.*, in *Flavor Science and Technology*, M. Martens, G.A. Dalen, and H. Russwurm, Editors. 1987, Wiely: New York. p. 489-99.

58. Cordonnier, R. and C. Bayonove, *Etude de la Phase Prefermentaire de la Vinification: Extraction et Formation de certains Composlis de l'Arome; Cas des Terpenols, des Aldehydes et des Alcohols en C6.* Conn. Vigne. Vin., 1981. **15**: p. 269-86.

59. Mesias, J.L., et al., *Alcools et Aldehydes Herbaces (C6) dans les Raisins et les Mouts en Fermentation Alcoolique.* Reo. Franc. d'0enolog., 1983. **90**: p. 50-55.

60. Ramey, D., et al., *Effects of Skin Contact Temperature on Chardonnay Must and Wine Composition.* American Journal of Enology and Viticulture, 1986. **37**(99-106).

61. Ong, P.K.C. and T.E. Acree, *Similarities in the Aroma Chemistry of Gewurztraminer Variety Wines and Lychee (Litchi chinesis Sonn.) Fruit.* Journal of Agricultural and Food Chemistry, 1999. **47**(665-670).

62. Dubois, P., *Les aro ̂mes des vins et leurs de ́fauts.* Rev. Fr. Oenol., 1994. **146**: p. 39-50.

63. Oliva, J., et al., *Effect of Pesticide residues on the aromatic composition of red wines.* Journal of Agricultural and Food Chemistry, 1999. **47**: p. 2830-36.

64. Zugliano, M. and L. Moio, *Changes in the Concentration of Yeast-Derived Volatile Compounds of Red Wine during Malolactic Fermentation with Four Commercial Starter Cultures of Oenococcus oeni.* Journal of Agricultural and Food Chemistry, 2005. **53**: p. 10134-39.

65. Kawabe, T. and H. Morita, *Production of Benzaldehyde and Benzyl Alcohol by the Mushroom Polyporus tuberaster K2606.* Journal of Agricultural and Food Chemistry, 1994. **42**: p. 2556-60.

66. Etschmann, M.M.W., et al., *Biotechnological production of 2-phenylethanol.* Applied Microbiology and Biotechnology, 2002. **59**(1): p. 1-8.

67. Antonelli, A., et al., *Yeast Influence on Volatile Composition of WIne*s. Journal of Agricultural and Food Chemistry, 1999. **47**: p. 1139-44.

68. Mallouchos, A., et al., *Volatile Compounds of Wines Produced by Cells Immobilized on Grape Skins.* Journal of Agricultural and Food Chemistry, 2003. **51**: p. 3060-66.

69. Ferreira, V., et al., *Quantitative Gas Chromatography-Olfactometry Carried out at Different Dilutions of an Extract. Key Differences in the Odor Profiles of Four High-Quality Spanish Aged Red Wines.* Journal of Agricultural and Food Chemistry, 2001. **49**: p. 4818-24.

70. Vernin, G., et al., *Aromas of Mourvedre wines*, in *Shelf-life studies of Foods and Beverages*, G. Charalambous, Editor. 1993, Elsevier: Amsterdam. p. 923-44.

71. Aldave, L., *The shelf-life of young white wines*, in *Shelf-life Studies of Foods and Beverages*, G. Charalambous, Editor. 1993, Elsevier: Amsterdam. p. 923-9544.

72. Martia, M.P., et al., *Solid-Phase Microextraction and Gas Chromatography Olfactometry Analysis of Successively Diluted Samples. A New Approach of the Aroma Extract Dilution Analysis Applied to the Characterization of Wine Aroma.* Journal of Agricultural and Food Chemistry, 2003. **51**: p. 7861-65.

73. Ribereau-Gayon, P., et al., *Handbook of Enology, Volume I: Wine and Wine Making*. 2000, Chichester: John Wiley & Sons.

74. Buszewski, B. and M. Ligor, *Qualitative and quantitative analyses of volatile organic compounds in wines using SPME-GC.* Chemia Analityczna (Warsaw, Poland), 2001. **46**(2): p. 215-24.

75. Beltran, G., et al., *Influence of the timing of nitrogen additions during synthetic grape must fermentations in fermentation kinetics and nitrogen consumption.* Journal of Agricultural and Food Chemistry, 2005. **53**: p. 996-1002.

76. Bisson, L.F. *Influence of nitrogen on yeast and fermentation of grapes*. in *Proceedings of the International symposium on Nitrogen in Grapes and Wines*. 1991. Davis, CA: American Society of Econolgy and Viticulture.

77. Diaaz-Maroto, M.C., R.M. Schneider, and R. Baumes, *Formation Pathways of Ethyl Esters of Branched Short-Chain Fatty Acids during Wine Aging.* Journal of Agricultural and Food Chemistry, 2005. **53**: p. 3503-09.

78. Moio, L. and P.X. Etievant, *Ethyl anthranilate, ethyl cinnamate, 2,3-dihydrocinnamate, and methyl anthranilate: four important odorants identified in Pinot noir wines of Burgundy.* American Journal of Enology and Viticulture, 1995. **46**(3): p. 392-98.

79. Aubry, V., et al., *Quantitative determination of potent flavor compounds in Burgundy Pinot noir wines using a stable isotope dilution assay.* Journal of Agricultural and Food Chemistry, 1997. **45**(6): p. 2120-23.

80. Maicas, S. and J.J. Mateo, *Hydrolysis of terpenyl glycosides in grape juice and other fruit juice: a review.* Applied Microbiology and Biotechnology, 2005. **67**: p. 322-25.

81. Manitto, P., *Biosynthesis of Natural Products.* 1980, Chichester: Ellis Horwood.

82. Mateo, J.J. and M. Jimenez, *Monoterpenes in grapes juice and wines.* Journal of Chromatography A, 2000. **881**: p. 557-67.

83. Ebeler, S.E., *Analytical chemistry: Unlocking the secrets of wine flavor.* Food Reviews International, 2001. **17**(1): p. 45-64.

84. Strauss, C.R., B. Wilson, and P.J. Williams. *Flavour of non-muscat varieties.* in *Proceedings of sixth Australian wine industry technical conference.* 1987. Adelaide, South Australia: Adelaide: Australian Indestrial Publishers.

85. Mendes-Ferreira, A., M.C. Climaco, and A. Mendes-Faia, *The role of non-Saccharomyces species in releasing glycosidic bound fraction of grape aroma components - a preliminary study.* Journal of Applied Microbiology, 2001. **91**(1): p. 67-71.

86. Vas, G., et al., *Examination of aroma production kinetics of different commercial wine yeasts in fermenting Muscat Ottonel wines with the help of SPME head-space sampling and fast GC analysis.* Acta-Alimentaria., 1999. **28(2)**:: p. 133-40.

87. Ganga, M.A., et al., *Aroma improving in microvinification processes by the use of a recombinant wine yeast strain expressing the Aspergillus nidulans xlnA gene.* International Journal of Food Microbiology, 1999. **47**(3): p. 171-78.

88. Tomasi, D., et al., *Studies on precursors of varietal aroma in musts from the winemaking grape cv. Prosecco.* Enologo., 2000. **36**(6): p. 97-102.

89. Rapp, A. and H. Mandery, *Wine aroma.* Experientia, 1986. **42**: p. 873-84.

90. Francis, I.L., M.E. Tate, and P.J. Williams, *The effect of hydrolysis conditions on the aroma released from Semillon grape glycosides.* Australian Journal of Grape and Wine Research, 1996. **2**: p. 70-76.

91. Lopez, R., et al., *Analysis of the aroma intensities of volatile compounds released from mild acid hydrolysates of odourless precursors extracted from Tempranillo and Grenache grapes using gas chromatography-olfactometry.* Food Chemistry, 2004. **88**: p. 95-103.

92. Pigella, R., et al., *Pinot noir varietal characterization by polyphenols and aroma precursors study.* Rivista di Viticoltura e di Enologia, 1998. **51**(1): p. 45-62.

93. Winterhalter, P., M.A. Sefton, and P.J. Williams, *Volatile C13-Norisoprenoid compounds in Riesling wine are generated from multiple precursors.* American Journal of Enology and Viticulture, 1990. **41**(4): p. 277-83.

94. Strauss, C.R., et al., *Development of precursors of C13 nor-isoprenoid flavorants in Riesling grapes.* American Journal of Enology and Viticulture, 1987. **38**(1): p. 23-27.

95. Cheynier, V., et al., *Phenolic composition as related of red wine flavor*, in *Chemistry of Wine Flavor*, A.L. Waterhouse and S.E. Ebeler, Editors. 1998, American Chemical Society: Washington, DC. p. 124-41.

96. Ribereau-Gayon, P., et al., *Handbook of Enology, Volume II: the Chemistry of Wine Stabilization and treatments.* 2000, Chichester: John Wiley & Sons.

97. Stevenson, T., *The Sotheby's Wine Encyclopedia.* 1997, New York: Dorling Kindersley. 664.

98. Buchholz, K.D. and J. Pawliszyn, *Determination of phenols by solid-phase microextraction and gas chromatographic analysis.* Environmental Science and Technology, 1993. **27**(13): p. 2844-8.

99. Ancin, C., et al., *Extraction of volatile compounds in model wine from different oak woods: effect of SO2.* Food Research International, 2004. **37**(4): p. 375-83.

100. Chatonnet, P., C. Viala, and D. Dubourdieu, *Influence of Polyphenolic Components of Red Wines on the Microbial Synthesis of Volatile Phenols.* American Journal of Enology and Viticulture, 1997. **48**(4): p. 443-48.

101. Towey, J.P. and A.L. Waterhouse, *Barrel-to-Barrel Variation of Volatile Oak Extractives in Barrel-Fermented Chardonnay.* American Journal of Enology and Viticulture, 1996. **47**(1): p. 17-20.

102. Ibern-Gómez, M., et al., *Differences in Phenolic Profile between Oak Wood and Stainless Steel Fermentation in White Wines.* American Journal of Enology and Viticulture, 2001. **52**(2): p. 159-64.

103. Clarke, R.J. and J. Bakker, *Wine flavour chemistry*. 2004, New York, NY: Blackwell Publishing.

104. Winterhalter, P., B. Baderschneider, and B. Bonnlander, *Analysis, structure, and reactivity of labile terpenoid aroma precursor in Riesling wine*, in *Chemistry of wine flavor*, A.L. Waterhouse and S.E. Ebeler, Editors. 1998, American Chemical Society: Washington, DC.

105. Waterhouse, A.L. and J.P. Towey, *Oak lactone isomer ratio distinguishes between wine fermentaed in American and French oak barrels.* Journal of Agricultural and Food Chemistry, 1994. **42**: p. 1971-74.

106. Chatonnet, P., *Volatile and odoriferous compounds in barrek-aged wines: ompact of cooperage techniques and aging conditions*, in *Chemistry of wine flavor*, A.L. Waterhouse and S.E. Ebeler, Editors. 1998, American Chemical Society: Washington, DC. p. 180-207.

107. Tominaga, T., P. Darriet, and D. Dubourdieu, *Identification of 3-mercaptoethanol acetate in Sauvignon wine, a powerful aromatic compound exhibiting box-tree odor.* Vitis, 1996. **35**(4): p. 207-10.

108. Tominaga, T., et al., *Contribution of volatile thiols to the aromas of white wines made from several Vitis vinifera grape variety.* American Journal of Enology and Viticulture, 2000. **51**: p. 178-81.

109. Murat, M.L., T. Tominaga, and D. Dubourdieu, *Assessing the Aromatic Potential of Cabernet Sauvignon and Merlot Musts Used to Produce Rose Wine by Assaying the Cysteinylated Precursor of 3-Mercaptohexan-1-ol.* Journal of Agricultural and Food Chemistry, 2001. **49**: p. 5412-17.

110. Tominaga, T., et al., *A powerful aromatic volatile thiol. 2-furanmethanethiol, exhibiting roast coffee aroma in wines made from several Vitis vinifera grape varieties.* Journal of Agricultural and Food Chemistry, 2000. **48**(5): p. 1799-802.

111. Blanchard, L., T. Tominaga, and D. Dubourdieu, *Formation of furfurylthiol exhibiting a strong coffee aroma during oak barrel fermentation from furfural*

release by toased staves. Journal of Agricultural and Food Chemistry, 2001. **49**: p. 4833-35.

112. Darriet, P., et al., *Identification of powerful aromatic compound of Vitis vinifera L. var. Sauvignon wines: 4-mercapto-4-methylpentan-2-one.* Flavour and Fragrance Journal, 1995. **10**: p. 385-92.

113. Ferreira, A.C.S., et al., *Influence of Some Technological Parameters on the Formation of Dimethyl Sulfide, 2-Mercaptoethanol, Methionol, and Dimethyl Sulfone in Port Wines.* Journal of Agricultural and Food Chemistry, 2003. **51**(3): p. 727-32.

114. Moreira, N., P. Guedes de Pinho, and I. Vasconcelos, *Method for analysis of heavy sulphur compounds using gas chromatography with flame photometric detection.* Analytica Chimica Acta, 2004. **513**(1): p. 183-89.

115. Mestres, M., O. Busto, and J. Guasch, *Chromatographic analysis of volatile sulfur compounds in wines using the static headspace technique with flame photometric detection.* Journal of Chromatography, A, 1997. **773**(1 + 2): p. 261-69.

116. Pripis-Nicolau, L., et al., *Methionine catabolism and production of volatile sulphur compounds by Oenococcus oeni.* Journal of Applied Microbiology, 2004. **96**(5): p. 1176-84.

117. Mestres, M., O. Busto, and J. Guasch, *Headspace solid-phase microextraction analysis of volatile sulphides and disulphides in wine aroma.* Journal of chromatography. A, 1998. **808**(1-2): p. 211-8.

118. Mestres, M., et al., *Headspace solid-phase microextraction of sulphides and disulphides using Carboxen-polydimethylsiloxane fibers in the analysis of wine aroma.* Journal of chromatography. A, 1999. **835**(1-2): p. 137-44.

119. Mestres, M., et al., *Simultaneous analysis of thiols, sulphides and disulphides in wine aroma by headspace solid-phase microextraction-gas chromatography.* Journal of chromatography. A, 1999. **849**(1): p. 293-7.

120. Mestres, M., O. Busto, and J. Guasch, *Application of headspace solid-phase microextraction to the determination of sulphur compounds with low volatility in wines.* Journal of Chromatography, A, 2002. **945**(1-2): p. 211-19.

121. Majcenovic, A.B., et al., *Synthesis and Stable Isotope Dilution Assay of Ethanethiol and Diethyl Disulfide in Wine Using Solid Phase Microextraction. Effect of Aging on Their Levels in Wine.* Journal of Agricultural and Food Chemistry, 2002. **50**(23): p. 6653-58.

122. Majcenovic, A.B., et al., *SIDA of ethanethiol and its disulphide in wine using SPME: application to the study of the effect of oenological tannins.* Flavour Research at the Dawn of the Twenty-First Century, Proceedings of the Weurman Flavor Research Symposium, 10th, Beaune, France, June 25-28, 2002, 2003: p. 510-13.

123. Rauhut, D., et al., *Headspace GC-SCD monitoring of low volatile sulfur compounds during fermentation and in wine.* Analusis, 1998. **26**(3): p. 142-45.

124. Lestremau, F., V. Desauziers, and J.-L. Fanlo, *Headspace SPME followed by GC/PFPD for the analysis of malodorous sulfur compounds in liquid industrial effluents.* Analytical and Bioanalytical Chemistry, 2004. **378**(1): p. 190-96.

125. Li, K.-C. and D. Shooter, *Analysis of sulfur-containing compounds in ambient air using solid-phase microextraction and gas chromatography with pulsed flame photometric detection.* International Journal of Environmental Analytical Chemistry, 2004. **84**(10): p. 749-60.

126. Burbank, H.M. and M.C. Qian, *Volatile sulfur compounds in Cheddar cheese determined by headspace solid-phase microextraction and gas chromatography-pulsed flame photometric detection.* Journal of Chromatography A, 2005. **1066**: p. 149-57.

127. Jing, H. and A. Amirav, *Pulsed flame photometric detector - a step forward towards universal heteroatom selective detection.* Journal of Chromatography, A, 1998. **805**(1 + 2): p. 177-215.

128. Mestres, M., O. Busto, and J. Guasch, *Analysis of organic sulfur compounds in wine aroma.* Journal of chromatography. A, 2000. **881**(1-2): p. 569-81.

129. Tominaga, T., M.-L. Murat, and D. Dubourdieu, *Development of a Method for Analyzing the Volatile Thiols Involved in the Characteristic Aroma of Wines Made from Vitis vinifera L. Cv. Sauvignon Blanc.* Journal of Agricultural and Food Chemistry, 1998. **46**: p. 1044-48.

130. Rauhut, D. and H. Kurbel, *Identification of wine aroma defects caused by sulfur-containing metabolites of yeasts.* Oenologie 95, Symposium International d'Oenologie, 5th, Bordeaux, June, 1995, 1996: p. 515-19.

131. Tsai, I.-M., *Understanding aroma impacts of four important volatile sulfur compounds in Oregon Pinot noit wines*, in *Food Science and Technology.* 2006, Oregon State University: Corvallis. p. 210.

132. Rauhut, D., *Production of sulfur compounds*, in *Wine, Microbiology, and Biotechnology*, G.H. Fleet, Editor. 1993, Hardwood Acdamic: Chur. p. 183-223.

133. Spiropoulos, A., et al., *Characterization of hydrogen sulfide formation in commercial and natural wine isolates of Saccharomyces.* American Journal of Enology and Viticulture, 2000. **51**(3): p. 233-48.

134. Wang, X.D., J.C. Bohlscheid, and C.G. Edwards, *Fermentative activity and production of volatile compounds by Saccharomyces grown in synthetic grape juice media deficient in assimilable nitrogen and/or pantothenic acid.* Journal of Applied Microbiology, 2003. **94**(3): p. 349-59.

135. Hallinan, C.P., D.J. Saul, and V. Jiranek, *Differential utilisation of sulfur compounds for H2S liberation by nitrogen-starved wine yeasts.* Australian Journal of Grape and Wine Research, 1999. **5**(3): p. 82-90.

136. Thomas, D. and Y. Surdin-Kerjan, *Metabolism of sulfur amino acids in Saccharomyces cerevisiae.* Microbiology and Molecular Biology Review, 1997. **61**: p. 503-32.

137. Vermeulen, C., L. Gijs, and S. Collin, *Sensorial contribution and formation pathways of thiols in food: A Review.* Food Reviews International, 2005. **21**: p. 69-137.

138. Rauhut, D., et al., *Properties and differences of commercial yeast strains with respect to their formation of sulfur compounds.* Wein-Wissenschaft, 1996. **51**(3/4): p. 187-92.

139. Franson, P., *Sulfur Compounds in Winemaking: A Report from the Vinquiry Seminar on the Role of Sulfur Compounds in Wine.* Vineyard & Winery Management, 2004. **30**(2).

140. Acree, T.E., E.P. Sonoff, and D.F. Splittstoesser, *Effect of yeast strain and type of sulfur comound on hydrogen sulfide production.* American Journal of Enology and Viticulture, 1972. **23**: p. 6-9.

141. Schutz, M. and R.E. Kunkee, *Formation of hydrogen sulfide from elemental sulfur during fermentation by wine yeast.* American Journal of Enology and Viticulture, 1977. **28**(3): p. 137-44.

142. Thomas, C.S., et al., *Changes in elemental sulfur residues on Pinot noir and Cabernet Sauvignon Grape Berries during the growing season.* American Journal of Enology and Viticulture, 1993. **44**(2): p. 205-10.

143. Thomas, C.S. and R. Boulton, *The effect of elementar sulfur, yeast strain, and fermentation medium on hydrogen sulfide producation during fermentation.* American Journal of Enology and Viticulture, 1993. **44**: p. 211-15.

144. Vos, P.J.A. and R.S. Gray, *The origin and control of hydrogen sulfide during fermentation of grape must.* American Journal of Enology and Viticulture, 1978. **30**: p. 187-97.

145. Henschke, P.A. and V. Jiranek, *Hydrogen sulfide fomation during the fermentation of model grape must as affected by nitrogen compostion*, in *International Syposium on Nitrogen in grapes and wine.* 1991, American Society of Enology and Viticulture: Davis, CA. p. 172-84.

146. Giudici, P. and R.E. Kunkee, *The effect of nitrogen deficiency and sulfur-containing amino acids on the reduction of sulfate to hydrogen sulfide by wine yeasts.* American Journal of Enology and Viticulture, 1994. **45**(1): p. 107-12.

147. Moreira, N., et al., *Volatile sulphur compounds in wines related to yeast metabolism and nitrogen composition of grape musts.* Analytica Chimica Acta, 2002. **458**(1): p. 157-67.

148. Karagiannis, S. and P. Lanaridis, *The effect of various vinification parameters on the development of several volatile sulfur compounds in Greek white wines of the cultivars Batiki and Muscat of Hamburg.* American Journal of Enology and Viticulture, 1999. **50**(3): p. 334-42.

149. Estevez, P., M.L. Gil, and E. Falque, *Effects of 7 yeast strains on the volatile composition of Palomino wines.* International Journal of Food Science and Technology, 2004. **39**(1): p. 61-69.

150. Park, S.K., R.B. Boulton, and A.C. Noble, *Formation of hydrogen sulfide and glutathione during fermentation of white grape musts.* American Journal of Enology and Viticulture, 2000. **51**(2): p. 91-97.

151. Leglise, M., *Une initiation à la dégustation des grands vins.* J. Laffitte (Ed), Marseille, 1984.

152. Schreier, P., *Flavour composition of wines: a review.* CRC Crit REV Food Sci Nutr, 1979. **12**: p. 59-111.

153. Nykanen, L., *Aroma of Beer, Wine and Distilled Alcoholic beverages.* 185 ed. 1982, Dordrecht, The Netherands: D Reidel Publishing Co.

154. Nykanen, L., *Formation and occurrence of flavor compounds in wine and distilled alcoholic beverages.* American Journal of Enology and Viticulture, 1986. **37**: p. 84-96.

155. Noble, A.C., *wine flavour*, in *Understanding natural flavours*, J.R. Piggot and P.A. Blackie, Editors. 1994, Glasgow, UK. p. 228-42.

156. Ferreira, V., R. López, and J.F. Cacho, *Quantitative determination of the odorants of young red wines from different grape varieties.* Journal of The Science of Food and Agriculture, 2000. **80**(11): p. 1659-67.

157. Chisholm, M.G., et al., *Comparison of some French-American hybrid wines with White Riesling using gas chromatography-olfactometry.* American Journal of Enology and Viticulture, 1994. **45**(2): p. 201-12.

158. Moio, L., et al., *Production of representative wine extracts for chemical and olfactory analysis.* Italian Journal of Food Science, 1995. **7**(3): p. 265-78.

159. Moio, L., P. Schlich, and P. Etievant, *Acquisition and analysis of aromagrams of Chardonnay Burgundy wines.* Sciences des Aliments, 1994. **14**(5): p. 601-08.

160. Ferreira, V., et al., *The aroma of Grenache red wine: hierarchy and nature of its main odorants.* Journal of the Science of Food and Agriculture, 1998. **77**(2): p. 259-67.

161. Lo´pez, R., et al., *Identification of impact odorants of young red wines made with Merlot, Cabernet Sauvignon and Grenache grape varieties: a comparative study.* Journal of the Science of Food and Agriculture, 1999. **79**: p. 1461-67.

162. Kotseridis, Y., et al., *Differentiation of the aromas of Merlot and Cabernet Sauvignon wines using sensory and instrumental analysis.* Journal of agricultural and food chemistry, 2000. **48**(11): p. 5383-88.

163. Ferreira, A.C.S., J.-C. Barbe, and A. Bertrand, *3-Hydroxy-4,5-dimethyl-2(5H)-furanone: A Key Odorant of the Typical Aroma of Oxidative Aged Port Wine.* Journal of Agricultural and Food Chemistry, 2003. **51**(15): p. 4356-63.

164. Boido, E., et al., *Aroma composition of Vitis vinifera cv. Tannat: the typical red wine from Uruguay.* Journal of Agricultural and Food Chemistry, 2003. **51**(18): p. 5408-13.

165. Brander, C.F., R.E. Kepner, and A.D. Webb, *Identification of some volatile compounds of wine of Vitis vinifera cultivar Pinot noir.* American Journal of

Enology and Viticulture, 1980. **31**(1): p. 69-74.

166. Schreier, P., *Wine aroma composition: identification of additional volatile constituents of red wine.* Journal-of-Agricultural-and-Food-Chemistry, 1980. **28**: p. 926-28.

167. Schreier, P., F. Drawert, and K.O. Abraham, *Identification and determination of volatile constituents in Burgundy Pinot noir wines.* Lebensmittel-Wissenschaft-und-Technologie, 1980. **13**: p. 318-21.

168. Hjorth, H.B., *Descriptive analysis of two consecutive vintages of Oregon Pinot noir wines as effected by irrigation, tillage and nitrogen supplementation in the vineyard*, in *Food Science and Technology*. 2002, Oregon State University: Corvallis. p. 172.

169. Qian, M. and G. Reineccius, *Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry.* Journal-of-Dairy-Science., 2002. **85(6):**: p. 1362-69.

170. Van den Dool, H. and P.D. Kratz, *A generalization of the retention indexsystem including linear temperature programmed gasliquid partition chromatography.* Journal of Chromatography A, 1963. **11**: p. 463-71.

171. Girard, B., et al., *Vinification effects on the sensory, colour and GC profiles of Pinot noir wines from British Columbia.* Food Research International, 2001. **34**(6): p. 483-99.

172. Webb, A.D. and C. Muller, *Advances in Applied Microbiology*. 1972, New York: Academic Press. 75-146.

173. Hashizume, K. and T. Samuta, *Green odorants of grape cluster stem and their ability to cause a wine stemmy flavor.* Journal of Agricultural and Food Chemistry, 1997. **45**(4): p. 1333-37.

174. Hatanaka, A., *the biogeneration of green odour by green leaves.* Phytochemistry, 1993. **34**: p. 1201-18.

175. Kanellis, A.K. and K.A. Roublakis-Angelakis, *Grapes*, in *Biochemistry of fruit ripening*, G.B. Seymour, J.E. Taylor, and G.A. Tucker, Editors. 1993, Chapman & Hall. p. 189-234.

176. Dozon, N.M. and A.C. Noble, *sensory study of the effect of fluorescent light on a sparkling wine and its base wine.* American Journal of Enology and Viticulture, 1989. **40**: p. 265-71.

177. Garcia, A., et al., *Influence of a mixed culture with Debaryomyces vanriji and Saccharomyces cerevisiae on the volatiles of a Muscat wine.* Journal of Food Science, 2002. **67**: p. 1138-43.

178. Ferreira, V., et al., *Chemical characterization of the aroma of Grenache rose wines: Aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies.* Journal of Agricultural and Food Chemistry, 2002. **50**(4048-5054).

179. Charles, M., et al., *Potent aroma compounds of two red wine vinegars.* Journal-of-Agricultural-and-Food-Chemistry, 2000. **48**(1): p. 70-77.

180. Karagiannis, S. and P. Lanaridis, *Influence of white wine conservation conditions on the levels of volatile sulphur compounds.* Vitis, 2000. **39**(2): p. 71-78.

181. Winterhalter, P. and G.K. Skouroumounis, *Glycoconjugated aroma compounds: occurrence, role, and biotechnological transformation*, in *Advances in biochemical engineering biotechnology*, T. Schaefer, Editor. 1997, Springer: Berlin, Germany. p. 73-105.

182. Skouroumounis, G.K. and M.A. Sefton, *The formation of ß-damascenone in wine*, in *Carotenoid-derived aroma compounds*, P. Winterhalter and R.L. Rouseff, Editors. 2001, American Chemicak Society: Washington, DC. p. 285-99.

183. Delfini, C., et al., *Production of benzaldehyde, benzyl alcohol and benzoic acid by yeasts and Botrytis cinerea isolated from grape musts and wines.* Vitis, 1991. **30**(4): p. 253-63.

184. Wildenradt, H.L. and V.L. Singleton, *Production of aldehydes as a result of oxidation of polyphenolic compounds and its relation to wine aging.* American Journal of Enology and Viticulture, 1974. **25**(2): p. 119-26.

185. Grosch, W., *Review: Determination of potent odorants in foods by aroma extract dilution analysis (AEDA) and calculation of odour activity values (OAVs).* Flavour Frangrance Journal, 1994. **9**: p. 147-58.

186. Guth, H., *Comparison of different white wine varieties in odor profiles by instrumental analysis and sensory studies*, in *ACS Symposium Series: Chemistry of Wine Flavor*, A.L. Waterhouse and S.E. Ebeler, Editors. 1998, American Chemical Society: Washington, DC. p. 39-52.

187. Berger, Drawert, and Kollmannsberger, Z Lebensm Unters Forsch, 1989. **188**: p. 122-26.

188. Umano, K., et al., *Volatile Chemicals Identified in Extracts from Leaves of Japanese Mugwort (Artemisia princeps Pamp.).* Journal of Agricultural and Food Chemistry, 2000. **48**: p. 3463-69.

189. Karagul-Yuceer, Y., et al., *Characteristic Aroma Components of Rennet Casein.* Journal of Agricultural and Food Chemistry, 2003. **51**: p. 6797-801.

190. Pino, J.A., R. Marbot, and A. Bello, *Volatile Compounds of Psidium salutare (H.B.K.) Berg. Fruit.* Journal of Agricultural and Food Chemistry, 2002. **50**: p. 5146-48.

191. Klesk, K. and M. Qian, *Aroma Extract Dilution Analysis of Cv. Marion (Rubus spp. hyb) and Cv. Evergreen (R. laciniatus L.) Blackberries.* Journal of Agricultural and Food Chemistry, 2003. **51**(11): p. 3436-41.

192. Vichi, S., et al., *Solid-Phase Microextraction in the Analysis of Virgin Olive Oil Volatile Fraction: Characterization of Virgin Olive Oils from Two Distinct Geographical Areas of Northern Italy.* Journal of Agricultural and Food Chemistry, 2003. **51**(22): p. 6572-77.

193. Pino, J.A., R. Marbot, and C. Vazquez, *Characterization of Volatiles in Strawberry Guava (Psidium cattleianum Sabine) Fruit.* Journal of Agricultural and Food Chemistry, 2001. **49**: p. 5883-87.

194. Loapez, R., et al., *Impact Odorants of Different Young White Wines from the Canary Islands.* Journal of Agricultural and Food Chemistry, 2003. **51**: p. 3419-25.

195. Schieberle, P., *Primary odorants of pale lager beer. Differences to other beers and changes during storage.* Zeitschrift fuer Lebensmittel Untersuchung und Forschung, 1991. **193**: p. 558-65.

196. Schieberle, P., S. Ofner, and W. Grosch, *Evaluation of potent odorants in cucumbers (Cucumis sativus) and muskmelons (Cucumis melo) by aroma extract dilution analysis.* Journal of Food Science, 1990. **55**(1): p. 193-95.

197. Pino, J.A. and R. Marbot, *Volatile Flavor Constituents of Acerola (Malpighia emarginata DC.) Fruit.* Journal of Agricultural and Food Chemistry, 2001. **49**: p. 5880-82.

198. Kotseridis, Y. and R. Baumes, *Identification of Impact Odorants in Bordeaux Red Grape Juice, in the Commercial Yeast Used for Its Fermentation, and in the Produced Wine.* Journal of Agricultural and Food Chemistry, 2000. **48**: p. 400-06.

199. Choi, H.-S., *Character Impact Odorants of Citrus Hallabong [(C. unshiu Marcov ´ C. sinensis Osbeck) ´ C. reticulata Blanco] Cold-Pressed Peel Oil.* Journal of Agricultural and Food Chemistry, 2003. **51**: p. 2687-92.

200. Hirose, S., et al., *Volatile components of musky scent of orchids.* Flavour and Fragrance Journal, 1999. **14**: p. 183-84.

201. Beaulieu, J.C. and C.C. Grimm, *Identification of Volatile Compounds in Cantaloupe at Various Developmental Stages Using Solid Phase Microextraction.* Journal of Agricultural and Food Chemistry, 2001. **49**: p. 1345-52.

202. Jirovetz, L., D. Smith, and G. Buchbauer, *Aroma Compound Analysis of Eruca sativa (Brassicaceae) SPME Headspace Leaf Samples Using GC, GC-MS, and Olfactometry.* Journal of Agricultural and Food Chemistry, 2002. **50**: p. 4643-46.

203. Klesk, K., M. Qian, and R.R. Martin, *Aroma Extract Dilution Analysis of cv. Meeker (Rubus idaeus L.) Red Raspberries from Oregon and Washington.* Journal of Agricultural and Food Chemistry, 2004. **52**: p. 5155-61.

204. Allen, M.S., et al., *Contribution of methoxypyrazines to Sauvignon blanc wine aroma.* American Journal of Enology and Viticulture, 1991. **42**(2): p. 109-12.

205. Kolpan, S., B.H. Smith, and M.A. Weiss, *Exploring Wine: The Culinary Institute of America's Guide to Wines of the World.* 2001, New York: John Wiley & Sons Inc.

206. Eggers, N., et al., *Evaluation of closed loop stripping for the isolation of wine aroma compounds from aqueous solution.* American-Journal-of-Enology-and-Viticulture. 2003.

207. Mestres, M., O. Busto, and J. Guasch, *Headspace solid-phase microextraction analysis of volatile sulphides and disulphides in wine aroma.* Journal of Chromatography A, 1998. **808**(1-2): p. 211-8.

208. Rio, J.L.P.d. and J.A. Kennedy, *Development of Proanthocyanidins in Vitis vinifera L. cv. Pinot noir Grapes and Extraction into Wine.* American Journal of Enology and Viticulture, 2006. **57**(2): p. In progress.

209. Rychlik, M., P. Schieberle, and W. Grosch, *In compilation of odour thresholds, odour qualities and retention indices of key food odorants.* 1998, München, HSRG: Deutsche Forschungsanstalt für Lebensmittelchemie and

Institut für Lebensmittelchemie der Technischen Universität.

210. Rapp, A., et al., Unters. Forsch., 1985. **180**: p. 109-16.

211. Rapp, A. and J. Marais, *The shelf life of wine: changes in aroma substances during storage and ageing of white wines*, in *The shelf life of foods and beverages*, G. Charalambous, Editor. 1993, Elsevier: Amsterdam. p. 141.

212. Dugelay, I., et al., *Etude de l'origine du citronellol dans les vins.* J. Int. Sci. Vigne Vin., 1992. **26**: p. 177-84.

213. Hernandez, L.F., et al., *Beta-Glucosidase activity in a Saccharomyces cerevisiae wine strain.* Int J Food Microbiol, 2003. **80**: p. 171–76.

214. Fan, W. and M.C. Qian, *Headspace solid phase microextraction (HS-SPME) and gas chromatography-olfactometry dilution analysis of young and aged Chinese "Yanghe Daqu" liquors.* Journal of Agricultural and Food Chemistry, 2005. **53**(20): p. 7931-38.

215. Fan, W. and M. Qian, *Identification of aroma compounds in Chinese 'Yanghe Daqu' liquor by normal phase chromatography fractionation followed by gas chromatography/olfactometry.* Flavour and Fragrance Journal, 2006. **21**: p. 333-42.

216. Keller, M. *Grape Ripening and Determination of Grape Maturity.* in *33rd Annual New York Wine Industry workshop*. 2004. Geneva, NY: CSIRO Rnquiries.

217. Jackson, D.I. and P.B. Lombard, *Environmental and management practices affecting grape composition and wine quality - A review.* American Journal of Enology and Viticulture, 1993. **44**: p. 409-30.

218. Coombe, B.G. and P.G. Iland. *Grape berry development.* in *the Sixth Australian Wine Industry Conference*. 1987. Adelaide, South Australia.

219. Iland, P.G., et al. *Viticultural parameters for sustaining wine style.* in *the Eighth Australian Wine Industry Technical Conference*. 1993. Adelaide, South Australia.

220. Blasco, C., et al., *Comparison of solid-phase microextraction and stir bar sorptive extraction for determining six organophosphorus insecticides in honey by liquid chromatography-mass spectrometry.* Journal of Chromatography, A, 2004. **1030**(1-2): p. 77-85.

221. Juan-Garcia, A., et al., *Evaluation of solid-phase extraction and stir-bar*

sorptive extraction for the determination of fungicide residues at low-microg kg(-1) levels in grapes by liquid chromatography-mass spectrometry. Journal of chromatography. A, 2004. **1050**(2): p. 119-27.

222. Almeida, C., et al., *New perspectives on preparing samples for chromatographic analysis.* Quimica (Lisboa, Portugal), 2004. **95**: p. 69-77.

223. Fang, Y. and M. Qian, *Effect of Grape Maturity on Aroma Compounds in Pinot Noir Wines Determined by Stir Bar Sorptive Extraction -Gas Chromatography-Mass Spectrometry.* Journal of Agricultural and Food Chemistry, 2006: p. Submitted.

224. Fang, Y. and M. Qian. *Development of Flavor/Flavor Precursor in Pinot noir Grapes*. in *ASEV Annual Meeting*. 2004. San Diego: poster.

225. Benanou, D., F. Acobas, and M.R. De Roubin, *Optimization of stir bar sorptive extraction applied to the determination of odorous compounds in drinking water.* Water Science and Technology, 2004. **49**(9): p. 161-70.

226. Alvarez-Aviles, O., L. Cuadra, and O. Rosario, *Optimization of microwave-assisted extraction/stir bar sorptive extraction/thermal desorption/gas chromatography/mass spectrometry methodology for the organic chemical characterization of particulate matter.* Abstracts of Papers, 229th ACS National Meeting, San Diego, CA, United States, March 13-17, 2005, 2005: p. ANYL-259.

227. Gunata, Y.Z., et al., *The aroma of grapes. Localisation and evolution of free and bound fractions of some grape aroma components c.v. Muscat during first develoemnt and maturation.* J. Sci. Food Agric., 1985. **36**: p. 857-62.

228. Herrick, I.W. and C.W. Nagel, *The caffeoyl tartrate content of white Riesling wines from California, Washington and Alsace.* American Journal of Enology and Viticulture, 1985. **36**: p. 95-97.

229. Ewart, A.J.W. *Influence of vineyard site and grape maturity on juice and wine quality of Vitis vinifera, cv. Riesling*. in *Sixth Australian Wine Industry Conference*. 1987. Adelaide, South Australian.

230. Fang, Y. and M. Qian, *The development of volatile composition in Pinot noir grapes.* 2006: p. Drafted.

231. Schneider, R., et al., *Rapid analysis of grape aroma glycoconjugates using Fourier-transform infrared spectrometry and chemometric techniques.* Analytic Chemica Acta, 2004. **513**: p. 91-96.

232. Francis, I.L., M.A. Sefton, and P.J. Williams, *Sensory descriptive analysis of the aroma of hydrolysed precursor fractions from Semillon, Chardonnay and Sauvignon Blanc grape juices.* Journal of the Science of Food and Agriculture, 1992. **59**(4): p. 511-20.

233. Shure, K.B., *In vivo and in vitro studies of Vitis x labruscana cv. Concord flavor.* Dissertation Abstracts International, B., 1994. **55**(7): p. 2461-62.

234. Stefano, R.D., et al., *Glycosylated aroma precursors in red winemaking grape cv.* Enotecnico., 1998. **34**(3): p. 63-74.

235. Wilson, B., C.R. Strauss, and P.J. Williams, *Changes in Free and Glycosidically Bound Monoterpenes in Developing Muscat Grapes.* Journal of Agricultural and Food Chemistry, 1984. **32**: p. 919-24.

236. Nielsen, A.T. and S. Jonsson, *Trace determination of volatile sulfur compounds by solid-phase microextraction and GC-MS.* Analyst, 2002. **127**(8): p. 1045-9.

237. Nowak, A., et al., *Production of H2S and properties of sulfite reductase from selected strains of wine-producing yeasts.* European Food Research and Technology, 2004. **219**(1): p. 84-89.

238. Fang, Y. and M.C. Qian, *Sensitive quantification of sulfur compounds in wine by headspace solid-phase microextraction technique.* Journal of Chromatograp. A, 2005. **1080**(2): p. 177-85.