Changes in Free and Glycosidically Bound Monoterpenes in Developing Muscat Grapes

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Five phases were distinguished in the development of free and glycosidically bound monoterpenes in *Vitis vinifera* cv. Muscat of Alexandria. At berry set, high concentrations of free and bound geraniol were observed; approaching véraison, levels of all terpenes decreased; during sugar accumulation terpene concentrations increased and several monoterpenes reached peak levels in the overripe fruit. After véraison, linalool, geraniol, nerol, α-terpineol, and the furan linalool oxides were present mainly as glycosides. While the total concentration of glycosides increased with ripening, only free linalool showed development paralleling that of its glycoside. At grape maturity, the level of (*E*)-3,7-dimethylocta-1,5-diene-3,7-diol was greater than the total concentration of all other monoterpenes. Possibilities for innovations in wine making are highlighted by the findings that the concentrations of glycosides and odorless polyols exceeded those of the free flavor compounds and also that maximal terpene levels were found in fruit of commercial maturity.

It is now widely recognized that the characteristic taste and aroma of muscat grape varieties are associated with the presence in the fruit of various volatile monoterpene compounds (Ribéreau-Gayon et al., 1975). Experiments designed to assign specific varietal characteristics to non-muscat grape varieties by use of statistical analysis have also demonstrated the importance of monoterpenoid constituents to the aroma of many different cultivars (Schreier et al., 1976a; Rapp et al., 1982). Similar statistical analyses have established that fruit-derived monoterpenes also characterize specific varietal wines (Schreier et al., 1976c). These observations have stimulated much research into the chemistry and biochemistry of grape monoterpenes.

A classification of muscat grape monoterpenoids (Williams et al., 1981) demonstrated that, in addition to the free terpenes that included a number of polyhydroxylated linalool derivatives (polyols) (Williams et al., 1980b; Rapp and Knipser, 1979; Rapp et al., 1980), there existed in juice nonvolatile water-soluble precursor compounds. These precursor compounds have been elucidated as a mixture of disaccharide glycosides of several monoterpene alcohols (Williams et al., 1982a). The monoterpene composition of grapes and wine has since been rationalized through hydrolytic studies on these glycosides and the polyols (Williams et al., 1980a, 1982b).

Several groups (Di Stefano et al., 1983; Rapp et al., 1982; Versini et al., 1981; Terrier et al., 1972; Hardy, 1970; Bayonove and Cordonnier, 1970a,b, 1971) have shown interest in the effect of ripening on grape monoterpene composition. Additionally, the influences of some grape processing conditions on juice terpene content and composition have also been investigated (Bayonove et al., 1976; Kinzer and Schreier, 1980; Versini et al., 1981). Many of these surveys, however, were made without considering the presence or hydrolytic sensitivity of the glycosidic precursors and polyols.

With recent knowledge of the properties and behavior of grape monoterpenoids combined with renewed interest in the quality of the harvest, experiments have been conducted to determine the change in content with berry development of individual monoterpenoids in the free and glycosidically bound categories. This paper presents the results of these experiments.

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EXPERIMENTAL SECTION

Grape Samples. Fruit of the *Vitis vinifera* L. variety Muscat of Alexandria (syn. Muscat Gordo Blanco) was harvested from the Waite Agricultural Research Institute vineyard, Glen Osmond, South Australia. According to the climatic classification of Smart and Dry (1980), this vineyard is in a region that is defined as hot, moderately maritime, sunny, arid, and not humid. The first sampling was made on Dec 6, 1982 (day 1 in Figure 2) immediately after berry set, with subsequent samplings taken at appropriate intervals until April 4, 1983 (day 139) when the grapes were overripe. Véraison occurred in the week beginning Jan 16, 1983 (days 47–54) while sugar and acid stabilized in the berries after day 90.

The terminology used in this work to describe the stages of berry development is that of Winkler et al. (1974).

Sample Preparation. Fresh grapes (125 g) were picked off bunches harvested from each of four vines to give a total sample mass of 500 g. The grapes were blended with NaCl (350 g), and the homogenate was titrated with 1 M KOH solution to pH 7–8. The homogenate was centrifuged at 10000 rpm at 5 °C for 20 min, and the supernatant was decanted and retained. After the residue was resuspended in pH 7 phosphate buffer, centrifuged, and decantered the supernatants were combined to give a volume of 400–700 mL, saturated with NaCl, and finally checked to ensure that the pH was >7.

Isolation of Free Monoterpenes. The internal standard (1-octanol; 0.165 mg/mL in EtOH) was added to each juice sample before liquid–liquid extraction with Freon F11. The extractions were made at 30 °C for 72 h, with solvent changes every 24 h (Williams et al., 1981). The stripped juice was set aside for assay of bound monoterpenes, while the combined Freon extracts were concentrated to 60 mL by fractional distillation through a column of Fenske's helices. For GC analysis, 10 mL of the Freon extract was treated with pyridine (10 μL of a 1 μL/mL solution in Freon) and concentrated in a sharply tapered flask as previously described (Williams et al., 1980a).

Isolation of Bound Monoterpenes. Passage of the solvent stripped juice down a column of C_{18} reversed-phase adsorbent (470 × 15 mm) and subsequent elution with MeOH gave the monoterpene glycosides (Williams et al.,

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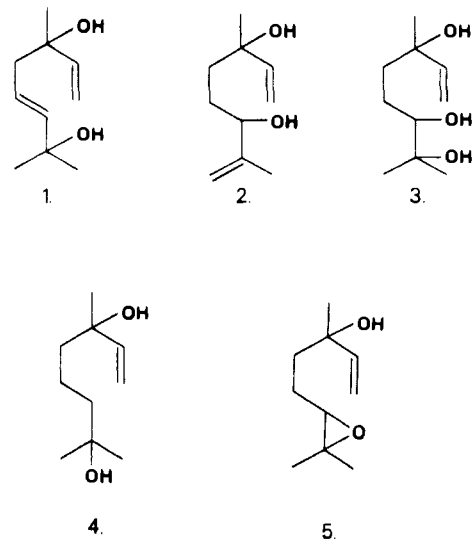

Figure 1. Monoterpenes referred to in this work.

1982c). The MeOH was removed in vacuo, yielding an aqueous residue (ca. 10 mL) as the substrate for enzymic hydrolysis. Rohapect C, a commercially available pectinase preparation from Rohm, previously shown to possess glycosidase activity (Williams et al., 1982a) (1 g), was made up in pH 5 buffer (200 mL). The bound monoterpene substrate was diluted with pH 5 buffer (20 mL) and enzyme solution (20 mL) added before incubation at 30 °C for 24 h. After addition of internal standard (1 mL), the enzyme hydrolysates were extracted and the extracts, containing the monoterpene aglycons, concentrated as described above. Control experiments demonstrated that thermally inactivated enzyme gave no volatile products from the substrate under these hydrolysis conditions.

Extracts of free and bound monoterpenes were analyzed by GC and GC–MS as described previously (Williams et al., 1982c).

Quantitative Analysis. A series of standard solutions of linalool, geraniol, α-terpineol, dienediol 1 (see Figure 1), *cis*-pyran linalool oxide, and the isomeric furan linalool oxides, ranging in concentration from 7.5 to 1000 μg/mL, was prepared by diluting aliquots of ethanolic stock solutions of each reference compound with pH 5 buffer to 50 mL. After addition of the internal standard, each solution was extracted with Freon F11, and the extracts were concentrated and analyzed as for the grape samples. Quantitative GC analyses were obtained with an electronic integrating recorder.

Reference Compounds. These materials were obtained as either commercial chemicals or donated samples, and all were at least 95% pure by GC.

RESULTS AND DISCUSSION

The procedures employed in this study allowed isolation of free monoterpenes by direct extraction of juice, and then, after subsequent enzymatic hydrolysis of the residual bound monoterpenes from their glycosides, the liberated aglycons were recovered by a second extraction. This enzymatic process for the determination of glycosidically bound monoterpenes was more convenient than a possible direct determination, e.g., GC analysis of the glycosidic fractions after trimethylsilylation. Direct determination of the aglycon composition would have been difficult because of the complexity of the mixture of the grape glycosides (Williams et al., 1982a). In the course of the above steps, and the ensuing GC analyses of the different monoterpene fractions, the following points of technique were found to be important. (1) The rapid hydrolysis of monoterpene glycosides and polyols at less than pH 4 necessitated adjustment of juices to near neutrality prior to solvent extraction (Williams et al., 1981). (2) Addition of salt to all juice samples inhibited any endogenous glycosidase activity and ensured that the free monoterpene levels were not enhanced by unwanted enzyme activity up to and during the course of the extraction. The glycosides were obtained salt free in the C_{18} reversed-phase isolation step and were then hydrolyzed enzymatically for determination of bound monoterpenes. (3) To observe the highly labile dienediol 1, it was found that all traces of acid had to be removed from the glassware and solvents before analysis. Accordingly, a small amount of pyridine was added to each Freon extract prior to concentration; failure to take this precaution gave low and nonreproducible figures for the level of this major grape monoterpenoid.

Free Monoterpenes (See Figure 2). The most abundant free monoterpene of the grapes before véraison was geraniol, which, at berry set, was present in the fruit at a level of 770 μg/kg. Of the other free compounds in the fruit at first sampling, significant levels of dienediol 1, linalool, nerol, and pyran linalool oxides were present. However, in the period from berry set to véraison, the concentrations of all free compounds fell to low levels. From véraison onward, only four monoterpenes, dienediol 1, linalool, and the pyran ring linalool oxides, began to accumulate to any significant extent in the berries. Dienediol 1 increased in concentration so rapidly that it dominated the free terpene distribution, and in the ripe grapes it attained a level that exceeded the collective concentrations of all other monoterpenoids of the fruit.

Increases in concentration did not occur with geraniol, nerol, or α-terpineol. The latter pair were never present in significant amounts during the development of the fruit, while geraniol, after showing a small rise with sugar accumulation, also fell, to less than 5 μg/kg in the ripe berries. The only other free monoterpenes of those studied to show any major changes in concentration were the furan linalool oxides, and of these two the trans isomer was present in the greatest concentration in the ripe fruit.

With regard to the oxidation level of the free monoterpenes of the juice, it is clear that the higher, linalool oxide, oxidation state compounds were most abundant in the ripe fruit. Linalool was the only lower oxidation level compound to increase with berry ripening, and the nexus between linalool development and the concentration increase of the pyran linalool oxides and dienediol 1 may reflect a biosynthetic connection between these monoterpenes of different oxidation levels. While the mechanism of the oxidation step remains unknown, it is difficult to interpret the significance, if any, of the cooccurrence of specific free monoterpenes. It is conceivable that free linalool is a substrate for the oxidation and that the products of the reaction, which could be dienediol 1 and the pyran linalool oxides, undergo glycosidic conjugation only after the oxidation step.

The high concentration of free geraniol observed in juice of the green berries also suggests a significant biosynthetic role for this compound in the fruit before but not after véraison.

Bound Monoterpenes (See Figure 2). At berry set, glycosidically bound monoterpenes were present in the fruit and the geranyl derivatives clearly predominated. During this stage of berry development, the relative abundance of the individual free and bound monoterpenes for the most part correlated well.

From berry set to véraison, the concentrations of glycosidically bound monoterpenes fell to their lowest levels

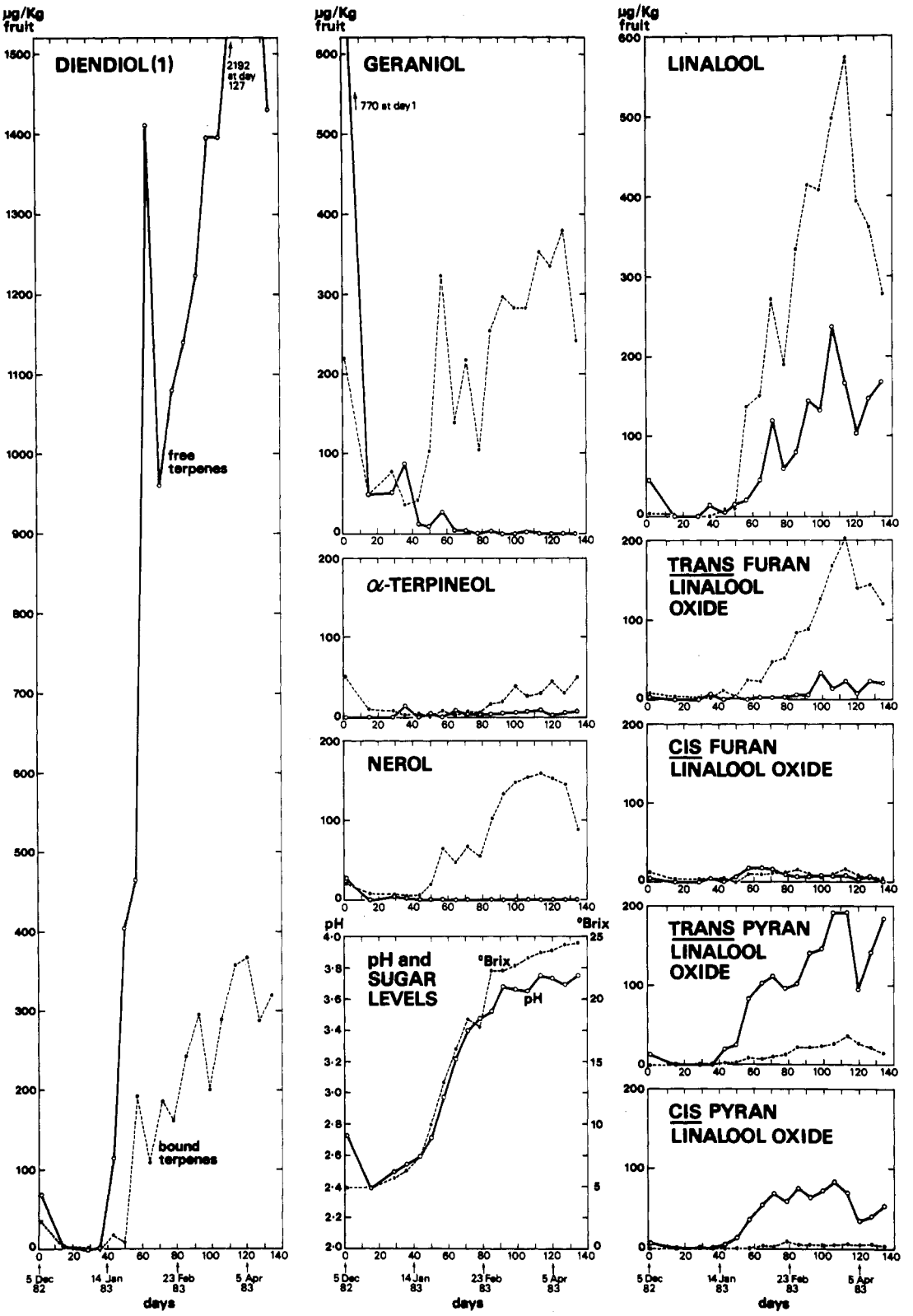

Figure 2. Changes in juice concentration of free (solid lines) and glycosidically bound (broken lines) monoterpenes in developing Muscat of Alexandria grapes. Juice pH and sugar levels are shown in the bottom center graph. Berry set was complete by the first week of December and véraison occurred between day 47 and day 54 of the survey.

of the entire sampling period. Substantial increases in concentration of glycosides of linalool, geraniol, nerol, dienediol 1, and *trans*-furan linalool oxide occurred during véraison. Over a 40-day period from day 50, the major monoterpene glycosides exhibited dynamic changes in concentration, with most compounds showing three rises and two falls. There was no obvious climatic variable to account for this perturbation, with neither significant rain nor abnormal temperature variations occurring in the period. It would not be unexpected, however, that significant changes in the biosynthesis of glycosides could accompany periods of such high metabolic activity of the fruit and vine. At the completion of sugar accumulation, glycosides of all the monoterpenes analyzed continued to increase in concentration, suggesting that biosynthesis of the glycosides was a process independent of sugar translocation.

In contrast to the free monoterpenes, geraniol oxida-

tion-state compounds dominated as glycosides in the juice at maturity, with linalyl and geranyl derivatives most significant. Of the higher oxidation state monoterpene glycosides, the dienediol 1 derivatives reached their highest concentration in the overripe fruit. However, this level of bound dienediol 1 was less than 20% of the concentration of free dienediol 1 in the juice at this time. With regard to the linalool oxide glycosides, a noteworthy feature was the imbalance in distribution of the various stereoisomeric forms. As free compounds the *cis*-furan linalool oxide was less abundant than the trans isomer, and this difference was much more apparent in their glycosides, with the cis/trans ratio varying from 5/1 at véraison to 30/1 in the overripe fruit. Similarly, the stereoisomeric forms of the pyran linalool oxide glycosides were also unequal in the juice of the ripening berries, with the trans isomer more abundant than the cis.

Apart from free dienediol 1, the juice of the mature grapes contained a higher total concentration of monoterpenes in the glycosidically bound form than as free compounds. This was the case even though the isomeric pyran ring linalool oxides were also present in higher concentrations as free compounds than as glycosides after day 50 of the study.

Finally, the peaking of the major glycosides in the later samplings may have reflected a metabolic decline of the compounds in the overripe fruit. The last samples were taken well past commercial picking time, when the vine was entering dormancy.

Relationship between Free and Glycosidically Bound Monoterpenes. The data in Figure 2 show a general similarity in development of free and glycosidically bound monoterpenes in the fruit but little correlation between the behavior of the two forms for most individual monoterpenes. Thus, with the exception of the high concentration of both forms of linalool in the mature fruit and also the situation in the green berries in which free and glycosidically bound geraniol were simultaneously abundant, there was no other obvious connection between the free monoterpenes and their glycosides.

It seems that many individual glycosides are synthesized from aglycons that exist in only a very small concentration as free compounds in the fruit. Also, glycosides of primary alcohols would be expected to be formed enzymatically more readily than those of secondary and tertiary alcohols, and this may help to explain the presence of neryl and geranyl glycosides in the fruit and the absence of free nerol and geraniol.

Origins and Development of Certain Monoterpenoids and Related Compounds. *Linalool Oxides.* Earlier work (Williams et al., 1980a) has shown that one source of the furan linalool oxides in muscat grape juice was acid hydrolysis of triol **3** (see Figure 1), but the appearance of the four linalool oxides among the enzyme-liberated monoterpenes established a glycosidic origin for these compounds as well. The possibility that these four oxides might not have arisen from their own glycosidic derivatives but indirectly from a glycoside of 6,7-epoxydihydrolinalool **5** (see Figure 1) was also explored (Strauss, 1983). Such a glycoside on enzyme hydrolysis would give an epoxy aglycon (**5**), which in turn could spontaneously cyclize to give the observed oxides. According to this model the uneven distribution observed for the various stereoisomeric forms of the four linalool oxides might then have been accounted for by the conditions of the cyclization reaction. However, experiments involving synthesis of β-D-glucopyranosides of the isomeric oxides as well as of 6,7-epoxydihydrolinalool **5** and comparison of these synthetic compounds with components in the glycosidic fraction of muscat grapes gave no evidence to demonstrate the existence of a glycosidic derivative of epoxide (**5**) and confirmed the presence of the linalool oxide glycosides in the grapes (Strauss, 1983).

Nerol Oxide and Hotrienol. Previous experiments have demonstrated that hotrienol and nerol oxide were hydrolysis products of dienediol 1 in heated muscat grape juice (Williams et al., 1980a). The analyses carried out for this survey confirmed that nerol oxide was not present naturally in Muscat of Alexandria grapes at any stage of berry development. The genesis of this previously reported grape component (Schreier et al., 1976b) can therefore be attributed exclusively to the hydrolysis of dienediol 1 during juice storage, processing, and analysis.

Hotrienol probably has a similar origin in the fruit although its natural occurrence cannot be totally excluded. All GC analyses of dienediol 1 showed a small amount of hotrienol present. Accordingly, the irregular pattern of appearance of hotrienol and its low overall concentration of never more than 50 μg/kg as either a free or bound monoterpene in the growing fruit suggests that this was also an unavoidable artifact of dienediol 1 breakdown during the analyses.

Citronellol, Geranial, and Neral. The isomeric citrals and citronellol were not observed as free terpenes at any stage of the berry development. These compounds were only detected among the enzyme-released monoterpenes from grapes harvested after day 80 of the survey. Although no quantitative figures were available for the concentration of citronellol, geranial, and neral, it was estimated that their levels never exceed that of glycosidically bound α-terpineol.

As a background to these observations a series of control experiments demonstrated that, with the exception of citronellol, all other primary, secondary, and tertiary monoterpene alcohols were totally liberated from a bound terpene fraction in 24 h with the commercial enzyme. Citronellol, together with the isomeric citrals, continued to increase in concentration with prolonged enzyme action. One other component, the degraded terpenoid 6-methylhept-5-en-2-one, also showed a similar behavior. Further studies confirmed that 6-methylhept-5-en-2-one was not formed degradatively from the citrals during the extended incubation nor were the citrals formed as artifacts by oxidation of geraniol.

At present no firm explanation can be given for the continued production of these carbonyl compounds or citronellol on extended enzyme action on grape glycoside fractions. However, Stevens (1970) observed oxidative enzymatic production of 6-methylhept-5-en-2-one and the citrals from polyene carotenes, and such a process could be operating here with the commercial enzyme. The substrate for such an oxidase could be those precursors of norisoprenoid compounds, which are known to be present in grape glycoside fractions (Williams et al., 1982c).

Dienediol 2, Enediol 4, 2-Phenethyl Alcohol, and Benzyl Alcohol. Detailed quantitative data were not obtained for free and glycosidically bound dienediol 2 or enediol 4 (see Figure 1) in the growing fruit. However, the pattern of behavior of these two polyols and their glycosidic derivatives in terms of peak heights in relation to the internal standard were recorded. Similar data were obtained for free and glycosidically bound benzyl alcohol and 2-phenethyl alcohol (Williams et al., 1983).

Dienediol 2 was more abundant free in the fruit than as its glycosidic derivatives, a circumstance analogous to that of dienediol 1. However the development of both free

and bound dienediol 2 occurred considerably later than that of any other monoterpene studied. These compounds were not detected in the juice before day 70 of the survey and showed significant increases in concentration only at the cessation of sugar accumulation. In their period of maximal concentration in the juice, i.e., from day 100 to the end of the survey, the ratio of free to bound dienediol 2 was greater than 4:1.

In contrast to the situation with dienediol 1 and dienediol 2, glycosidically bound enediol 4 was found at all times in greater amount than the free polyol. However, even at their maximum concentration, which occurred after day 90, enediol 4 and its glycosides were present at much lower levels than the dienediol 2 analogues. A possible genesis for enediol 4 in the berries may involve simple hydration of the 6,7 double bond of the aglycon moiety of linalyl glycosides. Such acid-catalyzed hydrations of linalool and derivatives have been observed previously by Baxter et al. (1978)

Free benzyl alcohol and 2-phenethyl alcohol were at low levels at all stages of berry development and were frequently undetectable. Glycosidically bound 2-phenethyl alcohol and benzyl alcohol were present at higher levels than their free forms after increasing in concetration during sugar accumulation. At all samplings after véraison, bound 2-phenethyl alcohol was more abundant than bound benzyl alcohol.

CONCLUSION

This study allows five different phases in the development of free and bound monoterpenes to be distinguished. High concentrations of free geraniol and its glycosidic derivatives were apparent at berry set. This was followed by a phase leading up to véraison in which the levels of all terpene compounds diminished. Then during sugar accumulation the concentrations of the major monoterpenes and derivatives fluctuated, and this phenomenon was most marked in the case of the glycosides. The fourth and fifth phases of development showed the various compounds reaching a maximal concentration followed by falling levels of many free and glycosidically bound monoterpenes in the overripe fruit.

Only more experimental data will confirm the generality of these observations and the influence that preharvest variables has on these patterns. Nevertheless, certain features are clearly evident from the trends established in this study.

First, on the basis of sugar and acid values the grapes were ready for harvest after day 90 of the survey. However, those monoterpenes that reached significant concentrations in the berries, both as free and bound species, were all to attain substantial levels up to day 120. This demonstrated that a higher concentration of flavor compounds could be established in the berries by leaving the fruit on the vine for extended periods and confirmed Hardy's (1970) conclusion that sugar and acid values do not give an accurate indication of ripeness from an aroma point of view.

Second, of the total monoterpenes in the juice after véraison there was a greater concentration present as flavorless glycosides and polyols than as free compounds. This clearly indicates that juice processing techniques, such as pH adjustment and heating to hydrolyze these flavorless forms, are appropriate to induce flavor in juice from the grapes.

Third, because of the high concentration of free dienediol 1 in the ripe fruit, acid-catalyzed hydrolytic methods are more important to the enhancement of free aroma compounds in the juice than possible enzymatic methods. A glycosidase, if active at juice pH, could liberate substantial quantities of linalool, geraniol, nerol, and *trans*-furan linalool oxide from their glycosidic derivatives. However, the major monoterpene of the grape, free dienediol 1, would be unaffected by this treatment.

Fourth, the limited number of free monoterpenes actually present in the juice and their relatively low total concentration reinforce the argument that the observed volatile compounds do not account for the perceived aroma of muscat grape juices (Etievant and Bayonove, 1983; Williams et al., 1981, 1980a). Obviously, the breakdown by hydrolysis of glycosides, polyols, and possibly other as yet unrecognized precursors in the grapes has an important influence on muscat aroma. This can occur by giving more numerous and/or larger quantities of volatiles among which trace amounts of minor but possibly potent aroma substances of the grape are to be found.

Fifth, the quantitative data for the development of muscat grape monoterpenes reported here gives base value information for less complex measures of terpene flavorants in grapes. Such an analytical technique for estimation of free and potentially volatile monoterpene flavorants has been recently developed (Dimitriadis and Williams, 1984).

Registry No. 1, 51276-34-7; 2, 51276-33-6; 4, 29210-77-3; 5, 15249-35-1; geraniol, 106-24-1; linalool, 78-70-6; nerol, 106-25-2; α-terpineol, 98-55-5; *trans*-furan linalool oxide, 34995-77-2; *cis*-furan linalool oxide, 5989-33-3; *trans*-pyran linalool oxide, 39028-58-5; *cis*-pyran linalool oxide, 14009-71-3; 2-phenethyl alcohol, 60-12-8; benzyl alcohol, 100-51-6.

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Received for review January 3, 1984. Accepted March 28, 1984.

Additional Volatiles of Black Tea Aroma

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The volatiles of aqueous infusions of two black tea qualities (Indian Broken; Darjeeling Orange Pekoe) were investigated. Aroma separation was carried out by standard controlled high-vacuum distillation followed by solvent extraction (pentane–dichloromethane, 2:1). The concentrated extracts were prefractionated in four fractions by adsorption chromatography on silica gel using a pentane diethyl ether gradient. In total, 133 volatiles not described as yet as black tea aroma constituents were identified by capillary gas chromatography and coupled capillary gas chromatography–mass spectrometry, among them 11 hydrocarbons, 30 esters, 25 alcohols, 48 carbonyls, 3 lactones, 3 phenols, and 13 volatiles of miscellaneous structures. These compounds are discussed from the aspect of their formation during black tea manufacturing.

Tea is one of the most widely consumed beverages in the world, and its popularity consists certainly of its pleasant flavor combined with its stimulating effects. There are several types of tea, e.g., green tea, oolong tea, and black tea, each with several subclassifications (Eden, 1976). All types are prepared from essentially the same material, the shoot tips of the tea plant, *Camellia sinensis*, L.

Analytical techniques, especially gas chromatography and mass spectrometry, have revealed that the aroma of tea is a complex mixture of mainly trace compounds. Many reviews of black tea aroma chemistry have been provided, summarizing more than 350 identified volatiles (Sanderson, 1972, 1975; Natarajan et al., 1974; Yamanishi, 1975, 1981; Howard, 1978), but which of the black tea aroma constituents are really essential in determining the character of this beverage is still unknown. Therefore, studies of black tea aroma are further extended. In this paper, some additional volatile constituents not described as yet as black tea volatiles (Van Straten and Maarse, 1983) will be described.

EXPERIMENTAL SECTION

Materials. Two black tea qualities, Indian Broken (IB) (grown at low altitude, harvested in late summer 1980) and Darjeeling Orange Pekoe (DOP) (Grown at high altitude, harvested in spring 1980), purchased from P. Schrader & Co., Bremen, West Germany, were used.

Solvents. All solvents used were analytical-grade materials and additionally purified by distillation.

Aqueous Tea Extract. An aqueous tea infusion was prepared as follows: 200 g of black tea was brewed for 3 min with 2 L of boiling water. After cooling (7 min) in an ice bath, the leaves were separated and pressed by hand with a cloth. The aqueous infusion (approximately 1.6 L) was placed in a 4-L three-neck bottle. After addition of internal standards (0.16 mg of alloocimene, 0.3 mg of methyl decanoate, 0.25 mg of 1-decanol, 0.25 mg of 1-dodecanol), high-vacuum distillation was started. In total, 1 kg of black tea was applied, i.e., repeating this step 5 times.

High-Vacuum Distillation. The sample (approximately 1.6 L) was temperature controlled at 40–50 °C in a water bath. Four cooling traps were connected in a row; traps 1 and 2 were mantel-cooled (−25 °C, CH_3OH–solid CO_2), and traps 3 and 4 were cooled with liquid nitrogen. The vacuum distillation took 2 h at 0.1–0.01 bar. The four traps were thawed, and the contents were combined (yield 1 L) and used for the following solvent extraction.

Solvent Extraction. The extraction with a pentane–dichloromethane (2:1) mixture was performed during 24 h (using fresh solvent after 12 h) (Drawert and Rapp, 1968). The extracts were concentrated to approximately 1 mL by means of a Vigreux column. In an experiment without any standard addition, the extract obtained after high-vacuum distillation–solvent extraction had the appropriate tea aroma properties.

Column Chromatography on Silica Gel. The concentrated extract from the high-vacuum distillations (1 mL) was fractionated on silica gel 60 (Merck), activity grade II, with a pentane–diethyl ether gradient (Schreier et al., 1979). Cooled (11–13 °C) glass columns, 2.0 cm i.d. × 45 cm, were used with an elution rate of 70 mL/h, and four fractions were obtained. Fraction I was eluted with 160 mL of pentane, fraction II was obtained by eluting with 160 mL diethyl ether–pentane (1:9 v/v), fraction III was eluted with 220 mL of diethyl ether–pentane (5:5 v/v), and fraction IV was obtained after elution with 200 mL of diethyl ether. All eluates were concentrated to 0.1 mL for gas chromatographic and gas chromatographic–mass spectrometrical investigation. The fractionation on silica gel destroyed the tea aroma complex; i.e., none of the fractions showed the aroma of the original extract.

Gas–Liquid Chromatography. A Carlo Erba Fractovap 4160 gas chromatograph equipped with a J & W fused silica wide-bore CW 20 M capillary column (30 m, 0.31 mm i.d.) was used. On-column injection with an air-cooled

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0021-8561/84/1432-0924$01.50/0