THE ENZYMATIC RELEASE OF GLYCOSIDICALLY-BOUND TERPENES IN MUST

by

LINDIE HELENE STRAUSS

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SUMMARY

Flavour in wines is perhaps the most important factor affecting wine quality, with monoterpenoids being among the compounds contributing to flavour. In grapes there exists glycosidically-bound forms of these monoterpenes, representing a latent source of aroma.

This project is a study on the ability of different enzymes to release these monoterpenes in Muscat d'Alexandrie and Weisser Riesling grapes.

Different commercial enzyme preparations were characterised with respect to their catalytic properties and fractionated by ion exchange chromatography in the most active fractions. Amongst those commercial preparations tested, Rohapect C was found to be the most effective at conditions prevalent during wine making.

These purified fractions were added to the above mentioned grapes to determine their effect on the release of different monoterpenes. Even though the enzymes were active on synthetic substrates, limited release of terpenes from must could be detected.

Possible causes for this apparent inability to release monoterpenes were investigated. Product inhibition due to the presence of high concentrations of glucose in the must appeared to be the main cause of limited enzyme activity.

In an attempt to overcome this problem, glucose in the above mentioned cultivars was oxidised to gluconic acid by glucose oxidase, prior to attempting monoterpene release by Rohapect C. Although no marked increase in the release of total terpenes occured, a significant increase in the concentrations of some individual terpenes could be observed. The effect of this on wine quality remains to be ascertained.

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MATERIALS AND METHODS

LIST OF ABBREVIATIONS

DMAPP	Dimethylallyl pyrophosphate
FVT	Free volatile terpene
GPP	Geranyl pyrophosphate
HPLC	High pressure liquid chromatography
IPP	Isopentenyl pyrophosphate
MVA	Mevalonic acid
MVAPP	Mevalonic acid pyrophosphate
NPP	Neryl pyrophosphate
PVT	Potentially volatile terpene
SDS	Sodium dodecylsulphate

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PART 1

REVIEW ON THE ENZYMATIC RELEASE OF BOUND TERPENES IN GRAPES AND MUST

1.1 INTRODUCTION

Flavour in wines, as with all foods and beverages, results from the interaction of the chemical constituents within the wine with the chemical senses of the person consuming it, namely the sense of taste and smell (Williams, 1985). While the number of taste sensations human beings can distinguish on the palate is limited, the sense of smell is very sensitive. Approximately 550 aroma components have been identified in wine and grapes. Science is trying to introduce some rationale to what is understood by flavour quality in wines, through interpreting the sensory characteristics in terms of the chemical and physical properties of the wine. The human being, however, is still the ultimate evaluator of flavour (Williams, 1982) and the ultimate criteria on which wine quality must be evaluated must be the response it evokes in the drinker (Williams et al., 1984a).

The flavour in grapes is perhaps the most important factor affecting wine quality (Webb, 1981). One of the aromas that has a marked effect on wine quality is the so-called muscat aroma. There is a close relationship between the aroma of muscat grapes and the content of volatile monoterpenoids in the fruit. Therefore, the taste and aroma of muscat berries are attributed to the presence of these compounds.

Important studies by Cordonnier and Bayonove (1974) suggested that in addition to the free volatile monoterpenes of the grapes there exist, in Muscat d'Alexandrie, non-volatile, acid-labile, bound forms of these compounds. It is important to recognise that substantial quantities of grape monoterpenes are bound in the fruit as glycosides or are present as free polyols. In these forms the compounds are odourless but they do represent a latent source of aroma.

In this reserve pool of aroma lies the potential to enhance and greatly improve the fruity character of the wine, but then breakdown of the glycosides and hydrolysis of polyols of these odourless monoterpenes and derivatives will have to be achieved. To assess the aroma potential, as well as the aroma of any batch of fruit, it is clear that the status of the free and glycosidically-bound terpenes must be known (Bayonove et al., 1983). Limited studies on Muscat d'Alexandrie and Weisser Riesling grapes have demonstrated that from one half to two thirds of certain monoterpenes are bound as glycosides in the growing berries. As the berry ripens, changes in the ratio of free and bound species, as well as sharp increases in the absolute concentrations of individual monoterpenes occur (Williams et al., 1983a; Marais, 1985; Marais, 1988).

1.2 TERPENES IN GRAPES

All terpenoids possess characteristic branched chain carbon skeletons with a variety of different functional groups such as hydroxyls, ethers and ketones. The monoterpenoids in particular have potent but pleasant fruity aromas and are common constituents of many flowers, fruits, essential oils and fragrances (Williams et al., 1983a). Williams (1982) has shown that there is a strong correlation between the content of monoterpenoids in grapes and the degree of muscat flavour of the fruit. It was observed that whilst none of the individual compounds studied had sensory properties identical with muscat character, a combination of these volatile monoterpenoids was essential for muscat grape aroma. It was concluded that since other aromatic, but non-muscat grape varieties, such as Weisser Riesling, also contain terpenoids, but in lesser amounts, the flavour of these non-muscat varieties also appears to be controlled by monoterpenoids. Typical aroma descriptions of some important terpenes are floral, roselike (geraniol, nerol, rose oxides), coriander (linalool), camphoraceous (linalool oxides), green (nerol oxide) and herbaceous (Etievant et al., 1983; Marais, 1983; Williams et al., 1984a).

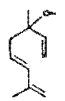
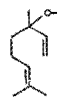
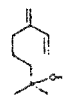
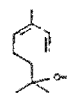
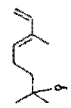
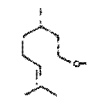
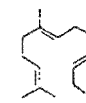
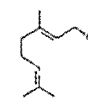
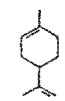
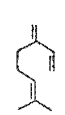
The aroma of a muscat grape is influenced by the form, as well as quantity, in which the terpenes are found in the berry. Cordonnier and Bayonove (1974) have shown that the terpenes are partly free and volatile and partly bound and non-volatile. Different categories of monoterpenes have been shown to

exist in muscat grapes; namely free volatile monoterpenes, free polyhydroxylated monoterpenes (polyols) and glycosidic derivatives of the two former monoterpenoid types (Williams et al., 1981).

The free volatiles consist mainly of linalool, geraniol, nerol, furan and pyran forms of the linalool oxides, ∝-terpineol, hotrienol and citronellol (Table 1). As these have relatively low aroma threshold values, they are also the major terpene aroma compounds of the fruit (Ribéreau-Gayon et al., 1975). The odourless polyols appear to be derived from four "parent" monoterpenes - linalool, citronellol, nerol and geraniol by hydration and oxidation reactions.

The monoterpene glycosides consist of a glycosidic mixture of β-rutinosides (i.e. 6-O-∝-L-rhamnopyranosyl-β-D-glucopyranosides and 6-O-∝-L-arabinofuranosyl-β-D-glucopyranosides) of predominantly geraniol, nerol and linalool with traces of ∝-terpineol. The bound terpenes are glycosides, in which form they are the precursors of the free terpenes. The main free terpenols are linalool, geraniol, nerol, hotrienol, ∝-terpineol and citronellol. The four linalool oxides as well as some of the polyols also exist in the bound form in the fruit (Strauss et al., 1984).

The different categories of monoterpenes in muscat grapes can be differentiated on the basis of solubility. Two major groups can be distinguished, i.e. those terpenes which are soluble in an organic solvent and those which are left in the juice after solvent extraction (Di Stefano and Ciolfi, 1983). The former can be subdivided into a pentane-soluble fraction and a Freon-soluble fraction (Usseglio-Tomasset and Di Stefano, 1979). The pentane soluble terpenoids are made up predominantly of geraniol, linalool, the pyran linalool oxides and nerol. They are part of the group of "free terpenes". The freon-soluble fraction of the solvent-soluble group contains the free polyols. The organic solvent insoluble terpenes are highly water soluble, non-volatile and are precursors of almost all the free terpenes, including the free polyols. Most of the known volatile monoterpenoid flavourants of the grape are derived by acid or enzyme catalysed hydrolysis from monoterpene precursors (Williams et al., 1981).

Terpene	Structure	Grapes	Wine
Hotrienol		X	X
Linalool		X	X
Myrcenol		X	
Nerol		X	X
cis-Ocimenol		X	
trans-Ocimenol		X	
Citronellol		X	X
Farnesol			X
Geraniol		X	X
Limonene		X	X
Myrcene		X	X

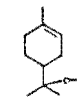
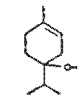
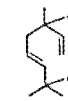
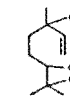
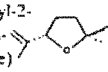
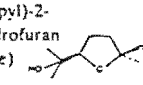
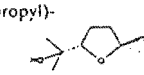
Terpene	Structure	Grapes	Wine
α-Terpineol		X	X
Terpinen-4-ol		X	X
3,7-Dimethylocta-1,5-dien-3,7-diol		X	X
3,7-Dimethylocta-1,7-dien-3,6-diol		X	X
3,7-Dimethyloct -1-en-3,6,7-triol		X	X
3,7-Dimethyloct -1-en-3,7-diol		X	X
trans-5-Isopropenyl-2-methyl-2-vinyl-tetrahydrofuran (trans-Anhydrofuran linalool oxide)		X	X[1]
cis-5-(2-Hydroxyisopropyl)-2-methyl-2-vinyl-tetrahydrofuran (cis-Furan linalool oxide)		X	X
trans-5-(2-Hydroxyisopropyl)-2-methyl-2-vinyl-tetrahydrofuran (trans-Furan linalool oxide)		X	X
cis-Rose oxide		X	X
trans-Rose oxide		X	X
Nerol oxide		X	X

Table 1: Monoterpenes and sesquiterpenes in grapes and wines (Ter Heide, 1968; Williams et al., 1980; Marais, 1983).

1.2.1 **Biosynthesis of Monoterpenes and Sesquiterpenes**

Monoterpenes (C_{10}) and sesquiterpenes (C_{15}) are the lower molecular weight representatives of the terpenoid family of compounds. They comprise two or three isoprene units respectively and are characteristic compounds of many essential oils. Mono- and sesquiterpenes are derived from mevalonic acid (MVA)(Cori, 1983).

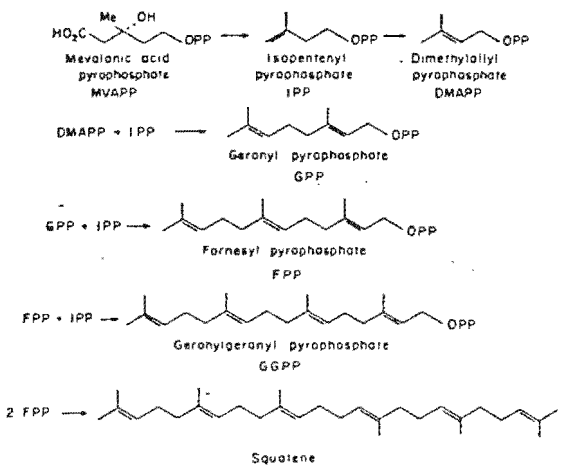


Figure 1.1 Biosynthesis of acyclic terpenoid intermediates from mevalonic acid pyrophosphate (Croteau, 1975).

Mevalonic acid pyrophosphate (MVAPP) is first converted to isopentenyl pyrophosphate (IPP), which is then isomerised to dimethylallyl pyrophosphate (DMAPP) (Fig. 1). This 1,4 condensation reaction is catalysed by prenyltransferases or prenylsynthetases. Condensation of IPP and DMAPP yields geranyl pyrophosphate, the first C_{10} isoprenoid compound. Further condensation of geranyl pyrophosphate with IPP yields farnesyl pyrophosphate, the first C_{15} isoprenoid, which then gives rise to geranyl-geranyl pyrophosphate, the precursor of the diterpenoids,by addition of one more IPP unit. Two units of farnesyl pyrophosphate can condense to yield squalene, the precursor of the triterpenes (Banthorpe et al., 1972; Croteau, 1975; Goodwin, 1979; Cane, 1983). The monoterpenes can be divided into four structural categories: irregular, acyclic, cyclopentanoid and cyclohexanoid. The irregular monoterpenes comprise a relatively small group of compounds formed by the head-to-head fusion of C_5 units. The three remaining structural types are constructed by the head-to-tail fusion of C_5 units, typified by the condensation of DMAPP and IPP to geranyl

pyrophosphate (GPP). It is, therefore, suggested that GPP is an intermediate in the biosynthesis of the acyclic, cyclopentanoid and cyclohexanoid monoterpenes (Cane, 1983). GPP has been shown to undergo cyclisation without loss of hydrogen (Croteau, 1975). While GPP could function as the direct precursor of cyclopentanoid monoterpenes and of most acyclic monoterpenes, neryl pyrophosphate is likely to function as the immediate precursor of the cyclohexanoid monoterpenes because the cis-geometry at the $\Delta 2$ position permits cyclisation to the cyclohexane ring. Numerous studies of the non-enzymic solvolysis of neryl and geranyl phosphates show that neryl derivatives readily cyclisise (primarily to α-terpineol), while the geranyl derivatives do not (the major product is the acyclic terpene alcohol, linalool). Thus, although GPP is a key intermediate in the biosynthesis of many terpenoid compounds, the cis-isomer, neryl pyrophosphate (NPP), is the most likely direct precursor of the cyclohexanoid monoterpenes (Croteau, 1975). Linalyl pyrophosphate serves as an alternative substrate for these cyclases (Cane, 1983).

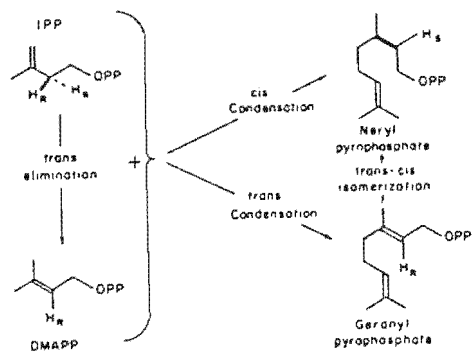


Figure 1.2 Two possible biosynthetic pathways leading to neryl pyrophosphate (Croteau, 1975).

There are two probable pathways for the biosynthesis of NPP (Fig. 1.2). The first pathway is the "cis-condensation" of IPP and DMAPP to yield NPP directly (analogous to the "trans-condensation" involved in GPP biosynthesis). The second route is the classical trans-condensation of IPP and DMAPP to GPP, followed by trans-cis isomerisation to NPP. The direct isomerisation of GPP would appear to be the most efficient pathway to NPP (Croteau, 1975).

Linaloyl pyrophosphate has also been suggested as an acyclic precursor of cyclic monoterpenes. The mechanisms of the non-enzymic cyclisation of NPP and linaloyl pyrophosphate are similar, although ∝-terpineol is formed at a somewhat lower yield from linaloyl PP than from NPP (Croteau, 1975). Hotrienol is almost totally formed by dehydration of (E)-3,7-dimethyl-1,5-octadiene-3,7-diol (Rapp et al., 1984).

The pathway for biosynthesis of linaloyl pyrophosphate has not been established. One possible biosynthetic route is suggested by the fact that linalool is the major product formed on acid hydrolysis of GPP. A novel condensation of IPP and DMAPP has also been suggested for the formation of linaloyl pyrophosphate (Croteau, 1975). In developing the biogenetic isoprene rule in 1953, Ruzicka suggested a scheme for the formation of cyclic monoterpenes from acyclic precursors based on carbonium ion mechanisms (Banthorpe et al., 1972).

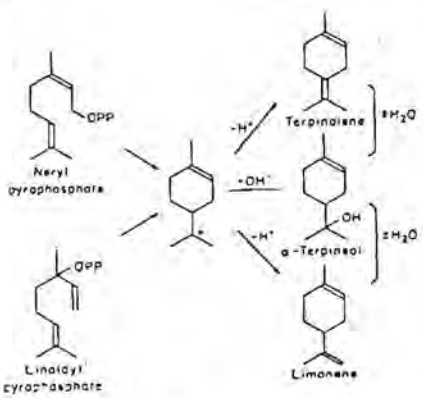


Figure 1.3 Postulated cyclisation of neryl pyrophosphate and linaloyl pyrophosphate to p-menthane monoterpenes (Croteau, 1975).

Cyclisation of both neryl pyrophosphate and linaloyl pyrophosphate gives rise to the same carbonium ion intermediate, which can be stabilised by the loss of a proton to give limonene or terpinolene, or by capture of a hydroxyl ion to give ∝-terpineol (Fig. 1.3). Although relatively little is known about the biosynthesis of sesquiterpenes, they are almost certainly derived from the C_{15} precursor, farnesyl pyrophosphate. Farnesyl pyrophosphate can occur in four geometric isomers: cis Δ^2, cis Δ^6; cis Δ^2, trans Δ^6; trans Δ^2, cis Δ^6; and trans Δ^2, trans Δ^6. Most hypothetical schemes for the biosynthesis of cyclic sesquiterpenes imply cis Δ^2, trans Δ^6 - and trans Δ^2, trans Δ^6 - farnesyl pyrophosphate as precursors, although there is little supporting evidence for this assumption. It does seem reasonable, however, to suggest that the pathways of sesquiterpene biosynthesis are similar to those of monoterpene biosynthesis (Croteau, 1975).

1.2.2 The Glycosidic Aroma Precursors

Early studies on the precursor compounds indicated these to be glycosidic derivatives of monoterpenes although not simply β-D-glucosides. It was determined that the precursors of both Muscat Gorda Blanco (Muscat d'Alexandrie) and Weisser Riesling were a complex mixture of β-rutonosides and 6-O-α-L-arabinofuranosyl-β-D-glucupyranosides of several monoterpene alcohols, predominantly geraniol, nerol and linalool. The disaccharide glycosides of precursors is a mixture made up of glucose, arabinose and rhamnose (Williams et al., 1982a; Strauss et al., 1984).

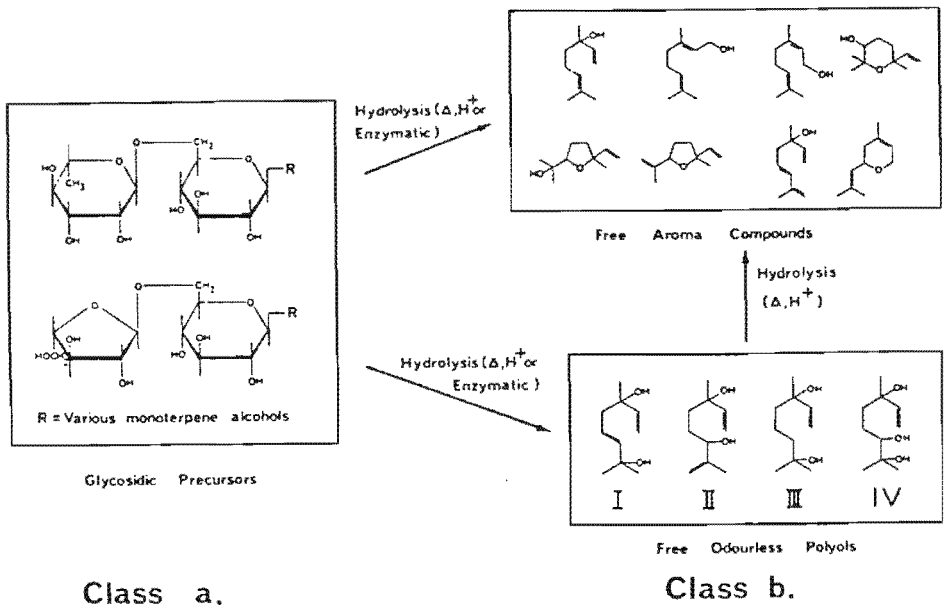


Figure 1.4 Categories of monoterpenes in grapes (Williams et al., 1983a)

Similar disaccharide conjugation is involved in the higher oxidation state terpene precursors, i.e. the linalool oxides (Fig. 1.4). The grape contains a diversified enzymatic mechanism which constitutes a dynamic transformation in the berry and manifests itself in such ways as, for instance, the formation of terpenols from precursors to produce geraniol and nerol (Cordonnier and Bayonove, 1979). It is possible to extract from must free monohydroxyl, dihydroxyl and trihydroxyl terpene compounds. With a subsequent extraction with ethyl acetate it is possible to extract a compound which, during steam distillation and in an acid medium at pH 3, produces linalool, α-terpineol, nerol, geraniol, arabinose and small quantities of a hexose. This compound most probably has the structure of linalyl arabinoside (Di Stefano and Castino, 1983). Williams et al. (1982c) put forward a disaccharide structure. The study

of this glycoside is important since it seems to be the compound on which the quality of Asti Spumante depends. This compound is constantly hydrolysed depending on the temperature and pH; it generates linalool, which in turn undergoes cyclisation to ≺-terpeneol, which is of smaller organoleptic importance (Di Stefano et al., 1983). The other glycosides present in must (which generate the products of decomposition of diendiol 1 and triol) have less clear function. The first one is not very stable and leads to hotrienol, which may contribute to the Moscato aroma. The second one is much more stable. It generates the furan linalool oxides A and B of little organoleptic importance. All the compounds generating terpene alcohols can be extracted from the must or wine using 2-methyl-propanol-1 (Di Stefano and Castino, 1983).

The preponderance of linalool and the observation that it is transformed into ∝-terpineol, geraniol and nerol as a result of heating in an acid medium, suggests a linalool precursor, rather than a precursor of the other terpenic alcohols (Usseglio-Tomasset and Di Stefano, 1980; Di Stefano, 1982).

∝-Terpineol is considered a major product of the acid-catalysed hydrolysis of the glycosides, while only trace amounts of its glycosides occur in grapes. ∝-Terpineol is also a product of acid-catalysed cyclisation of linalool, geraniol and nerol (Rapp et al., 1984).

The linalool oxidation state monoterpene glycosides is a glycoside mixture of β-rutinosides and 6-O-∝-L-arabinofuranosyl-β-D-glucopyranosides of predominantly geraniol, nerol and linalool, with smaller amounts of ≺-terpineol. 2-Phenylethanol and benzyl alcohol precursors in the grapes exist as a mixture of β-D-glucopyranosides and diglycosides (Williams et al., 1983b).

1.2.3 Distribution of free and glycosidically-bound monoterpenes in grapes

Generally speaking, the skins alone provide more than half the volatile constituents of the berry and, together with the pulp, about 70%. Thus, the aromatic potential of the grape is essentially found in the skins and the cellular debris of the pulp (Cordonnier and Bayonove, 1981). The distribution between solid and liquid phase of the grape berry differs for different chemical compounds. The terpenols, geraniol and nerol, are mostly found in the skin, whereas linalool is almost equally distributed between the juice on one hand

and the skin and cellular debris on the other hand. Hotrienol occurs mainly in the must while the skins contain only traces. Therefore, the composition of the aroma of the grape is not the same in all the constituent parts of the berry. It follows that the aroma of the skin and the aroma of the juice can differ, not only in intensity, but also in quality (Cordonnier and Bayonove, 1979; Wilson et al., 1986). The pressing and maceration of the berries with the juice causes an increase in terpenic compounds. The concentration of terpenols is two to four times higher in juice obtained by pressing than that of free-run juice. The relative concentrations of different terpenols also vary with the extent of pressing of the grapes. During pressing there is a relative enrichment of nerol and geraniol which is explained by the fact that these two terpenols are mostly found in the berry-skin (Kinzer and Schreier, 1980; Cordonnier and Bayonove, 1981; Marais and Van Wyk, 1986).

It was found by Wilson et al. (1986) and Gunata (1984) that glycosylation effectively distributes all the monoterpenes throughout the berry. The writers suggested that the hypodermal cells of the berry might be sites of biosynthesis as well as storage of geraniol and that this compound could play a fundamental role in monoterpene biosynthesis in the grape. The water solubility of the glycosidic forms could account for their wide distribution in the grape. With this knowledge it is important to qualify the perception that the aroma of the grape is concentrated in the skin. The high concentration of free linalool in the muscat juices, as well as the presence of potential flavourants in other parts of the grape, emphasises the importance of processes other than skin-contact for enhancement of aroma (Strauss et al., 1986).

Skin-contact is a technique which can be used to extract additional aroma compounds such as terpenes from grape skins during winemaking. The aroma intensity and quality of wines from cultivars with skins rich in terpenes can, therefore, be enhanced. Skin-contact may also result in the extraction of skin compounds detrimental to wine quality, such as certain phenols and acetamides. The temperature at which skin-contact is applied is also of critical importance, as higher extraction temperatures result in increases in concentrations of phenols and N-(3-methylbutyl)-acetamide (Marais and Rapp, 1988).

Maceration of the crushed grapes has the same qualitative but less marked effect on the differential extraction of nerol and geraniol as has pressing. The extraction of terpenols appears to be less linked to the duration of maceration

than to the initial alcohol concentration. Ethanol acts as an extraction solvent, even in small amounts (Cordonnier and Bayonove, 1981). Freezing of the grapes allows one to recover a large amount of nerol and geraniol mainly found in the skins. In this case cellular breakdown, as a result of freezing, facilitates transfer (Usseglio-Tomasset and Di Stefano, 1980).

From a practical point of view, these results emphasise the technological importance of the grape skin. The skin is really the noble part of the grape, both because of the specific compounds it contains and because of its richness in these compounds. Fully exploiting the aromatic potential thus depends on the application of methods capable of encouraging exchanges between the solid parts of the grape and the juice (Usseglio-Tomasset and Di Stefano, 1979; Usseglio-Tomasset and Di Stefano, 1980; Cordonnier and Bayonove, 1981; Etievant and Bayonove, 1983).

1.2.4 **Formation and effect on Terpenoids by Yeasts**

Banthorpe et al. (1972), in an elegant review of the biosynthesis of monoterpenes, summarise the meagre information on biosynthesis of terpenes by yeasts. The route to cholesterol and squalene in yeasts, starting with acetate, goes through mevalonate, geraniol and nerol, thence to higher molecular weight products. Details of the conversions are uncertain in several areas.

It is not known whether Saccharomyces cerevisiae produces terpenes while fermenting grape juice to wine (Fagan et al., 1981). There is, however, evidence of terpene formation by other yeast strains. In aerobic shake culture the yeast, Kluyveromyces lactis, which is fairly closely related to S. cerevisiae used in wine fermentation, produced free citronellol, linalool and geraniol (Drawert and Barton, 1978). According to Schreier (1984) his group found that several "wild" yeasts produced small quantities of linalool, myrcene, limonene and α-terpineol.

According to Abdurazakova et al. (1982), the analysis of pure grape must revealed the presence of nine terpenoid components. Cultivation of wine yeast (Saccharomyces vini and Hanseniaspora apiculata) in must increased the number of terpenoid components to 14, suggesting the biogenesis of terpenoids by yeasts. The intensity of terpenoid biosynthesis was dependent

on the yeast strain and cultivation conditions. Under conditions of submerged cultivation, the biosynthesis of isoprenoid compounds was intensified. The role of terpenoids in enhancing the aroma of wine was emphasized. Linalool, cis-nerol, trans-nerolidol and trans, trans-farnesol were produced in low concentrations by the wine yeast Saccharomyces fermentati growing as a film for ten weeks on the surface of a simulated fino sherry, containing ethanol as the only volatile carbon-containing compound (Fagan et al., 1981).

Grossmann et al. (1987) found that yeasts from the genus Hansenula have the ability to form β-glucosidase and this enzyme has the ability to release glycosidically-bound terpenes. A secondary effect of this genus unfortunately is the formation of esters that causes off-flavours in wine. Hansenula strains selected not to have this problem were less efficient in releasing bound terpenes (Grossmann and Rapp, 1988).

Molnar et al. (1981) showed that the concentrations of the terpene compounds, linalool, linalyl acetate and α-terpineol, increased only slightly in wine during storage on yeast, either intact or disintegrated. On the other hand, the concentrations of β-ionone, cis-farnesol and trans-farnesol, the three characteristic products of yeast autolysis, increased considerably.

It is well known that microorganisms are able to synthesise, transform and degrade substances with very complicated structures. Apart from this, microorganisms also produce very active enzyme systems, which are capable of catalysing chemical reactions with foreign substances, when the latter are added to a culture solution in which the organism is grown (Tressl et al., 1978). S. cerevisiae, the yeast species almost exclusively employed in modern winemaking technology, produces only trace amounts of farnesol. It does not produce any terpene derivative used in the varietal classification of grapes with GC-MS (Hock et al., 1984). Rapp et al. (1984) also found a slight rise in furanoid concentration and occasionally there is a slight rise in the concentration of nerol oxide with prolonged fermentation time. Concentrations of citronellol and hydroxycitronellol increase during fermentation, while its acetate does not change.

1.3 ENZYMES IN ENOLOGY

Enzymes are proteins that are very substrate specific in their catalytic action. They catalyse only well defined reactions and are not consumed during the process. Once the reaction is over, the enzyme is released and ready to catalyse the next reaction. Enzymes can theoretically be used endlessly but the presence of certain inhibiting substances reduces their active period (Villettaz, 1984).

When studying the structure of the glycosidic terpene precursors it is apparent that specific enzymes would have to be present in the grape in order to cleave these precursors to release the free monoterpenes. However, the isolation of significant quantities of monoterpene glycosidic precursors from finished wines indicate that such specific endogenous glycosidases were not very active during must processing and fermentation. Nevertheless, it may be possible to treat juice with exogenous enzymes having an appropriate glycosidase activity and so enhance the aroma of musts. Such activity is found in some commercial pectinase preparations, but the enzymes responsible for this dissacharide glycoside splitting function may need to be enriched so that the required activity is still significant under juice processing conditions, i.e. pH 3,2 - 3,8 at 10 - 15ºC and in the presence of free SO_2 (Williams et al., 1983b). An important aspect of the enzymatic release of monoterpenes is that the terpene remains intact during hydrolysis and will enhance aroma after this release. However, the enzymatic technique will not form aroma compounds from the polyols and only a hydrolytic technique, using heat and acid, will accomplish this. For total aroma extraction a combination of enzymatic and thermal techniques may be necessary (Williams et al., 1983a).

1.3.1 **Pectolytic Enzymes**

A group of enzymes called the pectolytic enzymes break down pectic substances of high molecular mass. In this group, distinction is made between depolymerising enzymes (hydrolases and lyases) and non-depolymerising enzymes (pectinmethylesterases). Depending on

whether they hydrolyse the pectin at the end of the chain (exo) or within the chain (endo), hydrolases are classified as exopolygalacturonases or endopolygalacturonases. The endopolygalacturonases break the pectin into oligomers of galacturonic acid with a rapid decrease in viscosity. In contrast, exopolygalacturonases attack the pectin chains at their ends with a slow decrease in viscosity. These hydrolases generally display a preference for moderately esterified substrates (Villettaz, 1984).

The commercial preparations of pectolytic enzymes are usually extracted from Aspergillus niger cultures. These preparations contain a mixture of enzymes with different pectolytic activities (pectinmethylesterases, lyases, hydrolases), as well as some related activities such as cellulases and hemi-cellulases. The ratio of these enzymes may differ from one commercial product to the next, because of differences between the manufacturing techniques. Experiments conducted on Sauvignon have shown that the addition of pectolytic enzymes to crushed grapes increased wine quality because of an increase in primary aroma. It was, however, not established whether this enrichment resulted from better aroma extraction or from the release of certain bound aromatic fractions (Villettaz, 1984). The amount of solids is slightly increased with the addition of enzymes to crushed grapes, as more solids are released from the grapes. As there is an increasing demand for fruity, young wine, the enzyme manufacturers have endeavoured to develop enzyme preparations that will have a good pectin splitting activity with little secondary activity (Haupt, 1981).

Commercial enzymes have been used to catalyse the hydrolysis of monoterpene disaccharide glycosides to identify the aglycone monoterpenes and to determine the relative ratios of the various terpene alcohols present in grapes. One of the enzymes employed is a commercial pectinase preparation, Rohapect C, which was demonstrated to possess potent glycosidase activity with geranyl-β-D-glucopyranose. Rohapect C exhibited a tenfold activity over emulsin, a β-glucosidase, and appeared to completely hydrolyse the substrate. The monoterpenes produced by both enzymes were geraniol, nerol and linalool and in the case of Rohapect C they were released in a ratio of 3:1:2. Whilst trace quantities of α-terpineol and citronellol were also observed with the Rohapect C, no other monoterpenoids were detected (Williams et al., 1982b; Williams et al., 1983b; Wilson et al., 1984b).

Aryan et al. (1987) found that one of the major advantages of using Rohapect C was, except for the ability to produce more free monoterpenes than any other enzyme tested, the ability to release the tertiary alcohols,

linalool and ∝-terpineol and the furan linalool oxides. The grape enzymes and almond emulsin were largely incapable of producing these compounds.

Pectinol and buckwheat rhamnodiastase (isolated from germinated buckwheat seeds) are other pectolytic enzymes used for this purpose. They have a special affinity for p-nitrophenyl-β-D-rutinoside, but differ in their ∝-glycosidasic activities that is absent in the grape (Bayonove et al., 1983).

Monosaccharides were found to inhibit the catalytic activity of the pectolytic enzymes quite strongly. Substrate hydrolysis is reduced to only 5% of normal at a concentration of 100 g/l glucose. From a cellar technological point of view it is better to only add the enzyme used for hydrolysis of glycosidically bound terpenes towards the end of fermentation when most of the sugar has been fermented (Grossmann and Rapp, 1988).

1.3.2 β-Glucosidase

β-Glucosidase is one of the components of cellulase, which catalyses the hydrolysis of cellobiose to glucose. It is inhibited by glucose. As glucose accumulates during the reaction, cellobiose will amass, and since cellobiose is a strong inhibitor of the other enzymatic components of cellulase, the rate of cellulose hydrolysis will be reduced drastically (Woodward and Arnold, 1981). The problem of the inhibition of β-glucosidase by glucose can be overcome if glucose is removed from the reaction mixture as soon as it is formed. This may be achieved, for example, when cellulose hydrolysis and the subsequent conversion of glucose to ethanol are carried out in a single step. The enzymatic hydrolysis of cellobiose was inhibited completely by glucose with approximate values of 0,5mM for cellobiose. The inhibition of β-glucosidase appeared to be pH dependent, with maximal inhibition at pH 4,8. No inhibition was observed at pH 6,5 or pH 3,5 (Woodward and Arnold, 1981). β-Glucosidase is very sensitive to low pH values. At pH 2,7 β-glucosidase of Trichoderma viride lost about 75% of its original activity and at pH 2,0 about 90% (Herr, 1979). This data suggests that the competitive inhibition of β-glucosidase by glucose is dependent on the concentration of an acidic group in its protonated form, present at the active site of the enzyme, which is essential for maximum enzyme activity. This may be a carboxylate group, since it has been shown to be present in the active site of β-glucosidase from

Aspergillus wentii. It was found that both anomers of glucose inhibited β-glucosidase, but that the α-form was more inhibitory than the β-form (Berghem and Petersson, 1974; Gong et al., 1977; Shewale and Sadana, 1978; Woodward and Arnold, 1981).

To identify the aglycone monoterpenes and to determine the relative ratios of the various terpene alcohols present, an enzyme catalysed hydrolysis was undertaken with almond β-glucosidase (emulsin) EC 3.2.1.2. The emulsin showed only weak activity towards natural grape precursors. The monoterpenes produced were geraniol, nerol and linalool (Cordonnier and Bayonove, 1974; Williams et al., 1982b).

According to Amerine and Joslyn (1970) the two most important sugars present in grapes are D-glucose and D-fructose, occuring in approximately equal amounts. The total sugar concentration is approximately 200 g/l. Fructose is considerably sweeter than glucose. During grape maturation the fructose to glucose ratio increases from about 0,5 to 1,0. The ratio of glucose to fructose tended to be lower in a warm season than in two cool seasons. The high concentration of glucose may be the reason for the low activity of β-glucosidase (Woodward and Arnold, 1981). This possibility was confirmed by Grossmann et al. (1987) who found that the inhibitory effect of fructose was much less than that of glucose. A sugar concentration of less than 10 g/l fructose was found to have no inhibitory effect and even at 25 g/l 85% of the enzyme activity remained. Glucose, on the other hand, caused only half of the enzyme activity to remain at a concentration of 10 g/l. Only at a concentration of less than 5 g/l glucose, did the enzyme activity remain intact. This is also the reason why the writers suggest that enzyme additions should only be made at the end of fermentation with the further advantage of using the fermentation heat as well.

1.3.3 **Enzymes in Grapes**

Grape juice stabilised by an antiseptic and kept at room temperature shows an increase in methanol content and a decrease in viscosity (Villettaz, 1984). At pasteurisation none of these changes occur. These observations, therefore, attest to the presence of pectinmethylesterase and polygalacturonase activities. Endopolygalacturonase activity increases as the fruit ripens and it appears to be characteristic of ripe grapes. The concentration of pectinmethylesterase in the grape is higher than that of polygalacturonase.

Its activity decreases during alcoholic fermentation. The pectinmethylesterase is located mainly in the grape skin, the pulp containing two to three times less, while the activity in the pips is practically nil. The pectinmethylesterase activity in grapes seems to vary from one cultivar to the next. Heat treatment causes a decrease in its activity, all the more so if the temperature is increased. The enzyme sensitivity to heating increases when the pH decreases (Villettaz, 1984).

Cordonnier and Bayonove (1981) have shown the marked effect that certain natural hydrolytic activities of the grape can have on the release of some aromatic muscat compounds. Activities of the β-D-glycosidase type are able to strengthen the aromatic profile of muscat type cultivars by solubilising the bound aromatic compounds. These activities also seem to be present in some pectolytic enzyme preparations. It would be interesting to determine exactly which part of the increase in aromatic intensity is derived from the operation of pectinases and which part from that of glycosidases (Villettaz, 1984).

Enzymatic extracts of grapes are obtained from acetonic powders with the method of Cordonnier and Dugal (1968). The acetonic powder extracts of grapes are endowed with activity towards p-nitrophenyl-β-D-glucopyranoside and other substrates of the β-glucosidases (Cordonnier and Dugal, 1974).

Bayonove et al., (1983) demonstrated that grape juices incubated for some time at 10°C and 30°C and at pH 3,5 and 5,0 showed an increase in concentrations of nerol and geraniol. The increase of these two terpenols was greater in must incubated at pH 5,0 than that incubated at pH 3,5. The same tendency occured at a temperature of 30°C compared to 10°C. These conditions had little effect on the linalool concentration. The release of nerol and geraniol is highest at pH 5,0. At lower pH, where acid hydrolysis occurs, and at more alkaline pH, less of these terpenols are found. Linalool shows little variation. The different behaviour of linalool can be as a result of an absence of linalylic precursors, or their smaller availability to the enzymes of the grape, or even to the high concentration of free linalool in the juices in relation to nerol and geraniol, which could partly conceal the linalool increase (Bayonove et al., 1983).

Aryan et al. (1987) have also isolated and partially purified endogenous grape glucosidases. Two β-glucosidases, a β-galactosidase and two weak

α-glucosidases were found. The β-glucosidases, which were located mainly in the juice of the grape, had increased activity with berry maturity. They both had optimal activity at pH 5,0 and were relatively tolerant to up to 10% ethanol.

The β-glucosidase activity towards p-nitrophenyl-β-D-glucopyranoside and other β-glucosidase substrates is maximal at approximately 40°C and at pH 5,0. At the temperatures and pH values usually encountered in oenology , however, it can still be important and can, therefore, play a technological role. The maximum grape glycosidase activity occurs at the same pH as the maximal production of terpenols in the juices (Bayonove et al., 1983). Enzymes hydrolyse geranyl-β-D-glucoside which can be encountered in the glycosidic fraction of the grape (Williams et al., 1982). Hydrolysis of this fraction produces geraniol (Cordonnier and Bayonove, 1974) and nerol (Williams et al., 1982b). Tested with various glycosidic substrates, enzymes of the grape shows only β-D-glycosidic activity. It hydrolyses p-nitrophenyl-β-D-rutinoside from its glycosidic coupling α-L-rhamnopyranosyl-β-D-glucopyranose. This is one of the glucosidic couples of the glycosides of the grape (Williams et al., 1982b). Thus, the specificity of the glycosidasic material of the grape is not in contradiction with the nature of the glycosides which it contains. It is, therefore, able to hydrolyse all or part of the glycosides (Bayonove et al., 1983).

Aryan et al. (1987), however, found that the β-glucosidases isolated from grapes were inhibited by glucose and it was also found to be inactive towards glycosides of tertiary alcohols such as linalool and the furan ring linalool oxides. Their study into the properties of the grape enzymes makes it clear that there is no practical advantage in seeking to exploit the grape's native β-glucosidase activity for the purpose of releasing glycosidically-bound flavourants during juice processing or winemaking.

1.3.4 Conversion of glucose to gluconic acid by glucose oxidase

Commercial preparations of the enzyme glucose oxidase from moulds such as Aspergillus and Penicillium have been available for many years. This enzyme has been used for removal of oxygen and/or glucose from products such as beer, carbonated beverages, fruit juices, wine and various dried fruit products for stabilisation of colour and flavour (Ough, 1975).

The enzyme is an aerobic dehydrogenase which catalyses the oxidation of glucose to gluconic acid. In the process molecular oxygen is reduced to hydrogen peroxide. For this reason commercial preparations frequently contain considerable amounts of another enzyme, catalase, which removes the hydrogen peroxide generated (Heresztyn, 1987). The enzyme reactions can be outlined as follows:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

The net reaction is:

$$2\text{Glucose} + O_2 \xrightarrow[2)\text{Catalase}]{1)\text{Glucose oxidase}} 2\text{Gluconic acid}$$

A continuous supply of oxygen is necessary for the reaction to proceed. The level of SO_2 in the juice is also important as it may react with the H_2O_2 to produce SO_4^{2-} and H_2O. It may also inhibit the action of the two enzymes (Ough, 1975; Heresztyn, 1987)

Glucose oxidase is most active at pH 5,5 - 6,0 and at 30 - 35°C. It is stable between pH 4,5 and 7,0 (Whitaker, 1972; Windholz, 1976).

Gluconic acid has been found to occur naturally in wines in Bordeaux and California as a result of Botrytis infection of the grapes (McCloskey, 1974).

Villettaz (1987) has suggested some applications of the glucose oxidase enzyme system, i.e. the production of low alcohol wines and the production of more balanced wines.

The demand for low alcohol wines has risen noticeably during the last few years with several technologies proposed for the production of such wines. The technologies proposed involve expensive equipment and the quality of the wines obtained has not always been satisfactory.

For production of low alcohol wines by enzymatic treatment of grape juice, the concept is that the glucose fraction contained in grape juices be converted to gluconic acid by treatment with glucose oxidase and catalase.

Since the gluconic acid is not converted to alcohol by wine yeasts, the wine obtained after fermentation of the treated juice contains less alcohol than conventionally made wine. The excess of acid (gluconic acid) contained in the low alcohol wine can be removed by chemical deacidification, until satisfactory organoleptic properties are obtained (Villettaz, 1987).

1.4. TERPENE GLYCOSIDES AND ACID HYDROLYSIS

The most important factor in determining the rate of acid hydrolysis is the nature of the sugar moiety (Harborne, 1965). The monosaccharides can be placed in the following order, based on ease of hydrolysis, L-rhamnose = L-arabinose > D-glucose ≈ D-galactose>D-glucuronic acid. The resistance of O-glucuronides (or O-glucosiduronic acids) to hydrolysis is a well-known phenomenon. The position of substitution is also important: 3-glycosides are hydrolysed more rapidly than 4-glycosides which are, in turn, hydrolysed more quickly than 7-glycosides (Harborne, 1965).

By extracting Moscato bianco must or wine with ethyl acetate, linalyl arabinoside is extracted which, when suspended in a solution at pH 3 and steam distilled, generates linalool, ∝-terpineol, nerol, geraniol, arabinose and a small quantity of a hexose. Since the above terpene alcohols convert into ∝-terpineol and not vice-versa, it is concluded that the quantity of linalool which converts into ∝-terpineol varies with the distillation conditions. Linalyl arabinoside is continuously hydrolysed depending on temperature and pH; it generates linalool, which in turn cyclises into ∝-terpineol, which is of smaller organoleptic importance (Di Stefano and Castino, 1983). The other glycosides present (which on hydrolysis release 3,7-dimethylocta-1,5-dien-3,7-diol and 3,7-dimethyloct-1-ene-3,6,7-triol) have a less clear function. The diol is not very stable and leads to hotrienol which may contribute to the Moscato aroma. The triol is much more stable. It generates the furan linalool oxides A and B which are of little organoleptic importance (Di Stefano and Castino, 1983).

Thus, there appears to be a pH-dependant interrelationship between several of the grape monoterpenes. For example, the isomeric ocimenols and myrcenol appear to be formed hydrolytically in juice at pH 1,0 at the expense of linalool, nerol and geraniol, which preferentially forms at pH 3,0. Structural studies on precursors of the linalool oxidation state monoterpenes of Vitis

vinifera have shown these to be a glycosidic mixture of rutinosides and 6-O-L-arabinofuranosyl- β-D-glycopyranosides of predominantly geraniol, nerol and linalool together with much smaller amounts of α-terpineol (Williams et al., 1982b; Williams et al., 1982c). The monoterpene glycosides were hydrolysed at pH 1,0 and pH 3,2. The latter value is typical for the pH of juice from mature grapes, while pH 1,0 was used to ensure complete hydrolysis of all precursor components. On hydrolysis at pH 3,2, linalyl-, geranyl- and neryl-β-D-glucoside each gave the same major products, linalool and α-terpineol. Nerol, 3,7-dimethyloct-1-ene-3,7-diol and 2,6,6-trimethyl-2-vinyltetrahydropyran were lesser components from all these reactions, as were the hydrocarbons limonene and terpinolene. Additionally, several compounds were observed in the hydrolysis of linalyl and geranyl glucosides that were not given by the neryl derivative. These included geraniol, (E)- and (Z)-ocimene, α-terpinene and myrcene. α-Terpinyl-β-D-glucoside gave no acyclic monoterpenes. α-Terpineol was its predominant hydrolysis product at pH 3,2. Hydrolytic studies at pH 1,0 on precursor fractions show a very different pattern of volatiles (Williams et al., 1982b; Williams et al., 1982c). At pH 1,0 a partial break down of the monoterpenes occurs, particularly linalool and geraniol. This results in the production of new substances. The results of acid hydrolysis are, therefore, difficult to interpret, owing to the modifications it causes, as the acid instability of monoterpenes has been known for some time (Cordonnier and Bayonove, 1974).

Williams et al. (1982b, 1982c) found that the free terpenes in muscat juice corresponded with that obtained from mild, warm hydrolysis (70°C) at the pH of juice of the glycosidic extracts from juice or of synthetic glycosidic precursors. This suggested that acid hydrolysis is the main process of breaking down disaccharide monoterpenic glycosides. Under the conditions under which the must was obtained, it is this process which is considered the most probable (Bayonove et al., 1983). Marais and Van Wyk (1986), however, found that heat treatment contributed to intensifying the terpene-like character and enhancement of overall wine quality.

As can be seen from the literature, there is a close relationship between the flavour of muscat juice and the content of volatile monoterpenoids in the fruit. In addition to these free volatile flavoured monoterpenes of the grape, there exists non-volatile, acid labile glycosidically-bound forms of these compounds,

as well as polyols, that are flavourless. This serves as a reserve pool of aroma and has the potential to enhance and greatly improve the fruity character of the grape. More than 70% of the volatile constituents of the berry are provided by the skin and pulp. During fermentation the contribution of the yeasts to the terpene concentration is negligible. In this study enzymatic hydrolysis of the glycosidic terpenes will be investigated, as this is a more natural method that will not disturb the natural composition of the aroma compounds, as compared to the other methods of hydrolysis, namely acid hydrolysis and heating (Bayonove et al., 1983).

PART 2

MATERIALS AND METHODS

2.1 ENZYMES USED

Seven enzyme preparations were studied for their ability to release terpenes from their bound glycosidic form. The enzymes employed were the following:

2.1.1 β-Glucosidase from sweet almonds

A commercial enzyme supplied by Boehringer-Mannheim.

2.1.2 Pectinase

A commercial pectinase (Rohapect C) from Rohm possessing potent glycosidase activity.

2.1.3 SP 249

An experimental enzyme preparation from Nova. The activity complex of this preparation may be divided into three main groups viz: pectolytic activity, cellulolytic activity and hemicellulolytic activity. This preparation also contains minor proteolytic activity.

2.1.4 Pectinex Ultra SP

A pectolytic enzyme preparation from Nova Ferment.

2.1.5 Pectinex XL

A pectolytic enzyme preparation from Nova Ferment.

2.1.6 Limonex

A pectinesterase enzyme from Enzymes SA working optimally at 30°C at a pH of 2,1 to 2,5.

2.1.7 Grape hydrolytic enzymes

These enzymes were obtained by extraction of Muscat d'Alexandrie grapes from the 1986 vintage frozen at -30°C until needed. The method of Cordonnier and Dugal (1968) using acetonic powders was used (2.4.1).

2.2 LINEARITY STUDIES

In order to determine the amount of enzyme to use during assays and further work, linearity studies were done.

Assays were done according to the method of Cordonnier et al. (1975) by adding the enzyme to a substrate of 0,004 M 4-nitrophenol-β-D-glucopyranoside (Merck) in 0,04 M acetate buffer pH 4.0, incubating at 40°C for 40 minutes and adding an equal volume of 0,2 M Na_2CO_3 to stop the enzymatic reaction. The intensity of the yellow colour of the 4-nitrophenol was then measured at 400 nm.

Pure grape hydrolytic enzyme (80 mg) was dissolved in 1 ml 0,04 M acetate buffer, pH 4,0. β-Glucosidase and Rohapect C (0,5 mg of each) were dissolved in 1,5 ml of the acetate buffer. The liquid enzyme preparations, i.e. Ultra SP, SP 249 and XL were diluted by adding 5 µl enzyme preparation to 1000 µl. Different volumes were incubated with the substrate during the assay in a linear configuration.

2.3 ACTIVITY AND STABILITY STUDIES

Temperature and pH activity and stability studies were conducted on the above mentioned enzymes in order to determine the activity and stability under winemaking conditions, i.e. 15°C and pH 3,5.

For the pH stability and activity studies, different buffer systems at 0,04 M were used to obtain the required pH range. During the pH stability tests, the different enzymes were incubated in the above mentioned buffers at different pH levels ranging from pH 2,5 to 6,0 for 1 hour at 37°C. Substrate was subsequently added, followed by a further incubation time of 30 minutes at 37°C. The remaining activity was determined by reading the absorbance of the 4-nitrophenol at 400 nm.

During the pH activity tests the different enzymes were incubated in substrate dissolved in the above mentioned buffers in the pH range from 2,5 to 6,0 for the normal assay period of 30 minutes.

The temperature stability tests were performed by incubating the different enzymes in 0,04 M acetate buffer pH 4, at a range of temperatures from 4 to 60°C for 1 hour. Substrate was added afterwards, followed by the normal assay procedure.

The temperature activity tests were conducted by following the normal assay procedures for the different enzymes at a range of temperatures from 4 to 60°C.

2.4 ISOLATION AND PURIFICATION OF THE ENZYMES

2.4.1 Isolation of grape hydrolytic enzymes

These enzymes were obtained by extraction of Muscat d'Alexandrie grapes from the 1986 vintage frozen at -30°C until needed. The method of Cordonnier and Dugal (1968) using acetonic powders was used.

The extraction was done as quickly as possible at -5°C in a cold room. Grapes (300 g) at -1°C was coarsely crushed with a mortar. The material was covered with a volume of acetone at -5°C equal to four times the weight of grapes used. The mixture was processed in a homogeniser for one minute. After the addition of a second volume of acetone equal in volume to the first, the mixture was again homogenised and thereafter left to rest for half an hour.

The precipitate was recovered by vacuum filtration on a Büchner funnel. It was washed twice with 96% ethanol at -5°C by each time processing the

precipitate in the homogeniser for 1 minute in a volume of ethanol equal to four times the equal weight of material used. This was washed twice with ether and then dried as quickly as possible on a cold bench at -5°C.

This crude enzyme extract was purified further (Gunata, 1984) by dissolving 15 g of crude enzyme (obtained from 300 g Muscat d'Alexandrie grapes) in 250 ml 0,1 M phosphate-citrate buffer pH 5 at 2°C. To this was added 17 ml Tween 20 and 2,7 g ascorbic acid. The pH was adjusted to 5,0 using 1 M NaOH, homogenised at 2°C and 15 g Polyclar AT was added. The mixture was stirred for 15 minutes at 2°C. Afterwards it was centrifuged at 2°C at 25 000 g for 20 minutes. The supernatant was the crude enzyme extract which could be freeze dried.

The crude enzyme extract was further purified by precipitating the protein with 80% $(NH_4)_2SO_4$. The precipitate was obtained by centrifuging the solution at 25 000 g at 2°C for 15 minutes. The precipitate was redissolved in 70 ml 0,1 M phosphate-citrate buffer pH 5,0 at 2°C. The solution was dialysed against 10 l of 0,01 M pH 5,0 phosphate-citrate buffer at 2°C for 12 hours. The retentate was freeze dried and this was the pure grape hydrolytic enzyme that was used in further studies.

2.4.2 High Pressure Liquid Chromatography (HPLC)

HPLC was done on the different enzyme preparations using a Waters Liquid Chromatograph in order to determine the homogeniety of the protein preparations.

DEAE anion exchange was done using a 0,05 M Tris buffer pH 7,6 with a NaCl gradient of 0,0 to 1,0 M and a flow rate of 1,5 ml/minute.

DEAE anion exchange was performed on the pure grape hydrolytic enzymes using a 0,05 M Tris pH 7,6 buffer system with a NaCl gradient of 0,0 to 1,0 M and a pump rate of 1,5 ml/min.

Further enzyme purification was done on a DEAE Protein Pak ion exchange column using a 0,05 to 1,0 M NH_4HCO_3 buffer system. The advantages of this buffer system are that there are no dialysis or pH problems.

1 mg of β-glucosidase was dissolved in 100 µl NH_4HCO_3 and 10 µl was fractionated on this column with the above mentioned buffer system.

2,2 mg Rohapect C was dissolved in 100 µl starting buffer and 10 µl was fractionated.

100 µl Pectinex XL was diluted to 1000 µl with starting buffer and 10 µl was fractionated.

100 µl Ultra SP was diluted to 1000 µl with 0,05 M NH_4HCO_3. Gradient 8 on the apparatus from 100% A to 50% B was used for the elution.

100 µl SP 249 was diluted to 1000 µl with 0,05 M NH_4HCO_3. Gradient 8 on the apparatus from 100% A to 50% B was used for the elution.

2.4.3 SDS Polyacrylamide Gel Electrophoresis

SDS (sodium dodecylsulphate) polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). This electrophoresis was performed to determine the purity of the active enzyme fractions obtained from HPLC.

2.4.3.1 Stock Solutions

a) Running Gel

A : 30% acrylamide (w/v)
0,8% N,N'-methylene bisacrylamide (w/v) in H_2O

B : 1,125 M Tris-HCl pH 8,8
0,3% sodium dodecylsulphate (SDS) (w/v)

C : 10% ammonium persulphate in H_2O (w/v)

D : TEMED

b) Stacking Gel

A : 30% acrylamide (w/v)
0,8% N,N'-methylene bisacrylamide (w/v) in H_2O

B : 0,375 M Tris-HCl pH 8,8
0,3% sodium dodecylsulphate (w/v)

C : 10% ammonium persulphate (w/v) in H_2O (made up fresh daily)

D : TEMED

c) Sample Application Buffer

0,0625 M Tris HCl pH 6,8
2% SDS (w/v)
10% glycerol (w/v)
0,001% bromophenol blue (w/v)

d) Tray Buffer

0,025 M Tris HCl pH 8,3
0,192 M glycine
0,1% SDS (w/v)

2.4.3.2 Gel Preparation

The separating gel consisted of 15% (w/v) acrylamide, 0,4% (w/v) N,N'-methylene bisacrylamide, 0,375 M Tris-HCl pH 8,8, 0,1% (w/v) ammonium persulphate and 0,6% (v/v) TEMED. The gel was poured leaving enough space for a stacking gel.

Distilled water was carefully layered onto the SDS gel solution to obtain a straight interface.

The stacking gel consisted of 3% (w/v) acrylamide and 0,125 M Tris-HCl pH 8,8. The separating gel was allowed to polymerise overnight and the stacking gel for 30 - 45 minutes.

Electrophoresis was carried out overnight at 15 mA.

2.4.3.3 Staining and Destaining

Gels were stained in a mixture of water/acetic acid/methanol (4:1:5) containing 0,25% Coomassie Brilliant Blue, for 1 - 3 hours.

Destaining was achieved in a solution containing water/acetic acid/methanol (8:1:1). Destaining was done by diffusion, changing the buffer regularly until the gel background became clear. This could be achieved in a day.

The gels were stored in 5% glycerol, covered.

2.4.4 **Column Chromatography**

The enzyme preparations in powder form were dissolved in starting buffer (0,05 M NH_4HCO_3) and dialysed overnight against starting buffer, while the liquid preparations were dialysed as is. After dialysis the enzymes were freeze dried and dissolved in starting buffer just before chromatography. The individual fractions were measured at 280 nm. The activity of the peaks was determined (2.2) and the active fractions were pooled and freeze dried.

2.4.4.1 Ion Exchange Chromatography

The enzyme preparations that were dialysed or desalted on Sephadex and freeze dried were fractionated on a Whatman DE-52 column (16 x 300mm) with a linear 0,05 to 0,5 M NH_4HCO_3 gradient. The NH_4HCO_3 gradient was 500 ml 0,05 M NH_4HCO_3 to 500 ml 0,5 M NH_4HCO_3. The sample was dissolved in a small volume of 0,05 M NH_4HCO_3. Approximately 5 g of crude protein was placed on a DE column per run. The flow rate was 4 ml/minute. The individual fractions were measured at 280 nm. The activity of the peaks was determined as in 2.2. The active fractions were pooled and freeze dried.

These active fractions were refractionated on the DE-52 column by changing the gradient to 0,05 to 0,25 M NH_4HCO_3 in an attempt to improve the fractionation.

The flow rate was maintained at 4 ml per minute using a peristaltic pump. The column was flushed with 0,5 M NH_4HCO_3 after every run.

2.4.4.2 Gel Exclusion Chromatography

Sephadex G15 (16 x 300mm) was used for desalting the enzyme preparations before going onto the ion exchange column. The eluant was 0,05 M NH_4HCO_3.

2.5 DETERMINATION OF THE FREE AND POTENTIALLY FREE TERPENES

2.5.1 Enzyme Quantitation

The glycosidic activity of the commercial enzyme preparations was unknown. The amounts of the different active enzyme fractions to be used were determined by using a β-glucosidase standard curve. According to Rapp (personal communication, 1987) 300 mU/ml enzyme must be added to grape must for maximal terpene hydrolysis. The mass of the different commercial enzyme preparations to be added were determined by using this standard curve.

2.5.2 Determination of the free (FVT) and potentially volatile (PVT) monoterpenes in grapes

The method as developed by Dimitriadis and Williams (1984) was used to determine the terpenes.

2.5.2.1 Grapes

Grapes of the cultivars Muscat d'Alexandrie and Weisser Riesling were harvested at full maturity, when having reached an approximate sugar-acid ratio of 2,5 and an approximate sugar concentration of 20^{0}B (20 g sucrose/100 g water) and destemmed by hand. Only healthy berries were clipped from the stem at the pedicel junction. The grapes were frozen at -30^{0}C until needed.

2.5.2.2 Treatments

The following alternatives were investigated in fourfold for both cultivars.

2.5.2.2.1 Skin contact

a) No skin contact, in other words after the grapes were macerated the pulp was immediately strained through muslin gauze. The above mentioned method was then followed to determine the FVT and PVT in both cultivars.

b) Twenty hour skin contact, in other words the macerated grapes were

left for 20 hours at 10 and 20°C and afterwards the same procedure as in 2.5.2.2.1(a) was followed.

2.5.2.2.2 Enzyme treatments

a) β-Glucosidase : 300 mU/ml (43,2 mg) was added to 400 ml must and left for skin contact of 20 hours at 10 and 20°C. Afterwards the same procedure as in 2.5.2.2.1(a) was followed for both cultivars.

b) Rohapect C : 300 mU/ml (166,8 mg) was added to 400 ml must and afterwards the same procedure as in 2.5.2.2.2(a) was followed for both cultivars.

c) Ultra SP : 1 g was added to 400 ml must and afterwards the same procedure as in 2.5.2.2.2(a) was followed for Muscat d'Alexandrie.

d) β-Glucosidase : 300 mU/ml (43,2 mg) was added to 400 ml Muscat d'Alexandrie must of which the pH had been adjusted to 5. Subsequently the same procedure as in 2.5.2.2.2(a) was followed.

2.5.2.3 Terpene isolation from juice

The grape berries (500 - 600 g) were defrozen and macerated in a Waring blender and the resulting pulp strained through muslin gauze. The gauze was squeezed to remove the remaining juice from the skins and seeds.

The pH of 400 ml juice was adjusted to 6,6 - 6,8 by the addition of 20% NaOH. The juice was transferred to a 1 l three necked flask fitted with a sealed thermometer, addition funnel and splash head/steam delivery tube. The juice was slowly heated to 95°C by means of a heating mantle. At this temperature steam was blown in from a 2 l flask fitted with a 60 cm safety tube and polythene connecting tube. A short double surface condenser was used to condense the volatiles.

The first 100 ml of distillate contained essentially all of the FVT and was collected in a graduated receiver. Without interrupting the steam flow, the receiver was changed and 10 ml of 40% H_3PO_4 was rapidly added via the addition funnel.

A further 150 ml of distillate was collected from the juice for the determination of PVT.

Both the FVT and PVT distillates were well mixed and 10 ml of each were taken for the colorimetric determination.

2.5.2.4 The colorimetric determination

a) Reagent solutions

Linalool stock solution:
50 mg linalool, dissolved in 10 ml ethanol and diluted to 50 ml with water. This was diluted 1 in 10 to give the standard solution.

2% (w/v) vanillin in concentrated H_2SO_4 was prepared and stored in a glass stoppered brown bottle at 0 - 4°C. The solution is extremely corrosive and exothermic when mixed with water and should be handled with utmost care.

b) Preparation of standard curve

Pyrex test tubes fitted with screw capped silicone rubber seals were used as reaction vessels. Solutions (200 to 2000 µl) containing 20, 50, 100, 150 or 200 µg linalool were made up to 10 ml with distilled water. A blank was prepared with 10 ml of water alone. To each precooled tube was added 5 ml of vanillin in H_2SO_4 while the contents were agitated in an ice bath. The colour was developed by heating the tubes in a water bath at 60 $\pm$ 1°C for 20 minutes. After cooling at 25°C for 5 minutes, the optical density (OD) was read at 608 nm.

c) Terpene quantitation

The same assay procedure as described above was used for the FVT and PVT distillates. Levels of monoterpenes in the distillates were read as ug of linalool from the standard curve.

$$\text{FVT or PVT} = \frac{A \times B}{C \times D} \text{ mg/l (as linalool)}$$

where A = µg of linalool read from the standard curve; B = volume of juice collected; C = volume of juice distilled; D = volume of aliquot taken for the determination (10 ml).

2.5.3 Capillary Gas Chromatography for the determination of Specific Terpenes in the FVT distillates

A gas chromatographic technique, as developed by Rapp et al. (1976) and adapted to local conditions by Marais (1986), was used for the determination of individual terpenes in the FVT distillates obtained from 2.5.2.3

2.5.3.1 Extraction technique

FVT distillate (50 ml) was diluted to 250 ml before cooling to 0°C to prevent emulsification. The internal standard, 0,5 ml 2-ethyl hexanol (80 µg/l), was added to 250 ml of the sample and mixed for 30 seconds.

Freon 11 (20 ml) was poured into the extraction apparatus and a tuft of sylilated glass wool placed on the freon surface to prevent emulsification during extraction. The sample containing the internal standard was then carefully poured into the extraction apparatus.

The extraction unit was installed with its bottom in ice and the collecting funnel and a condenser, through which coolant at approximately -5°C was circulated, was fitted to the extraction unit.

A 25 ml pear-shaped collecting flask, containing 20 ml freon, was fitted to the extraction unit and immersed in a waterbath at 35°C. Extraction was done for 20 hours at a controlled room temperature of 19 to 20°C.

2.5.3.2 Concentration of freon extracts

A Vigreux (270 x 20 mm) and an air condenser (550 x 13 mm) were installed onto the flask containing the extract to facilitate reflux. The flask was held in a waterbath at 35°C, the air cooler controlled at 20°C or lower and the extract concentrated to approximately 2 ml.

After concentration the flask was placed in solid CO_2 to freeze out possible traces of water. The dry extract was then transfered to a 3 ml pear-shaped flask with a tapered tip by means of a Pasteur pipette. A small air condenser (220 x 8 mm) with a spiral made of 1 m x 0,5 mm Teflon strip was fitted onto the flask and the concentration was continued under partial reflux to approximately 0,1 ml. Concentration time was approximately 45 minutes. Extracts were stored at -12°C prior to analysis.

2.5.3.3 Gas chromatographic conditions

Gas Chromatograph	Hewlett Packard 5880A with automatic dual integrators.
Column	50 m x 0,31 mm (i.d.), Carbowax 20 M fused silica capillary column (Hewlett Packard).
Injection temperature	200°C
Detector	Flame ionization
Detector temperature	250°C
Temperature program	60°C for 10 minutes 60°C to 120°C at 1°C/min 190°C for 30 min
Carrier gas	Helium
Column flow rate	1,5 ml/min
Split flow rate	120 ml/min
Split ratio	90 : 1
Septum purge	6 ml/min
Hydrogen flow rate	30 ml/min
Air flow rate	300 ml/min
Injection volume	1 µl
Overall analysis time	170 minutes

2.5.3.4 Calibration of terpenes

The response factor for the internal standard (f_a) was also used for the terpenes analysed and terpene concentrations were consequently calculated as relative concentrations. The internal standard calibration method, used by the HP 5880 computer, entails the following calculation.

$$\text{Concentration (b)} = \frac{\text{area(b) x factor(b) x concentration(a)}}{\text{area(a) x factor(a)}}$$

a = Internal standard

b = Unknown compound

2.6 ENZYME INHIBITION

2.6.1 Product inhibition

Beer's law for the effect of grape juice concentration on absorbance was measured through making a range of dilutions of Muscat d'Alexandrie grape juice with water and adding an equal volume of Na_2CO_3 and measuring the absorbance at 400 nm.

To test for the effect of different concentrations of grape juice on the activity of Rohapect C and Ultra SP, a range of dilutions of grape juice with 0,04 M NaOAc pH 3,5 buffer was made. Substrate was dissolved in the dilutions to obtain 0,004 M 4-nitrophenyl-β-D-glucopyranoside. Assays were done as in 2.2.

2.6.2 Metal ion effect

A range of Muscat d'Alexandrie grape juice dilutions were made with 0,04 M citrate pH 3,5 buffer and to this was added 0,004 M substrate. Thereafter the normal assay for Rohapect C was followed.

2.6.3 Enzyme inhibition by sugar

A range of solutions of 0 to 100 g/l of each of glucose and fructose was made in 0,004 M substrate. The normal assay procedure (2.2) was followed afterwards.

2.7 PREPARATION OF GRAPES

Healthy Muscat d'Alexandrie and Weisser Riesling berries were destemmed as in 2.5.2.1. Afterwards only the grapes with a sugar concentration from 20 to 22°B were selected.

This was done using the method of Singleton et al. (1973). Two containers with sugar concentrations of 20°B and 22°B respectively were prepared. The separation was done by placing some grapes in the container at the highest sugar concentration. The grapes that floated were transfered to the container with the lower sugar concentration. The grapes that sank in the second solution, had a sugar concentration of between 20 and 22°B.

Strainers were used for the transfer of grapes to prevent the transfer of solution. Throughout the separation process, the sugar concentration in the solutions was monitored using a refractometer to ensure that the sugar concentrations were kept at the right levels.

After separation, the grapes were rinsed and carefully dried before they were weighed and frozen in 1 kg quantities at -30°C until use.

2.8 DETERMINATION OF FREE VOLATILE AND POTENTIALLY VOLATILE TERPENES IN MUST WHERE THE GLUCOSE HAD BEEN OXIDISED TO GLUCONIC ACID

2.8.1 Quantitation of glucose oxidase

Volumes of 0,5, 1 and 2% of an experimental glucose oxidase/catalase enzyme preparation (obtained from Enzymes SA) , was added to 20 ml Weisser Riesling must that was obtained by macerating thawed grapes (1988 vintage) and filtering through muslin gauze. Oxygen was bubbled through at a rate of 30 ml/min at 20°C for 24 hours. The rate of oxidation of glucose was measured using the Boehringer-Mannheim enzyme test kit.

After fairly successful initial oxidation of glucose in must, it was found that oxidation stopped at about 50 g/l glucose. Addition of a similar volume of enzyme preparation after 7 hours completed the oxidation in 24 hours.

A preparation of glucose oxidase/catalase was prepared to attempt to overcome this problem. Pure glucose oxidase from Aspergillus niger (Fluka) and catalase from A. niger (Merck) was prepared to such a concentration that 100 g/l glucose would be oxidased in 20 hours.

2.8.2 Determination of the Free and Potentially Free Terpenes

Grapes of the cultivars Muscat d'Alexandrie and Weisser Riesling of the 1988 vintage, that had been selected to have a sugar concentration of between 20 and 22°B, were used. Eight ml of the enzyme preparation was added to 500 g grapes that had been thawed and macerated. The glucose oxidase/catalase preparation obtained from Enzymes SA was used on

Weisser Riesling. Since the quantity of enzyme was at a premium, the prepared glucose oxidase/catalase was used on Muscat d'Alexandrie. Oxygen was bubbled through at a rate of 700 ml/min at 20°C. After 7 hours a second addition of 8 ml enzyme was made. The glucose concentration was determined using the Boehringer-Mannheim enzyme test kit. After 24 hours, when the glucose had reached a concentration of approximately 10 g/l, 300 mU/ml (166,8 mg) Rohapect C was added and the must left for a further 20 hours at 20°C. Thereafter, the same procedure as in 2.5.2 was followed to determine the free volatile and potentially volatile terpenes. The individual terpenes of the FVT fraction were determined as in 2.5.3.

PART 3

RESULTS

3.1 ENZYME STUDIES

3.1.1 Linearity studies

In order to determine the amount of the different enzymes to use during assays and further work, linearity studies were done and the results are displayed in figure 3.1.

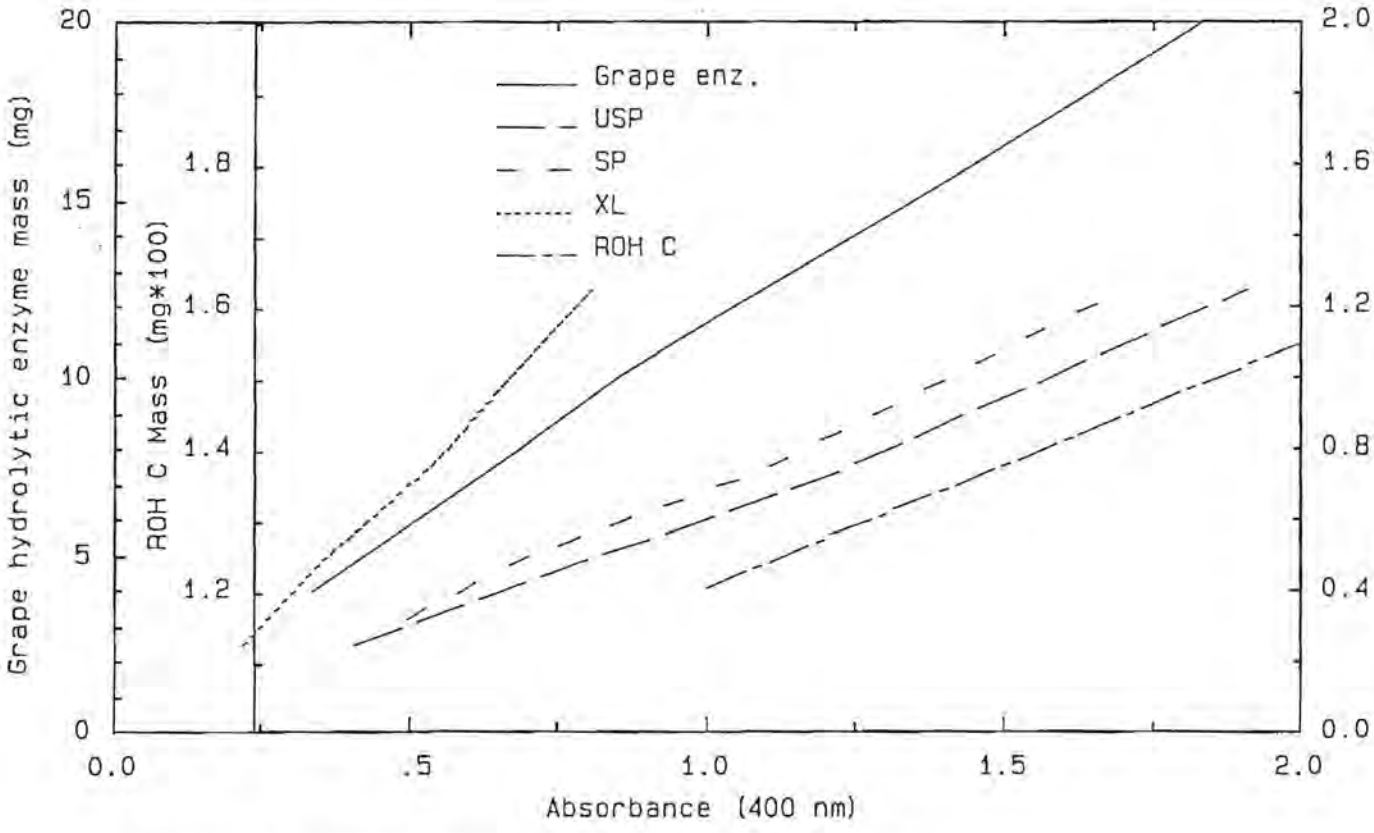


Figure 3.1 Linearity of the enzymes used in the study.

- Grape hydrolytic enzymes
- Rohapect C
- Ultra SP
- SP 249
- XL

3.1.2 Activity and stability studies

Temperature and pH activity and stability studies were conducted on the above mentioned enzymes in order to determine the activity and stability under winemaking conditions, i.e. 15°C and pH 3,5 (Fig. 3.2 to 3.5)

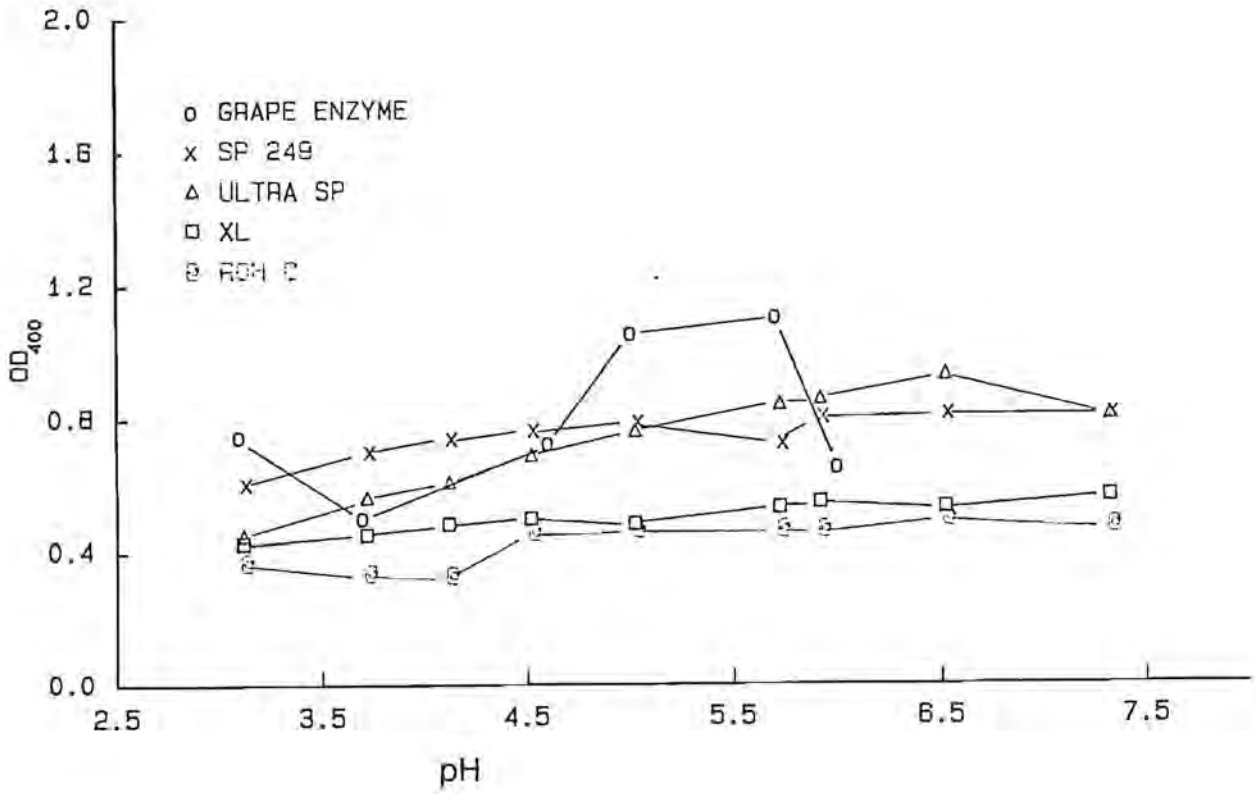


Figure 3.2 pH stability of different enzymes

- Grape hydrolytic enzymes
- Rohapect C
- Ultra SP
- SP 249
- XL

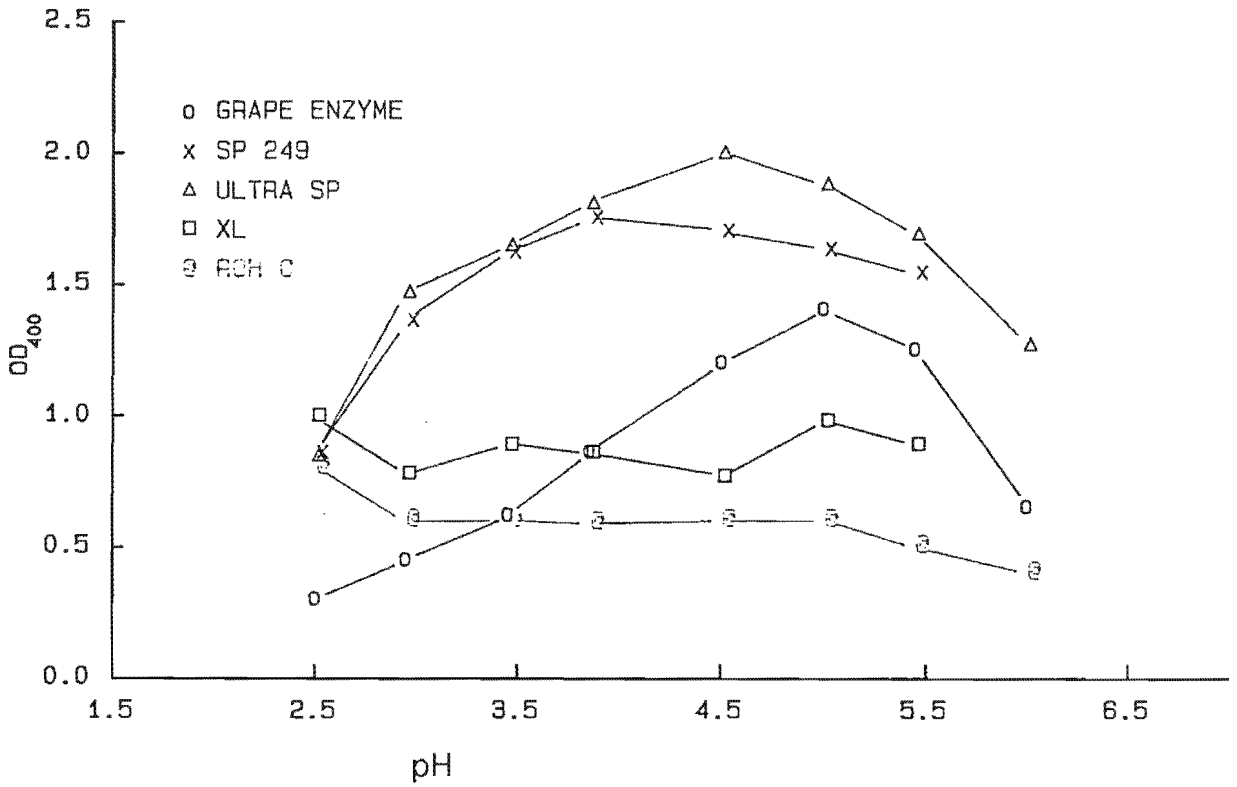


Figure 3.3 pH activity of different enzymes

- Grape hydrolytic enzymes
- Rohapect C
- Ultra SP
- SP 249
- XL

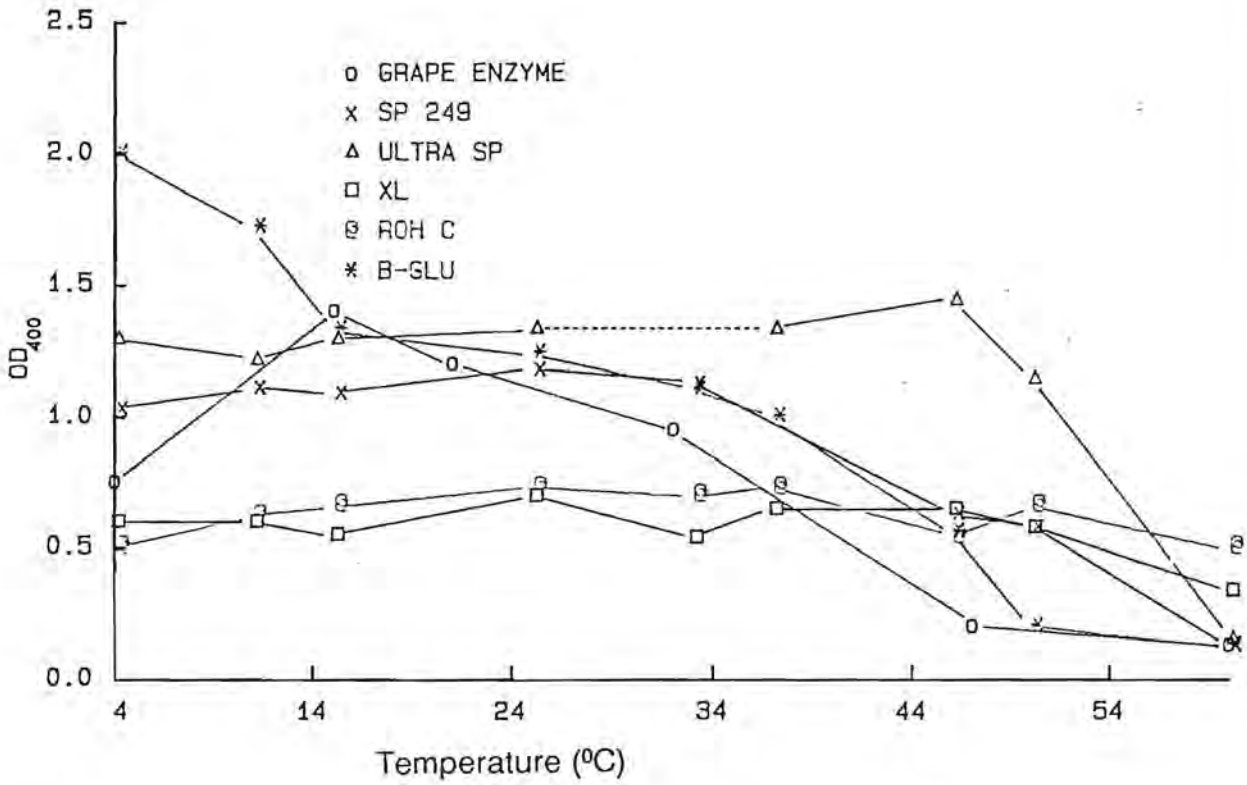


Figure 3.4 Temperature stability of different enzymes

- Grape hydrolytic enzymes
- β-glucosidase from sweet almonds
- Rohapect C
- Ultra SP
- SP 249
- XL

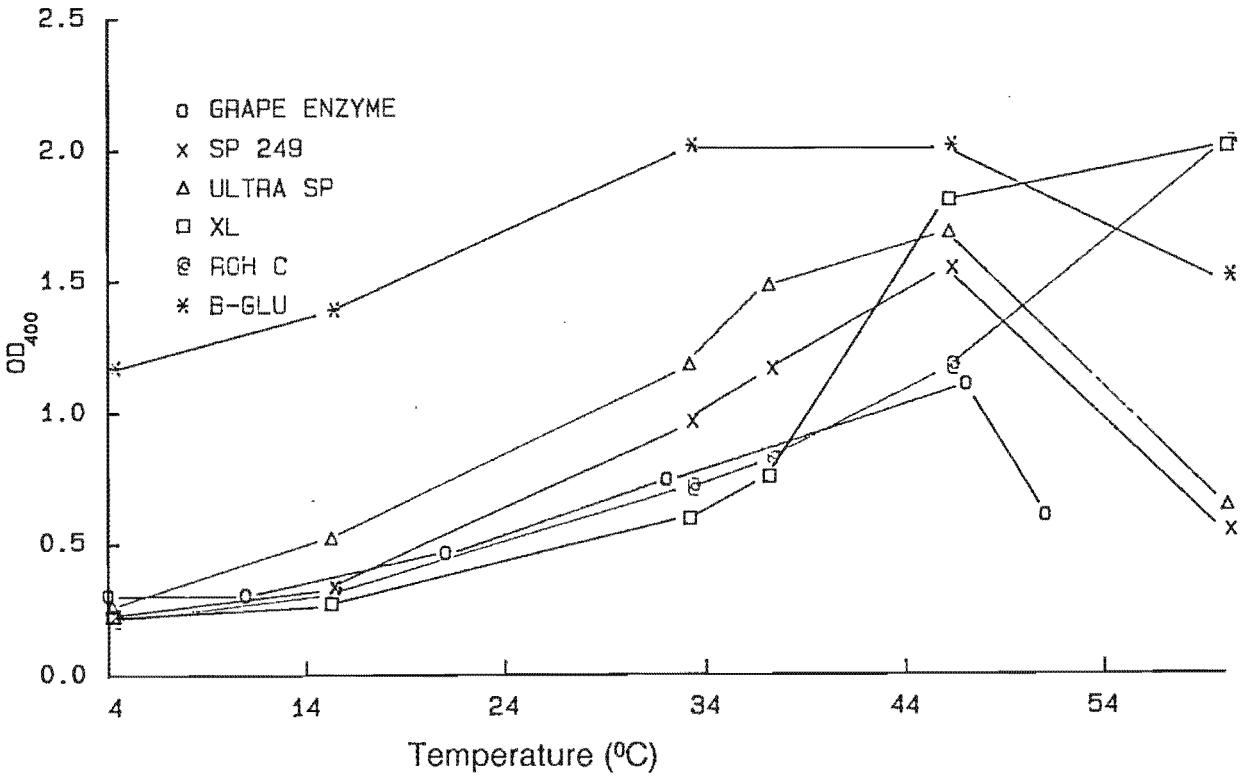


Figure 3.5 Temperature activity of different enzymes

- Grape hydrolytic enzymes
- -β-glucosidase from sweet almonds
- Rohapect C
- Ultra SP
- SP 249
- XL

3.1.3 High pressure liquid chromatography (HPLC) on the different enzymes

HPLC was performed on the different enzyme preparations as a method to screen the purity of the preparations.

DEAE anion exchange was performed on the pure grape hydrolytic enzymes using a 0,05 M Tris pH 7,6 buffer system with a NaCl gradient of 0,0 to 1,0 M and a pump rate of 1,5 ml/min. Four major peaks were obtained with activity in only the first peak (Fig. 3.6)

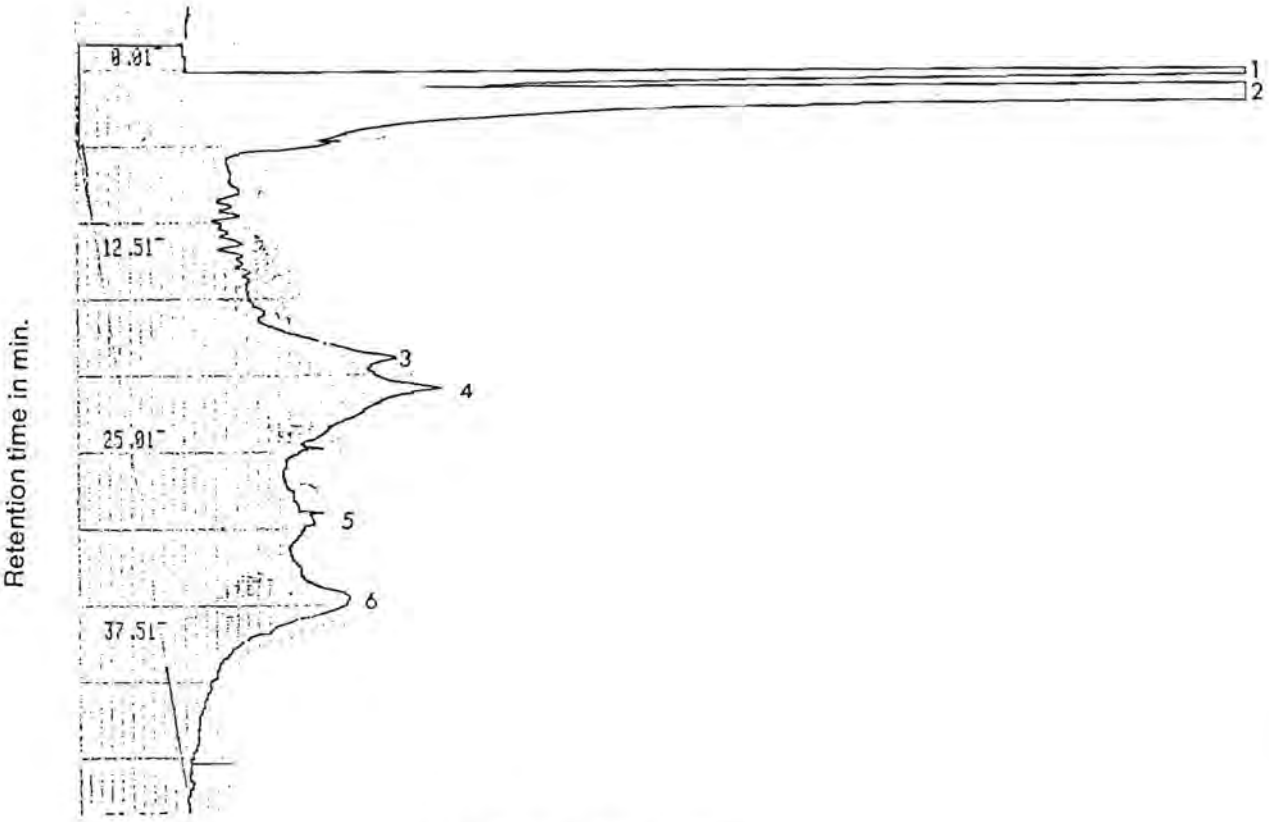


Figure 3.6 DEAE anion exchange of the pure grape hydrolytic enzymes with a linear gradient of 0,0 - 1,0 M NaCl in 0,05 M Tris pH 7,6.

The sample (100 mg) was dissolved in 1 ml Tris pH 7,6. The total gradient volume was 75 ml and the flow rate 1,5 ml/min.

Six major peaks were obtained with activity in the first.

For further enzyme purification a DEAE protein pak ion exchange column was used with a 0,05 to 1,0 M NH_4HCO_3 buffer system (Fig. 3.7 to 3.10).

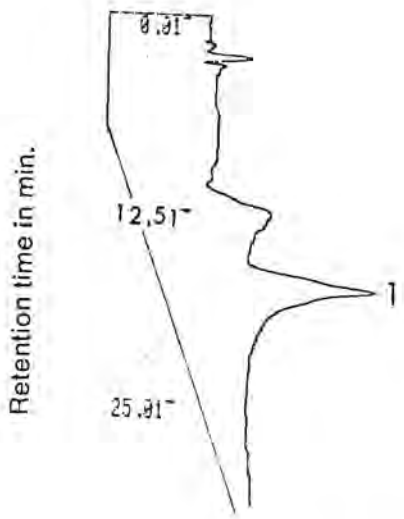


Figure 3.7 Elution pattern of β-glucosidase from sweet almonds from a protein pak ion exchange column with a linear gradient of 0,05 - 1,0 M NH_4HCO_3

1 mg of sample was dissolved in 100µl 0,05 M NH_4HCO_3 buffer. Total gradient volume was 45 ml and the flow rate 1,5 ml/min.

β-Glucosidase had only one major peak and all activity was concentrated in that peak.

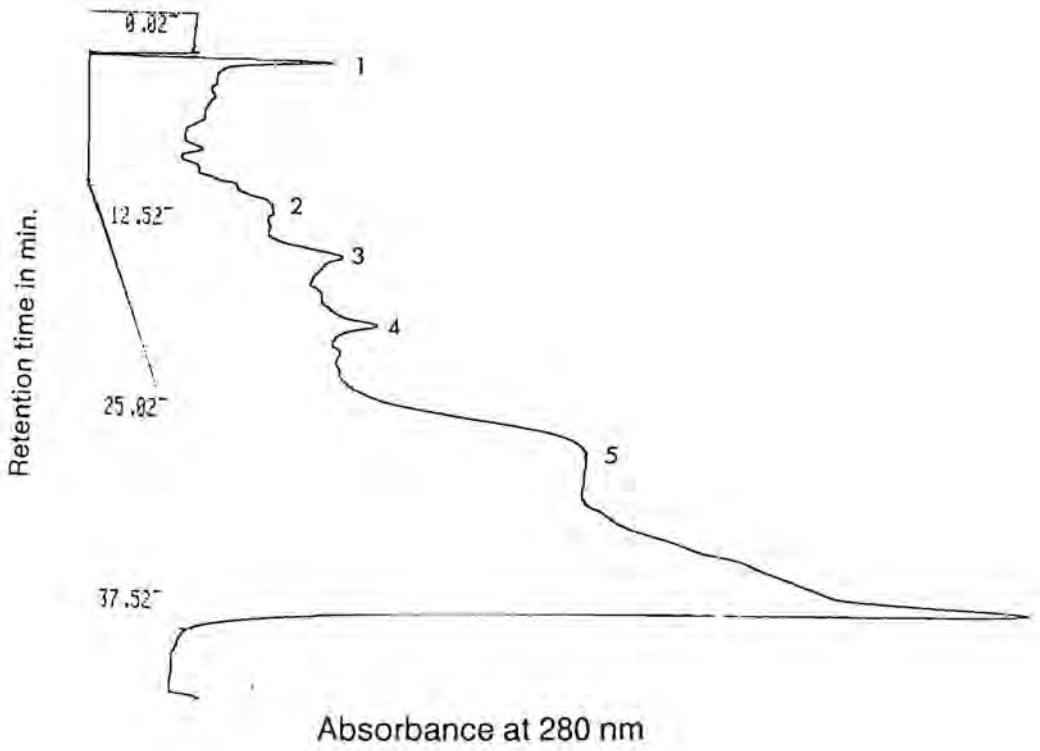


Figure 3.8 Elution pattern of Rohapect C from a protein pak ion exchange column with a linear gradient of 0,05 - 1,0 M NH_4HCO_3

2,2 mg Rohapect C was dissolved in 100 µl 0,05 M NH_4HCO_3 buffer. Total gradient volume was 75 ml and the flow rate 1,5 ml/min.

Five major peaks were obtained from the fractionation with activity found in the third peak.

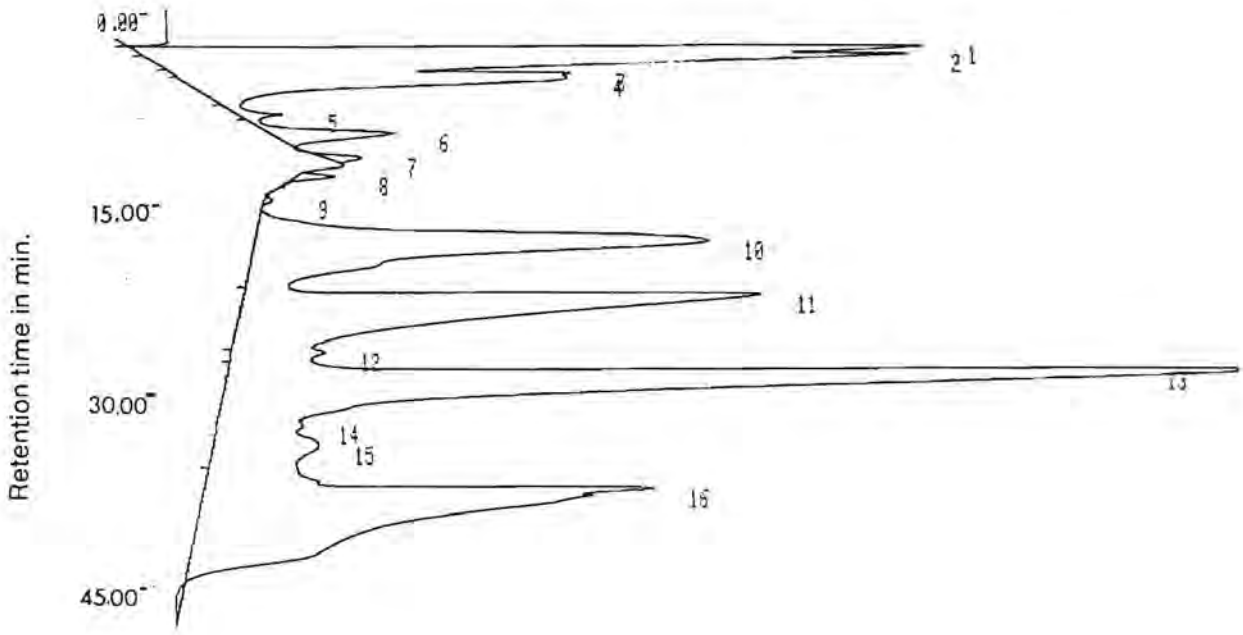
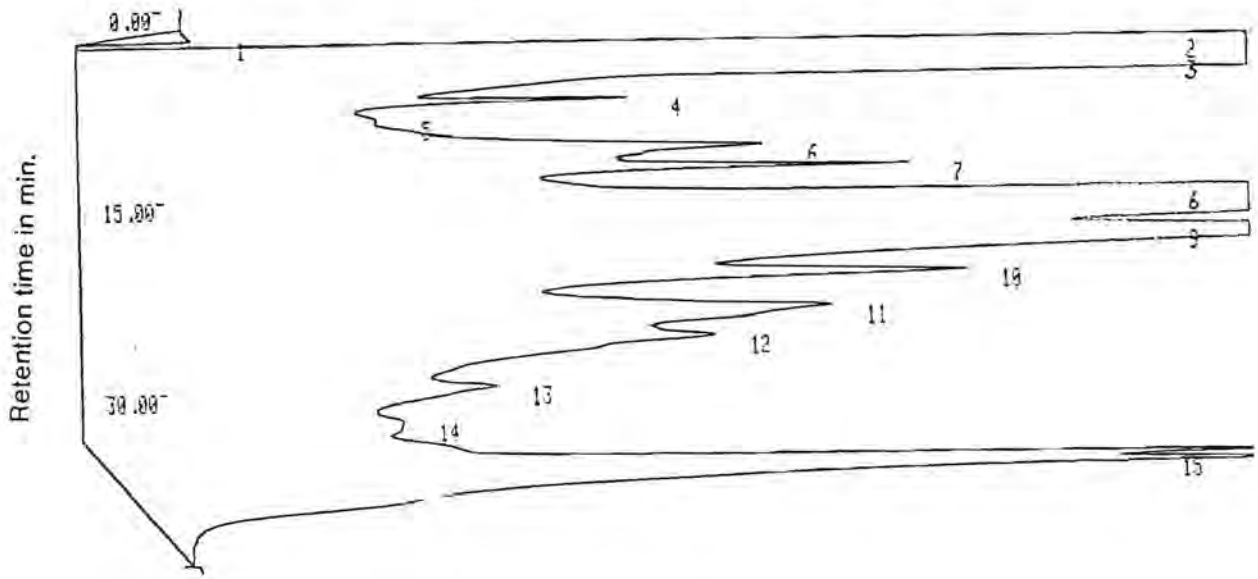


Figure 3.9 Elution pattern of Pectinex XL from a protein pak ion exchange column with an isocratic gradient of 30 ml 0,05 M NH_4HCO_3 buffer and 1,0 M NH_4HCO_3 buffer.

100 µl Pectinex XL was diluted to 1000 µl with 0,05 M NH_4HCO_3 buffer. 50 ml 0,05 M NH_4HCO_3 was pumped through followed by 0,5 M NH_4HCO_3 at a flow rate of 1,5 ml/min.

A major Pectinex XL peak with activity was detected (13) and five minor peaks with no activity.



Absorbance at 280 nm

Figure 3.10 Elution pattern of Ultra SP from a protein pak ion exchange column using gradient 8 of 100% 0,05 M NH_4HCO_3 to 50% 0,5 M NH_4HCO_3 at a flow rate of 1,0 ml/min.

100 µl Ultra SP was diluted to 1000 µl with 0,05 M NH_4HCO_3. Flow rate was 1,0 ml/min with a total gradient volume of 30 ml.

Ultra SP was fractionated into 16 peaks with peak 9 and 10 the major peaks. Activity was concentrated in peak 6 to 10.

3.1.4 Electrophoresis of the different enzymes

Protein polyacrylamide gel electrophoresis (Laemmli, 1970) was done on the different active enzyme fractions to determine the homogeneity of the different active fractions. The active fractions as obtained from HPLC were lyophilysed. NaOAc buffer 0,04 M, pH 5 (50 ul) was added to each fraction followed by 17 ul loading buffer and the sample boiled for 2 - 3 minutes. Each fraction (30 µl) was loaded onto the gel and electrophoresed for 20 hours at 15 mA.

From the gel it was clear that β-glucosidase, as represented by the a single band, was a pure preparation. The other active fractions, however, displayed more than one band and were not pure (results not shown).

3.1.5 Column chromatography of the enzymes

3.1.5.1 Gel Exclusion Chromatography

Gel exclusion chromatography on Sephadex G-15 was used for the desalting (instead of dialysis) of the enzyme preparations. Typical separations are shown in figures 3.11 to 3.13.

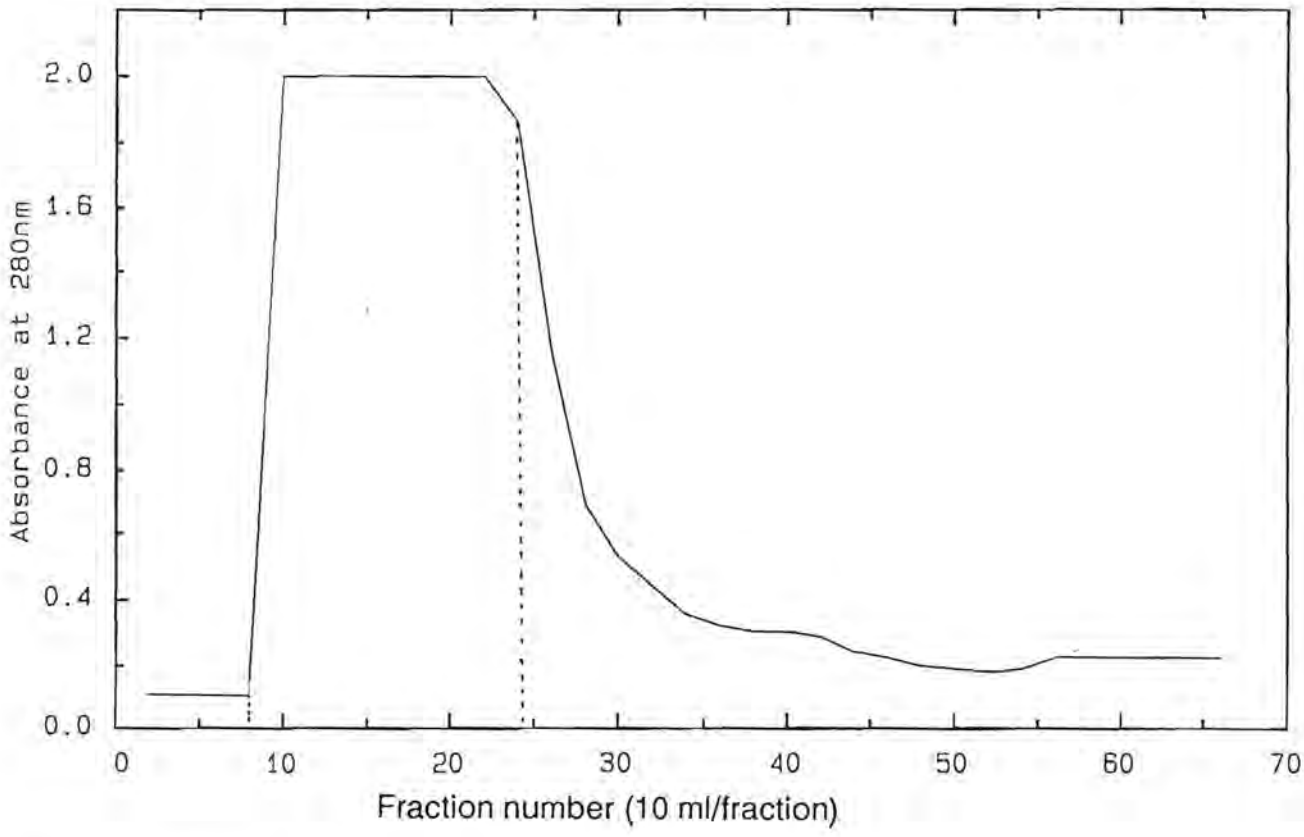


Figure 3.11 Fractionation of Rohapect C
Column : 16 x 300 mm Sephadex G-15 with a 0,05 M NH_4HCO_3 buffer as eluant, flow rate : 4 ml/min., total volume : 1000ml. Dotted lines indicate the fraction cuts.

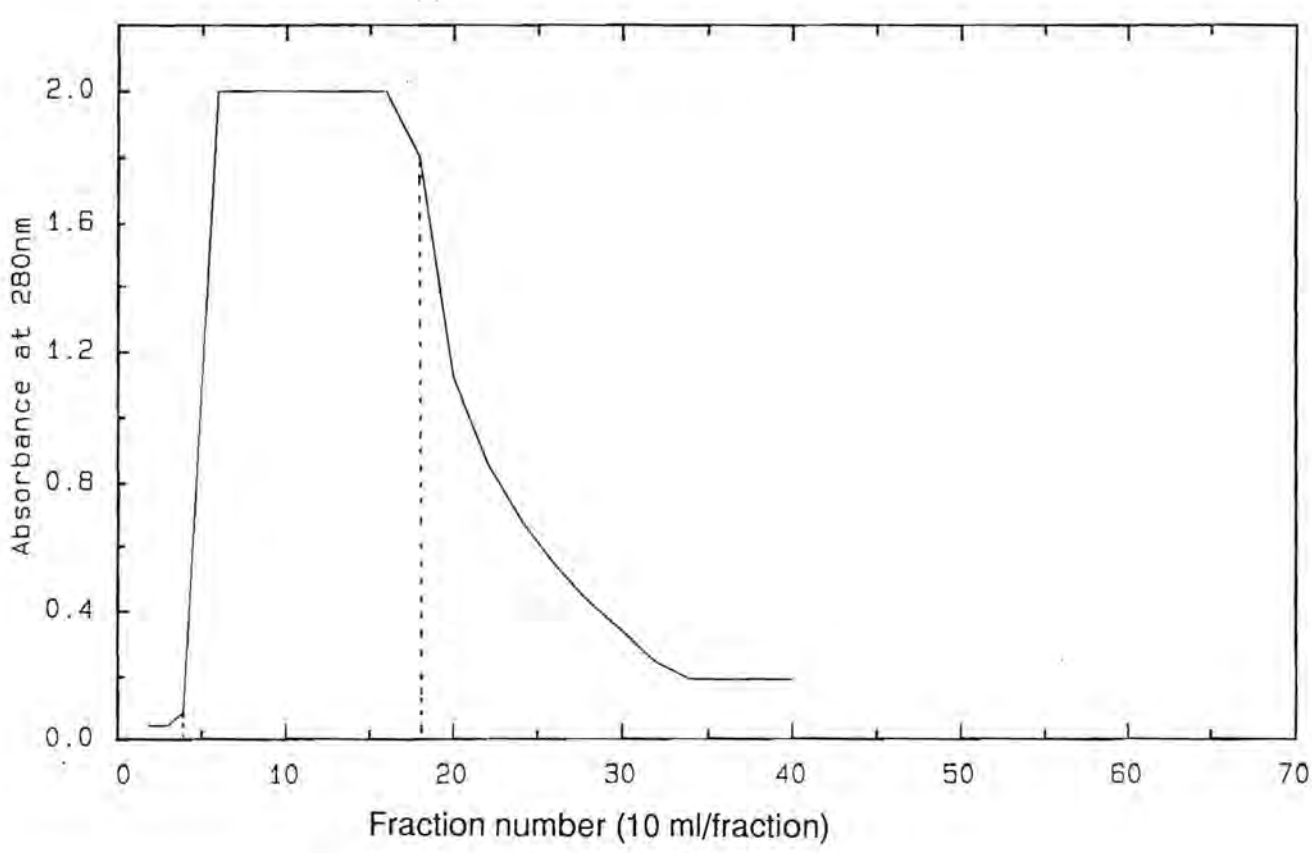


Figure 3.12 Fractionation of Pectinex XL

Column : 16 x 300 mm Sephadex G-15 with a 0,05 M NH_4HCO_3 buffer as eluant, flow rate : 4 ml/min., total volume : 1000ml. Dotted lines indicate the fraction cuts.

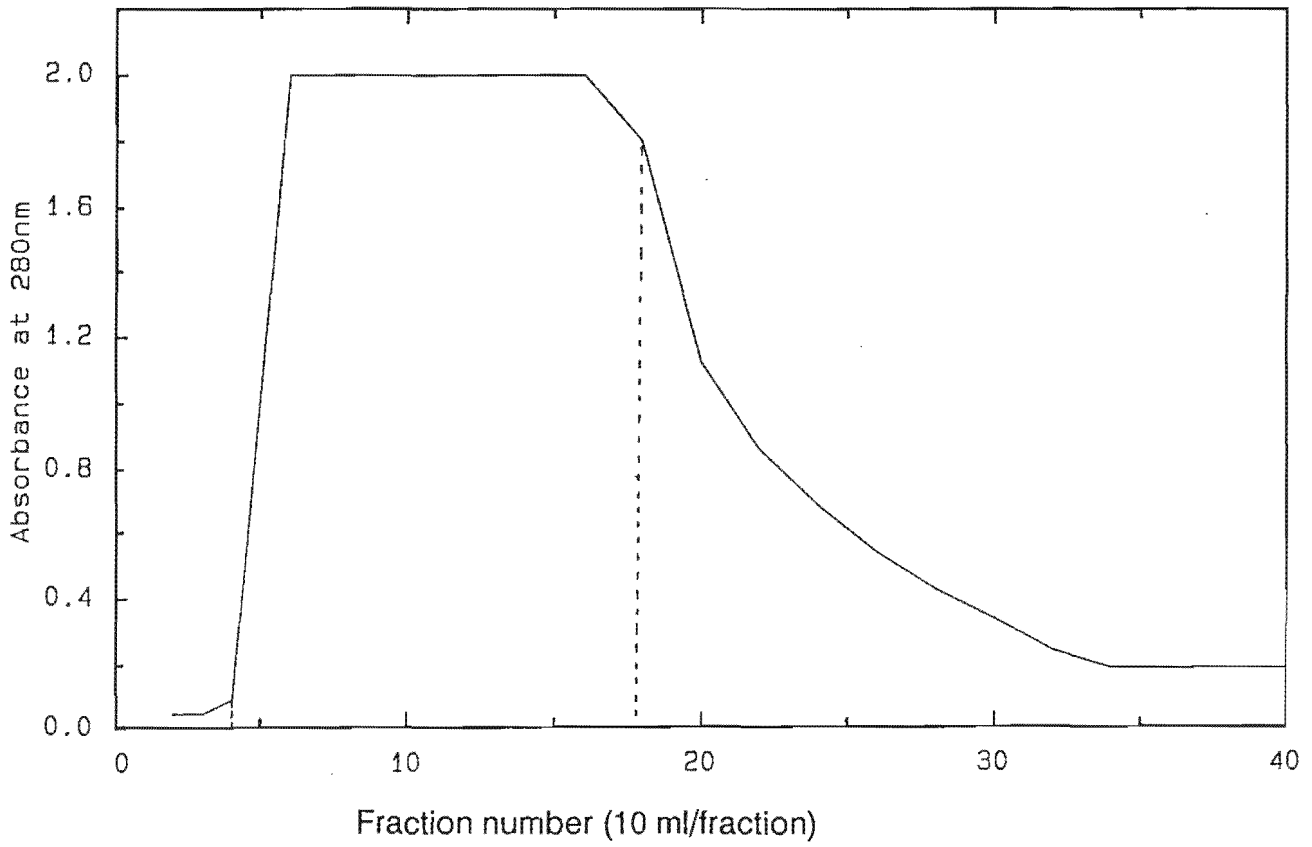


Figure 3.13 Fractionation of Ultra SP

Column : 16 x 300 mm Sephadex G-15 with a 0,05 M NH_4HCO_3 buffer as eluant, flow rate : 4 ml/min., total volume : 1000ml. Dotted lines indicate the fraction cuts.

3.1.5.2 Ion Exchange Chromatography

DE anion exchange chromatography was applied to separate the enzyme preparations into the different protein fractions using their differences in charge rather than molecular weight to separate them. Typical separations of the enzyme preparations are shown in figures 3.14 to 3.16

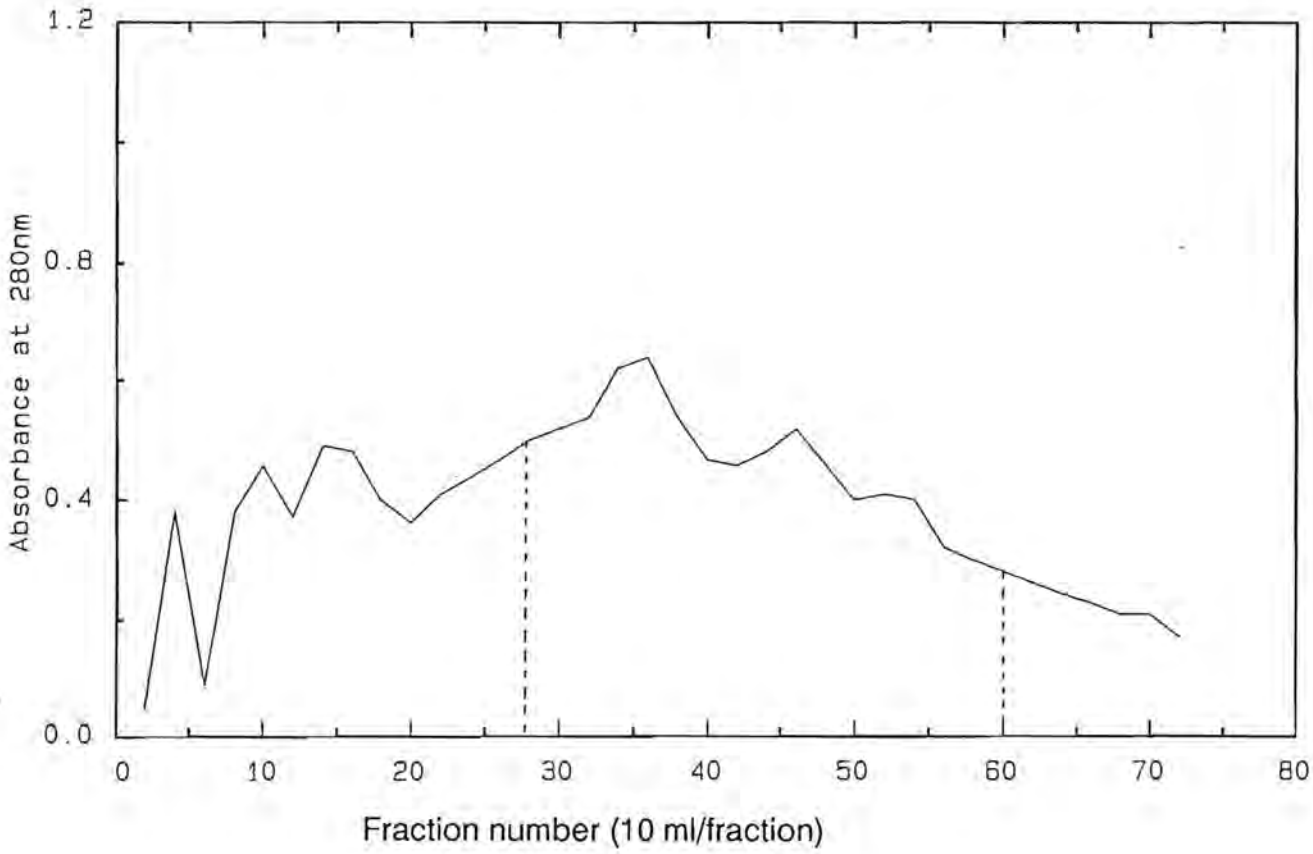


Figure 3.14 Fractionation of Rohapect C

Column : 16 x 300 mm DE-52 with a linear 0,05 to 0,5 M NH_4HCO_3 gradient as eluant, flow rate : 4 ml/min., total volume : 1000ml. Dotted lines indicate the fraction cuts.

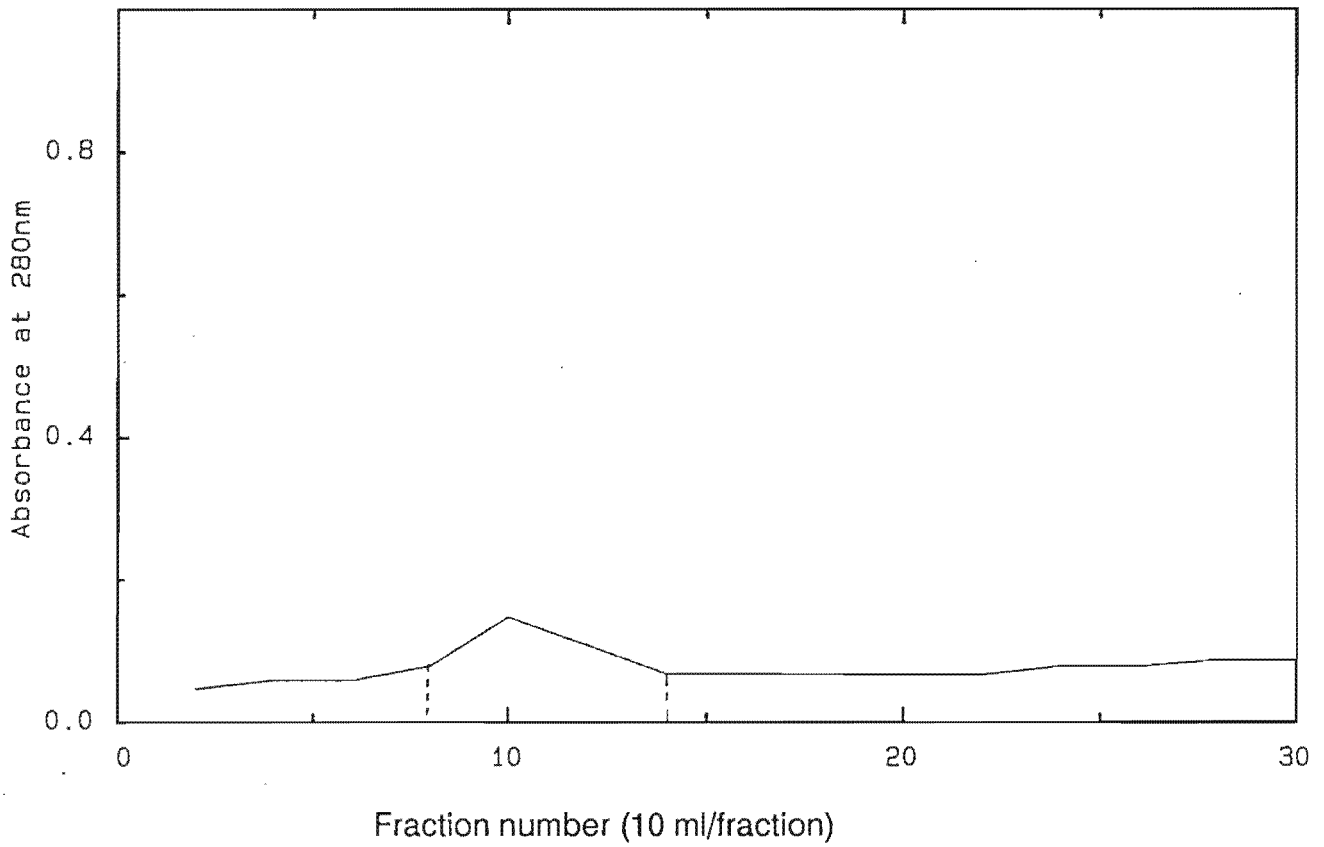


Figure 3.15 Fractionation of Pectinex XL

Column : 16 x 300 mm DE-52 with a linear 0,05 to 0,5 M NH_4HCO_3 gradient as eluant, flow rate : 4 ml/min., total volume : 1000ml. Dotted lines indicate the fraction cuts.

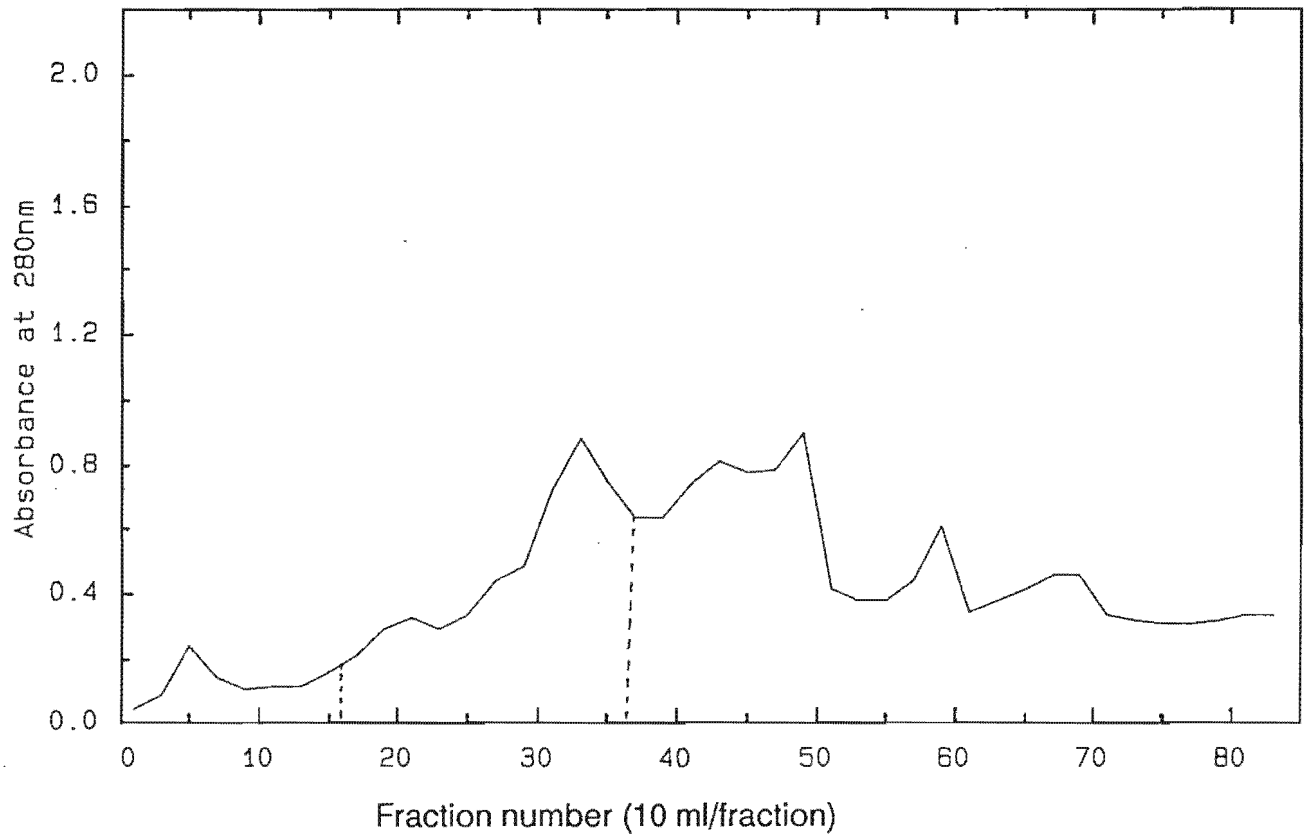


Figure 3.16 Fractionation of Ultra SP

Column : 16 x 300 mm DE-52 with a linear 0,05 to 0,5 M NH_4HCO_3 gradient as eluant, flow rate : 4 ml/min., total volume : 1000ml. Dotted lines indicate the fraction cuts.

By changing the gradient the active fractions were purified to a larger extent as shown in figures 3.17 to 3.19.

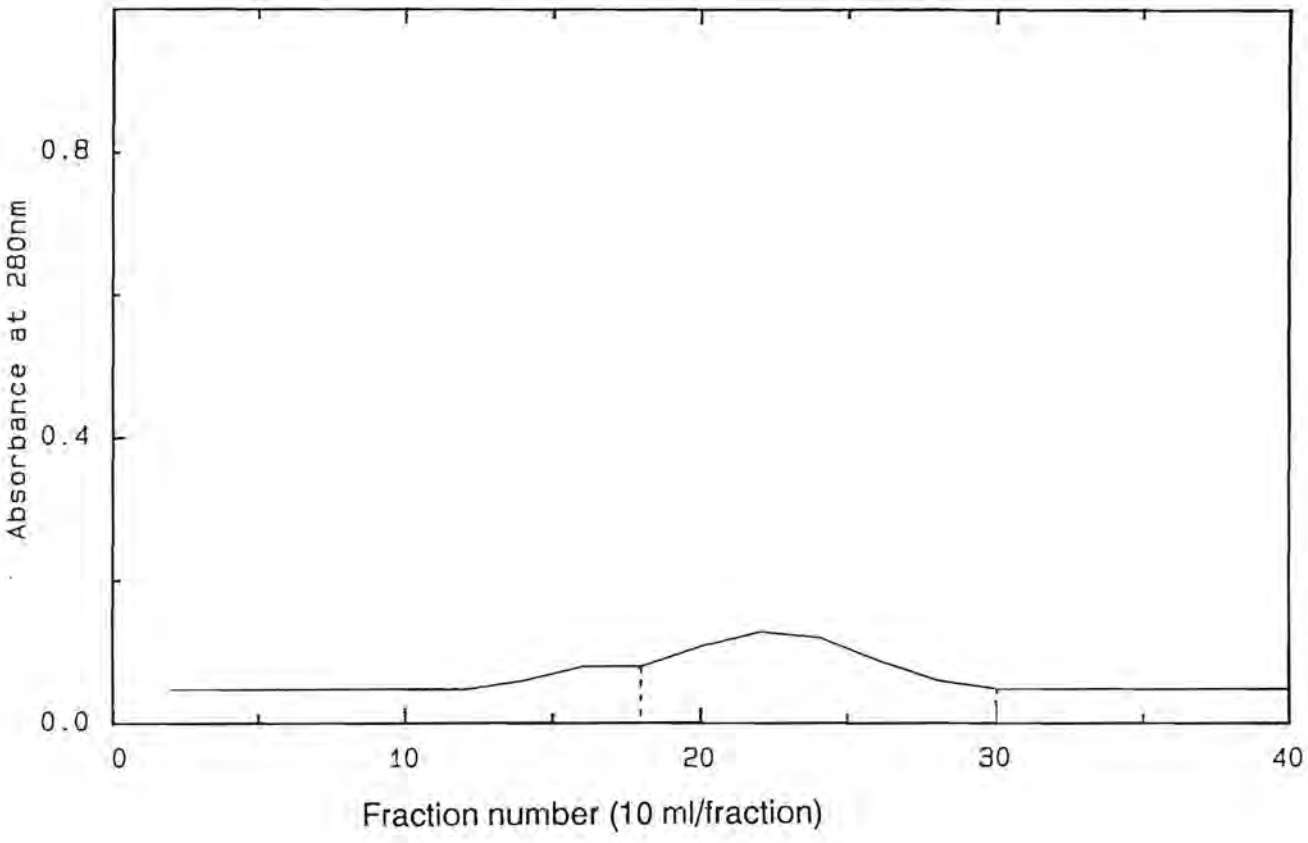


Figure 3.17 Fractionation of Rohapect C

Column : 16 x 300 mm DE-52 with a linear 0,05 to 0,25 M NH_4HCO_3 gradient as eluant, flow rate : 4 ml/min., total volume : 1000ml. Dotted lines indicate the fraction cuts.

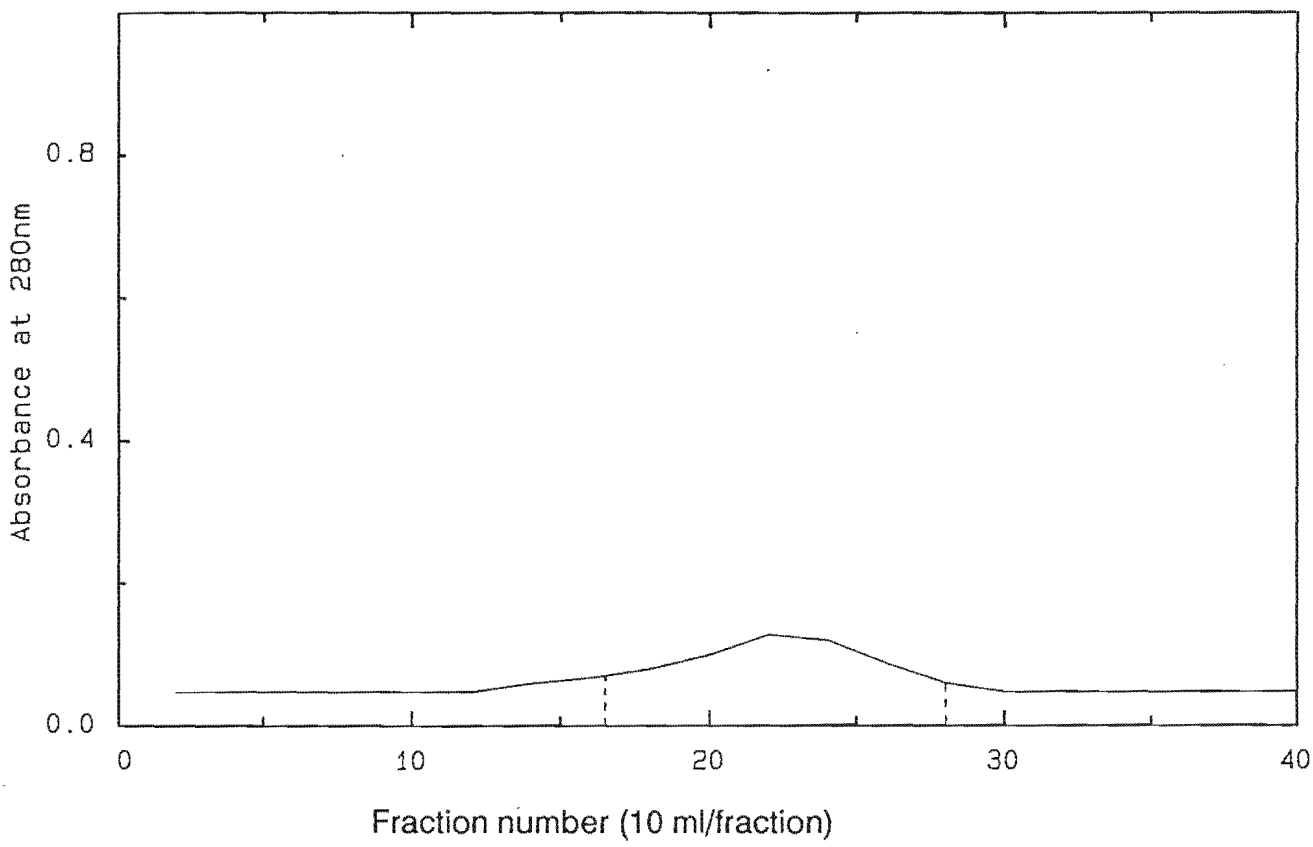


Figure 3.18 Fractionation of Pectinex XL

Column : 16 x 300 mm DE-52 with a linear 0,05 to 0,25 M NH_4HCO_3 gradient as eluant, flow rate : 4 ml/min., total volume : 1000ml. Dotted lines indicate the fraction cuts.

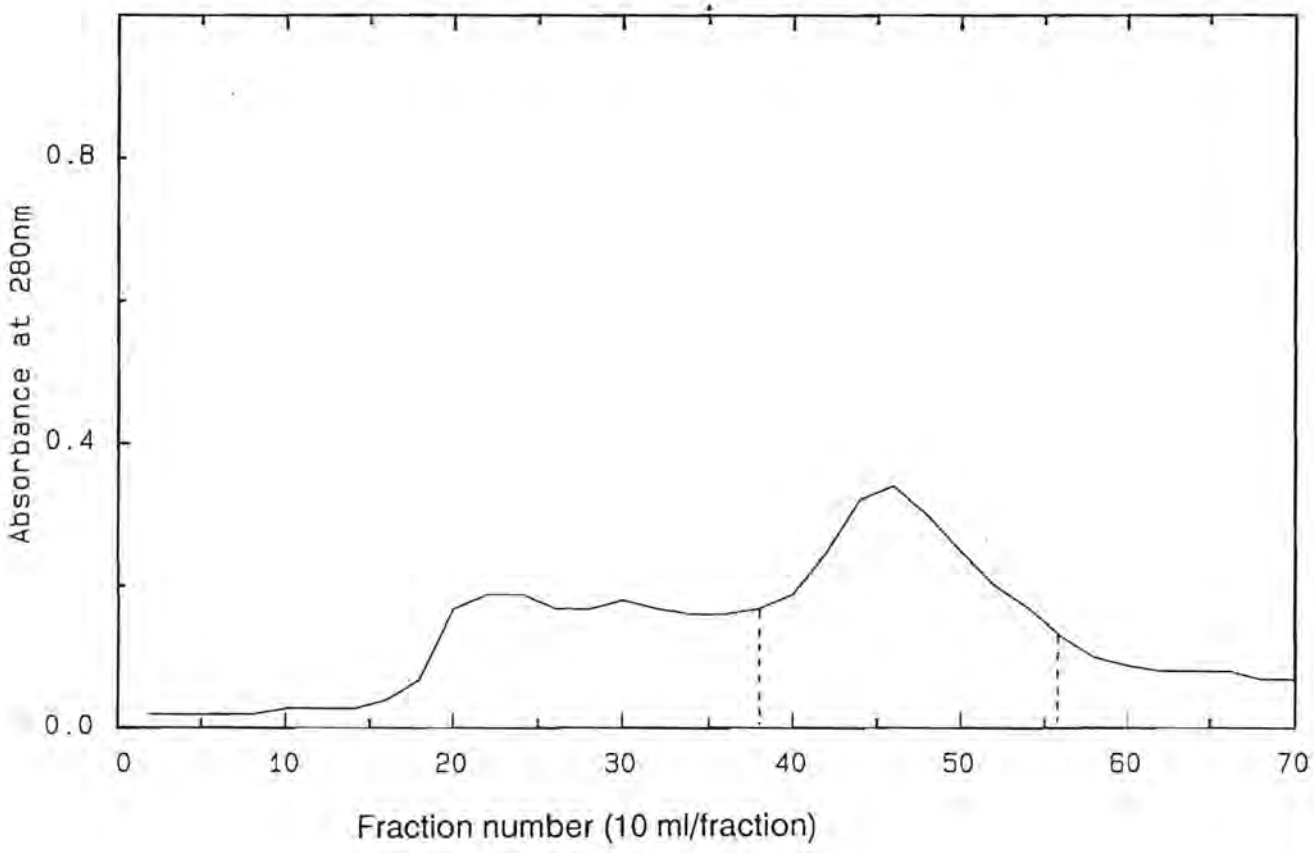


Figure 3.19 Fractionation of Ultra SP

Column : 16 x 300 mm DE-52 with a linear 0,05 to 0,25 M NH_4HCO_3 gradient as eluant, flow rate : 4 ml/min., total volume : 1000ml. Dotted lines indicate the fraction cuts.

These pure fractions were used for further studies on the hydrolysis of the glycosidically-bound terpenes in the grape musts.

3.2 DETERMINATION OF FREE AND POTENTIALLY FREE TERPENES

3.2.1 Enzyme quantitation

The glycosidic activity of the commercial enzyme preparations was unknown. The amount of the different active enzyme fractions to be used were determined by using a β-glucosidase standard curve. The mass of the different commercial enzyme preparations to be added were determined by using this standard curve (Table 3.1).

Table 3.1 Determination of the mass of Rohapect C, Ultra SP and Limonex necessary for maximal terpene hydrolysis using a β-glucosidase standard curve

β-glucosidase:

Mass (mg) Units (mU) OD (405 nm)	0,833 2,31 0,0760	2,033 5,71 0,3988	4,167 11,55 1,1787	8,33 23,14 2,68

Rohapect C:

Mass (µg) OD (405 nm)	1,2 0,0581	3,0 0,1332	6,0 0,2750	12,0 0,4925

Ultra SP:

Mass (µg) OD (405 nm)	130 0,1732	520 0,7570	750 1,1821	1000 1,5252

Limonex:

Volume (µl) OD (405nm)	1,0 0,0095	2,5 0,0153	5 0,0184	20 0,0503

The values obtained were the average of duplicate assays.

The best straight line for the β-glucosidase standard curve was determined using linear regression and was found to be

y =2,149 + 7,87x
with r = 0,999
where y = mU and x = OD

The β-glucosidase standard curve was used to determine the mass of Ultra SP, Limonex or Rohapect C (Table 3.1) that had to be added to 400 ml grape must in order to release the maximal amount of terpenes. The activity needed was 300 mU/ml.

Rohapect C:
6,0 µg = 0,2750 OD's
= 4,313 mU
300mU/ml = 300 mU/4,313 mU x 6,0 µg
= 0,417 mg/ml
166,8 mg Rohapect C was therefore needed in 400 ml must.

Ultra SP:
750 µg = 1,1821 OD's
= 11,5421 mU
300mU/ml = 300mU/11,5421 mU X 750 µg
= 19,49 mg
7,79 g Ultra SP was needed in 400 ml must.

Limonex:
5 µl = 0,0184 OD's
= 2,29 mU
300 mU = 130,79 µl
52,32 ml Limonex was needed in 400 ml must.
= 13% v/v

3.2.2 Determination of Free (FVT) and Potentially Volatile (PVT) Monoterpenes in Grapes

The method developed by Dimitriadis and Williams (2.5.2) was used for determining the FVT and PVT of the different treatments (2.5.2.2).

The results from the above treatments are displayed in table 3.2. and figures

3.20 and 3.21. The results are the average of quadruplicate assays. The replicates were increased to four due to the weak repeatability of the results. This weak repeatability can be the result of non-random sampling and because berries with differing degrees of ripeness can be found on the same bunch, especially in the case of Muscat d'Alexandrie. It is known that the concentration of terpenes in grapes is a function of ripeness (Marais, 1985).

3.2.3 Capillary Gas Chromatography for the determination of specific terpenes in the FVT distillates

The gas chromatographic technique as adapted by Marais (1986) for local conditions was used for the determination of individual terpenes in the FVT distillates.

A typical chromatogram indicating the relevant terpene components is displayed in figure 3.22. As many as possible terpenes were analysed initially. Eventually only those terpenes that are known to contribute to grape aroma were used in reporting on the results. The results are displayed in table 3.3 and figures 3.23 and 3.24 and are the average of quadruplicate assays.

Table 3.2 The effect of the enzyme treatments on the FVT and PVT concentrations in grapes.

Treatments	10°C				20°C			
	FVT		PVT		FVT		PVT	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
A. **Muscat d'Alexandrie**								
1. No skin contact	1,48	0,14	4,28	0,86				
2. Skin contact (20 hrs)	2,02	0,14	5,283	0,50	1,70	0,14	4,79	0,21
3. β-Glucosidase + skin contact (20 hrs)	1,86	0,21	5,23	0,43	1,79	0,07	4,82	0,43
4. Rohapect C + skin contact (20 hrs)					2,32	0,21	5,61	0,50
5. Ultra SP (1 g) + skin contact (20 hrs)					1,71	0,14	4,55	0,43
6. β-Glucosidase + skin contact (pH 5)					2,26	0,14	4,81	0,50
B. **Weisser Riesling**								
1. No skin contact	0,37	0,07	1,24	0,07				
2. Skin contact (20 hrs)	0,51	0,14	1,07	0,14	0,23	0,07	0,93	0,14
3. β-Glucosidase + skin contact (20 hrs)	0,46	0,21	1,04	0,14	0,37	0,07	1,13	0,07
4. Rohapect C + skin contact (20 hrs)					0,631	0,07	1,37	0,07

FVT : Total Free volatile terpenes expressed as mg/l Linalool

PVT : Total Potentially volatile terpenes expressed as mg/l Linalool

$\bar{x}$: The average of four treatments

s : Standard deviation

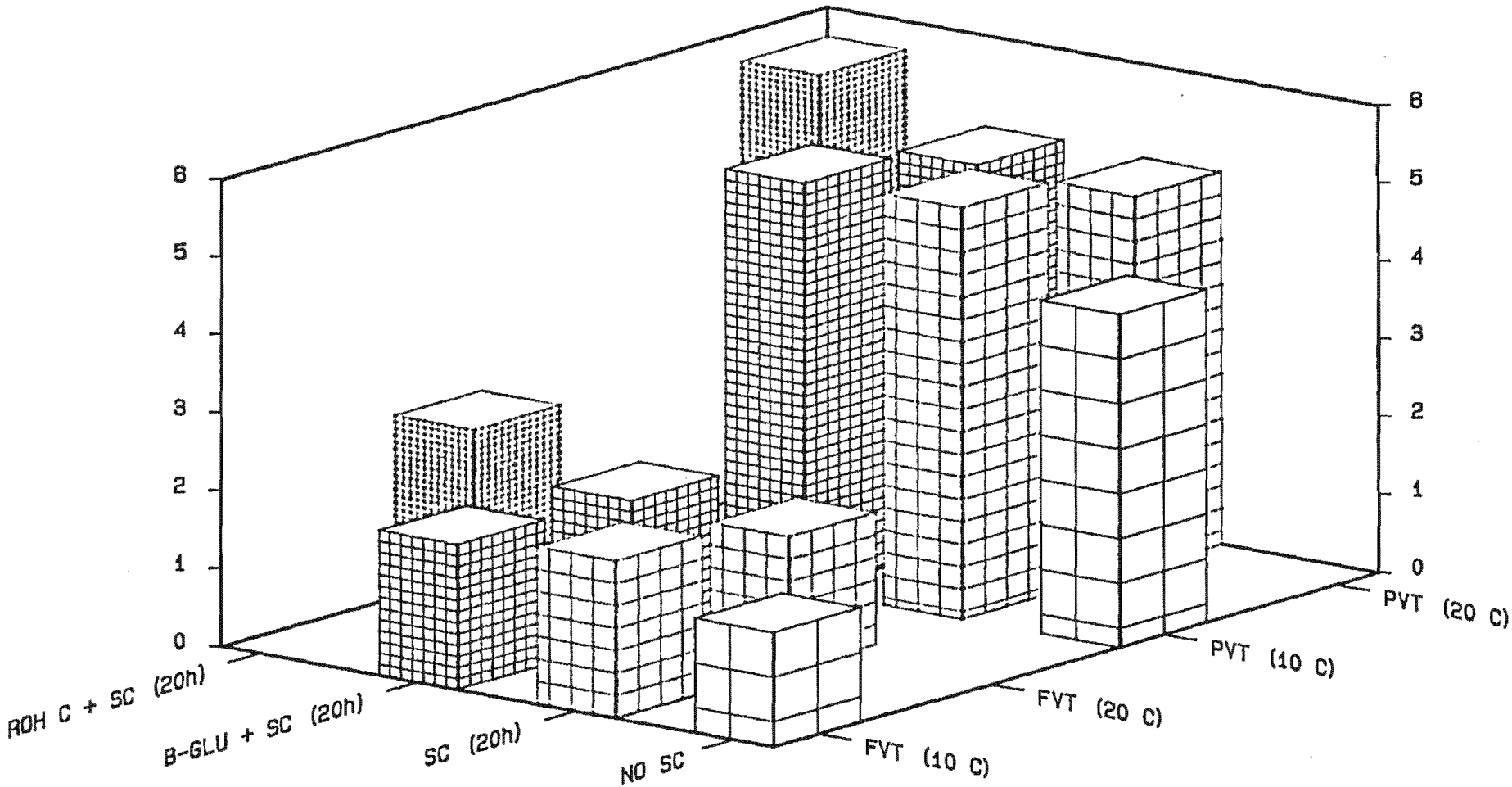


Figure 3.20 The effect of enzyme treatments on the FVT and PVT concentrations in Muscat d'Alexandrie.
SC - Skin Contact
No SC - No Skin Contact

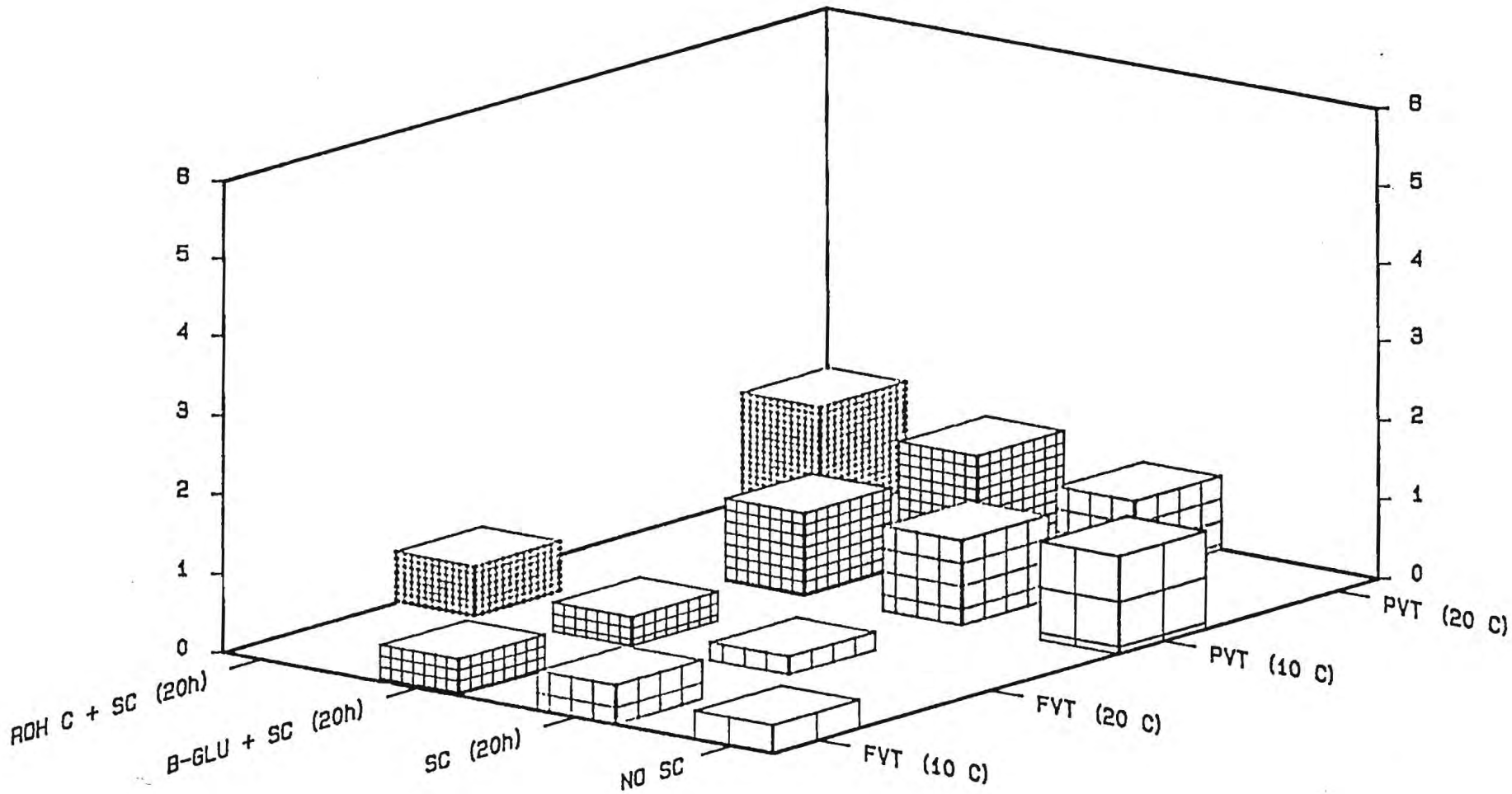


Figure 3.21 The effect of enzyme treatments on the FVT and PVT concentrations in Weisser Riesling
SC - Skin Contact
No SC - No Skin Contact

Table 3.3 The effect of enzyme treatments on the release of different terpene components

Treatments	Terpene Components											
	Linalool		Hotrienol		∝ -Terpineol		Citronellol		Nerol		Geraniol	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Muscat d'Alexandrie												
No skin, no enzyme	132,42	6,70	4,77	1,68	4,89	1,07	3,52	1,03	60,90	14,34	488,78	135,51
Skin 10^{0}C, no enzyme	181,26	11,33	7,64	0,95	9,17	2,13	7,60	2,83	109,51	25,11	728,54	200,05
Skin 20^{0}C, no enzyme	92,64	17,24	5,48	2,30	8,11	3,49	6,74	2,14	91,37	15,96	529,52	133,05
Skin 10^{0}C, β-glucosidase	213,06	26,76	3,11	0,83	7,86	2,14	5,53	2,62	85,68	35,30	689,06	179,45
Skin 20^{0}C, β-glucosidase	127,12	21,40	5,61	1,80	7,21	1,60	5,84	1,75	85,76	21,18	530,26	162,02
Skin 20^{0}C, Rohapect C	200,53	37,76	3,94	0,54	4,46	3,13	7,33	3,40	122,89	45,22	877,99	353,32
Weisser Riesling												
No skin, no enzyme	7,42	1,01	0,79	0,18	1,36	0,11	-	-	2,78	0,78	18,54	2,24
Skin 10^{0}C, no enzyme	10,21	4,39	0,55	0,58	0,83	0,23	0,60	0,17	5,55	0,93	25,11	1,08
Skin 20^{0}C, no enzyme	7,79	0,85	0,90	0,31	1,59	0,30	0,49	0,32	3,29	1,33	16,69	4,95
Skin 10^{0}C, β-glucosidase	6,30	0,28	0,90	0,46	1,19	0,15	0,52	0,14	4,91	1,10	25,15	4,36
Skin 20^{0}C, β-glucosidase	5,88	0,88	9,13	2,61	0,61	0,22	0,80	0,02	2,72	1,97	15,75	7,45
Skin 20^{0}C, Rohapect C	9,33	0,32	1,12	0,21	1,22	0,35	1,98	0,07	4,86	1,35	23,29	6,16

$\bar{x}$: The average of four treatments
s : Standard deviation

Terpene concentrations are in terms of relative concentration units.

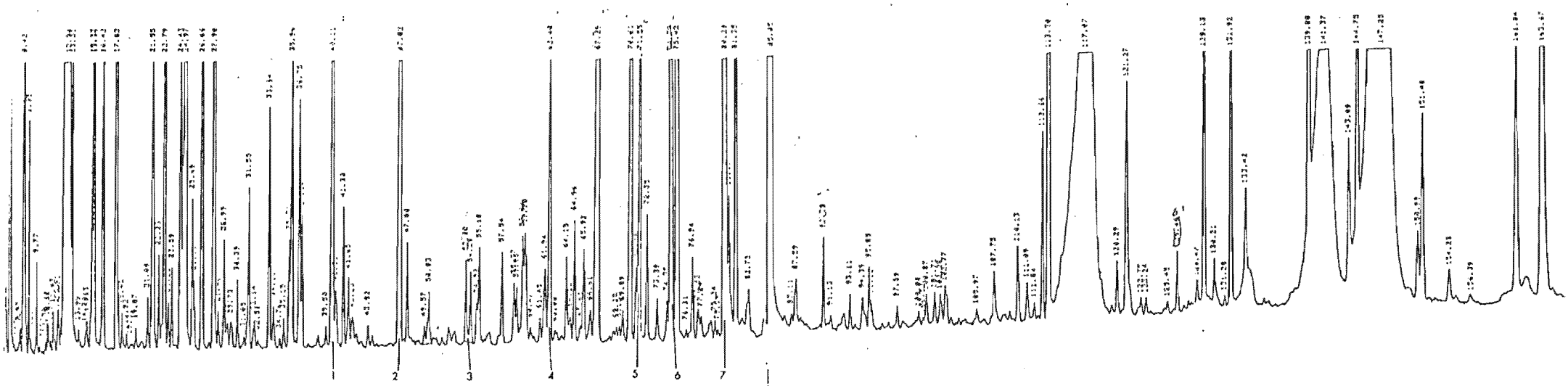


Figure 3.22 Chromatogram of Freon 11 extract of the FVT fraction of Muscat d'Alexandrie
1 = 2-Ethyl hexanol; 2 = Linalool; 3 = Hotrienol; 4 = ⊀-Terpineol; 5 = Citronellol; 6 = Nerol; 7 = Geraniol.

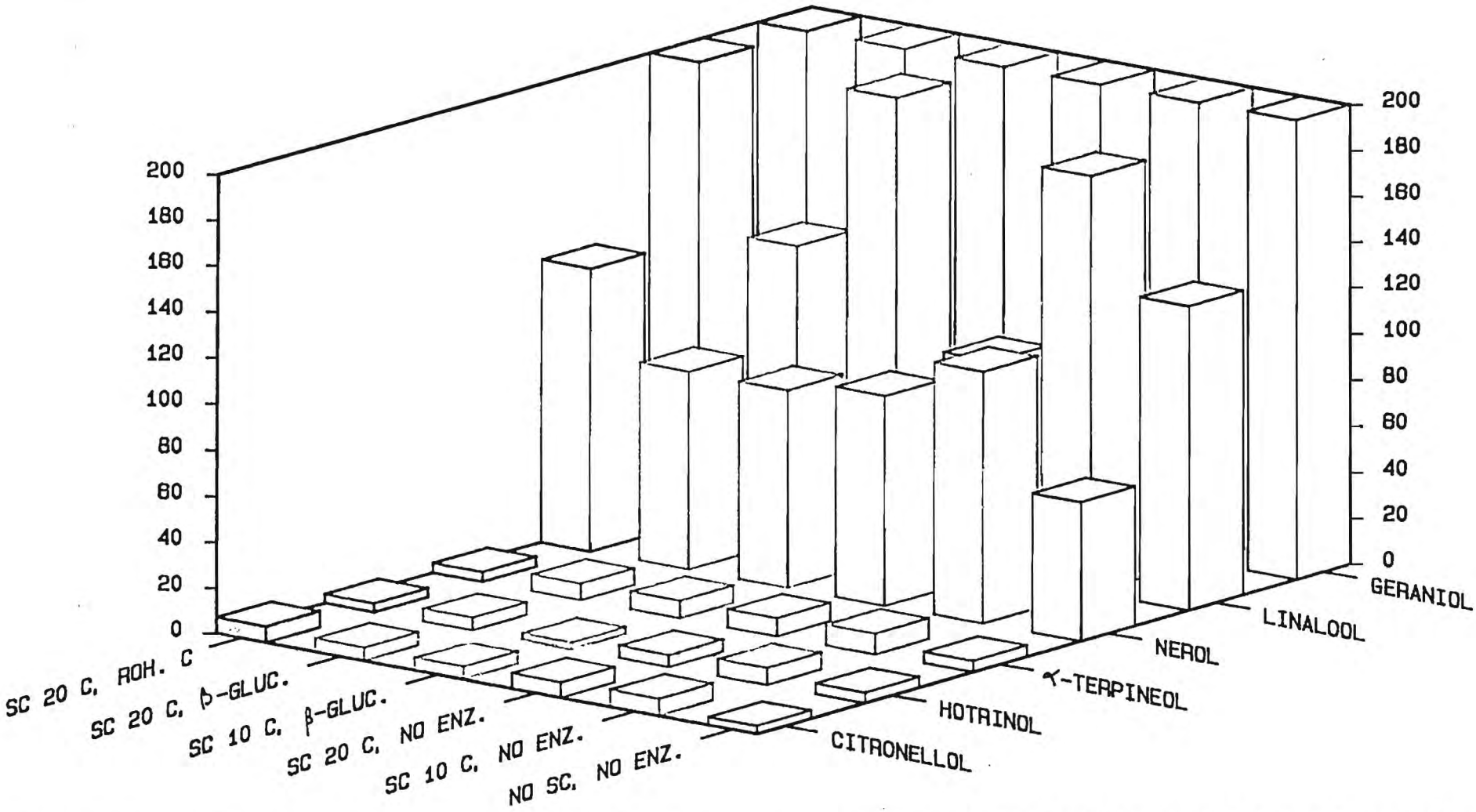


Figure 3.23 The effect of enzyme treatments on the release of different terpenes in the FVT fraction of Muscat d'Alexandrie
SC - Skin Contact
No SC - No Skin Contact
Terpene concentrations are in terms of relative concentration units.

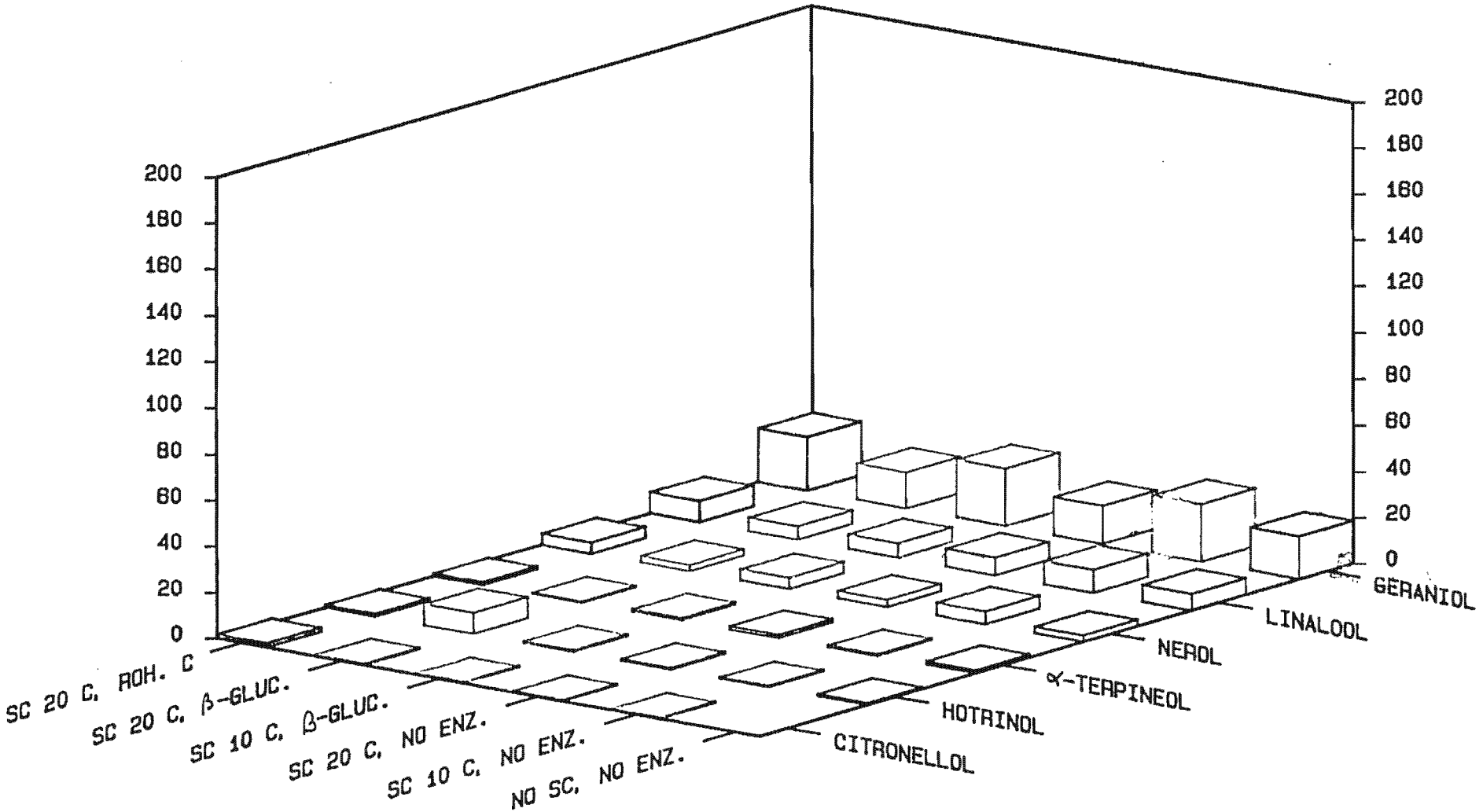


Figure 3.24 The effect of enzyme treatments on the release of different terpenes in the FVT fraction of Weisser Riesling
SC - Skin Contact
No SC - No Skin Contact
Terpene concentrations are in terms of relative concentration units.

3.3 ENZYME INHIBITION

3.3.1 Product inhibition

The obeyence of Beer's law for the effect of different grape juice concentrations on absorbance is demonstated in figure 3.25.

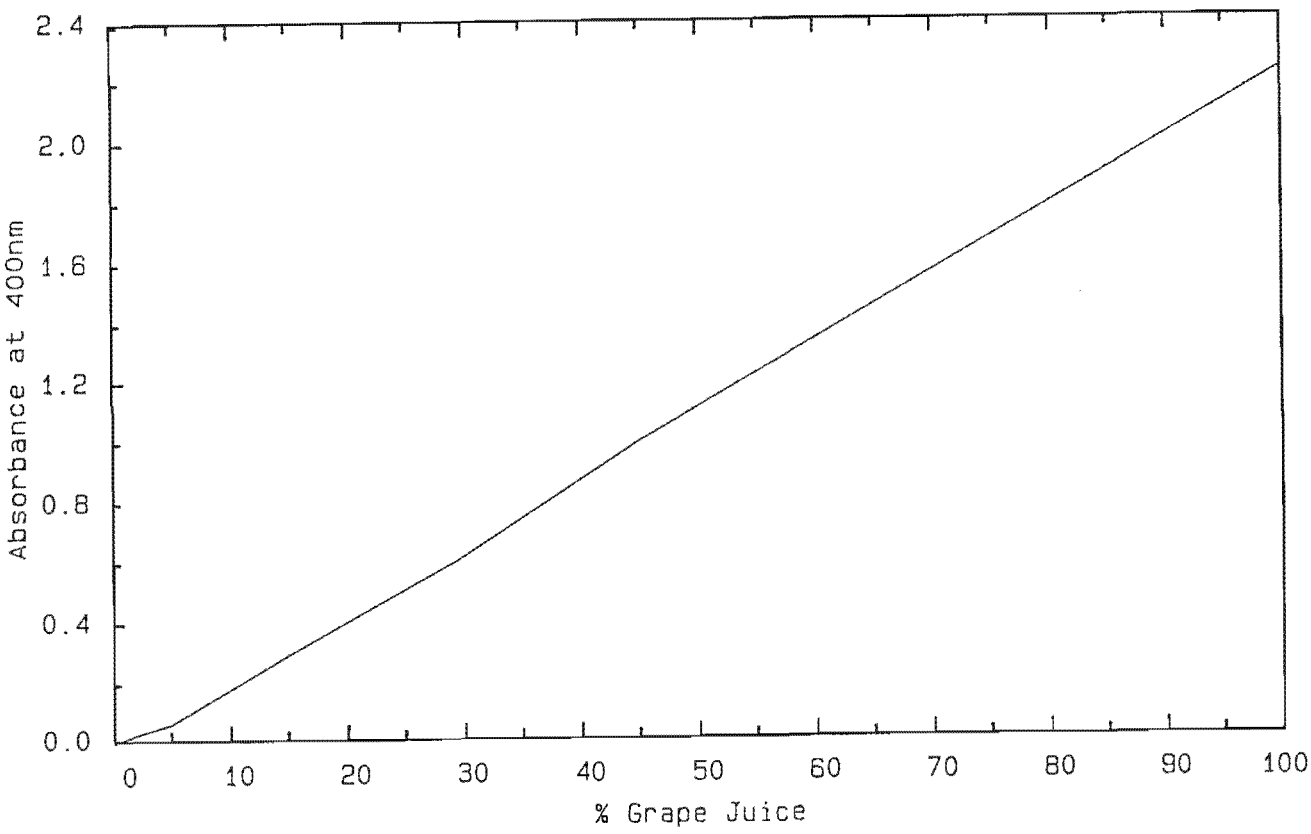


Figure 3.25 The obeyence of Beer's law for the effect of grape juice concentration on absorbance

The effect of different grape juice concentrations on the activity of Rohapect C and Ultra SP is demonstated in figure 3.26

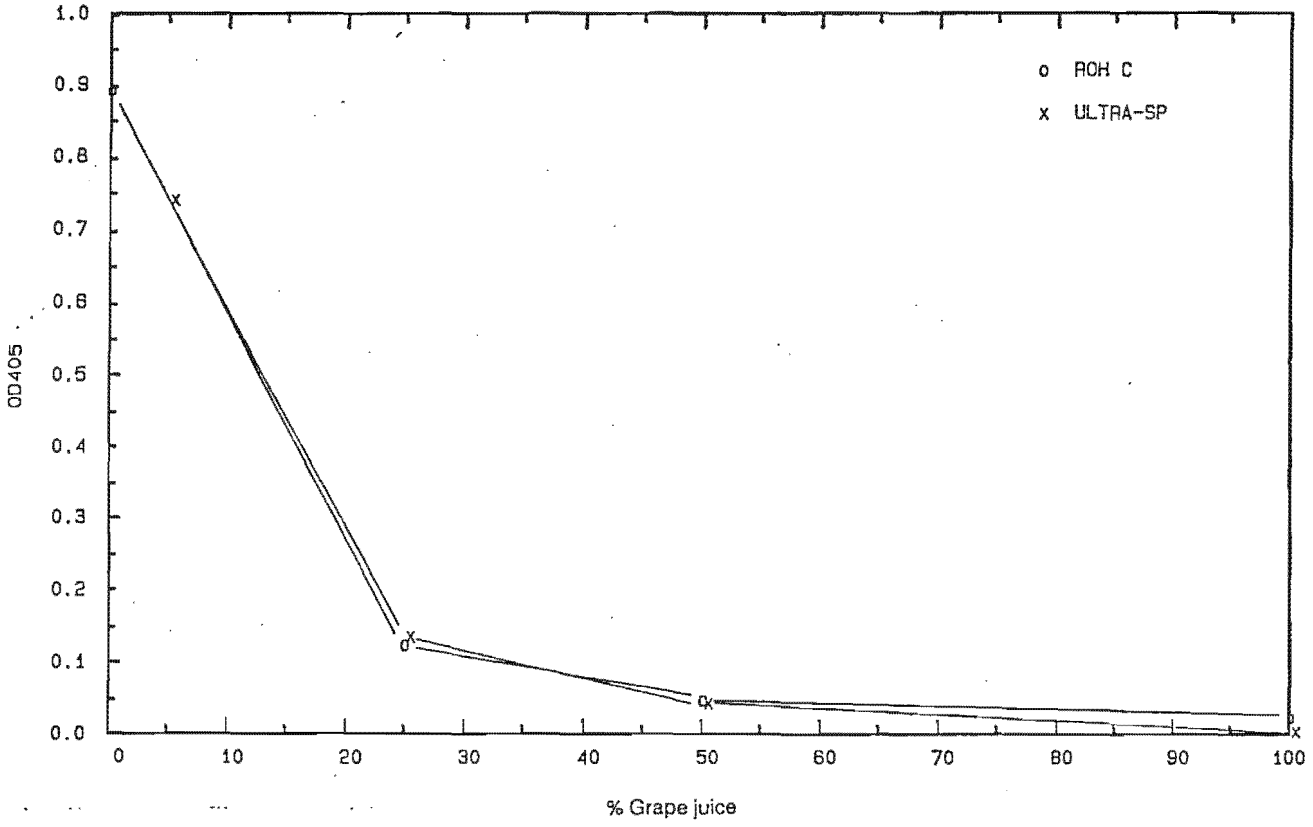


Figure 3.26 The effect of different concentrations of grape juice on enzyme activity

- Rohapect C
- Ultra SP

3.3.2 Metal ion effect

The effect of chelating metal ions in grape juice on the activity of Rohapect C is shown in figure 3.27.

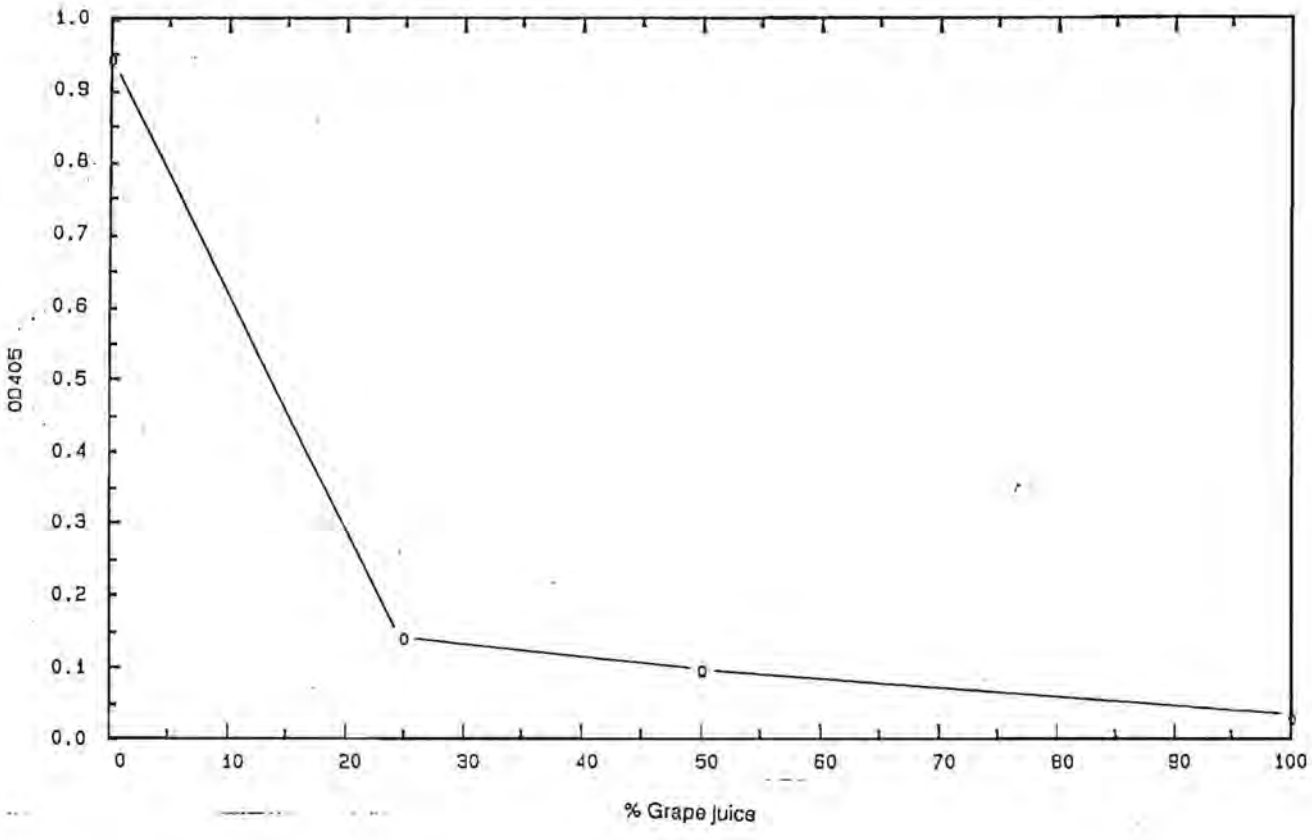


Figure 3.27 Metal ion effect on Rohapect C

3.3.3 Enzyme inhibition by sugar

The effect of different concentrations of glucose and fructose on Rohapect C is demonstrated in figure 3.28.

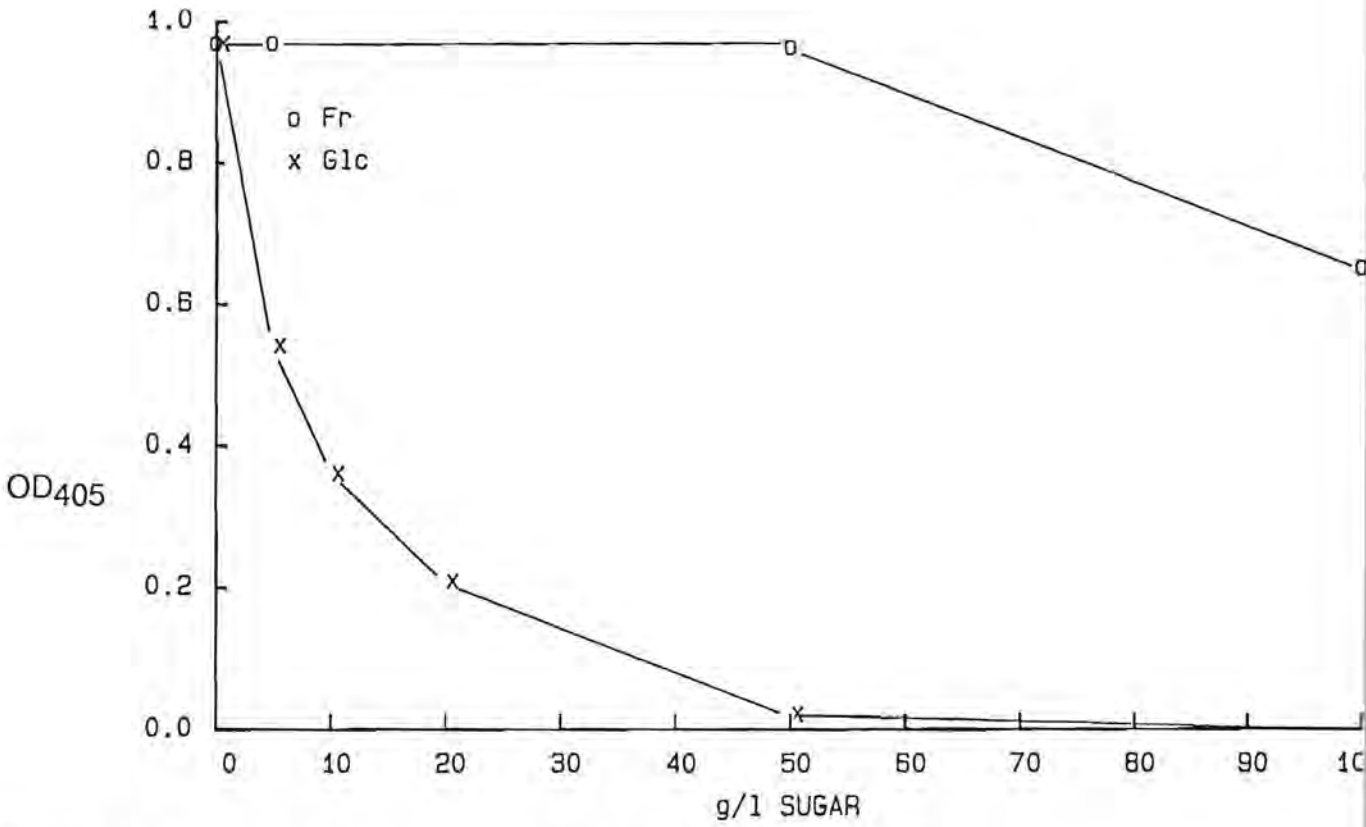


Figure 3.28 The effect of sugar concentration on enzyme activity

- glucose
- fructose

3.4 DETERMINATION OF FREE VOLATILE AND POTENTIALLY VOLATILE TERPENES IN MUST WHERE THE GLUCOSE HAD BEEN OXIDISED TO GLUCONIC ACID

3.4.1 Quantitation of glucose oxidase

The effect of different concentrations of a glucose oxidase/catalase preparation on the oxidation of glucose in Muscat d'Alexandrie must is demonstrated in figure 3.29.

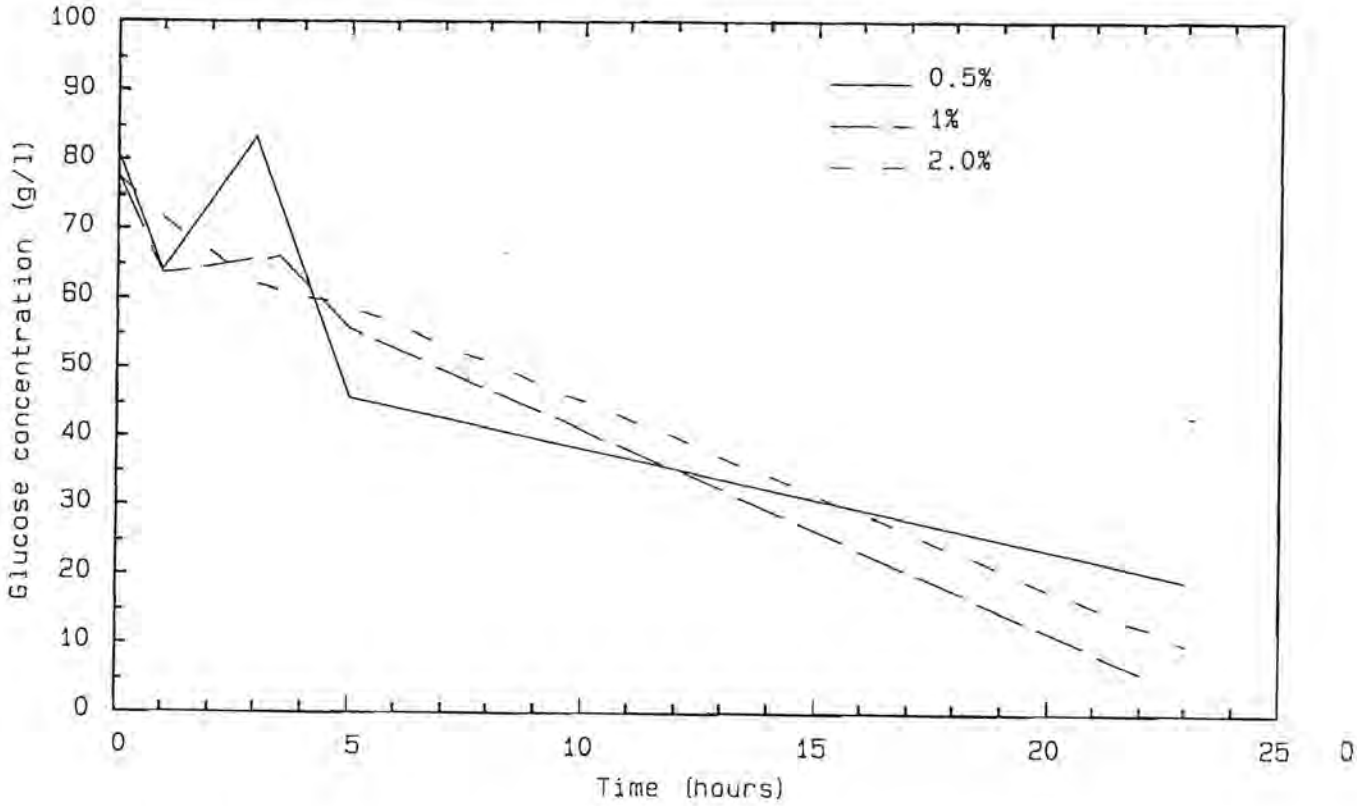


Figure 3.29 The effect of different concentrations glucose oxidase/catalase on oxidation of glucose

- 0,5 % glucose oxidase
- 1,0 % glucose oxidase
- 2,0 % glucose oxidase

A glucose oxidase/catalase preparation was prepared by using the enzymes mentioned in 2.8.1. The assumption was made that at winemaking conditions of pH 3,5 and 15°C, the activity of the enzymes would be half optimal. The glucose oxidase contained 180 U/mg enzyme with an activity of oxidising 1 umol glucose/min at pH 7 and 25°C. The activity of the catalase is 8500 U/mg. A preparation was prepared containing 10 000 U of both enzymes in 10 ml water giving an activity of 1 U/µl water.

3.4.2 Determination of the Free and Potentially Free Terpenes

The method developed by Dimitriadis and Williams (2.5.2) was used for determining the FVT and PVT of different treatments (2.8.2).

The results of the above treatments are displayed in table 3.4. The results are the average for duplicate assays with the enzyme treatment and triplicate assays for the controls.

Table 3.4 The effect of glucose oxidase and Rohapect C treatments on FVT and PVT concentrations in must.

Treatments	FVT	PVT
A. **Muscat d'Alexandrie**		
1. Control	1,98	4,83
2. Glc oxidase/cat + Rohapect C	1,25	2,72
B. **Weisser Riesling**		
1. Control	0,57	1,08
2. Glc oxidase/cat + Rohapect C	0,74	0,62

FVT : Total Free Volatile terpenes expressed as mg/l linalool
PVT : Total Potentially Volatile terpenes expressed as mg/l linalool

3.4.3 Capillary Gas Chromatography for the determination of specific terpenes in the FVT distillates

The gas chromatographic technique as adapted by Marais (1986) was used for the determination of the different terpenes in the FVT distillates of different treatments.

The results are displayed in table 3.5 and are the average of triplicate assays for the controls and duplicate assays for enzyme treatments.

Table 3.5 The effect of glucose oxidase and Rohapect C treatments on the release of different terpene components

Treatments	Terpene Components					
	Linalool	Hotrienol	⍺-Terpineol	Citronellol	Nerol	Geranic acid
Muscat d'Alexandrie						
1. Control	228,79	8,30	31,64	40,37	156,66	9,40
2. Glc oxidase/catalase + Rohapect C	222,78	155,43	78,47	8,06	88,53	342,45
Weisser Riesling						
1. Control	4,35	0,49	1,80	3,51	2,31	0,95
2. Glc oxidase/catalase + Rohapect C	14,03	3,36	6,22	0,75	1,21	0,41

Terpene concentrations are in terms of relative concentration units.

PART 4

DISCUSSION

4.1 ENZYME STUDIES

From the results of the temperature stability studies (Fig. 3.4) it was clear that all the enzymes remained stable up to 35°C. Rohapect C remained stable up to 45°C. At 60°C there was hardly any remaining activity in any of the enzyme preparations. At wine making temperatures of 10 to 20°C all the enzymes displayed good stability.

From the temperature activity studies (Fig. 3.5) it was clear that the enzymes were optimally active at about 40°C after which they started denaturing. At wine making temperatures the enzymes displayed some activity though considerably reduced.

All the enzymes were relatively stable in the pH range of 3,0 to 7,5 (Fig. 3.2) and were stable at must pH of about 3,5. The pH activity (Fig. 3.3) of grape enzymes, Ultra SP and SP 249 increased up to a pH of 4,5 and 5,0 respectively, after which denaturation became apparent. The activity of Rohapect C and Pectinex XL was relatively stable over the pH range.

From these studies it was clear that the grape hydrolytic enzymes isolated from Muscat d'Alexandrie grapes displayed weak activity and relative stability at the temperature and pH conditions prevalent during wine making. This supports the findings by Bayonove et at. (1984) and Aryan et al. (1987) that glycosidases from Muscat d'Alexandrie are maximally active at pH 5,0 and 45°C. It was thus decided not to include this enzyme in further studies.

High pressure liquid chromatography was performed on the different enzyme preparations to screen the purity of the preparations. Using DEAE anion exchange it was clear that the only enzyme preparation used that was pure was the almond -glucosidase. This was confirmed by electrophoresis. Grape hydrolytic enzymes displayed four peaks with one active peak. Rohapect C had five peaks with activity in the third. Pectinex XL had a major active peak, while Ultra SP and SP 249 had 16 peaks with activity in peak 9 and 10. It was clear from the chromatograms that Ultra SP and SP 249 was

well as in figures 3.23 and 3.24. From these results it is clear that the measured terpene concentrations for both cultivars were higher during skin contact at 10 than at 20°C.

The addition of β-glucosidase increased only the linalool concentration in Muscat d'Alexandrie at both temperatures. There was a decrease in terpene concentration of all other measured terpenes in comparison with only skin contact treatment. Rohapect C, however, increased the linalool concentration as well as the concentrations of the terpenes that occur mostly in grape skins, namely citronellol, nerol and geraniol.

In the case of Weisser Riesling, however, treatment with β-glucosidase resulted in a decrease in α-terpineol and linalool concentrations with a small increase in hotrienol concentration. The other terpene concentrations remained more or less constant. Treatment with Rohapect C resulted in an increase in all terpene concentrations with the exception of α-terpineol.

4.3 ENZYME INHIBITION

From the results obtained up to this point it was clear that the addition of enzymes, even the most active enzyme, was not very successful in the release of bound terpenes. This led to the suspicion that enzyme inhibition might be a problem.

It was found that grape juice concentration obeys Beer's law in that concentration is directly related to absorbance with an increase in absorbance with increasing concentration.

It was found that the addition of grape juice led to a decrease in enzyme activity. The addition of 25% grape juice led to a decrease in Rohapect C activity to only 14% of the original (Figure 3.26) and at 100% grape juice the enzyme displayed virtually no activity. Ultra SP had only 18% of original activity left at 25% grape juice and virtually none at 100%.

Metal ions are very often needed for enzyme activity or might be responsible for the inactivation of enzymes, especially Cu^{+} or Cu^{2+}-ions. It was, therefore, tested if the presence or absence of metal ions might not be responsible for the lesser activity of the enzymes at higher grape juice concentrations. Citrate buffer of the same molarity and pH as the normal

the same enzyme preparation and this was at a later date confirmed by the suppliers. Using a NH_4HCO_3 gradient was advantageous as no dialysis was required. From these findings it was decided to fractionate the enzyme preparations on a DE-52 anion exchange column in order to obtain the most active enzyme fractions for further work.

4.2 DETERMINATION OF FREE AND POTENTIALLY FREE VOLATILE TERPENES

By using a β-glucosidase standard curve it was determined that 166,8 mg Rohapect C, 7,79 g Ultra SP or 52,32 ml Limonex was needed in 400 ml must for the required 300 mU/ml enzyme activity.

With both Ultra SP and Limonex this exceeded catalytic quantities, especially if it is taken into consideration that in the case of Ultra SP this is the most active fraction selected from anion exchange. It was, therefore, decided not to include Limonex or Ultra SP in further work.

From table 3.2 and figures 3.20 and 3.21 it was clear that skin contact increased the free volatile terpenes (FVT) with about 38% in both Muscat d'Alexandrie and Weisser Riesling. It is interesting to note that in both cultivars more terpenes are released at 10 than at 20°C.

From these results it is also clear that β-glucosidase was not successful as an enzymatic treatment as these conditions are extremely unfavourable for effective enzyme activity.

Work on Rohapect C had only been done at 20°C. At this temperature very promising results were obtained with an increase of 36% in FVT in the case of Muscat d'Alexandrie and 170% in Weisser Riesling.

Figure 3.22 is an example of a chromatogram obtained from a Freon 11 extract of the FVT fraction of Muscat d'Alexandrie. The internal standard, 2-ethyl hexanol, as well as only the following terpenes are indicated: linalool, hotrienol, α-terpineol, citronellol, nerol and geraniol. These terpenes were selected from the chromatogram as they are the organoleptically most important terpenes.

The results obtained by using GC-techniques are displayed in table 3.3, as

acetate buffer was used to test for this effect, as citrate acts as a biological chelating agent. The enzyme activity, however, displayed the same tendencies as with only the addition of grape juice as can be seen in figure 3.27.

Grossmann et al. (1987) and Aryan et al. (1987) found that glycosidases are strongly inhibited by glucose and to a lesser extent by fructose. Experimental work was done to determine if this effect resulted in the same tendencies as previously reported. Glucose and fructose occurs in a ratio of 1:1 in must, with more or less 100 g/l of each sugar at 20^{0}B. Glucose and fructose were added to substrate and it was found that at a glucose concentration of 10 g/l, Rohapect displayed only 36% of its initial activity and at 50 g/l, glucose virtually no activity remained (Figure 3.28). In the case of addition of fructose, Rohapect C activity only started decreasing at 50 g/l and at 100 g/l fructose 65% of original activity remained. It is, therefore, clear that glucose was responsible for the inhibitory effect that occured.

4.4 DETERMINATION OF FREE VOLATILE AND POTENTIALLY VOLATILE TERPENES WHERE GLUCOSE HAD BEEN OXIDISED TO GLUCONIC ACID

With the results obtained thus far, it was decided to lower the glucose concentration in must in order to attempt to increase enzyme activity. The novel idea of Villettaz (1987) was employed by oxidising the glucose to gluconic acid by means of glucose oxidase. The hydrogen peroxide that is a by-product of the reaction is reduced with catalase. It was initially found that an enzyme concentration of 1% was sufficient to oxidise glucose to less than 10 g/l in 20 hours (Figure 3.28). With the actual experimental work it was found that 8 ml enzyme in 500 g macerated grapes with a reinoculation of a further 8 ml enzyme after 7 hours, gave the best results and was able to oxidise glucose to less than 10 g/l in 24 hours. Throughout it was found that the rate of bubbling oxygen through was critical. A rate of 700 ml/min gave good results.

From the results of the FVT and PVT determinations (Table 3.4) it was clear that in the case of Muscat d'Alexandrie both FVT and PVT concentrations were lower after enzyme treatment than the control. In the case of Weisser Riesling, however, enzyme treatment resulted in more effective release of

terpenes with the FVT concentration higher and the PVT concentration lower. These results cannot be compared directly with our previous findings as it might be due to differences in the grapes.

The results from the gas chromatography indicated that enzyme treatment was effective in increasing linalool, ∝-terpineol and hotrienol concentrations and decreasing citronellol, nerol and geranic acid in the case of Weisser Riesling. In the case of Muscat d'Alexandrie linalool concentration remained relatively stable, while hotrienol and ∝-terpineol concentrations increased. As was the case in Weisser Riesling, citronellol and nerol concentrations decreased. Geranic acid displayed a very marked increase. These increases and decreases might be the result of oxidation during treatment as well as conversion of terpenes mutually.

4.5 CONCLUSION

From this study on the effectiveness of enzymatic release of glycosidically-bound terpenes, it was clear that Rohapect C was the most effective in releasing terpenes. Due, however, to glucose inhibition, this effectiveness was not very high. After oxidation of glucose to gluconic acid before further treatment with Rohapect C, there was still no really marked increase in free terpene concentrations, though some of the individual terpenes displayed considerable increases. Wine will, however, have to be made in order to determine if wine aroma can be increased in this way, especially with the view of making more flavourful low alcohol wines.

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