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BARD

FINAL REPORT

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**The Effect of Viticultural Practices,
Environmental Factors and Enological
Treatments on Specific Grape and Wine
Volatiles and on Wine Flavor**

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C. ABSTRACT

Free and bound monoterpenes steadily accumulated during development of Muscat and Symphony grapes. Fluctuations in concentration of terpenes during development of grapes correlated with climatic changes, however, free monoterpenes were less responsive to climatic changes than bound. Approximately 90 % of the terpenes were bound with 10% free; both forms were equally distributed between the skin and mesocarp. The level of free terpenes in must increased (and bound terpenes decreased) during fermentation and wine aging. At the levels found in wines, terpene glycosides did not contribute to bitterness. The monoterpene glycosides of eight *Vitis vinifera* cultivars were shown to consist of β -disaccharides of 6-O- α -rhamnopyranosyl- β -D-glycopyranoside. A positive correlation was found between terpene content and wine aroma of non-muscat wines. Pectinol addition to Sauvignon blanc must had no affect on monoterpene content. Lowering pH of must to 3.1 raised the free and reduced the bound monoterpene content, whereas flash heating reduced both. In vitro grown berries synthesized monoterpene glycosides; transport of monoterpene glycosides from leaves to berries also occurred. An endo- β -glucosidase was developed which can hydrolyze glycosides in wines and juices. Previously unreported, iso-geraniol was identified in Muscat Roy wines.

D. OBJECTIVES

Objectives proposed in original BARD proposal:

1. To evaluate the role and contribution of monoterpenes to the aroma and flavor of Muscat, Muscat-related and non-Muscat grape cultivars.
2. To determine the effect of specific treatments on specific volatiles and wine flavor
3. To develop maturation indices at each experimental site
4. To determine the influence of specific viticultural practices and climate on the concentration of free and bound monoterpenes in grape berries.
5. To determine the site(s) of biosynthesis (leaves vs berries) and rate of accumulation and turnover of free monoterpenes and terpene glycosides
6. To identify non-terpenoid "impact" volatiles in non-Muscat varieties

In response to comments of reviewers, the objectives were modified. The reviewers suggested that the research be focussed on lab studies, eliminating field work which introduced too many variables. Both groups examined some aspects of all objectives except 3 and 4 which were given minimal attention in keeping with comments from the reviewers. Neither group was able to address Objective 6, since the major efforts of the research in both programs were directed towards the study of terpenes and/or of Muscat or Muscat related grapes.

E. BARD FINAL REPORT

Introduction

As demonstrated previously by Williams et al (1981), in Muscats and non-Muscats, a large percentage of the monoterpenes are present in the grapes and must as odorless glycosides. When converted to the free odorous terpenes, these precursors can produce concentrations which are well above sensory thresholds. In this project, the Israeli and US investigators examined several different issues, as described in the objectives, to examine the formation and distribution of free and bound terpenes and study factors affecting their conversion to free terpenes in wines.

U.S.A.

1. EXPERIMENT 1. Investigation of the contribution of glycosides to bitterness and as aroma precursors in wine.

1.1. METHODS and MATERIALS

The principal investigator (US) was on sabbatical leave in 1986-7 at the Australian Wine Research Institute in Adelaide, Australia where sensory evaluation of terpenes and terpene glycosides extracted from wines was conducted. Because of the unique bitterness of Muscat and related aromatic wines, which are high in terpene glycosides, the contribution to wine flavor of these compounds was studied. Experimental details and results for this research are provided in Noble et al 1987, 1988, but are briefly presented here. The terpene glycosides extracted from the Chardonnay and Muscat of Alexandria wines were evaluated at amounts equal to the concentration at which they were found in wine (Level 1) and at two times the original concentration (Level 2). The terpene glycosides were assessed in water, a model solution and in the parent wine by the duo-trio test by 13 trained judges.

Terpene glycosides were isolated from Muscat of Alexandria and Chardonnay wines. To evaluate the potential contribution to wine flavor of the terpene glycosides, the glycosidic fraction was hydrolyzed enzymatically. This hydrolyzed extract was added at single, double and triple the concentrations that were present in the starting wines. The aroma of the hydrolyzed volatiles was evaluated by duo-trio difference tests as described above by the same 13 judges.

1.2 RESULTS (Expt. I).

As shown in Table 1, the glycosides could only be detected in water. In the model solution or in wine, the glycosidic material could not be detected even with addition of twice the

TABLE 1. Summary of results for tasting of glycosides in water, model solution and wine. Number of correct responses for difference tests (n=26, except as noted^a).

Level	Chardonnay			Muscat		
	Water	Model	Wine	Water	Model	Wine
1	19 *	16 NS	7 ^a NS	19 *	12 NS	5 ^a NS
2	-- ^b	12 NS	6 ^a NS	--	14 NS	15 NS

^an=13. ^bTest not performed.

NS, * denote no significance and $p < 0.05$.

concentration in the starting wine. The taste of the aqueous samples containing the glycosides was generally described by the judges as a faint floral taste. No bitterness was reported.

Subsequently, in water the taste of ten times the original concentration of the glycosidic fraction was evaluated informally. It was faintly bitter, equivalent to approximately the bitterness of 2 ppm quinine. Clearly, the unique bitterness of Muscat wines is not produced by the terpene glycosides. The results of the evaluation of the contribution of hydrolyzed glycosides to the aroma of wine are summarized in Table 2. Addition of the volatiles from the glycosides extracted from the Chardonnay wine produced a significantly different aroma in water at single and double strength. However, only at triple strength was a significant difference in the aroma of the Chardonnay wine produced. Further, triple-strength addition of the Chardonnay aglycons produced NO change in the aroma of Muscat wine.

In contrast, the Muscat aglycons had a much more potent contribution to aroma. At single strength, the volatiles liberated from the Muscat aglycons produced a significantly different aroma in the model solution and in Chardonnay wine. However, it required addition of a double strength amount of the Muscat aglycons to produce a detectable difference in Muscat wine aroma.

TABLE 2. Summary of results for evaluation of aroma of aglycons in water, model solution and wines. Number of correct responses for difference tests (n = 26, except as noted^a).

Level	Chardonnay Aglycons		
	Wine		
	Model	Chardonnay	Muscat
1	20 **	-- ^b	--
2	26 ***	15 NS	--
3	--	20 **	9 ^a NS

Level	Muscat Aglycons		
	Wine		
	Model	Chardonnay	Muscat
1	20 **	20 **	15 NS
2	26 ***	20 **	20 **
3	--	13 ^a ***	21 **

^an=13.

^bTest not performed.

NS, **, *** denote no significance, p<0.01, p<0.001, respectively.

2. EXPERIMENT II. Distribution of free and bound forms of monoterpenes during maturation of Muscat of Alexandria and Symphony grapes.

2.1 MATERIALS AND METHODS

2.1.1. Materials

Grapes from *Vitis vinifera* L. cvs. Muscat of Alexandria (Muscat) and Symphony, a cross between Grenache gris and Muscat of Alexandria, were evaluated in the 1988 season. Three adjacent rows, each with 23 vines, were used for the experimental blocks for each variety. The

grapes were sampled bi-weekly starting July 12, 1988, approximately 2.3 weeks before véraison until a week after harvest (Sept. 16) for Muscat and at the time of "normal harvest" (Aug. 31) for Symphony grapes. On each sampling date, from each vine, 10 berries were removed from each of ten clusters for berry chemical analysis; the remaining berries were stored at -17 C for about 3-4 months until used for terpene analysis. A total of 40-50 grams of frozen berries were taken from the middle of each of ten frozen clusters for row 1 and row 3. The skins were carefully peeled off the frozen grapes using forceps. The skins and deseeded mesocarp fractions were homogenized separately with NaCl and adjusted to pH 7, centrifuged and the supernatant stored at -23oC until extracted.

2.1.2. Winemaking

Wines were prepared in triplicate in 20 l lots using standard winemaking practices. Grapes were crushed and pressed using a basket press and 50 ppm SO₂ was added to the must. After settling (with no pectinase), the racked must was split into three replicate fermentations, inoculated with 1-2% v/v yeast (Montrachet) and fermented at 16oC. After fermentation and racking, wines were filtered and 12 ppm SO₂ was added at bottling.

2.1.3. Analyses of grape and wine composition

After the 100 berry samples were weighed, whole berries without pedicels were homogenized, filtered and centrifuged. The supernatant was used for analysis of pH, titratable acidity (TA), soluble solids (oBrix), and potassium (K⁺) by methods described in Amerine and Ough (1980).

Wines were analyzed for % ethanol, pH, titratable acidity, phenolics, and residual sugars (glucose and fructose). With the exception of residual sugars, all these analyses were performed as described by Amerine and Ough (1980). Residual sugars in wines were analyzed by HPLC with cation exchange column (Bio-Rad).

2.1.4. Analyses of Monoterpenes

2.1.4.1. Extraction

Free terpenes were extracted from the homogenates (corresponding to 250g of mesocarp or from 60g of skins) or 30 ml of juice or wine in 50ml Freon 11 using a continuous liquid/liquid extraction system (Williams et al., 1981). The extractions were made at 30oC for 72 hrs, with solvent changed every 24 hrs. The pooled solvent was concentrated and stored til analyzed at -10 C

For determination of bound monoterpenes, filtered homogenates (corresponding to 30-50 grams of mesocarp or skins), filtered juice or dealcoholized wines were loaded on a column packed with 9 grams of Davisil C18 reversed-phase adsorbent (90-130µm) (Alltech associates). Sugar and acids retained on the C18 RP adsorbent were washed out with 100ml of distilled/deionized water, then bound monoterpenes were eluted with 50ml of double distilled methanol. The methanolic eluate was evaporated to dryness and resuspended in 25ml citrate-phosphate buffer, pH 5. The aqueous glycosidic residue was washed with Freon 11 to remove residual free terpenes and then evaporated to remove any residual Freon 11 solvent. A

0.02 g aliquot of β -glucosidase EL (an experimental enzyme from Röhm, W. Germany) was incubated in the glycosidic solution at 40°C for 24 hours. The hydrolyzed terpenes were extracted and concentrated as described above.

2.1.4.2. Quantification and identification

A Hewlett-Packard gas chromatograph Model 5890A equipped with a flame ionization detector and on-column injection system was used. Supelcowax-10 bonded phase capillary column (60 M X 0.32mm I.D., film thickness, 0.50 μ m) protected by a 1.0 meter X 0.32mm I.D. deactivated fused silica pre-column (J&W Scientific) was used. The operating conditions were the following: carrier gas flow (He), 1.3ml/min; column head pressure, 13 psi; hydrogen flow, 37ml/min; air flow, 364ml/min; make-up gas (N₂), 30.5ml/min; purge vent, 58ml/min; injector temperature, 45°C; detector temperature, 250°C. Oven temperature was held at 50°C 10 min then increased to 185°C at 2°C/min, and held for 25 min. A 1 μ l of final concentrate was injected directly into the capillary column.

Maxima 820TM (Dynamic Solutions) was used for acquiring and processing the signal from the flame ionization detector of HP 5890A GC. Quantification of the components was also carried out by Maxima 820, by comparing a peak response of known amount of internal standard extracted, concentrated, and analyzed with the sample. These data were expressed as μ g/Kg skin or mesocarp or μ g/L juice or wine using mean response factors for each terpene. For components found in grapes and wines for which authentic standards were not available, a mean response factor of 0.7428 was used.

Monoterpenes were identified by comparing the mass spectra and retention times of authentic monoterpenes. Confirmed identification was made by spiking the samples with the known compounds and comparing retention times and mass spectra. Identification of compounds for which authentic chemicals were unavailable was made by comparing the mass spectra and retention times of the samples with data previously reported by others. A VG-Trio 2; quadrupole mass spectrometer (VG Instruments, England) interfaced by jet separator to a Hewlett Packard 5890A gas chromatography equipped with an on-column injector (J&W Scientific) was used. A 60 M X 0.32mm I.D., film thickness 0.25 μ m DB-WAX capillary column (J&W Scientific) was used. Injection volume was 1 μ l. The gas chromatographic conditions were same as described previously except carrier gas flow (linear velocity) which was 40cm/sec. The ion source temperature was 250°C and the mass spectra were taken over the m/z range from 30-300, utilizing an ionizing voltage of 70 eV.

2.1.5. Recovery studies

2.1.5.1. Free monoterpenes

Authentic monoterpene compounds in a model solution were extracted and chromatographed as described previously. The recovery efficiency was very close to 100 % for each of the terpenes examined.

2.1.5.2. Glycosides

The recovery efficiency from the C18 column was determined using

p-nitrophenyl- β -glucopyranoside (PNG) (Sigma chemical) with β -glucosidase (Aryan et al., 1987). PNG was dissolved in pH 5 citrate-phosphate buffer, and 10 ml (6363 μ g PNG/10ml buffer) was pumped through the C18 column. PNG eluted from a C18 column and a control solution of PNG were incubated for 48 hr. The recovery, determined by measuring absorbance at 405 nm of the released p-nitrophenolate anion from p-nitrophenyl- β - glucopyranoside, was 100%.

2.1.5.3. Reproducibility of bound terpene analysis

The reproducibility of the method for the determination of bound terpenes using C18 RP chromatography was examined using by Muscat juice. Filtered must samples (30ml) were loaded on the C18 columns, rinsed and eluted as above. Following enzyme hydrolysis, hydrolyzed aglycons were extracted with Freon 11 and gas chromatographed described as previously outlined. Concentrations of individual terpenes were expressed as μ g/Kg juice. Results of determinations, which were performed in triplicate, are shown in Table 3 for the 14 compounds which included 10 major terpenes. The mean coefficient of variation was 6.26% and the mean standard deviation was 5.72.

Table 3. Reproducibility of the analysis of bound terpenes from Muscat juice. Concentration of terpenes in $\mu\text{g/kg}$ juice, mean and standard deviation (SD) (n=3).

Component	Mean	SD
Ethyl octanoate	29.62	2.61
cis-furan Linalool oxide	3.13	0.20
Nerol oxide	2.04	0.24
Linalool	339.88	12.35
Hotrienol	3.13	0.41
-Terpineol	30.53	4.10
trans-pyran Linalool oxide	22.51	4.16
cis-pyran Linalool oxide	3.13	0.25
Citronellol	8.29	2.18
Nerol	83.72	9.21
Geraniol	599.34	28.03
Benzyl alcohol	60.61	3.73
Phenyl ethanol	56.45	6.16
Diendiol-I	37.21	6.48

2.1.5.4. Comparison of enzyme activity

The efficiency in hydrolyzing glycosidic substrates of Rohapect CTM (Röhm, W. Germany), a purified pectinase with β -glucosidase activity, was compared with β -glucosidase EL (Röhm), a crude pectinase but less purified than commercially available Rohapect C. To 65ml (prepared using 11.8 g of the skins for Rohapect C) and 40 ml (5.5 g of skins for β -glucosidase) of the buffered skin homogenates from Muscat berries prepared as described previously, was added 0.02g and 0.01g, respectively of Rohapect C and β -glucosidase EL. The solutions, prepared in duplicate were incubated at 40°C for 24 hrs. The hydrolyzed aglycons were extracted with Freon 11 for 72 hrs and analyzed by gas chromatography as described previously. In Table 4, the amounts of selected aglycons hydrolyzed by the two enzymes are shown. As illustrated by these results, the relative activity of Rohapect C ranges from 0 to 8 % that of β -glucosidase EL, with an overall average relative activity of 3.2%.

Table 4. Comparison of β -glucosidase activity of Rohapect C and β -glucosidase EL. Concentrations of hydrolyzed aglycons from glycosidic fraction of Muscat grape skins expressed as $\mu\text{g/1kg}$ whole berries.

Components	β -glucosidase EL	Rohapect C	R.A. (%) ^a
Linalool	364	7	1.9
-Terpineol	19	tb	0
t-pyran Linalool oxide	9	-c	0
c-pyran Linalool oxide	3	-	0
Nerol	114	3	2.6
Geraniol	369	18	4.9
Diendiol-I	37	0	8.1
Benzyl alcohol	13	t	0
Phenyl alcohol	39	3	7.8

a R.A. (%): Relative activity of Rohapect C as compared to EL

b trace : < 1 $\mu\text{g/kg}$ berries. c - : Not detected.

2.1.6. Enzymatic hydrolysis of bound terpenes in wine

To preliminarily explore the use of the β -glucosidase EL to hydrolyze bound terpenes in wine, β -glucosidase EL (0.33g/l) was added to the Muscat wine. The wine headspace was flushed with nitrogen gas and stored in 750-ml bottles at 10 C for 4 months, with control wine to which no enzyme was added. At the end of the storage interval, the free and bound monoterpenes in both wines were evaluated as described above.

2.2. RESULTS. Expt. II

2.2.1. Analyses of grape composition

As shown in Figure 1, berry weight and volume increased to the 4th sampling date, then slowly decreased before increasing again to a maximum on 7th sampling date for Muscat. For Symphony grapes, the final harvest occurred on the 5th date (Aug. 31), which had lower berry volume and weight than observed on the 4th sampling date. For several days before the 5th sampling date, the temperature was high, spiking for two days at 106 F. This coincides with the drop in berry volume and weight observed here.

For both varieties, from the first sampling dates which were 20 and 23 days before véraison for Symphony and Muscat, respectively, until the final sampling, the oBrix, and pH continually increased, while titratable acidity (TA) decreased with a sudden drop after 2nd sampling date (Figure 2). The sudden jump of oBrix and pH on 6th and 7th sampling dates in Muscat may be result of the concentration effect by hot temperatures prior to those days.

2.2.2. Identification of volatiles in grapes and wines

About 43 peaks were found in extracts of the skins of which 15 peaks were identified, including 8 monoterpenes as shown in Table 5. Over 53 peaks were separated from the Freon 11 concentrate of mesocarp and 26 peaks were identified among them, which included 14 monoterpenes including monoterpene alcohols, aldehydes, and diendiols were found among them (Table 6). For Muscat wine about 100 peaks were separated of which 62 peaks were identified (Table 7). Most of the identified components have already been found in Muscat and related grapes and wines. However, in addition to compounds previously observed in Muscat grapes, myrcen-2-ol, geranial, and neral were identified from the fresh Muscat grapes. Myrcen-2-ol, was previously not reported in fresh grape juice but was a significant constituent of heated juices (Rapp et al., 1984; Williams et al., 1980a). The headspace composition of the heat-induced products (15 min at 70°C) of the Muscat juice contained myrcen-2-ol, suggesting that it could have arisen directly from linalool as they share the same oxidation level (Williams et al., 1980a). In this study about 40 μ g/kg and 3 μ g/kg from the fresh Muscat skin and mesocarp, respectively were found.

Figure 1. Changes of berry weight and juice volume per 100 berries in Muscat (top) and Symphony (bottom). Arrow at abscissa indicates véraison.

Figure 2. Changes in °Brix, pH, and Titratable acidity (g/100 ml).
Arrow at abscissa indicates veraison.

Table 5. Major volatile components identified in the skins of Muscat of Alexandria (6th sampling date, Sept. 9) and criteria for their identification.

Components	Scan no.	ID	Peak no.
2-Ethyl cyclobutanol	408	GC-MS	1
1-Butanol	601	GC-MS, GC	2
2-Hexenal	846	GC-MS	3
1-Hexanol	1478	GC-MS	4
(Z)-3-Hexen-1-ol	1595	GC-MS	5
2-Octanol (Int Std)	1765	GC-MS, GC	6
Nerol oxide	2039	GC-MS, GC	7
Linalool	2259	GC-MS, GC	8
t-Pyran linalool oxide	2919	GC-MS, GC	9
Citronellol	3035	GC-MS, GC	10
Nerol	3142	GC-MS, GC	11
Geraniol	3304	GC-MS, GC	12
Benzyl alcohol	3359	GC-MS, GC	13
2-Phenyl ethanol	3468	GC-MS, GC	14
Diendiol-I	3633	GC-MS, GC	15
Myrcen-2-ol	4670	GC-MS	16

GC-MS : Gas chromatography-mass spectrometry.

GC : Retention time of authentic samples by GC

Peak no : Peak numbers corresponds to the chromatograms not shown.

Table 6. Major volatile components identified in mesocarp of Muscat of Alexandria (6th sampling date, Sept. 9) and criteria for their identification.

Components	Scan no.	ID	Peak no.
n-Hexanal	397	GC-MS	1
1-Butanol	587	GC-MS	2
2-Hexenal	829	GC-MS	3
3-Hydroxy-2-Butanone	1110	GC-MS	4
1-Hexanol	1463	GC-MS	5
(Z)-3-hexen-1-ol	1580	GC-MS	6
Cyclohexanol	1677	GC-MS	7
2-Octanol (Int std)	1750	GC-MS, GC	8
t-Furan linalool oxide	1793	GC-MS, GC	9
7-Methyl-3,4,-octadiene	1855	GC-MS	10
c-Furan linalool oxide	1907	GC-MS, GC	11
Nerol oxide	2015	GC-MS, GC	12
Linalool	2243	GC-MS, GC	13
Hotrienol	2400	GC-MS, GC	14
Butyrolactone	2431	GC-MS	15
Neral	2670	GC-MS	16
Geranial	2852	GC-MS	17
t-Pyran linalool oxide	2903	GC-MS, GC	18
, -dimethyl benzyl alc.	2970	GC-MS,	19
c-Pyran linalool oxide	2999	GC-MS, GC	20
Citronellol	3020	GC-MS, GC	21
Nerol	3124	GC-MS, GC	22
Geraniol	3287	GC-MS, GC	23
Benzyl alcohol	3341	GC-MS, GC	24
2-Phenyl ethanol	3449	GC-MS, GC	25
Diendiol-I	3616	GC-MS, GC	26
Myrcen-2-ol	4651	GC-MS	27

GC-MS : Gas chromatography-mass spectrometry

GC : Retention time of authentic samples by gas chromatography

Peak no : Peak numbers corresponds to the chromatogram not shown.

Table 7. Major volatile components identified wine from Muscat of Alexandria and criteria for their identification.

Components	Scan no.	ID	Peak no.
	218	GC-MS	1
Propyl acetate	240	GC-MS	2
2-Methyl propyl acetate	283	GC-MS	3
Ethyl butanoate	310	GC-MS	4
Unknown	364	GC-MS	5
2-Methyl-1-propanol	427	GC-MS	6
3-Methyl-1-butanoic acetate	501	GC-MS	7
1-Butanol	588	GC-MS	8
3-Methyl-1-Butanol	836	GC-MS	9
Ethyl hexanoate	938	GC-MS	10
Unknown	1023	GC-MS	11
Hexyl acetic acid	1091	GC-MS	12
Unknown	1197	GC-MS	13
(Z)-3-hexen-1-ol acetate	1273	GC-MS	14
4-Methyl-1-pentanol	1347	GC-MS	15
1,3-Butanediol	1395	GC-MS	16
1-Hexanol	1463	GC-MS	17
(E)-3-Hexen-1-ol	1496	GC-MS	18
3-Ethoxy-1-propanol	1534	GC-MS	19
(Z)-2-Hexen-1-ol	1576	GC-MS	20
2-Methyl cyclopentanol	1641	GC-MS	21
2-Octanol (Int. Std)	1747	GC-MS, GC	22
Ethyl octanoate	1770	GC-MS, GC	23
2-ethyl-1-hexanol	2017	GC-MS, GC	24
2-Thioxo-4-imidazolidinone	2073	GC-MS	25
3-H-Ethyl butanoate	2096	GC-MS	26
2,2'-thiobis-ethanol	2111	GC-MS	27
1,3- or 2,3-Butanddiol	2209	GC-MS	28
Linalool	2239	GC-MS, GC	29
2-Methyl propanoic acid	2293	GC-MS	30
1,3-Butanediol	2349	GC-MS	31
Butyrlactone	2428	GC-MS	32
Hotrienol	2460	GC-MS, GC	33
Butanoic acid	2506	GC-MS, GC	34
Ethyl decanoate	2559	GC-MS	35
1,3-Propanediol, diacetate	2612	GC-MS	36
4,4,5-trimethyl-1,3-dioxan-5-ol	2634	GC-MS	37
3-Methyl butanoic acid	2653	GC-MS	38
Diethyl butanedioate	2688	GC-MS	39
-Terpineol	2750	GC-MS, GC	40
3-(Methylthio)-1-propanol	2813	GC-MS	41
t-pyran Linalool oxide	2899	GC-MS, GC	42
3-Hydroxy butanol	2953	GC-MS	43
4-Methyl-2-pentanethiol	2964	GC-MS	44
1,4-Butanediol, diacetate	3035	GC-MS	45

Table 7. Continued.

Components	Scan no.	ID	Peak no.
2-(2-butoxyethoxyl-ethanol	3090	GC-MS	46
Ethyl-2-phenyl acetate	3131	GC-MS	47
Hexanoic acid	3254	GC-MS, GC	48
Geraniol	3286	GC-MS, GC	49
Benzyl alcohol	3334	GC-MS, GC	50
Propyl acetate	3378	GC-MS	51
2-Phenyl ethanol	3444	GC-MS, GC	52
Diendiol-I	3609	GC-MS, GC	53
Pentyl-2-methyl-2-propenoate	3886	GC-MS	54
Octanoic acid	3924	GC-MS	55
1-(2-H-5-methyl phenyl)-ethanone	4280	GC-MS	56
t-tetrahydroxy-5-methyl-	4367	GC-MS	57
Ethyl undecanoate	4492	GC-MS	58
Decanoic acid	4534	GC-MS	59
1,2,3-Propanetriol	4632	GC-MS	60
2-Methyl-3-penten-1-ol	4705	GC-MS	61
	4831	GC-MS	62

GC-MS: Gas chromatography-mass spectrometry. GC: By GC retention
Peak no : Peak numbers corresponds to the chromatogram not shown.

2.2.3. Changes and distribution of monoterpenes

2.2.3.1. Changes in monoterpenes during ripening

Variation was seen between the field replications: for example on the 6th and 7th sampling dates, the difference in geraniol in Muscat mesocarp between replicate (row) analyses was 100 and 300 ug/Kg, respectively. The data for the two different rows were pooled, and the mean of these two analyses reported below.

In Figures 3a and b, the concentrations of the major terpenes in the skin and in the mesocarp at each sampling date are shown for Muscat grapes. In Figure 3c, changes in aromatic alcohols are plotted. For Symphony grapes, the distribution of the major terpenes (Figures 4a and b) and aromatic alcohols (Figure 4c) are similarly illustrated. The most abundant aromatic alcohols observed in this study are benzyl alcohol and 2-phenyl ethanol (Figures 3c and 4c). They were very low in concentration in both varieties. The highest level observed was for 2-phenyl ethanol (14 µg/kg in mesocarp of Muscat). In both varieties, fairly low concentrations of free or bound terpenes were present before véraison.

For both varieties of grapes, the levels of free monoterpenes increased slowly throughout development, while the bound terpenes increased very significantly. To summarize this information, the totals of the three major terpenes present in the skin and mesocarp free and bound at each sampling date are provided in Tables 8 and 9. Given the thresholds for sensory detection of monoterpenes in sugar-water model solution (Noble et al, 1987) and the levels of free monoterpenes found in the grapes, the only monoterpenes which were found at harvest at levels near thresholds are geraniol, linalool, and nerol. These three have been

Figure 3a. Change in concentration of free and bound forms of linalool (top), geraniol (middle), and nerol (bottom) in mesocarp (MC) and skin of Muscat grape. Arrow at abscissa indicates véraison.

Figure 3b. Change in concentration of free and bound forms of trans-pyran linalool oxide (t-LI-P) and α -terpineol (α -TER) in mesocarp (MC) and skin of Muscat grape. Arrow at abscissa indicates véraison.

Figure 3c. Change in concentration of free and bound forms of benzyl alcohol (BENZ) and 2-phenyl ethanol (PHENALC) in mesocarp (MC) and skin of Muscat grape. Arrow at abscissa indicates véraison.

Figure 4a. Change in concentration of free and bound forms of linalool (top), geraniol (middle), and nerol (bottom) in mesocarp (MC) and skin of Symphony grape. Arrow at abscissa indicates véraison.

Figure 4b . Change in concentration of free and bound forms of trans-pyran linalool oxide (t-LI-P) and α -terpineol (α -TER) in mesocarp (MC) and skin of Symphony grape. Arrow at abscissa indicates véraison.

Figure 4c . Change in concentration of free and bound forms of benzyl alcohol (BENZ) and 2-phenyl ethanol (PHENALC) in mesocarp (MC) and skin of Symphony grape. Arrow at abscissa indicates véraison.

Table 8. Total concentration in mesocarp (MC) and skin of free and bound forms of the three monoterpenes in Muscat: linalool, nerol, and geraniol ($\mu\text{g}/\text{kg}$) ($n=2$).

Date	Free		Bound	
	MC	Skin	MC	Skin
7/12	21.7	4.2	62.0	172.5
7/25	3.5	14.0	55.5	83.5
8/08	12.5	13.7	192.0	212.5
8/22	73.0	30.0	409.5	288.5
9/06	49.5	62.0	771.0	448.0
9/09	75.0	58.5	744.5	384.5
9/16	122.0	87.5	1011.52	889.0

Table 9. Total concentration in mesocarp (MC) and skin of free and bound forms of the three monoterpenes in Symphony: linalool, nerol, and geraniol ($\mu\text{g}/\text{kg}$) ($n=2$).

Date	Free		Bound	
	MC	Skin	MC	Skin
7/12	3.0	2.1	67.5	80.4
7/25	2.0	8.5	51.0	70.0
8/08	15.5	42.0	232.5	97.0
8/22	39.5	10.5	430.5	171.5
8/31	53.0	23.5	319.0	319.0

previously reported to be the most abundant and important contributors to the flavor of Muscat berries (Rapp et al., 1984; Gunata et al., 1985a; Wilson et al., 1986). All three monoterpenes (linalool, geraniol, and nerol) continually increased even a week after "normal harvest" date (Sept. 9), and the rate of increases observed between 6th (Sept. 9) and 7th sampling date (Sept. 16) were more rapid than those observed at any other developing stages. However, all free monoterpenes actually present in the grapes were below published aroma threshold levels in sugar-water model solution and their relatively low total concentration may question previous claims that volatile monoterpenes account for the varietal character of Muscat grapes (Etievant et al., 1983; Williams et al., 1981; Wilson et al., 1984).

The highest concentration of bound diendiol-I was found pre véraison in mesocarp for both Muscat ($283\mu\text{g}/\text{kg}$) and Symphony ($215\mu\text{g}/\text{kg}$). It is interesting to note the highest level of diendiol-I in early stages, which then slowly decreased. At final sampling dates (Sept. 16 and

Aug. 31 for Muscat and Symphony, respectively), about half of total bound diendiol-I was found in the skins of both varieties. This is contrast to the results of others (Williams et al., 1985; Wilson et al., 1984) in which bound diendiol-I increased continually and reached a maximum concentration about 30 days after "normal harvest" and only then decreasing.

Sugar accumulation at 6th (Sept. 9) and 7th sampling dates (Sept. 16) in Muscat almost plateaued as shown in Figure 1, however, three major monoterpenes (geraniol, linalool, and nerol) increased even after sugar accumulation was slowed (Figure 3a, 4a), suggesting that the accumulation of monoterpenes are independent of sugar accumulation. Generally, in both varieties the three most important bound monoterpenes (linalool, nerol, and geraniol) steadily increased during berry development and the amount of these bound monoterpenes was about 8-9 times higher than free terpenes at the time of harvest in Muscat and Symphony.

As shown in Figures 3a and 4a, there was a drop in bound terpene accumulation after the 4th sampling date, with a subsequent rise in Muscat by the last sampling dates. The decrease in concentration of the major bound terpenes suggests a response by the vine to the hot spell when grapes were dehydrated. Since there was no increase in corresponding increase in free terpenes, the hydrolyzed terpenes were probably metabolized or volatilized.

Muscat and Symphony had very similar patterns of distribution and concentrations of free and bound terpenes for the first 4 sampling dates. (An unscheduled early harvest following the 4th assay curtailed the Symphony experiment at an earlier maturity level than that for the Muscat.) In Table 10a, the concentration and distribution of total free and bound terpenes are shown for the Muscat and Symphony on the the last date at which they can be compared. Except for the difference in distribution of the bound terpenes between the skin and mesocarp, the levels and patterns are very close. However, as shown in Table 10b, at the final harvest dates for the two varieties, the Muscat grapes have 45% more total bound terpenes. As was seen in the developmental data for the two varieties in Figures 3 and 4, the most rapid increase in the levels of terpenes came after the grapes reached over 17 Brix, after the 4th sample date. Throughout the development and at both harvest dates, both varieties showed fairly equal distribution of terpenes between the skin and mesocarp, however the bound terpenes were always found at concentrations 8 - 10 times those of the free compounds.

The levels of both free and bound forms of monoterpenes steadily increased during grape development, with the major accumulation occurring in the bound monoterpenes. A common pattern in the level of individual free monoterpenes found in both varieties was the lower levels in the skins versus higher levels in mesocarp. For both varieties at final harvest, the distribution patterns were generally similar even though the grapes were harvested at different maturities. About 4 to 5% of total terpenes (sum of the seven major terpenes) was found free in the skins in both grapes, while 12.7% (Symphony) and 8.3% (Muscat) of the total terpenes were found free in mesocarp. For Muscat, 87% of the total terpenes occurred in the bound form with 29.5% in the skins and 57.5% in the mesocarp. For Symphony, 83.2% of the total terpenes were bound, with the distribution reversed from that found in Muscat: 46.1% were in skins versus 37.1% in mesocarp. Despite the low proportion of skins (<10% by weight), the relatively high concentration of both free and bound terpenes in the skins can be a great

Table 10. Comparison of distribution in $\mu\text{g/kg}$ (and on a % basis in parenthesis) of total free and bound monoterpenes for Muscat and Symphony

a. At same sampling date (Aug. 22, 1988; 4th sampling date).

	Muscat (17.7°B)		Symphony (17.2°B)	
	Free	Bound	Free	Bound
Skin	36 (3.9%)	302 (32.4%)	14 (1.6%)	195 (22.7%)
Mesocarp	152 (16.3%)	443 (47.4%)	114 (13.3%)	537 (62.4%)
Total	188 (20.2%)	745 (79.8%)	128 (14.9%)	732 (85.1%)

b. At the last Harvest Date: for Muscat (Sept. 9, 6th sampling date) and Symphony (Aug. 31, 5th sampling date).

Location	Muscat (23.2°B)		Symphony (20.3°B)	
	Free	Bound	Free	Bound
Skin	64 (4.7%)	404 (29.5%)	41 (4.1%)	455 (46.1%)
Mesocarp	113 (8.3%)	788 (57.5%)	125 (12.7%)	367 (37.1%)
Total	177 (13.0%)	1192 (87.0%)	166 (16.8%)	822 (83.2%)

a : Linalool, Geraniol, Nerol, Nerol oxide, Citronellol, -Terpineol, trans-Pyran linalool oxide.

potential source for wine aroma. However, extended skin contact may have a deleterious effect on juice and wine flavor because of the extraction of undesirable components, such as phenolics. Further investigation is needed on ways to enhance the conversion of bound to free terpenes in wine.

To compare the juice data for this California Muscat of Alexandria with results for Muscats examined by other workers, the free and bound concentrations of linalool, α -terpineol, nerol and geraniol are shown in Figure 5. The data for Australian Muscat (Wilson et al., 1986) is very similar in levels and distribution to that reported here for the 1988 California Muscat (Park and Noble). The French Muscat (Gunata et al, 1985a) although showing twice the concentrations reported by the other studies shows a similar proportion of free and bound terpenes, and relative amounts of these four terpenes. The Israeli Muscat (Shoseyev, 1988) differs from the rest showing very low levels of free and bound geraniol.

Figure 5. Comparison of free and bound major terpenes in Muscat of Alexandria Grapes in Australia (Wilson et al, 1986), USA (Park, 1989), France (Gunata et al, 1985a), and Israel (Shoseyev (1988).

2.2.3.2. Distribution of free and bound monoterpenes in juices and wines

Generally, the juices contained fairly low levels of free monoterpenes in Muscat (118 μ g/kg) and Symphony (188 μ g/kg), while much higher levels of bound monoterpenes were found in Muscat (1389 μ g/kg) and Symphony (397 μ g/kg). Much higher levels of free monoterpenes were found in wines than juices: for Muscat (755 μ g/kg) and Symphony (641 μ g/kg); in contrast lower levels of bound monoterpenes in Muscat (1120 μ g/kg) was found. However, the decrease in bound monoterpenes in Symphony juice was not significant, whereas free monoterpenes increased from 188 μ g/kg in juice to 641 μ g/kg in wine, indicating other contributors, namely terpene diols, which were not included in the total concentration calculations.

To illustrate differences in distribution of free and bound terpenes between grape must at harvest and the finished wine, in Figure 6 are plotted the major terpenes in Muscat juice and wines. While all free terpenes, except geraniol increased in the wine above the levels found in juice, the most significant increase from juice to wine was free trans-pyran linalool oxide, in which only a trace (2 μ g/L) existed in juice as free whereas 336 μ g/L was found in the wine. The lack of apparent increase in free geraniol is probably explained by its conversion to other terpenes. Free hotrienol, α -terpineol, and diendiol-I, nerol oxide, and citronellol also increased in the wine.

Also shown in Figure 6 are the free and bound terpene concentrations in the same Muscat wine following 4 months of storage after the initial terpene analysis. A further increase in free terpenes and decrease in bound terpenes occurred, the result of acidic hydrolysis. Wine, to which β -glucosidase EL had been added showed a higher production of free terpenes over the same storage interval. Linalool and geraniol, monoterpenes with the lowest threshold, were almost completely converted from the bound to free forms with the addition of the glucosidase. As has been reported elsewhere (Aryan et al., 1987), grape glycosidase is inhibited by the presence of extremely low levels of glucose. Shoseyev et al.(1988) reported on the use of a β -glucosidase isolated from *A. niger* which was not inhibited by low levels of sugar, and apparently activated by ethanol. In this study, the enzyme was not inhibited by 0.07% glucose and 0.02% fructose in the Muscat wine. However, the enzyme was less effective in Symphony which contained the same level of glucose, but 0.13% fructose. As shown in Table 11, other wine compositional parameters could have influenced the activity of the enzyme.

Table 11. Chemical analyses of wines (n=2).

	pH	TAa	%Ethanol (v/v)	Phenolicsb (ppm)	R.S.c Glu(%) Fru(%)	
Muscat	3.49	0.45	12.22	185	0.066	0.019
Symphony	3.14	0.60	13.04	216	0.060	0.130

a : Titratable acidity expressed as g/100ml of tartaric acid.

b : Total phenolics expressed as ppm of gallic acid equivalent (GAE) .

c : Residual sugar, glucose and fructose (g/L) .

Figure 6. Free and bound terpenes in Muscat juice and wines at initial analysis (Wine), after 4 months storage at 10 C (4-MO), and after 4 months storage with added β -glucosidase (4-MO + ENZ).

Informal sensory evaluation of the wines, indicated that both enzyme treated Symphony and Muscat wines were more "floral" and had a higher intensity of "linalool" aroma. However, the enzyme possibly contained polyphenoloxidase enzymes as impurities since the enzyme treated wines were observed by visual inspection to have undergone browning in contrast to the control. In this study, (and in the research performed in Israel), the potential for increasing aroma by hydrolysis of bound terpenes has been demonstrated.

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E. BARD FINAL REPORT

ISRAEL

MATERIALS AND METHODS

1.1. Extraction, Isolation, Identification and Synthesis of Free and Bound Monoterpenes

1.1.1 Freon 11 extraction and GC-MS analysis

About 400 g of grapes were crushed and treated according to the method of Rapp et al. (1976). Freon 11 extraction was used regularly except for high efficiency extraction of Diols when Freon 11:methylene chloride 9:1 mixture was used.

The extracts were concentrated to 100 µl and 0.5 µl sample was injected to a GC (varian 3700) equipped with a capillary Supelcowax 10 column 60 m long, and .32 mm I.D. Helium served as a carrier gas at a flow rate of 1 ml/min and FID detector was employed. The temperature of both the detector and the injector was 230°C. Temperature program was initially 60°C for 5 minutes and then was gradually increased at a rate of 4°C per min up to 190°C. A GC-MS Finingen 4600 was used, ionization energy was 70 ev. The mass spectra of 27 terpenoids are presented in Appendix 1. An example of an Emerald Riesling wine extract is presented in Fig. 1.

1.1.2 Free and bound monoterpenes analysis

The analysis was performed according to Dimitriadis and Williams (1984). Linalool (purity 99.9%) was used for the establishment of a regression equation for free monoterpenes. The method was adopted for wine analysis using a correction factor for alcohol with concentrations made by using a model solution containing 6–15% alcohol and 200 µg linalool per l for free monoterpenes. a synthetic geranyl-β-rutinoside was used for the establishment of a regression equation for bound monoterpenes. The following equations were calculated.

$$A_F - \frac{(\% \text{ EtOH} \times 3.57)}{1000} \times 13830 = \text{Free Monoterpenes } (\mu\text{g/l}) \quad A_F = \text{O.D. at 608 nm (free)}$$

$$A_B \times 92250 = \text{Free Monoterpenes } (\mu\text{g/l}) \quad A_B = \text{O.D. at 608 nm (bound)}$$

The recovery of free monoterpenes was about 90% for linalool and about 40% for bound monoterpenes (using geranyl- β -rutinoside).

1.1.3 Isolation of monoterpene glycosides

Monoterpene glycosides were isolated using a C₁₈ preparative flash chromatography. One hundred ml of Rp-18 (40–63 μ m Lichroprep, Merck, Germany). The method was according to Williams et al (1982a).

1.1.4 Isolation of monoterpene glycosides by HPLC

Twenty mg of isolated glycosides (see 1.1.3) were dissolved in 1 ml of water and then with 6 ml (3 times) of Freon 11:methylene chloride 9:1). Samples of 2–5 μ l for analytical purposes and up to 250 μ l when a semipreparative chromatography was used. A Hewlett Packard HPLC (HP 1090) with a semi preparative column (Altech USA) 10 $\times$ 250 mm C₁₈-HL was employed. A gradient programme of water and acetonitril was used as follows:

Time	A%	B%
0	0	100
40	40	60
50	40	60
60	0	100

The glycosidic bond was read at 205 or 220 nm. The collected fractions were lyophilized and deuterized with D₂O. HNMR spectrum was produced by a Bruker 200 MHz (Germany).

1.1.5 Determination of the anomeric structure and identification of the sugar component of the monoterpene glycosides

Alditol acetates were prepared according to Harris et al. (1984). Samples of 0.5 μ l were injected to the GC-MS using a Supelcowax-10 60 m long I.D. 0.32 mm column. the injector's temperature was 240°C and the initial temperature was 180°C, increasing gradually to 220°C at a rate of 2°C per min, ionization energy was 70 ev. Standards for mass spectra were rutin, geranyl- β -rutinoside and p-nitrophenyl- α -L-arabinofuranoside which were converted to alditol acetates as described above.

1.1.6 Synthesis of geranyl- β -rutinoside and p-nitrophenyl- β -rutinoside and determination of the HNMR spectra

The synthesis of geranyl- β -rutinoside was done according to Shakhova et al. (1962) and Williams et al 1982 and p-nitrophenyl- β -rutinoside according to Bourbouze et al (1972) with certain modifications. the HNMR spectrum of p-nitrophenyl- β -rutinoside is presented in Fig. 2 and the chemical shifts are shown in Table 1.

The high coupling coefficient (7.8) of the hydrogen doublet and of carbon No. 1 in the glucose indicates the existence of a β -glucosidic bond. The chemical shift at 1.25 ppm of the hydrogen methyl doublets of carbon No. 6 of the rhamnose indicates its presence in the diglucoside rutinose (6-O- α -rhamnopyranosyl-D-glucopyranoside).

1.1.7 Synthesis of *cis* and *trans* Pyran and Furan linalool oxide and iso geraniol

6,7 Epoxidihydrolinalyl acetate (Fig. 7) was prepared according to Shoseyov (1988).

Iso geraniol (3,7 octadien-3,7-dimethyl-1-ol) was prepared according to the method of Arpino et al (1977) except that geraniol was oxidized by sodium bi chromate in sulfuric acid to obtain citral as a major product and Iso-geraniol as a secondary product. The mass spectra of the synthetic *cis* and *trans* pyran and Furan linalool oxide and the chromatogram and mass spectrum of the geraniol, Iso geraniol and geraniol are presented in Figs. 3, 4 respectively.

1.1.8 Enzymatic hydrolysis of monoterpene glycosides for isolation and identification of the terpenoid's aglycon.

Monoterpene glycosides isolated from grape juice as described above (1.1.3) were dissolved in 50 ml citrate buffer (20 mM, pH 3.4). The solution was stirred with Freon 11 methylene chloride mixture 9:1 for removing free-monoterpene residues. The solution was incubated with the endo- β -glucosidase B-1 (produced by *A. Niger*) under nitrogen atmosphere at 25°C for 24 h. Sodium chloride (10% w/v) was added and 3 octanol was used as an internal standard. Freon 11 liquid liquid extracts were concentrated and injected to GC-MS as described above (1.1.1).

2.1 WINE

2.1.1. Vinification treatments and sensory evaluation

Sauvignon blanc grapes from Aderet (inner coastal area) vineyard were harvested at 20° Brix and pH 3.6. Three treatments were tested: 1. pH adjustment by Tartrate addition; pectolitic enzyme (Pectinol - Rohm) and fast heating for 20 s at 85°C and then fast cooling to 40°C. The cooling and heating system is presented in Fig. 5. The heating bath was kept at 90°C and the cooling at 30°C. The enzyme treatment (100 ppm) and the pH adjustment were done 24 h prior to the heat treatment. Free and bound monoterpenes were assayed according to Dimitriades and Williams (1984). Sensory evaluation was done by the regular tasting panel of the Israel Wine Institute. The score was based on a weighted average of four criteria: appearance, aroma, taste and harmony using 1:2: 3.5:2 multiplication factors respectively on a 0–20 scale. A duo trio test was conducted for determining the effect of glucosides on bitterness. Red wines were tasted in dark glasses.

2.2 Monoterpene In Leaves and Berries

2.2.1 Seasonal pattern in leaves and berries

Two kg of Sauvignon blanc and Muscat of Alexandria grapes and 200 g leaves were sampled weekly from Hulda vineyard (inner coastal region). Both kinds of samples were homogenized and filtered through cheesecloth and pH, brix, free and bound monoterpenes were assayed.

2.2.2 Monoterpene tracing by ^{14}C mevalonic acid

Sixteen Muscat of Alexandria leaves opposite the clusters were selected on 4 vines. The main vein of each leaf was cut and 50 μl of DL ^{14}C (3) Mevalonic acid were injected by means of a syringe and a tygon tube. The specification of the solution was 12.75 mM, 40 mCi/mmol - (CEA, France) in 10 mM phosphate buffer at pH 5.9. The end of the tygon tubing was plugged and about 1 hour was allowed for a complete uptake of the solution. Four leaves and four bunches were sampled at 12, 24, 48 and 72 h. The leaves were kept at -30°C and free and bound

monoterpenes were assayed according to scheme 1. The β radiation was measured by a scintillation counter.

2.2.3 *In vitro* culture of berries and monoterpene glycosides biosynthesis

Plant material

Small berries of *Vitis vinifera* L. cv. Muscat of Alexandria (1–2 mm diameter) were harvested just after fruit set from a 14-year-old vineyard. Two different types of explants were prepared using berries with and without pedicels. Explants were surface-sterilized by a quick dip in 70% (v/v) ethanol followed by 3 or 10 min immersion in 1% (v/v) Sodium hypochlorite containing 0.02% (v/v) Triton X-100 as a wetting agent. The explants were then rinsed 4 times in autoclaved tap water.

Culture preparation

Each explant was planted separately on agar in a single 7 ml vial containing 3 ml Murashige and Skoog basal medium (Murashige and Skoog 1962) supplemented by 10 μ M benzyladenine (BA) with or without 1 μ M indoleacetic acid (IAA).

Explants were cultured for up to 4 months, at $26 \pm 1^\circ\text{C}$ under a 16 h photoperiod of cool white (Orsam Hellweiss, FRG) fluorescent light (10 W m^{-2}) with no change of medium or subculturing.

Experimental design

Three factorial two level experiments were conducted as follows: (1) pedicel's presence or absence; (2) immersion time in sodium hypochlorite, 3 or 10 min; and (3) with or without IAA supplement. Each treatment was replicated 20 times.

Cell fixation for SEM studies

The berries were fixed in 4% (v/v) glutaraldehyde in 0.01 M potassium phosphate, pH 7, for 24 h and then dehydrated 3 times with acetone: 30 min in 50% (v/v); 30 min in 70%; and 30 min in 100% acetone. Berries were air dried and further dried using a critical point drying apparatus (CPD polaron, E3000) for 60 min, glued to an aluminum carrier and coated with gold under vacuum.

Scheme 1. Procedure for free and bound monoterpene isolation from grape leaves and berries.

25 g Berries + 25 g NaCl or 2 g leaves + 10 ml H₂O + 1 g NaCl

Homogenization

centrifugation 10 min 10,000 rpm

3x10 ml L:L extraction F11 CH₂Cl₂ (9:1)

Bound

aqueous phase concentrated to
100 ml pH = 2.2 (by H₃PO₄)

40 ml steam distillation

3x10 ml L:L extraction F11

concentration to 2 ml, addition
of 15 ml lumax toluene (3:7)
 β -counting

Free

organic phase concentrated
to 2 ml + 100 ml H₂O
pH = 2.2 (H₃PO₄)

25 ml steam distillation

3x10 ml L:L extraction F11

concentration to 2 ml
addition of 15 ml lumax
toluene (3:7) β -counting

Feeding experiment

A 10 l steel cylinder containing 10 MPa compressed $^{14}\text{CO}_2$ labeled air (6.0 Bq ml^{-1}) was used for field leaf feeding experiments. The gas mixture was simultaneously fed through a manifold to 4 shoots enclosed in plastic bags, at a rate of 200 ml min^{-1} for 5 h. The outgoing gas was trapped in Ascarite (sodium hydroxide on non-fibrous silicate carrier) to prevent contamination. All shoots were under full sunlight during the 5 h feeding period. Clusters located at the shoot bases at the end of the veraison (color change) ripening stage (not directly exposed to the $^{14}\text{CO}_2$) were cut off 24 h after the beginning of the feeding period and brought to the laboratory.

The berries were separated and crushed in a Waring blender for 30 s, and filtered through cheesecloth. The juice was then centrifuged for 30 min in a swinging bucket at 20,000 g. The supernatant was filtered through $0.45 \mu\text{m}$ Millipore membrane (Bedford MA, USA) and then passed through a C_{18} reversed phase adsorbent column by flash chromatography. The free sugars and acids were eluted with water and the column was then regenerated with methanol (Williams et al. 1982). This procedure was repeated 3 times. The aqueous solution was lyophilized and 2.7 g of the crystals were resuspended in 20 ml of water and liquid extracted (3 times) with Freon 11.

The 20 ml extract containing about 1680 Bq ml^{-1} (^{14}C labeled) was combined with 20 ml of double strength MS liquid medium and 20 berries, which were grown previously for 60 days *in vitro*, were incubated in a flask under agitation for 48 h under environmental conditions as described above. The berries were recultured in agar medium for 48 h and then crushed, filtered through cheesecloth and $0.45 \mu\text{m}$ membrane and the glycosidic fraction was then isolated according to Williams et al. (1982b). The glycosidic fraction was separated on TLC silica gel 60 plate using 3:1:1 (v/v) n-propanol: ethyl acetate: water, as a developing solvent and 5:5:2 (v/v) 2% diphenylamine in acetone: 2% (v/v) aniline in acetone and 85% (w/v) H_3PO_4 as a spray reagent. geranyl- β -rutinoside, rutinose and geraniol were used as markers.

Geranyl- β -rutinoside was synthesized and rutinose was prepared by deacetylation of hepta-o-acetylrutinoside as described by Shoseyov et al. (1988). Its spectroscopic data, MS and ^1H -NMR was identical with that reported by Williams et al. (1982a). The TLC plate was transversely divided into 11 equal strips and the silica of each was scraped off, collected on a filter paper and eluted by methanol into scintillation vials. A scintillation solution, 70% (v/v) toluene and 30% (v/v) Lomax (Lumac, Schaesberg, The Netherlands) was added and the samples counted in a Beckman b-scintillation counter.

2.4 Endo- β -Glucosidase from *Aspergillus niger*: Selection, Production and Utilization

Aspergillus niger strain selection

A. niger was grown from spores on a medium containing 0.4% monoterpene glycosides isolated and purified from Muscat of Alexandria wine (see below) as the sole carbon source; $(\text{NH})_2\text{SO}_4$ 0.5 g/l, $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$ 0.2 g/l, MgSO_4 0.2 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g/l, $\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$ 0.001 g/l, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001 g/l, pH 3.4 adjusted by HCl. Following incubation for several weeks in an agitating incubator at 100 rpm and 30° , the slow-growing mycelium was crushed by ultratourax and incubated for a further 3 days. The resulting massive mycelium sporulated after several days and served as a source for the *A. niger* strain used.

Enzyme production by *A. niger*

The selected strain was grown in a 10 l fermentor with the same medium, rutin (Quercetin-3- β -rutinoside) serving as an inexpensive sole carbon source. Temperature was kept at 35° , agitation 200 rpm and aeration at 10 l/min. After 48 hr the growing medium was filtered through cheesecloth, then through a 0.45μ nitrocellulose membrane.

The filtrate was ultrafiltrated and concd to 20 ml with a 20,000 M_r cut-off membrane.

For large volumes a Pelicon ultrafiltration device was used. The concentrate was kept in a citrate buffer (20 mM pH 3.4) at 4°C . Dialysis against pure water, freezing and lyophilization did not reduce the enzyme's activity obtained.

2.4.3 Enzyme purification by affinity chromatography

(a) Column preparation. Three kinds of columns were used: 1. *p*-aminophenyl- β -D-glucopyranoside sepharose (PAPGS). 2. α -aminocaproic *p*-aminophenyl- β -D-glucopyranoside sepharose (EAPGS). 3. Tyramine *p*-amino-phenyl- β -D-glucopuranoside Upergit C (TPAPGU).

The first two columns were prepared according to Cuatrecasas (1970). The third column was prepared as follows: Tyramine 274 mg was dissolved in 20 ml of DMF and mixed with 20 ml of 1 M K_2HPO_4 containing 2 g preswollen Upergit C for 48 hr at room temp. The mixture was filtered through glass filter paper and the gel was washed first with DMF and then with 0.2 M Na_2CO_3 soln. Dialysed *p*-aminophenyl- β -D-glucopyranoside (100 μ mol) was coupled through azo-linkage to the tyramine Upergit C gel according to Cuatrecasas [1970] resulting in an orange-coloured product. Each column (standard Pasteur pipette) contained 2 ml of the gel. (b) Enzyme separation. One ml (0.5 mg/ml protein) was loaded onto each column and eluted.

Affinity chromatography

Affinity chromatography was performed on three kinds of columns (Fig. 7). The PAPGS and the EAPGS columns split the protein into two portions, one prior to and one following the NaCl shock, whereas the third column, TPAPGU, adsorbed the whole protein and released the entire enzyme in a single fraction following the NaCl shock. The TPAPGU column yielded the highest specific activity of the enzyme. The M_r of the enzyme, as determined by gel filtration, was 120,000D.

2.4.4. Endo- β -glucosidase activity assay and characterization

Enzyme assay

β -glucosidase activity was assayed by incubating 25 μ l of enzyme preparation in 1 ml of 1mM PNPG (4-nitro phenyl- β -D-glucopyranoside) for 2 min at 35°. The reaction was terminated by the addition of 200 μ l 1 M Na_2CO_3 and the A was read at 399 nm.

A. niger β -glucosidase activity was characterized using standard procedures for the determination of pH, ionic strength, temperature, EtOH response curves, K_M , V_{max} and K_I for

glucose. Also examined were the effects of various concentrations of grape juice (Muscat of Alexandria), sucrose, fructose and SO₂ on the enzyme activity under standard conditions.

Isoelectric focusing on polyacrylamide gel

Analytical isoelectric focusing was performed on 0.5 mm polyacrylamide gel (T = 5%; C = 3%) containing 2.4% (w.v) Ampholine (pH 2.4–5) using a multiphor unit LKB 2117 at 2000 V and 25 W for 1 hr. The slab was sliced longitudinally into three strips. One strip was cross-sectioned into 0.5 cm slices, which were immersed in 1 ml of H₂O for several hours and the pH gradient was then established. The second strip was immersed in 100 mg/l X-glue (5-bromo-4-chloro-3-indolyl β -D-glucopyranoside) for several hours and the β -glucosidase activity was visualized as described above. The third strip was stained with a general protein stainer, Coomassie Brilliant Blue 250-R.

The sub units mol wt was determined using two dimensional electrophoresis-first dimension isoelectric focusing pH 3.0–10.0 and the second dimension horizontal ultrathin SDS polyacrylamide pore gradient gel electrophoresis using prestained ml wt markers kit (MW-SDS blue, Sigma Inc.). The procedure was used according to the LKB application note 320 and carried out on a 2117 multiphor II (LKB produkter).

1.4.5. Immobilization of the fungus by Ca alginate

One l of medium containing *A. niger* B-1 was prepared as described above. The liquid medium was homogenized using an ultra tourax for 10 S. The homogenized solution was mixed with sterilized Na alginate 1% (w/v) and rutin was added to a final concentration of 0.4% (w/v). The mixed solution was pumped by a peristaltic pump into a pasteur pipet at a rate sufficient to form drops. The drops fell into a 50 mM CaCl₂ solution and the beads formed were dug out and rinsed in citrate buffer (20 mM, pH - 3.4). The immobilization procedure is based on the method of Kierstan and Coughlan (1968). One l of beads served in the continuous reactor as shown in Fig. 8. The fermentation was performed at 35°C, substrate supply rate was 1 1/24h and aeration 1 1/min. Fifteen ml samples were collected continuously. The β -glucosidase activity was determined using PNPG (P-nitrophenyl- β -D-glucopyranoside) as a substrate.

1.4.6 Proteolytic activity assay

The proteolytic activity in the fermentation medium was determined using kit No. 500-0011 (Bio Red, USA). The method is based on formation of a concentric ring of hydrolyzed casein on the agar medium plate. The pH was adjusted to 3.4 using citric acid and in a few cases to pH 5.0, 5.5, 6.0 and 7.0.

In order to determine the nature of the proteinase, specific proteinase inhibitors were used; PMSF (phenylmethylsulfonyl fluoride) for inhibiting serin proteinase and PCMB (para-chloro-mercury benzoic acid) for specific inhibition of cysteine proteinases. PMSF was dissolved in DMF. The final concentration of the DMF in the reaction system was 1%. PCMB was dissolved in 1 mM NaOH. The enzymes volume in the agar well was 2 mM. The reaction time was 30 h at 35°C. The reaction was stopped by 10% acetic acid.

2.4.7 Immobilization of the enzyme to acrylic polymer and to ground corn stores particles

Polexzyme-O-(Rohm Germany) beads on a polyacrylamide base activated by oxiran rings were used for immobilizing the enzyme. The procedure was as follows: Forty mg of protein in 10 ml K_2HPO_4 , (1 M – at pH 6.5). One g beads were added to the solution which was shaken for 48 h at room temperature. The beads were rinsed twice in 50 ml NaCl and twice with citrate buffer at 4°C. The leachate was collected and dialyzed against distilled water and then against citrate buffer. The activity of b-glucosidase and protein content were determined in the leachate as described above. The immobilized enzymes activity, K_m , optimal pH and half life time in 60°C were compared with those of free enzyme. Corn (maize) stovers were dried in the oven at 110°C, ground and sifted to 500–1000 μ particles.

Two methods were used for immobilizing the enzyme to the cellulose particles.

1. The method is based on the work of Vallat et al. (1985).

One g corn particles were oxidized in 20 ml $NaClO_4$ (0.2 M) in the dark for 24 h at room temperature using continuous shaking. The solution was decanted and the sediment was

aminated by adding 20 ml ethylenediamine (2.7 M). The solution was shaken again for 24 h and decanted, chemical reduction was done by 3 h shaking with 20 ml Sodium-Cyano-Borohydride (5 g/l in 50 mM phosphate buffer at pH 6). The solution was shaken for 3 h and then decanted. The particles were then activated by adding 3% glutaraldehyde in pyrophosphate buffer (50 mM pH - 6.5). The solution was shaken for 24 h, decanted and the particles were transferred to 20 ml citrate buffer (20 mM pH - 6.5) containing 40 mg protein. The solution was then shaken for 24 h and the beads were rinsed in NaCl solution and buffer similar to what was described above for the poloxzyme-O.

Glucose (1 M) was added in one of the experiments at the immobilization stage in order to test the possibility of eliminating the binding by blocking the active site and thereby maintaining a better activity of the enzyme.

2. The second method consisted of oxidizing the cellulose (ground stover) by NaIO_4 as above for obtaining 2,3 dialdehyde cellulose. A further oxidation for obtaining carboxylic acid was performed by NaClO_2 . One hundred ml NaClO_2 0.2 M was prepared by dissolving 1.81 g in 75 ml water and adding 20 ml acetic acid (5 M) and brought to 100 ml by H_2O . One g poly 2,3 dialdehyde cellulose was treated for 72 h in 50 ml of sodium chlorite 0.2 M at room temperature. The beads were then separated and rinsed in iced water. Fifty ml HCl (0.1M) at 0°C were then added for 30 min. The beads were separated and kept in iced water for 24 h to remove the acid residue. The beads were then separated and lyophilized. Distilled thionyl chloride was added to the particles in order to obtain the chloride. One g of particles were introduced into a 50 ml flask equipped with a condenser and CaCl_2 trap. One ml distilled thionyl chloride was added to the solution and refluxed for one h until all the SO_4 and HCl were depleted. The flask was cooled and connected to a condenser and an evacuated trap.

The SOCl_2 residue was distilled and its residue was removed by adding a few portions of benzene which was distilled to form azeotrope. Twenty ml of acetate buffer (50 mM, pH - 6.5)

containing 40 mg protein were then added to the beads. The mixture was shaken at 40°C for 4 h. At the end of the reaction the beads were rinsed, as described above for the other methods. The activity of b-glucosidase and protein content of the bound enzyme and the leachate were assayed as described elsewhere (Shoseyov et al. 1988).

2.5 Headspace Analysis

A syringe of 10 ml volume driven by a motor and controlled by an electronic circuit which enables automatic sampling was used. A scheme of the sampling device is presented in Fig. 9. The 10 ml headspace sample was cryogenically focused using a process which is not detailed in this report, and then injected to a GC (Varian 3700). The temperature of the FID detector was 230°C, and a 30 m long capillary column (0.53 mm I.D) DB-5 (J&W) was employed. GC-MS analysis of the headspace samples were conducted.

RESULTS

Isolation and Identification of Free and Bound Monoterpenes and of the Sugar component in the Glycosidic Fraction

The free and bound composition of 8 *Vitis Vinifera* cultivars was studied and 24 monoterpenes were identified by GC-MS. (The mass spectra and the empirical formulas are presented in Appendix 1.) The profiles of the free monoterpenes was found to be very similar to that of the bound in all cultivars (Fig. 10). There are however, considerable differences between profiles of different cultivars. Ten monoterpenes and 2 additional aroma compounds 2 benzyl alcohol and 2 phenyl ethanol - were selected for demonstrating the free and bound aroma profile of the 8 cultivars used in this study. Linalool was the major aroma compound in all the muscat varieties whereas geraniol was the highest in cv. Emerald Riesling. Sauvignon Blanc had high concentrations of a terpineol and geraniol whereas cv. Semillon was characterized by high concentrations of Trans pyran linalool oxide and geraniol. Among the muscat cultivars, Muscat Roy had a relatively high citronellol content. As a rule all the muscat cultivars contain higher concentrations of monoterpenes. Among the non muscat cultivars containing lower monoterpene

concentrations, Emerald Riesling had the highest monoterpenes concentrations - whereas French Colombard had the lowest. Among the muscat cultivars, Muscat of Alexandria had the highest and Muscat Frontignon, the lowest monoterpene content, linalool in particular. However, this cultivar had a relatively high bound monoterpene content particularly linalool and thus, a very high aroma potential. Terpen-diol 1 (3,7 dimethyl-1,5-octadien-3,7-diol), which is a precursor for a few aroma compounds, was found to be high in cv Muscat of Alexandria and Emerald Riesling, particularly in its free form, cv French Colombard had the lowest concentrations of this terpen (Table 2). Iso geraniol was found only in Muscat Roy, p-menth-1-en-9-ol was found in Emerald Riesling, Muscat Roy, Muscat of Alexandria and Sauvignon Blanc and not in French Colombard and Semillon. 3,7 dimethyl-1-octen-3,7-diol was found only in Sauvignon blanc and Muscat of Alexandria. Comparison of Figs. 10,11 shows that the monoterpene profile of musts and wines within each cultivar is similar and so are the profiles of the free and bound monoterpenes. These results are further strengthened by the fact that the wines were not always made from the corresponding musts. Fig. 12 shows the sugar composition and their anomeric structure of glycosides isolated from wines by HPLC using a C₁₈ reverse phase semi-preparative column. The fractions marked by X were analyzed by NMR (after deuteration) and then alditol acetates were prepared and were identified by GS-MS. The absorption spectra of all samples had a sharp peak at 210 nm (glycosidic bond). The quality of separation was confirmed by spectra taken at the beginning, middle and the end of each peak. Geranyl- β -rutinoside was synthesized and served as a marker in chromatographical separations of monoterpene glycosides and for determining the chemophysical properties of the enzyme. The NMR spectrum of the synthetic glycoside was compared to that of the free sugar (rutinose) and the aglicon (geraniol), and the synthetic geranyl- β -glucopyranoside (Table 3). The good correspondence between the chemical shifts of the glycosides and the compounds served for comparison proves the success of the synthesis. The high coupling coefficient (7.3 Herz) of the hydrogen in carbon No. 1 of the glucose proves the existence of the β -glucosidic bond between the rutinose and the geraniol. The NMR spectrum of

the rutinose shows the 2 doublets of the first hydrogen of carbon No. 1 of the glucose, one having a chemical shift of 4.59 ppm and a coupling coefficient of 7.9 Herz (b structure) and the second having a chemical shift of 5.18 ppm and a coupling coefficient of 3.8 Herz (a structure) since the reducing edge of rutinose performs a mutarotation in the D₂O solution. The NMR spectra of the various compounds are shown in Appendix 1.

From the NMR spectra (Table 3) it is evident that a doublet having a chemical shift of 4 ppm appeared in all cases corresponding to the Hydrogen in carbon No 1 of the glucose. The coupling coefficient of 7.3 Herz proves the existence of the b-glycosidic bond. In cv.'s Sauvignon Blanc (peak No. 1), Chardonnay (peak 2) and Muscat of Alexandria (peaks 2.6) a doublet with a chemical shift of 1.25 ppm corresponding to a methyl on carbon 6 in rhamnose (a typical spectrum is presented in appendix 1). All samples used for alditol acetate preparation contained 2,3,4 tri-o-methyl-rhamnitol-1,5-D acetate which proves the existence of rhamnose (pyran structure) bound through carbon 1 to the aglycon and through carbon No. 6 to rhamnose, namely to the disaccharide rutinose. In other cases, 2,3,5 three-o-methyl-arbitol-1,4-D-acetate was found which proves the existence of arabinose (in a Furan structure) bound through carbon No. 1. Here again is a disaccharide in which the arabinose is connected to glucose through carbon No. 6 of the glucose (The mass spectra of the alditol acetates and their retention times are presented in Fig. 18. In one case, alditol acetates were prepared from the entire glycoside fraction of Muscat Roy wine and in addition to the above mentioned sugars also 1,3,4,6 - tetra-o-methyl-mannitol-2,5-D-acetate was identified. This indicates the presence of mannose (pyran structure) bound through carbon No. 2.

Effect of Cultural Practices and Vinification Procedures on Monoterpene Content In the Must and on the Wine Quality

A positive correlation was found between the monoterpene content of the must and the aroma of the wine in non Muscat cultivars. Representative examples are presented in Fig. 14 for cv. French Colombard and Emerald Riesling. A positive correlation was found between

monoterpene content and wine aroma in Cabernet Sauvignon. Negative correlations were found between the free monoterpene content and the crop load, tannin content and pH and positive correlations between free monoterpene content, TSS and K. (data not shown).

Vinification Treatments

Fast heating of must reduced both the bound and the free monoterpene content probably due to oxidation. Lowering the pH to 3.1 reduced the bound monoterpene content and increased slightly the free monoterpenes. The pectolytic enzyme pectinol (Novo Inc.) did not affect the monoterpene content at all. A positive interaction between pH lowering and fast heating on the reduction of the bound monoterpene content was evident (Table 4).

Effect of Monoterpene Glycosides on Wine Bitterness

A duo trio sensory evaluation of glycosides isolated from Muscat of Alexandria wine by C₁₈ reverse phase column was conducted. Ten mg/l of glycosides were added to dry Sauvignon blanc wine or to pure water. A significant increase in bitterness was evident in both cases. When a synthetic geranyl- β -rutinoside was added, no increase in bitterness was evident. Furthermore, addition of an endo- β -glucosidase which efficiently hydrolized the glycosides isolated using C₁₈ reverse phase column did not reduce the bitterness. It seems therefore that the glycosidic fraction isolated by C₁₈ reverse phase column contains some impurities which contribute to a bitter taste.

Free and Bound Monoterpenes in Leaves and Berries Throughout the Season

In both varieties studied, Sauvignon Blanc and Muscat of Alexandria, the free and bound monoterpene content was higher in leaves than in the berries particularly at the beginning of the season (fig. 15).

The monoterpene content of both bound and free forms was higher in Muscat of Alexandria than in Sauvignon Blanc throughout the season. Despite the variations it is clear that both forms of monoterpenes increase continuously in the leaves and the berries throughout the season.

However, the free monoterpenes in the berries of Muscat of Alexandria peaked at 23° brix and pH 3.9 and then declined, whereas in Sauvignon Blanc they peaked at 15° brix and pH 3.03

before declining. It seems therefore that there is no necessary linkage between the sugar and monoterpene metabolism in the berries.

Monoterpene Transport from Leaves to Berries

When leaves were fed with ^{14}C mevalonic acid it was found that the radioactivity of the free and bound monoterpenes in the leaves dropped sharply from 12 h to 72 h following the feeding (Fig. 16). A concomitant increase in the glycosides content was found. The radioactivity in the free monoterpenes of the berries was stable throughout the experimental period whereas the bound have increased continuously. It seems therefore that monoterpenes are synthesized in the leaves and then transported to the berries in a bound form.

Monoterpene Glycosides Metabolism In *In Vitro* Grown Detached Berries

In order to study the metabolism of monoterpenes in the berries a method for *in vitro* growing of berries was developed. No one has so far reported such a method to the best of our knowledge.

The presence of the pedicel did not affect the level of contamination of the berries and has positively affected their viability (Table 5). Addition of auxin to the medium increased the viability in 3 out of 4 pairs of treatments. Dissection of the pedicel induced callus formation regardless of auxin addition. Addition of auxin to the medium caused callus formation only when the pedicel was not removed (Table 6). In berries with pedicels which were grown on auxin containing medium the pedicel was swollen due mainly to intensive growth of the parenchymatic tissue (Plate 4-1). The intensive growth of the parenchymatic tissue, as evident from the electron microscope pictures, formed pressures on the conducting tissue and a consequent burst (plate 1-5,6). Dipping of the fruitlets in sodium hypochlorite caused oxidation of the external tissue. Parenchymatic tissue emerged and continued to expand (plates 2,3) when the greater part of the skin split, berries which were partly split became lopsided due to the parenchymatic tissue growth (plate 4). Berries which did not split were green and regular and tripled their size within 60 days but did not reach a size of a normal berry (plate 5). Cell structure in the green, regular berries was normal, whereas the

split berries developed callus-like cells (plates 3,6) callus has emerged from the stylar end of all berries after a few weeks (plate 7). Berries which grew in a medium containing ^{14}C labelled free sugars and organic acids (without monoterpenes) have synthesized ^{14}C labelled monoterpene glycosides (plate 8). It seems therefore that berries are capable of synthesizing monoterpenes in addition to the monoterpene glycosides transported from the leaves.

Endo- β -Glucosidase from *Aspergillus niger*

Isolation and characterization

The β -glucosidase activity was found in both the homogenized mycelium and the filtrate of the media of the specific *A. niger* B1 grown on rutin or monoterpene glycosides as a sole carbon source (Table 7). The activity of the supernatant enzyme was higher when the fungus was grown on rutin than that of monoterpene glycosides but the sum of activities of the homogenate and the supernatant was similar for rutin and monoterpene glycosides media. The difference in the activity distribution might be attributed to the limited solubility of the rutin in water. However it is evident that rutin can serve as a cheap substitute for monoterpene glycosides. The enzyme was active at a wide range of pH with an optimum at 3.4 (Fig. 17), increasing the ionic strength of the substrate solution from 0.08 to 0.3 M citrate buffer reduced the activity. Standard activity assays were conducted at 20 mM at the range where the enzyme was highly active (Fig. 18). The enzyme was active at a wide range of temperatures with an optimum at 65°C (Fig. 19). Its stability was high and no activity was lost after one week at 50°C (Table 8). The half life time at 60°C was 2 h. Ethanol enhanced the activity of the enzyme at the entire range of 0–50% ethanol. Optimum activity was found at 9% ethanol, at this concentration the activity was doubled in comparison to control (fig. 20). The K_m of the enzyme was 1 mM for p-nitrophenyl- β -D-glycopyranoside (PNPG) as a substrate and 1.2 mM for geranyl- β -rutinoside indicating high affinity of the enzyme for the natural substrate. V_{max} was lower for geranyl- β -rutinoside as compared to PNPG (Table 8). Probably since the p-nitrophenyl separates easier than the monoterpene aglycon. nitrophenyl competitively inhibited the enzyme (Fig. 28, A,B), the K_i for glucose was 40 mM showing a low

affinity of the enzyme to glucose and to its ability to function in the presence of high concentrations of glucose (in the order of a few hundred mMolar). SO_2 at 200 ppm, (grape juices and wines contain SO_2 as a preservative at concentrations up to 100 ppm), fructose and sucrose at 1 M concentrations hardly inhibited the enzyme's activity whereas glucose at similar concentrations completely inhibited the enzyme completely (Fig. 22). Glucose at 0.25 M inhibited the enzyme by 81%, grape juice at 21° brix inhibited the enzyme completely whereas 1% juice inhibited the enzymes activity about 40%. It seems that most of the inhibition of the enzyme in the juice was due to the glucose since 25% juice contain 0.14 M glucose which corresponds to the same degree of inhibition (Fig. 22). The isoelectric focusing of the enzyme was $\text{pI} = 3.9$ from the zymogram of the β -glucosidase activity in isoelectric focusing, polyacrylamid gel electrophoresis (Plate 9). The molecular weight of the enzyme was 120 k Dalton as determined by gel filtration whereas in non denaturative gradient polyacrylamid electrophoresis gel a value of 168 k Dalton was determined (plate 10). The molecular weight of the subunits was assayed in a two dimensional electrophoresis, first isoelectric focusing and then SDS gradient polyacrylamid gel electrophoresis. Four identical subunits, 42 k Dalton each, were identified.

The enzyme was concentrated and purified by ultrafiltration. In this experiment 7.5 l filtrate were concentrated to 52 ml. Fifty percent of the total enzymes activity was lost but the specific activity of the enzyme increased from 9.43 to 245 mili units per mg namely the enzyme was concentrated 26 fold (Table 9).

Four different types of affinity chromatography were used (Fig. 23). Glucose served as a ligand in 3 cases whereas rutinose was used in one case. A and B are sepharose columns whereas C is a column on acrylic basis. The differences between the first couple is in the spacers length. Using ϵ Amino Caproic acid to better expose the ligand improved the separation capability but the best results were obtained by column C (*p*-aminophenyl- β -rutinoside bound to tyramin). An attempt to isolate the enzyme on sepharose with a caproic acid arm and *p*-amino- β -rutinoside ligand failed due to low affinity between the ligand and the enzyme). Various glycolitic activities of

the enzyme after ultrafiltration were assayed using glycosidic derivatives of *p*-nitrophenol as substrates (Table 10). It was found that the enzymatic preparation contains *b*-glucosidase activity, a rhamnosidase and α -glucosidase. A preference for degrading the pyran form over the furan form of α -arabinoside was evident probably due to the fact that the fungus was grown on rutin containing α -rhamnose in a furan form. Application of the enzyme on synthetic geranyl- β -rutinoside and the separation of the products enabled the identification of rutinose, glucose, rhamnose and geraniol (not shown) verifying the endo-glucosidic activity of the enzyme.

Immobilization of Mycelia for Enzyme Production In a Continuous Reactor and Characterization of the Appearance Time of an Acid Cystein Proteinase

The viability of the immobilized mycelia in Ca alginate beads was studied by adding rutin to the sodium alginate mixture thereby filling up the beads with yellow coloured chromophore of the quercetin. After 24 h of incubation the yellow colour disappeared and the beads became colourless and transparent due to the enzyme activity in the centre of each bead. In another experiment, rutin was added to a system containing beads with mycelia and rutin. After 24 h the medium was clarified and after additional 24 h the yellow color disappeared from the beads as well and the entire system became transparent (Plate 11). These results indicate that the enzyme is excreted out of the beads in spite of its large molecular weight (168 K D). A *b*-glucosidase activity was also found in medium samples of the continuous reactor. There is however still a possibility that the enzyme is excreted out of mycelia which broke away from the beads into the medium. Scanning electron microscope studies did not support this possibility and show a smooth layer of Ca alginate without any external mycelia. The advantage of the continuous reactor over the batchwise is illustrated in Fig. 24. The *b*-glucosidase activity peaked after 48 h in the batchwise reactor and declined to zero after a few additional hours. In the continuous reactor the activity increased rapidly to a constant level and levelled off for 10 days after which the experiment was terminated, due to technical reasons. The reasons for the reduction in the *b*-glucosidase activity in the filtrate were studied and it was found that the fungus excretes a proteinase when the level of

the rutin declines. The proteinase is not inhibited by phenyl methyl sulfonyl fluorine (PMSF) - a specific inhibitor for serin proteinases (Plate 13 4D) but rather by *p*-chloro argentum benzoic acid (PCMB) known to be a specific inhibitor of cystein proteinase (plate 14 EF). The proteinase was inactive at pH 6.0 and up. It may be concluded that the fungus excretes an acid cystein proteinase. A periodic sampling of filtrates from both batch and continuous fermentors revealed that proteolytic activity first appeared in the batchwise fermentation mode 48 h after the incubation and reached a maximum after 72 h (plate 13 A,B), whereas no proteolytic activity was found in the continuous reactor during the entire 10 days of the experimental period.

Immobilization of Glucosidase to Acrylic Polymer and to Cellulose

Comparison of the enzyme characteristics in various immobilized forms shows an advantage of the enzyme binding to the acrylic polymer over that of the cellulose. The immobilization of the enzyme to cellulose through cellulosic acid chloride had an advantage over the binding through gluteraldehyde. The k_m of the enzyme bound to cellulose (corn clover) via gluteraldehyde was reduced to 0.42 mMolar as compared to 1mMolar in the free enzyme and in the other immobilized forms of the enzyme. The immobilization to the acrylic polymer reduced the V_{max} whereas immobilization via acid chloride increased the V_{max} 8 fold as compared with the free enzyme. The pH optimum of the enzyme remained unchanged due to the immobilization and was fixed at 3.4. The enzymes stability at 60°C improved due to the immobilization and retained a full activity even after 4 h at 60°C.

Effect of the Immobilized Enzyme on Wine Quality

Two g of poloxyzyme-o-beads containing 20 mg b-glucosidase were added to one l of Muscat of Alexandria wine. The wine was shaken in an N atmosphere for 72 h at 25°C. A Freon 11 extract injected into a GC-MS revealed a considerable increase in linalool, terpen-4-ol, α -terpeneol, geraniol, Nerol and *cis* furan linalool oxide (Fig. 25).

Sensory evaluation showed an increase in the wine aroma and most of the panel tasters found that the wine aroma and taste were improved during the incubation with the enzyme. After

72 h the wine was filtered and the enzyme activity was assayed and found to be fully active.

Other Enzyme Activities

Enzyme addition to ultrafiltrated grape juice cv. Muscat of Alexandria for 24 h caused juice clarification (plate 15). This was the consequence of pectolytic activity according to enzyme activity assays we have conducted (not shown). Anthocyanin isolated from Cabernet Sauvignon wine using C₁₈ column was added to a Ca alginate immobilized fungus containing medium. After 24 h the red color disappeared due to anthocyanase activity (plate 14). No such activity was noted when extracellular filtrate was added. Thus, anthocyanase activity was evident only when the fungus itself was in contact with the medium. No significant reduction in color was noted when free or immobilized enzyme was added to red wine.

Headspace Analysis

Ten ml of head space gas phase were sampled from juices, wines and alcoholic beverages. This volume was injected into GC after cryogenic focusing. The details of the method for cryogenic focusing are not presented in this report. Some profiles of grape juice (Fig. 26). Cabernet Sauvignon wine (Fig. 27) and Remi Martin Cognac (Fig. 28) are presented. Some volatile compounds including monoterpenes were identified (data not presented).

Effect of the Endo- β -glucosidase on passion fruit juice

Passion fruit (*Passiflora edulis*) juice at 10°brix pH 2.45 was treated by 6 enzyme units per l for 24 h at 25°C. Four treatments were applied. 1. No SO₂ no enzyme. 2. No SO₂ plus enzyme 3 plus SO₂ (110 ppm) no enzyme 4. plus SO₂ plus enzyme. Sensory evaluation of the juice revealed improvement of the aroma. GC-MS analysis of Freon 11 extracts showed that the enzyme increased the content of the benzaldehyde and benzy alcohol. Benzaldehyde is known to be a product of cyanogenic glucoside hydrolysis whereas benzyl alcohol is an aromatic compound found in many fruit juices and wines. This enhancement in aroma compound occurred regardless of the presence of SO₂ (Fig. 29). SO₂ is a common preservative used in wines and juices and the insensitivity of the enzyme to these compounds is of commercial importance.

In another experiment where a higher sensitivity was achieved an increase in linalool (3.2 fold) was found in addition to benzaldehyde and benzyl alcohol (Fig. 30).

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HNMR Chemical shifts (200 MHz)
(ppm)

	Glu (C-1)	Rha (C-6)	Phe (C-2,6)	Phe (C-3,5)
p-Nitrophenyl-(⁶ -O- α -L-rhamnopyranosyl- β -D-glucopyranoside)	1H 5.34d [7.8]	3H 1.15d [6.2]	2H 7.24d [9.35]	2H 8.28d [9.25]
p-Nitrophenyl- β -D-glucopyranoside	1H 5.34d [7.9]	-	2H 7.23d [9.33]	2H 8.25d [9.33]

* Rutinose

[] = Coupling factor in Herz, d - dublet

Table 1: Chemical shifts and coupling factors of NMR spectrum of P-Nitrophenyl- β -Rutinoside and P-Nitrophenyl- β -D-Glucopyranoside.

The content of free and bound terpenediol-I in musts of several cultivars.
(μ gr/liter)

	Muscat of Alexandria	Muscat Frontignac	Muscat Roy	French Colombard	Semillon	Sauvignon Blanc	Emerald Riesling
Free	1377	223	301	3	86	257	1147
Bound	478	449	48	32	123	161	54
Total	1855	672	349	35	209	418	1201

Table 2. Free and bound Terpen Diol 1 in grape juices of various cultivars of the coastal area. Extraction of the free monoterpenes was done by Freon 11, Methylene chloride 9:1. Bound Terpen Diol 1 was assayed after enzymatic hydrolysis.

HNMR Chemical shifts (200 MHz)

(ppm)

	Rha (C-8)	Gln (C-1)	Ger (C-1)	Ger (C-2)	Ger (C-4,5)	Ger (C-6)	Ger (C-8)	Ger (C-9)	Ger (C-10)
6-O- α -L-rhamnopyranosyl-D-glucopyranose (rutinose)	3H 1.25d [6.1]	$\frac{1}{2}$ H 5.18d [3.8] $\frac{1}{2}$ H 4.59d [7.9]	-	-	-	-	-	-	-
2,6-Octadien-3,7-dimethyl-1-ol (s) (geraniol)*	-	-	2H 4.15d [7.0]	1H 5.42t	4H 2.06 m	1H 5.10 m	3H 1.69 s (abolder)	3H 1.61 s	3H 1.68 s
Geranyl- β -D-glucopyranoside	-	1H 4.42 [7.9]	2H 4.31 m	1H 5.38 m	4H 2.10 m	1H 5.14 m	3H 1.65 s (abolder)	3H 1.60 s	3H 1.68 s
Geranyl- β -rutinose	3H 1.25d [6.0]	1H 4.40d [7.3]	2H 4.28d [7.6]	1H 5.33 m	4H 2.09 m	1H 5.14 m	3H 1.66 s (abolder)	3H 1.59 s	3H 1.66 s

[] - Coupling factor in Hz

s - singlet

d - doublet

t - triplet

m - multiplet

* Geraniol dissolved in CDCl₃ and the spectrum was taken by Bruker 250 MHz.

Table 3. Chemical shifts and coupling coefficients in NMR spectra of Rutinose, Geranyl-b-D-Glucopyranoside and Geranyl-b-Rutinose. All compounds except Geraniol were duetured in 100% D₂O and the NMR spectrum were produced using a Brooker 200 MH.

	pectinol		pH		fast heating	
	without	with	3.6	3.1	without	with
main effect						
	696 (868)	519 (868)	506 (a1000)	714 (b736)	799 A (a1056)	417 B (b680)
with	-	-	434 (928)	605 (808)	577 (984)	462 (752)
pectinol						
without	-	-	570 (a1072)	823 (b664)	1020 A (a1128)	373 B (b608)
3.6	570 (1072)	434 (928)	-	-	557 (1072)	448 (928)
pH						
3.1	823 (668)	605 (808)	-	-	1041 A (a1040)	387 B (b432)
fast heating						
with	373 (608)	462 (752)	448 (a928)	387 (b432)	-	-
without	1020 (1128)	577 (984)	557 (1072)	1041 (1040)	-	-

Table 4. Effect of fast heating, pH adjustment and pectinol on free and bound Monoterpenes in Sauvignon Blanc juice. Free Monoterpenes (without parenthesis) bound Monoterpenes (in parenthesis). Values are μg linalool/l. $p=0.05$ letter separation for horizontal comparisons.

Table 5. Effect of IAA in the growing medium, immersion time in NaOCl and pedicel cut off viability and contamination of in vitro grown grape berries.

Immersion time (min)	IAA supplement	pedicel presence	contamination (%)	berry viability (green berries number out of twenty)
3	+	+	60	7
3	-	+	55	6
10	+	+	30	1
10	-	+	50	2
3	+	-	55	7
3	-	-	90	0
10	+	-	30	2
10	-	-	20	0

Table 6. Callus formation as affected by pedicel presence and IAA supplement

pedicel presence	IAA supplement	Callus formaiton (%)
-	+	11(19)*
+	+	82(22)
-	-	89(18)
+	-	91(23)

*The total number of berries from which the percent was calculated

Table 7. The effect of the carbon source on extra and endo cellular enzyme activity

Enzyme source	linalool equivalent mg/l
Supernatant of <i>A. niger</i> on monoterpene glycosides medium	7.26
Homogenate of <i>A. niger</i> on monoterpene glycosides medium	23.72
Supernatant of <i>A. niger</i> on a rutin medium	13.28
Homogenate of <i>A. niger</i> on a rutin medium	19.19

Fig. 17. Effect of pH on the Endo-b-Glucosidase activity.

Table 8. Physiochemical properties of *Aspergillus niger* endo β -glucosidase preparation

Property

K_M (PNPG)	1.0 mM
V_{max} (PNPG)	$0.22 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{prot}$
K_M (geranyl β Rutinoside)	1.25 mmol
V_{max} (geranyl β Rutinoside)	$0.08 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{prot}$
K_I (Glucose)	40 mM
Temperature activity optimum	65°C
Temperature stability half life at 60°	2hr
activity after 7 days at 50°	100%
Molecular weight	120,000
pH activity optimum	3.4
Isoelectric point	pI=3.9
Activity between pH 1.5-5	>50%
Activity at 12°C	>5%
Activity in the presence of 0-50% ethanol	>150%
Activity in the presence of 1M sucrose	90%
Activity in the presence of 1M Fructose	90%
Activity in the presence of 200ppm SO_2	100%

Table 9. Concentration and purification of β -glucosidase by 20,000 mol wt cut-off ultrafiltration membrane.

	Filtrate volume (ml)	Total activity (U)	Recovery (%)	Total protein (mg)	Specific activity (U/mg prot)	Purification factor (folds)
Initial	7500	14.50	100	1537.5	9.43	—
Final	52	7.23	50	29.4	246	26

U = μ mol p-nitrophenol / min

Table 10. Specific activity of enzyme preparation with different substrates

Substrate	Activity mU/mg protein
*PNP- β -D-glucopyranoside	680
PNP- α -D-glucopyranoside	20
PNP- α -L-rhamnopyranoside	1050
PNP- α -L-arabinofuranoside	2250
PNP- α -L-arabinopyranoside	1200
**Geranyl- β -rutinoside	80
(6-O- α -L-rhamnopyranosyl- β -D-glucopyranoside)	—

*PNP=p-Nitrophenyl

**Pico mol geraniol/min / mg protein

 Immobilization to

Property	Free	Polexyzyme	Corn stover* A	Corn stover* B
Protein immobilization (%)	–	82	<1	5.4
Activity immobilization (%)	–	10	<1	5.4
K_M (mM)	1.0	1.0	0.42	1.0
V_{max} (**U/mg prot)	0.22	0.16	0.11	1.67
Activity after 2h at 60°C (%)	50	100	–	100
Optimal pH activity (pH) [–]	3.4	3.4	–	3.4

*A – Immobilization using glutardialdehyde.

*B – Immobilization using acid chloride cellulose.

**U – μ mol p-Nitrophenol/min. p-nitrophenyl- β -D-glucopyranoside as a substrate.

Table 11. Effect of the immobilization method on binding efficiency and the chemophysical properties of the enzyme.

Fig. 1. GC chromatogram of Freon 11 extract (liquid-liquid) of Emerald Riesling.

H.N.M.R.

GERANYL- β -RUTINOSIDE

H.N.M.R.

p-NITROPHENYL- β -RUTINOSIDE

Fig. 3. Mass spectrum of synthetic *cis* and *trans* Pyran and Furan Linalool oxides.

Fig. 4. GC chromatogram and mass spectrum of Iso geraniol. 1-Geraniol 2-Iso Geraniol 3-Geraniol.

Fig. 5. Device for fast heating of grape juice. a-Grape juice. b-peristaltic pump. c-Heat exchanger. d-Water bath (90°C) e-Water bath (30°C) f-Grape juice.

Fig. 6. Feeding of ^{14}C Mevalonic acid through the main vein of grape blades.

Fig. 7. Chemical structures of the compounds used for filling the affinity chromatography columns.

I - *p*-Aminophenyl- β -D-Glucopyranoside sepharose. II- *p*-Aminophenyl- β -D-Glucopyranoside *e*-Amino Capriol-Sepharose. III *p*-Aminophenyl- β -Rutinoside *e*-Amino-Caproyl-Sepharose. IV- *p*-Aminophenyl- β -D-Glucopyranoside-Tyramin-“Upergit C”

Fig. 8. Production of β -glucosidase in a continuous reactor by *A. Niger B-1* mycelium immobilized in Ca Alginate beads.

A - Aquarium pump. B - Flowmeter. C - Air filter. D - Airlift fermentor. E - Peristaltic pump. F - Magnetic stirrer. S - Substrate container. II - Fraction collector.

Fig. 9. Schematic drawing of sampling and injecting device for GC headspace analysis.

Fig. 10 Free and bound monoterpenes following enzymatic hydrolysis of grape musts from the coastal area. 1. Cis-furan linalooloxide. 2. Trans furan linalool oxide. 3. Linalool. 4. Hotrienol. 5. α -Terpineol. 6. Cis firan linalool oxide. 7. Trans firan linalool oxide. 8. Citronellol. 9. Nerol. 10. Geraniol. 11. Benzyl alcohol. 12. 2 phenylthanol.

Fig. 11 GC-MS analysis of bound wine monoterpenes after enzymatic hydrolysis. I. Muscat of Alexandria. II. Muscat Roy. III. Emerald Riesling, IV. French Colombard. V. Semillon. VI. Sauvignon blanc.

-
1. Trans-furan-Linalooloxide. 2. cis-furan-Linalooloxide. 3. *U.I. monoterpene. 4. U.I. monoterpene. 5. Linalool. 6. Hotrienol. 7. P-Menth-1-en-9-ol. 8. α -Terpineol. 9. U.I. monoterpene. 10. trans-pyran-Linalooloxide. 11. Cis-pyran Linalooloxide. 12. Citronellol. 13. U.I. monoterpene alcohol. 14. U.I. monoterpene. 15. Nerol. 16. Iso-geraniol. 17. U.I. monoterpene. 18. Geraniol. 19. U.I. monoterpenediol. 20. Benzyl alcohol. 21. 2-Phenyl ethanol. 22. U.I. monoterpene. 23. 3,7-Dimethyl-1,5-octadien-3,7-diol (terpenediol I). 24. 1,8-Terpin. 25. 3,7-Dimethyl-1-octen-3,7-diol. 26. U.I. monoterpenediol. 27. 3,7-Dimethyl-2,7-octadien-1,6-diol.

*U.I. - unidentified.

III - אמרלד ריזלינג

IV - פרנש קולומבר

Fig. 12 Chromatograms and absorption spectra in UV of Monoterpene Glycosides isolated from wine by HPLC using a semi preparative C₁₈ column. I. Muscat of Alexandria. II-Emerald Riesling. III-Sauvignon blanc. IV-French Colombard. V-Chardonnay. The peaks marked by X were collected for HNMR and GC-MS

Fig.13. GC-MS analysis of alditol acetates of the glycosidic fraction of Muscat Roy wine.

Fig. 14. The relationship between free Monoterpene content of juice and sensory evaluation score of the wine. I-Emerald Riesling. II-French Colombard.

Fig. 15, Free and bound monoterpenes in leaves and berries and pH and *Brix of berries throughout the season:

A - Muscat of Alexandria, B - Sauvignon blanc.

Fig. 16. Radioactivity of free and bound terpenoids isolated from leaves and berries as a function of time after ^{14}C mevalonic acid feeding into leaves.

Fig. 18. Effect of ionic strength on the Endo-b-Glucosidase activity.

Fig. 19. Effect of temperature on the Endo-b-Glucosidase activity.

Fig. 20. Effect of Ethanol on the Endo-b-Glucosidase activity

Fig. 21. Lineweaver-Burk plot for determining the Endo-b-Glucosidase inhibition by Glucose A- $f(1/c) = 1/V_o$ B $f(c) = \text{slope}$ $c = \text{Glucose concentration}$, $V_o = \text{initial velocity}$

Fig. 22. Effect of grape juice, Glucose, Sucrose and Fructose on the enzyme activity

Fig. 23. Purification of the Endo- β -Glucosidase using affinity chromatography

Fig 24. Activity of endo β -glucosidase in continuous and batchwise mode fermentation as a function of time. Both systems contained A. Niger B-1 mycelium immobilized in Ca Alginate beads.

Fig. 25 Effect of immobilized (to polyezyme) endo β -glucosidase on the free monoterpene content of Freon 11 extract A - Enzyme treated. B - Control. Muscat Roy wine.

Fig. 26. Headspace analysis of Muscat of Alexandria juice.

Fig.27. Headspace analysis of Cabernet Sauvignon wine

Fig.28. Headspace analysis of Remi Martin Cognac.

Fig. 29. Effect of endo β -glucosidase and SO_2 on free volatiles in passion fruit juice (*passiflora edulis*). 1. Unidentified. 2. Internal standard (3 oc-

Fig.30. Effect of endo β -glucosidase on the aroma profile of passion fruit. 1 - Benzaldehyde. 2 - Internal standard (3 - octanol). 3 - Linalool. 4 - Benzyl alcohol.

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H.N.M.R.

RUTINOSE

H.N.M.R.

Sauvignon Blanc

NQ 1

m-Terpineol
C₁₀H₁₈O (154)

Plate 1: Effect of Auxin added to the medium on the anatomical structure of the pedicel of in vitro grown berries. Scanning Electron Microscope pictures of non treated (1,2,3) and treated (4,5,6) pedicels.

Plate 3: SEM picture of a splitted in vitro grown berry.

1

Plate 6: Electron Microscope picture of a dissected in vitro grown berry after 60 days in culture.

Plate 7: Callus outgrowth from the stylar end of a berry grown in vitro.

Plate 8: Radioactivity of Monoterpene Glycosides isolated by C₁₈ column from in vitro grown berries and separated on TLC (silica gel).
(1) Rutinose; (2) Glycosidic fraction isolated on C₁₈ column;
(3) Synthetic Geranyl- β -Rutinoside; (4) Geraniol.

Plate 11: Ca Alginate beads containing *A. Niger* B-1 mycelium and Rutin.
1-zero time. 2-After 24h. 3-After 48h.

Plate 9: Isoelectric focusing polyacrylamide gel electrophoresis of the Endo- β -Glucosidase. Right Coomassie Brilliant Blue 250-R protein staining, left X-Glue zymogram.

Plate 10: Molecular weight determination of Endo- β -Glucosidase by non-denaturing polyacrylamide gel electrophoresis. Right Coomassie Brilliant Blue 250-R protein staining, left X-Glue zymogram.

Plate 13: Proteinase assay on Casein-Agar gel (pH=3.4). A - Sampled after 96 h. of batchwise fermentation mode. B - Sampled after 48 h. of batchwise fermentation mode. C - As in A but with Serin Proteinase inhibitor (PMSF) dissolved in Dimethyl Formate. D - As in A but with Dimethyl Formate only. E - As in A but with Cysteine Proteinase inhibitor (pcmb) dissolved in 1mM NaOH. F - As in A but with 1mM NaOH only. The final concentration of all inhibitors was 2mM.

Plate 12: Scanning Electron Microscope picture of an immobilized Ca Alginate mycelium containing bead.

F. Description of Cooperation

The work of the two groups was very complementary. Both groups worked independently using different approaches to evaluate the overall objectives. Bravdo visited the USA several times, but Noble was unable to visit Israel. The two researchers were in contact by electronic mail. PhD student Shoseyev (supported by this BARD grant in Israel) communicated with the MS student Park (supported by the BARD grant in the USA). Having the very different exercises available for reference in the two countries is beneficial to this project, where no one approach will provide answers. In working with flavor in an agricultural crop, often it is the interaction of different projects that suggests new directions.

G. Evaluation of the Research Achievements

U.S.A.

The objectives of this study were to evaluate factors affecting terpene accumulation and evaluate the contribution of monoterpenes to wine flavor. To follow the accumulation and localization of free and bound terpenes, Muscat of Alexandria and Symphony grapes were examined. During maturation, terpenes accumulated in the bound form, at levels 6 - 10 times those of the free compounds. Although the terpenes are reported to be synthesized in plastids in the epidermis, roughly equal distribution of the free and bound terpenes were observed between the skin and the mesocarp.

Some fluctuations in total concentrations during development of grapes were observed and correlate with climatic changes. High temperature and low humidity during one interval is suggested to have interfered with terpene synthesis. Despite a decrease in berry volume, the concentrations of several bound terpenes decreased following several days above 100F, suggesting the importance of environment. Symphony, a cross between Muscat of Alexandria and Grenache gris, had a very similar pattern of terpene accumulation and distribution to Muscat.

On fermentation of wine and on subsequent storage, the bound terpenes hydrolyzed to provide higher levels of the free terpenes, which contribute to the distinct, floral aroma of Muscat and related wines. While the terpene glycosides were shown to not be responsible for the relatively high intensity of bitterness in Muscat, the contribution of hydrolyzed terpenes to wines low in initial terpene content increases the intensity of the floral character. As demonstrated in studies both in Israel and the USA, β -glucosidase enzymes may play a role in enhancing the aroma of varieties, containing terpene glycosides.

G. Evaluation of the Research Achievements

ISRAEL

Objective 1 was achieved though not completed. Profiles of bound and free monoterpenes were established and the similarity of their pattern found, strengthened the approach consisting of utilizing the bound monoterpenes for aroma enrichment of wines and juices of Muscat as well as non Muscat varieties. The methodologies and instrumentation developed enable us to extend the study for various grapes and wines originating at various climatic regions.

Objective 2 was achieved even beyond the expectations. Studies of various treatments such as flash heating, pH adjustment and commercial pectinol did not yield significant improvements. On the other hand we succeeded in producing an endo- β -glucosidase which hydrolyzes monoterpene glycosides in juices and wines. The development of the enzyme required heavy investments and the key foundation contributed to this subject. The enzyme was patented and is now on the way to commercial utilization.

Objectives 3, 4 were not achieved due to limitation imposed by BARD's reviewing committee. They recommended concentrating on laboratory studies only.

Objective 5 was achieved to a certain extent. $^{14}\text{CO}_2$ and ^{14}C Mevalonic acid feeding experiments revealed that monoterpene glycosides are biosynthesized in the leaves and are transported to the berries. In addition a method for in vitro growing of berries developed in this project enabled us to prove that the berries are capable of biosynthesizing monoterpene glycosides.

Objective 6 was only partially achieved. A positive correlation between aroma sensory evaluation and free monoterpenes was found. Head space analysis system developed in our lab (patent pending) enabled us to collect considerable information concerning aroma profiles of wines and musts. Not enough data are yet available for utilizing this fast method for the characterization of wines and juices of various cultivars and climatic regions.

Benefit to Agriculture

This project resulted in significant contribution to Agriculture and to the wine industry. The endo- β -glucosidase developed may greatly improve the quality of Muscat and non Muscat varieties particularly in hot regions. This is of course a direct contribution to winegrape growing and the wine industry.

The methodologies developed in this project open new possibilities for further contribution. Cultural practices which enhance the biosynthesis of bound monoterpenes may now have a greater importance than the production of free monoterpenes, particularly under warm climatic conditions. It is evident that the specific endo- β -glucosidase enriches wine aroma not only of monoterpenes but of other aglycons such as benzyl alcohol and 2-phenylethanol.

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