Journal of Chromatography, 235 (1982) 471–480
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,327

USE OF C_{18} REVERSED-PHASE LIQUID CHROMATOGRAPHY FOR THE ISOLATION OF MONOTERPENE GLYCOSIDES AND NOR-ISOPRENOID PRECURSORS FROM GRAPE JUICE AND WINES

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(Received August 31st, 1981)

SUMMARY

Glycosidic derivatives of monoterpene flavourants of grapes and wines can be preparatively isolated by selective retention on a C_{18}-bonded reversed-phase adsorbent. These components can thus be concentrated by a factor of 20,000 in a single chromatographic step. Class separation of monoterpene glycosides at different oxidation levels can also be achieved on the reversed-phase adsorbent.

The process has also led to the discovery of precursors of 2-phenylethanol, benzyl alcohol, damascenone, vitispirane and 1,1,6-trimethyl-1,2-dihydronaphthalene. Additionally, many other related nor-isoprenoid compounds not previously known in grapes have been observed.

INTRODUCTION

It is now understood that terpene flavourants of grapes are derived from a pool of non-volatile precursor compounds in the fruit[1]. Indirect evidence suggested that these compounds are glycosidic derivatives of monoterpenes, although not simply β-D-glucosides[2]. Recent investigations in this laboratory have confirmed this proposal[3]. During the course of research on this topic techniques for the isolation of terpene glycosides in amounts sufficient for structural elucidation have been required.

Glycosides of non-iridoid monoterpenes have been recognised relatively recently as plant constituents and a variety of techniques have been used to isolate these hydrophilic compounds. Croteau and Martinkus[4], after lyophilizing aqueous leaf extracts, used thin-layer chromatography (TLC), ion-exchange chromatography and gel permeation chromatography to purify neomethyl-β-D-glucoside. Francis and Allcock[5] isolated β-D-glucosides of geraniol, nerol and citronellol from aqueous extracts of rose petals by solvent extraction followed by silica gel chromatography and

0021-9673/82/0000–0000/$02.75

preparative thin-layer electrophoresis. Subsequently, Banthorpe and Mann[6] applied the same techniques to petals of *Tanacetum vulgare*. Similarly, Sakata and Mitsui[7], Bohlmann and Grenz[8], and Tschesche *et al.*[9] extracted plant material directly with organic solvents and, after clean-up of the extracts on silica gel, conventional chromatographic procedures were used to isolate β-D-glucosides and derivatives of several monoterpenoids.

Unfortunately these techniques were either inappropriate or found to be unsuitable for the isolation of monoterpene glycosides from grape juice and wines. In the case of grape juice, the presence in aqueous solution of large amounts of glucose and fructose, together with other free sugars, makes the problem of separation of small amounts of glycosides extremely difficult. On the other hand, the isolation from wines in which most of the sugars have been removed by fermentation, is also complicated by the presence of glycerol and the isomeric butane-2,3-diols. Several techniques were tried unsuccessfully, including solvent extraction and subsequent TLC, gel filtration of both juice and wines and ion-exchange chromatography of juice on a column of cation exchanger in the calcium form[10] and on an anion exchanger in the bisulphite form[11,12].

Eventually it was found that chromatography of juice or dealcoholised wine on a C_{18}-bonded reversed-phase adsorbent allowed separation of monoterpene glycosides free from other polar components, *i.e.* sugars, organic acids, glycerol, etc. The latter components were totally eluted from the column with water. This technique has also facilitated the separation of grape glycosidic precursors of monoterpenes at the linalool oxidation level from those at the higher linalool oxide oxidation state. Furthermore, precursors of the grape and wine volatile constituents, vitispirane[13], 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN)[14] and damascenone[15,16] have also been isolated by C_{18} reversed-phase chromatography. It is now recognised that these last components co-occur with a number of other thirteen-carbon constituents not previously identified in grapes.

EXPERIMENTAL

Isolation of isoprenoid precursors on reversed phase

Juice or dealcoholised wine was peristaltically pumped down a glass column (470 × 15 mm I.D.) containing C_{18} reversed-phase adsorbent (Applied Science Labs., State College, PA, U.S.A.; Hi-flosil, 80–100 mesh) (40 g) at rates up to 400 ml/h, depending on the viscosity of the solution. After loading, the column was flushed with a volume of water equal to three times the volume of the applied sample. Retained material was then eluted with methanol (250 ml), and this fraction concentrated to dryness *in vacuo* and stored in an evacuated desiccator over KOH pellets. In a typical experiment 100 mg of concentrate was obtained from 2 l of grape juice.

Separation of isoprenoid precursors on reversed phase

A concentrate (*ca.* 50 mg), prepared as described above, was dissolved in water (*ca.* 10 ml) and peristaltically pumped, at 50 ml/h, down a glass column (80 × 5 mm I.D.) containing C_{18} reversed-phase adsorbent (3.5 g). The column was flushed with water (25 ml), 20% aqueous acetic acid (15 ml), water (25 ml), 30% aqueous acetic acid (15 ml), water (25 ml) and methanol (25 ml). The 30% aqueous acetic acid

fraction and subsequent water wash were pooled, concentrated *in vacuo* and stored as above. The methanol eluate was similarly concentrated and stored.

Preparative isolation

For the isolation of linalool oxidation state glycoside precursors used in structural elucidation studies[3], the above processes were combined.

Muscat of Alexandria grape juice was centrifuged (16,500 *g*, 15 min) and the clear supernatant (15.75 l) passed down the larger C_{18} reversed-phase column. The methanol eluate, after evaporation, was then rechromatographed on the small column of the bonded phase. Acetic acid fractions from the second column, after removal of solvent, were set aside for future study. The residue, eluted from the small column with methanol, was concentrated to give highly active material (100 mg) which yielded predominantly linalool oxidation state monoterpenoids on hydrolysis.

Hydrolysis of fractions

Dried samples (*ca.* 1 mg) of 30% acetic acid fraction or methanol fraction from the reversed-phase columns, were taken up in water (2 ml). Each sample was adjusted to either pH 1 or pH 3 (glass electrode) with 1% aqueous perchloric acid. The solutions were washed with cold Freon F11 (2 × 15 ml) to ensure removal of any volatiles prior to hydrolysis. Then each acidified solution was heated on a steam bath at 100°C for 20 min, cooled, and re-extracted with cold Freon (2 × 15 ml). The organic extracts of the hydrolysates were made up to 30 ml and one half of this taken and concentrated as previously described[17] for analysis by gas chromatography (GC) or GC–mass spectrometry (MS).

GC and GC–MS

Analytical GC was performed on a Perkin-Elmer Sigma 2 instrument using a glass support-coated open tubular (SCOT) column (96 m × 0.5 mm I.D.) of SP-1000 liquid phase on Chromosorb R support. The column was operated isothermally at 50°C for 10 min and then programmed at 1°C/min to 180°C and held at the upper temperature for 20 min. Nitrogen carrier gas was used at a linear velocity of 19 cm/sec.

GC–MS analyses were made on a Finnigan 4021 GC–MS data system. The chromatograph was equipped with a SP-1000, glass SCOT column (105 m × 0.5 mm I.D.) with helium as carrier gas at a linear velocity of 39 cm/sec. Injections were made with a 10:1 split at an injector temperature of 250°C. The column was held at 60°C for 10 min, programmed at 1°C/min to 180°C and held at this temperature for 20 min. Electron impact mass spectra were taken at 70 eV, scanning upwards from m/z 35 to m/z 350 each second, with a 0.1-sec delay between each scan.

RESULTS AND DISCUSSION

Clarified juice from Muscat of Alexandria grapes was passed through a column packed with C_{18} reversed phase. Hydrolysis of the eluent did not produce volatile monoterpenes thus confirming the absence of monoterpene precursors[1] in the column effluent. Water washing of the bonded phase did not elute any of the precursors, indicating efficient retention of these compounds by the C_{18} reversed phase. However,

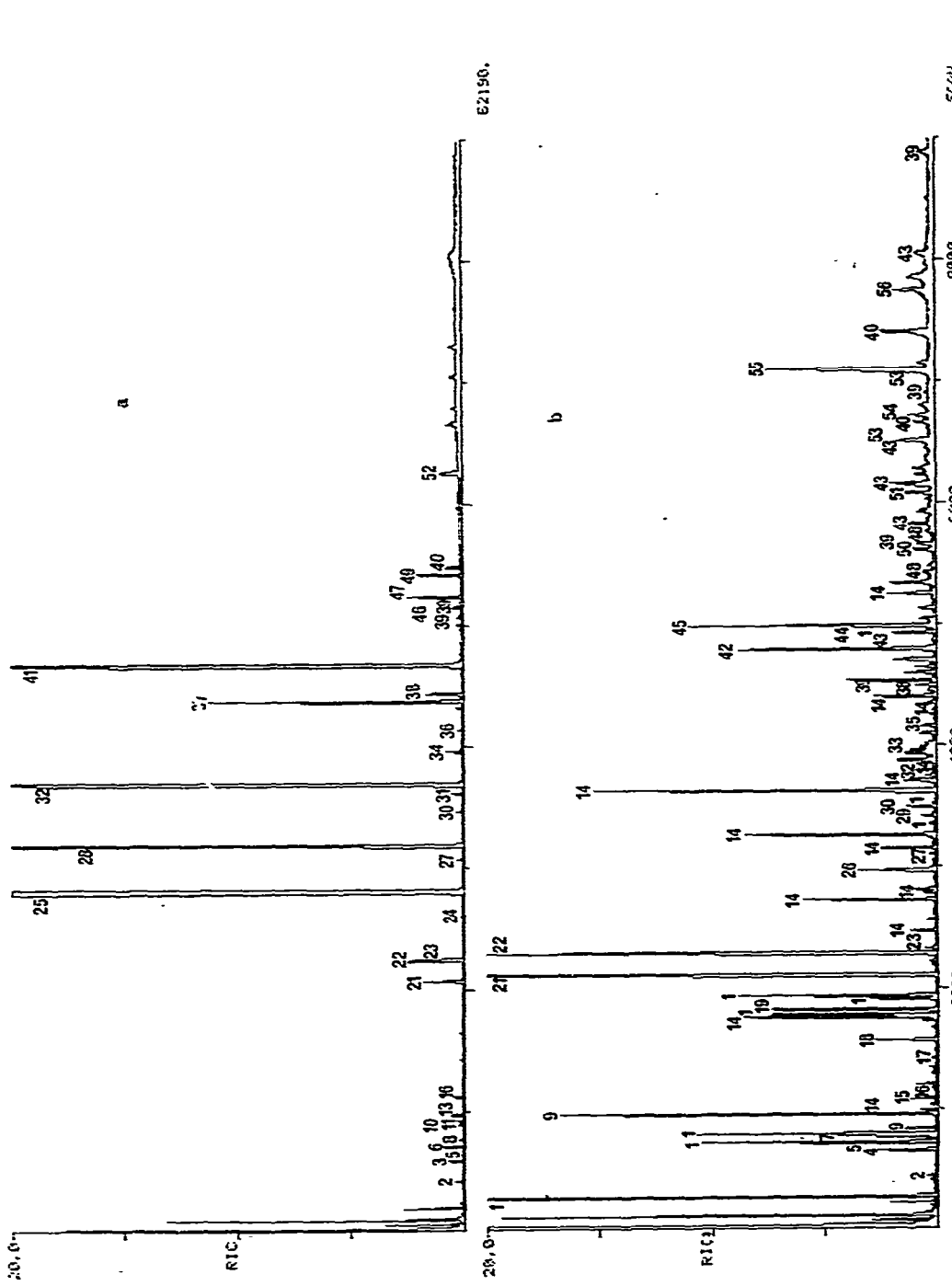


Fig. 1. Reconstructed ion chromatogram from the GC–MS data system of Freon extracts of the total material selectively retained by C_{18} reversed phase from Muscat of Alexandria grape juice, after elution with methanol, and (a) hydrolysis at pH 3, (b) hydrolysis at pH 1. Peak identities are in Table I and GC–MS conditions in the Experimental section.

TABLE I

COMPOUNDS IDENTIFIED IN CHROMATOGRAMS SHOWN IN FIGS. 1 AND 2

Peak No.	*Assignment**	*Evidence for assignment***	*Refs.*
1	A monoterpenoid at the linalool oxidation state		
2	2,6,6-Trimethyl-2-vinyltetrahydropyran	A	17
3	Myrcene	B,C	18,19
4	1,4-Cineole	B,C	20,21
5	α-Terpinene	B,C	18,19
6	Limonene	B,C	18,19
7	1,8-Cineole	B,C	20,21
8	*trans*-5-Isopropenyl-2-methyl-2-vinyltetrahydrofuran	A	17
9	An isomeric 2,2-dimethyl-5-(1-methylpropenyl) tetrahydrofuran	A	17
10	*Z*-Ocimene	B,C	18,21
11	*cis*-5-Isopropenyl-2-methyl-2-vinyltetrahydrofuran	A	17
12	γ-Terpinene	B,C	18,19
13	*E*-Ocimene	B,C	18,21
14	A monoterpenoid at the linalool oxide oxidation state		
15	*p*-Cymene	B,C	18,21
16	Terpinolene	B,C	18,19
17	A trimethylbenzene	D	
18	*n*-Hexanol	A	14
19	3,5,5- or 2,6,6-Trimethylcyclohex-2-enone	B	21,22
20	4-Isopropenyltoluene	D	
21	*trans*-Furan linalool oxide	A	17
22	*cis*-Furan linalool oxice	A	17
23	Nerol oxide	A	17
24	Isomeric vitispiranes	A	13
25	Linalool	A	17
26	Terpinen-1-ol	B	21
27	Terpinen-4-ol	A,B	21
28	Hotrienol	A	17
29	A trimethyltetrahydronaphthalene	B,D	23
30	*Z*-Ocimenol	A	17
31	*E*-Ocimenol	A	17
32	α-Terpineol	A	17
33	1,1,6-Trimethyl-1,2-dihydronaphthalene	A	14
34	*trans*-Pyran linalool oxide	A	17
35	2,2,6-Trimethylcyclohexane-1,4-dione	B	21
36	*cis*-Pyran linalool oxide	A	17
37	Nerol	A	17
38	Damascenone	A	24
39	A C_{13} nor-isoprenoid compound at the damascenone oxidation level		
40	An unknown nor-isorpenoid		
41	Geraniol	A	17
42	Benzyl alcohol	A	21
43	A C_{13} nor-isorpenoid compound at the vitispirane oxidation level		
44	An unknown C_{14} nor-isoprenoid hydrocarbon		
45	2-Phenylethanol	A	14
46	*Z*-2,6-Dimethylocta-3,7-diene-2,6-diol	B,D	25

(Continued on p. 476)

TABLE I *(continued)*

Peak No.	*Assignment**	*Evidence for assignment***	*Refs.*
47	*E*-2,6-Dimethylocta-3,7-diene-2,6-diol	A	25
48	A 1,16-Trimethyldihydronaphthalene	B,D	23
49	3,7-Dimethyloct-1-ene-3,7-diol	A	25
50	Megastigma-4,7,9-trien-3-one	B	26
51	*Z*-4-(2,3,6-Trimethylphenyl)but-3-en-2-one	B,D	27
52	3,7-Dimethylocta-1,7-diene-3,6-diol	A	25
53	A megastigma-4,6,8-trien-3-one	B	26
54	4-(2,3,6-Trimethylphenyl)butan-2-one	B	27
55	*E*-4-(2,3,6-Trimethylphenyl)but-3-en-2-one	B	27
56	4-(2,3,6-Trimethylphenyl)butan-2-ol	B	28

* Many components from the hydrolyses in Figs. 1 and 2 remain unidentified, and where no interpretative information from the mass spectrum was available, peaks were left unassigned. However, where the class of compounds could be distinguished, it has been so designated.

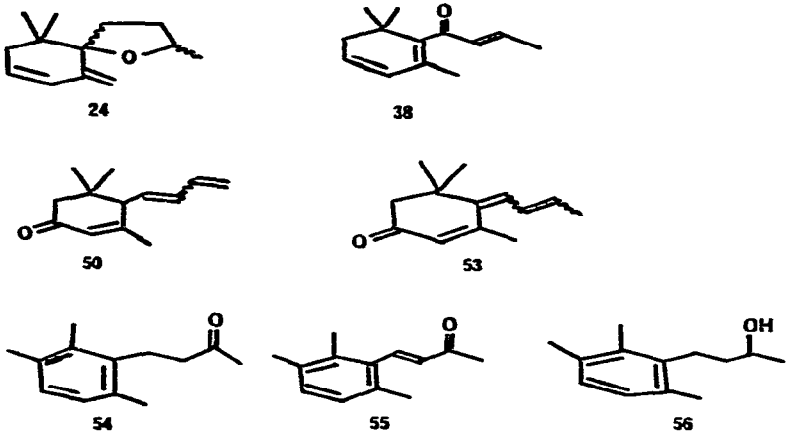
** A = proven previously in this laboratory by spectral and chromatographic comparison with reference material; B = mass spectrum consistent with that of published data; C = retention time consistent with that of published data; D = tentative assignment based on similarity of mass spectral data with those of related compounds.

the washing procedure did remove all the major interfering juice constituents, *i.e.* free sugars and organic acids. Finally, elution of the column with methanol yielded a fraction which, on solvent removal and desiccation, gave a dark residue. This material, after dissolving in water and Freon extraction, was found to contain no free terpenoids or other volatile compounds. Hydrolysis at pH 3, followed by extraction and GC analysis, demonstrated that the C_{18} reversed-phase material liberated monoterpenes with a pattern similar to that seen in whole juice[17] (see Fig. 1a). Thus precursors of those monoterpenes which occur free in muscat juice were isolated and concentrated by a factor of typically 20,000. Furthermore, examination of the products indicated that compounds at both the linalool and linlool oxide oxidation levels were present. This implied that more than one monoterpene precursor was in the juice initially.

The important nor-isoprenoid grape volatiles vitispirane (24)[13] and damascenone (38)[16] were also identified in this pH 3 hydrolysate of the C_{18} reversed-phase material (see Table I). Previous work[29] had shown that vitispirane concentration increased with maturation in wines. Vitispirane formation appeared to be hydrolytic rather than oxidative, implying the presence of a precursor of this compound in grapes and wines. Similarly, work by Masuda and Nishimura[15] indicated that damascenone (38) also has a precursor in grapes, but this latter compound has remained unisolated. The present study has revealed an additional pair of isomeric compounds (peaks 39 in Fig. 1a) related to the above C_{13}-constituents. These unknown new components, which were detected in this pH 3 hydrolysis, have also been observed in heated muscat grape juice headspace samples[30].

The extent of occurrence of these C_{13} and related nor-isoprenoid grape con-

stituents was not apparent however until the C_{18} reversed-phase fraction was hydrolysed at pH 1 (Fig. 1b). Extraction and GC analysis showed that a significant proportion of the volatiles produced was of this nor-isoprenoid category (Table I). Whilst some of these compounds have only been tentatively identified at present, excellent MS evidence for the isomeric megastigmatrienones (50) and (53)[26] and the aromatic compounds (54)[27], (55)[27] and (56)[28] was obtained. In addition, the presence of hydrocarbon TDN[14] was confirmed and other closely related trimethyl dihydronaphthalenes indicated (peaks 48), along with what appears to be a C_{14} homologue (peak 44). These nor-isoprenoid compounds are usually regarded as degradation products of carotenoids[31] and such a genesis may well hold in grapes. Nevertheless, it is possible that the formation of aromatic compounds like TDN, ketones (54) and (55) and alcohol (56) involved extensive rearrangement under acidic conditions and their presence may not be diagnostic of specific precursors.



Work on these grape nor-isoprenoid compounds and their precursors, as well as the mechanisms by which the volatile constituents listed in Table I are derived from their precursors, is in progress. It will be particularly interesting to determine if the grape nor-isorpenoid precursors are glycosidic derivatives similar to those recently isolated from tobacco[32].

In addition to the nor-isoprenoid compounds in the reversed-phase material, other constituents, such as 2-phenylethanol and benzyl alcohol, were also liberated from non-volatile precursors by hydrolysis at pH 1. These compounds, which are often abundant in wines[33], are now recognised as having a derivation from grapes directly.

Significant differences can be seen in the pattern of volatile monoterpenoids produced from the reversed-phase material by hydrolysis at pH 1 and 3. It is apparent that the more acidic conditions bring about extensive rearrangement of monoterpenoids at both oxidation levels. Many of these rearrangement products have yet to be firmly identified. The influence of hydrolytic conditions on the pattern of volatiles obtained from grape monoterpene glycosides will be the subject of a separate study.

To examine further the potential of the C_{18} reversed-phase chromatographic technique to fractionate precursors of grape flavourants, material concentrated from Rhine Riesling grape juice by the method described above was rechromatographed

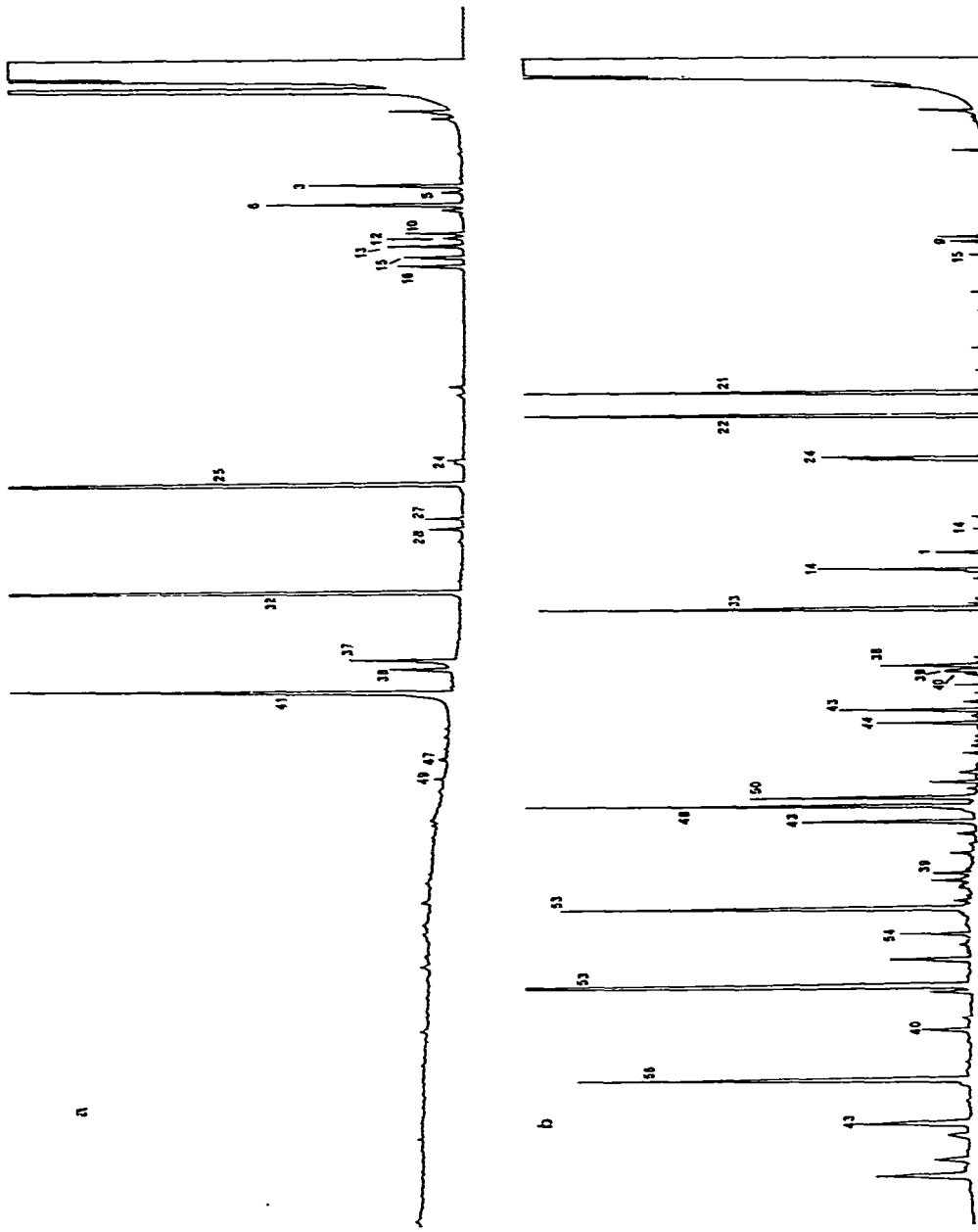


Fig. 2. Analytical gas chromatograms of Freon extracts of material selectively retained by C_{18} reversed phase from Rhine Riesling grape juice, after (a) final elution of the column with methanol and hydrolysis at pH 3, and (b) pre-elution of the column with 30% acetic acid and hydrolysis at pH 1. Peak identities are in Table I, and analytical GC conditions in the Experimental section.

on a small column of the bonded phase. After application of an aqueous solution of precursor concentrate to a short column of C_{18} reversed phase, the column was washed with water and then eluted with aqueous acetic acid. Fig. 2b shows the GC analysis of volatiles obtained by pH 1 hydrolysis of material eluted from the column with 30% acetic acid. It can be seen that monoterpenoids present in the chromatogram are principally at the higher (linalool oxide) oxidation level. By contrast, the chromatogram in Fig. 2a shows the products obtained by pH 3 hydrolysis of residual material eluted from the short C_{18} reversed-phase column with methanol, after the above 30% acetic acid fraction. Here monoterpenoids predominate and the majority of them are at the lower (linalool) oxidation state. Thus chromatography on the bonded C_{18} reversed phase allows class separation of glycosidic precursors of monoterpenes at different oxidation levels.

The chromatogram in Fig. 2b demonstrates that the fraction eluted with 30% acetic acid was also particularly rich in precursors of the grape nor-isoprenoids.

It is of interest that all of these precursor compounds have been isolated from both Rhine Riesling and Muscat of Alexandria grape varieties. Possibly significant differences have been observed between the patterns of volatiles produced on hydrolysis of the various C_{18} reversed-phase fractions from each variety. This is particularly true for that fraction giving the nor-isoprenoid products.

This technique of selective retention of monoterpene glycosides onto a bonded C_{18} reversed-phase column has also been applied to large volumes of dealcoholised wine concentrate prepared from Muscat of Alexandria as well as juice from this variety. The same column has been used repeatedly, by simply washing the bonded phase with water after elution of the target compounds and before application of the next batch. Thus a total of 45 l of dealcoholised wine concentrate and juice has been processed in batches of up to 15 l. This has enabled the isolation of quantities of glycosidic material giving linalool oxidation state monoterpenes on both acidic and enzymatic hydrolysis (similar to material giving the products seen in Fig. 2a). Further purification of this material, after acetylation, has allowed a complete spectral and chemical investigation of the monoterpene glycoside structures[3].

Presumably the hydrophobic inrteraction of the terpene moeity of the glycosides with the alkyl portion of the bonded phase accounts for the highly selective retention of these compounds on the C_{18} reversed phase. A similar phenomenon must also apply to the grape nor-isorpenoid, 2-phenylethanol and benzyl alcohol precursors. Application of the selective retention of terpene glycoside derivatives from aqueous solutions of polar interfering substances should be of use for the isolation of this class of compound from a variety of sources, *i.e.* other fruit juices and extracts of plant tissues, etc. This should facilitate research on this important group of plant constituents.

ACKNOWLEDGEMENT

We thank the South Australian Department of Agriculture, Lindemans Wines Pty. Ltd., and S. Smith & Sons Pty. Ltd. for generously donating samples of wines and grape juices. We are also indebted to Dr. D. J. Casimir, C.S.I.R.O. Division of Food Research for preparing wine concentrates. Bush Boake Allen Australia Ltd., Dragoco Pty. Ltd., Firmenich SA, Givaudan Pty. Ltd., and Naarden International are also thanked for gifts of several flavour chemicals used as reference compounds.

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