Chromatographic separation and partial identification of glycosidically bound volatile components of fruit

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ABSTRACT

Synthetic monoterpenic and aromatic β-D-glucosides and β-D-rutinosides were separated by Fractogel TSK HW-40 S chromatography according to their molecular size and interactions occurring between the aglycone moiety and the gel matrix. Under these conditions terpenyl rutinosides were eluted before the homologous glucoside derivatives. In the two classes, monoglucosides and rutinosides, aromatic glycosidically bound components had lower retention times than the corresponding terpenyl components. The use of over-pressure layer chromatography (OPLC) allowed the separation of glucoside and rutinoside derivatives, and in these two classes aromatic and several monoterpene compounds were well separated. Based on these results, the separation of glycosidically bound volatile components isolated from grapes (Muscat of Alexandria) and apricot (Rouge du Roussillon) was undertaken using a three-step process. Silica gel fractionation allowed the separation of mono- and diglycosidic fractions present in apricot. Fifteen peaks were obtained from grape extracts by Fractogel chromatography, and under the same conditions sixteen and eleven peaks were detected in monosaccharidic and disaccharidic fractions, respectively isolated from apricot. The glycosidic fractions isolated by gel chromatography were purified by preparative OPLC, and some of them were sufficiently pure for a subsequent structural analysis. Moreover, partial identification of glycosidically bound volatile components separated by these two methods may be achieved using retention data when standard compounds are available; on the other hand, enzymatic hydrolysis of the isolated pure fractions gives information in the absence of reference compounds.

INTRODUCTION

The presence of glycosidically bounds volatile components such as monoterpenic, aromatic and aliphatic alcohols, phenols, isoprenoids or polyols in plants and more precisely in fruits such as grapes [1–3], papaya [4], passion fruit [5], apricot and mango (6) has been reported. These non-volatile components, which are considered by flavour chemists to be aroma precursors, are present in plants in complex mixtures. Their isolation and their partial separation before analysis are generally performed by selective adsorption either on activated charcoal [7] or on organic hydrophobic adsorbents such as reversed-phase C_{18} or Amberlite XAD-2 [8]. According to Croteau *et al.* [9], a combination of hydrophobic interactions and gel-permeation chromatography on Bio-Gel P-2 provides a selective procedure for the purification of

monoterpenyl glucosides and galactosides. In some instances pretreatment with insoluble polyvinylpyrrolidone [10] or adsorption chromatography on silica gel [11,12] was used for sample clean-up. More recently, application of droplet countercurrent chromatography to these compounds has allowed the separation and the subsequent analysis of several minor constituents present in grape juice [10].

In our laboratory, the study of glycosidically bound volatile components present in fruits such as grapes, apricot, mango and passion fruit was undertaken using non-destructive methods such as high-performance liquid chromatography (HPLC) and soft ionization modes in mass spectrometry and tandem mass spectrometry [6,13,14]. For these studies further separations of complex mixtures obtained after extraction and prepurification steps were needed.

In this paper, chromatographic separations and partial identifications of glycosidically bound volatile compounds obtained by synthesis or isolated from grape and apricot fruits using gel chromatography on Fractogel TSK HW-40 S and preparative over-pressure layer chromatography (OPLC) are reported.

EXPERIMENTAL

Plant material
Mature, sound grapes (cultivar Muscat of Alexandria) were obtained from experimental vineyards of the Chambre d'Agriculture des Pyrénées Orientales, Rivesaltes, France. Apricots (cultivar Rouge du Rousillon) were gathered in the experimental orchard of the Institut National de la Recherche Agronomique, Manduel, France.

Grape berries and apricot halves were crushed at 5–10°C for 3 min. In order to decrease the viscosity of the apricot purée, the homogenate was treated for 90 min at 25°C with 3.5 g l^{-1} Pectinol D5 S (Röhm, Darmstadt, F.R.G.) and 0.2 g l^{-1} cellulase (Sigma, St. Louis, MO, U.S.A.). The hydrolysis of grape glycosidically bound terpenes present in or added to grape juice was checked under the same conditions. Clear juices were obtained by two successive centrifugations at 2500 g for 30 min and 50 000 g for 15 min.

Isolation of glycosidic fraction
The glycosidic fraction was obtained according to the procedure described by Williams *et al.* [15] and prepurified by treatment with insoluble polyvinylpyrrolidone, Polyclar AT (Serva, Heidelberg, F.R.G.) [10] A 10-ml volume of the raw glycosidic fraction was poured in a 100 × 10 mm I.D. column filled with Polyclar AT in methanol, elution was performed with 50 ml of methanol and the solution was concentrated to 2 ml under vacuum.

Silica gel chromatography
The concentrate obtained after methanol evaporation of the solution collected from the Polyclar AT column was separated on Chromagel 60 Å C.C. silica gel (230–400 mesh) (Solvants, Documentation, Synthèse, Peypin, France) in a 450 × 15 mm I.D. column. Elution was effected successively with 150 ml of chloroform, 440 ml of chloroform–acetonitrile–32% ammonia (15:85:10, v/v) and 660 ml of the same mixture (12.5:87.5:12.5, v/v). Fractions of 8 ml were collected and the presence of glycosides was checked by thin-layer chromatography (TLC).

Thin-layer chromatography

TLC was performed on 0.2-mm precoated silica plates (Kieselgel 60; Merck, Darmstadt, F.R.G.) with ethyl acetate–isopropanol–water (65:30:15, v/v/v) as eluent. After evaporation of the solvent, mono- and disaccharidic derivatives were revealed using N-(1-naphthyl)ethylenediamine dihydrochloride (Nediac reagent; Merck). Under these conditions monosaccharides were diversely coloured whereas monoglucoside and rutinoside compounds were violet-red and carmine red, respectively.

Fractogel TSK HW-40 S gel chromatography

Fractions isolated by silica gel chromatography and containing heterosidic compounds as indicated by TLC were diluted with distilled water and chromatographied on a Fractogel TSK HW-40 S (0.250–0.040 mm) Superformance column (600 × 26 mm I.D.) (Merck). Elution was performed with distilled water at 8 bar pressure using a Milton-Roy pump at a flow-rate of 3 ml min^{-1}. Eluted compounds were detected at 210 nm using a Varian UV 50 detector, 10-ml fractions being collected. All the fractions corresponding to one peak on the chromatogram were combined and water was eliminated by azeotropic distillation after addition of acetonitrile. The resulting concentrates were dissolved in 2 ml of methanol and stored at 4°C until use.

The column was calibrated using carbohydrates of increasing molecular weight: glucose (180), maltose (342), maltotriose (504) and stachyose (666); in this instance the UV detector was operated at 190 nm.

Over-pressure layer chromatography (OPLC)

A Chrompres 25 apparatus (Radiomatic Instruments et Consommables I) was used. Fractions obtained by gel chromatography, concentrated to 0.05 ml, were spoted on 0.2-mm precoated 20 × 20 cm silica plates (Kieselgel 60; Merck) and eluted with ethyl acetate–*tert.*-amyl alcohol–acetic acid–water (18:1:1:1, v/v) at a flow-rate of 0.75 ml min^{-1}. The elution time was 20 min for monoglucosides and 50 min for disaccharidic derivatives. After evaporation of the solvent, heterosidic compounds were revealed using Nediac reagent.

When OPLC was used in the preparative mode the residue obtained by solvent elimination after the gel chromatographic step was lined in the same plate as that used for the analytical mode, and elution was performed under the same conditions except that the reagent was spread only on the edges of the plates and strips corresponding to glycosidic compounds were scraped off and extracted with methanol.

Gas chromatography (GC)

A Varian 3300 apparatus fitted with a flame ionization detector and a silica capillary column containing CP-Sil 5 CB5 (methylsilicone) (Chrompack, Middelburg, The Netherlands) (50 m × 0.225 mm I.D.) was used. The column was programmed from 70 to 140°C at 2°C min^{-1} then from 140 to 250°C at 10°C min^{-1}; the carrier gas was hydrogen at 1.2 ml min^{-1}. The chromatograph was coupled with a Shimadzu CR 3A integrator. Authentic samples of terpenic alcohols were used for identification purposes.

Mass spectrometry

Desorption chemical ionisation (DCI) spectra in the positive-ion mode were obtained with a ZAB-HF mass spectrometer (Laboratoire d'Analyse du CNRS, Vernaison, France). Ammonia was used for ionization and argon for collision.

Synthesis of reference compounds

The reference compounds geranyl, neryl, α-terpinyl and citronellyl glucosides and rutinosides were obtained by condensation of α-bromoacetylated monosaccharides and alcohols catalysed by silver oxide [16]. Tetraacetylated α-bromoglucose and hexaacetylated α-bromorutinose were obtained according to refs. 17 and 18, respectively. The method of Paulsen *et al.* [19] was used for deacetylation of peracetylated β-D-glucosides.

Peracetylated precursors and monoterpenyl glycosides were purified by silica gel chromatography. For peracetylated compounds the excess of alcohol was eliminated using *n*-hexane–ethyl acetate (80:20, v/v) and the peracetylated compounds were eluted with the same mixture (50:50, v/v). The residue obtained after deacetylation was purified on the same column; dichloromethane–methanol mixtures (first 95:5, v/v, and then 60:40, v/v) were used as eluents; the heterosides were isolated in the second fraction, as indicated by TLC.

Enzymatic hydrolysis

Aspergillus niger pectinase preparation (Sigma) containing β-D-glucosidase, α-L-arabinase and α-L-rhamnosidase activities was partially purified by ultrafiltration (PM 10 Diaflo) and exclusion chromatography on Fractogel TSK HW-55 S before use [20]. A 0.05-ml volume of 0.1 M phosphate–citrate buffer (pH 5), 0.02 ml of partially purified pectinase containing 40 g l^{-1} of freeze-dried preparation were added to the residue obtained by elimination of the solvent under a stream of nitrogen from 0.01 ml of a methanolic solution of glycosides. The reaction was performed at 25°C with stirring in a hermetically sealed flask; after 30 min the reaction was stopped by addition of 0.5 ml of dichloromethane and chilling to 0°C. The organic layer was separated, dried over anhydrous sodium sulphate and filtered. The solvent was evaporated under a smooth stream of nitrogen and the concentrate was analysed by gas chromatography.

RESULTS AND DISCUSSION

Separation of synthetic glycosides

Fractogel TSK HW-40S chromatography. Chromatography of thirteen synthetic monoterpenyl and aromatic β-D-glucopyranosides and β-D-rutinosides (MW 270–462) on a Fractogel TSK HW-40 S column previously calibrated with standard carbohydrates (MW 180–660) is shown in Fig. 1. Twelve well resolved peaks, corresponding to thirteen standard glycosidically bound components, are detected; only two compounds, 2-phenylethanol β-D-glucoside and α-terpinyl β-D-rutinoside, lare coeluted. As a rule, monoterpenyl β-D-rutinosides are eluted before monoterpenyl β-D-glucosides according to their respective molecular weights; in these two classes the elution sequence relative to the monoterpenic moiety is linalool, α-terpineol, nerol and geraniol. The more apolar aromatic derivatives, benzyl and 2-phenylethyl glucosides
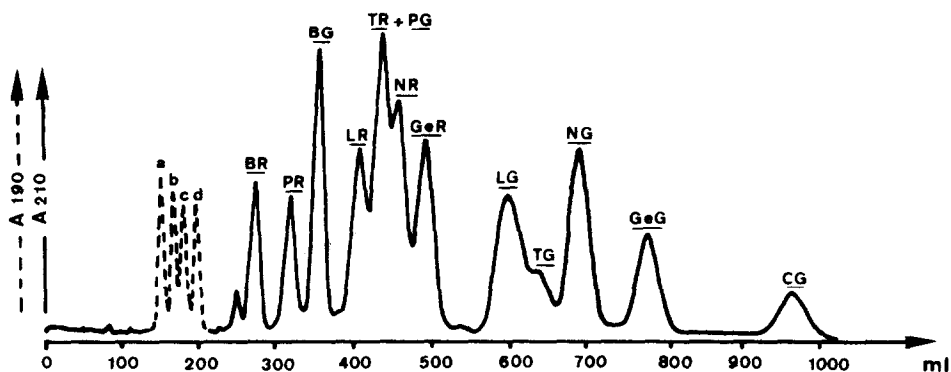
Fig. 1. Fractogel TSK HW-40 S chromatography of a mixture of synthetic glycosidically bound volatile components and of standard carbohydrates. Mobile phase, water at 3 ml min⁻¹; pressure, 8 bar; UV detection at 210 (————) and 190 (--------) nm. a = Stachyose; b = maltotriose; c = maltose; d = glucose; B = benzyl; P = 2-phenylethyl; L = linalyl; T = α-terpinyl; N = neryl; Ge = geranyl; C = citronellyl; R = rutinoside; G = glucoside.

and rutinosides, have lower elution volumes than the corresponding terpenyl compounds.

On the other hand, the elution volumes of glycosides relative to those of carbohydrates with comparable molecular weights show clearly that gel permeation is not the only phenomenon involved in the separation of these compounds. Indeed, the elution volumes of β-D-glucosides differ appreciably from that of maltose whereas the elution volumes of β-D-rutinosides are greater than that of maltotriose. As stated for the purification of monoterpenyl glycosides on Bio-Gel P-2 [9] or of flavonol glycosides [21] and proanthocyanidins [22] on Fractogel TSK HW-40 S, hydrophobic interactions of the aglycone moiety of glycosides with the gel matrix retard selectively the different compounds according to the hydrophobicity of this moiety. Under these conditions the compounds are eluted according to the length of the carbohydrate chain and the hydrophobicity of the aglycone.

Over-pressure layer chromatography. The chromatogram obtained by OPLC for the thirteen standard compounds considered above is given Fig. 2. This technique, previously used for the separation of compounds having related structures [23–26], is more accurate and gives better resolution than TLC. On the one hand it allows a good separation of glucoside and rutinoside derivatives, and on the other, among these two classes, monoterpenyl compounds are well separated from aromatic compounds. More particularly, linalyl derivatives are separated from the other terpenyl compounds present in the mixture.
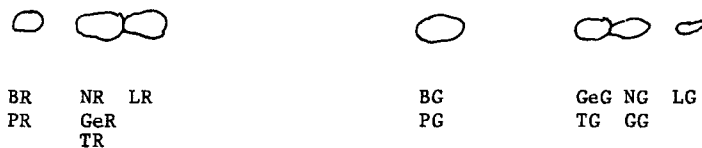

Fig. 2. OPLC of synthetic glycosidically bound volatile components on 0.2-mm silica gel (Kieselgel 60). Eluent, ethyl acetate–*tert.*-amyl alcohol–acetic acid–water (18:1:1:1, v/v) at a flow-rate of 0.75 ml min⁻¹. Nediac reagent was used for detection. Notation for glycosidically bound components as in Fig. 1.

Separation of fruit glycosidically bound volatile components

Silica gel pre-fractionation. A prefractionation of heterosidic pools obtained from grapes and apricots by adsorption on reversed-phase C_{18} and purified by treatment with Polyclar AT was performed using silicagel chromatography. Two apricot fractions containing essentially monoterpene monoglucosides and diglycosides and one grape fraction containing essentially monoterpene diglycosides, as indicated by TLC, were collected.

Fractogel TSK HW 40-S chromatography. On the Fractogel TSK HW-40 S chromatogram obtained for the fraction isolated from grapes (Fig. 3), fifteen peaks are detected and collected for subsequent OPLC separation. Two of these peaks, 7 and 9, have the same retention times as linalyl and geranyl β-D-rutinosides previously identified among grape glycosidically bound components [3]. In the absence of standard compounds, arabinoglucosides, also present in muscat cultivars [3], were not identified, but the presence of these compounds was suspected in peaks 8 and 10 according to the carmine red colour developed in TLC or OPLC using Nediac reagent. TLC of the different fractions collected showed that some of them are not revealed with this reagent and are not glycosidically bound components. Pyrophosphate [27,28] and other non-sugar derivatives [29,30] have been previously reported in plants.

With apricot the first fraction obtained after silica gel chromatography and containing essentially monosaccharide derivatives gives sixteen peaks (Fig. 4) in Fractogel chromatography; in five of these peaks, linalyl (peak 9), α-terpinyl (peak 10), neryl (peak 11) and geranyl (peak 12) glucosides were tentatively identified on a retention time basis. Benzyl glucoside was present in one another silica gel fraction.

In the fraction containing principally diglycosidic derivatives (Fig. 5) peak 7 has the same elution time as peak 8 detected in grape extract and suspected to be an arabinoglucoside derivative. These results are in good agreement with those previously reported concerning the presence in apricot of monoterpene glucosides in addition to monoterpene diglycosides [6]. The possibility of partial hydrolysis of diglycosides occurring during the juice liquefaction step was discarded on the basis of the low hydrolysis rate of apricot diglycosides by pectinol [6]. On the other hand the fact that rutinosides or arabinoglucosides present in grape juice or added to grape juice are not hydrolyzed under the conditions used for the recovery of apricot glycosidically bound derivatives agrees with this finding.
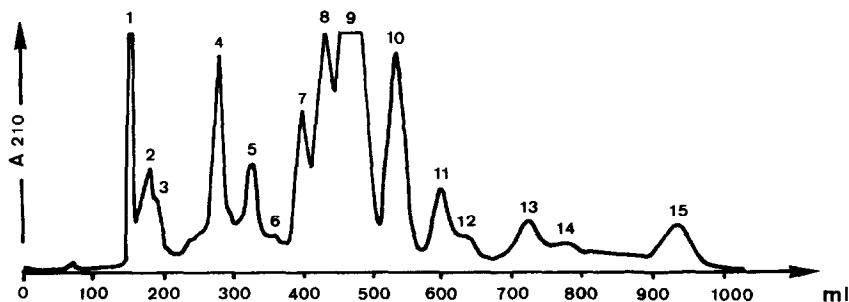

Fig. 3. Fractogel TSK HW-40 S chromatography of grape glycosidically bound fraction obtained after silica gel separation. Mobile phase, water at 3 ml min^{-1}; pressure, 8 bar; UV detection at 210 nm.
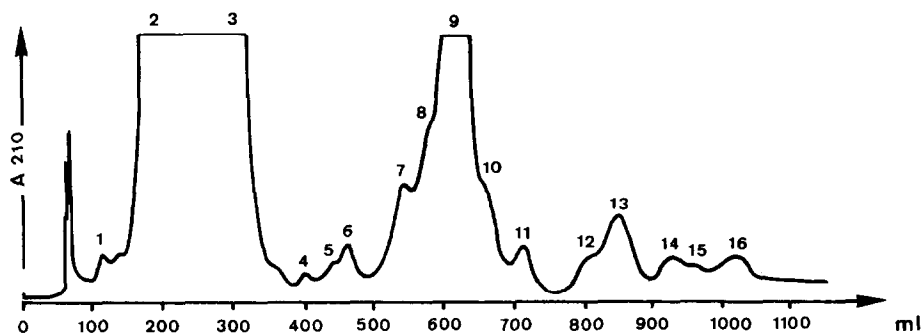
Fig. 4. Fractogel TSK HW-40 S chromatography of apricot monosaccharidic fraction obtained after silica gel separation. Mobile phase, water at 3 ml min^{-1}; pressure, 8 bar; UV detection at 210 nm.

Additionally, the reported data show that chromatography on silica gel is an efficient technique for the separation of glycosidically bound terpenes in two classes, mono- and diglycosidic derivatives.

Over-pressure layer chromatography. The grape heterosidic fractions 7, 8, 9 and 10 isolated by filtration on Fractogel were separated by preparative OPLC. Ninety different fractions were so isolated, but only the quantitatively most important and giving a specific saccharide coloration with Nediac reagent were investigated. The analytical OPLC of these fractions is shown in Fig. 6; linalyl (spot 7-1) and geranyl (spot 9-4) β-D-rutinosides are confirmed as grape juice components. In order to identify the other compounds separated, total enzymatic hydrolysis was applied and the results obtained by GC for the aglycone moiety and by TLC for the saccharidic moiety are reported Table I. The identification of linalool, α-terpineol, nerol and geraniol on the one hand and glucose, arabinose and rhamnose on the other agree with the presence of linaly and geranyl rutinosides or glucorhamnosides and of linalyl, α-terpinyl, neryl and geranyl arabinoglucosides or glucoarabinosides.

A preliminary study using DCI positive-mode mass spectrometry [6] was undertaken in order to obtained information on the sequence of the saccharidic
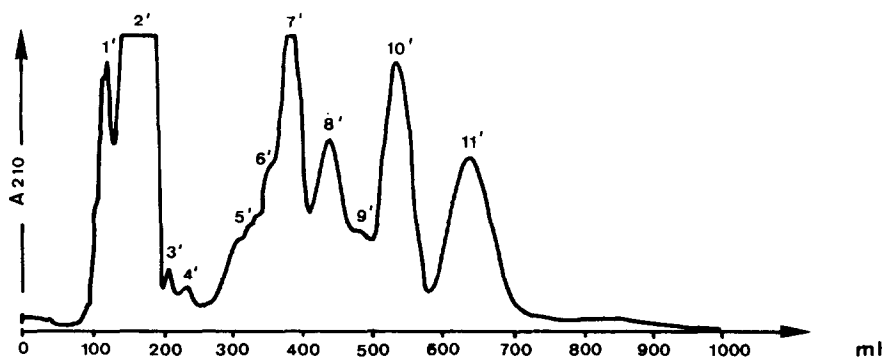

Fig. 5. Fractogel TSK HW-40 S chromatography of apricot disaccharidic fraction obtained after silica gel separation. Mobile phase, water at 3 ml min^{-1}; pressure, 8 bar; UV detection at 210 nm.
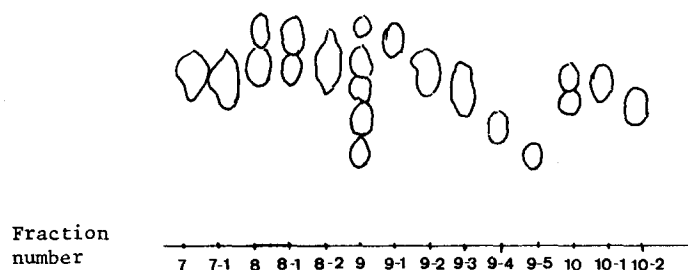
Fig. 6. Analytical OPLC of grape glycosidically bound fractions separated by preparative OPLC on 0.2-mm silica gel (Kieselgel 60). Eluent, ethyl acetate–*tert.*-amyl alcohol–acetic acid–water (18:1:1:1, v/v) at a flow-rate of 0.75 ml min^{-1}. Nediac reagent was used for detection.

moiety. For compounds 7-1 and 9-4 the spectrum obtained (Fig. 7) shows the presence of the molecular ion of m/z 480 $(M + NH_4)^+$ and fragment ions of m/z 334 (OH $\sim$ Glu $\sim$ O$\sim$Agl$+NH_4)^{+,a}$, 326 (Rut $\sim$ NH$_3)^+$, 180 (Glu $\sim$ NH$_3)^+$ and 164 (Rha $\sim$ NH$_3)^+$. These results are indicative of the sequence rhamnose–glucose–aglycone but no information concerning the nature of the aglycone moiety is available, the fragment ions of m/z 137 and 154 characteristic of this moiety being present in all instances with approximately the same relative abundance.

TABLE I

AGLYCONE AND SACCHARIDIC MOIETIES IDENTIFIED BY GC AND TLC AFTER ENZYMATIC HYDROLYSIS OF GRAPE GLYCOSIDICALLY BOUND COMPOUNDS SEPARATED BY OPLC

Fraction	Aglycone moiety (GC)	Saccharidic moiety (TLC)
7-1	Linalool	Glucose + rhamnose
8-2	Linalool	Glucose + arabinose
9-2	α-Terpineol	Glucose + arabinose
9-3	Nerol	Glucose + arabinose
9-4	Geraniol	Glucose + rhamnose
10-2	Geraniol	Glucose + arabinose

For the other grape heterosidic compounds isolated by OPLC, the following fragments were found in the mass spectra (Fig. 8): m/z 466 $(M + NH_4)^+$, 334 (OH$\sim$Glu $\sim$ O$\sim$Agl $+ NH_4)^+$, 312 (Ara$\sim$Glu $\sim$ NH$_3)^+$, 180 (Glu $\sim$ NH$_3)^+$ and 150 (Ara $\sim$ NH$_3)^+$. These fragments are characteristics of the sequence arabinose–glucose–aglycone. As reported by Williams *et al.* [3], the mass spectrometric determinations indicate the presence of rutinosides and arabinoglucosides in grapes.

The analytical OPLC of apricot fractions obtained by preparative OPLC, 9-2, 10-1, 11-1 and 12-1 for glucosidic compounds and 7'-1 for disaccharidic compounds, is shown Fig. 9. The results concerning the relative migration data and the results

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^{a} $\sim$ = Bond. This symbol is used in order to distinguish between a bond and the sign minus.
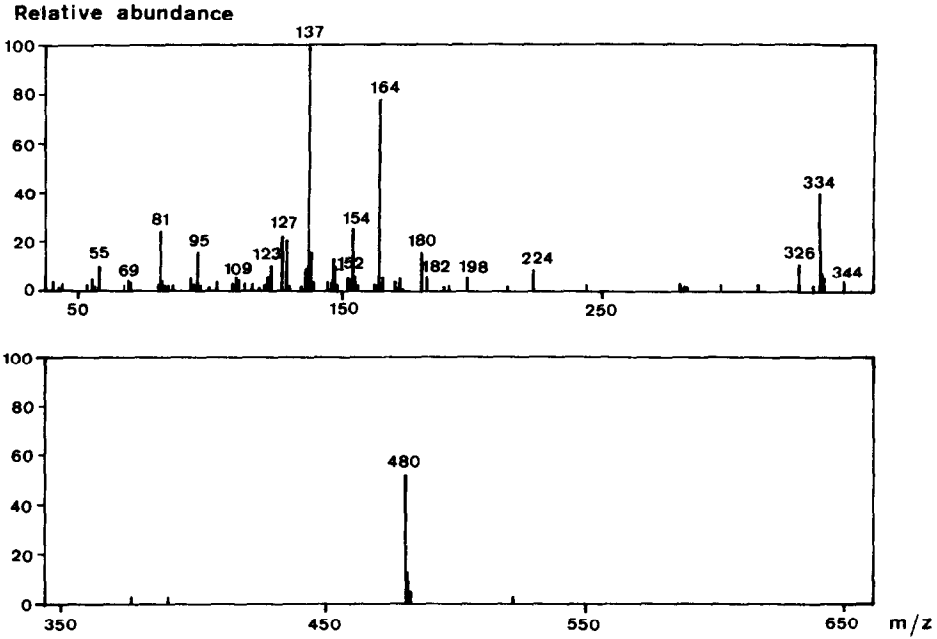
Relative abundance

Fig. 7. DCI in positive mode (NH₃/NH₄⁺) mass spectrum of OPLC 9-4 grape fraction (geranyl β-D-rutinoside).

Relative abundance
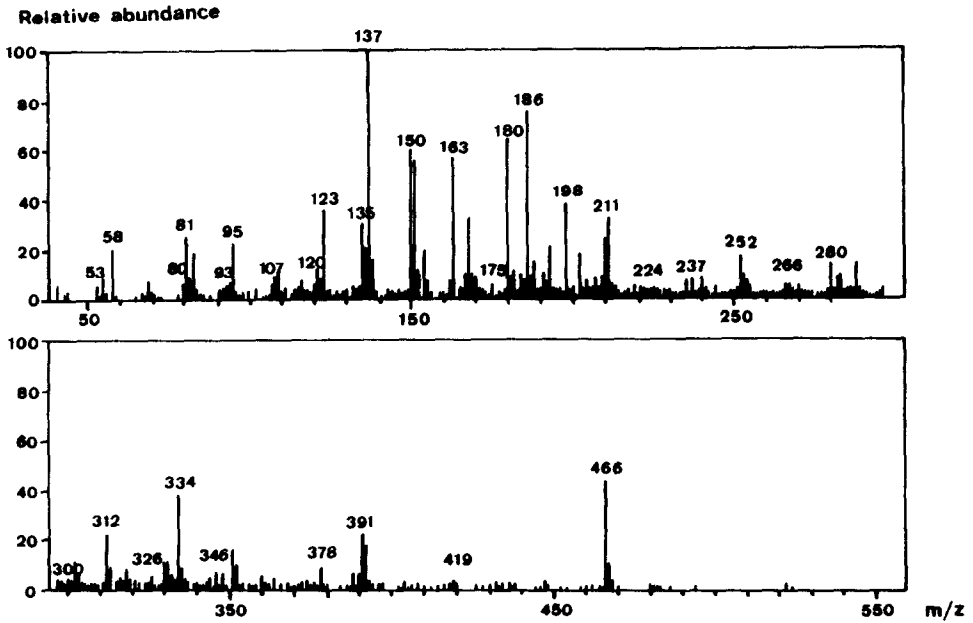
Fig. 8. DCI in positive mode (NH₃/NH₄⁺) mass spectrum of OPLC 8-2 grape fraction (linayl arabinoglucoside)

Fraction
number   9-2   10-1 11-1 12-1   7'-1

Fig. 9. Analytical OPLC of apricot glycosidically bound fractions obtained after preparative OPLC on
0.2-mm silica gel (Kieselgel 60). Eluent, ethyl acetate–*tert.*-amyl alcohol–acetic acid–water (18:1:1:1, v/v) at
a flow-rate of 0.75 ml min⁻¹. Nediac reagent was used for detection.

obtained by GC and TLC after enzymatic hydrolysis confirmed the tentative
identification of linaly (9-2), α-terpinyl (10-1), neryl (11-1) and geranyl (12-1)
glucosides. The identification of linalool by GC and of arabinose and glucose by TLC
after total enzymatic hydrolysis of fraction 7'-1 agrees with the presence of linalyl
arabinoglucoside or glucoarabinoside, the former certainly being the actual com-
pound, as indicated by mass spectrometry.

As established from Figs. 6 and 7, the purity of most of the fractions studied was
sufficient for subsequent ulterior HPLC or improved mass spectrometric analysis in
order to obtain more information on the structure of these compounds and
particularly on the nature of the aglycone moiety.

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