The phytochemistry of blackcurrant flavour and aroma

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THE PHYTOCHEMISTRY OF BLACKCURRANT

FLAVOUR AND AROMA

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Good reasons must perforce give way to better.

JULIUS CAESAR

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ABSTRACT

Chapter 1 reviews the results of previous investigations into the composition of blackcurrant leaf, bud and fruit oils, and the limited literature relating to changes in blackcurrant fruit volatiles during the fruit maturation and subsequent processing. A comprehensive table is presented summarising the occurrence of the two hundred and fifty seven compounds reported in blackcurrant leaf, bud and fruit oils.

Chapter 2 presents the experimental procedures used to isolate blackcurrant leaf oil and fruit volatiles, and the gas chromatographic and combined gas chromatographic - mass spectrometric methods of analysis employed. Using these methods the leaf and fruit oils of three blackcurrant cultivars were examined. Changes in blackcurrant fruit volatiles during ripening and distribution of these compounds within the fruit was also investigated. Experimental procedures used to demonstrate the presence of non-volatile terpene precursors are presented, together with details of labelling studies using [2 - ^{13}C] mevalonate.

Chapter 3 presents the collective results of these investigations.

Chapter 4 discusses the identification of twenty seven compounds not previously found in blackcurrant leaf oil, and changes in the composition of the oil when isolated by steam distillation. These changes have been attributed to acid catalysed rearrangements and oxidation of the terpenes and the products of lipoxidation. Only quantitative differences where observed between the leaf oils and fruit volatiles of the three cultivars studied. The distribution of terpenes in blackcurrant fruit showed distinct compartmentation, monoterpene olefins being found mainly in the epidermis and monoterpene alcohols in the

pericarp. Terpenol glycosides have been tentatively identified in blackcurrant fruit for the first time, the concentration of which increases during ripening. Quantitative changes in the concentration of terpene compounds occurs during ripening, accompanied by a corresponding increase in the relative percentage of monoterpene alcohols. The study of monoterpene biosynthesis in blackcurrant fruit using [2 - ^{13}C] mevalonate was largely unsuccessful because of low incorporation of the labelled precursor.

CONTENTS

CHAPTER 1

Review of Blackcurrant Flavour and Aroma Chemistry to 1986

Blackcurrant plants belong to the genus *Ribes*, derived from an Arabic word meaning acid-tasting. The plant family to which they belong may be known as *Saxifragaceaa*, of which many members are rock plants or *Grossulariaceae*, a Latinized botanic word from the French for gooseberry, *groseille*. Until such time as there is a consensus of opinion, the choice is arbitrary and depends upon the system of botanic nomenclature followed. Varying sorts of *Ribes* occur throughout the world and apart from currants and gooseberries there are many flowering currant varieties popular for late spring and early summer floral displays. Botanically the blackcurrant is specified as *Ribes nigrum* (Latin: *nigrum*, black) whilst red and white currants are derived from three *Ribes* species, R. *petraeum*, R. *vubrum* and R. *vulgare*. These are 5 to 6 feet high shrubs native to Europe and western Asia.

Although the blackcurrant may be found growing wild in Britain it is not thought to be native, but to have escaped from gardens. Commercially, blackcurrants are grown extensively in central and northern Europe, England and Australasia. In Britain, blackcurrants are cultivated mainly in Kent, Norfolk and Worcestershire (1), and are used mainly for canning, jamming and juice production, and a measure of this activity is shown by the fact that in 1984 the annual production of blackcurrants in Britain was 36,000 tonnes. There are now over forty different varieties of blackcurrant grown worldwide, but in Britain most blackcurrants grown commercially are either Baldwin, Wellington XXX or Ben Lomond.

In some regions, especially in Burgundy, France, the bushes are pruned during the winter months, and the cuttings which already possess the hibernating buds are harvested to produce blackcurrant bud absolute. Blackcurrant bud absolute is obtained in 2-4% yield from alcoholic extraction of the buds. The absolute which is a dark green paste, contains green pigments, predominantly chlorophyll, a complex acid fraction, unsaponifiable material and approximately fifteen per cent essential oil (42) which is isolated from the absolute by steam distillation.

Previously blackcurrant bud oils were isolated by benzene extraction followed by steam distillation (2,41) but this has now been totally superseded by alcoholic extraction. Both the blackcurrant bud absolute and oil are extremely expensive owing to the high labour intensity of the bud harvest, and therefore only find use in high quality fragrances and cosmetics, and at low levels in blackcurrant flavourings.

Analysis of blackcurrant bud oil was carried out long before any aroma analyses were carried out on blackcurrant fruit. In 1907 Schimmel (2) noted the presence of *p*-cymene in blackcurrant bud essential oil, then in 1937 Glichitch and Igolen (3) reported the existence of a number of terpenes and terpene derivatives, notably β-pinene, sabinene, caryophyllene, cadinene, mono and sesquiterpene alcohols and a mixture of terpineols. Their work was carried out by fractional distillation followed by chemical and physical analysis.

In 1951 McGlumphy (4) reviewed the results of the early aroma analysis carried out by Schimmel and Glichitch, and this stimulated Mehlitz and Matzik (6,7) to continue this work. They then reported the existence of formic, acetic, *n*-butyric, glycollic and salicylic acids in blackcurrant juice and also the presence of ethanol and acetaldehyde. The presence of formic and acetic acid was confirmed by Smith (8) in 1957.

With the advent of commercially available gas chromatographs, flavour research and aroma analysis was subsequently carried out by many other groups worldwide and, from 1963 onwards, a large number of papers appeared relating to the analysis of blackcurrant leaf, bud and fruit aroma. The first gas chromatographic analysis of blackcurrant leaf oil was performed in 1963 by Andersson and co-workers (11). Here blackcurrant leaves (var. Brödtorp) were distilled at atmospheric pressure to obtain the essential oil in 0.017% yield. This was then analysed using three different stationary phases, and additionally, fractions were collected and infrared spectra obtained from them. Further identification was carried out by combined gas chromatography/mass spectrometry. From this work 17 compounds were conclusively identified: α-pinene, myrcene, oct-1-en-3-ol, car-3-ene, *p*-cymene, *m*-cymene, limonene, cis-β-ocimene, β-phellandrene, 1-methyl-4-isopropenylbenzene, linalool, terpinene-4-ol, methyl salicylate, geraniol, citronellyl acetate, caryophyllene, and humulene.

In 1964 the first gas chromatographic analysis was carried out on blackcurrant fruit aroma independently by Andersson and co-workers (12) and by Spanyar (9,10). Andersson and co-workers used the

same techniques as had been used for the analysis of the leaf oils and, following pentane extraction, the yield of fruit oil was 9.1ppm of the fresh weight (0.00091% m/m), some twenty times less than the level of essential oil in blackcurrant leaves. Twenty five compounds were identified in the fruit oil, including all the compounds found in blackcurrant leaves except linalool, geraniol and 1-methyl-4-isopropenylbenzene, and in addition γ-terpinene, terpinolene, *trans*-β-ocimene, camphene, citronellol, α-terpineol, *p*-cymen-8-ol, benzaldehyde, methyl benzoate, ethyl benzoate and *cis*-3-hexen-1-ol.

The work reported by Spanyar in the same year recorded none of the compounds reported by Andersson and co-workers, but identified ethanol, butan-1-ol, pentan-1-ol and their acetate esters, none of which had been previously found in blackcurrant. Spanyar's work was carried out on ether/ pentane extracts of blackcurrant distillates, followed by gas chromatography on a 1.2m x 0.4m glass column packed with 15% dinonyl phthalate on 80-100 mesh Celite run isothermally at 75^{o}C.

In 1966 Andersson and von Sydow (13) extended their work to the lower boiling compounds of blackcurrant fruit. The low boiling compounds were vacuum distilled from blackcurrant juice, this was followed by solvent extraction and concentration. The subsequent GC analysis identified 25 components of which 19 were reported for the first time. These were predominately alcohols; methanol, propanol, 2-methyl propanol, 3-methyl butanol, hexanol, butan-2-ol, pentan-2-ol, 2-methyl-3-buten-2-ol, 3-methyl-2-buten-1-ol, 1-penten-3-ol and 2-methyl butanol; carbonyl compounds: hexanal,

acetone, 2-butanone, pentanal and 2,3-butanedione, methyl acetate, ethyl-*n*-butyrate, plus 1,8-cineole and styrene. Although many of these compounds have characteristic aromas, Andersson and von Sydow concluded that the characteristic odour of blackcurrants is dependent on the compounds mainly found in the higher boiling fraction.

Similar work carried out by Prillinger and Horwatitsch in 1965 (14) found many of the compounds reported by Andersson and von Sydow and tentatively identified propionic, isobutyric and isovaleric acids, isopropyl acetate, *n*-propyl acetate, isopentyl acetate and ethyl-*n*-hexanoate. In 1966 Andersson and von Sydow (15) examined the qualitative and quantitative composition of the essential oils of six botanical varieties of blackcurrants, and the changes in the essential oil composition of one variety (Brödtorp) during ripening. The varieties examined were Brödtorp, Wellington XXX, Silvergieters Zwarte, Cotswold Cross and two hybrids Wellington XXX x Brödtorp and Cotswold Cross x Brödtorp. Cotswold Cross was found to have three times more essential oil than the other varieties. Silvergieters Zwarte and Wellington XXX, which are of similar botanical origins, were qualitatively and quantitatively very similar. Compared with these two varieties both Brödtorp and Cotswold Cross contained much more caryophyllene. Brödtorp was characterized by low concentrations of γ-terpinene and terpinen-4-ol, and Cotswold Cross by a low concentration of 3-carene. Most of these differences were expressed in the relevant hybrid.

Analysis of the essential oils of Brödtorp fruit harvested over a four week period showed a slow increase in total oil during

ripening, but little change in its composition. An analysis of blackcurrant aroma was also carried out by Kulesza (16, 17) and Pribela (18) during 1966 and 1967 but only three new carbonyl compounds were identified; propanal, *n*-butanal and 3-hydroxy-2-butanone. The aroma analysis carried out on blackcurrant leaves, buds and fruit was reviewed by Nursten *et al* (19) in 1967 and by Gierschnner *et al* (20) in 1968.

During 1969 research groups working in England, Spain and France published analytical work on the volatiles of fresh fruit and a commercial distillate, blackcurrant juice concentrate, and blackcurrant bud oil respectively. Gasco *et al* (21) working in Spain analysed blackcurrant juice concentrate volatiles by gas chromatography for the first time as part of a survey of fruit concentrate volatiles. Seventeen compounds were identified: acetaldehyde, isobutanal, isopropyl formate, propan-2-ol, ethyl acetate, ethanol, *n*-propyl formate, methyl propionate, 2-pentanone, butan-2-ol, propan-1-ol, isobutanol, ethyl *n*-butyrate, isoamyl acetate, 2-methyl-1-butanol, 3-methyl-1-butanol and ethyl hexanoate. Of these iso and *n*-propyl formates, propan-2-ol, isobutanal, 2-pentanone and methyl propionate had not been previously reported, however identification was based on retention times alone.

Latrasse (22) examined a benzene extract of blackcurrant buds by gas chromatography and identified nine terpene compounds by their retention times: Δ^3-carene, myrcene , limonene, *p*-cymene, β-phellandrene, linalol, geraniol, citronellyl acetate and caryophyllene. Latrasse also isolated volatiles from fresh fruit by gas displacement and condensation at -78°C, which were subsequently analysed by gas chromatography. The following

compounds were reported: methanol, ethanol, isobutanol, acetaldehyde, pentanal, ethyl acetate, butyl formate, ethyl valerate, isoamyl isobutyrate, ethyl formate and butyl formate, of which the last four were found for the first time.

One of the most exhaustive studies of blackcurrant volatiles to be carried out was that of Nursten and Williams (23, 34), published in 1969. Examination of a commercial distillate revealed over 150 components of which twenty compounds were conclusively identified by gas chromatography, infrared spectroscopy and mass spectrometry. A further thirty four components were tentatively identified. Separation of the volatiles was carried out on four different stationary phases, using either F.I.D. or thermal conductivity detection. Infrared spectra were recorded from fractions trapped from the G.C. runs using a spectrophotometer fitted with a beam condenser and ultramicro cavity cell. The following new compounds were identified from this study: α-terpinene, 3-methyl-2-butanone, *cis*-2-hexen-1-ol, *trans*-2-hexen-1-ol, 2-ethyl butan-1-ol, octan-4-ol, isobutyl acetate, isobutyl propionate, methyl *n*-butyrate, isobutyl *n*-butyrate, methyl hexanoate and a pentenol of unknown structure.

Further support for identification of some of the alcohols was obtained by reacting the essence with acetic anhydride and noting the shift of the alcohol peaks to their corresponding acetates. This study was followed by an examination of fresh fruit volatiles isolated by climbing film evaporation (24). Baldwin blackcurrants were used for the preparation of the main batch of essence.

Analysis of the extracts obtained was carried out using the same methods as previously used for the commercial distillate. A total of twenty four compounds were identified, ten of these were confirmed by infrared spectroscopy. 2-ethyl-butan-1-ol was identified for the first time. Distillates from fruit picked in two consecutive years showed marked differences. The total yield of essence in 1964 was 60µl/100Kg fresh weight of fruit, and in 1965 was 144µl/100Kg of fruit. The differences between the two essences were mainly quantitative. Although the presence of *trans*-2-hexen-1-ol and terpinen-4-ol as major components, together with methyl benzoate, methyl *n*-hexanoate, butan-1-ol, ethanol and *trans*-2-hexenal was common to both distillates and the commercial distillate, 1,8-cineole clearly present in the commercial and 1965 distillate was swamped by *trans*-2-hexenal in the 1964 distillate.

Considerable differences in the composition of the volatiles isolated by Anderson *et al* (11) and Nursten and Williams (24) were tentatively explained by varietal differences, differences in soil and climate in which the bushes were grown, time of harvest and method of storage, but the method of extraction was considered the most probable cause.

In 1967 Kuusi & Kuusi (25) investigated the characterization of blackcurrant aroma using three different methods. The methods were: aroma number (assay of volatile reducing substances), aroma analysis by gas chromatography, and organoleptic evaluation. No consistent and reliable correlation was found between the results of the different methods used, and it was noted that climate and growing locality had a marked effect on the fruit volatiles.

The use of capillary gas chromatography to analyse blackcurrant volatiles was first reported in 1971 by von Sydow and Karlsson (26). Blackcurrant volatiles from unheated and heated fruit were isolated by solvent extraction or headspace precolumn concentration techniques followed by capillary gas chromatography and mass spectrometry. Eighty nine compounds were identified: eighty two in the unheated sample and eighty eight in the heated sample, constituting eighty six per cent and eighty per cent respectively of the solvent free concentrates. Absolute concentrations of seventeen compounds were determined in the head space of unheated and heated blackcurrants. The main effect of heating was the formation of furan derivatives and an increase of dimethyl sulphide, 2-methyl propanal and 2-methyl butanal concentrations. There were large variations in the proportions of the monoterpenes, but obviously little degradation of the monoterpene molecules themselves. Caryophyllene, one of the major components in the "normal" extract, is decreased on heating by about 50%.

The enhanced resolution obtained by the use of capillary columns led to the identification of a further thirty eight new compounds: *n*-hexane, 2-methyl butanal, 3-methyl butanal, 3-hexanone, 3-methyl-2-butenal, furfural, α-fenchene, 2-pentyl furan, (+)-4-carene, *n*-butyl benzene, linalool oxide, nonanal, rose oxide, carveol, geranial, 4-methyl acetophenone, decanal, *m*-4,6-menthadien-8-ol, 1-*p*-menthan-9-al, piperitenone, α-terpinyl acetate, γ-elemene,

dendrolasin, methyl formate, carbon disulphide, 2-methyl furan, benzene, trichloroethylene, chloroform, dimethyl disulphide, ethyl benzene, *o*,*m* and *p*-xylene, *p*-mentha-1(7)8-diene, methylethylbenzene, 3-octanone, hexyl acetate, dimethylethylbenzene and dimethylstyrene.

Following this extensive analytical work, von Sydow and Karlsson (27) investigated the effect of heating upon odour quality. The sensory properties of heated and unheated blackcurrants were investigated in two ways. By a direct sensory evaluation of the berries, unheated and heated at different time-temperature conditions using an odour quality assessment technique (the human nose!) and an evaluation of intensity and quality of the gas chromatographic eluates from runs of head space samples of low-temperature distillates from unheated and strongly heated berries. Six odour qualities increased (musty/mouldy, burnt/smoky, sickly, warm, sharp/pungent and cooked vegetable) and eight decreased (fruity, floral, green, citrus, aromatic, fragrant, cool and sweet) with increasing heat treatment. These changes in intensity corresponded well with observed chemical changes (26).

In 1971 Latrasse and Demaizieres (28) investigated the oxidation of the monoterpene fraction of blackcurrant bud oil using thin layer chromatography, gas chromatography and measurements of oxygen absorption using a Warburg apparatus (29). Latrasse and Demaizieres demonstrated the absorption of oxygen by the bud oil, and detected a number of monoterpene peroxides by TLC, which then underwent progressive polymerization. The principal hydroperoxide identified was that of Δ^3-carene, for which an autoxidation process was proposed. In an attempt to isolate the

characteristic "catty" blackcurrant aroma compound Williams (30,31) analysed pentane extracts of blackcurrant buds (var. Baldwin and Raven A) by gas chromatography, mass spectroscopy and odour assessment. The"catty" aroma was found to be associated with compounds eluting between 120°C and 125°C on the column used for this study (10% SE30). The only compounds identified in that region were monoterpene hydrocarbons, none of which have even a remote blackcurrant aroma.

Similar work was carried out in 1971 by Fridman *et al* (32) who found limonene to be the major compound present in the particular blackcurrant bud oil which they examined. Two years later Karlsson-Ekström and von Sydow (33) published further details of correlations between instrumental and sensory data from unheated and heated blackcurrants. The methods used were the same as previously reported (26, 27) but a computer facilitated the correlation of the data obtained. From this study it was concluded that the sensory changes of aroma which take place on heating can be very well correlated with a decrease in terpene hydrocarbons, the formation of dimethyl sulphide and the increase of some aliphatic aldehydes: ethanal, propanal, 2-methyl propanal and 2-methyl butanal.

This work was extended to examine the influence of some other processing parameters on the aroma of blackcurrants (34). It was found that when a juice is pressed from blackcurrant mash, the monoterpene hydrocarbon levels decrease considerably. In addition there were also further decreases in the concentrations of the monoterpene hydrocarbons and increases in the concentrations of the

aliphatic aldehydes, dimethyl sulphide, 2-methyl-3-buten-2-ol and benzene derivatives, when a juice or mash was heated in a 'closed' system under vacuum corresponding to boiling temperatures of 85°C and 70°C, the changes were larger at 85°C. When the volatiles of heated juice were compared with the volatiles of heated mash, the same changes occur, but the decrease in the concentration of terpenes was more significant.

Blackcurrant bud oils from eighteen different varieties were examined by Latrasse (35, 43) in 1974 and 1976. The bud oil was extracted by steam distillation following hydro alcoholic maceration and analysed by gas chromatography, infrared spectroscopy and mass spectrometry. Three groups were distinguished amongst the cultivars examined. The first group containing 6 cultivars, was found to have 75% sabinene in the extracted oil. The second and largest group of nine cultivars, yielded an oil containing 58% sabinene, 21% Δ^3-carene and 10% terpinolene. The oil composition of the final group of three cultivars, contained no sabinene but predominantly Δ^3-carene at 36%, β-phellandrene at 20% and 15.5% terpinolene. The flavour of the third group oils was considered to be the best, followed by the oil of the second group of cultivars.

Although not strictly aroma compounds, aromatic acids can contribute to the flavour of various foods (36). In 1975, Stöhr and Herrmann (37) reported the identification of nine phenolic acids in blackcurrants at levels up to 110ppm. The highest levels were of caffeic, *p*-coumaric, ferulic and gallic acids, with traces of protocatechuic acid, syringic acid, gentisic acid, salicylic acid and 4-hydroxy benzoic acid. The levels of the phenolic acids

decreased during ripening relative to the fresh weight, except protocatechuic acid which appeared mostly in the last stage of ripening.

Over the next few years, further attempts were made to identify the elusive characteristic blackcurrant aroma compound. In 1978 Lewis *et al* (38) identified pulegone for the first time in blackcurrant bud oil, a compound closely related to *p*-menthane-8-thiol-3-one which had recently been isolated from buchu oil (39, 40) and has a characteristic blackcurrant odour. Evidence was also obtained for a compound of molecular weight 186 with mass spectral data similar to that of *p*-methane-8-thiol-3-one. Earlier the same year Hussein (47) associated the blackcurrant aroma with a high boiling fraction, but failed to identify any compound in that region to account for the aroma. No further details of this work were available since access to this thesis was denied.

The most extensive analysis of blackcurrant juice aroma to be carried out in recent years was that of Latrasse *et al* in 1982 (48). Volatiles were obtained by vacuum distillation of blackcurrant juice, and also by hydroalcoholic infusion of blackcurrants. The distillate from the juice was extracted with dichloromethane and concentrated under vacuum. The hydroalcoholic infusions were extracted with Freon II and also concentrated under vacuum. Both concentrates were fractionated on silica columns, fractions being eluted off with solvents of increasing polarity.

The fractions were analysed by combined gas chromatography - mass spectrometry and organoleptic assessment of the eluate. Only the polar fractions of the volatiles eluted from the silica column with

dichloromethane had the blackcurrant odour and the principal aroma appeared to be associated with the low boiling portion of the fractions. In this region of the chromatogram several compounds were identified: 2,3-butanedione, ethyl-*n*-butyrate, 1,8-cineole, linalol, limonene-4-ol, α-terpineol and terpinene-4-ol. These compounds and an unidentified compound of 'catty' aroma were considered important to the blackcurrant aroma. Two varieties, *Noir de Bourgogne* and *Royal de Naples*, were found to be richer in those compounds, and this reflected in the quality of the flavour and aroma.

Other secondary components which were considered important were methylacetophenone,citronellol, geraniol, damascenone and a compound with a jam-like aroma. In addition to the identification of this important fraction, a total of one hundred and twelve compounds were recognised, forty two of which were new. These are: fenchol, limonene-8-ol, *cis* and *trans* piperitol, 1-octen-2-ol, 3-hexanol, 3-methyl-hexan-2-ol, 4-isopropyl cyclohexanol, heptan-1-ol, nonan-1-ol, cuminalcohol, cyclohexanol, phenylethanol, furfurol, cuminaldehyde, 1-octen-3-one, 3-cyclocitral, 1,3-dimethylcyclohexen-3-yl methyl ketone, 2-heptenal, 2-octenal, 2-nonenal, 2-decenal, 2-4-heptadienal, nonadienal, camphor, damascenone, tiglaldehyde, carvone, piperitone, neryl acetate, geranyl acetate, 4-acetoxy-1,8-menthadiene, methyl *cis*-jasmonate, γ-nonalactone, *o*-cresol, carvacrol, vanillin, a methoxybenzylpyrazine, allylphenol, ledol and umbellulone.

A further compound of molecular weight 220 was found in the two cultivars *Noir de Bourgogne* and *Royal de Naples*, its mass spectral fragmentation pattern suggested a sesquiterpene alcohol.

At the International Symposium on Food Flavours held in Paris in 1982, Williams (49) reviewed the work carried out up to 1982 on the attempted identification of the characteristic blackcurrant aroma. Examination of blackcurrant juice head-space volatiles by Adam (50) in 1983 found predominately aliphatic and terpene alcohols, esters and furfural. Only nine compounds were identified.

At the 9th International Essential Oils Congress held in Singapore in 1983, Kerslake and Menary (51) presented the results of their work on blackcurrant bud oil. Blackcurrant buds (*var.* White bud a local selection of Baldwin) were macerated with petroleum ether and extracted overnight. The bud oil was vacuum distilled from the concrete obtained from the petroleum extract, and fractionated on a silica gel column. Fractions were eluted with increasing levels of diethyl ether in *n*-pentane, and analysed by gas chromatography and mass spectrometry. Aroma profiles were collated using a glass capillary OV101 column eluting into a sniffer port.

This study identified three regions which were considered important in the overall aroma. The first region was found to have a steely, spicy aroma, the second region with a similar boiling point to that reported by Williams (31) contained the catty note, and the third region contained the rich, jammy blackcurrant aroma. No compounds could be identified which accounted for these smells. Twelve new compounds were identified. Continued work by this group (52) was reported in 1985. The same techniques were used to isolate and analyse the bud oils, except that high performance liquid chromatography was used to separate the

monoterpene hydrocarbons, alcohols and sesquiterpene hydrocarbons.

Although a total of one hundred and twenty three compounds were separated, and sixty six identified, twenty three for the first time, the regions of the chromatogram giving rise to the steely, catty and jammy blackcurrant aromas remained unidentified. The new compounds identified by this group, including the twelve identified in the previous publication, were: tricyclene, *n*-propyl benzene,isopropyl benzene, β-thujene, 1-ethyl-2-methyl benzene, 1,2,3-trimethylbenzene, 3,5,5-trimethylhexanol, nonan-2-one, menthone, *cis*-*p*-menth-1,8-diol, napthalene, sabinene hydrate, undecan-2-one, β-terpinyl acetate, methyl undecanoate, alloaromadrene, germacrene-D, γ-cadinene,β-cadinene, β-elemol, γ-elemol, caryophyllene epoxide and humulene epoxide.

Kerslake and Menary followed this work by a study of varietal differences of blackcurrant bud extracts (53). The composition of extracts from ten cultivars grown in southern Tasmania were examined as previously described. The patterns of association of the thirty four components were analysed by principal co-ordinate analysis, the co-ordinates being plotted in four dimensions. A close association between oils extracted from selections belonging firstly to the Goliath and Baldwin groups, and secondly to the Boskoop and French groups was shown. Thirty three of the thirty four compounds used in the analysis were terpenes, the other was 2-nonanone.

At the International Fruit Juice Union Symposium held in Tel Aviv in 1984, Bricout *et al* (54) presented the results of their research

on the aroma of blackcurrant juice from eleven different cultivars used in the traditional French alcoholic beverages. The volatiles were extracted by passing helium through an aqueous fruit homogenate and collecting the eluted compounds on 80-100 mesh Tenax. The volatiles were eluted from the Tenax with a small volume of redistilled diethyl ether, and analysed by combined gas chromatography - mass spectrometry. Eighteen compounds including nine terpenes and seven esters were identified and used to compare the eleven cultivars. The results showed a marked difference in the abundance of monoterpene hydrocarbons and butyrate esters between all eleven cultivars and an even larger variation between fruit harvested in 1982 and that harvested in 1983.

In the last few years, there has been growing interest in the isolation and use of blackcurrant absolute for flavours and fragrances. Some preliminary work was carried out by Fellows (44) and Latrasse (45) from 1974 to 1977 but the most detailed analysis of the absolute was reported by Wytenhove (42) in 1985. Absolutes from French, English and mixed blackcurrant buds were isolated, and the volatile oil obtained by steam distillation of the absolute. Gas chromatographic analysis of the oil revealed no new compounds, but revealed significant quantitative differences in the oils. At the end of 1985, a total of 257 compounds had been reported in blackcurrant leaf, bud or fruit oils (Table I), in fifty one separate publications.

This study investigated in detail the composition of blackcurrant leaf oil isolated by low temperature solvent extraction and by steam distillation, using gas chromatography and combined gas

chromatography and mass spectrometry. Using the same techniques the effect of various isolation methods was examined with respect to the terpene fraction of the fruit volatiles of the three most commonly grown cultivars in Britain, Baldwin, Ben Lomond and Wellington XXX.

Using these same three cultivars, the changes in the terpene fraction of the fruit volatiles during ripening were examined using low temperature solvent extraction followed by combined gas chromatography/mass spectrometry using single ion monitoring to avoid the use of concentration or fractionation techniques. In addition to these studies, the biosynthesis of monoterpenes in blackcurrant fruit was investigated, using stable isotopes and mass spectrometry to detect the labled compounds.

TABLE I

Compound	Occurrence		
	Leaf	Bud	Fruit
Hydrocarbons			
n-Hexane	-	-	26
Methyl butene	-	30,31	-
Cyclohexene	-	30,31	-
α-Thujene	-	51	12,26
β-Thujene	-	52	-
β-Pinene	-	3,22,30,31,32,42,51,52	26,32,54
α-Pinene	-	22,28,30,31,42,51,52	12,26,27,54
Camphene	-	28,30,31	12,23
Sabinene	-	3,30,31,35,42,51,52	23
Myrcene	11	22,28,30,31,34,42,51,52	12,23,24,26,54
α-Phellandrene	-	22,28,30,31,51,52	23,24,26,27
β-Phellandrene	11	22,28,30,31,35,42,51,52	12,23
Limonene	11	22,28,30,31,32,35,42,51,52	12,23,26,32,54
Δ^3-Carene	11	22,28,30,31,32,34,35,42,51,52	12,27,32
Δ^4-Carene	-	-	26
α-Terpinene	-	30,31,42,51,52	23,26,27,50,54
γ-Terpinene	-	30,31,42,51,52	12,23,24,26,54
trans-β-Ocimene	11	42,51,52	12,23,26,54
cis-β-Ocimene	11	30,31,42,51,52	12,23,26,27,54
Terpinolene	-	30,31,34,35,42,51,52	12,26,27
α-Fenchene	-	-	26
p-Mentha-1(7),8 diene	-	-	26,31

	Leaf	Bud	Fruit
β-Caryophyllene	11	3,22,30,31,32,42,51,52	12,24,26,32
Δ-Cadinene	11	3,30,31	26
γ-Cadinene	-	52	-
Humulene	11	42,51,52	12,26
β-Elemene	-	30,31,42,51,52	-
γ-Elemene	-	51,52	26
α-Copaene	-	42,52	-
Alloaromadrene	-	52	-
Germacrene D	-	51,52	-
Benzene	-	30,31,52	26
Toluene	-	30,31	24,26
Ethylbenzene	-	30	26
1,2-Dimethylbenzene	-	51,52	26
1,3-Dimethylbenzene	-	51,52	26
1,4-Dimethylbenzene	-	51,52	26
Vinylbenzene	-	28	13,26
Propylbenzene	-	52	-
Trimethylbenzene	-	51,52	-
2-Methyl-1-ethylbenzene	-	51,52	26
p-Cymene	11	2,3,22,28,30,31,34,42,51,52	12,23,26,54
m-Cymene	11	-	12
1-Methyl-4-isopropenylbenzene	11	30,31,34	12,23,26,27
n-Butylbenzene	-	-	26
t-Butylbenzene	-	-	26
Dimethylvinylbenzene	-	-	26

	Leaf	Bud	Fruit
Dimethylethylbenzene	-	30	26
Napthalene	-	51,52	-
Alcohols			
Methanol	-	-	13,14,18,22,23,26
Ethanol	-	-	9,10,13,14,18,21, 22,23,24,26,50
Propan-1-ol	-	-	13,14,21,23
Propan-2-ol	-	-	21
Butan-1-ol	-	51	9,10,14,13,23,24, 48
Butan-2-ol	-	-	13,14,21
2-Methyl-propan-1-ol	-	51	13,14,18,21,22,23 24
Pentan-1-ol	-	-	9,10,13,14,23,48
Pentan-2-ol	-	51	13,14,23
2-Methyl-butan-1-ol	-	51	14,21
3-Methyl-butan-1-ol	-	-	13,14,18,21,23,24, 50
1-Penten-3-ol	-	-	13,26
2-Methyl-3-buten-2-ol	-	-	13,23,26,27,34,50
3-Methyl-2-buten-1-ol	-	-	13
Hexan-1-ol	-	-	13,23,24,26,48,54
Hexan-3-ol	-	-	48
cis-2-Hexenol	-	-	23,26,48
trans-2-Hexenol	-	-	23,24
cis-3-Hexenol	-	-	13,48

	Leaf	Bud	Fruit
2-Ethyl-butan-1-ol	-	-	23
Heptan-1-ol	-	-	48
3-Methyl-hexan-2-ol	-	-	48
2-Octenol	-	-	48
Octan-4-ol	-	-	23
1-Octen-3-ol	ll	42,52	12,13,23,48
2-Ethyl-hexan-1-ol	-	52	24,48
Octadienol	-	-	48,31
Nonan-1-ol	-	-	48
3,5,5-Trimethylhexanol	-	52	-
Cyclohexanol	-	-	48
4-isoPropylcyclohexanol	-	-	48
Linalol	ll	22,30,31,51,52	23,48
Geraniol	ll	3,22	23,48
Sabinol	-	3	-
Citronellol	ll	30,31	12,23,26,48
α-Terpineol	-	30,31,32,51,52	12,23,24,26,32,48
Terpinen-4-ol	ll	30,31,42,51,52	12,13,23,24,26,32, 48,50
Carveol	-	-	26
m-4,6-Menthadien-8-ol	-	30,31	26
Limonen-4-ol	-	-	48
cis-Piperitol	-	-	48
trans-Piperitol	-	51,52	48

	Leaf	Bud	Fruit
cis-*p*-Menth-2-ene-1,8-diol	-	52	-
β-Elemol	-	51,52	-
γ-Elemol	-	51,52	-
Phenol	-	3	-
Benzyl alcohol	-	-	48
Phenyl ethanol	-	-	48
p-Cresol	-	-	48
o-Cresol	-	-	48
p-Cymen-8-ol	11	52	23,26,48
Eugenol	-	-	48
Allyl phenol	-	-	48
Cuminalcohol	-	-	48
β-Napthol	-	3	-
Furfuryl alcohol	-	-	48
Acids			
Formic	-	-	5,6,7,8
Acetic	-	52	5,6,7,8,14,18
Propanoic	-	-	14
n-Butanoic	-	-	5,6,7,14,18
2-Methylpropanoic	-	-	14
3-Methylbutanoic	-	-	14
Glycollic	-	-	5,6,7
Salicylic	-	-	5,6,7,37
Caffeic	-	-	37
p-Coumaric	-	-	37
Ferulic	-	-	37

	Leaf	Bud	Fruit
Gallic	-	-	37
Protocatechuic	-	-	37
Syringic	-	-	37
Gentisic	-	-	37
4-Hydroxybenzoic	-	-	37
Carbonyls			
Acetaldehyde	-	-	13,18,21,22,23,26, 27,34
Propanal	-	-	18,26,27,34
Butanal	-	-	23
Pentanal	-	-	13,22,26,34
2-Methylpropanal	-	-	21,27,34
2-Methylbutanal	-	-	26,27,34
3-Methylbutanal	-	-	26
3-Methyl-2-butenal	-	-	26
2-Methyl-2-butenal	-	-	48
Hexanal	-	-	13,26,27,48,54
trans-2-Hexenal	-	-	23,24,26,34
Heptanal	-	-	12,48
2-Heptenal	-	-	48
2,4-Heptadienal	-	-	48
Octanal	-	-	48
2-Octenal	-	-	48
Nonanal	-	-	26,27,34
2-Nonenal	-	-	48
Nonadienal	-	-	48
Decanal	-	-	26,48

	Leaf	Bud	Fruit
2-Decenal	-	-	48
Propanone	-	-	13,18
2-Butanone	-	-	13,23
2,3-Butanedione	-	-	13,16,17
3-Hydroxy-butan-2-one	-	-	23
2-Pentanone	-	-	21
3-Methyl-butan-2-one	-	-	23
2-Hexanone	-	-	24,48
3-Hexanone	-	-	26,48
2-Methyl-pentan-3-one	-	-	48
2-Heptanone	-	-	48
3-Methyl-2-hepten-4-one	-	-	48
3-Octanone	-	42	26,48
1-Octen-3-one	-	-	48
2-Nonanone	-	51,52	-
2-Undecanone	-	51,52	-
3-Cyclocitral	-	-	48
Geranial	-	-	26
1-*p*-Menthan-9-al	-	-	26
Piperitenone	-	-	26
Pulegone	-	38	-
Piperitone	-	-	48
Camphor	-	-	48
Menthone	-	51,52	-
Carvone	-	52	48
Umbellulone	-	-	48

	Leaf	Bud	Fruit
Benzaldehyde	11	52	23,26,48
p-Methoxybenzaldehyde	-	-	48
Cuminaldehyde	-	-	48
Vanillin	-	-	48
Acetophenone	-	-	48
4-Methylacetophenone	-	-	26,48
Damascenone	-	-	48
2 or 3-Methylcyclopentanone	-	-	48
1,4-Dimethylcyclohex-3-enyl methyl ketone	-	-	48
Methylfurylketone	-	-	48
Furfural	-	-	26,27,50
<u>Esters</u>			
Methyl formate	-	-	26
Methyl acetate	-	-	13,14,18,24
Methyl propionate	-	-	21
Methyl *n*-butyrate	-	-	23,48,50,54
Methyl-*trans*-2-butenoate	-	-	48
Methyl *n*-hexanoate	-	-	23,24,48,54
Methyl acetoacetate	-	-	48
Methyl-3-acetoxy butyrate	-	-	48
Methyl undecanoate	-	51,52	-
Methyl hexadecanoate	-	30,31	-

	Leaf	Bud	Fruit
Methyl *cis*-jasmonate	-	-	48
Methyl benzoate	-	-	12,23,24,26,48
Methyl salicylate	11	-	12,22,23,48
Ethyl formate	-	-	22,26
Ethyl acetate	-	-	9,10,13,14,18,21, 22,23,24,26,34,50
Ethyl *n*-butyrate	-	-	13,14,18,21,24,26, 48,50,54
Ethyl *n*-valerate	-	-	22
Ethyl *n*-hexanoate	-	-	14,21,23,24,48,54
Ethyl-3-acetoxy butyrate	-	-	48
Ethyl-2-hydroxy butyrate	-	-	48
Ethyl benzoate	-	-	12,23,26,48
Ethyl *n*-octanoate	-	-	21
Ethyl *cis*-9-octandecenoate	-	30,31	-
n-Propyl formate	-	-	21
isoPropyl formate	-	-	21
n-Propyl acetate	-	-	14
isoPropyl acetate	-	-	14,23
n-Butyl formate	-	-	22
n-Butyl acetate	-	-	9,10,13,14,23,48, 54
i-Butyl acetate	-	-	23,48,54
i-Butyl propionate	-	-	23
i-Butyl *n*-butyrate	-	-	23

	Leaf	Bud	Fruit
n-Pentyl acetate	-	-	9,10,23,48
i-Pentyl acetate	-	-	14,18,21,23,24,26, 48,54
i-Pentyl *n*-butyrate	-	-	22
i-Pentyl i-valerate	-	-	21
isoPentenyl acetate	-	-	48
3-Methyl-2-butylacetate	-	-	48
Hexyl acetate	-	-	26,48
cis-3-Hexenyl acetate	-	-	48
cis-2-Hexenyl acetate	-	-	48
Citronellyl acetate	11	22,30,31,42,51,52	12,23,26,48
α-Terpinyl acetate	-	-	26,48
Bornyl acetate	-	30,31,51,52	48
Linalyl acetate	-	-	48
Neryl acetate	-	-	48
Geranyl acetate	-	51,52	48
β-Terpinyl acetate	-	51,52	-
Terpinen-4-yl acetate	-	51,52	-
Citronellyl formate	-	51,52	-
Benzyl acetate	-	-	48
Sulpher Compounds			
Carbon disulphide	-	-	26
Dimethyl sulphide	-	-	26

	Leaf	Bud	Fruit
Dimethyl disulphide	-	-	27,34
Benzothiazole	-	-	48
Halocarbons			
Chloroform	-	-	26
Trichloroethylene	-	-	26
Furans			
Furan	-	-	27
2-Methyl furan	-	-	26
Methyl-isoprop-furan	-	-	48
2-Pentyl furan	-	-	26,27
(3-(4,8-Dimethyl-3,7-nonadienyl)-furan	-	-	26
Lactones			
γ-Butyrolactone	-	-	48
γ-Hexalactone	-	-	48
γ-Nonalactone	-	-	48
Δ-Decalactone	-	-	48
Epoxides			
Linalool oxide	-	-	26,48
Rose oxide	-	-	26,48
Carene oxide	-	-	48
Caryophyllene epoxide	-	51,52	-
Humulene epoxide	-	51,52	-

	Leaf	Bud	Fruit
Miscellaneous			
1,8-Cineole	-	-	13,24,26,48
4-Acetoxy-1,8-menthadiene	-	-	48
5,6-Epoxy-β-ionone	-	-	48
Sabinene hydrate-		52	-
2,3-Dimethyl maleicanhydride	-	-	48
2-Methyl-2-butenolide	-	-	48
Dihydroactinidiolide	-	-	48
Methylbenzylpyrazine	-	-	48

CHAPTER 2

Experimental Procedures

Materials

Pure terpene standards were obtained variously from Aldrich Chemical Co., Fluka A.G., S.C.M. Organics, Bush Boake Allen Ltd. and International Flavours and Fragrances Ltd. β-Terpineol and γ-terpineol were provided as generous gifts from Firmenich & Co. The purity of the standards was determined by gas chromatography, and in all instances was better than 98%. The standard compounds were not purified further before use, but were stored under nitrogen at -25°C in the dark to prevent deterioration.

Analar methanol and dichloromethane (B.D.H. Chemicals) were redistilled prior to use, and their purity checked by gas chromatography after concentrating a sample 1000 times. The glycosidic enzyme used was Pectinol C obtained from Rohm and Haas which was used as supplied. The enzyme was stored desiccated at -20°C. (2-^{13}C) Mevalonic acid lactone (99 atom %) was obtained from MSD Isotopes Ltd., and used as supplied. All other reagents were of at least Analar purity, used as supplied, and stored under conditions specified by the supplier.

Fruit samples were either supplied by commercial growers or by Luddington Experimental Horticultural Station. All were stored at -25°C prior to analysis. Blackcurrant leaves and stems were kindly supplied by Luddington Experimental Horticultural Station, and either used fresh or stored at -25°C. All fruits, leaves and stems used for comparative studies were obtained from three to five year old bushes grown at the same location.

Methodology

Isolation of blackcurrant leaf oil

Blackcurrant bud oil was isolated either by steam distillation or by low temperature solvent extraction. Isolation of the volatiles by steam distillation was carried out on 75g samples of blackcurrant leaves using a modified Clevenger-type apparatus (fig. I) as described in the European Pharmacopoeia (56). Glass distilled water was used for the distillation, and the distillate was extracted three times with one volume of redistilled dichloromethane. The extracts were then bulked, dried with anhydrous sodium sulphate, and the volume reduced to 7.5cm^3 by careful distillation using a short (20cm) Vigreux column. The extract was stored under nitrogen at -25^{o}C in sealed hypovials.

In order to investigate the effect of steam distillation on the composition of the blackcurrant leaf oil, the leaf oils were also isolated by low temperature solvent extraction. All solvent and equipment used was thoroughly cleaned and then cooled to -25^{o}C before use. Blackcurrant leaves (10g) were ground with solid carbon dioxide using a ground glass pestle and mortar, then homogenised with 100cm^3 redistilled dichloromethane for two minutes, using a Silverson homogeniser fitted with a micro disintegration head (Silverson Machines Ltd.). The extract was transferred to cooled glass centrifuge tubes and centrifuged at 5000g for ten minutes at -25^{o}C. The supernatent liquid was decanted off and the residue rehomogenised with a further 50cm^3 of cooled dichloromethane, and centrifuged as before. The supernatents were combined, dried over anhydrous sodium sulphate and the volume carefully reduced to 1cm^3 using a micro distillation apparatus.

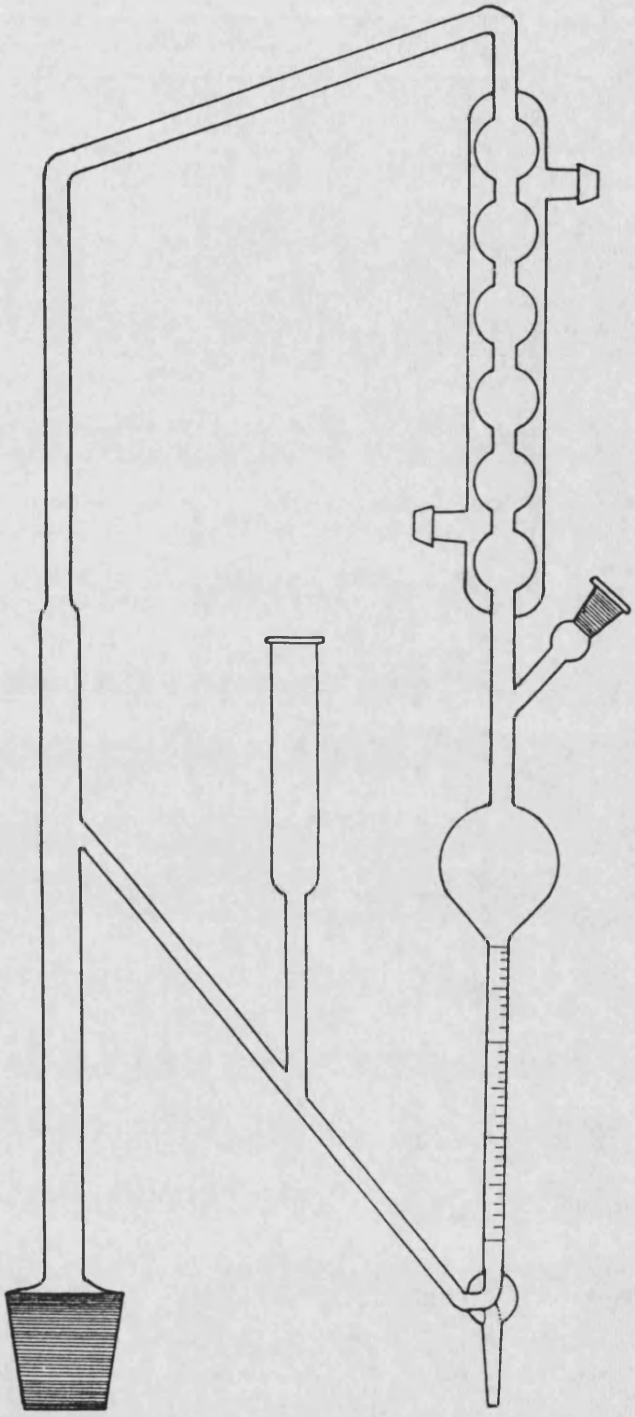


Fig. I. Modified Clevenger still as described in the European Pharmacopia (56), and used for steam distillation of blackcurrant leaf oil

The extracts thus obtained were also stored in a sealed hypovial under nitrogen at -25^{o}C.

Isolation of blackcurrant fruit volatiles

Blackcurrant fruit volatiles were isolated using four different methods: (a) low temperature solvent extraction, (b) alcoholic extraction, (c) vacuum distillation, and (d) distillation at reduced pressure, after depectinisation and juice extraction processes which simulate the commercial process. The method used for low temperature solvent extraction was similar to that previously employed for the extraction of blackcurrant leaves. Blackcurrants (20g) were gound with solid carbon dioxide, using a ground glass pestle and mortar, then homogenised with 200cm^{3} of cooled dichloromethane as previously described. The extract was transferred to a cooled (-25^{o}C) funnel, and allowed to separate into two layers during the course of ten minutes. The lower organic extract was passed through a previously dichloromethane washed silicone impregnated paper (Whatman IPS). The aqueous residue was rehomogenised with a further 100cm^{3} of cooled dichloromethane and separated as before into organic and aqueous phases. The bulked dichloromethane extracts were dried over anhydrous calcium sulphate, which for this extract was found to be more efficient than anhydrous sodium sulphate. The dried dichloromethane extract was carefully reduced to 2cm^{3} using micro distillation apparatus. Initially the dichloromethane extract was analysed without any prior concentration, but subsequent trials showed that careful concentration did not affect the composition of the extract.

Alcoholic extraction of the fruit was carried out at $4^{o}C$ using redistilled methanol. Blackcurrants (20g) were homogenised with $70cm^3$ cooled methanol ($4^{o}C$) using a precooled micro disintegration head as previously described. The homogenate was transferred to a $100cm^3$ volumetric flask. Then the disintegration head and beaker were rinsed with methanol, and this added to the homogenate. The volume was finally adjusted to $100cm^3$. After thoroughly mixing, the extract was transferred to centrifuge tubes and centrifuged at 5000g for 10 minutes at $4^{o}C$. The supernatent fluid was decanted off and stored in sealed hypovials under nitrogen at $-25^{o}C$.

Extraction of blackcurrant aroma by vacuum distillation was carried out by homogenising 100g thawed blackcurrants with $100cm^3$ of distilled water using a 25mm disintegrating head. The all glass still was assembled as shown in fig. II. Here the first trap was cooled with ice and water, and the second and third traps with carbon dioxide/acetone slush. The main condenser was cooled with water at $8-10^{o}C$. The still was operated at a pressure of 25mm Hg and the flask temperature was not allowed to exceed $30^{o}C$. The homogenate was stirred continuously during the distillation. The main distillate and the contents of the cold traps were pooled (total $104cm^3$) and 30g Analar sodium chloride added. When the sodium chloride has completely dissolved, the distillate was extracted three times with $50cm^3$ redistilled dichloromethane. The dichloromethane extracts were bulked, dried with anhydrous calcium sulphate, and the volume carefully reduced to $10cm^3$. The residue was stored at $-25^{o}C$ in hypovials under nitrogen.

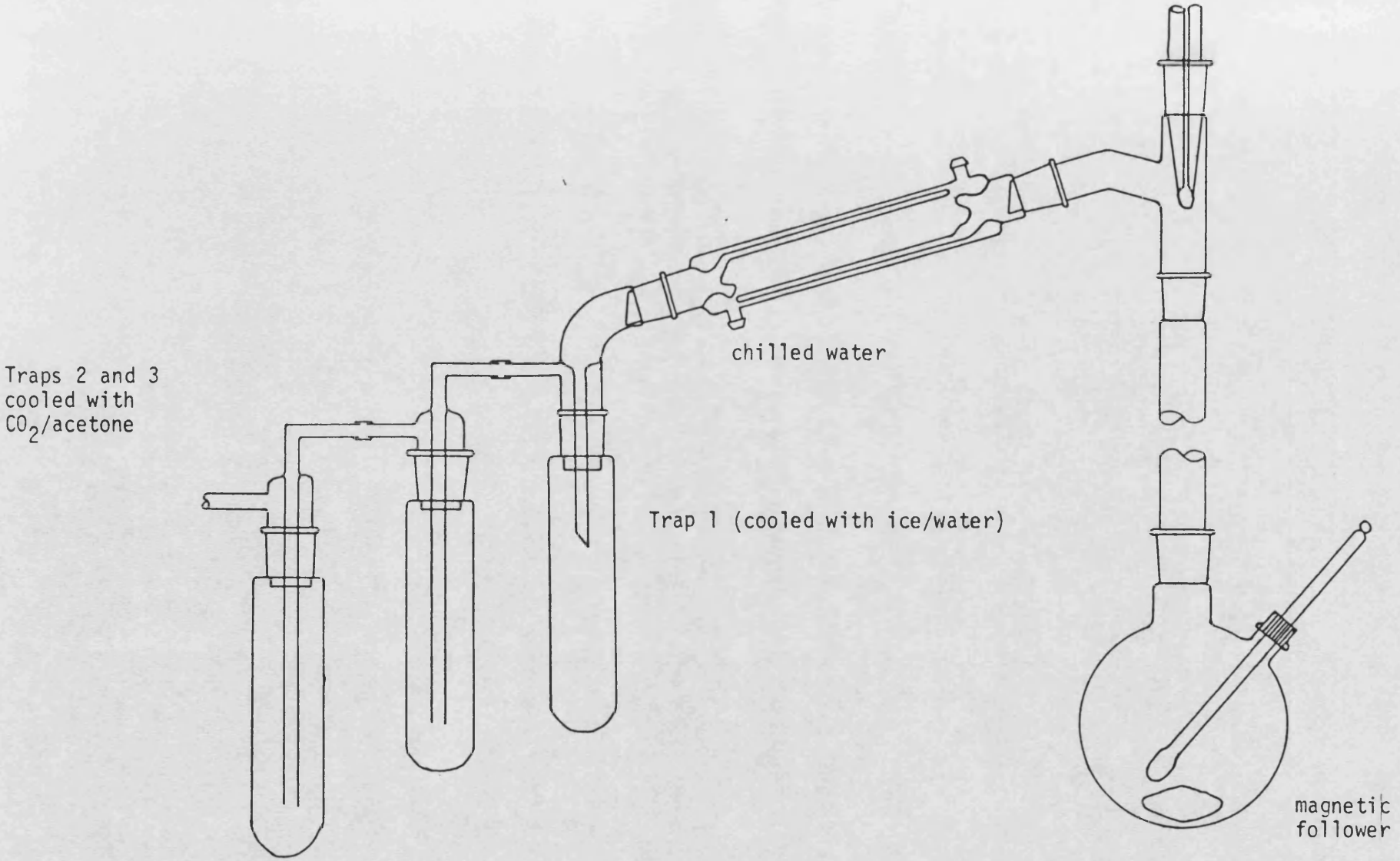


Fig. II. All glass still used for vacuum distillation of blackcurrant volatiles

The last method of isolation is similar to that used at Barnett and Foster Ltd. for the production of commercial aroma concentrates (Fig. III). Frozen blackcurrants (500g) were allowed to thaw at room temperature and then crushed between two stainless steel rollers, set 3mm apart. The product was then heated to $50^{o}C$ in a $1000cm^3$ Pyrex beaker and stirred slowly at $50^{o}C$ using an external water bath. A depectinising enzyme, Pectinol C (Rohm & Haas) was added at a rate of 0.05% m/m, and the pulp was held at $50^{o}C$ for two hours to allow depectinisation to occur.

This event is characterised by a decrease in pulp viscosity and the pulp is judged to be depectinised when a sample of juice pressed from the pulp gives no gelatinous precipitate when mixed with 2 volumes of propan-2-ol. After depectinisation, the juice was pressed from the pulp through a fine muslin in a laboratory screw press, and collected. The yield of juice was $360cm^3$ (72%). A portion of the juice ($160cm^3$) was distilled in the same way as previously described for the extraction by vacuum distillation except that the pressure was maintained at 360mm Hg. This resulted in a pulp temperature of $80-82^{o}C$, which simulates commercial conditions. Also in accordance with production conditions, a sixteenth fraction ($10cm^3$) was taken. To this fraction was added 3g sodium chloride, and when this had completely dissolved, the distillate was extracted three times with $10cm^3$ portions of redistilled dichloromethane. The three extracts were combined and dried over calcium sulphate, before being carefully reduced in volume to $16cm^3$.

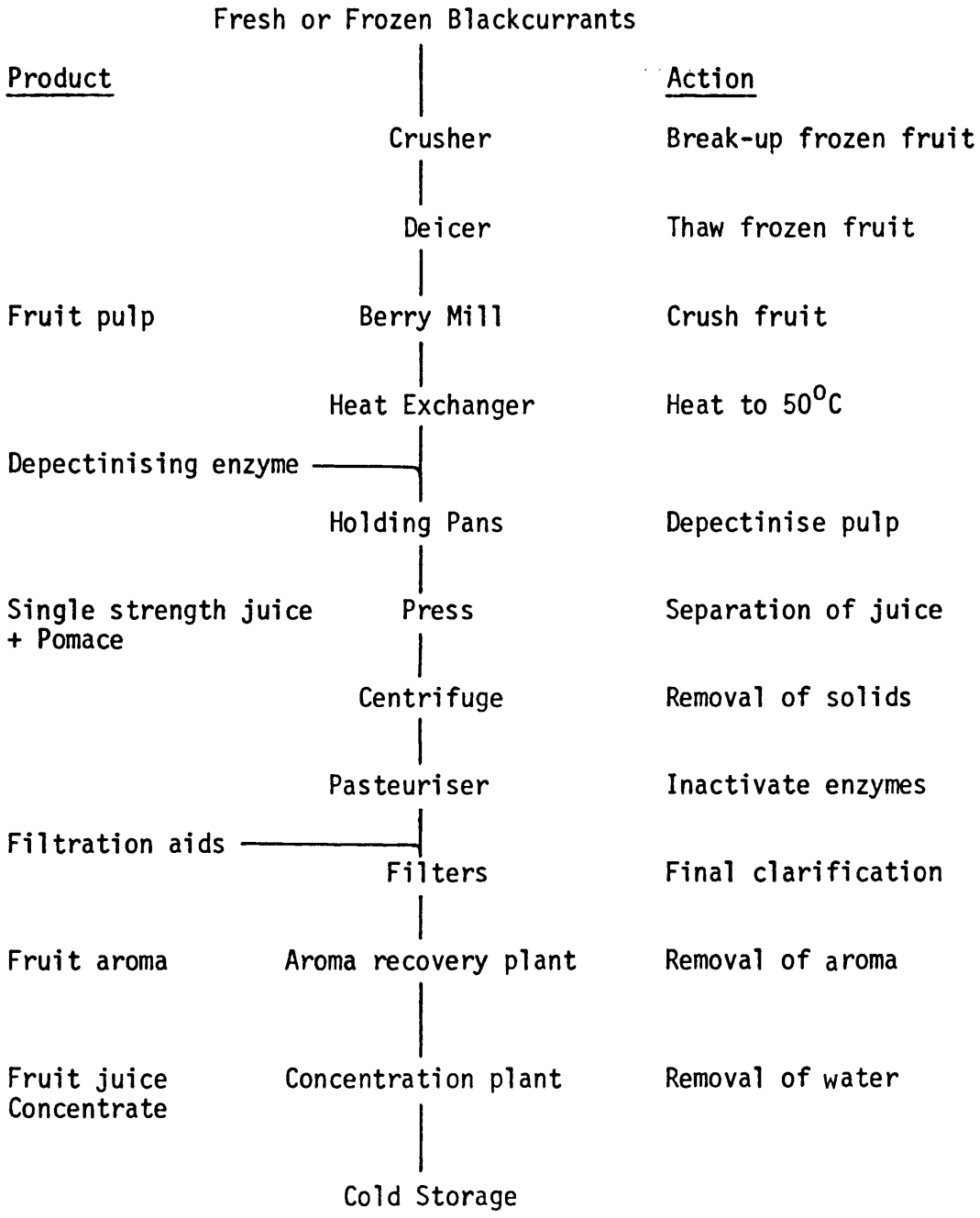


Fig. III. Scheme for the commercial production of blackcurrant juice and aroma concentrates

All the fruit aroma extracts were carried out on fruit from which all strig, leaf and stalks had been removed. The micro still used to remove dichloromethane from the fruit extracts is shown in figure IV. Using low temperature solvent extraction blackcurrant aroma was isolated from Baldwin, Ben Lomond and Wellington XXX blackcurrants at various stages of ripening, and isolated from separated skin, flesh and seeds to ascertain changes in aroma composition during ripening and distribution of aroma compounds in the fruit.

The appearance of high levels of monoterpene alcohols in the commercially processed blackcurrants, indicated either oxidation of monoterpene hydrocarbons during processing or the presence of non-volatile precursors. Non-volatile precursors such as polyhydroxylated terpenes or terpene glycosides have been reported in grapes (58-65), tobacco (66-70), peppermint (57) and passionfruit (71). In order to eliminate oxidation as much as possible during processing, the commercial process on a laboratory scale was carried out with all operations being performed in an atmosphere of nitrogen. The presence of monoterpene glycosides was investigated by treating the aqueous residue from the low temperature dichloromethane extract with a glycosidase. The aqueous residue was suspended in two volumes ($40cm^3$) 0.1M phosphate buffer pH 5.0 (for details of reagent preparation, see appendix I) and homogenised with a Silverson micro disintegration head as previously described. The homogenate was briefly evaporated by the rotary film technique to remove any residual dichloromethane which might inhibit enzyme activity. 0.1% m/v Pectinol C (0.06g) was added in $1cm^3$ of phosphate buffer and the homogenate incubated at 30^oC for 24 hours.

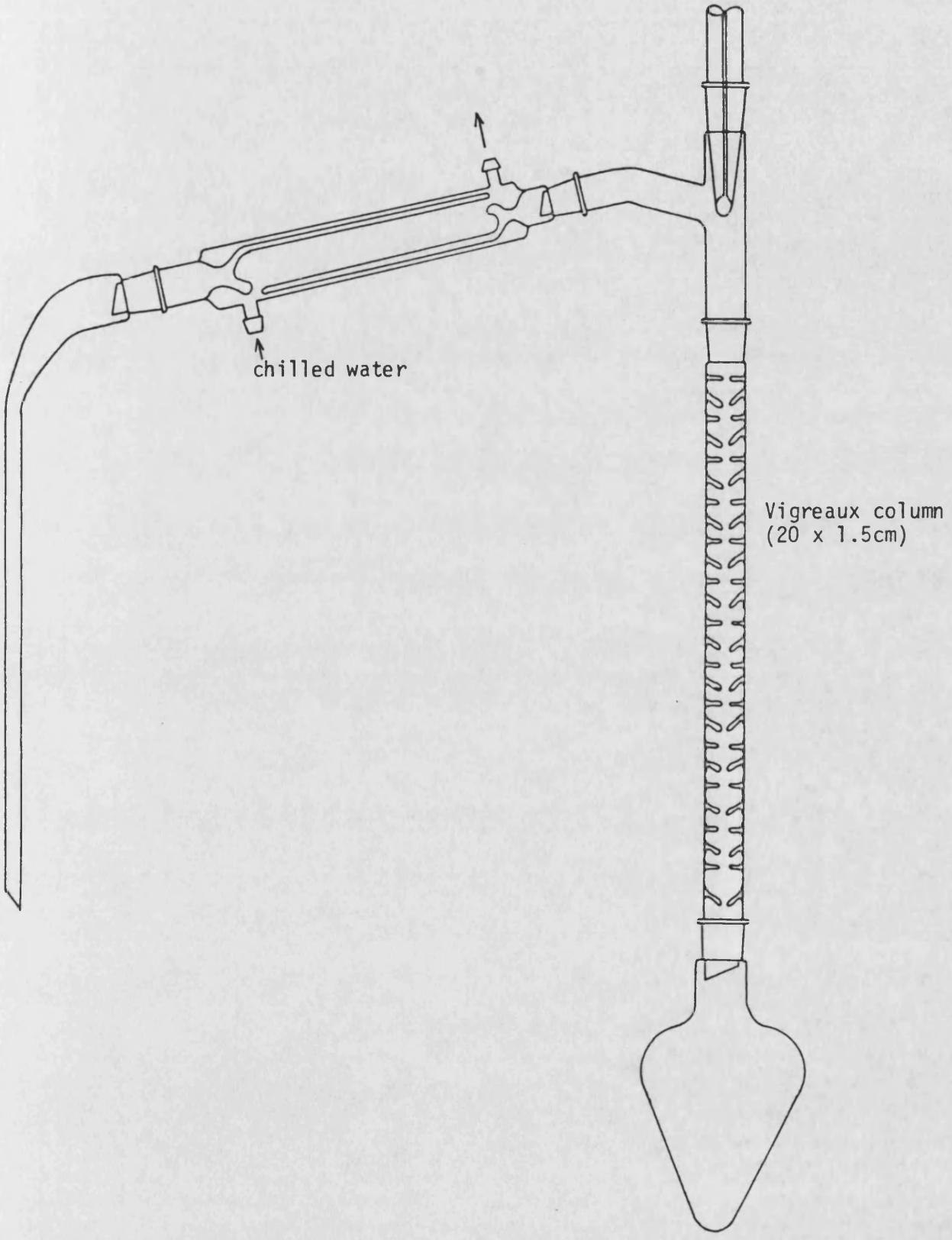


Fig. IV. Micro still used for removal of dichloromethane from blackcurrant extracts

After incubation the homogenate was extracted with three 60cm^3 portions of redistilled dichloromethane. The extracts were bulked, dried over anhydrous calcium sulphate and the volume carefully reduced to 2cm^3. The concentrated extract was again stored in a hypovial at -25^{o}C under nitrogen.

Glycosides were also isolated from blackcurrant juice by reverse phase liquid chromatography, then hydrolysed by the addition of a glycosidase. Blackcurrants (500g) were thawed and crushed as previously described and the juice expressed immediately, followed by centrifugation at 15000g for twenty minutes. A Pyrex glass column (20 x 1cm i.d.) was slurry packed with Licroprep RP-18 (15g) in methanol, then washed with methanol (500cm^3) and glass distilled water (500cm^3). Clarified blackcurrant juice (50cm^3) was diluted with an equal volume of distilled water then passed through the Licroprep column. The column was washed with distilled water (1000cm^3), then the absorbed compounds were eluted from the column using Analar methanol (500cm^3). The methanol was removed by rotary film evaporation, and the residue dissolved in pH 5.2 phosphate buffer (25cm^3). The glycoside solution was extracted with three 10cm^3 portions of redistilled dichloromethane to remove any volatiles. After extraction the aqueous glycoside solution was treated with 0.1% m/v Pectinol C (0.025g) as previously described. The final extract of the aglycons was carefully concentrated to 5cm^3, and again stored in a hypovial at -25^{o}C under nitrogen.

[2-^{13}C] Mevalonate feeding experiments

Blackcurrant stems (*ca.* 150g) with 4 or 5 strigs of fruit were picked from 3 year old bushes when the fruit was fully formed but

still green (1st - 3rd week of June 1985), since earlier experiments showed the highest level of terpenes occurred at this stage of ripening.

The stems were defoliated, cut under sterile water and immediately immersed in a solution of [2-^{13}C] mevalonate (0.1m mol) and glucose (0.3m mol) in 10cm^3 sterile water. Immediately before use, the mevalonic acid lactone was converted into the potassium salt by incubating the lactone with an excess of aqueous potassium bicarbonate at 37^{o}C for 1 hour. The mevalonate solution was followed with 0.5cm^3 sterile water after which the stems were fed with a nutrient solution (see Appendix I) supplemented with glucose (1mg (cm^3)$^{-1}$) and adjusted to pH 7.8. Fruits were picked at regular intervals over the following 10 days, and the volatiles extracted.

Because of the expected low incorporation of mevalonate, a slightly different method of extraction was used. The isolation was carried out by low temperature solvent extraction as previously described, except a solution of anisalcohol (10mg (cm^3)$^{-1}$ in methanol) was added prior to extraction. 10µl of anisalcohol solution was added to 20g blackcurrants which resulted in a level of 1mg.ℓ^{-1} in the concentrated dichloromethane extract. The use of the internal standard was considered necessary if accurate assessment of mevalonate incorporation was to be obtained.

Analysis of blackcurrant leaf and fruit volatiles

Blackcurrant leaf oils were initially analysed by gas chromatography with a flame ionisation detector. Analysis was carried out under the following conditions.

Chromatograph	:	Pye Unicam 204
Column (1)	:	50m x 0.22mm i.d. vitreous silica capillary, coated with BP1 (≡OV-101) (film thickness 0.15 μm)
(2)	:	50m x 0.22mm i.d. vitreous silica capillary, coated with BP20 (≡PEG20M) (film thickness 0.15 μm)
Column temperature	:	$50^{o}C$ to $200^{o}C$ at $2^{o}C\ min^{-1}$ BP1 $60^{o}C$ to $200^{o}C$ at $3^{o}C\ min^{-1}$ BP20
Carrier gas	:	Hydrogen
Carrier gas velocity	:	$30cm.\ sec^{-1}$
Injector (1)	:	Split 100:1 (Scientific Glass Engineering)
(2)	:	Splitless (Scientific Glass Engineering)
Injection temperature	:	$200^{o}C$
Injection volume	:	0.1 - 1μl
Detector	:	Flame ionisation
Detector temperature	:	$250^{o}C$
Recorder	:	Philips PM8251 linear chart recorder.

Initial identification was carried out by calculating the Kovats retention index (72) of the peaks, and tentative identification of the compounds was made by reference to prepublished retention data (73). Further confirmation was obtained by standard addition of reference compounds. This was carried out on polar (BP20) and non-polar (BP-1) columns.

Final confirmation of the components of blackcurrant leaf oil was obtained by combined gas chromatography/mass spectrometry, using a Pye Unicam 104 chromatograph directly vacuum coupled to a Micromass 12B mass spectrometer (VG Analytical Ltd.). The mass spectrometer was linked to VG2000 data system. The chromatographic and mass spectrometer conditions are as follows.

Chromatograph	:	Pye Unicam 104
Column (1)	:	50m x 0.22mm i.d. vitreous silica coated with BP20 (≡PEG20M) (film thickness 0.15μm)
(2)	:	50m x 0.22mm i.d. vitreous silica coated with BP1 (≡OV-101) (film thickness 0.15μm)
Carrier gas	:	Helium
Column temperature	:	$60^{o}C$ to $200^{o}C$ at $3^{o}C\ min^{-1}$ (BP20) $50^{o}C$ to $200^{o}C$ at $2^{o}C\ min^{-1}$ (BP1)
Carrier gas velocity	:	30cm. $sec.^{-1}$
Injector	:	Splitless (Scientific Glass Engineering)
Injector temperature	:	$200^{o}C$
Interface	:	Column as above
Interface temperature	:	$200^{o}C$
Mass spectrometer	:	Micromass 12B
Ionisation	:	Electron impact
Ionisation energy	:	70 eV
Acceleration voltage	:	4 kV
Focusing	:	single sector magnetic
Detection	:	electron mutliplier
Multiplier voltage	:	2.5 kV.

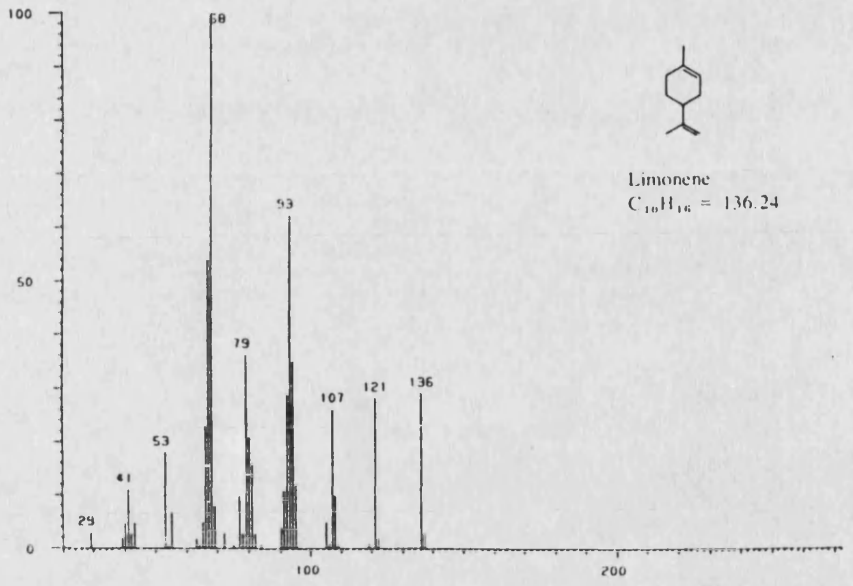
The amplifier setting depended on the concentration of volatiles in the dichloromethane extracts, and was set independently of the data system. The scan duration, law and range was controlled from the data system, and was normally set as follows.

Scan duration : 1 second

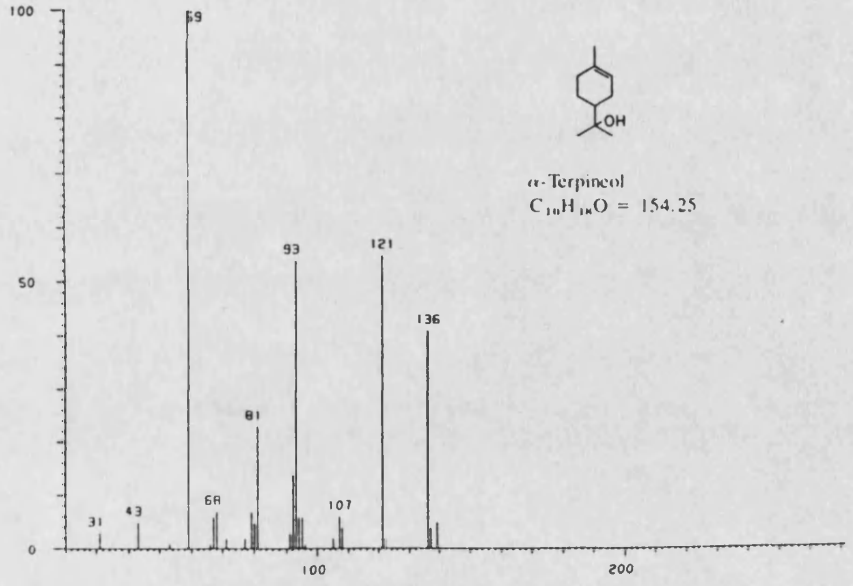
Scan law : linear, decreasing mass

Scan range : 300 - 20 A.M.U.

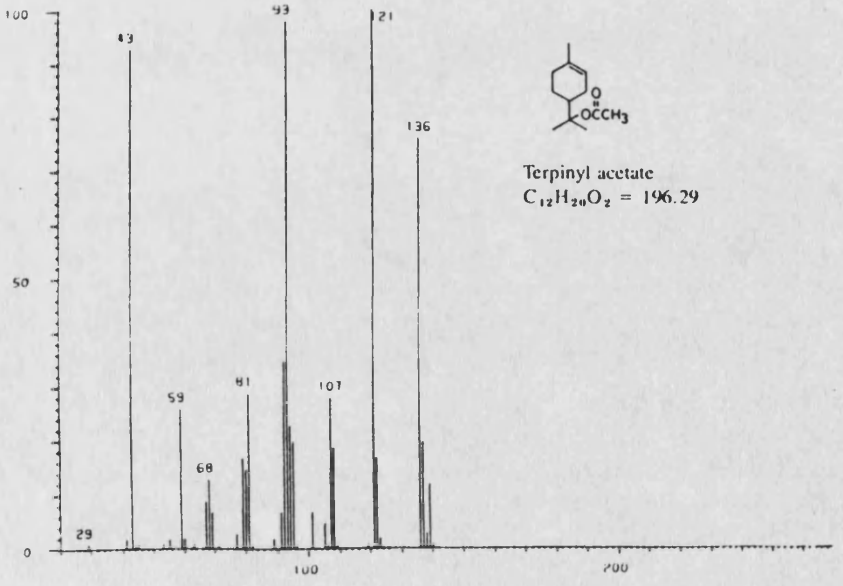
The scan data was acquired by the data system, and when the data collection was completed chromatograms and mass spectra were reconstructed by the data system. Because of the low level of volatiles in the blackcurrants, very little data could be obtained by gas chromatography or conventional mass spectrometry using the data system. To obtain analytical data on blackcurrant extracts, combined gas chromatography/mass spectrometry was used with single ion monitering. The mass spectrometer conditions were the same as previously described, except that the mass being monitored was tuned manually using reference compounds. The output from the amplifier was fed into a linear chart recorder. Using this method selected compounds could be detected down to a level of $1\mu g.\ell^{-1}$. The detection limit varied between compounds, and was related to the intensity and mass of the fragment ions. One of the most important groups of compounds in blackcurrants is the terpenes. Most monoterpene hydrocarbons, alcohols and esters exhibit strong ions at *m/z*93 and *m/z*136 (Fig. V). Using these two ions, calibration mixtures of reference compounds were prepared in dichloromethane ($1mg.\ell^{-1}$), and single ion chromatograms were obtained at both masses. Blackcurrant extracts were run under the same conditions, to obtain qualitative and quantitative data. Quantitative data was based on comparison of peak heights of standard mixtures and sample extracts



Limonene
$C_{10}H_{16}$ = 136.24



α-Terpineol
$C_{10}H_{18}O$ = 154.25



Terpinyl acetate
$C_{12}H_{20}O_2$ = 196.29

Figure V. Examples of monoterpene spectra showing m/z93 and m/z136 used for single ion monitoring.

using both *m*/z93 and *m*/z136, and qualitative data on retention times on both BP1 and BP20 columns compared to retention times of reference compounds. Wherever possible complete mass spectra were obtained. The extracts from the feeding experiments were analysed in the same way except masses *m*/z138 and *m*/z94, the molecular ion and M-44 fragment respectively (fig. VI), were used in order to detect labled terpenes. Qualitative data was again obtained by comparison with retention times of unlabled terpene standards, but due to the low level of label incorporation quantitative data was calculated with reference to the internal standard (anisyl alcohol). The levels of labled compounds were too low for complete mass spectra to be obtained.

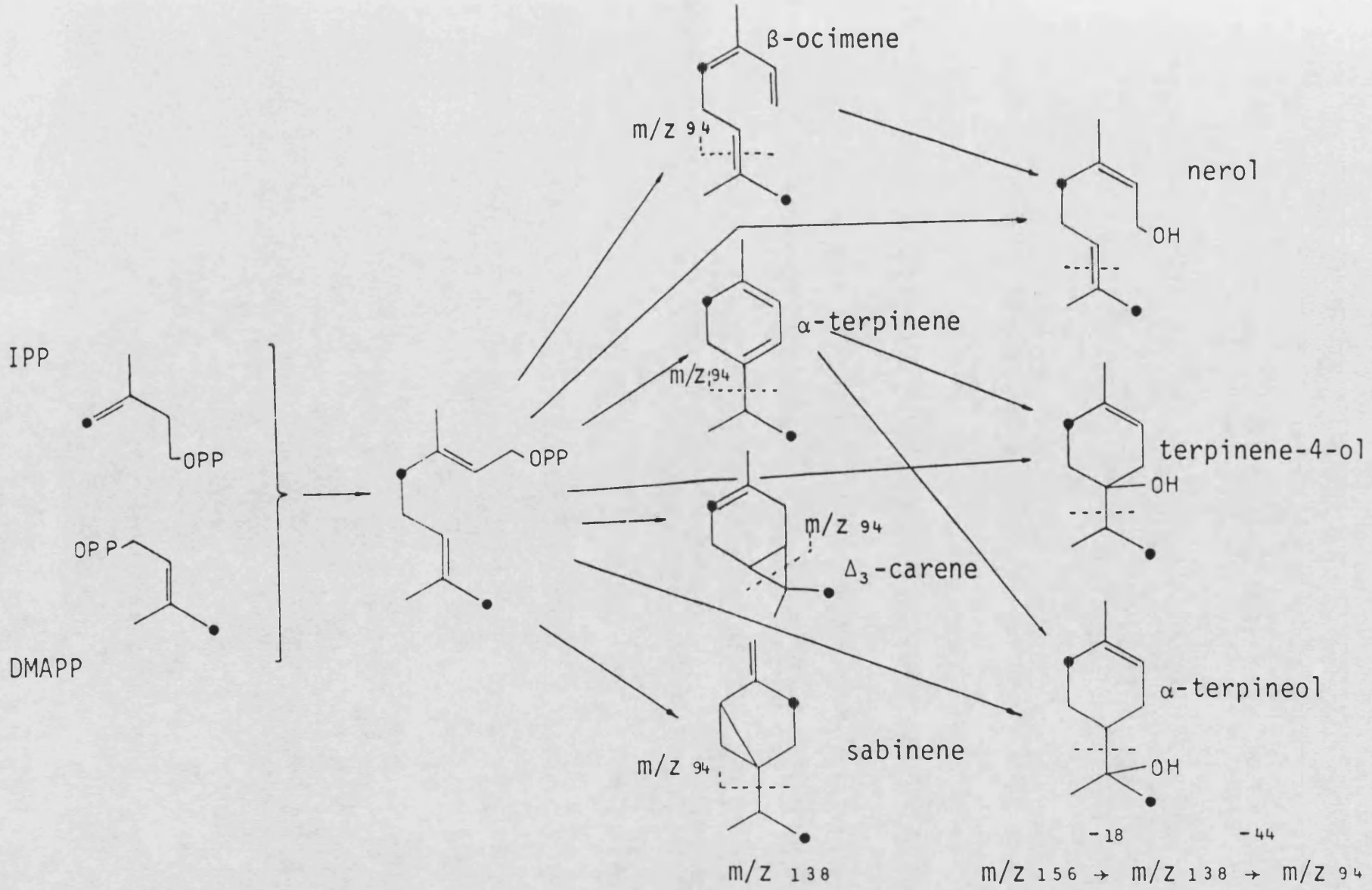


Fig. VI. Fragmentation patterns of ^{13}C labled monoterpenes

CHAPTER 3

Analytical Results

Blackcurrant leaf oil

Blackcurrant leaf oil was obtained from Wellington XXX, Baldwin and Ben Lomond leaves by steam distillation and low temperature solvent extraction, followed by analysis using capillary gas chromatography, combined capillary gas chromatography/mass spectrometry and combined gas chromatography/mass spectrometry using single ion monitoring at *m/z*93 and *m/z*136 for terpene compounds.

The chromatograms obtained from the gas chromatographic analysis of the leaf oils are shown in figures VII to XII, and the concentration of the identified compounds is given in tables II and III. In all instances compounds were identified by capillary gas chromatography/ mass spectrometry using both BP-1 and BP-20 columns, since on each column a number of compounds co-elute, but were resolved on the other column. The chromatographic and mass spectral data was acquired by the data system and reconstructed after the analyses were complete.

The reconstructed total ion chromatogram of steam distilled Ben Lomond leaf oil obtained on a BP-20 column is shown in figure XIII. Single ion chromatograms of the same leaf oils are shown in figures XIV to XIX, and the level of the terpene compounds calculated from these chromatograms are given in tables IV and V.

The figures for both the gas chromatographic analyses and the single ion analyses are mean figures of four separate analyses. In all instances the coefficient of variation was less than five per cent.

Peak identification - steam distilled Ben Lomond leaf oil

1	*trans*-2-hexenal	29	*trans*-β-terpinyl acetate
2	*cis*-3-hexenal	30	citronellyl acetate
3	α-thujene	31	β-caryophyllene
4	α-pinene	32	humulene
5	camphene	33	germacrene-D
6	sabinene + 1-octen-3-ol	34	Δ-cadinene
7	β-pinene	35	α-nerolidol
8	myrcene	36	Unknown m.w. 204 (3)
9	α-phellandrene + hexyl acetate	37	caryophyllene oxide
10	Δ^3-carene	38	humulene oxide
11	α-terpinene	39	Unknown m.w. 220 (4)
12	*p*-cymene	40	" " " (5)
13	β-phellandrene	41	" " " (6)
14	*d*-(+)-limonene	42	" " " (7)
15	*cis*-β-ocimene		
16	*trans*-β-ocimene		
17	γ-terpinene		
18	*trans*-linalool oxide		
19	terpinolene		
20	linalol		
21	Unknown m.w. 154 (1)		
22	*cis*-verbenol		
23	unknown m.w. 152 (2)		
24	*p*-cymen-8-ol		
25	terpinen-4-ol		
26	α-terpineol		
27	geraniol		
28	*cis*-β-terpinyl acetate		

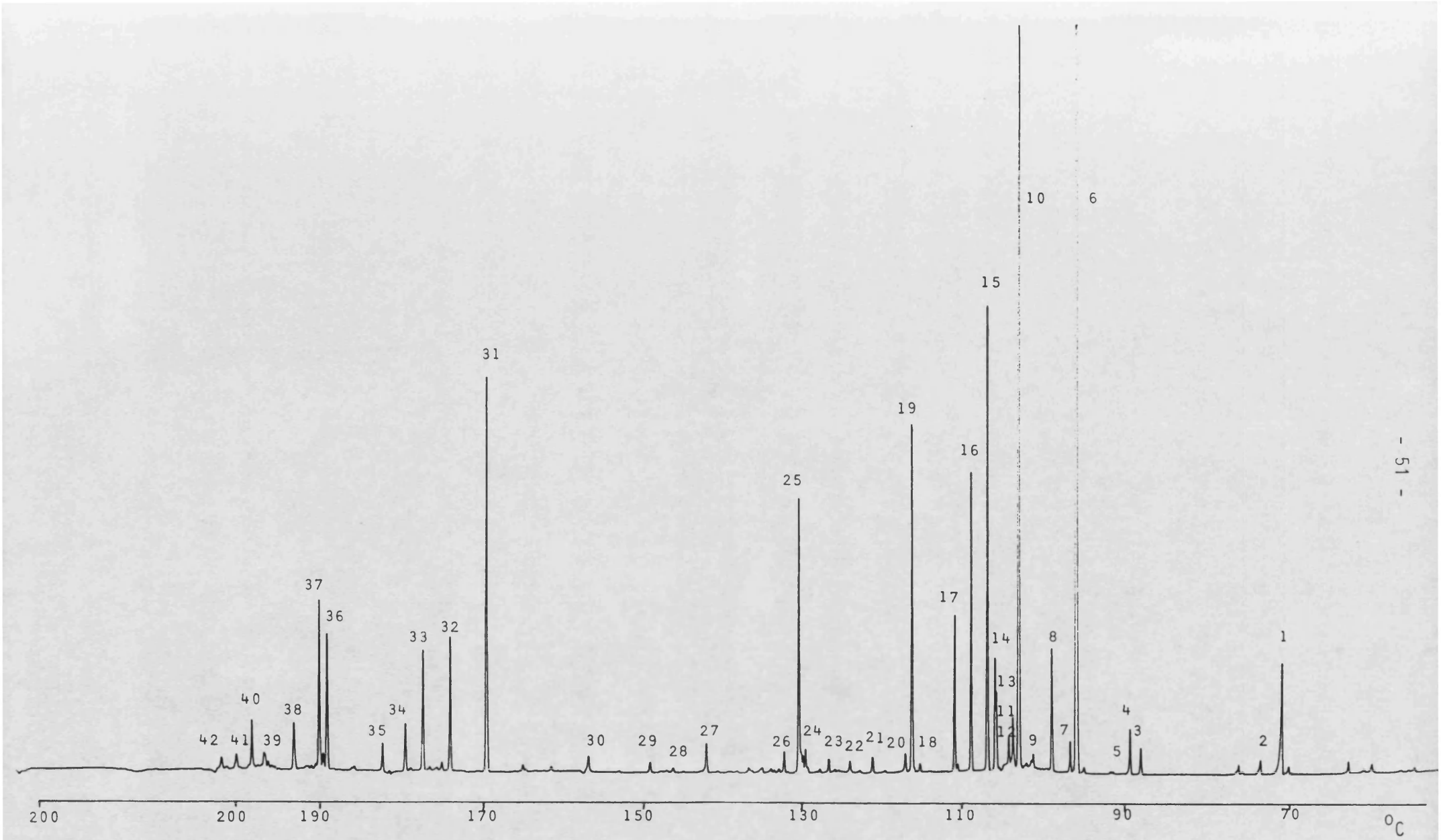


Fig. VII Steam distilled Ben Lomond leaf oil (BP-1 column)

Peak identification - steam distilled Baldwin leaf oil

1	*trans*-2-hexenal	29	terpinen-4-ol
2	*cis*-3-hexenol	30	α-terpineol
3	α-thujene	31	Unknown
4	α-pinene	32	"
5	camphene	33	"
6	sabinene + 1-octen-3-ol	34	"
7	β-pinene	35	"
8	myrcene	36	geraniol
9	α-phellandrene + hexyl acetate	37	Unknown
10	Δ^3-carene	38	"
11	α-terpinene	39	"
12	*p*-cymene	40	"
13	β-phellandrene	41	β-caryophyllene
14	*d*-(+)-limonene	42	Unknown
15	*cis*-β-ocimene	43	humulene
16	*trans*-β-ocimene	44	germacrene-D
17	γ-terpinene	45	Δ-cadinene
18	*trans*-sabinene hydrate	46	Unknown m.w. 204 (3)
19	*trans*-linalool oxide	47	caryophyllene oxide
20	terpinolene	48	Unknown
21	linalol	49	humulene oxide
22	Unknown	50	Unknown
23	Unknown m.w. 154 (1)	51	Unknown m.w. 220 (5)
24	*cis*-verbenol	52	" " " (6)
25	Unknown m.w. 152 (2)	53	" " " (7)
26	Unknown		
27	Unknown		
28	*p*-cymene-8-ol		

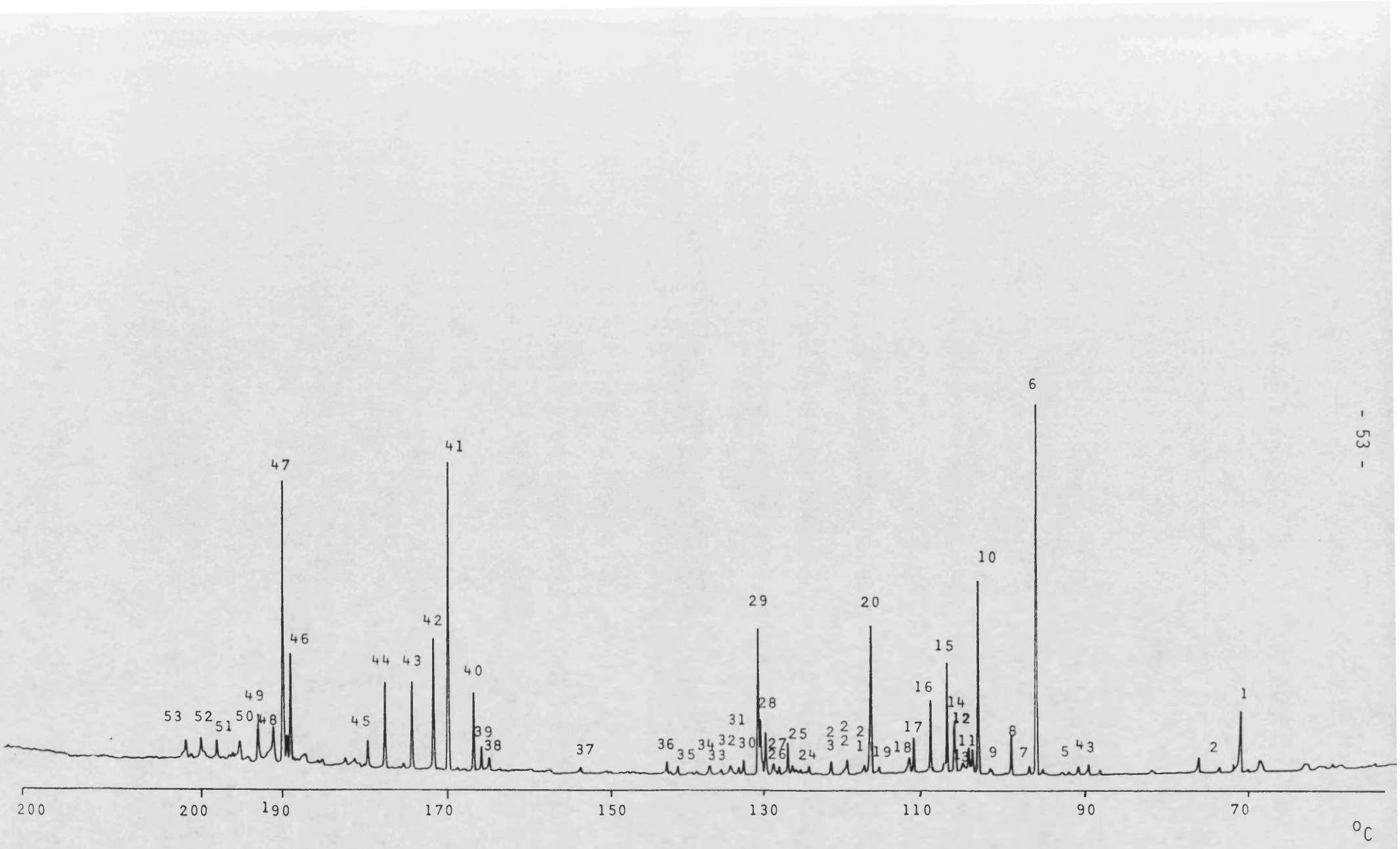


Fig. VIII Steam distilled Baldwin leaf oil (BP-1 column)

Peak identification - steam distilled Wellington leaf oil

1	*trans*-2-hexenal
2	*cis*-3-hexenol
3	α-thujene
4	α-pinene
5	camphene
6	sabinene + 1-octen-3-ol
7	β-pinene
8	myrcene
9	α-phellandrene + hexyl acetate
10	Δ^3-carene
11	α-terpinene
12	*p*-cymene
13	β-phellandrene
14	*d*-(+)-limonene
15	*cis*-β-ocimene
16	*trans*-β-ocimene
17	γ-terpinene
18	*trans*-sabinene hydrate
19	*trans*-linalool oxide
20	terpinolene
21	linalol
22	Unknown m.w. 154 (1)
23	*cis*-verbenol
24	Unknown m.w. 152 (2)
25	terpinen-4-ol
26	α-terpineol
27	Unknown
28	"
29	geraniol
30	*cis*-β-terpinyl acetate
31	*trans*-β-terpinyl acetate
32	citronellyl acetate
33	Unknown
34	β-caryophyllene
35	Unknown
36	humulene
37	alloaromadrene
38	germacrene-D
39	Δ-cadinene
40	α-nerolidol
41	Unknown m.w. 204 (3)
42	caryophyllene oxide
43	humulene oxide
44	Unknown m.w. 220 (4)
45	" " " (5)
46	" " " (6)
47	" " " (7)

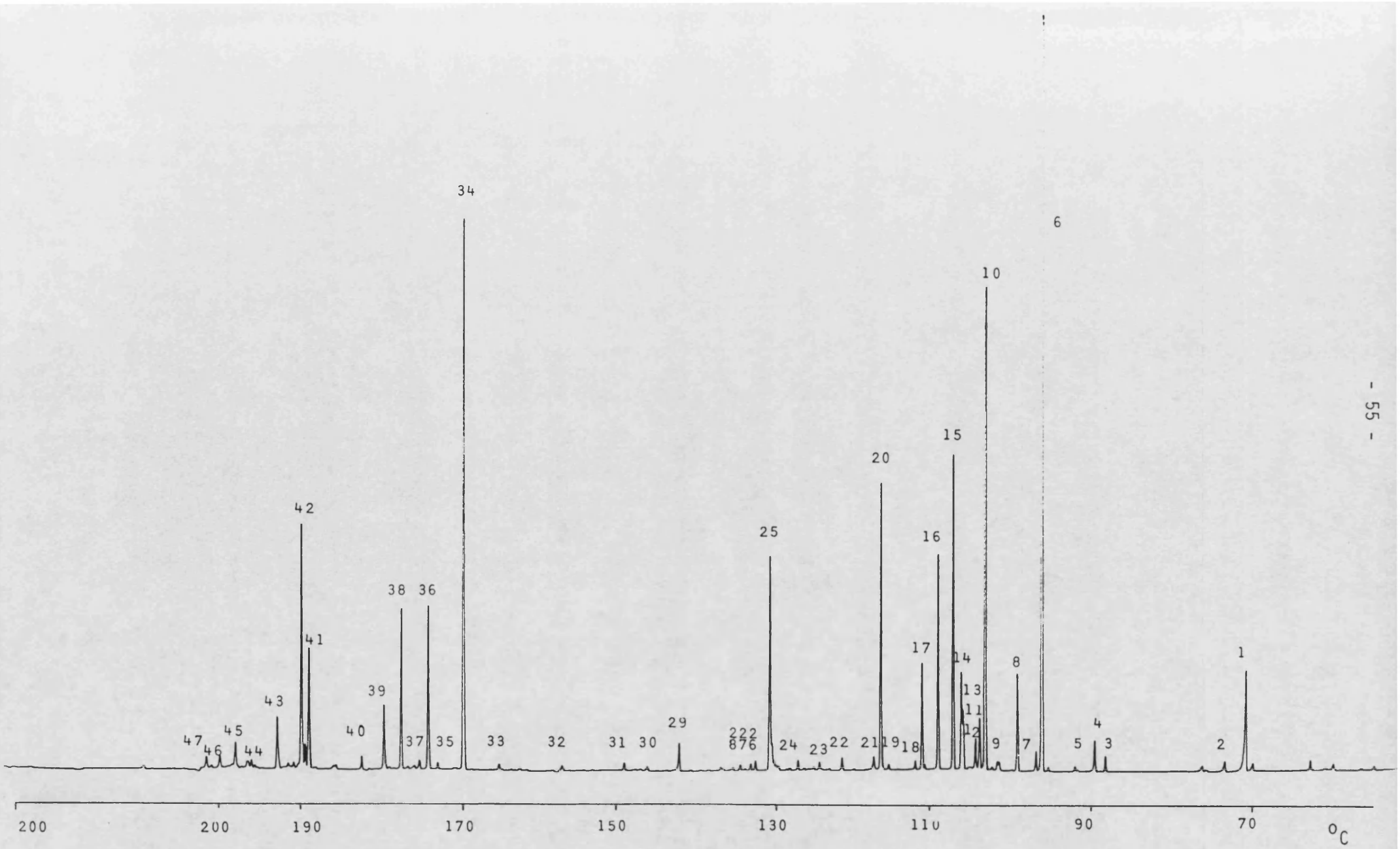


Fig. IX Steam distilled Wellington XXX leaf oil (BP-1 column)

Peak identification - solvent extracted Ben Lomond leaf oil

1	*trans*-2-hexenal	29	humulene
2	α-thujene	30	alloaromadrene
3	α-pinene	31	germacrene-D
4	camphene	32	γ-elemene
5	sabinene + 1-octen-3-ol	33	Δ-cadinene
6	β-pinene	34	α-nerolidol
7	myrcene	35	Unknown
8	α-phellandrene + hexyl acetate	36	Unknown
9	Δ^3-carene	37	Unknown m.w. 204 (3)
10	α-terpinene	38	caryophyllene oxide
11	*p*-cymene	39	humulene oxide
12	β-phellandrene	40	Unknown m.w. 220 (7)
13	*d*-(+)-limonene	41	Unknown
14	*cis*-β-ocimene		
15	*trans*-β-ocimene		
16	γ-terpinene		
17	*trans*-sabinene hydrate		
18	terpinolene		
19	linalool		
20	*p*-cymen-8-ol		
21	terpinen-4-ol		
22	α-terpineol		
23	Unknown		
24	citronellol		
25	geraniol		
26	*cis*-β-terpinyl acetate		
27	citronellyl acetate		
28	β-caryophyllene		

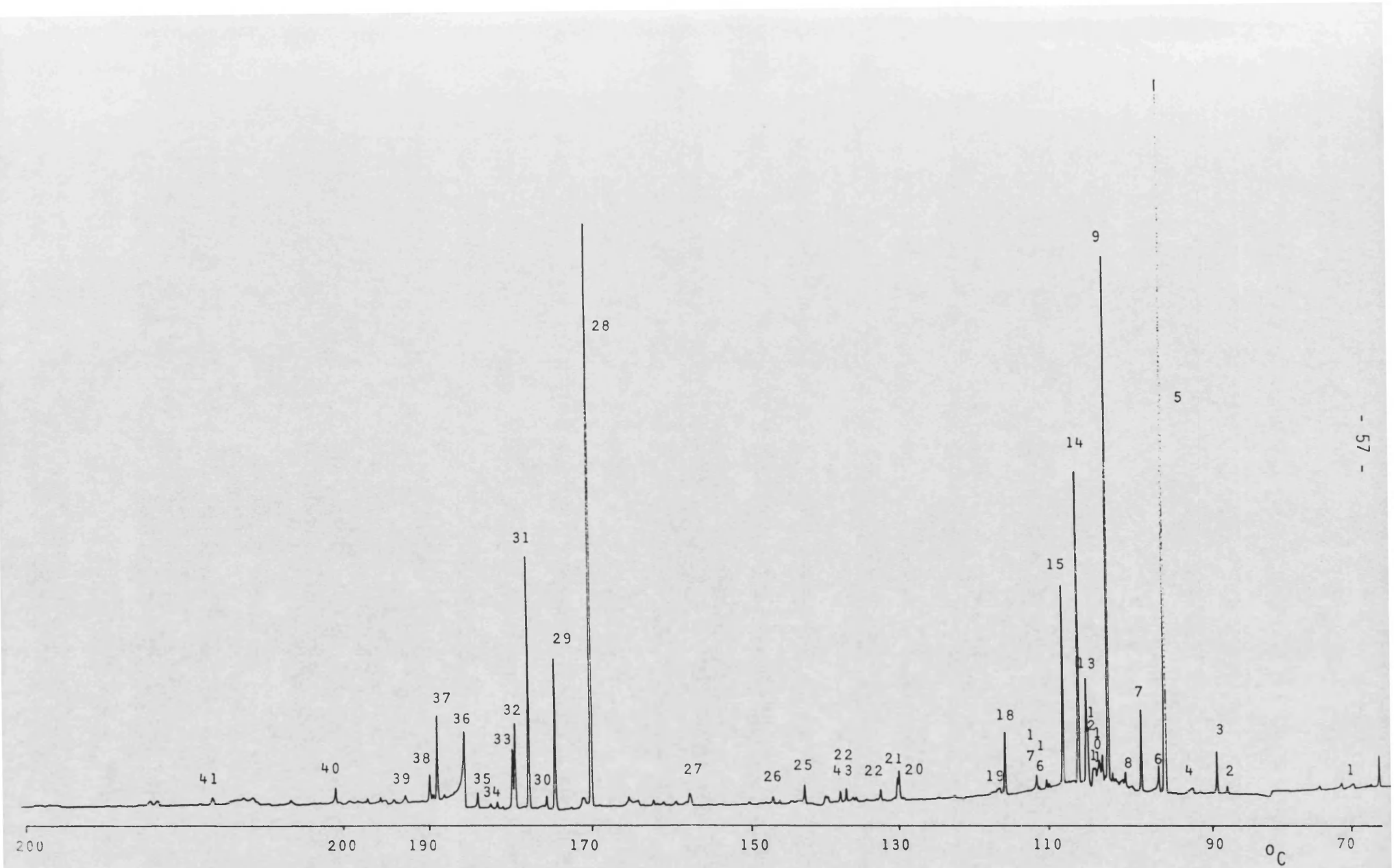


Fig. X Solvent extracted Ben Lomond leaf oil (BP-1 column)

Peak identification - solvent extracted Baldwin leaf oil

1	*trans*-2-hexenal	29	citronellol
2	α-thujene	30	Unknown
3	α-pinene	31	Unknown
4	Unknown m.w. 136 (8)	32	geraniol
5	sabinene	33	*cis*-β-terpinyl acetate
6	β-pinene	34	Unknown
7	myrcene	35	citronellyl acetate
8	α-phellandrene	36	Unknown
9	Unknown m.w. 136 (9)	37	Unknown
10	Δ^3-carene	38	β-caryophyllene
11	α-terpinene	39	humulene
12	*p*-cymene	40	alloaromadrene
13	β-phellandrene	41	germacrene-D
14	*d*-(+)-limonene	42	γ-elemene
15	*cis*-β-ocimene	43	Δ-cadinene
16	*trans*-β-ocimene	44	α-nerolidol
17	Unknown	45	Unknown
18	γ-terpinene	46	Unknown
19	*trans*-sabinene hydrate	47	Unknown m.w. 204 (11)
20	Unknown m.w. 154 (10)	48	Unknown m.w. 204 (3)
21	*trans*-linalool oxide	49	caryophyllene oxide
22	terpinolene	50	Unknown m.w. 204 (12)
23	linalol	51	" " 220 (4)
24	*trans*-β-terpineol	52	" " 220 (6)
25	*p*-cymen-8-ol	53	" " 220 (7)
26	terpinen-4-ol	54	Unknown
27	α-terpineol	55	Unknown
28	Unknown	56	Unknown

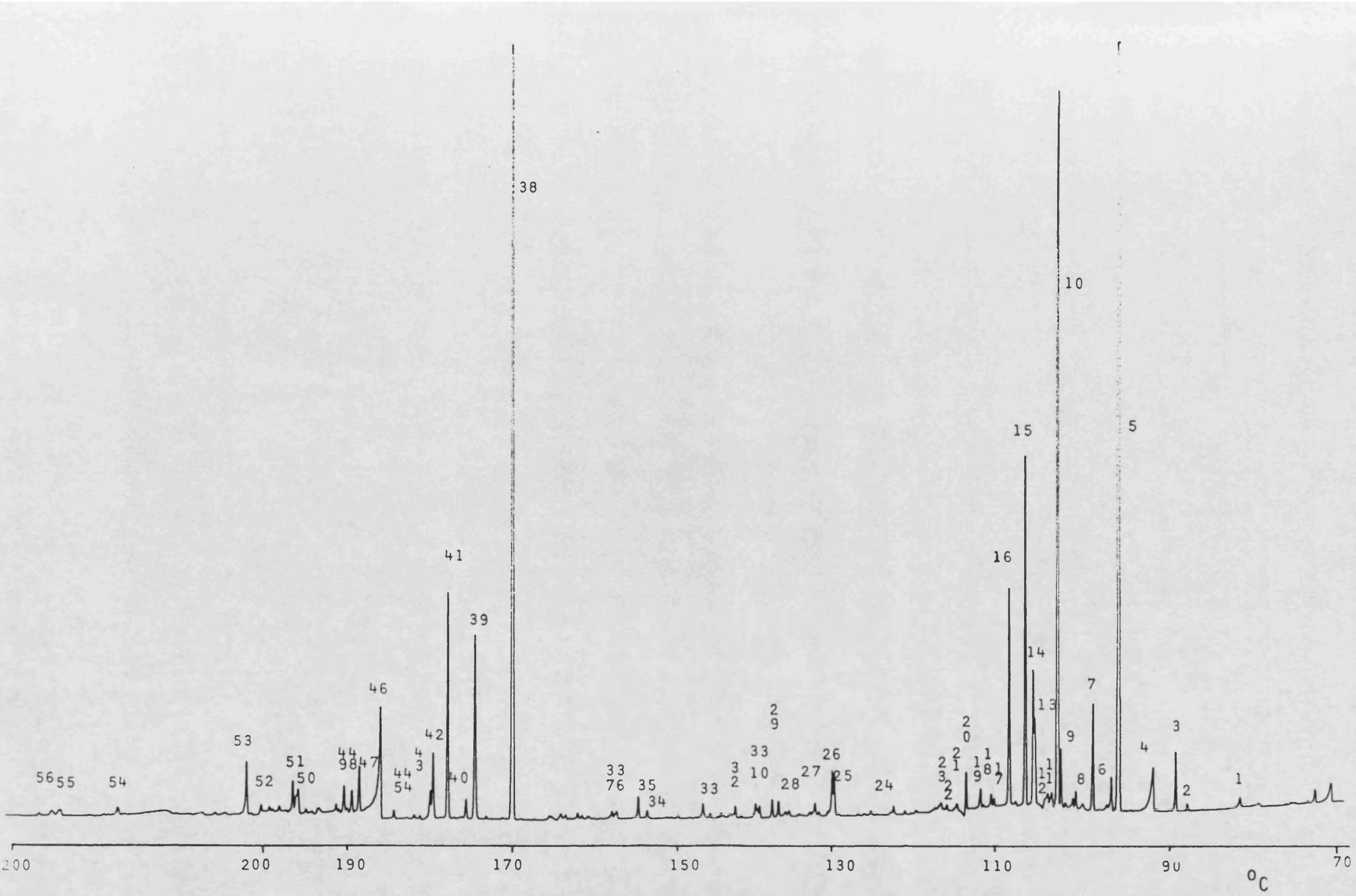


Fig. XI Solvent extracted Baldwin leaf oil (BP-1 column)

Peak identification - solvent extracted Wellington XXX leaf oil

1	*trans*-2-hexenal	29	geraniol
2	α-thujene	30	Unknown
3	α-pinene	31	citronellyl acetate
4	camphene	32	Unknown
5	sabinene	33	Unknown
6	β-pinene	34	β-caryophyllene
7	myrcene	35	humulene
8	α-phellandrene	36	alloaromadrene
9	Unknown m.w. 136 (9)	37	germacrene-D
10	Δ^3-carene	38	γ-elemene
11	α-terpinene	39	Δ-cadinene
12	β-phellandrene	40	Unknown
13	*d*-(+)-limonene	41	Unknown
14	*cis*-β-ocimene	42	Unknown m.w. 204 (11)
15	*trans*-β-ocimene	43	Unknown m.w. 204 (3)
16	Unknown	44	caryophyllene oxide
17	γ-terpinene	45	Unknown
18	*trans*-sabinene hydrate	46	Unknown m.w. 204 (12)
19	Unknown m.w. 154 (10)	47	" " 220 (4)
20	terpinolene	48	" " 220 (7)
21	linalol	49	Unknown
22	*p*-cymen-8-ol	50	"
23	terpinen-4-ol	51	"
24	α-terpineol		
25	Unknown		
26	citronellol		
27	Unknown		
28	Unknown		

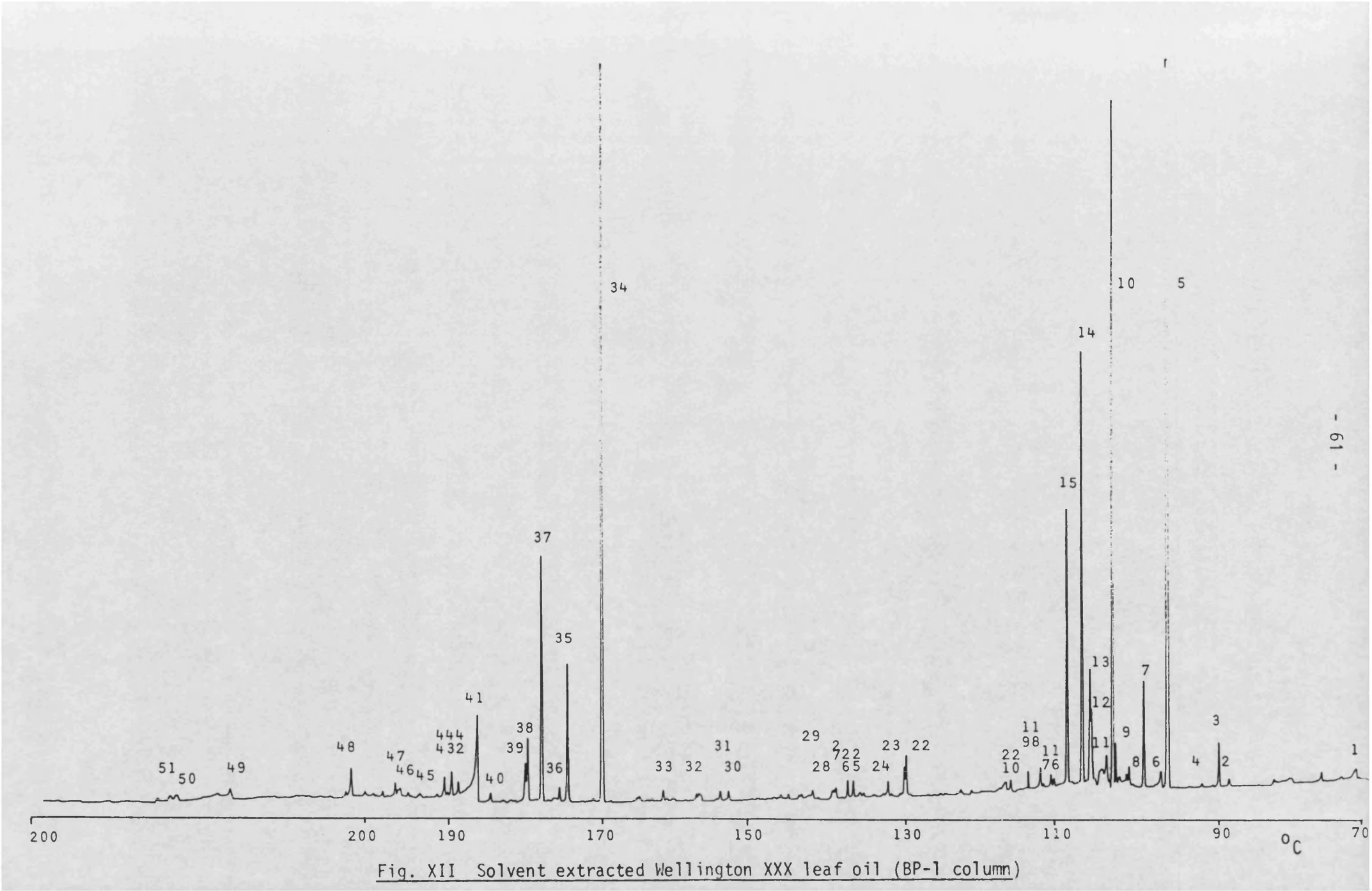


Fig. XII Solvent extracted Wellington XXX leaf oil (BP-1 column)

Peak identification - total ion chromatogram of steam distilled Ben Lomond leaf oil

1 α-pinene
2 β-pinene
3 sabinene
4 Δ^3-carene
5 *d*-(+)-limonene
6 β-phellandrene
7 *trans*-2-hexenal
8 *cis*-β-ocimene
9 γ-terpinene
10 *p*-cymene
11 terpinolene
12 Unknown
13 *n*-hexyl acetate
14 *cis*-3-hexenol
15 1-octen-3-ol
16 linalol
17 *cis*-β-terpineol
18 β-caryophyllene + terpinen-4-ol
19 Unknown
20 *trans*-β-terpineol
21 Unknown
22 humulene
23 α-terpinyl acetate
24 α-terpineol
25 germacrene-D
26 neryl acetate
27 Unknown
28 Δ-cadinene
29 Unknown
30 geraniol
31 Unknown
32 caryophyllene oxide
33 humulene oxide
34 Unknown

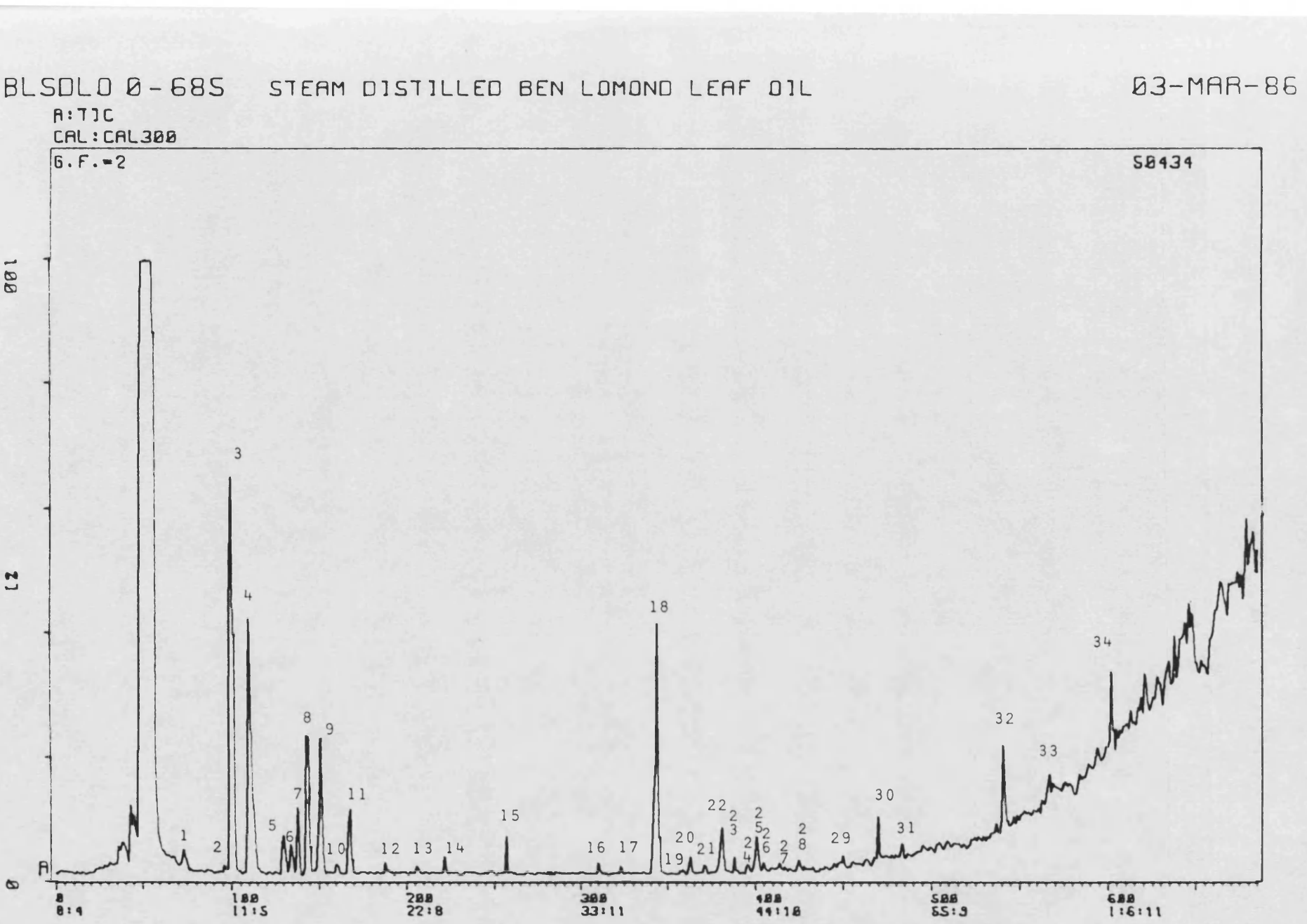


Fig. XIII. Total ion chromatogram of steam distilled Ben Lomond leaf oil (BP-20 column)

Table II. Concentration of volatile components of blackcurrant leaves (mg/kg) calculated from steam distilled oils

Compound	Wellington XXX	Baldwin	Ben Lomond
trans-2-hexenal	4.00	1.19	4.25
cis-3-hexenol	0.38	0.13	0.50
α-thujene	1.01	0.09	1.50
α-pinene	1.56	0.21	2.00
camphene	0.32	0.08	0.16
sabinene	40.00	7.67	53.3
1-octen-3-ol	0.79	0.27	1.01
β-pinene	0.83	0.14	1.19
myrcene	2.70	0.69	4.04
α-phellandrene	0.39	0.13	0.52
n-hexyl acetate	0.41	0.27	0.50
Δ^3-carene	17.90	3.57	28.50
α-terpinene	1.95	0.42	1.83
p-cymene	0.21	0.75	1.81
d-(+)-limonene	3.65	0.94	4.00
cis-β-ocimene	10.00	1.71	14.34
β-phellandrene	2.50	0.40	3.20
trans-β-ocimene	6.86	1.11	9.20
γ-terpinene	4.86	0.78	6.86
terpinolene	36.00	9.20	42.40
trans-sabinene hydrate	0.67	0.56	n/d
trans-linalool oxide	0.44	0.27	0.67

linalool	1.00	0.27	1.35
cis-verbenol	0.44	0.33	0.78
p-cymen-8-ol	5.45	5.45	5.45
terpinen-4-ol	13.40	3.60	16.80
α-terpineol	0.69	1.16	1.62
geraniol	2.90	0.64	2.90
cis-β-terpinyl acetate	0.09	n/d	0.79
trans-β-terpinyl acetate	0.28	n/d	1.61
citronellyl acetate	0.13	0.33	3.30
β-caryophyllene	46.50	12.97	32.40
humulene	14.50	3.33	9.76
alloaromadrene	1.03	n/d	n/d
germacrene-D	12.50	3.50	9.00
Δ-cadinene	5.00	3.50	3.75
α-nerolidol	1.26	1.00	2.25
caryophyllene oxide	30.80	12.57	14.57
humulene oxide	4.57	2.01	4.00
Total	277.97	81.24	292.11

n/d = not detected

Table III. Concentration of volatile components in blackcurrant leaves (mg/kg) calculated from solvent extracted oil

Compound	Wellington XXX	Baldwin	Ben Lomond
trans-2-hexenal	1.25	0.63	1.90
α-thujene	0.81	0.78	0.82
α-pinene	4.96	6.38	4.61
camphene	0.39	0.39	0.39
sabinene	153.25	153.25	116.67
1-octen-3-ol	0.09	0.02	0.04
β-pinene	1.63	2.97	2.38
myrcene	8.77	8.51	6.91
α-phellandrene	1.97	1.97	1.64
n-hexyl acetate	0.57	0.55	0.61
Δ^3-carene	64.58	63.98	49.11
α-terpinene	2.44	1.52	2.44
p-cymene	1.70	1.70	2.27
d-(+)-limonene	10.29	11.76	9.41
β-phellandrene	7.33	8.67	6.00
cis-β-ocimene	34.59	26.76	24.75
trans-β-ocimene	21.72	16.41	15.91
γ-terpinene	1.25	1.42	1.42
trans-sabinene hydrate	3.33	2.78	3.33
trans-linalool oxide	0.27	n/d	1.11
terpinolene	4.00	2.00	2.00
linalool	1.70	1.13	1.13

trans-β-terpineol	n/d	1.12	n/d
p-cymen-8-ol	28.40	29.55	20.45
terpinen-4-ol	4.00	7.00	3.25
α-terpineol	2.91	2.33	2.33
citronellol	5.73	5.21	4.16
geraniol	2.42	3.22	4.84
cis-β-terpinyl acetate	n/d	5.21	2.20
citronellyl acetate	2.50	4.16	1.67
β-caryophyllene	226.00	229.73	123.60
humulene	26.19	33.33	28.57
alloraomadrene	1.11	1.11	1.11
germacrene-D	48.75	50.00	42.50
γ-elemene	18.20	30.10	17.80
Δ-cadinene	12.50	16.25	12.50
α-nerolidol	1.31	1.20	2.20
caryophyllene oxide	4.28	5.71	5.71
humulene oxide	0.63	0.63	0.63
Total	711.82	739.44	528.37

Table IV. Concentration of volatile terpenes in blackcurrant leaves (mg/kg) calculated from single ion monitoring(*m/z*93) analysis of distilled oils

Compound	Wellington XXX	Baldwin	Ben Lomond
α-pinene	2.65	0.46	3.76
camphene	0.10	n/d	0.19
sabinene	69.30	11.60	93.3
β-pinene	1.12	0.15	1.90
myrcene	4.57	0.76	5.71
α-phellandrene	0.22	n/d	0.19
Δ^3-carene	30.10	6.39	47.4
α-terpinene	2.09	n/d	0.85
d-(+)-limonene	1.79	0.46	2.75
cis-β-ocimene	26.87	5.00	38.13
trans-β-ocimene	1.00	0.06	0.63
γ-terpinene	13.05	1.89	18.52
terpinolene	39.09	1.63	41.8
β-phellandrene	2.43	0.32	3.65
linalol	0.39	0.19	1.17
trans-β-terpineol	0.40	n/d	0.53
cis-β-terpineol	0.06	n/d	0.13
terpinene-4-ol	17.50	5.80	27.4
α-terpineol	0.80	0.67	2.13
geraniol	2.28	n/d	4.00
α-terpinyl acetate	0.32	n/d	0.64

neryl acetate	1.71	n/d	2.00
β-caryophyllene	40.00	10.28	51.42
humulene	6.67	1.65	11.03
germacrene-D	9.80	2.40	8.10
Δ-cadinene	4.10	n/d	3.80
caryophyllene oxide	26.90	10.40	14.01
humulene oxide	5.80	n/d	5.21
Total	311.06	55.12	390.22

Table V. Concentration of volatile terpenes in blackcurrant leaves (mg/kg) calculated from single ion monitoring (*m/z*93) analysis of solvent extracted oil

Compound	Wellington XXX	Baldwin	Ben Lomond
α-pinene	3.98	5.05	5.05
camphene	0.25	0.25	0.25
sabinene	122.98	122.1	101.20
β-pinene	1.25	2.50	2.32
myrcene	7.03	5.62	5.51
α-phellandrene	0.19	0.28	0.28
Δ^3-carene	51.80	50.10	42.20
α-terpinene	n/d	n/d	n/d
d-(+)-limonene	4.25	4.96	4.96
cis-β-ocimene	44.44	33.90	41.47
trans-β-ocimene	0.79	0.98	0.79
γ-terpinene	17.30	12.80	15.80
terpinolene	1.56	0.78	12.50
β-phellandrene	4.56	5.03	4.08
linalol	0.25	n/d	0.50
α-terpineol	2.31	2.69	1.54
geraniol	2.88	5.76	3.84
β-caryophyllene	91.25	126.25	56.25
humulene	12.50	24.6	13.28
germacrene-D	20.20	23.43	19.99

Δ-cadinene	7.21	9.20	7.90
caryophyllene oxide	2.91	n/d	n/d
Total	401.08	436.28	340.90

Peak identification - mass fragmentogram (*m*/z93) of distilled Ben Lomond leaf oil

1 α-pinene
2 camphene
3 β-pinene
4 sabinene
5 Δ^3-carene
6 myrcene
7 α-phellandrene
8 α-terpinene
9 *d*-(+)-limonene
10 β-phellandrene
11 *cis*-β-ocimene
12 *trans*-β-ocimene
13 γ-terpinene
14 terpinolene
15 linalol
16 *cis*-β-terpineol
17 β-carophyllene + terpinen-4-ol
18 *trans*-β-terpineol
19 Unknown
20 humulene
21 α-terpinyl acetate
22 α-terpineol
23 germacrene-D
24 neryl acetate
25 Δ-cadinene
26 geraniol
27 caryophyllene
28 humulene oxide
29 Unknown

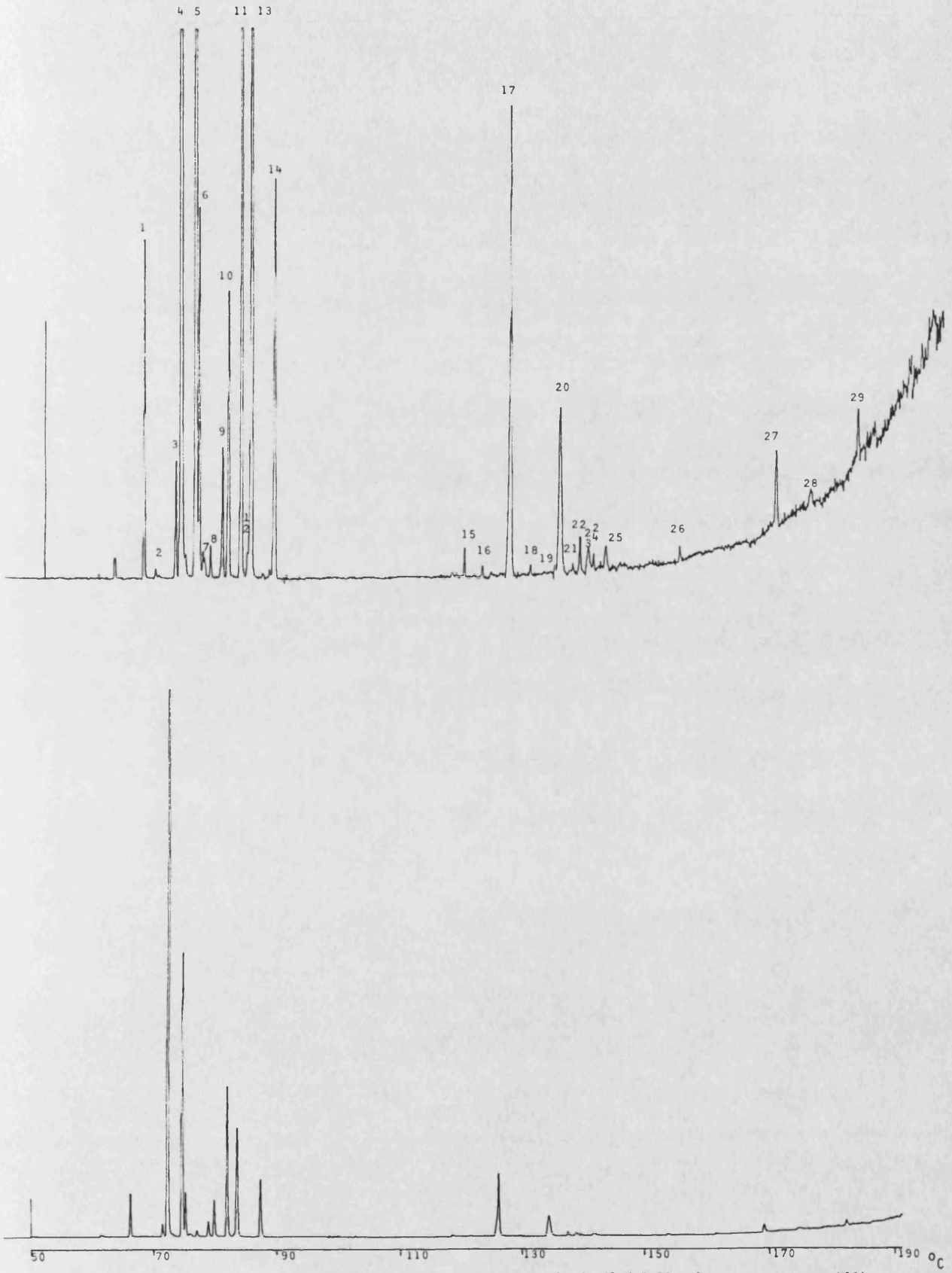


Fig. XIV Mass fragmentogram (m/z93) of steam distilled Ben Lomond leaf oil (BP-20 column, upper trace X10)

Peak identification - mass fragmentogram (*m*/z93) of distilled Baldwin leaf oil

1 α-pinene

2 β-pinene

3 sabinene

4 Δ^3-carene

5 myrcene

6 *d* -(+)-limonene

7 β-phellandrene

8 *cis*-β-ocimene

9 γ-terpinene

10 terpinolene

11 linalol

12 *cis*-β-terpineol

13 β-caryophyllene + terpinen-4-ol

14 humulene

15 α-terpineol

16 germacrene-D

17 neryl acetate

18 Unknown

19 caryophyllene oxide

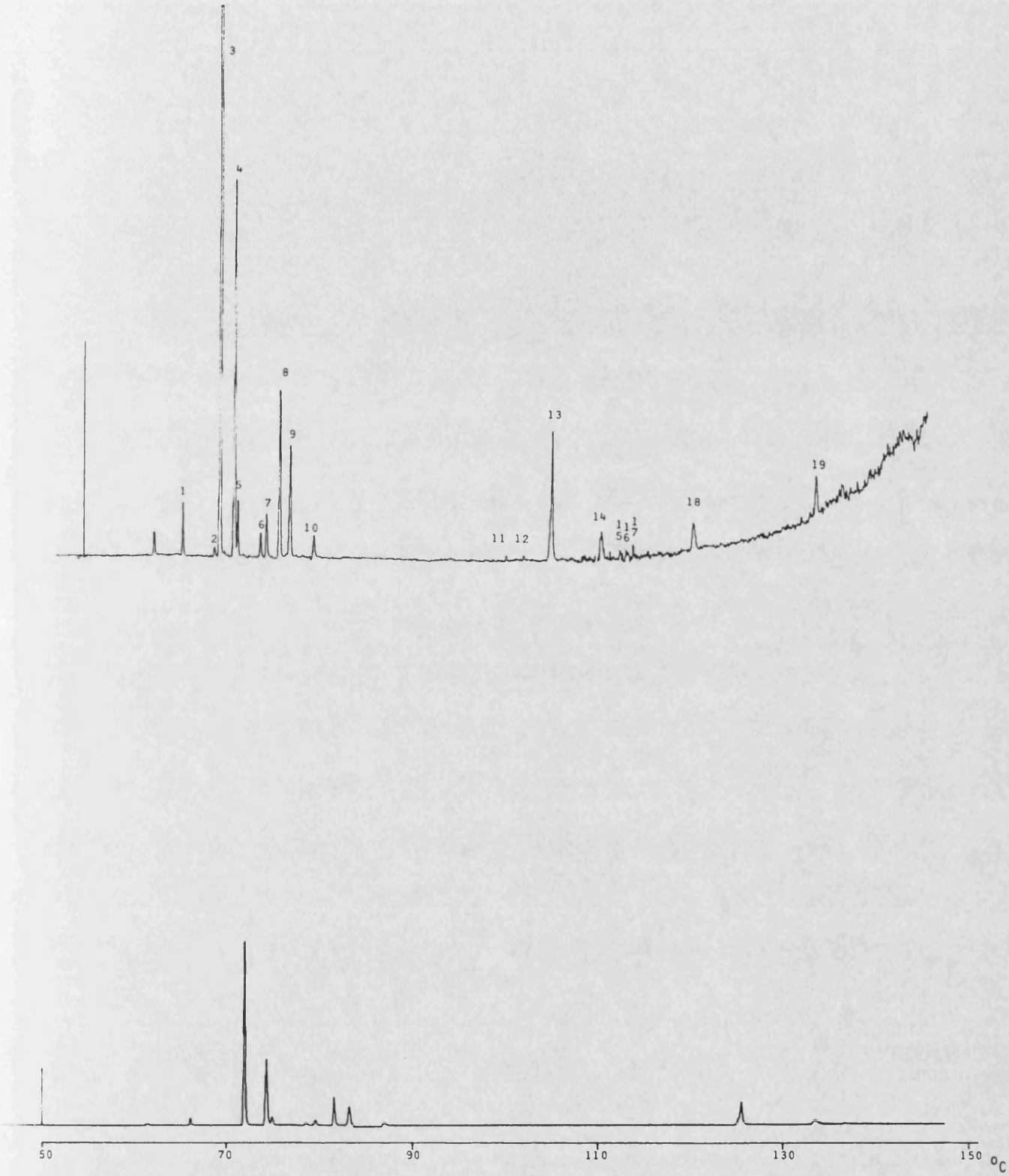
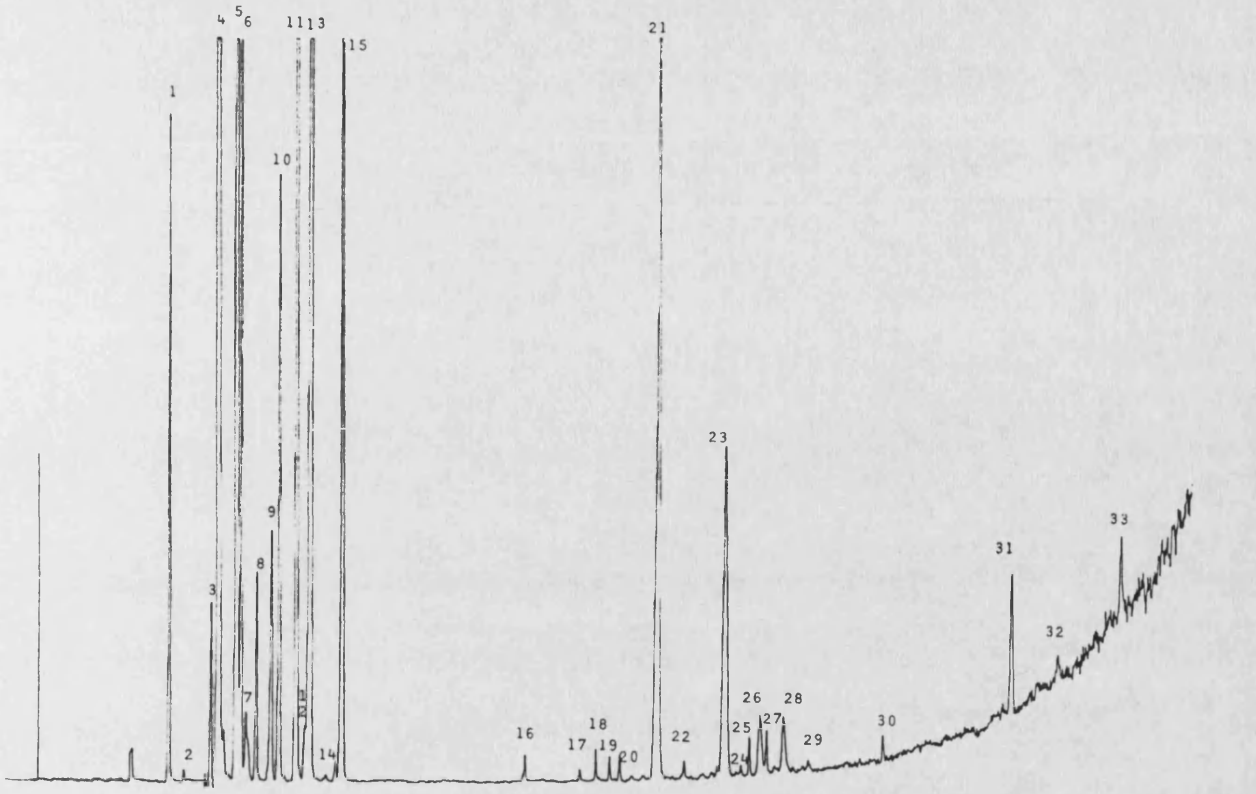


Fig. XV Mass fragmentogram (*m/z*93) of steam distilled Baldwin leaf oil (BP-20 column, upper trace X10)

Peak identification - mass fragmentogram (*m*/z93) of distilled Wellington XXX leaf oil

1 α-pinene
2 camphene
3 β-pinene
4 sabinene
5 Δ^3-carene
6 myrcene
7 α-phellandrene
8 α-terpinene
9 *d*-(+)-limonene
10 β-phellandrene
11 *cis*-β-ocimene
12 *trans*-β-ocimene
13 γ-terpinene
14 Unknown
15 terpinolene
16 Unknown
17 Unknown
18 linalol
19 Unknown
20 Unknown
21 β-caryophyllene + terpinene-4-ol
22 *trans*-β-terpineol
23 humulene
24 α-terpinyl acetate
25 α-terpineol
26 germacrene-D
27 neryl acetate
28 Δ-cadinene
29 Unknown
30 geraniol
31 caryophyllene oxide
32 humulene oxide
33 Unknown



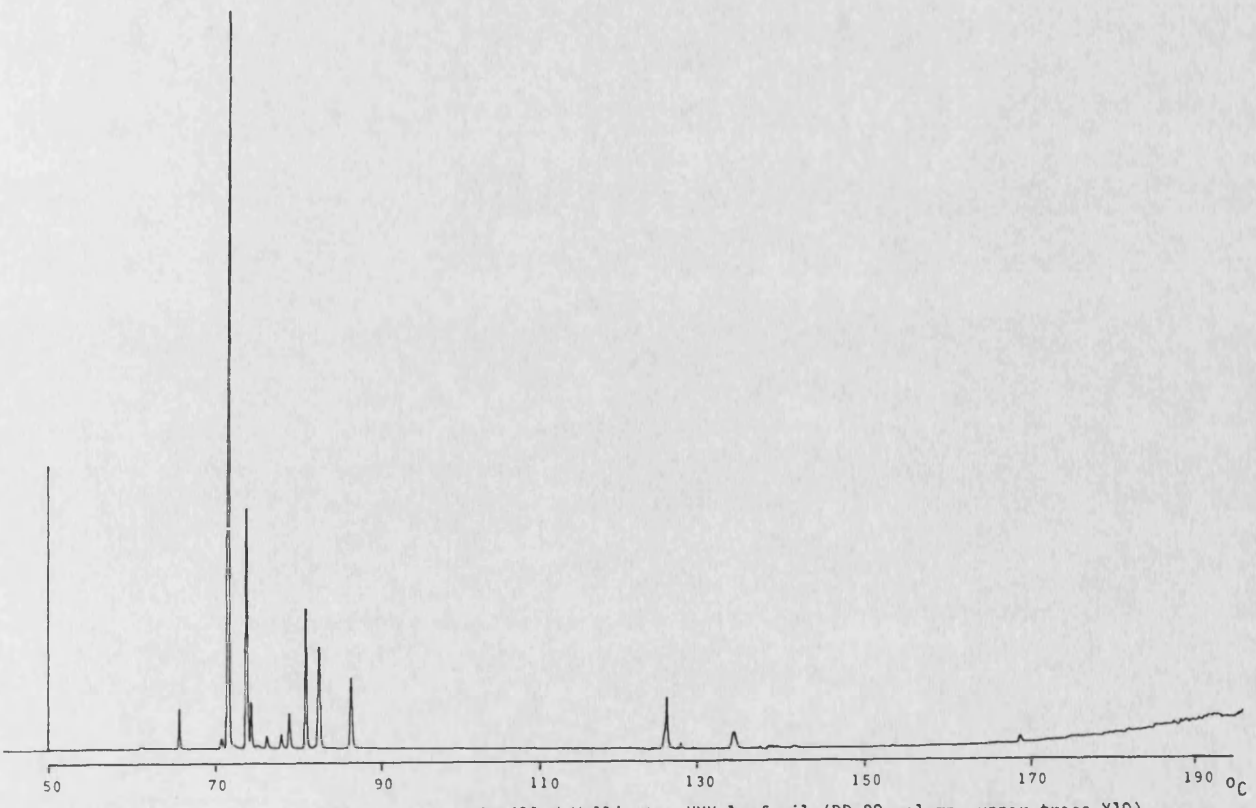


Fig. XVI Mass fragmentogram (m/z93) of steam distilled Wellington XXX leaf oil (BP-20 column, upper trace X10)

Peak identification - mass fragmentogram (*m*/z93) of solvent extracted Ben Lomond leaf oil

1 α-pinene

2 camphene

3 β-pinene

4 sabinene

5 Δ^3-carene + myrcene

6 *d*-(+)-limonene

7 β-phellandrene

8 *cis*-β-ocimene

9 *trans*-β-ocimene

10 γ-terpinolene

11 terpinolene

12 Unknown

13 Unknown

14 β-caryophyllene + terpinen-4-ol

15 humulene

16 α-terpineol

17 germacrene-D

18 Δ-cadinene

19 geraniol

20 caryophyllene oxide

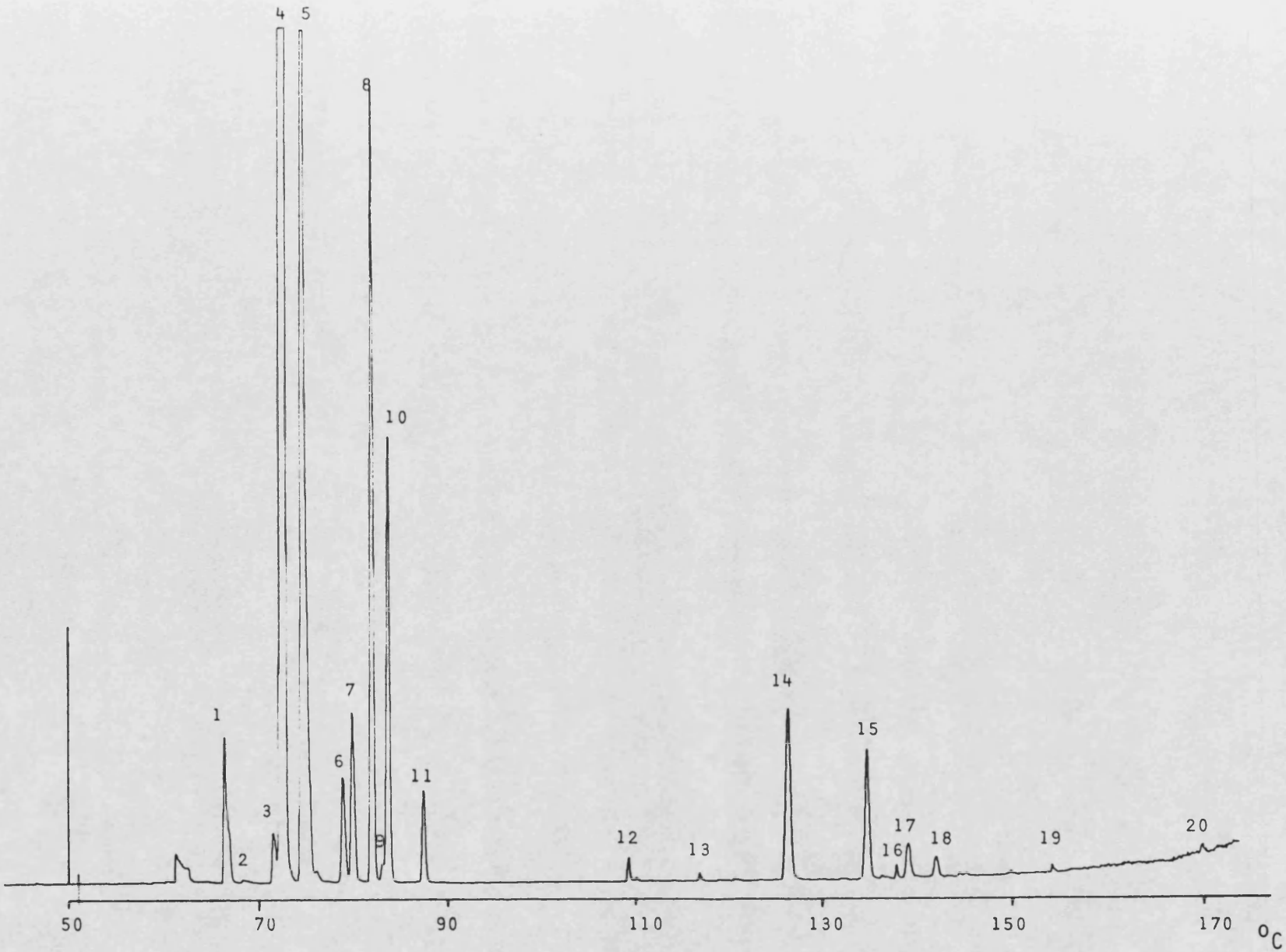


Fig. XVII Mass fragmentogram (m/z93) of solvent extracted Ben Lomond leaf oil (BP-20 column)

Peak identification - mass fragmentogram (*m*/z93) of solvent extracted Baldwin leaf oil

1 α-pinene

2 camphene

3 β-pinene

4 sabinene

5 Δ^3-carene + myrcene

6 *d*-(+)-limonene

7 β-phellandrene

8 *cis*-β-ocimene

9 *trans*-β-ocimene

10 γ-terpinene

11 terpinolene

12 Unknown

13 Unknown

14 β-caryophyllene + terpinen-4-ol

15 humulene

16 α-terpineol

17 germacrene-D

18 Δ-cadinene

19 Unknown

20 Unknown

21 geraniol

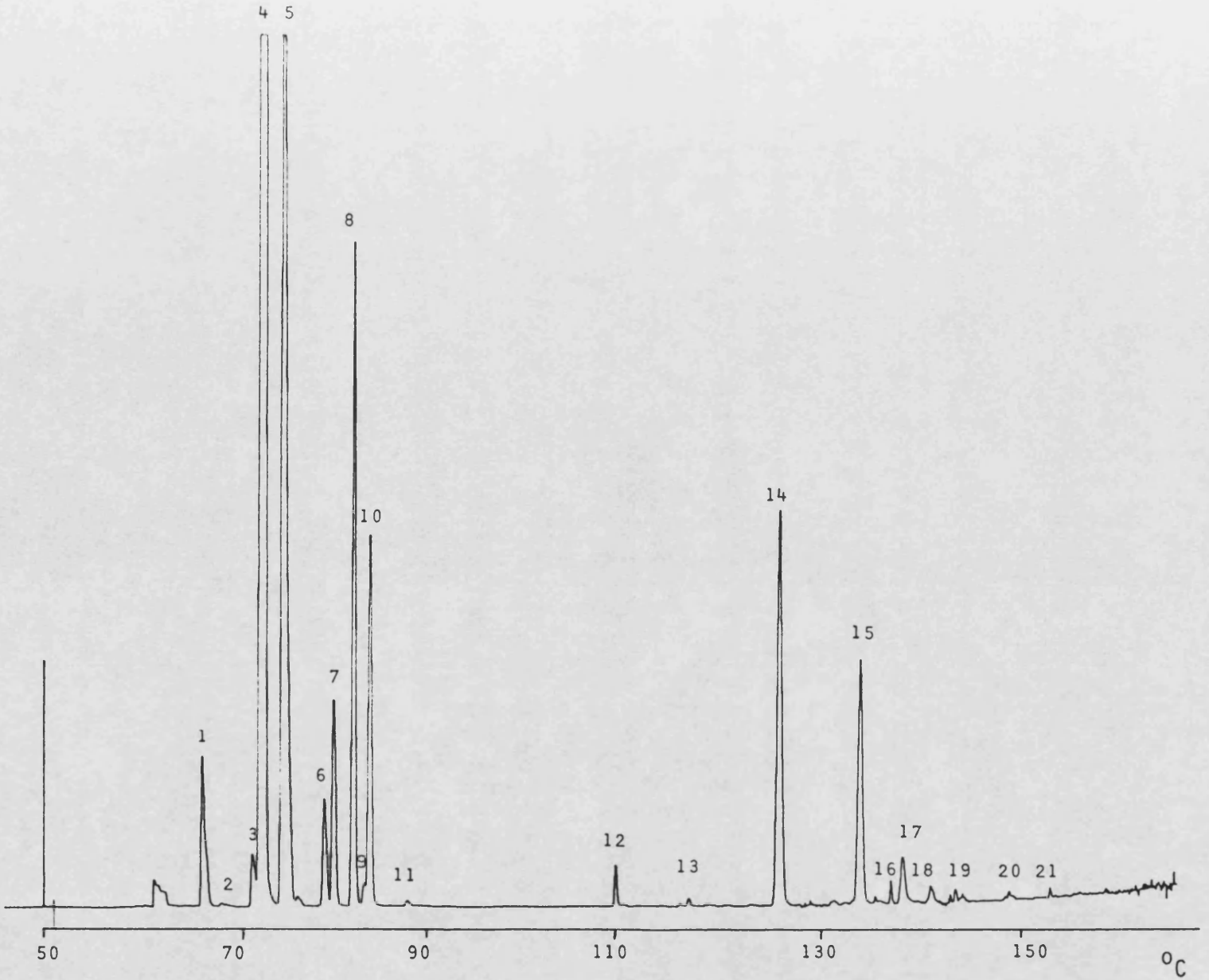


Fig. XVIII Mass fragmentogram (*m*/z93) of solvent extracted Baldwin leaf oil (BP-20 column)

Peak identification - mass fragmentogram (*m*/z93) of solvent extracted Wellington XXX leaf oil

1 α-pinene

2 camphene

3 β-pinene

4 sabinene

5 Δ^3-carene + myrcene

6 *d*-(+)-limonene

7 β-phellandrene

8 *cis*-β-ocimene

9 *trans*-β-ocimene

10 γ-terpinene

11 terpinolene

12 Unknown

13 Unknown

14 β-caryophyllene + terpinen-4-ol

15 humulene

16 α-terpineol

17 germacrene-D

18 Δ-cadinene

19 Unknown

20 Unknown

21 geraniol

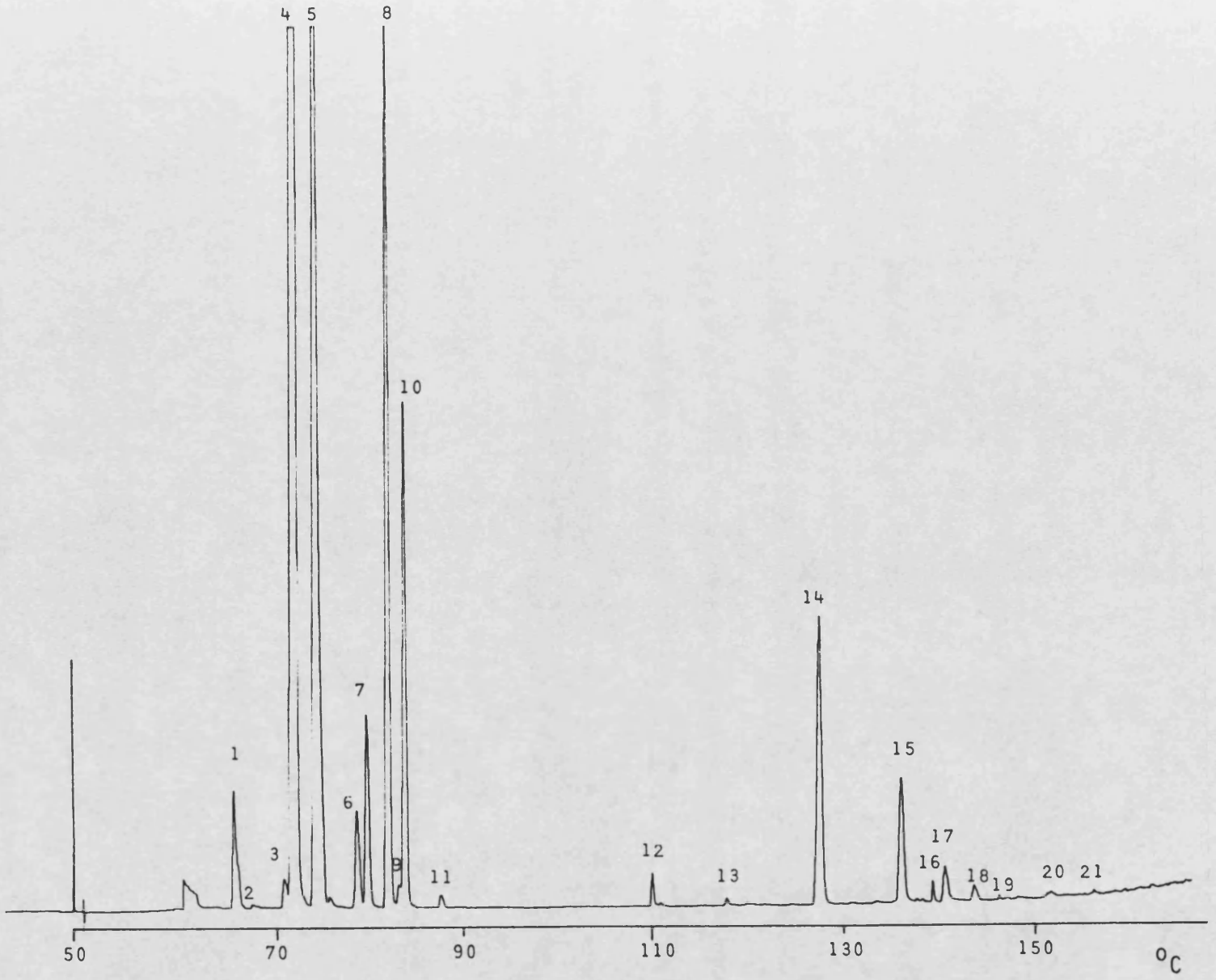


Fig. XIX Mass fragmentogram (m/z93) of solvent extracted Wellington XXX leaf oil (BP-20 column)

Using ripe blackcurrant fruit (var. Ben Lomond), volatiles were isolated by solvent extraction, vacuum distillation and distillation at atmospheric pressure. The resulting extracts were analysed by combined gas chromatography/mass spectrometry using single ion monitoring at *m*/z93 and *m*/z136 to determine the effect of the method of extraction on the composition of the terpene compounds. The mass fragmentograms obtained using *m*/z93 and a BP-1 (OV-101 equivalent) column are shown in figures XX to XXII. The concentration of the individual compounds as found by the three extraction methods is given in table VI. The levels are expressed in µg/kg fresh weight of fruit and are mean values of three analyses.

The use of single ion monitoring to examine the terpene fraction of the fruit volatiles is essential if the use of complex separation methods is to be avoided. Figure XXIII shows the complexity of solvent extracted Wellington XXX fruit volatiles analysed by capillary gas chromatography, and the position of the major terpene compounds. In contrast the mass fragmentogram of the same extract (figure XXIV) shows the terpene compounds very clearly.

Using single ion monitoring at *m*/z93 and *m*/z136 the terpene fractions of solvent extracted volatiles of three cultivars were examined. Figures XXIV to XXVI show the differences between the terpene fractions of Ben Lomond, Baldwin and Wellington XXX cultivars. The level of the individual compounds in each cultivar is shown in table VII. These figures are mean values of three separate analyses, and are expressed in µg/kg fresh fruit weight.

Peak identification - mass fragmentogram (*m*/z93) of solvent extracted Ben Lomond volatiles

1 α-thujene
2 α-pinene
3 camphene
4 sabinene
5 β-pinene
6 myrcene
7 α-phellandrene
8 Δ^3-carene
9 *d*-(+)-limonene
10 *cis*-β-ocimene
11 *trans*-β-ocimene
12 γ-terpinene
13 *trans*-sabinene hydrate
14 terpinolene
15 linalol
16 *cis*-β-terpineol
17 *trans*-β-terpineol
18 Unknown
19 terpinen-4-ol
20 Unknown
21 α-terpineol
22 *trans*-piperitol
23 Unknown
24 fenchyl acetate
25 geraniol
26 Unknown
27 bornyl acetate
28 Unknown
29 β-caryophyllene
30 Unknown
31 humulene
32 Unknown
33 caryophyllene oxide

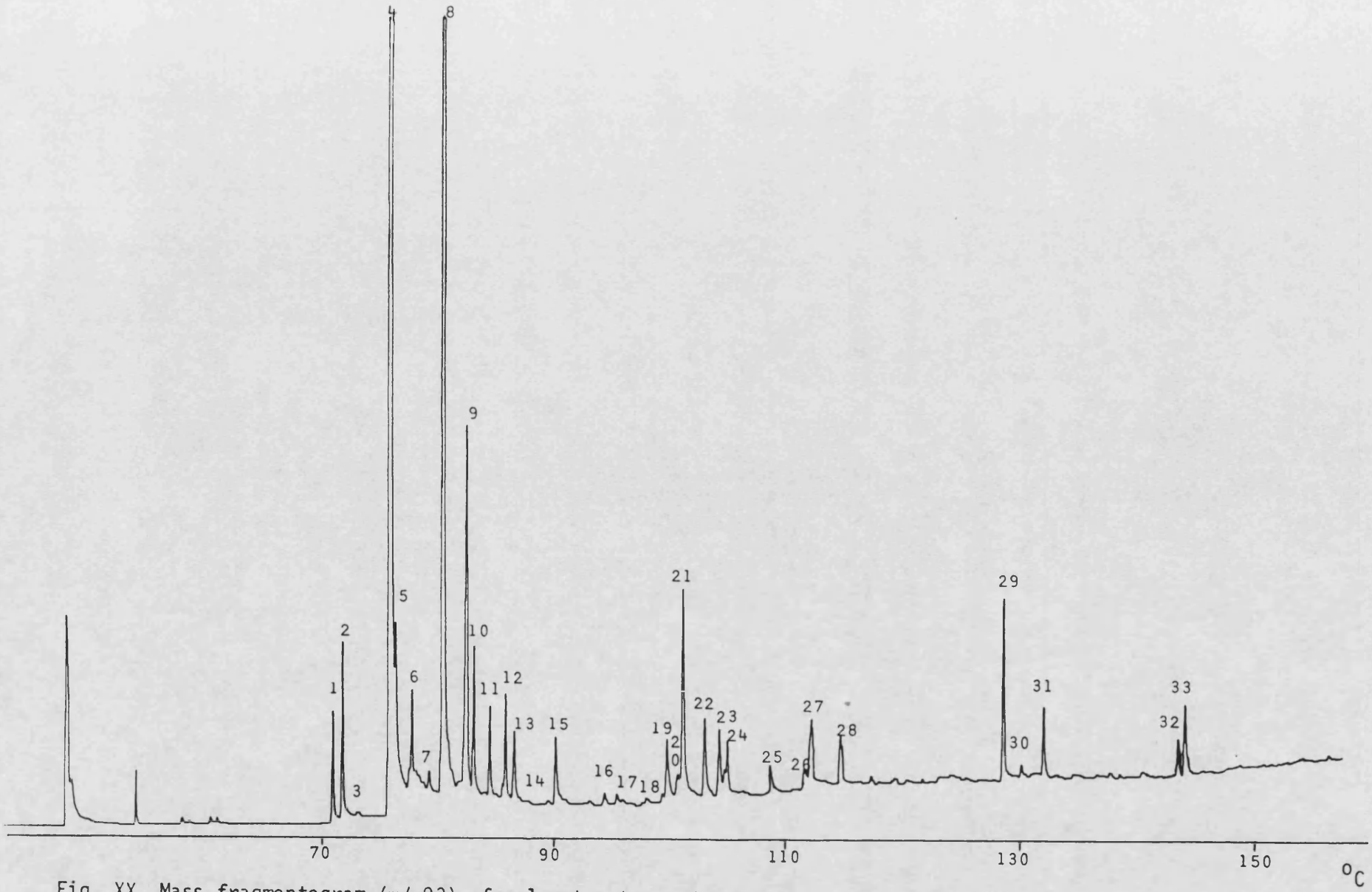


Fig. XX Mass fragmentogram (*m/z*93) of solvent extracted Ben Lomond volatiles (BP-1 column)

Peak identification - mass fragmentogram (*m*/z93) of vacuum distilled Ben Lomond volatiles

1 α-pinene
2 sabinene
3 β-pinene
4 myrcene
5 α-phellandrene
6 Δ^3-carene
7 α-terpinene
8 *d*-(+)-limonene
9 *cis*-β-ocimene
10 *trans*-β-ocimene
11 γ-terpinene
12 *trans*-sabinene hydrate
13 *cis*-linalool oxide
14 *trans*-linalool oxide
15 terpinolene
16 linalol
17 Unknown
18 Unknown
19 *cis*-β-ocimene
20 *trans*-β-ocimene
21 allo ocimene
22 Unknown
23 Unknown
24 terpinen-4-ol
25 α-terpineol
26 γ-terpineol
27 Unknown
28 *trans*-piperitol
29 Unknown
30 Unknown
31 Unknown
32 nerol
33 geraniol
34 linalyl acetate
35 Unknown
36 bornyl acetate
37 Unknown
38 α-terpinyl acetate
39 neryl acetate
40 geranyl acetate
41 β-caryophyllene
42 Unknown
43 humulene
44 Δ-cadinene
45 caryophyllene oxide

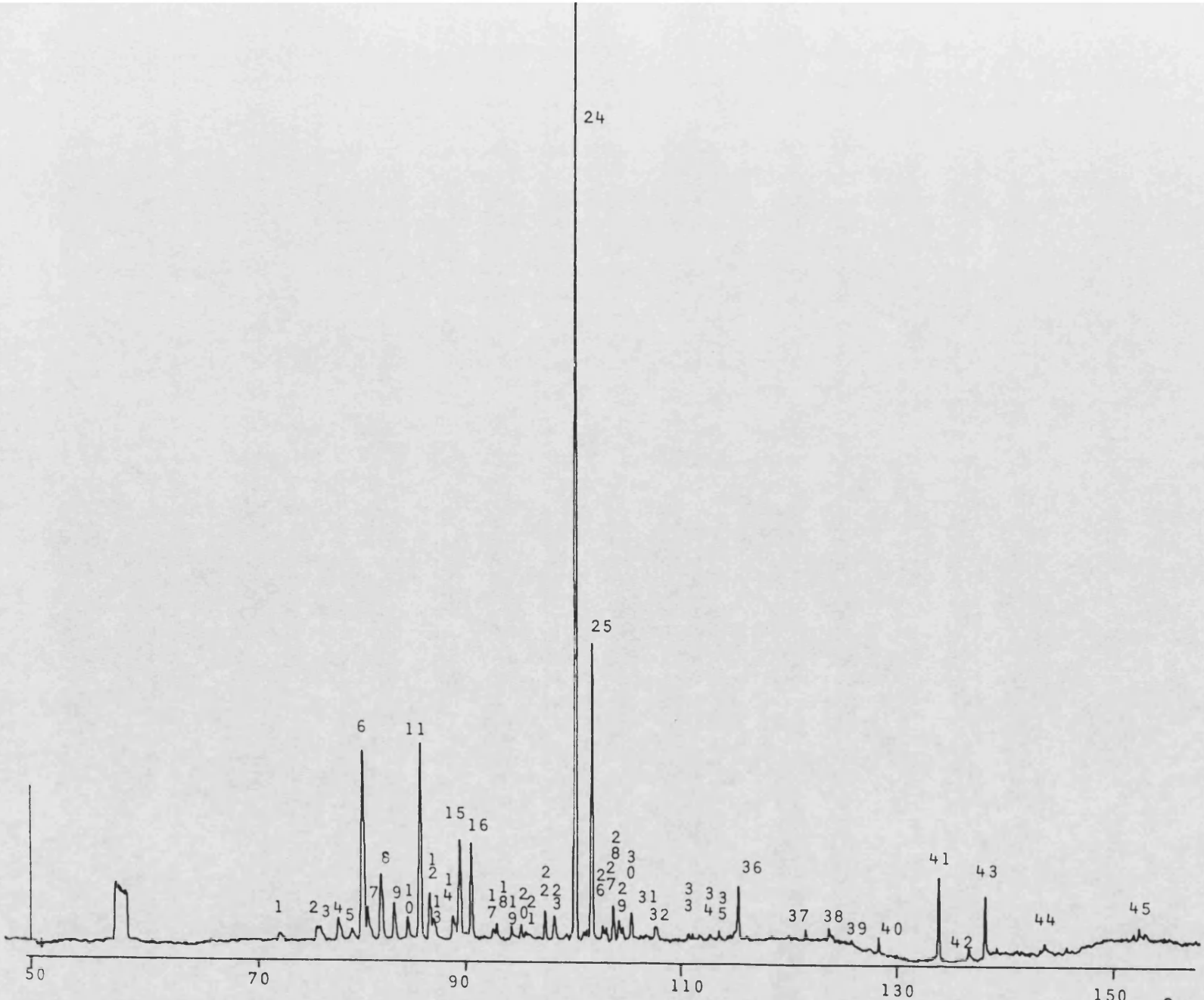


Fig. XXI Mass fragmentogram (*m/z*93) of vacuum distilled Ben Lomond volatiles (BP-1 column)

Peak identification - mass fragmentogram (*m*/z93) of Ben Lomond volatiles distilled at atmospheric pressure

1	α-thujene	29	Unknown
2	α-pinene	30	α-terpineol
3	sabinene	31	γ-terpineol
4	β-pinene	32	*trans*-piperitol
5	myrcene	33	Unknown
6	α-phellandrene	34	Unknown
7	Δ^3-carene	35	Unknown
8	α-terpinene	36	nerol
9	*d*-(+)-limonene	37	geraniol
10	β-phellandrene	38	Unknown
11	*cis*-β-ocimene	39	bornyl acetate
12	*trans*-β-ocimene	40	Unknown
13	γ-terpinene	41	α-terpinyl acetate
14	*trans*-sabinene hydrate	42	neryl acetate
15	*cis*-linalool oxide	43	geranyl acetate
16	*trans*-linalool oxide	44	β-caryophyllene
17	terpinolene	45	Unknown
18	linalol	46	humulene
19	fenchol	47	alloaromadrene
20	Unknown	48	Δ-cadinene
21	*cis*-β-terpineol	49	α-nerolidol
22	Unknown	50	Unknown
23	*trans*-β-terpineol	51	caryophyllene oxide
24	Unknown		
25	Unknown		
26	Unknown		
27	terpinen-4-ol		
28	Unknown		

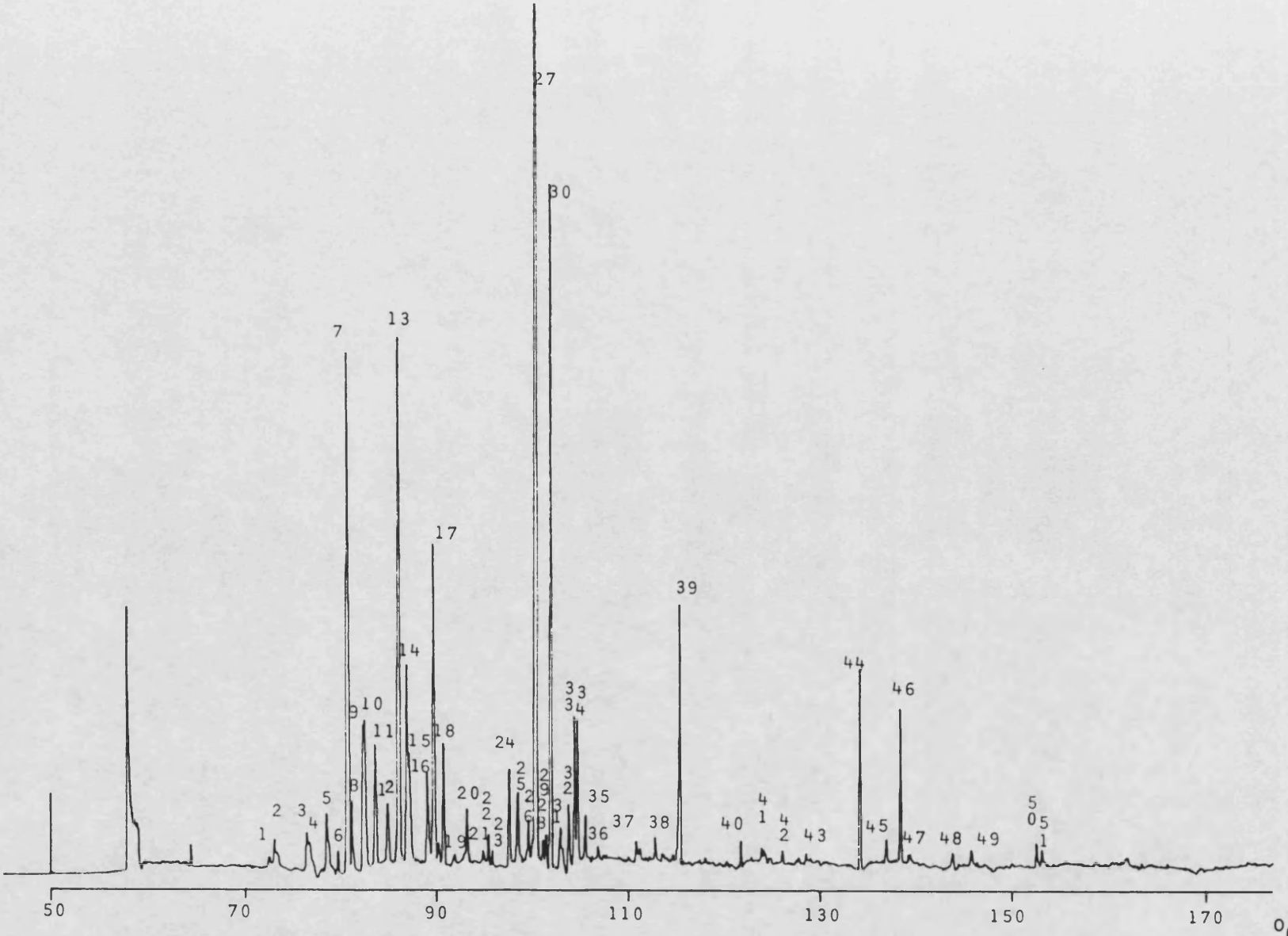


Fig. XXII Mass fragmentogram (*m*/z93) of Ben Lomond volatiles distilled at atmospheric pressure (BP-1 column)

1. Δ^3-carene
2. *d*-(+)-limonene
3. linalol
4. α-terpineol

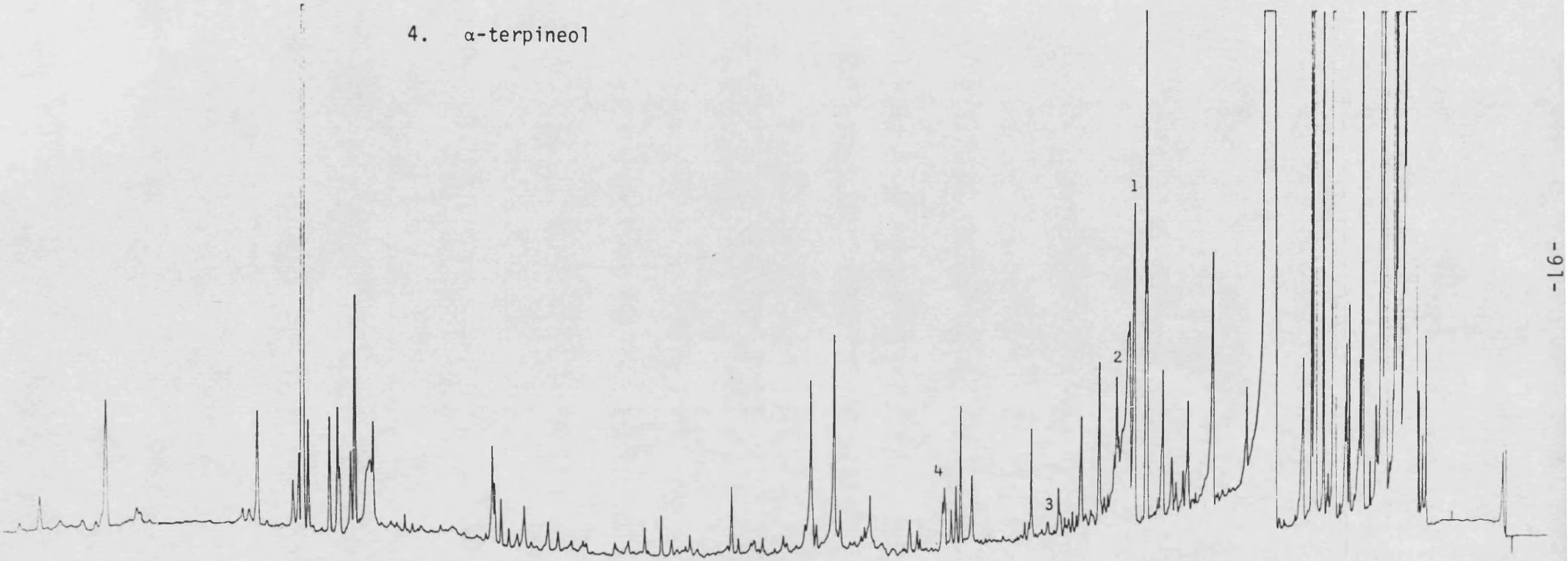


Fig. XXIII FID chromatogram of solvent extracted Wellington XXX fruit volatiles (BP-20 column)

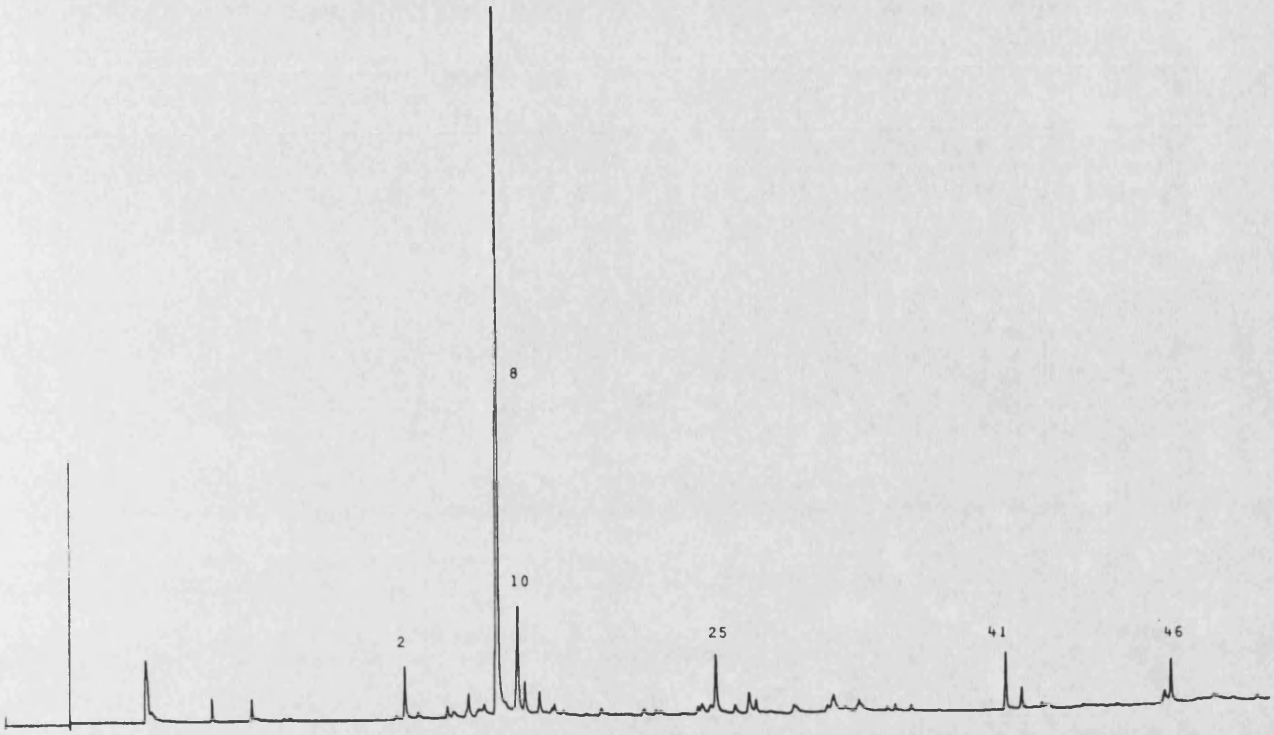
Table VI. Concentration (µg/kg fresh weight) of terpene compounds in blackcurrants (var. Ben Lomond) isolated by solvent extraction, vacuum distillation and distillation at atmospheric pressure.

Compound	Solvent extraction	Vacuum distillation	Atmospheric distillation
α-thujene	49.6	-	4.0
α-pinene	36.9	3.0	8.0
camphene	2.2	-	-
sabinene	1450.0	4.0	13.0
β-pinene	46.3	4.0	4.0
myrcene	48.9	7.0	20.0
α-phellandrene	29.3	2.0	7.0
Δ^3-carene	649.0	247.0	79.0
α-terpinene	-	11.0	38.0
d-(+)-limonene	174.3	185.0	51.0
β-phellandrene	-	-	43.0
cis-β-ocimene	97.2	16.0	63.0
trans-β-ocimene	58.3	10.0	31.0
γ-terpinene	39.7	59.0	183.0
trans-sabinene hydrate	46.0	22.0	101.0
cis-linalool oxide	-	12.0	40.0
trans-linalool oxide	-	20.0	88.0
terpinolene	2.2	175.0	679.0
linalol	44.2	53.0	80.0
fenchol	-	-	15.0

cis-β-terpineol	11.1	7.0	7.0
trans-β-terpineol	11.1	7.0	9.0
allo ocimene	2.0	5.0	-
terpinen-4-ol	40.2	567.0	667.0
α-terpineol	211.3	167.0	437.0
γ-terpineol	-	8.0	26.0
trans-piperitol	21.0	12.5	28.0
fenchyl acetate	46.0	-	-
nerol	-	11.0	11.0
geraniol	92.5	8.0	50.0
linalyl acetate	-	2.0	-
bornyl acetate	42.0	19.0	103.0
α-terpinyl acetate	-	4.0	4.0
neryl acetate	-	3.0	8.0
geranyl acetate	-	5.0	7.0
β-caryophyllene	131.0	35.0	96.0
humulene	32.8	18.0	57.0
alloaromadrene	-	-	16.0
Δ-cadinene	-	16.0	32.0
α-nerolidol	-	-	26.0
caryophyllene oxide	188.0	33.0	44.0

Peak identification - mass fragmentogram of solvent extracted Wellington XXX volatiles

1 α-thujene
2 α-pinene
3 camphene
4 sabinene
5 β-pinene
6 myrcene
7 α-phellandrene
8 Δ^3-carene
9 α-terpinene
10 *d*-(+)-limonene
11 *cis*-β-ocimene
12 *trans*-β-ocimene
13 Unknown
14 γ-terpinene
15 *trans*-sabinene hydrate
16 Unknown
17 linalol
18 *cis*-β-terpineol
19 *trans*-β-terpineol
20 allo ocimene
21 Unknown
22 Unknown
23 terpinen-4-ol
24 Unknown
25 α-terpineol
26 *trans*-piperitol
27 Unknown
28 fenchyl acetate
29 Unknown
30 geraniol
31 Unknown
32 Unknown
33 Unknown
34 bornyl acetate
35 Unknown
36 Unknown
37 α-terpinyl acetate
38 Unknown
39 Unknown
40 Unknown
41 β-caryophyllene
42 Unknown
43 humulene
44 Δ-cadinene
45 Unknown
46 caryophyllene oxide
47 Unknown



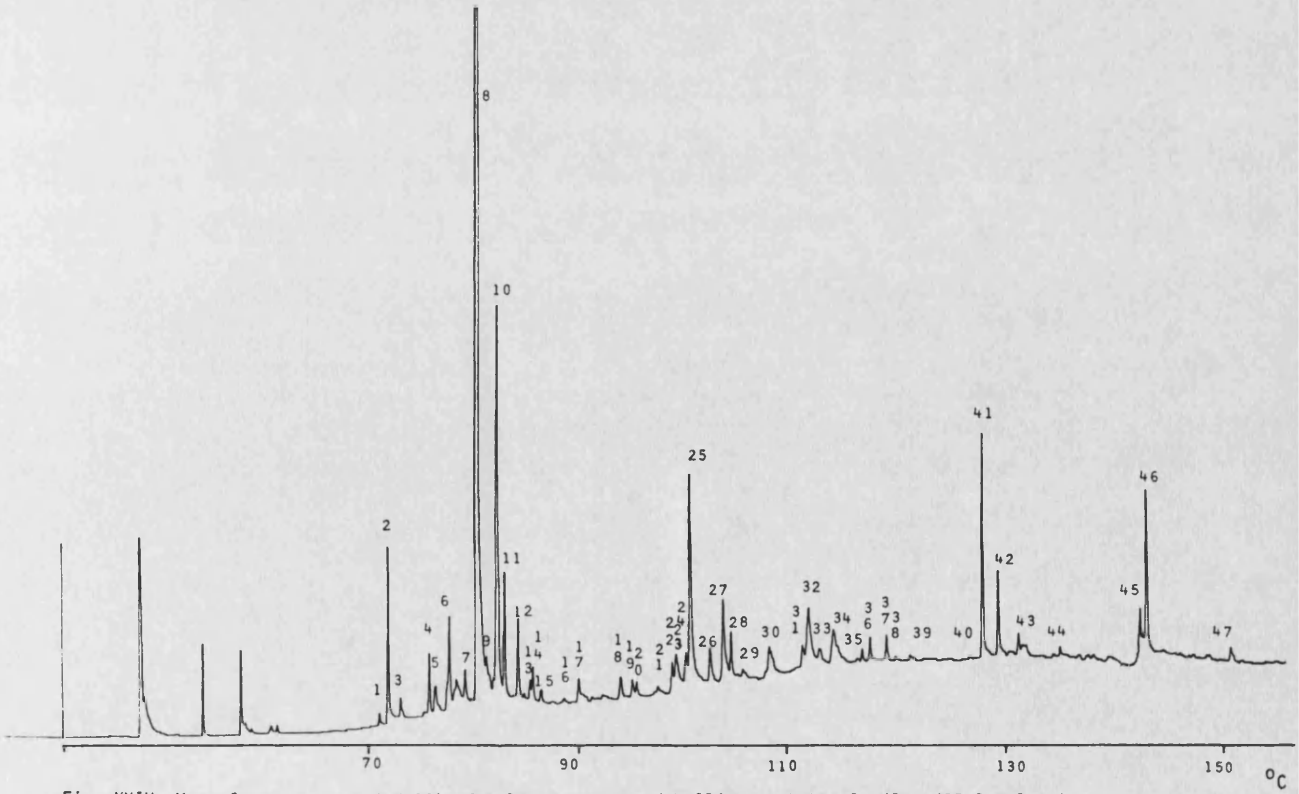
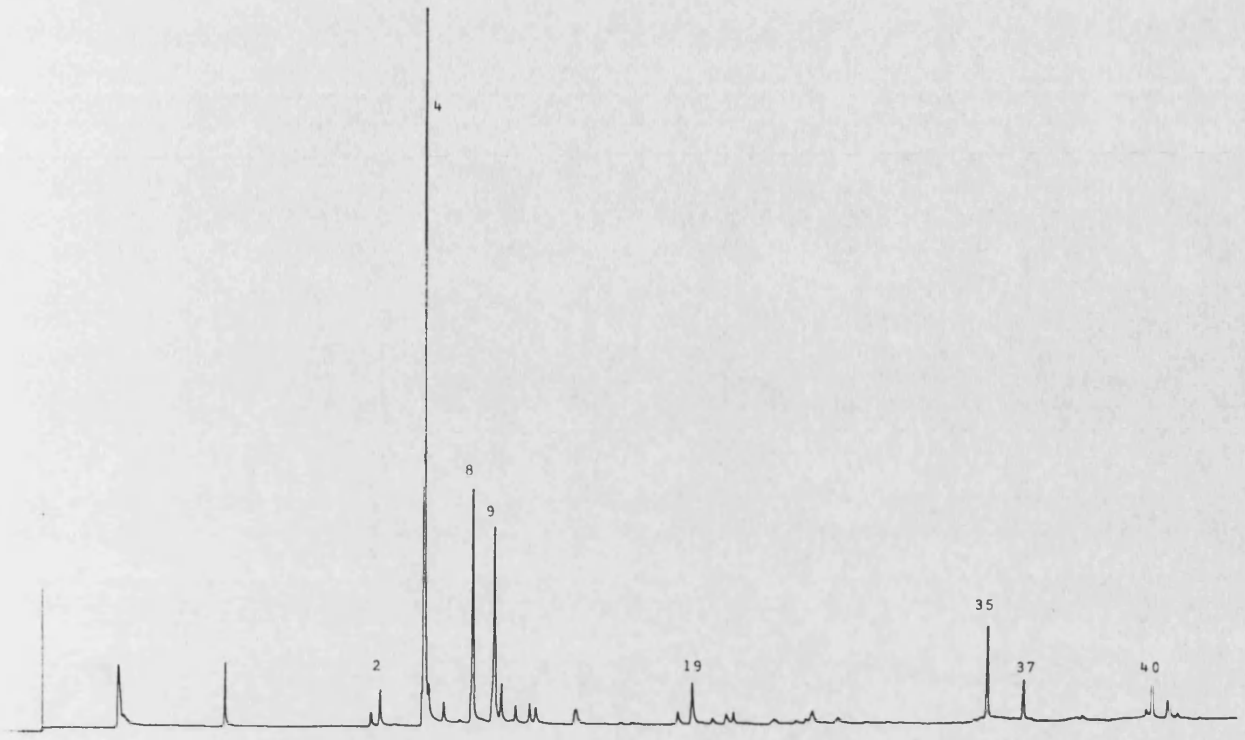


Fig. XXIV Mass fragmentogram (*m/z*93) of solvent extracted Wellington XXX volatiles (BP-1 column)

Peak identification - mass fragmentogram of solvent extracted Baldwin volatiles

1 α-thujene
2 α-pinene
3 camphene
4 sabinene
5 β-pinene
6 myrcene
7 α-phellandrene
8 Δ^3-carene
9 *d*-(+)-limonene
10 *cis*-β-ocimene
11 *trans*-β-ocimene
12 γ-terpinene
13 *trans*-sabinene hydrate
14 terpinolene
15 linalol
16 *cis*-β-terpineol
17 *trans*-β-terpineol
18 terpinen-4-ol
19 α-terpineol
20 γ-terpineol
21 *trans*-piperitol
22 Unknown
23 fenchyl acetate
24 geraniol
25 Unknown
26 Unknown
27 bornyl acetate
28 Unknown
29 Unknown
30 Unknown
31 α-terpinyl acetate
32 neryl acetate
33 geranyl acetate
34 Unknown
35 β-caryophyllene
36 Unknown
37 humulene
38 alloaromadrene
39 Unknown
40 caryophyllene oxide
41 Unknown
42 humulene oxide



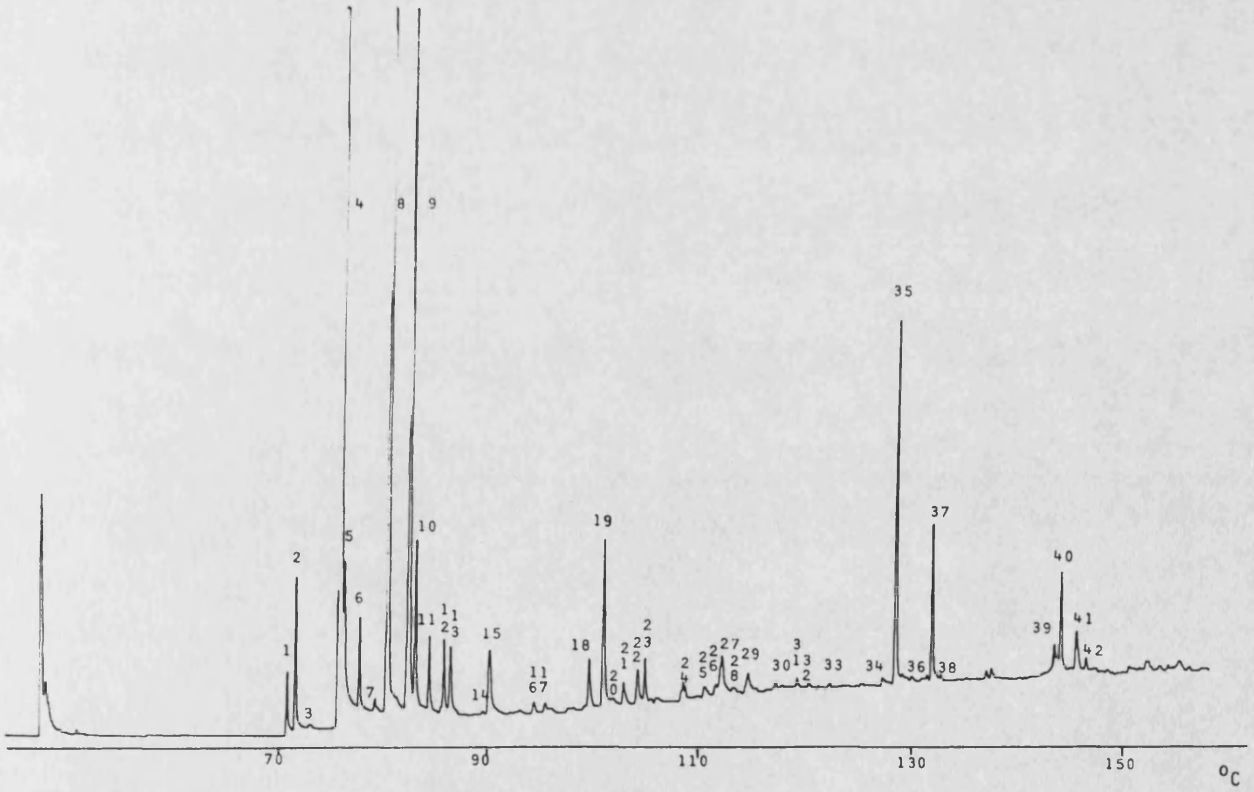
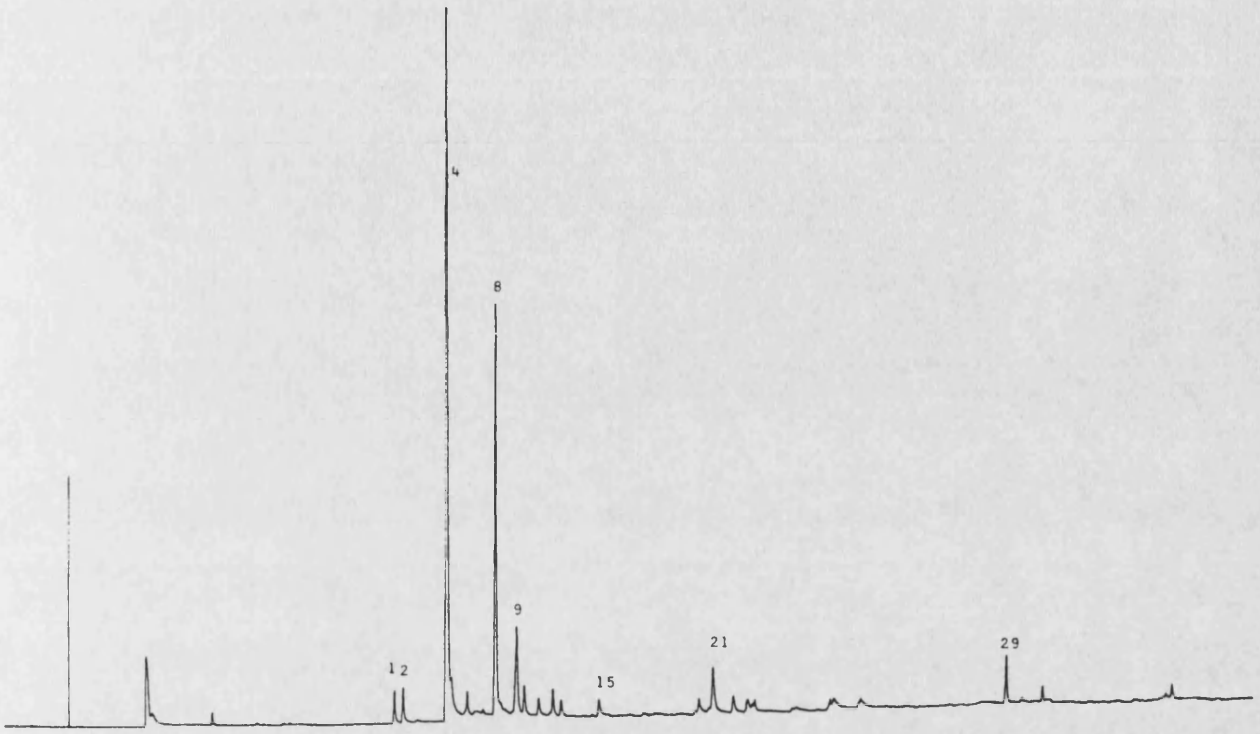


Fig. XXV Mass fragmentogram (m/z93) of solvent extracted Baldwin volatiles (BP-1 column)

Peak identification - mass fragmentogram (*m*/z93) of solvent extracted Ben Lomond volatiles

1 α-thujene
2 α-pinene
3 camphene
4 sabinene
5 β-pinene
6 myrcene
7 α-phellandrene
8 Δ^3-carene
9 *d*-(+)-limonene
10 *cis*-β-ocimene
11 *trans*-β-ocimene
12 γ-terpinene
13 *trans*-sabinene hydrate
14 terpinolene
15 linalol
16 *cis*-β-terpineol
17 *trans*-β-terpineol
18 Unknown
19 terpinen-4-ol
20 Unknown
21 α-terpineol
22 *trans*-piperitol
23 Unknown
24 fenchyl acetate
25 geraniol
26 Unknown
27 bornyl acetate
28 Unknown
29 β-caryophyllene
30 Unknown
31 humulene
32 Unknown
33 caryophyllene oxide



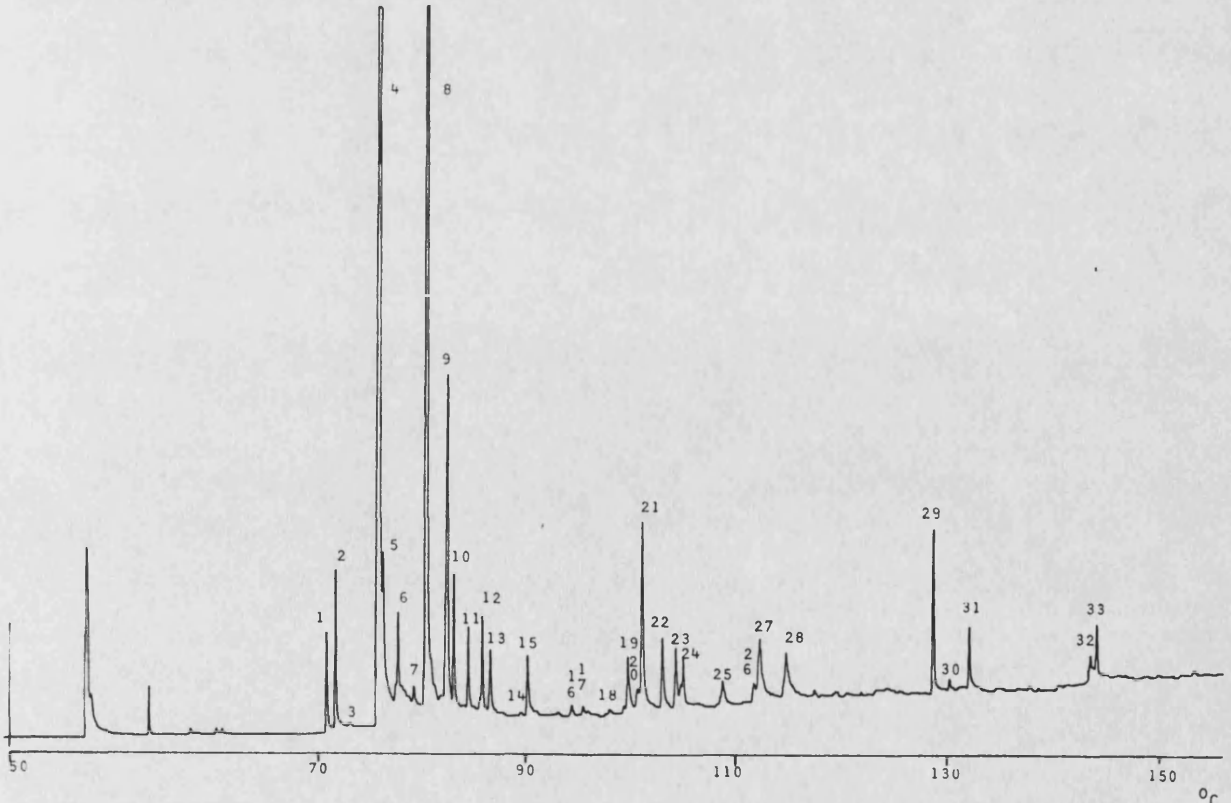


Fig. XXVI Mass fragmentogram (*m/z*93) of solvent extracted Ben Lomond volatiles (BP-1 column)

Table VII. Concentration of terpene compounds in three blackcurrant cultivars (µg/kg fresh weight).

Compound	Ben Lomond	Baldwin	Wellington XXX
α-thujene	49.6	36.9	5.8
α-pinene	36.9	42.2	37.6
camphene	2.2	2.2	9.9
sabinene	1450.0	1866.7	27.5
β-pinene	46.3	59.8	20.9
myrcene	48.9	72.9	50.4
α-phellandrene	29.3	28.5	53.2
Δ^3-carene	649.0	665.9	1454.0
α-terpinene	-	-	38.3
d-(+)-limonene	174.3	205.3	188.4
cis-β-ocimene	97.2	141.6	79.2
trans-β-ocimene	58.3	64.9	52.8
γ-terpinene	39.7	37.9	12.8
trans-sabinene hydrate	46.0	45.5	7.6
terpinolene	2.0	2.0	-
linalol	44.2	59.6	15.6
cis-β-terpineol	11.1	18.0	21.8
trans-β-terpineol	11.1	13.5	16.8
allo ocimene	2.0	-	4.2
terpinen-4-ol	40.2	45.8	18.2
α-terpineol	211.3	237.7	212.8
trans-piperitol	21.0	7.5	10.5

fenchyl acetate	46.0	42.0	42.0
geraniol	92.4	96.2	95.7
bornyl acetate	42.0	25.2	38.1
α-terpinyl acetate	-	8.1	4.2
neryl acetate	-	4.0	-
geranyl acetate	-	4.0	-
β-caryophyllene	131.0	294.0	177.2
humulene	32.8	84.7	12.9
alloaromadrene	-	2.7	-
Δ-cadinene	-	-	5.3
caryophyllene oxide	188.0	376.1	647.6
humulene oxide	-	41.8	-

In order to examine the changes in terpene composition of the volatiles during ripening, fruit was picked at regular intervals between June and August. The volatiles were solvent extracted and analysed by combined gas chromatography/mass spectrometry using single ion monitoring as previously described. Figures XXVII to XXVIX show the changes in the terpene fraction during ripening of Wellington XXX blackcurrants picked during 1984. Table VIII summarises the concentration of the individual compounds during ripening, expressed in µg/kg fresh fruit weight. The figures are again means of three analyses.

In most species the flavour compounds are not evenly distributed (74-76) throughout the fruit, and are often concentrated in the epidermal layers (77,78). In order to examine the distribution of terpene compounds in blackcurrant fruit, seeds and the epidermal layer were separated from the pericarp, and the volatiles isolated from the individual parts by solvent extraction. The chromatograms obtained from analysis of these extracts by gas chromatography/mass spectrometry using single ion monitoring at *m/z*93 and *m/z*136 are shown in figure XXX, and the concentration of these compounds in table VIX. The figures are mean figures in µg/kg wet weight of duplicate analyses.

During a study of processing parameters and their effect on the aroma composition of blackcurrants, it was observed that the addition of pectin hydrolysing enzymes resulted in an increase in certain terpene alcohols and olefins. The presence of terpene glycosides

Peak identification - mass fragmentogram of unripe Wellington XXX fruit volatiles

1	α-thujene
2	α-pinene
3	camphene
4	sabinene
5	β-pinene
6	myrcene
7	α-phellandrene
8	Δ^3-carene
9	α-terpinene
10	*d*-(+)-limonene
11	*cis*-β-ocimene
12	*trans*-β-ocimene
13	Unknown
14	γ-terpinene
15	*trans*-sabinene hydrate
16	terpinolene
17	linalol
18	Unknown
19	Unknown
2o	*cis*-β-terpineol
21	*trans*-β-terpineol
22	allo ocimene
23	Unknown
24	Unknown
25	terpinen-4-ol
26	α-terpineol
27	*trans*-piperitol
28	Unknown
29	fenchyl acetate
30	geraniol
31	Unknown
32	bornyl acetate
33	Unknown
34	"
35	"
36	"
37	"
38	"
39	α-terpinyl acetate
40	neryl acetate
41	geranyl acetate
42	β-caryophyllene
43	humulene
44	alloaromadrene
45	Unknown
46	"
47	"
48	caryophyllene oxide
49	Unknown
50	"
51	"

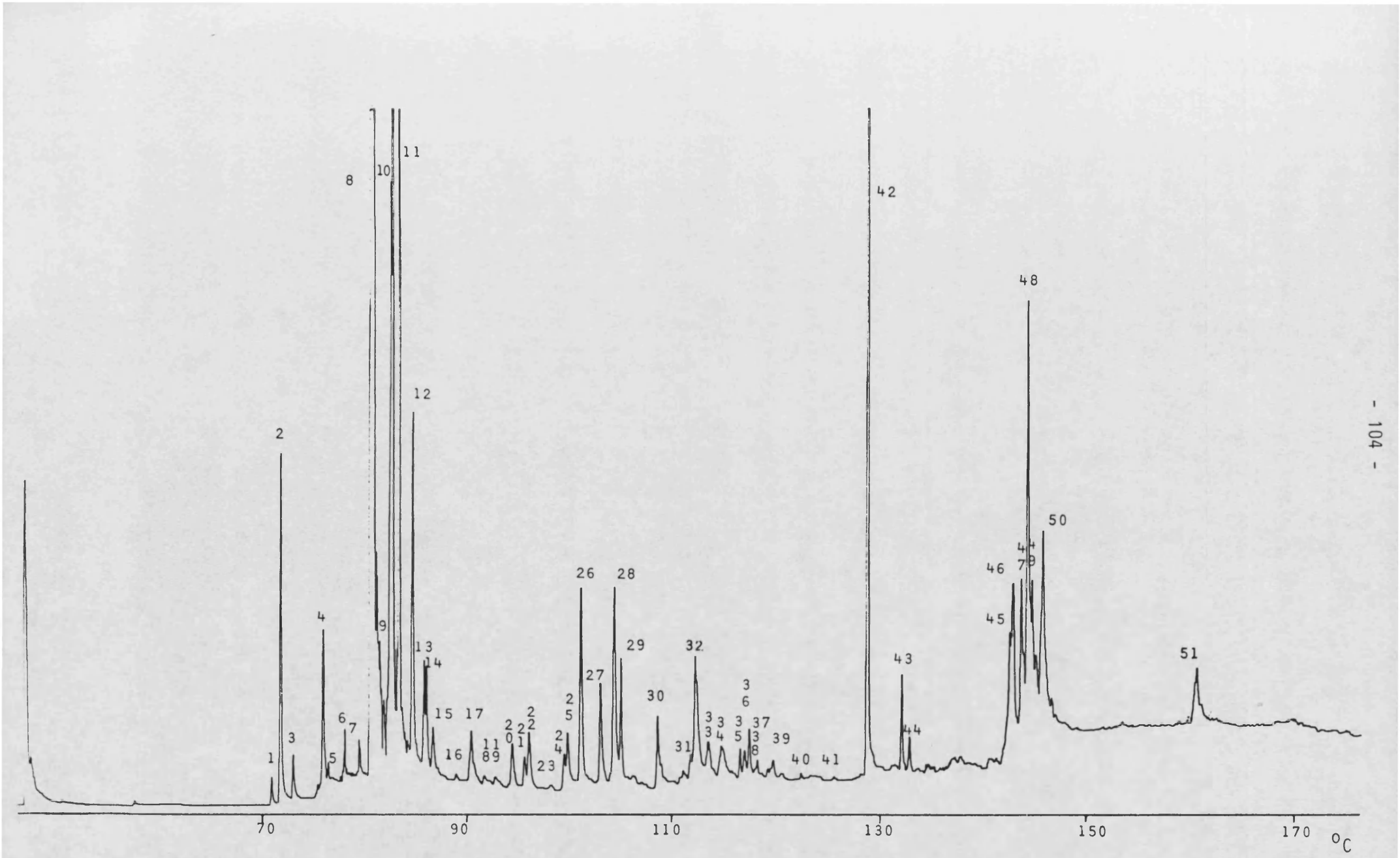


Fig. XXVII Mass fragmentogram (*m/z*93) of unripe Wellington XXX fruit volatiles (BP-1 column)

Peak identification - mass fragmentogram of half ripe Wellington XXX fruit volatiles

1	α-thujene	29	Unknown
2	α-pinene	30	bornyl acetate
3	camphene	31	Unknown
4	sabinene	32	"
5	β-pinene	33	"
6	myrcene	34	"
7	α-phellandrene	35	"
8	Δ^3-carene	36	α-terpinyl acetate
9	*d*-(+)-limonene	37	neryl acetate
10	*cis*-β-ocimene	38	Unknown
11	*trans*-β-ocimene	39	β-caryophyllene
12	Unknown	40	Unknown
13	γ-terpinene	41	humulene
14	*trans*-sabinene hydrate	42	alloaromadrene
15	terpinolene	43	Δ-cadinene
16	Unknown	44	Unknown
17	linalol	45	Unknown
18	*cis*-β-terpineol	46	Unknown
19	*trans*-β-terpineol	47	caryophyllene oxide
20	allo ocimene	48	Unknown
21	Unknown	49	humulene oxide
22	Unknown		
23	terpinen-4-ol		
24	α-terpineol		
25	*trans*-piperitol		
26	Unknown		
27	fenchyl acetate		
28	geraniol		

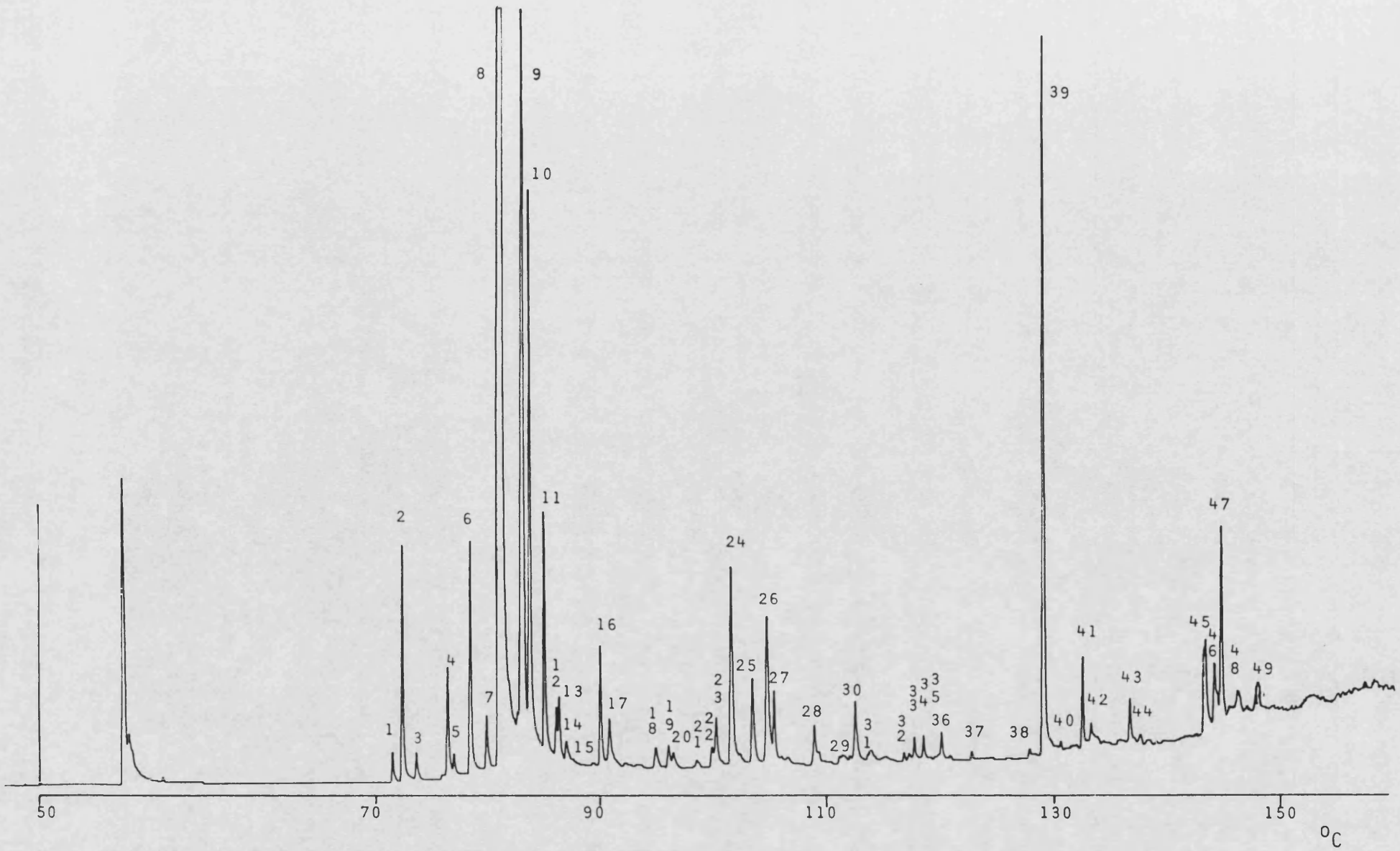


Fig. XXVIX Mass fragmentogram (*m*/z93) of half ripe Wellington XXX fruit volatiles (BP-1 column)

Peak identification - mass fragmentogram of solvent extracted ripe Wellington XXX fruit volatiles

1	α-thujene	29	Unknown
2	α-pinene	30	geraniol
3	camphene	31	Unknown
4	sabinene	32	Unknown
5	β-pinene	33	Unknown
6	myrcene	34	bornyl acetate
7	α-phellandrene	35	Unknown
8	Δ^3-carene	36	Unknown
9	α-terpinene	37	α-terpinyl acetate
10	*d*-(+)-limonene	38	Unknown
11	*cis*-β-ocimene	39	Unknown
12	*trans*-β-ocimene	40	Unknown
13	Unknown	41	β-caryophyllene
14	γ-terpinene	42	Unknown
15	*trans*-sabinene hydrate	43	humulene
16	Unknown	44	Δ-cadinene
17	linalol	45	Unknown
18	*cis*-β-terpineol	46	caryophyllene oxide
19	*trans*-β-terpineol	47	Unknown
20	allo ocimene		
21	Unknown		
22	Unknown		
23	terpinen-4-ol		
24	Unknown		
25	α-terpineol		
26	*trans*-piperitol		
27	Unknown		
28	fenchyl acetate		

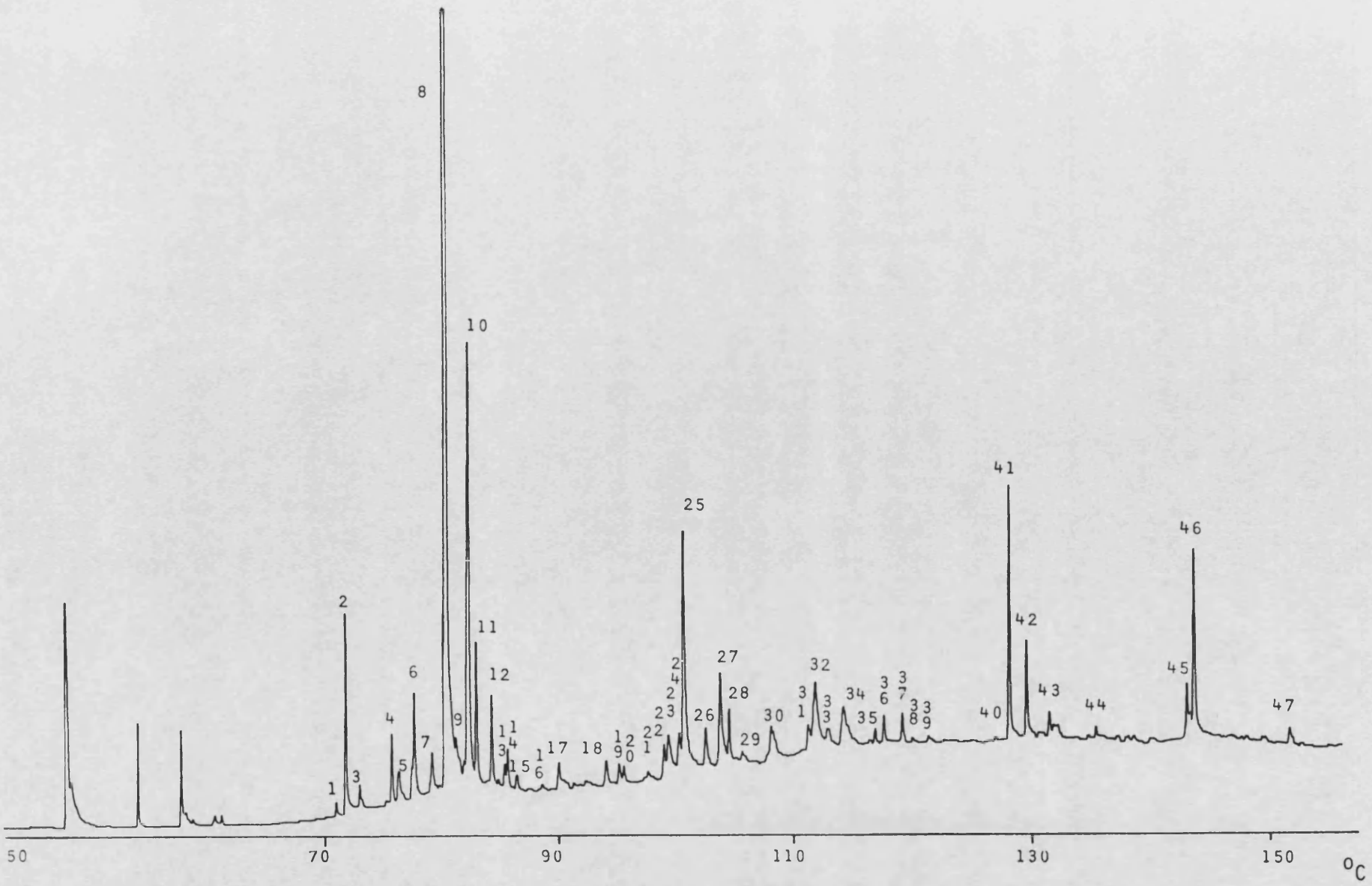


Fig. XXVIX Mass fragmentogram (*m*/z93) of full ripe Wellington XXX fruit volatiles (BP-1 column)

Table VIII. Concentration of terpene compounds in Wellington XXX fruit volatiles at three stages of ripening.(µg/kg fresh weight)

Compound	Unripe	Half ripe	Full ripe
α-thujene	11.3	14.4	5.8
α-pinene	83.8	60.7	37.6
camphene	18.0	13.2	9.9
sabinene	62.5	49.8	27.5
β-pinene	11.1	14.3	20.9
myrcene	23.4	124.3	50.4
α-phellandrene	42.8	82.6	53.2
Δ^3-carene	3622.0	3038.0	1454.0
α-terpinene	267.7	27.5	38.3
d-(+)-limonene	524.5	288.6	188.4
cis-β-ocimene	524.6	296.4	79.2
trans-β-ocimene	168.2	134.2	52.8
γ-terpinene	20.2	16.8	12.8
trans-sabinene hydrate	30.4	11.4	7.6
terpinolene	11.3	6.1	-
linalol	26.9	28.1	15.6
cis-β-terpineol	41.4	20.9	21.8
trans-β-terpineol	26.6	22.8	16.8
allo ocimene	23.1	4.2	4.2
terpinen-4-ol	26.2	28.5	18.2
α-terpineol	186.9	224.3	212.8
trans-piperitol	31.5	24.0	10.5

fenchyl acetate	105.5	52.5	42.0
geraniol	121.6	78.2	95.7
bornyl acetate	83.6	39.2	38.1
α-terpinyl acetate	16.8	29.4	4.2
neryl acetate	5.8	8.8	-
geranyl acetate	5.9	-	-
β-caryophyllene	620.8	527.7	177.2
humulene	64.5	54.8	12.9
alloaromadrene	22.6	9.7	-
Δ-cadinene	-	21.2	5.3
caryophyllene oxide	1671.2	710.3	647.6
humulene oxide	-	83.5	-

Peak identification - mass fragmentogram (*m*/z93) showing the distribution of terpenes in blackcurrant fruit

1 α-thujene
2 α-pinene
3 camphene
4 sabinene
5 β-pinene
6 myrcene
7 α-phellandrene
8 Δ^3-carene
9 *d*-(+)-limonene
10 *cis*-β-ocimene
11 *trans*-β-ocimene
12 γ-terpinene
13 *trans*-sabinene hydrate
14 terpinolene
15 Unknown
16 linalol
17 Unknown
18 *cis*-β-terpineol
19 *trans*-β-terpineol
20 Unknown
21 terpinen-4-ol
22 α-terpineol
23 γ-terpineol
24 *trans*-piperitol
25 Unknown
26 fenchyl acetate
27 nerol
28 geraniol
29 linalyl acetate
30 bornyl acetate
31 β-caryophyllene
32 Unknown
33 humulene
34 alloaromadrene
35 germacrene-D
36 γ-elemene
37 Δ-cadinene
38 Unknown
39 α-nerolidol
40 Unknown
41 caryophyllene oxide
42 humulene oxide

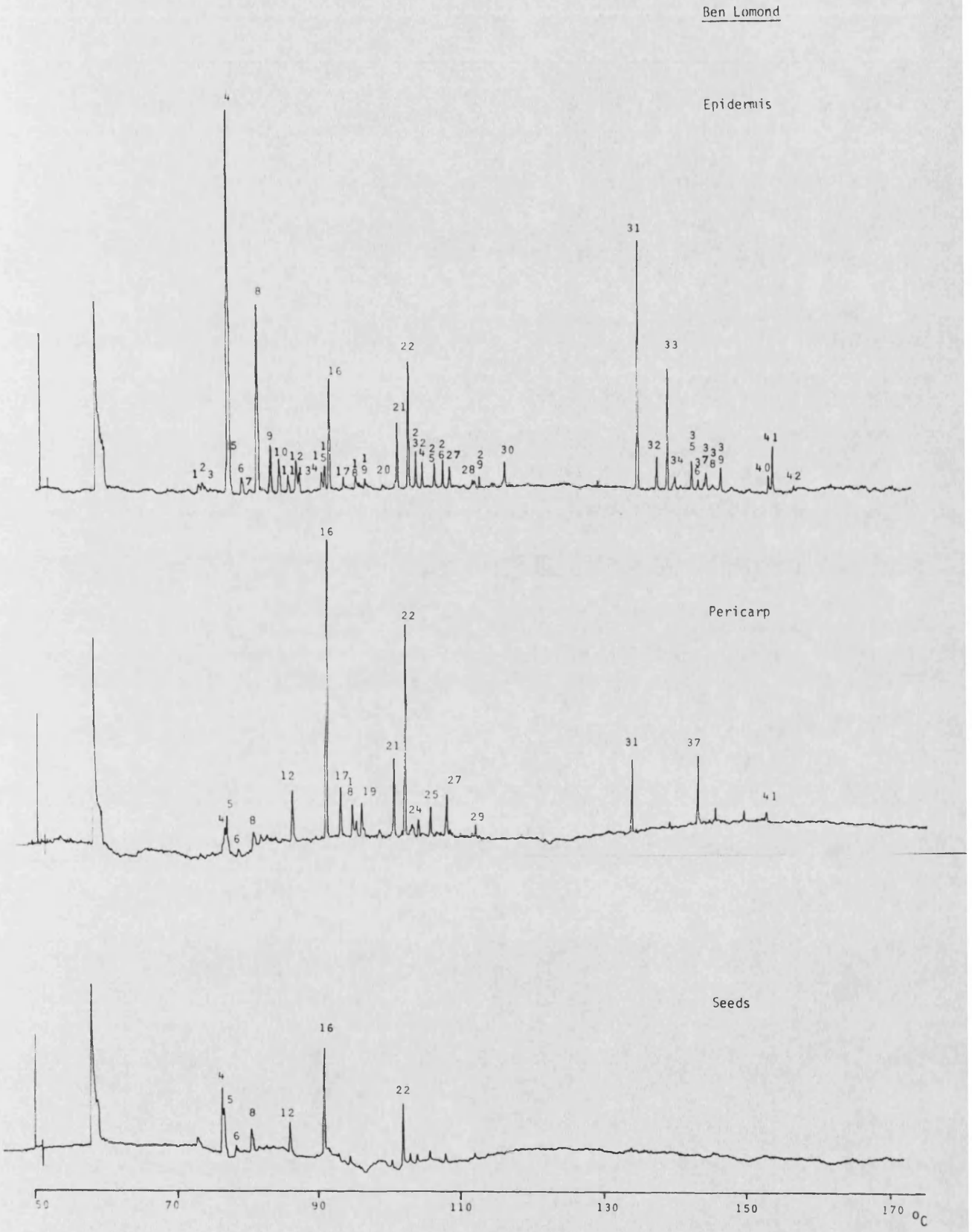


Fig.XXX Mass fragmentogram (*m/z*93) showing the distribution of terpenes in blackcurrant fruit (BP-1 column)

Table IX. Distribution of terpenes in Ben Lomond blackcurrant fruit (µg/kg fresh weight).

Compound	Epidermis	Pericarb	Seeds
α-thujene	13	-	-
α-pinene	15	-	30
camphene	11	-	-
sabinene	887	59	152
β-pinene	112	85	65
myrcene	40	20	27
α-phellandrene	6	-	3
Δ^3-carene	605	46	73
d-(+)-limonene	151	20	21
cis-β-ocimene	122	-	-
trans-β-ocimene	61	-	-
γ-terpinene	74	120	74
terpinolene	250	-	-
linalol	487	1313	463
cis-β-terpineol	59	131	-
trans-β-terpineol	36	143	-
terpinen-4-ol	232	283	30
α-terpineol	543	926	260
γ-terpineol	156	39	39
fenchyl acetate	270	-	-
nerol	151	283	57
geraniol	125	-	-

linalyl acetate	31	31	16
bornyl acetate	70	-	-
trans-sabinene hydrate	69	-	-
trans-piperitol	70	-	-
β-caryophyllene	806	231	9
humulene	304	-	-
alloaromadrene	42	17	-
germacrene-D	84	-	-
γ-elemene	30	-	-
Δ-cadinene	50	191	-
α-nerolidol	80	50	-
caryophyllene oxide	888	167	-
humulene oxide	83	41	-

has already been demonstrated in a wide variety of fruits (57-71) and, since the pectin hydrolysing enzyme preparation was known to have glycosidase activity, it was suspected that the increase in terpene alcohols and indirectly the terpene olefins was associated with the presence of terpene glycosides.

The mass fragmentogram obtained from the solvent extract after glycosidase treatment of Ben Lomond aqueous residue is shown in figure XXXI. Although a very complex chromatogram was obtained, only terpinen-4-ol, α-terpineol and their corresponding olefins were identified.

Using the method previously described, the level of terpenes alcohols and olefins present after glycosidase treatment was determined in fruit at different stages of maturity. The results are presented in table X.

In order to obtain a clearer understanding of the biosynthesis of terpenes in blackcurrant fruit, studies were carried out using ^{13}C mevalonate. Using the methods previously described labelled /-2-^{13}C-/ mevalonate was fed to unripe, halfripe and full ripe blackcurrants. Since the concentration of terpenes was highest in unripe fruit, it was concluded that the biosynthetic pathways leading to the generation of terpene compounds were at their most active at this time, and thus unripe fruit was studied initially.

The position of the ^{13}C label is shown in figure VI. Using single

1. *d*-(+)-limonene
2. γ-terpinene
3. terpinen-4-ol
4. α-terpineol

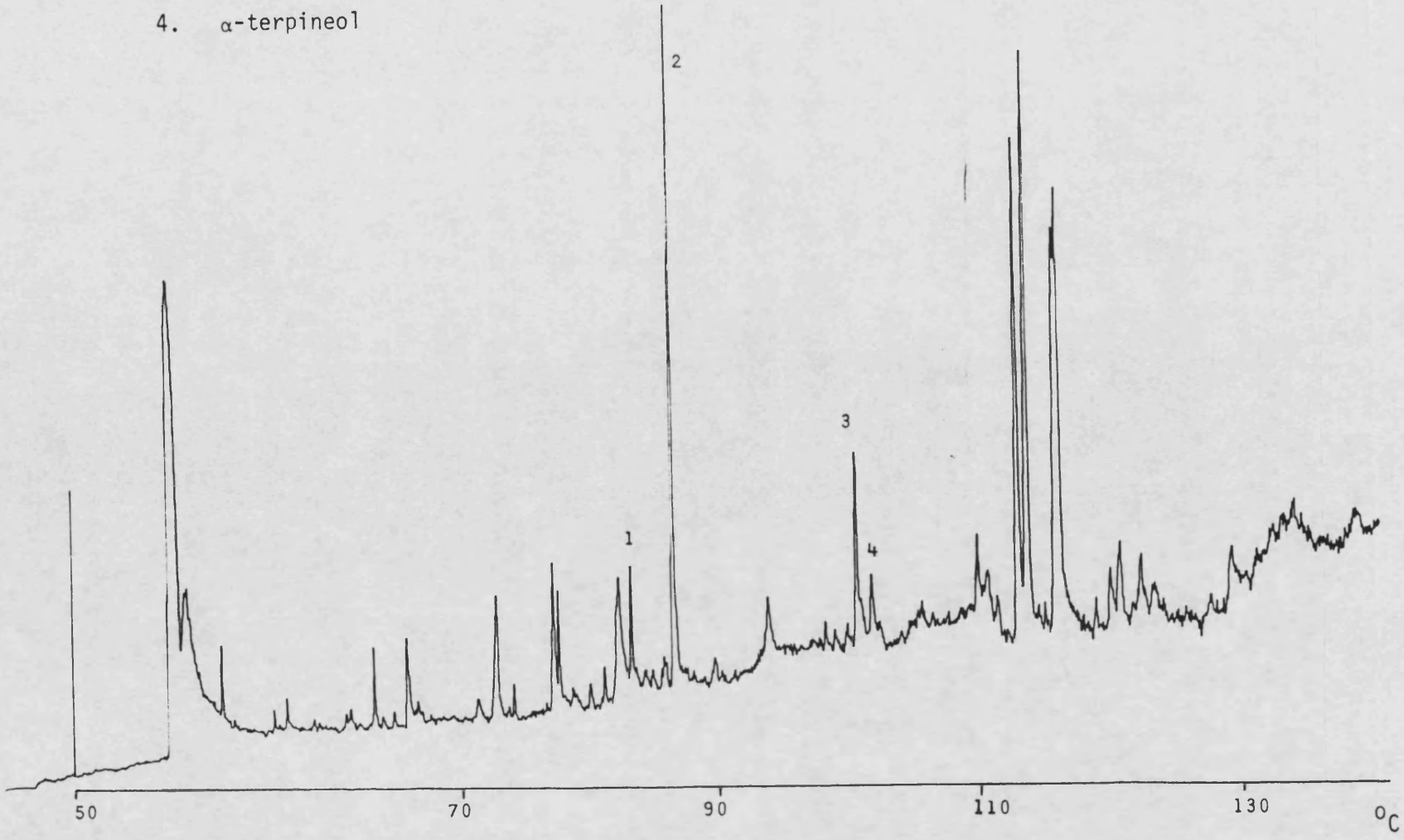


Fig XXXI Mass fragmentogram (*m*/z93) of terpene aglycons present after glycosidase treatment of Ben Lomond Lomond aqueous extract (BP-1 column)

Table X: Concentration of terpene alcohols and olefins after glycosidase treatment at different stages of maturity (mg/kg fresh weight)

Date (1984)		*d*-(+)-limonene	γ-terpinene	terpinen-4-ol	α-terpineol	total
June	1	0.20	1.30	0.40	0.10	2.0
"	15	0.10	0.62	0.22	0.06	1.1
"	29	0.14	0.93	0.34	0.09	1.5
July	13	0.14	1.12	0.35	0.09	1.7
"	27	0.30	2.66	0.82	0.22	4.0
August	10	0.33	3.29	1.18	0.30	5.1
"	24	0.45	4.86	1.81	0.48	7.6

ion mass spectrometry at *m*/z94 and *m*/z138 the uptake of the label was monitored on a daily basis after administration of the /2-^{13}C-/ mevalonate. A typical mass fragmentogram obtained from this analysis is shown in figure XXXII. Because of the low uptake of label, each analysis was carried out in triplicate and the results averaged. Only a significant increase was observed in seven terpene compounds. The results of this analysis are shown in tables XI and XII. These results are discussed in detail in the following section.

Peak identification - mass fragmentogram (*m/z*94) of labelled Ben Lomond fruit volatiles and internal standard

1. α-thujene
2. α-pinene
3. sabinene
4. myrcene
5. Δ^3-carene
6. *d*-(+)-limonene
7. *cis*-β-ocimene
8. *trans*-β-ocimene
9. γ-terpinene
10. linalol
11. terpinen-4-ol
12. α-terpineol
13. anisyl alcohol - internal standard
14. β-caryophyllene
15. α-humulene

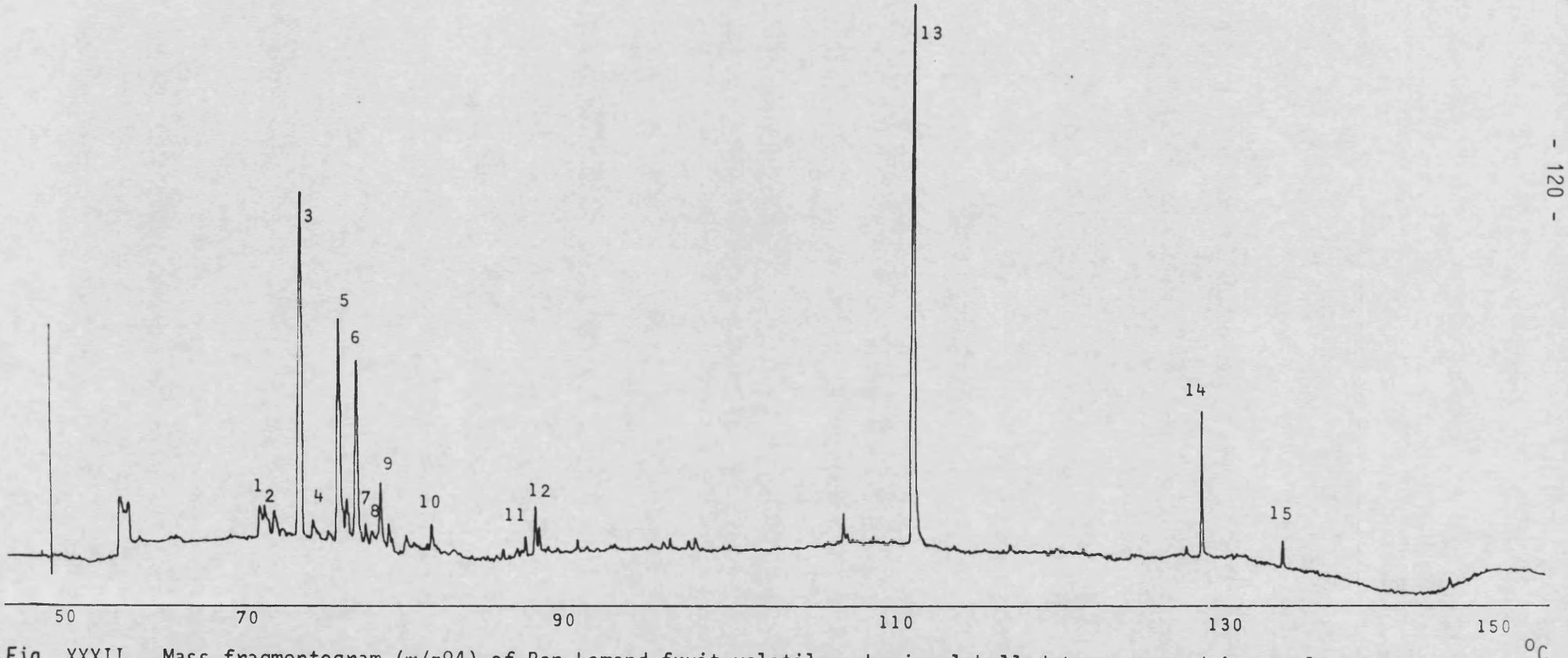


Fig. XXXII Mass fragmentogram (*m*/z94) of Ben Lomond fruit volatiles showing labelled terpenes and internal standard (anisyl alcohol)

Table XI: Incorporation of /2-^{13}C/-mevalonate into terpene compounds. (Mean values of three analyses. Results in µg/kg fresh unripe fruit)

Compound	0	1 day	2 days	3 days	4 days	5 days	6 days
m/z94							
sabinene	1550	+ 1.0	+ 6.0	+ 8.0	+14.0	+16.0	+16.1
Δ^3-carene	640	+ 0.5	+ 2.5	+ 3.0	+ 5.0	+ 5.5	+ 5.5
d-(+)-limonene	185	-	+ 0.5	+ 1.0	+ 1.8	+ 2.0	+ 2.2
γ-terpinene	41.5	-	-	+ 0.4	+ 0.8	+ 1.2	+ 1.2
linalol	45	-	-	+ 0.5	+ 0.9	+ 1.1	+ 1.2
α-terpineol	205	-	+ 0.8	+ 1.1	+ 1.8	+ 2.0	+ 2.2
β-caryophyllene	135	-	+ 0.5	+ 0.9	+ 2.0	+ 2.0	+ 2.0
total	2801.5	+ 1.5	+10.3	+14.9	+26.3	+29.8	+30.4
m/z138							
sabinene	1550	-	-	+ 0.5	+ 1.0	+ 3.0	+ 3.8
Δ^3-carene	640	-	-	-	+ 0.5	+ 1.0	+ 1.3
d-(+)-limonene	185	-	-	-	-	+ 0.5	+ 0.6
total	2375	-	-	+ 0.5	+ 1.5	+ 4.5	+ 5.7

Table XII: Incorporation of /2-^{13}C/-mevalonate into terpene compounds in ripe, halfripe and full ripe fruit after six days (levels in µg/kg fresh weight, calculated from m/z94 data)

Sample	sabinene	Δ^3-carene	d-(+)-limonene	α-terpineol	β-caryophyllene
Unripe day 0	1550	640	185	205	135
Unripe day 6	+16.1	+ 5.5	+ 2.2	+ 2.2	+ 2.0
Halfripe day 0	1150	485	143	160	102
Halfripe day 6	+ 9.5	+ 3.5	+ 1.5	+ 1.7	+ 1.3
Full ripe day 0	550	201	75	90	54
Full ripe day 6	+ 3.0	+ 1.0	+ 0.5	+ 1.0	+ 0.3

CHAPTER 4

Discussion of Results and Conclusions

Blackcurrant leaf oil

The isolation and analysis of the blackcurrant leaf oils has resulted in a significantly improved knowledge of the compounds found in these oils. Previous analysis of blackcurrant leaf oil carried out by Andersson and co-workers in 1963 (II) identified twenty one compounds in steam distilled leaf oil. Seventeen of these compounds were also found in this study, however benzaldehyde, methyl salicylate, *m*-cymene and 4-isopropenyltoluene were not identified.

A total of forty four compounds were identified by combined gas chromatography/mass spectrometry and confirmed by comparison with authentic standards using polar (BP.20≡PEG20M) and non-polar (BP.1≡OV-101) columns. Of the twenty three compounds found for the first time in blackcurrant leaves only β-terpineol has not previously been reported in blackcurrant fruit, leaves or buds.

The following compounds have been identified in blackcurrant leaf oil for the first time: α-thujene, β-pinene, camphene, sabinene, α-phellandrene, α-terpinene, γ-terpinene, terpinolene, *trans*-sabinene hydrate, *cis* and *trans*-β-terpineol, α-terpineol, verbenol, *trans*-linalool oxide, *cis* and *trans*-β-terpinyl acetate, neryl acetate, α-terpinyl acetate, alloaromadrene, germacrene-D, γ-elemene, α-nerolidol, caryophyllene epoxide, humulene epoxide-II, *trans*-2-hexenal, *cis*-3-hexenol and *n*-hexyl acetate.

Andersson and co-workers reported a yield of steam distilled leaf oil of 170mg/kg in Brödtorp blackcurrant leaves, which is of the same order as found in this study (80-290mg/kg). The most significant difference between this study and that of Andersson and co-workers is the total absence of sabinene in the oil isolated by Andersson's group. Although a very significant decrease in the sabinene level of steam distilled oil compared to solvent extracted oil was noted in this study, sabinene was still the most abundant monoterpene in the steam distilled leaf oils of all three cultivars examined.

Although it is possible that the absence of sabinene in the oil isolated by Andersson and co-workers was the result of acid catalysed hydration during distillation, none of the expected products of sabinene hydration were present except for a low level of terpinen-4-ol. Since all of the products of sabinene hydration, α-terpinene, γ-terpinene, terpinolene and terpinen-4-ol were found at greatly increased levels in the steam distilled oils isolated during this study, it may be concluded that Brödtorp leaf oil does not contain an appreciable quantity of sabinene. This conclusion is supported by the work of Latrasse and Lantin (35) who found that blackcurrant bud oil (var. Brödtorp) belongs to a family of cultivars in which the bud oils are devoid of sabinene.

Apart from this obvious difference, the compounds identified in this study are present at approximately the same levels as reported by Andersson and co-workers.

The yields of blackcurrant leaf oil from the three cultivars studied were very similar when separated by solvent extraction (530 - 730mg/kg) but a much lower yield of oil was noted from blackcurrant leaves var. Baldwin when isolated by steam distillation.

Table XIII

Cultivar	mg/kg oil isol. by solv. extraction	mg/kg oil isol. by steam distillation	pH of distillation water
Baldwin	739	81	4.1
Ben Lomond	528	292	4.7
Wellington XXX	712	277	4.5

The yield of all the leaf oils was much lower when isolated by steam distillation and initially this was thought to be a function of distillation time. However, increasing the distillation time from six hours to fifteen hours only increased the relative yield by a further seven to nine per cent.

The difference in yield between steam distilled and solvent extracted oils appears to be linked to the acidity of the distillation water. The lower pH resulting in a lower yield of oil by steam distillation. Thus either polymerisation or decomposition into water soluble products must be suspected.

Since the yield of oil has been calculated by summing the concentration of individual compounds, the yield of steam distilled oil is slightly higher than shown in table XIII since there are a small number of unidentified components, but this can at best only increase the yield by 25-50mg/kg.

Examination of the chromatograms of the steam distilled and solvent extracted leaf oils isolated in this study shows a number of major differences.

In all three varieties studied the levels of α-thujene, sabinene, α-pinene, β-caryophyllene, humulene, germacrene-D and γ-elemene were found to be markedly decreased, whereas the concentration of γ-terpinene, terpinolene, terpinen-4-ol, caryophyllene oxide and humulene oxide were correspondingly increased.

The acid catalysed hydration of sabinene and α-thujene has been studied by a number of research groups (74-81). The results of their work have shown that the hydration of sabinene and α-thujene proceeds through a common carbonium ion (Fig. XXXIII) to yield a mixture of α-terpinene, γ-terpinene, terpinolene and terpinen-4-ol, by either elimination of a proton to yield one of the olefins or addition of a nucleophile in this instance OH^- from water to give terpinen-4-ol.

Similar rearrangements of sabinene and α-thujene have been recognised in steam distilled pine (82,83), marjoram (84), angelica root (85) and juniperberry oils (86); in all cases a large increase in the concentration of terpinen-4-ol was observed, accompanied by an increase in at least one of the olefins. The actual distribution of hydration products appears to be related to the pH of the water used in the distillation (83), a lower pH resulting in a higher level of olefins. The pH of the

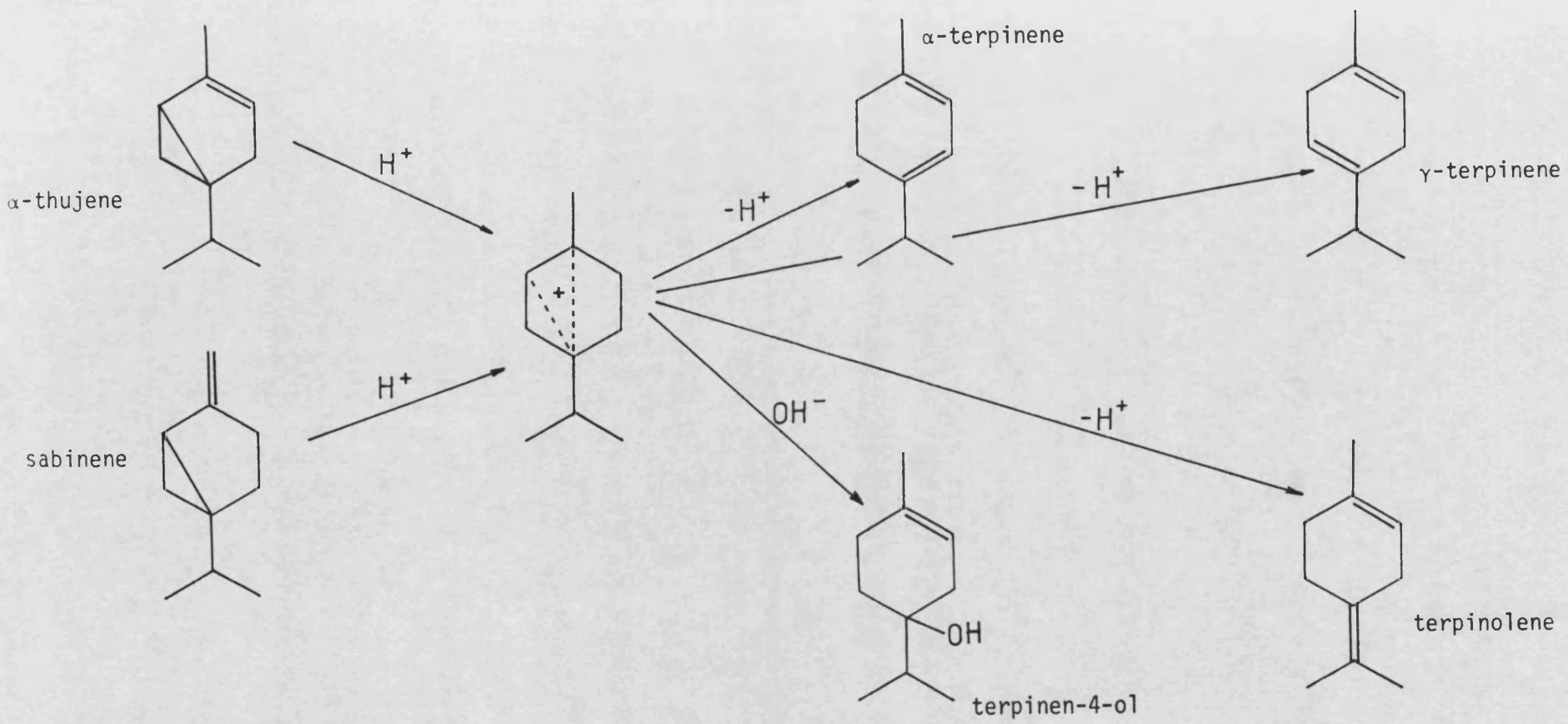
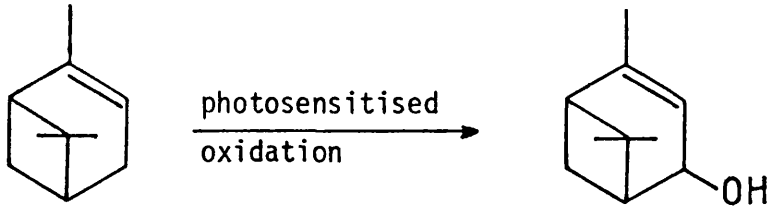


Figure XXXIII. Acid catalysed hydration of sabinene and α-thujene

distillation water after isolation of blackcurrant leaf oil was 3.4 which is lower than much of the previously reported work on other botanical materials (82-86), and this resulted in the production of a whole spectrum of hydration products.

The concentration of *trans*-sabinene hydrate was also found to be reduced by 70-80% in steam distilled blackcurrant leaf oils. *Trans*-sabinene hydrate undergoes similar rearrangements to sabinene and α-thujene. Taskinen (81) reported the distribution of acid catalysed rearrangement products of *trans*-sabinene hydrate. It is suggested that the reaction proceeds through a carbonium-ion intermediate resulting from protonation and loss of water. This species then undergoes addition of an hydroxyl ion from water, or elimination of a proton to yield terpinen-4-ol,α-terpinene or γ-terpinene (Figure XXXIV). Taskinen observed a degree of epimerisation proceeding through the carbonium-ion intermediate, but the product, *cis*-sabinene hydrate, was not observed in this study.

A marked reduction of α-pinene was also found in the steam distilled leaf oils, together with an increase in verbenol, an alcohol derived from α-pinene. The photosensitised oxidation of α-pinene



α-pinene verbenol

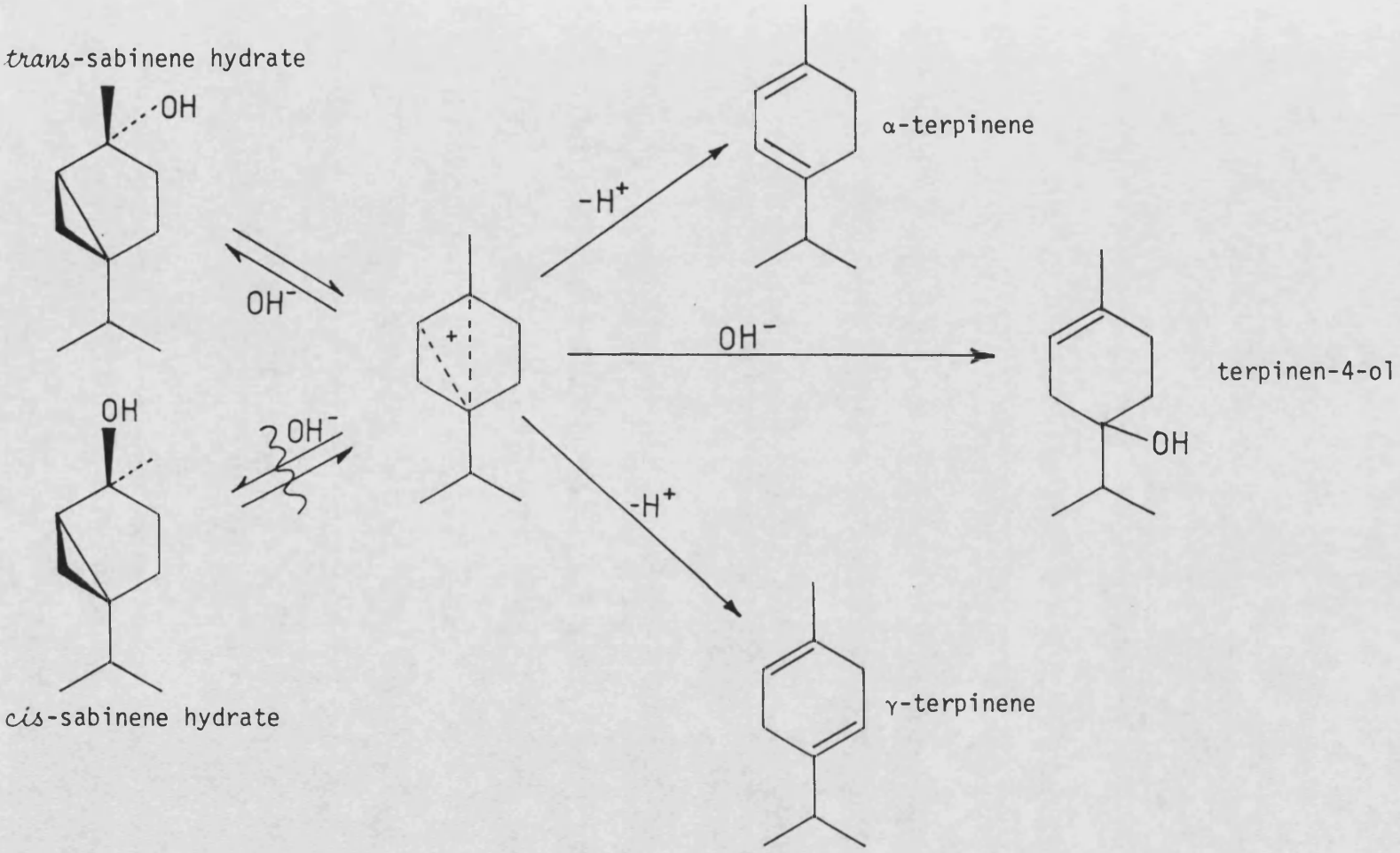


Figure XXXIV. Acid catalysed rearrangements of *trans*-sabinene hydrate

to verbenol has been previously reported (87) to occur during the isolation of essential oils by steam distillation. The level of verbenol will only account for approximately 50% of the decrease in α-pinene, but α-pinene undergoes a wide range of acid catalysed reactions (88-91) in this instance probably resulting in *p*-menthane derivatives, since no increase in bornane or fenchane derivatives was observed (Fig. XXXV). This result would be consistent with the observations of Williams and Whittaker (89, 91).

The remainder of the monoterpenes remained at approximately the same relative concentration in both the steam distilled and solvent extracted oil, except for citronellol, which was completely absent from steam distilled blackcurrant leaf oil, *p*-cymen-8-ol which was significantly reduced in steam distilled oil and citronellyl acetate of which only 10% of that present in solvent extracted oil survived steam distillation.

Unlike linalol, geraniol or nerol, citronellol does not cyclize or rearrange readily in the presence of acid (92-94) due to the hydrogenation of the 6,7-bond. However citronellol will readily oxidise to citronellal which will undergo cyclisation to isopulegol (95) but neither these products or any others were detected to account for the decrease in citronellol and citronellyl acetate, since terpene esters have been shown to undergo rapid hydrolysis during steam distillation (96-100) followed by rearrangement of the alcohol.

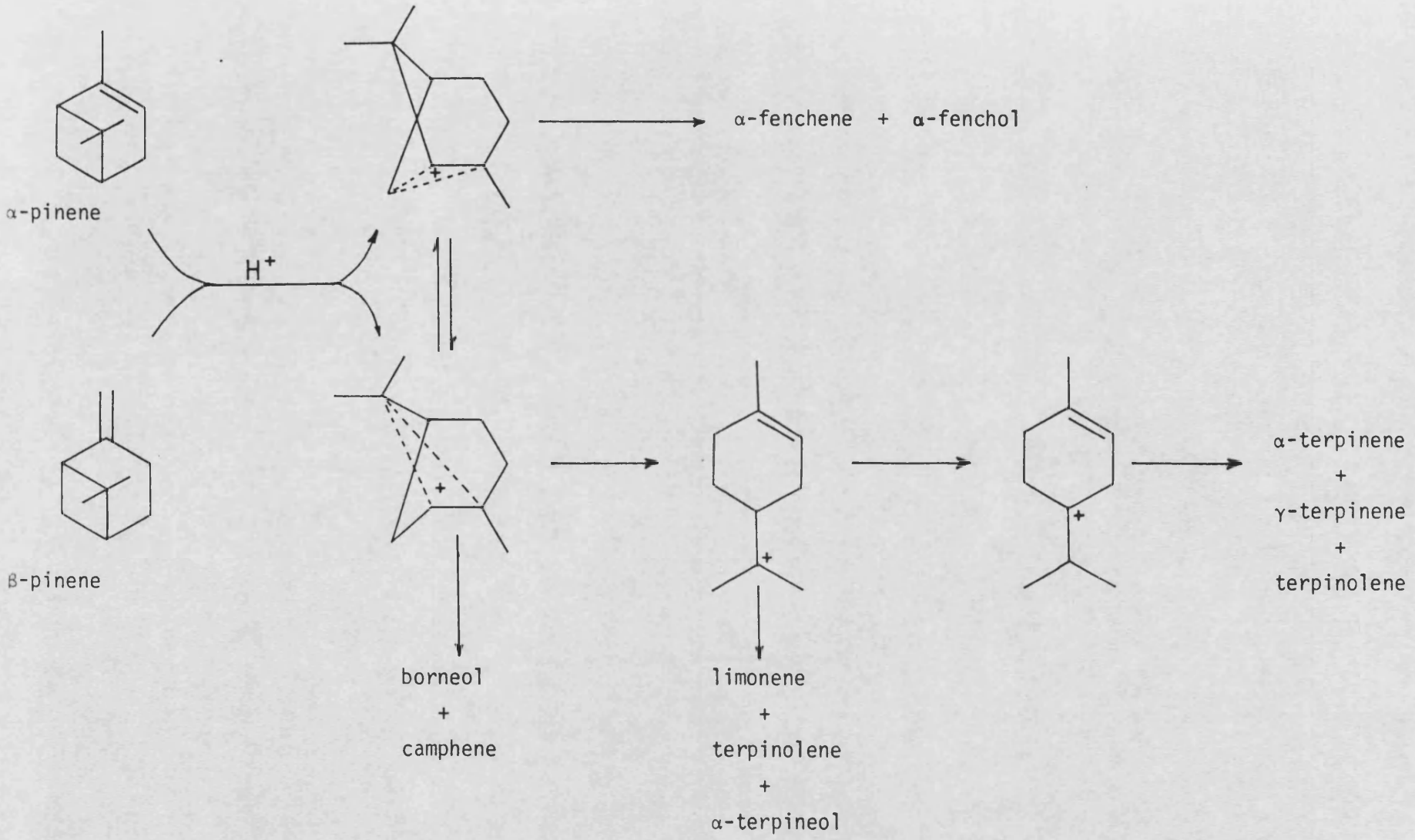
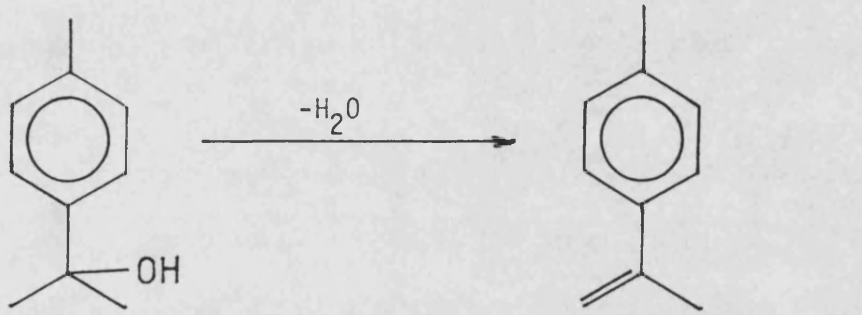


Figure XXXV. Acid catalysed hydration of α-pinene and β-pinene

The significant reduction of *p*-cymen-8-ol in steam distilled leaf oils may be the result of dehydration of this compound to *p*,α-dimethylstyrene. The dehydration of *p*-cymen-8-ol to



p-cymen-8-ol 4-α-dimethylstyrene

4-α-dimethylstyrene has been demonstrated by a number of workers (156-158) and shown to proceed under mildly acidic conditions at room temperature. Therefore it is not unreasonable to expect the dehydration of *p*-cymen-8-ol to proceed during the steam distillation of blackcurrant leaf oil.

The sesquiterpene fraction of the leaf oils also showed major rearrangements during steam distillation. The six sesquiterpene hydrocarbons identified in the leaf oil were all greatly reduced in concentration during steam distillation, and related sesquiterpene alcohols and epoxides increased significantly. Caryophyllene epoxide and humulene epoxide-II were the major products, together with a number of unidentified sesquiterpene alcohols or epoxides and an unidentified sesquiterpene hydrocarbon.

The oxidation of caryophyllene and humulene has been extensively studied (101-106) and has been shown to occur during essential oil

distillation (106, 107, 52). Although three epoxides of humulene are possible (Fig. XXXVI) only humulene epoxide-II was identified in this study probably because the ($\Delta^{1,2}$) double bond is the most strained (108-111).

Isomerisation of both humulene epoxide-II and caryophyllene epoxide to humulenol-II and caryophyllenol-II respectively could not be conclusively demonstrated, since neither of these compounds was positively identified. However, one of the unknown compounds (Unknown 4) has a spectrum very similar to that reported for humulenol-II by Pickett *et al* (106) and may indeed be this compound since only the ion intensities vary from the reported spectrum. These observations are consistent with those of Tressl *et al* (107) and Pickett (106) who both found that humulene epoxide-II was formed at ten times the rate of humulene epoxide-I and only found humulenol-II was present in distilled hop oils.

Epoxidation is only one of a number of acid-catalysed reactions and rearrangements which caryophyllene and humulene undergo (112-115). Some of these rearrangements are shown in figure XXXVII. The formation and stereochemistry of clovene and caryolan-1-ol have been rationalised by Aebi *et al* (116) and the mechanism for the formation of neoclovene was deduced by Parker *et al* (117). In this study however, only the epoxides were conclusively identified in steam distilled leaf oils.

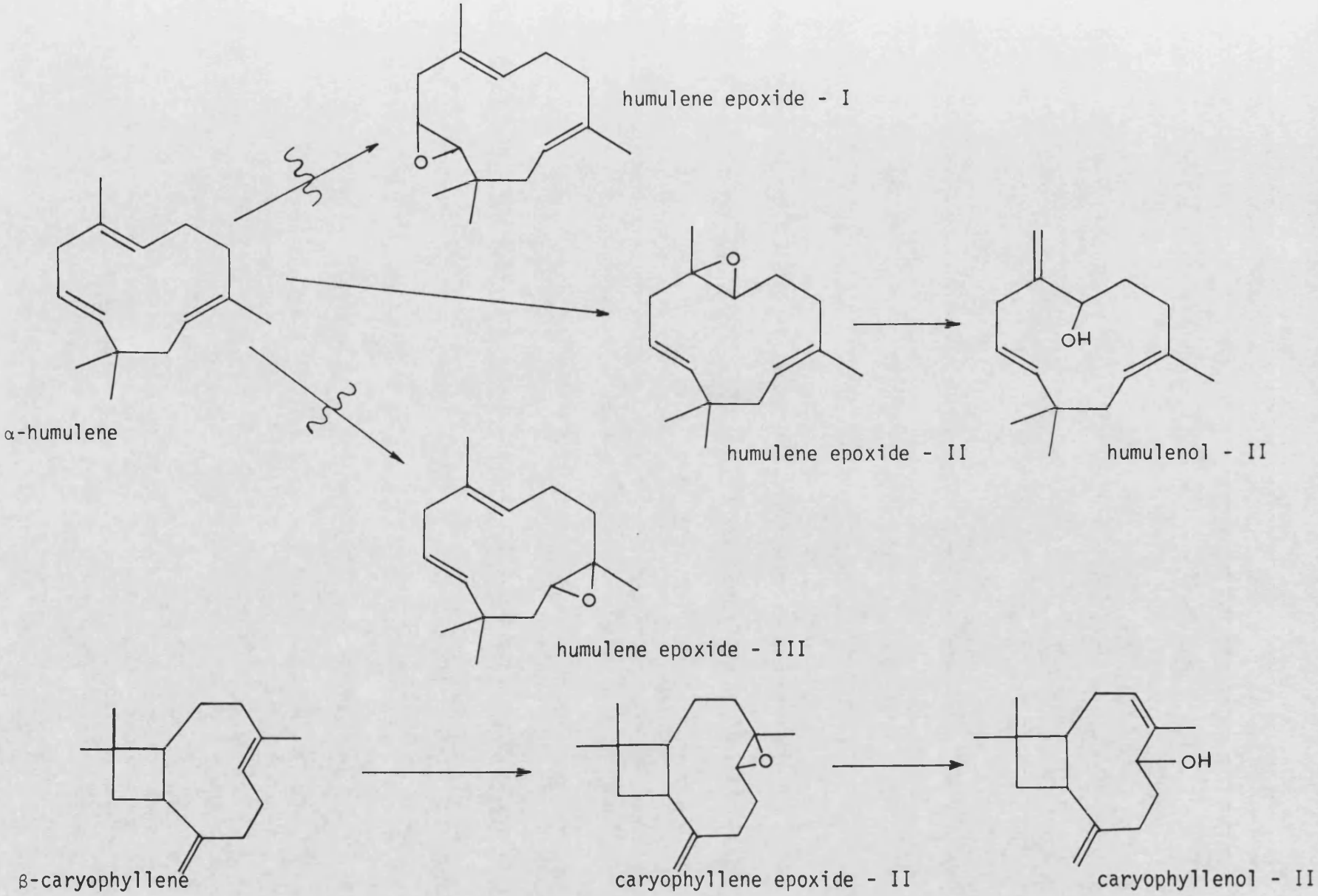


Figure XXXVI. Oxidation of humulene and β-caryophyllene

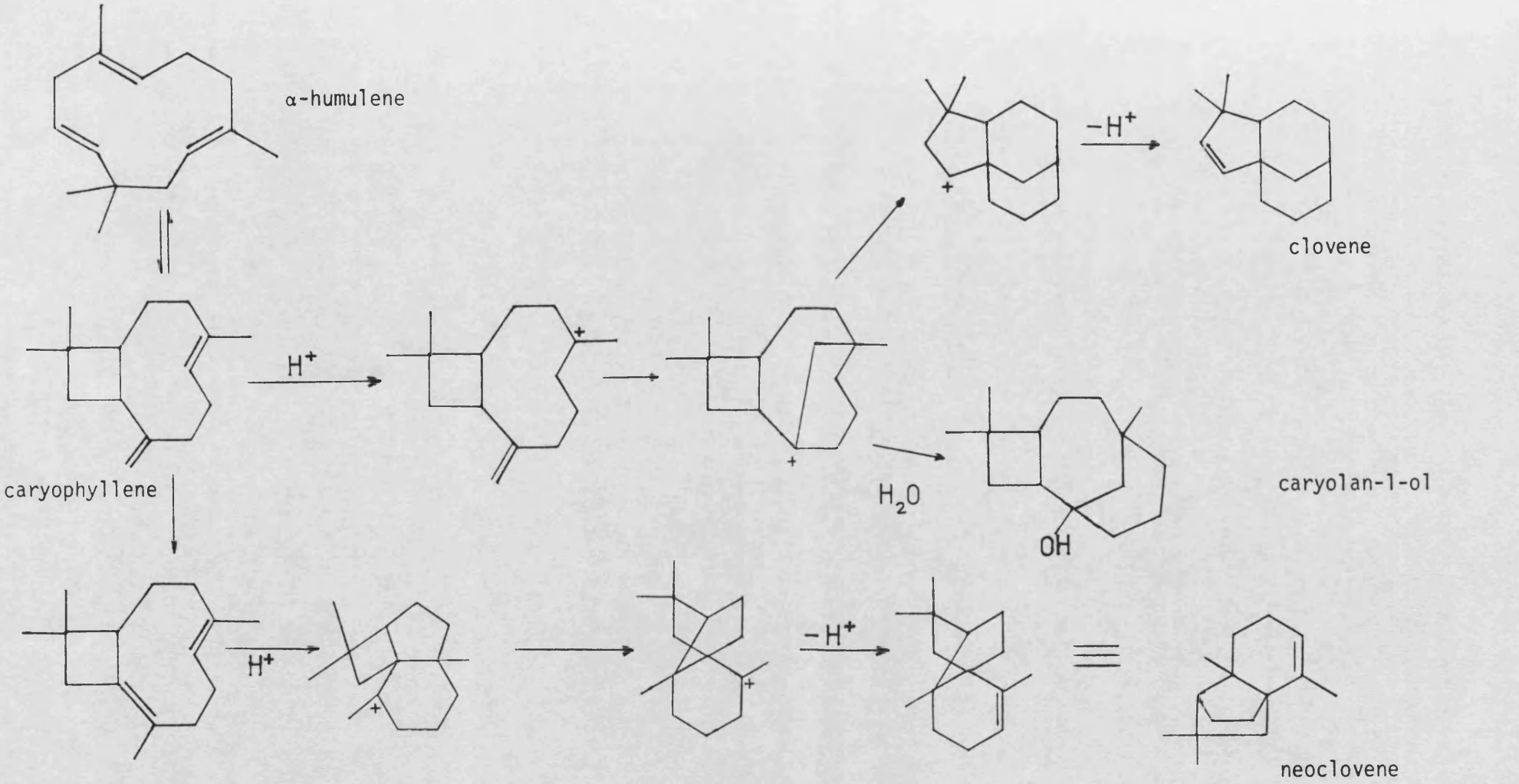


Figure XXXVII. Acid catalysed rearrangements of caryophyllene

The relative concentrations of alloaromadrene and Δ-cadinene were unchanged during steam distillation, but the levels of germacrene-D and γ-elemene were reduced by 70-100%. In all the oils isolated the γ-elemene present at 17-30mg/kg in solvent extracted oils was completely destroyed during steam distillation.

Germacrene-D is known to rearrange to cadinene and muurolene isomers under acidic conditions (118) and to β-bourbonene in the additional presence of light (119, 120). Tressl *et al* (106) postulated a reaction scheme to explain the transformation of germacrene-D to γ-elemene and alloaromadrene which proceeds via germacrene-B or bicyclogermacrene respectively. Since bicyclogermacrene may also be an intermediate in the synthesis of Δ-cadinene and γ-elemene, it is proposed that bicyclogermacrene is formed from germacrene-D and subsequently rearranges to Δ-cadinene, alloaromadrene and γ-elemene (figure XXXVIII).

γ-Elemene, Δ-cadinene and alloaromadrene may then undergo epoxidation to cadinene epoxide-I and II and alloaromadrene epoxide. Although not identical to the published spectra (107) unknown compound 6 may be alloaromadrene epoxide. This scheme may account for the decrease in germacrene-D and the maintenance of Δ-cadinene and alloaromadrene levels in steam distilled leaf oils, but if γ-elemene is formed via bicyclogermacrene it is subsequently destroyed. If this is the case then it would explain the discrepancy between the decrease in germacrene-D and only small

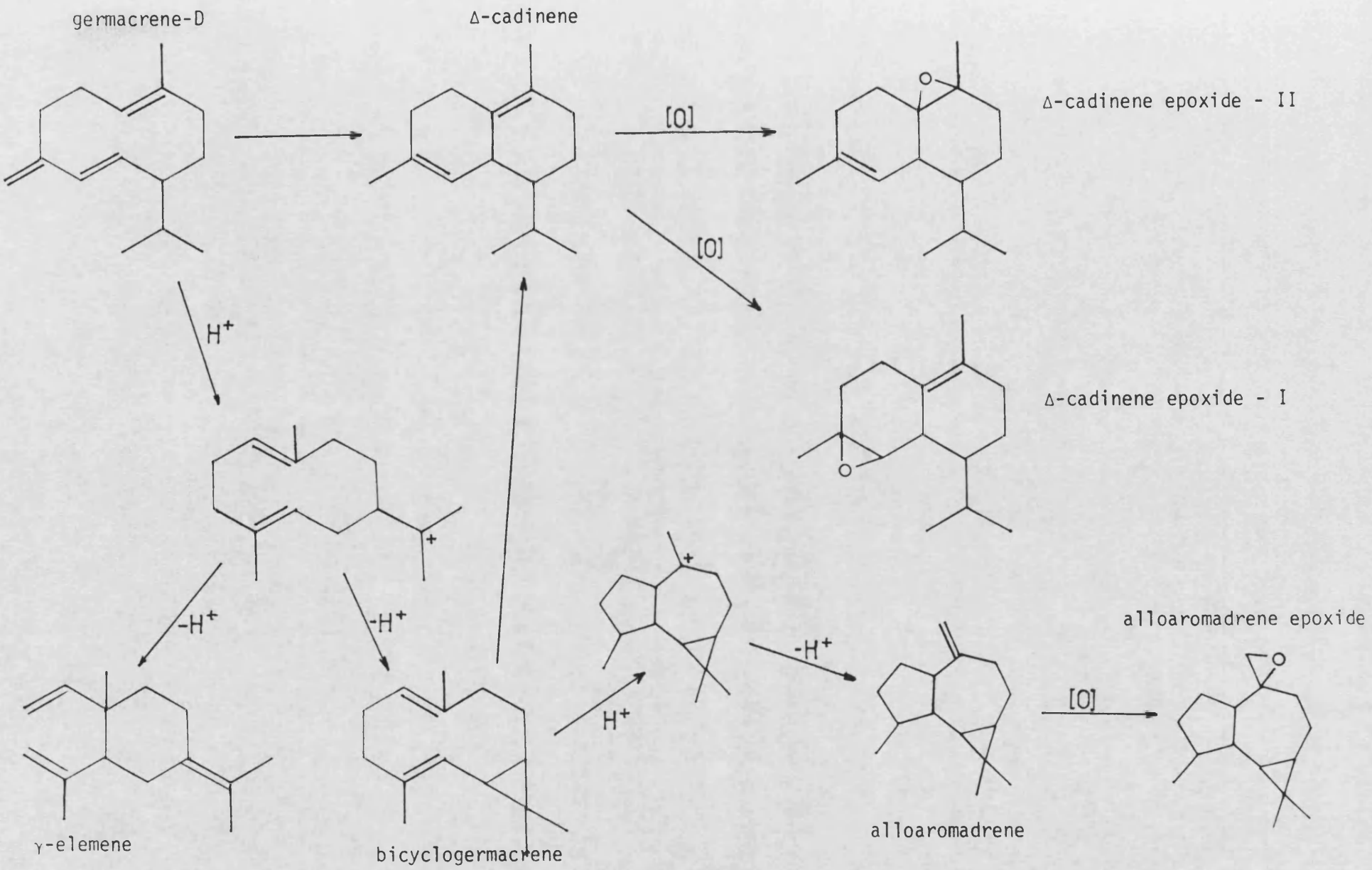


Figure XXXVIII. Acid catalysed rearrangements of germacrene-D and bicyclogermacrene

relative increase in Δ-cadinene, alloaromadrene and the tentatively identified alloaromadrene epoxide.

The structure of γ-elemene allows epoxidation to proceed readily and although not positively identified in this study may be one of the unidentified compounds (m.w.220).

A study of isolation techniques applied to pine oil by Koedam *et al* (83) found that β-elemene and Δ-cadinene were both completely destroyed during steam distillation, but no related products were found. In a recent study (52) β-elemol and γ-elemol were tentatively identified in blackcurrant bud oil which had been fractionated on silica gel. A number of studies (83, 118, 121, 122) have shown that silica gel can often produce artefacts similar to those obtained during distillation, but in this study neither of the elemols were detected.

The only aliphatic compounds detected in blackcurrant leaf oil were *trans*-2-hexenal, *cis*-3-hexenol, 1-octen-3-ol and *n*-hexyl acetate; of these only 1-octen-3-ol has previously been reported in blackcurrant leaf oil. The concentration of *n*-hexyl acetate was slightly reduced in steam distilled oil, presumably by hydrolysis although no *n*-hexanol was detected. This was probably because *n*-hexanol was found to co-elute was β-terpinyl acetate on polar (BP-20) columns and with *trans*-2-hexenal on non-polar (BP-1) columns. Moreover the presence of *n*-hexanol by single ion mass spectrometry is not easy and no special procedures were carried out to ensure

its recognition.

The concentration of *trans*-2-hexenal, *cis*-3-hexenol and 1-octen-3-ol was at least ten times higher in steam distilled blackcurrant leaf oils, compared to solvent extracted leaf oil. All three compounds arise from enzymic or non-enzymic splitting of unsaturated fatty acids particularly linoneic and linoleic.

Enzymic formation of hydroperoxides from polyunsaturated fatty acids is catalysed by lipoxygenase in the presence of oxygen (124), and this is followed by cleavage of the hydroperoxide by an aldehyde lyase. This scheme has been demonstrated in a wide variety of fruit, vegetables, leaves and seeds (125-134). Enzymic cleavage of the 13-hydroperoxide leads to the formation of hexenal and *cis*-3-hexenal. In plants the *cis*-3-double bond is often isomerised to the conjugated *trans*-2 derivative, either by an isomerase (136, 135) or non-enzymically (137).

In the presence of oxygen, fresh leaves which are damaged or macerated in any way produce large quantities of hexanol, *trans*-2-hexenal, *cis*-3-hexenal and *cis*-3-hexenol (138, 139). This is of particular importance in the formation of tea flavour (140-143). *cis*-3-Hexenol is formed from *cis*-3-hexenal by the action of alcohol dehydrogenase. This enzyme has been isolated and studied in tea leaves and a number of fruits (144-150).

The formation of 1-octen-3-ol from linoleic acid has been studied

in mushroom homogenates (151-154), and shown to involve the stereospecific reduction of 1-octen-3-one to (-)1-octen-3-ol. In contrast the autoxidation of linoleic acid results in the formation of racemic 1-octen-3-ol (151). Although it was not possible to determine the stereochemistry of the 1-octen-3-ol found in blackcurrant leaf oil, it may be assumed that this compound arises from the autoxidation of linoleic acid, since the enzymic formation of 1-octen-3-ol has only been demonstrated in basydiomycetes and some fungi such as *Penicillium* and *Aspergillus Sp.* (155).

It is evident from this study of blackcurrant leaf oil that enzymic and autoxidation of polyunsaturated fatty acids to volatile aldehydes and alcohols is minimised during low temperature solvent extraction, and that even the short time between the leaves thawing and reaching a temperature at which the enzymes are inactivated allows significant formation of *trans*-2-hexenal, *cis*-3-hexenol and 1-octen-3-ol. A more representative essential oil profile is therefore obtained through low temperature solvent extraction.

In addition to the forty four compounds identified in this study, there were at least another fifty minor components. Mass spectra were obtained on twelve of these compounds (Appendix II), the remainder being at a level below which reliable mass spectrometric data could be obtained. Two of these unknown compounds, four and six, have already been discussed and may be humulenol-II and alloaromadrene epoxide respectively.

The remaining spectra represent four different compound groups. Spectra eight and nine are monoterpene olefins with a molecular weight of 136. The predominance of lower mass ions in spectrum eight would indicate an unstable ring system, or an acyclic monoterpene not stabilized by a conjugated double bond system. This spectrum is quite unlike any published terpene mass spectrum (159 -162). Spectrum nine is very similar to that of camphene (159), but this is not supported by retention data. However, a bicyclic monoterpene is indicated.

The spectra of unknown compounds one, two and ten indicate monoterpene alcohols or ketones of molecular weight 154, 152 and 154 respectively. Spectrum ten is very similar to that of *trans*-β-terpineol, but elutes well before this compound. The spectrum of unknown compound one, shows a predominence of ions below mass ninety three indicating a substantially unstable molecule. A non-aromatic monocyclic terpene alcohol is indicated with the hydroxyl group attached to the ring (163, 164).

The spectrum of unknown compound two shows only a very small peak at *m*/z = M-18 indicating that this is a ketone (165). Strong ions at *m*/z110 and 79 indicate a monocyclic terpene with the keto group on the ring (165).

Mass spectra of unknown compounds three, eleven and twelve show them to be sesquiterpenes of molecular weight 204. Although none of these spectra accurately match any published sesquiterpene mass spectra

(160-162, 166), spectrum eleven is similar to the recorded spectrum of clovene, which is not entirely unexpected since clovene is a product of acid catalysed rearrangement of caryophyllene, and this compound occurs at a much greater level in steam distilled leaf oils.

The spectrum of unknown twelve has similarities with that of β-elemene. This compound has previously been reported in black-currant bud oil (52). Reference compounds were not available to confirm these tentative identifications. The spectrum of unknown compound three is quite unlike any published sesquiterpene spectra. The base peak, *m/z*57, and low intensity of higher mass ions indicates a very substantially unstable structure, with a labile C_4H_9 fragment, thus an acyclic or monocyclic sesquiterpene is indicated.

The final two unknowns, spectra five and seven, both show molecular ions at 220. Spectrum seven shows a M-18 ion at 202 with an intensity of twenty per cent, which would indicate a sesquiterpene alcohol, whereas spectra five exhibits only three per cent M-18, (*m/z*202), which suggests that this may be an epoxide. Spectrum seven may be an isomer of cadinol, since published spectra of several cadinol isomers (162) compare reasonably with this spectrum. No identification can be suggested for compound five.

Analysis of the blackcurrant leaf oils by single ion mass spectrometry showed a number of quantitative and qualitative differences. Table XIV shows the concentration of terpenes in Ben Lomond leaf oil

Table XIV. Concentration of terpene compounds in distilled Ben Lomond leaf oil calculated from single ion monitoring on BP-1 and BP-20 columns and by GLC using a BP-1 column.(mg/kg fresh weight)

Compound	GLC on BP-1	SIM (*m/z* 93) on BP-1	SIM (*m/z* 93) on BP-20
α-pinene	2.00	2.12	3.76
camphene	0.16	0.19	0.19
sabinene	53.30	50.10	93.30
β-pinene	1.19	1.20	1.90
α-thujene	1.50	1.05	n/d
myrcene	4.04	4.80	5.71
α-phellandrene	0.52	0.45	0.19
Δ^3-carene	28.50	25.70	47.40
α-terpinene	1.83	1.90	0.85
d-(+)-limonene	4.00	4.22	2.75
cis-β-ocimene	14.34	15.05	38.13
trans-β-ocimene	9.20	9.00	0.63
γ-terpinene	6.86	7.15	18.52
terpinolene	42.40	42.00	41.80
β-phellandrene	3.20	3.30	3.65
linalol	1.35	1.40	1.17
trans-β-terpineol	n/d	n/d	0.53
cis-β-terpineol	n/d	n/d	0.13
terpinen-4-ol	16.80	17.01	27.4
α-terpineol	1.62	1.50	2.13
geraniol	2.90	3.10	4.00
α-terpinyl acetate	n/d	n/d	0.64

neryl acetate	n/d	n/d	2.00
trans-linalool oxide	0.67	0,50	n/d
cis-verbenol	0.78	0.81	n/d
cis-β-terpinyl acetate	0.79	0.63	n/d
trans-β-terpinyl acetate	1.61	1.42	n/d
citronellyl acetate	3.30	n/d	n/d
β-caryophyllene	32.40	34.66	51.42
α-humulene	9.76	10.15	11.03
germacrene-D	9.00	9.06	8.10
Δ-cadinene	3.75	3.91	3.80
α-nerolidol	2.25	2.01	n/d
caryophyllene epoxide	14.57	14.40	14.01
humulene epoxide-II	4.00	4.01	5.21
Total	292.11	275.66	390.22

analysed by single ion monitoring using BP-1 and BP-20 columns and analysis by gas chromatography using a BP-1 column. The calculated levels using either gas chromatography or single ion mass spectrometry and a BP-1 (non-polar) column are very similar, but when the analysis is carried out on a BP-20 (polar) column large discrepancies occur, which indicates the presence of catalytic sites on the column.

The monoterpene esters appear to undergo hydrolysis resulting in the absence of *cis* and *trans*-β-terpinyl acetate, and the appearance of *cis* and *trans*-β-terpineol. α-Terpinyl acetate and neryl acetate were also present when a BP-20 column was used, and these may arise from transesterification of α-terpineol and geraniol. Citronellyl acetate is not detected by single ion monitoring at *m/z* 93.

Verbenol and *trans*-linalool oxide are also absent in leaf oil analysed on BP-20 columns, a concurrent increase in α-pinene may indicate that these two compounds undergo on-column dehydration. The ratio of *cis* and *trans*-β-ocimene was markedly affected by changing the column, and appears to isomerise to the *cis* isomer when analysed on a polar column. The *trans* isomer being almost completely absent.

Most of the remaining terpene compounds show little change when analysed on BP-20 (polar) columns, except for those compounds present at levels in excess of 15mg/kg. Of these all except terpinolene were found at twice or three times the level found when a non-polar column was employed. This may be due to an adsorption effect, which

reduces the peak heights of the calibration standard in which all the compounds are at 10mg/kg resulting in high levels being calculated. Compounds of a concentration of 15mg/kg or less, would therefore experience the same adsorptive effect and the peak heights would be reduced by the same amount as the standard mixture, resulting in approximately correct results being obtained.

Blackcurrant fruit volatiles

Isolation of blackcurrant fruit volatiles

The analysis of blackcurrant fruit volatiles first carried out by Andersson (12) and Spanyar (9,10) involved almost all known methods of isolation, to arrive at a concentrated extract of blackcurrant volatiles suitable for chromatographic analysis.

In this work using combined gas chromatography - mass spectrometry and single ion monitoring at *m/z*93 and *m/z*136, it was hoped that direct analysis of the fruit volatiles would be achieved without the need to raise the temperature above -10°C, or subject the isolated aroma to any fractionation techniques, other than the analytical separation.

Blackcurrant fruit volatiles were isolated from ripe Ben Lomond fruit by low temperature solvent extraction using redistilled dichloromethane, vacuum distillation and distillation at reduced pressure (to stimulate production conditions) and the resulting extracts analysed by single ion monitoring at *m/z*93 and *m/z*136 to study changes in the terpene fraction of the volatiles. The results are shown in figures XX to XXII and table VI.

Many of the rearrangements observed during the distillation of blackcurrant leaf oil are mirrored in the distillation of the fruit volatiles. α-Thujene and sabinene are reduced from 49.6 and 1450.0 µg/kg respectively to 4.0 and 13.0 µg/kg on distillation at

reduced pressure and to 4.0µg/kg sabinene only after vacuum distillation. As observed in leaf oil an increase in γ-terpinene, terpinolene and terpinen-4-ol accompanies the decrease in sabinene and α-thujene.

Compound	Solvent extract	vac. distillate	80°C distillate
sabinene	1450.0	4.0	13.0
α-thujene	49.6	n/d	4.0
γ-terpinene	39.7	59.0	183.0
terpinolene	2.2	175.0	679.0
terpinen-4-ol	40.2	567.0	667.0

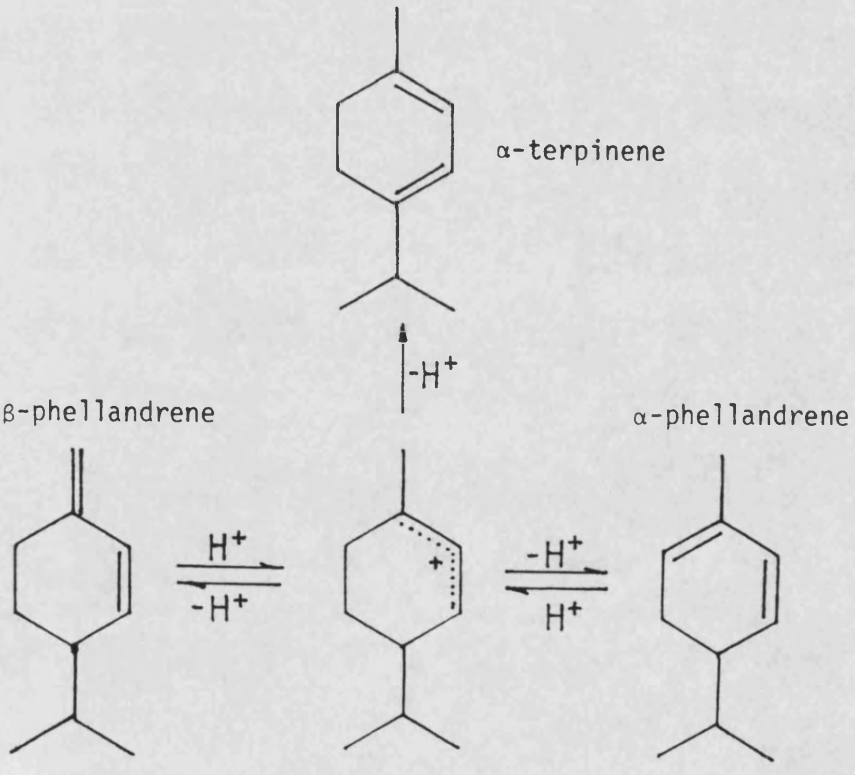
In contrast to leaf oil distillation, the concentration of *trans*-sabinene hydrate increased during 80°C distillation from 46.0µg/kg to 101.0µg/kg. This may be due to autoxidation since in contrast with leaf oil *d*-(+)-limonene is also greatly reduced, with a corresponding increase in α-terpineol (89).

α-Pinene and β-pinene were also found to be greatly reduced in volatile extracts from vacuum or 80°C distillates but in contrast to leaf oil distillations no verbenol was formed, and it is assumed that α-pinene undergoes acid catalysed hydration as shown in figure XXXV. This may be because the pH of fruit distillates is at least one pH unit lower than leaf distillates.

Δ^3-Carene, unchanged by distillation of leaf oil, is reduced by sixty

per cent during vacuum distillation of blackcurrant fruit and by eighty eight per cent during 80°C distillation. Studies carried out by Rudakov (168) and Arbuzov (169) showed that Δ^3-Carene can easily undergo cyclopropane ring opening to yield α-terpinene, limonene, terpinolene and sylvestrene (figure XXXIX). Sylvestrene has been shown to undergo rapid oxidation to 8-hydroxy-*m*-cymene (87). Both α-terpinene and terpinolene are found to increase during distillation and therefore may arise from the hydration of Δ^3-carene or sabinene.

α-Terpinene may also arise from acid catalysed rearrangement of α-phellandrene (167), as can β-phellandrene. This would account for the decrease in α-phellandrene and the increase in β-phellandrene and α-terpinene in distilled blackcurrant volatiles.



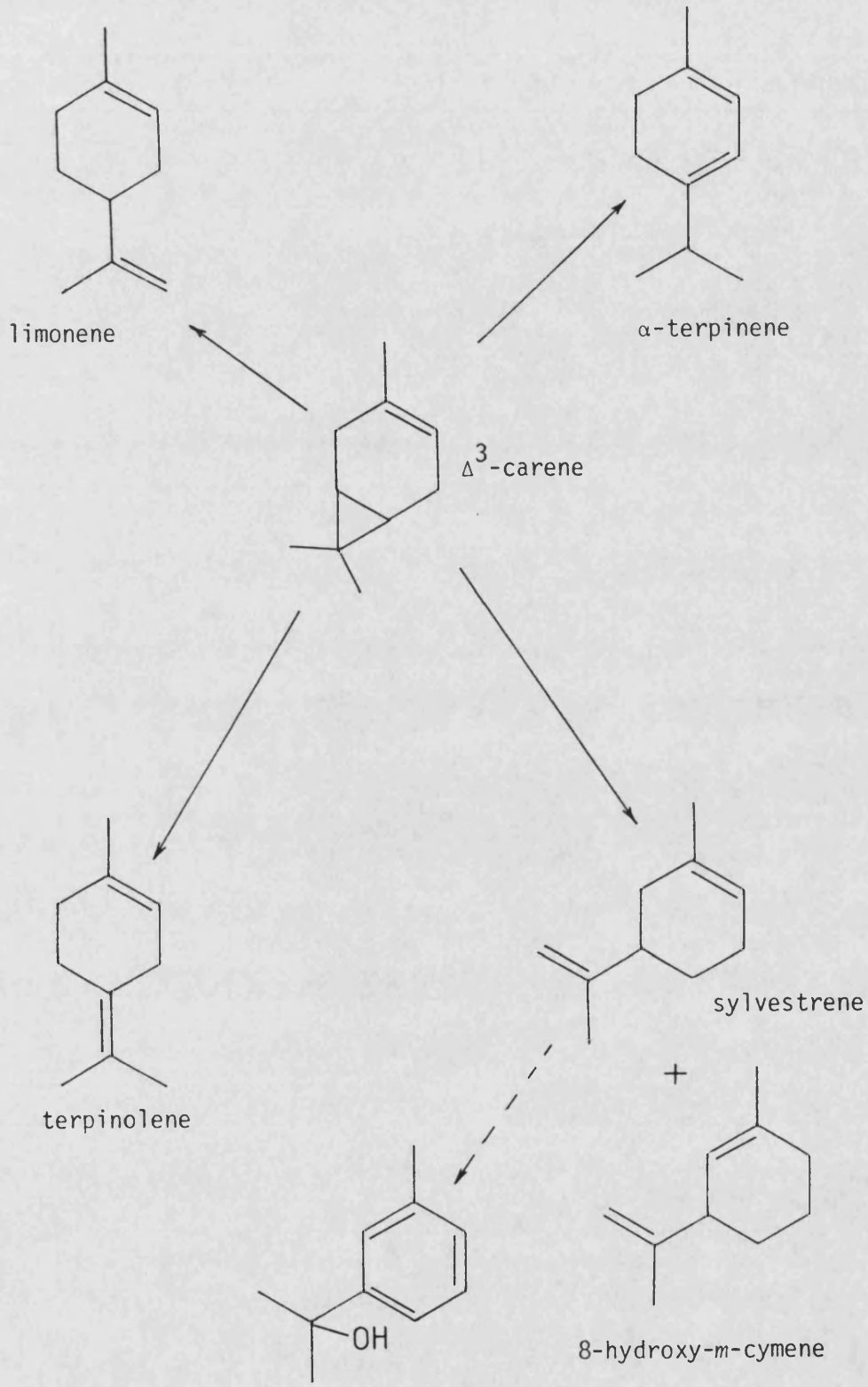


Figure XXXIX Acid catalysed rearrangements of Δ^3-carene.
After Arbuzov *et al* (169)

The remaining *p*-menthane alcohols, show individual increases or decreases on distillation. γ-Terpineol appears on vacuum distillation and increases further on 80°C distillation, and this probably arises from acid catalysed hydration of terpinolene. In the same way piperitol is possibly the product of α-terpinene. *Cis* and *trans*-β-terpineol show little variation during distillation.

The four acyclic monoterpene olefins present, myrcene, *cis* and *trans*-β-ocimene and alloocimene, are substantially reduced in concentration by distillation at 80°C or under vacuum. This corresponds with increases in linalol, nerol, geraniol and linalool oxide, which are all probably formed by hydration of the carbonium ion according to the scheme shown in figure XXXX, the alcohols undergoing acyclic rearrangement as shown. In the case of linalool oxide, linalol may cyclise to give the pyran oxide (170, 171). Alternatively the oxide may arise from mild acid hydrolysis of the enediol (59).

The formation of linalol and linalool oxides has been studied extensively in grapes and grape juice (59). The formation of linalool oxide was found to be exclusively formed from the enediol and it was proposed that derivatives of linalol, nerol and geraniol observed to increase during heating of grape juice may all arise from monoterpene polyols. It is therefore possible that these compounds also exist in blackcurrants and blackcurrant juice.

The terpene esters identified in blackcurrant fruit volatiles

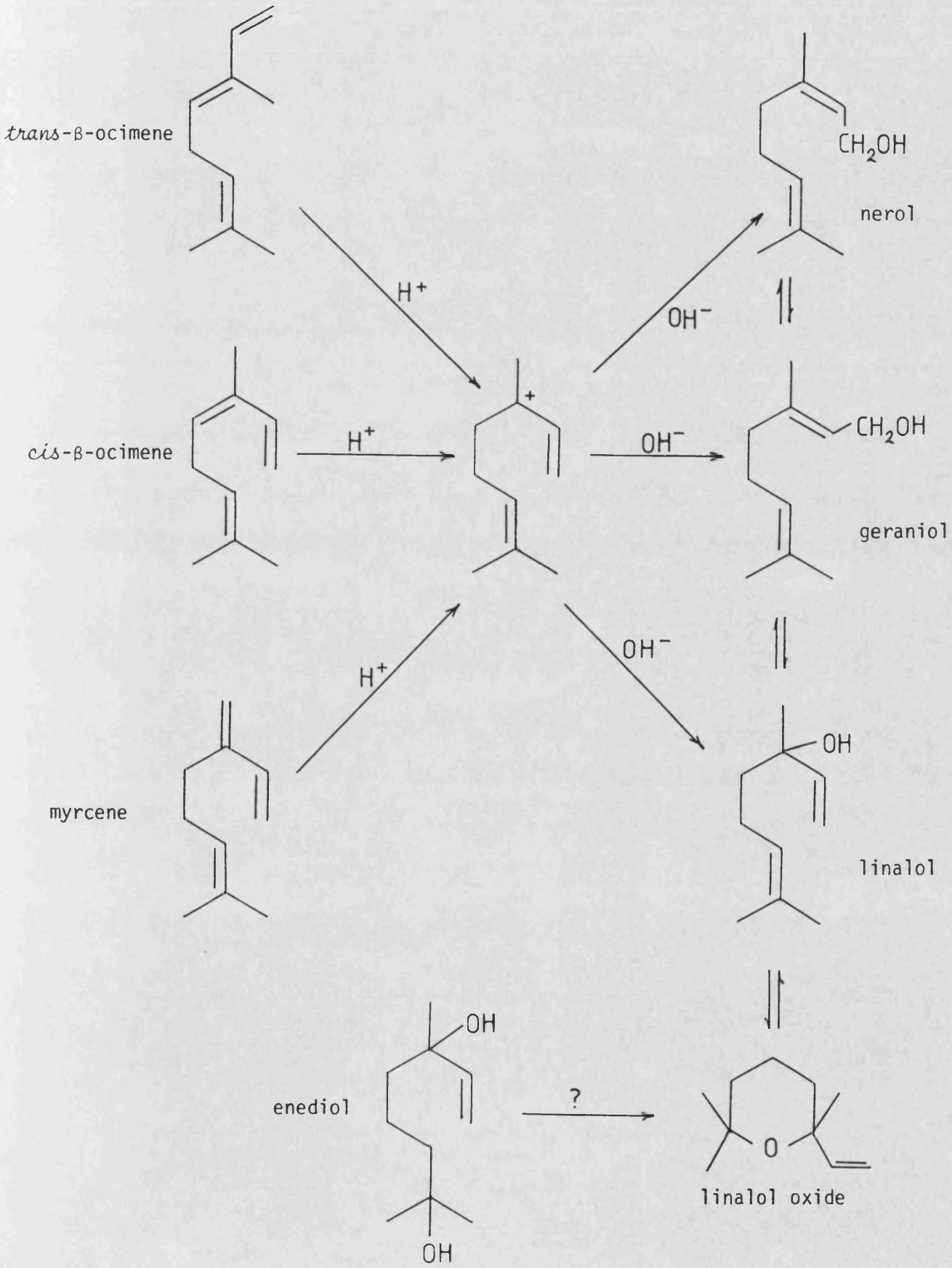


Figure XXXX Acid catalysed hydration of acyclic monoterpenes

appeared to show random changes although an overall increase in terpene esters was observed on distillation.

The sesquiterpene fraction of blackcurrant fruit volatiles did not show changes at all similar to those observed during the distillation of leaf oils. The concentration of both β-caryophyllene and caryophyllene epoxide was reduced on distillation in contrast to the leaf oils, which a reduction in β-caryophyllene resulted in an increase in caryophyllene epoxide.

Although the level of humulene decreased on vacuum distillation, humulene epoxide-II was not detected. Germacrene-D was not found in solvent extracts or distillates, but Δ-cadinene and alloaromadrene were found in distillates indicating that these are rearrangement products of caryophyllene or humulene. α-Nerolidol was only found in 80°C distillates.

These results are consistent with many of the observations of von Sydow and Karlsson (26), who found that of the monoterpenes identified α-thujene, α-pinene, β-pinene, myrcene, Δ^3-carene, limonene, β-phellandrene, *cis* and *trans*-β-ocimene decreased on heating, while α-phellandrene, α-terpinene and γ-terpinene increased. The only differences noted in our study were that α-phellandrene decreased on heating instead of increasing, and *vice versa* for β-phellandrene, and a much larger increase was noted in the concentration of γ-terpinene and terpinolene. This is undoubtedly due to the presence of high levels of sabinene which was absent in

the blackcurrant cultivar studied by von Sydow and Karlsson.

Our results agree with those of von Sydow who noted increases in all the monoterpene alcohols except β-citronellol (not detected in our study using S.I.M. at *m/z*93) and the appearance of linalool oxide when the fruit was heated. Again we also note slight increases in the level of monoterpene esters on heating.

Decreases in caryophyllene and humulene noted in our study were also observed by von Sydow and Karlsson, accompanied by increases in Δ-cadinene and γ-elemene on heating.

These changes which occur on vacuum or 80°C distillation may account for some of the anomalies in earlier work carried out on blackcurrant fruit distillates. The work carried out by Andersson and von Sydow (12, 13) described high levels of terpinen-4-ol, terpinolene and other terpene rearrangement products. In both of these studies the aroma was either subjected to steam distillation (12) or distillation at approximately 48°C (13).

The later study of volatile constituents of a commercial blackcurrant distillate and volatiles isolated from fresh fruit carried out by Nursten and Williams (23, 24) used predominately Baldwin blackcurrants. Our work using low temperature solvent extracts of the fruit of this cultivar has shown that sabinene is the major monoterpene present. However, this compound was not found in either of the earlier studies, but high levels of terpinen-4-ol and γ-terpinene were found. In

both of these studies the aroma was isolated by vacuum distillation.

A recent study of blackcurrant concentrate headspace volatiles (50) only identified two terpene compounds, terpinen-4-ol and α-terpinene, probably again due to the effect of the concentration temperature.

It was concluded from our work that low temperature solvent extraction resulted in a representative aroma isolate, and when single ion mass spectrometry was employed this could be analysed without further concentration.

Figures XXIII and XXIV show the advantage of single ion monitoring to study the terpene fraction of complex aroma extracts.

Varietal differences in the terpene fraction of blackcurrant fruit volatiles

The only previous study of varietal differences in blackcurrant fruit volatiles was carried out by Andersson and von Sydow in 1966 (15). Since then a number of workers (35, 43, 45, 53) have studied the characteristic chemical features in the bud oil of some cultivars, traced in their hybrids.

A key to established blackcurrant cultivars grown in the early 1960's was constructed by Todd (172), based on morphological observations, and this key has been confirmed by the studies carried out on the composition of blackcurrant bud oil.

The three varieties examined in this work, Baldwin, Ben Lomond and Wellington XXX, are the three most commonly grown varieties in England at the present time. Analysis of low temperature solvent extracts of ripe fruit by single ion mass spectrometry, showed the major difference to be the relative concentrations of Δ^3-carene and sabinene (Table VII).

In Baldwin and Ben Lomond fruit volatiles, the levels of sabinene are 1867 and 1450µg/kg respectively whereas in Wellington XXX the level is only 28µg/kg. In contrast the concentration of Δ^3-carene in Wellington XXX is 1454µg/kg and in Baldwin and Ben Lomond 666 and 649µg/kg respectively.

Terpene compounds associated with sabinene are also significantly decreased in Wellington XXX volatiles as shown in the following table.

Compound	Ben Lomond	Baldwin	Wellington XXX
α-thujene	49.6	36.9	5.8
sabinene	1450.0	1866.7	27.5
γ-terpinene	39.7	37.9	12.8
terpinolene	2.0	2.0	n/d
terpinen-4-ol	40.2	45.8	18.2
trans-sabinene hydrate	46.0	45.5	7.6

Similarly α-terpinene which may arise from opening of the cyclopropane ring of Δ^3-carene, increases in parallel with Δ^3-carene in Wellington XXX fruit volatiles.

These observations are in total contrast with the results obtained with blackcurrant buds. Latrasse and Lantin (35) found that Baldwin and Wellington XXX both contained 55-60% sabinene, 19-21% Δ^3-carene and 10% terpinolene and these results were confirmed in later work by Latrasse and Lantin (43, 45) and Kerslake (53).

However, our results are in agreement with those of Andersson and von Sydow (15) who found almost exactly the same pattern of terpenes in Wellington XXX as was found in this study, although it is possible that any sabinene present may have undergone acid catalysed hydration to terpinen-4-ol, γ-terpinene and terpinolene since somewhat higher levels of these compounds were found in

Wellington XXX compared to Brödtorp. The aroma concentrate had unfortunately been subjected to steam distillation.

Latrasse and Lantin (43) have proposed that the pattern of monoterpenes: sabinene,Δ^3-carene, limonene, β-phellandrene and terpinolene resulting in six distinct phenotypes, can be explained by the segregation of three major genes which they designated: T_1 for sabinene; T_2 for the Δ^3-carene - terpinolene pair and T_3 for the pair limonene - β-phellandrene (and maybe β-pinene).

However, the existence of the pairs: limonene - β-phellandrene and Δ^3-carene - terpinolene, is not evident from our analysis, and it is concluded that these compounds are controlled by individual genes rather than being paired to one gene, expression of the genes being controlled by physiological or biochemical factors. This would account for the difference in terpene composition of Wellington XXX fruit and buds or leaves.

The concentration of other bicyclic monoterpenes and their derivatives in the three cultivars studied shows a random occurrence, as shown in the following table.

Compound	Ben Lomond	Baldwin	Wellington XXX
α-pinene	36.9	42.2	37.6
β-pinene	46.3	59.8	20.9
camphene	2.2	2.2	9.9
fenchyl acetate	46.0	42.0	42.0
bornyl acetate	42.0	25.2	38.1

The distribution of acyclic monoterpenes and their derivatives also shows no conclusive pattern.

Compound	Ben Lomond	Baldwin	Wellington XXX
myrcene	48.9	72.9	50.4
cis-β-ocimene	97.2	141.6	79.2
trans-β-ocimene	58.3	64.9	52.8
allo ocimene	2.0	n/d	4.2
geraniol	92.4	96.2	95.7
linalol	44.2	59.6	15.6
neryl acetate	n/d	4.0	n/d
geranyl acetate	n/d	4.0	n/d

The monocyclic terpenes and their derivatives which have not already been discussed show an interesting contrast with bud or leaf oil.

Compound	Ben Lomond	Baldwin	Wellington XXX
α-phellandrene	29.3	28.5	53.2
d-(+)-limonene	174.3	205.3	188.4
cis-β-terpineol	11.1	18.0	21.8
trans-β-terpineol	11.1	13.5	16.8
α-terpineol	211.3	237.3	212.8
trans-piperitol	21.0	7.5	10.5
α-terpinyl acetate	n/d	8.1	4.2

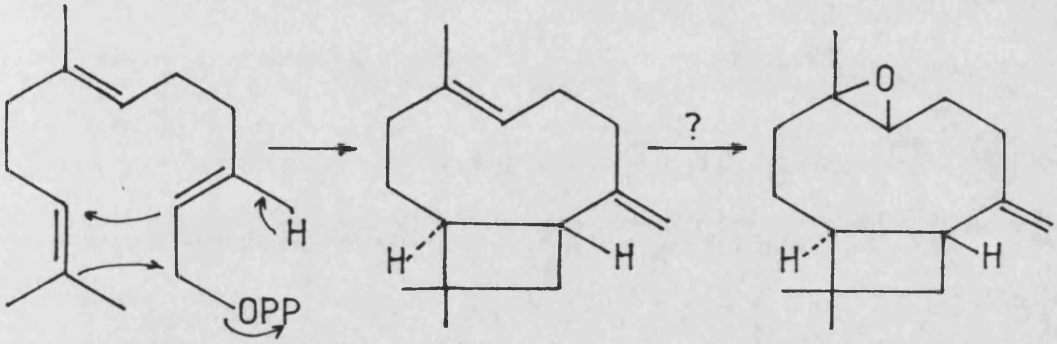
β-Phellandrene appears to be totally absent in blackcurrant fruit volatiles, and replaced by α-phellandrene. Since the concentration of limonene is essentially the same in all cultivars studied, this would seem to invalidate Latrasse and Lantin's proposal that limonene and β-phellandrene are paired on the same gene.

As a general rule we have found that the total level of monoterpene alcohols is much higher in fruit aroma extracts relative to the monoterpene olefins than in blackcurrant bud or leaf oil, and this is clearly demonstrated by the high concentration of α-terpineol in all three cultivars.

This observation raises a number of biosynthetic questions. Do α-terpineol and other terpene alcohols arise from hydroxylation of their corresponding olefins, or are the alcohols and olefins formed from a common ionic intermediate? Alternatively could the dienes be derived from the alcohol by dehydration? These options will be discussed in consideration with the results of the maturation and labelled mevalonate studies.

The pattern of sesquiterpenes found in the fruit volatiles is not in agreement with those reported by Latrasse and Lantin (43, 45). In both Baldwin and Wellington XXX cultivars, Latrasse and Lantin found α-humulene to be absent in the bud oil, but our work has shown that α-humulene occurs in the fruit volatiles of all three cultivars. α-Humulene was also found in the fruit volatiles isolated by Andersson (15).

The concentration of caryophyllene epoxide appears to be independent of the concentration of β-caryophyllene in all three cultivars studied, and since the biosynthesis of caryophyllene has been shown to involve the direct cyclization of *cis,trans*-farnesylpyrophosphate (173), it is suggested that caryophyllene undergoes secondary epoxidation, although no conclusive evidence can be offered.



cis,-*trans*-farnesyl pyrophosphate

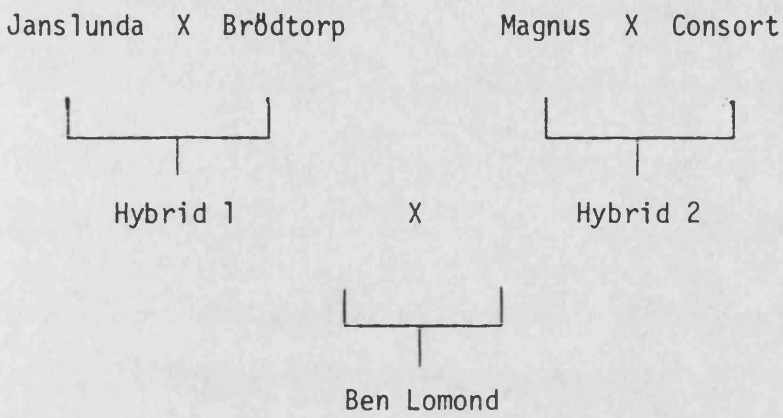
β-caryophyllene

β-caryophyllene epoxide

None of the sesquiterpenes identified in this study show any inter-varietal pattern.

This study has demonstrated that inter-varietal variation in mono and sesquiterpene composition is significantly different in black-currant fruit, buds and leaves, particularly with respect to carane, thujane and *p*-menthane derivatives. It is suggested that the biosynthesis of sabinene, Δ^3-carene, limonene, β-phellandrene and terpinolene are controlled by individual genes, rather than paired genes as suggested by Latrasse and Lantin.

Both Baldwin and Wellington XXX varieties are of indeterminate parentage, but the hybrid Ben Lomond, being a more recent variety, has known lineage and has been derived by the following hybridization (198):



Data on the volatile components of fruit, bud or leaf oils of Janslunda and Magnus varieties has not been published, and only Consort bud oils have been analysed (43). Ben Lomond fruit volatiles, bud and leaf oils contain sabinene as the major terpene, so the influence of Brödtorp is minimal since this variety is devoid of sabinene. The overall profile of Ben Lomond fruit volatiles, bud and leaf oil (since all three are comparable in this variety) is very similar to that reported for Consort by Latrasse and Lantin (43). However, without data on the other two varieties used for the hybridization, the precise influence of each varietal genotype cannot be deduced.

Distribution of terpene compounds in blackcurrant fruit

Compartmentation of terpenoid metabolism is well established (174-177) and this may be an important feature of regulation (178-181). It is now generally assumed that monoterpenes are synthesized within the secretory cells of oil glands (174, 175), and this severely limits *in vivo* studies of terpene biosynthesis.

The only exception to this compartmentation is in flower petals, where a lack of strict compartmentation has resulted in significant incorporation of exogenous mevalonate (182).

In order to investigate compartmentation of monoterpene synthesis and accumulation in blackcurrant fruit, the epidermis, pericarp and seeds were separated from individual berries at -25^{o}C, and the volatiles extracted from the separated portions, using the method previously described.

Combined gas chromatography - mass spectrometry using single ion monitoring gave the results shown in figure XXX and table IX. It is very clear from these results that the majority of terpene compounds are found in the epidermis, particularly the mono and sesquiterpene olefins, and that a relatively high level of terpene alcohols is found in the pericarp. The distribution of terpene groups is found in the following table.

Terpene group	Epidermis		Pericarp		Seeds	
	µg/kg	%	µg/kg	%	µg/kg	%
Monoterpene olefins	2347	33.5	350	8.1	445	33.7
Monoterpene alcohols	1928	27.5	3118	71.7	849	64.4
Monoterpene esters	371	5.3	31	0.7	16	1.2
Sesquiterpene olefins	1316	18.8	539	12.4	9	0.7
Sesquiterpene alcohols	80	11.0	50	1.2	n/d	
Sesquiterpene epoxides	971	13.9	208	4.9	n/d	
Total	7013	100	4346	100	1319	100

The distribution of monoterpene olefins, alcohols and esters indicates that the epidermis is the primary site of terpene synthesis, and the relatively higher concentration of monoterpene alcohols in the pericarp may reflect a separate biosynthetic site.

The concentration of mono and sesquiterpene olefins appears to show parallel distribution in blackcurrant fruit. A number of studies have shown that although mono and sesquiterpenes generally are found together, their biosynthesis shows significant differences.

Bernard-Dagan *et al* (183) have shown that the synthesis of monoterpenes in pine needles is concentrated in the epithelial cells of the resin ducts, whereas sesquiterpenes are synthesized in the whole needle. Similar studies carried out on the biosynthesis of mono and sesquiterpenes in peppermint (173, 184) showed that labelled mevalonate was incorporated into caryophyllene and other

sesquiterpene olefins much more extensively than into monoterpenes, and Croteau and Loomis suggested that the site of sesquiterpene biosynthesis must be more accessible than the site of monoterpene biosynthesis. This uneven distribution is also observed in *Citrus* sp., which often show higher concentrations of sesquiterpenes in the juice oil than in the peel oil, notably the high concentration of valencene in orange juice oil (185 - 188).

These results indicate that in blackcurrant fruit the mono and sesquiterpene olefins may be synthesized at the same site. The relative proportion of caryophyllene : caryophyllene epoxide and humulene : humulene epoxide remains constant throughout the fruit, secondary epoxidation of the olefins as discussed previously may account for this observation.

The presence of relatively high levels of monoterpene alcohols, predominately linalol and α-terpineol in the pericarp and olefins in the epidermis suggests that the site of synthesis of these groups of compounds is different, indicating that for example limonene and α-terpineol are synthesized by different mechanisms rather than α-terpineol being formed by secondary hydroxylation of limonene.

The synthesis of monoterpene olefins appears to be concentrated in cells associated with a high lipid content, such as cuticle wax, presumably allowing the export of terpenes from the site of synthesis. This prerequisite may not be required for terpene alcohols which are appreciably more soluble in aqueous environments

or could be transported from the site of synthesis as glycosides.

Studies on flavour location in Muscat grapes showed that the majority of volatile components are found in the grape-skin and cellular residues of the pulp. Geraniol and nerol were formed predominately in the grape-skin whereas linalol was found in the pulp and juice and to a lesser extent in the skins (189 - 192). The distribution of linalol in grapes is similar to the distribution we have found in blackcurrants.

High concentration of flavour components in the cuticle wax of fruit has been demonstrated in a wide variety of fruits including apples (193), Golden Egg Plums (194) and Cranberries (195).

Non-volatile terpene precursors in blackcurrant fruit

Reference has already been made to the possibility of linalool oxide being formed from an enediol precursor, in the same way that linalool oxide is formed in grapes and grape juice (59).

It has been observed that the levels of some terpene alcohols increase during the processing of blackcurrants (196), and this may be explained either by oxidation of corresponding olefins or by the presence of terpenol glycosides undergoing acid hydrolysis. Since this increase in terpene alcohols is observed even when additional ascorbic acid is added to the freshly pulped fruit, and when the pulp is sparged with nitrogen and held in a nitrogen atmosphere (196), it was concluded that the terpene alcohols either arise from enzymic hydroxylation due to cellular rupture or from the hydrolysis of terpenol glycosides.

Single ion analysis of extracts from glycosidase treated aqueous residue, isolated by the method previously described, indicates that terpenol glycosides may be present. The results of this study are shown in table X, and figure XXXXI.

The presence of both the terpene alcohol and its corresponding olefin may be the result of subsequent dehydration of the alcohol or enzymic hydrolysis of the glycoside either side of the ether linkage (Figure XXXXII). The glycosidase used (Pectinol C) is not a pure enzyme but a complex mixture of polysaccharide hydrolysing enzymes,

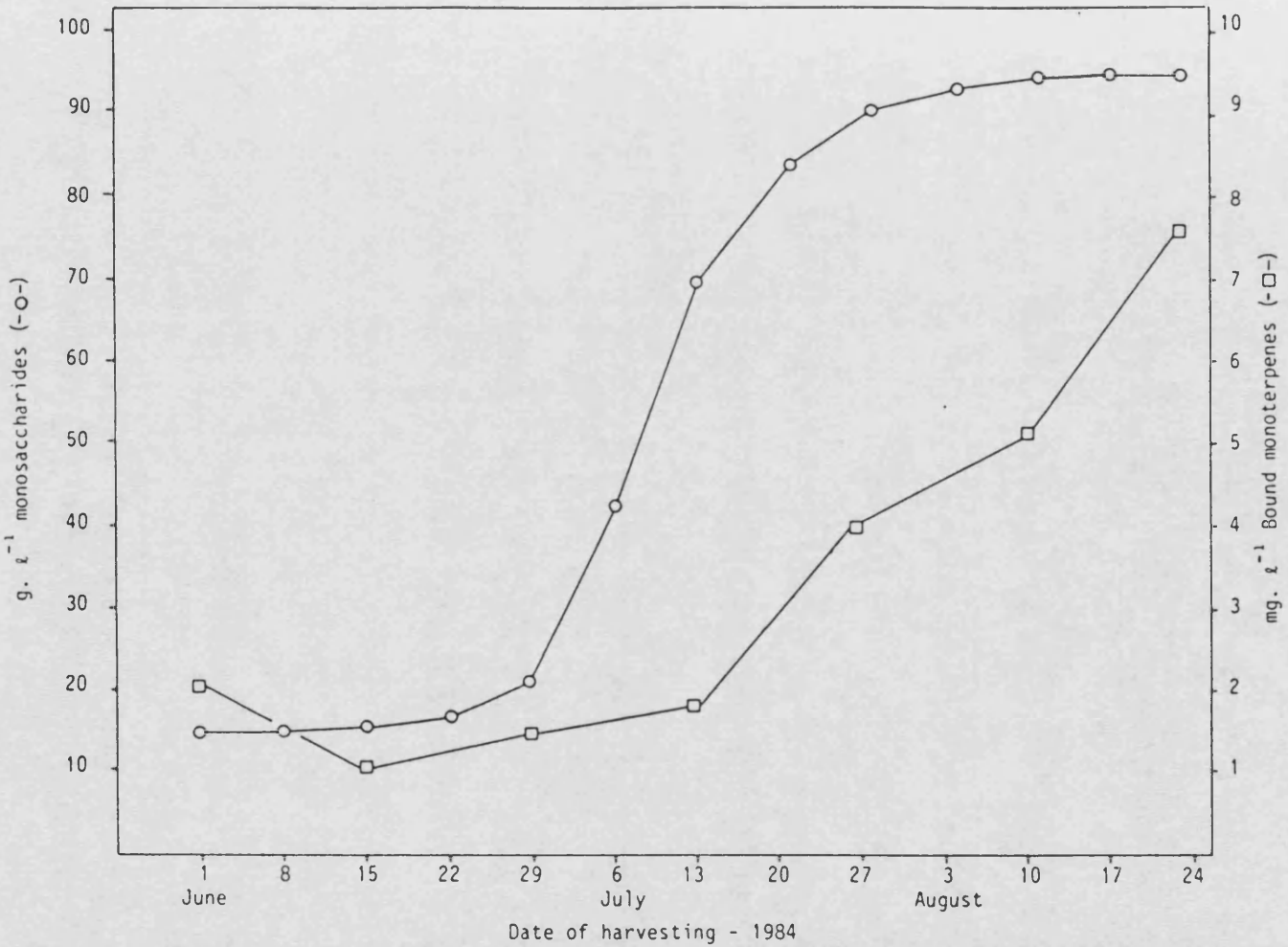


Figure XXXXI. Changes in the concentration of bound monoterpenes during ripening of Wellington blackcurrants.

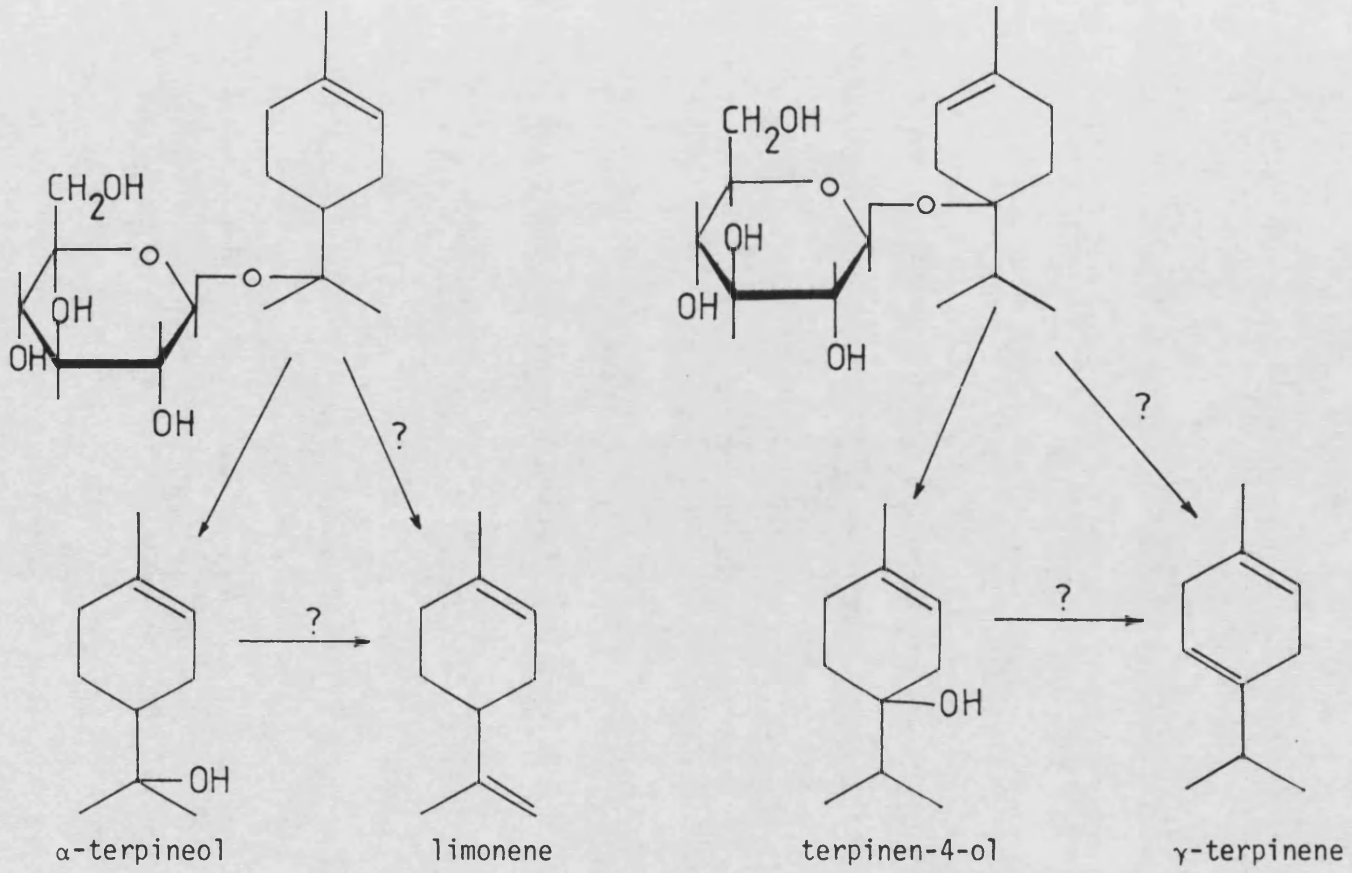


Figure XXXXII Proposed enzymic hydrolysis of terpene glycosides tentatively identified in blackcurrants

thus enzymes may be present which will catalyse hydrolysis of the glycoside either side of the ether linkage.

In recent studies of glycosides in grape (58 - 64) and passion fruit (71), an increase in both terpene alcohols and olefins was observed but only after the fruit or juice had been heated at pH 3.0 or lower.

Since neither enzymic dehydration of terpene alcohols or hydrolysis of glycosides to olefins is really plausible, it must be concluded that the olefins arise from thermal dehydration during incubation of the enzyme and glycoside.

The concentration of bound terpenes initially decreases as ripening progresses, but increases significantly after sugar accumulation begins (Figure XXXXI). A similar pattern of glycoside accumulation was observed by Williams *et al* (63) during the development of Muscat grapes. This increase in glycosides coincides with a decrease in monoterpene olefins and to a lesser extent monoterpene alcohols during ripening of blackcurrants, and the conversion of these compounds to their glycosides is indicated. However, this conversion appears to be specific for monocyclic terpenes.

The presence of only monocyclic terpenol glycosides is unexpected since linalol occurs at the same level as α-terpineol in the blackcurrant pericarp, and is found as its glycoside in other

species (63, 71). Studies carried out on the biosynthesis of menthol and neomenthol glycosides in peppermint leaf have shown that the transglucosylase is not a highly specific enzyme (197), and this is supported by the wide range of terpene glycosides found in Grape (63) and passion fruit (71). The increasing level of monosaccharides in the fruit appears to be linked to the synthesis of the glycosides in blackcurrant fruit (Figure XXXXI) but this may be coincidental, with the synthesis of glycosides being related to other physiological or biochemical changes.

In a separate study (196), glycosides of phenyl ethanol and benzyl alcohol have been tentatively identified by their products of glycosidase treatment.

Changes in terpene compounds during ripening of blackcurrant fruit

Analysis of volatiles from unripe (green), halfripe and full ripe Wellington XXX blackcurrants by single ion monitoring showed major quantitative changes in terpene concentration but few qualitative changes. The results are shown in table VIII and summarised below.

Terpene group	Unripe		Halfripe		Full ripe	
	µg/kg	%	µg/kg	%	µg/kg	%
Monoterpene olefins	5414.5	63.7	4166.9	67.8	2035.0	60.5
" alcohols	491.5	5.8	442.4	7.2	399.0	11.8
" esters	217.6	2.6	129.9	2.1	84.3	2.5
Sesquiterpene olefins	707.9	8.3	613.4	9.9	195.4	5.8
" epoxides	1671.2	19.6	793.8	13.0	647.6	19.4
Total	8502.7	100	6146.4	100	3361.3	100

The only previously reported study of changes in blackcurrant volatiles during ripening was that of Andersson and von Sydow (15). Their results showed the overall level of essential oil increased during ripening, with significant increases in mono and sesquiterpenes (Δ^3-carene, terpinolene and caryophyllene) decreases in monoterpene alcohols (terpinen-4-ol) and no change in terpene esters (citronnellyl acetate).

These contrasting results may be explained in a number of ways. The results of Andersson and von Sydow's work were based on

distilled volatiles, and this would have resulted in hydrolysis of any terpene glycosides. This study has shown that the concentration of glycosides increases as ripening progresses, and if the concentration of bound and free terpenes is summed then the overall level of terpenes increases slightly during ripening. The method of isolation employed by Andersson and von Sydow may also account for the rise in monoterpene olefins, resulting from acid catalysed dehydration of the corresponding alcohol, as was observed during the isolation of passion fruit volatiles (71).

The effects of growing locality and climate have been suggested as contributing to differences in the composition of blackcurrant volatiles (15, 24, 25) and work carried out on Brödtorp blackcurrants has confirmed that growing locality does produce quantitative differences in aroma volatiles (15).

Studies carried out on a wide variety of fruits have shown that generally the concentration of volatiles increases during ripening. During ripening the metabolism of the fruit changes to predominately catabolic pathways, decreasing the amounts of acids, poly- and oligosaccharides, leading to the formation of volatiles, and other changes.

Studies carried out on apples (199), pears (200, 201) and bananas (202) have demonstrated that the formation of volatiles is initiated by the climacteric rise in respiration, and reaches a maximum in the post-climacteric ripening phase. The dynamic nature of the

biogenesis of volatiles was demonstrated by the cyclic variation in the rate of synthesis of α-farnesene, butyl and hexyl acetate in ripening pears (201).

Analysis of volatiles isolated at different stages of ripening has shown that the concentration of volatiles increases during ripening of pears (200, 201), apples (199, 203), bananas (202, 204 - 207), papaya (208), cantaloupes (209), Arctic bramble (210), strawberries (211 - 214) and mangoes (215). All these fruits (except strawberries and Arctic bramble) show the climacteric pattern of evolution, and the increase in volatiles is predominantly due to increased synthesis of aliphatic esters.

Non-climacteric fruits such as cherries, citrus and grapes do not show the same respiratory behaviour, or the same changes in volatile compounds during ripening.

Recent studies of the changes in free and bound terpenes in Muscat grapes (63), has shown that there is an initial drop in free and bound terpenes reaching the lowest concentration at veraison, followed by a rapid increase in the level of glycosidically bound monoterpenes, particularly linalol, geraniol and nerol, and the level of free terpenes particularly linalol and trans-pyran oxide.

The increase in glycosidically bound terpenes coincided with monosaccharide accumulation as was observed in blackcurrants.

In another recent study of the changes in the volatile constituents of Rabbiteye blueberries during ripening (216), the level of terpene olefins and alcohols was found to decrease during ripening, with an increase in terpene esters at full ripeness. However these changes varied depending on the variety of Rabbiteye blueberries studied. No data regarding corresponding changes in sugar concentration or pH was given. Since the volatiles were isolated by steam distillation, the results obtained probably represent total terpene concentration, since any terpene glycosides would have been hydrolysed.

The changes in terpene composition of volatiles during ripening of blackcurrants which has been carried out in this study, and results reported by other workers, indicate that changes in the composition of the volatiles during ripening are a characteristic of the species and even of varieties of that species.

This study was carried out to investigate whether monoterpene olefins and alcohols arise independently from the acyclic precursor, or whether they are formed by sequential modification of a single intermediate.

From this study no association between related compounds, such as α-terpineol and limonene, can be deduced. The unrelated changes in concentration of related olefins and alcohols may support independent synthesis, but from these results this is purely speculative, and for this reason biosynthetic studies using ^{13}C labelled mevalonate were carried out.

Biosynthesis of terpenes from /2-^{13}C/ mevalonate

Since the highest level of terpene compounds was found in unripe fruit, initial studies were carried out using fruit at this stage of maturity. The results of this work are shown in Table XI.

Maximum /2-^{13}C/ mevalonate incorporation was reached after six days, but even this only represented incorporation of 0.41% of the applied label. Such low incorporation of mevalonate has been observed in almost all studies using this precursor (174 - 177), and is generally thought to be due to poor uptake of precursor, competition for precursor by other biosynthetic or degradative pathways and, most significantly, to compartmentation of monoterpene biosynthesis in sites not readily accessible to exogenous precursors (217).

It can be seen from the results of this study (Table XI), that incorporation of labelled mevalonate into the terpene fragment derived from isopentenyl pyrophosphate (IPP) proceeds more rapidly than incorporation in the fragment derived from dimethylallyl pyrophosphate (DMAPP) (see Figure VI). This phenomenon has been observed in a number of investigations using a variety of labelled precursors and administration techniques including cell free extracts (219 - 223) and is attributed to the condensation of dimethylallyl pyrophosphate from an endogenous metabolic pool, with isopentenyl pyrophosphate derived from the exogenous labelled precursor (174).

The only exception to this general observation is the incorporation of labelled mevalonate into flower petals, where equivalent labelling of both the isopentenyl pyrophosphate and dimethylallyl pyrophosphate units was found (218). The participation of endogenous pools and the compartmentation of biosynthetic sites may thus be related phenomena.

The unequal labelling of terpenes in blackcurrant fruit is further support for compartmentation of monoterpene synthesis, probably located in the blackcurrant fruit epidermis.

The essentially equal rate of incorporation of labelled mevalonate into all the terpenes found to be labelled is indicative of independent synthesis of the terpenes from a common precursor, since sequential modification would have resulted in sequential appearance of the precursor and product. The incorporation of labelled mevalonate into α-terpineol and limonene, for example, is identical both in rate and concentration.

The first scheme to be proposed for the formation of cyclic monoterpenes was that of Ruzicka *et al* in 1953 (224). According to this hypothesis, the neryl cation precursor, now thought to be the corresponding prenyl pyrophosphate (175), undergoes cyclization to the monocyclic α-terpinyl cation subsequently giving rise to the parent cations of the various structural types, via a series of internal additions, hydride shifts and rearrangements.

Termination of these ionic reactions is considered to proceed either by addition of a nucleophile such as OH^- from water to yield the corresponding alcohol or by elimination of a proton to form the corresponding olefin. Although the limitations of the scheme have been described (225), and modifications have been suggested (175, 227, 228), it is still considered a useful working hypothesis for the biosynthesis of monoterpenes.

Alternative schemes based on radical mechanisms have generated little practical or theoretical support (177, 226).

The labeled terpenes identified in this study, with the exception of linalol, can be formed within the framework proposed by Ruzicka *et al.*

Linalol may arise directly from geranyl pyrophosphate, and although such an enzymic transformation has not been demonstrated, the non-enzymic rearrangement of geranyl pyrophosphate to linalol has ample support (229 - 232). The possibility of linaloyl pyrophosphate as a precursor, not only for linalol, but also cyclic monoterpenes, has been investigated (233 - 237). Conclusive evidence for the role of this precursor has not been forthcoming. At present the formation of linalol from geranyl pyrophosphate is considered the most likely.

The biosynthesis of sabinene has been extensively investigated in *Tanacetum vulgare* (238 - 242) by Banthorpe and co-workers and in *Citrus limonium* by Cori and associates (243). Banthorpe and

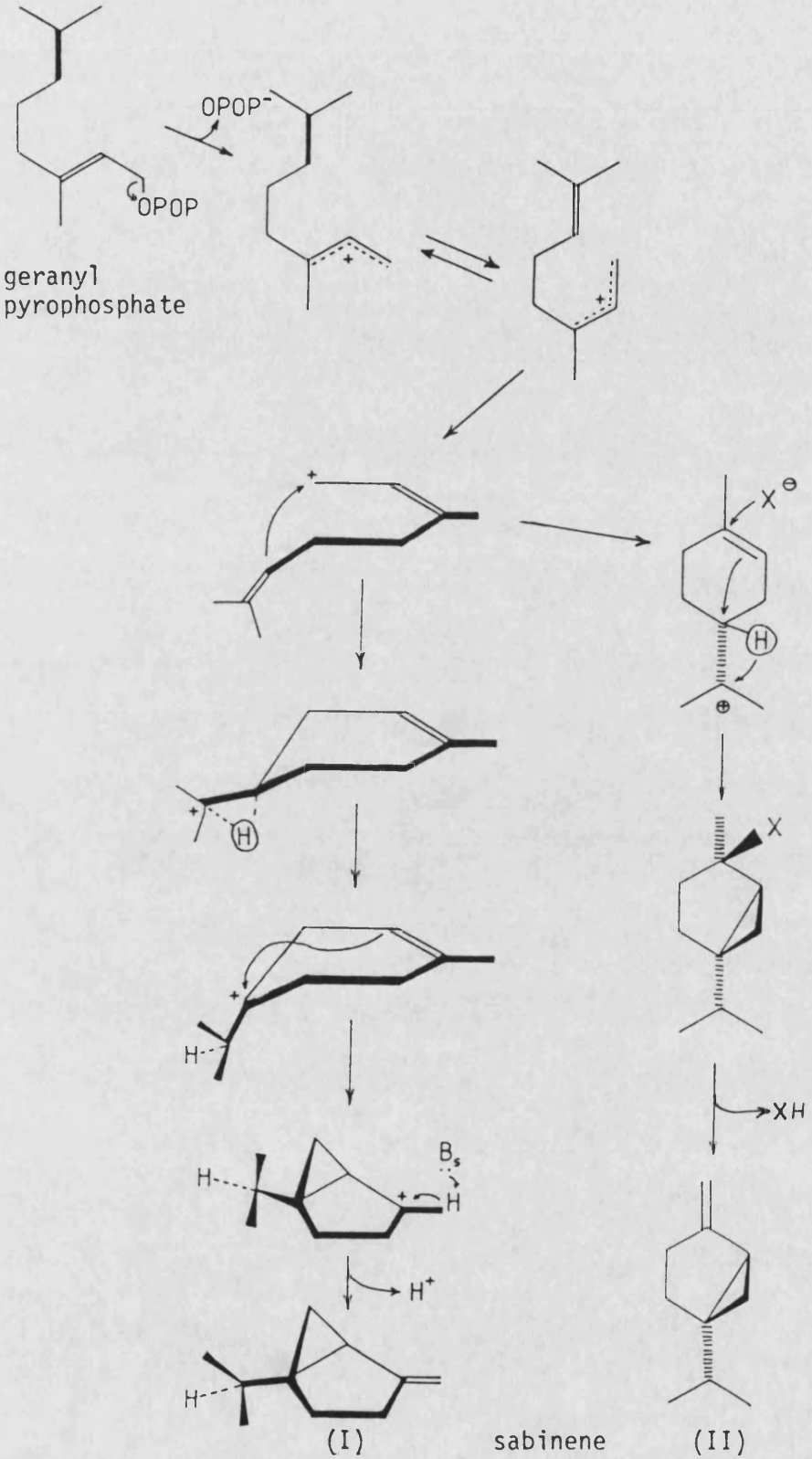


Figure XXXXIII. Proposed biosynthetic pathways to sabinene.
From (I) Cori *et al* and (II) Banthorpe *et al* (242)

co-workers have proposed a pathway for the biosynthesis of sabinene and related thujane monoterpenes directly from the α-terpinyl ion, whereas Cori and co-workers proposed an alternative route via both α-terpinyl and terpin-4-yl ions. However both schemes show a number of similarities (Figure XXXXIII), and since the species on which the studies was carried out are completely different, both pathways are probable.

The biosynthesis of Δ^3-carene has been studied in *Pinus* species using /2-^{14}C/ mevalonate (244, 245) and degradation of (+)-car-3-ene derived from this precursor showed the label at C-4 rather than C-2. If (+)-car-3-ene is derived from the α-terpinyl ion, the formation of the cyclopropane ring must be accompanied by migration of the double bond. Subsequent studies showed that the 2-*pro*-S-hydrogen of mevalonate is lost in the formation of the new double bond, which is accompanied by an unusual 1,2-shift of a proton to the site of the original double bond.

Since the stereochemistry of this *syn* interaction would be difficult to attain, an 'X-group' mechanism was proposed (Figure XXXXIV). It was considered that 'X' was an enzyme with two binding sites (Y and Z) which would accommodate the stereochemical requirements. The biosynthesis of car-3-ene has only been studied in *Pinus* species.

The alternative routes for the biosynthesis of limonene and α-terpineol have already been mentioned. Previous biosynthetic

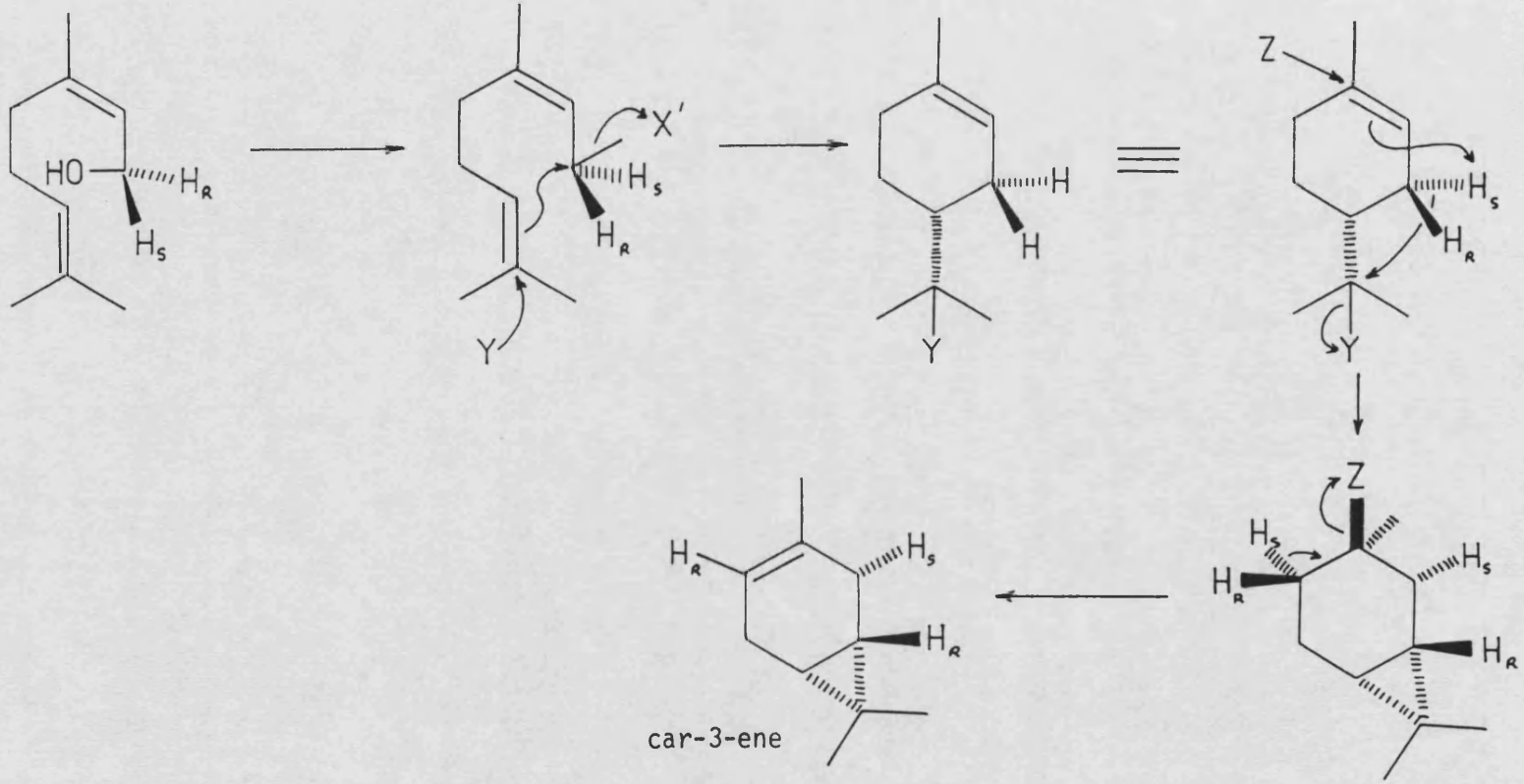


Figure XXXXIV Proposed 'X'-mechanism for the biosynthesis of car-3-ene. After Banthorpe *et al* (245)

studies of these two compounds using /2-^{14}C/ mevalonate are consistent with either independent biosynthesis or sequential modification.

Cell free extracts from peppermint were shown to convert neryl pyrophosphate to α-terpineol as the major cyclic product (246), but the same extracts produced limonene and terpinolene with substrate concentrations of α-terpineol (247) thus opening up the possibility that the dienes may be derived from α-terpineol by dehydration.

However cell free extracts from *Citrus* species (243, 248) produced limonene from neryl pyrophosphate or geranyl pyrophosphate with no α-terpineol detected from either precursor. A soluble enzyme preparation from sage leaves has been shown to catalyse the conversion of neryl and geranyl pyrophosphates to a number of monocyclic terpenes including limonene and α-terpineol (249). Isotopic dilution experiments indicated that α-terpineol, limonene, terpinolene and 1,8-cineole were derived independently, and subsequent studies (250, 251) have chromatographically resolved α-terpineol synthetase, limonene synthetase and 1,8-cineole synthetase activities, thus conclusively proving that distinct synthetases are responsible for the biosynthesis of these monoterpenes. However such independent synthetases have not been demonstrated in any other species as yet.

The parallel increase in α-terpineol and limonene in blackcurrants may be indicative of separate synthetase activities in this species.

γ-Terpinene has been shown by time course studies to be the key intermediate in the biosynthesis of the aromatic compounds *p*-cymene and its hydroxylated derivatives (252). The key enzyme, γ-terpinene synthetase has been isolated from *Thymus vulgaris* leaves (253) and the partially purified enzyme has been shown to convert /1-^{3}H/ geranyl and neryl pyrophosphates to γ-/3-H^{3}/-terpinene with equal efficiency. Attempts to separate the neryl and geranyl activities were unsuccessful which suggested that both were substrates for the same enzyme. The proposed mechanism is shown in figure XXXXV.

The biosynthesis of caryophyllene has already been discussed with reference to previous studies (173).

All of these biosynthetic studies have been carried out using species which have a high concentration of essential oil in specialised oil glands which exhibit strict compartmentation with respect to terpene synthesis. The somewhat higher incorporation of mevalonate in this study compared with that of the species to which reference has been made (0.4% vs. 0.01 - 0.1%) would indicate that the compartmentation in blackcurrant fruit although evident is considerably less rigid, and it cannot be assumed that the biosynthetic pathways previously discussed are necessarily those operating in blackcurrant fruit.

The low level of incorporation, and the natural ^{13}C abundance precluded a more detailed study of the biosynthetic mechanisms.

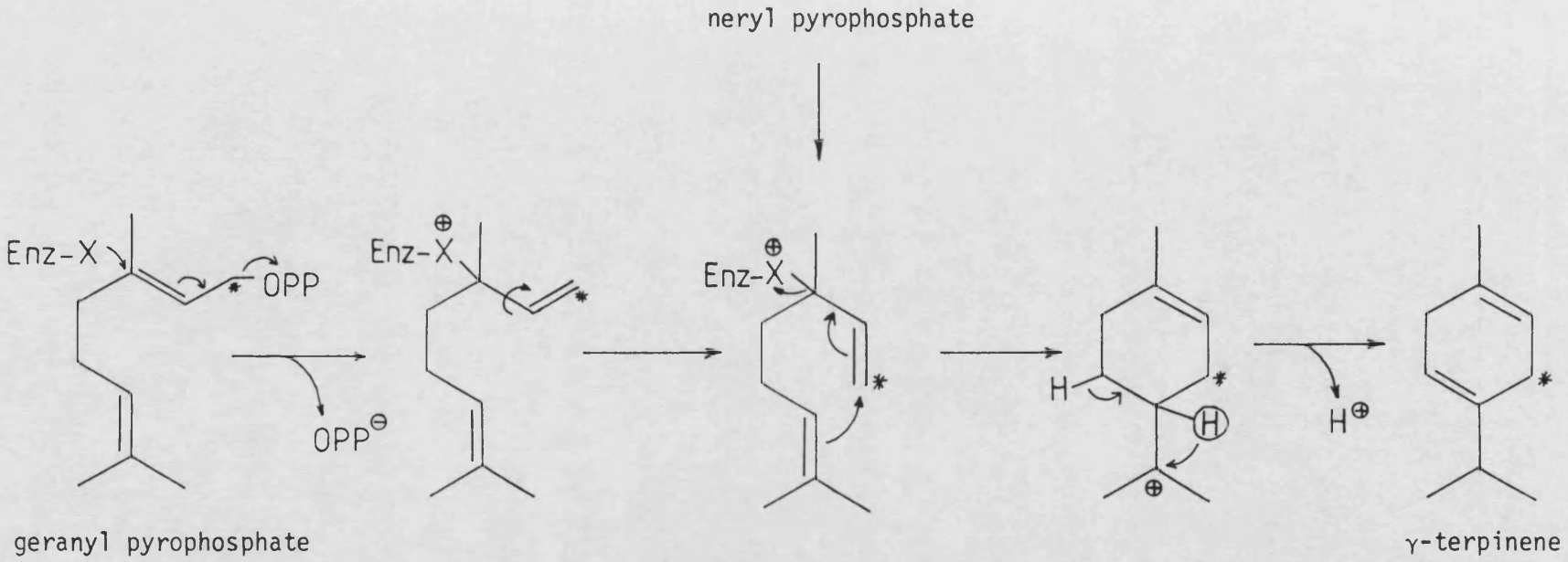


Figure XXXXV Proposed biosynthesis of γ-terpinene from geranyl or neryl pyrophosphate. After Poulose and Croteau (252).

Incorporation of /2-^{13}C/-mevalonate into the terpene fraction of blackcurrant volatiles at various stages of maturity was shown to decrease as the concentration of terpenes present decreased (Table XII). In unripe fruit 1.03% of total terpenes were labelled six days after /2-^{13}C/-mevalonate administration whereas in halfripe and full ripe fruit, only 0.86% and 0.59% respectively of the terpenes were labelled.

The presence of maximal label uptake after six days, indicates that contrary to studies in other species (182, 184, 239, 240), the rate of monoterpene turnover is relatively slow, although the decreasing concentration of terpenes during ripening is evidence of active catabolism during this period. The rate of mevalonate incorporation at later stages of maturity may appear to be lower because of a higher rate of catabolism of both endogenous and labelled terpenes.

Conclusions

Analysis of blackcurrant leaf oils isolated by either steam distillation or low temperature solvent extraction has resulted in the identification of a total of forty four compounds of which twenty seven had not previously been reported in blackcurrant leaves.

The differences observed between oils isolated by the two methods have been attributed to predominantly acid catalysed rearrangmeents, or oxidation of the terpenes, and lipoxidation of polyunsaturated fatty acids. The low temperature solvent extract is considered to reflect more accurately the oil composition of the leaves. Any differences observed between the leaf oils of the three cultivars studied were negligible and only of a quantitative nature.

By using a combination of single ion mass spectrometry and low temperature solvent extraction, it has been possible to analyse the terpene fraction of blackcurrant fruit volatiles without using any concentration or fractionation techniques, which have been shown to produce artefacts in the isolated volatiles.

Analysis of blackcurrant fruit volatiles from Ben Lomond, Baldwin and Wellington XXX has shown major differences between the cultivars with respect to the concentration of terpenes derived from carane, thujane and *p*-menthane, which is not observed in bud or leaf oils.

This has led us to propose that the biosynthesis of the major terpenes in blackcurrant volatiles, is directed by individual genes rather than a limited number of genes which has been proposed previously (43), and expression of these genes is controlled by physiological or biochemical factors.

The distribution of terpenes in blackcurrant berries has been shown to exhibit distinct compartmentation, with the principal site of biosynthesis of monoterpene olefins being located in the epidermis, and the monoterpene alcohols being formed mainly in the pericarp.

The presence of terpenol glycosides has been suggested by studies of glycosidase treatment products, and it is proposed that the terpene alcohols are solubilised by glycosylation in the pericarp. The concentration of bound terpenes has been shown to increase during ripening, and it is suggested that this may be the first stage of terpene catabolism.

Changes in the volatile constituents during ripening are confined to quantitative changes. The total concentration of free terpenes decreases during ripening, although there is a relative increase in terpene alcohols. The changes observed in free and bound terpenes has led us to propose that during ripening synthesis of terpene olefins is switched to terpene alcohols which subsequently undergo glycosylation before being catabolised.

The incorporation of ^{13}C labeled mevalonate, has indicated that there

is an endogenous pool of dimethylallyl pyrophosphate since the monoterpene fragment derived from isopentenyl pyrophosphate is labelled preferentially. The equal rate of label incorporation into structurally related terpenes such as α-terpineol and limonene has led us to propose that such compounds are derived from a common acyclic precursor but involve distinct synthetase activities.

This is the first study to be undertaken using a ^{13}C labelled precursor to attempt to follow monoterpene biosynthesis *in vivo*. Any further detailed investigation into the biosynthesis of terpene compounds using stable isotopes would first require a solution to the low incorporation of precursor into the almost inaccessible sites of terpene biosynthesis.

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Appendix 1

Reagents

Citrate/phosphate buffer pH 5.2

citric acid anhydrous	8.91 g
disodium hydrogen orthophosphate dihydrate	19.08 g
glass distilled water to	1000 cm^3

Nutrient solution

Inorganic salts:

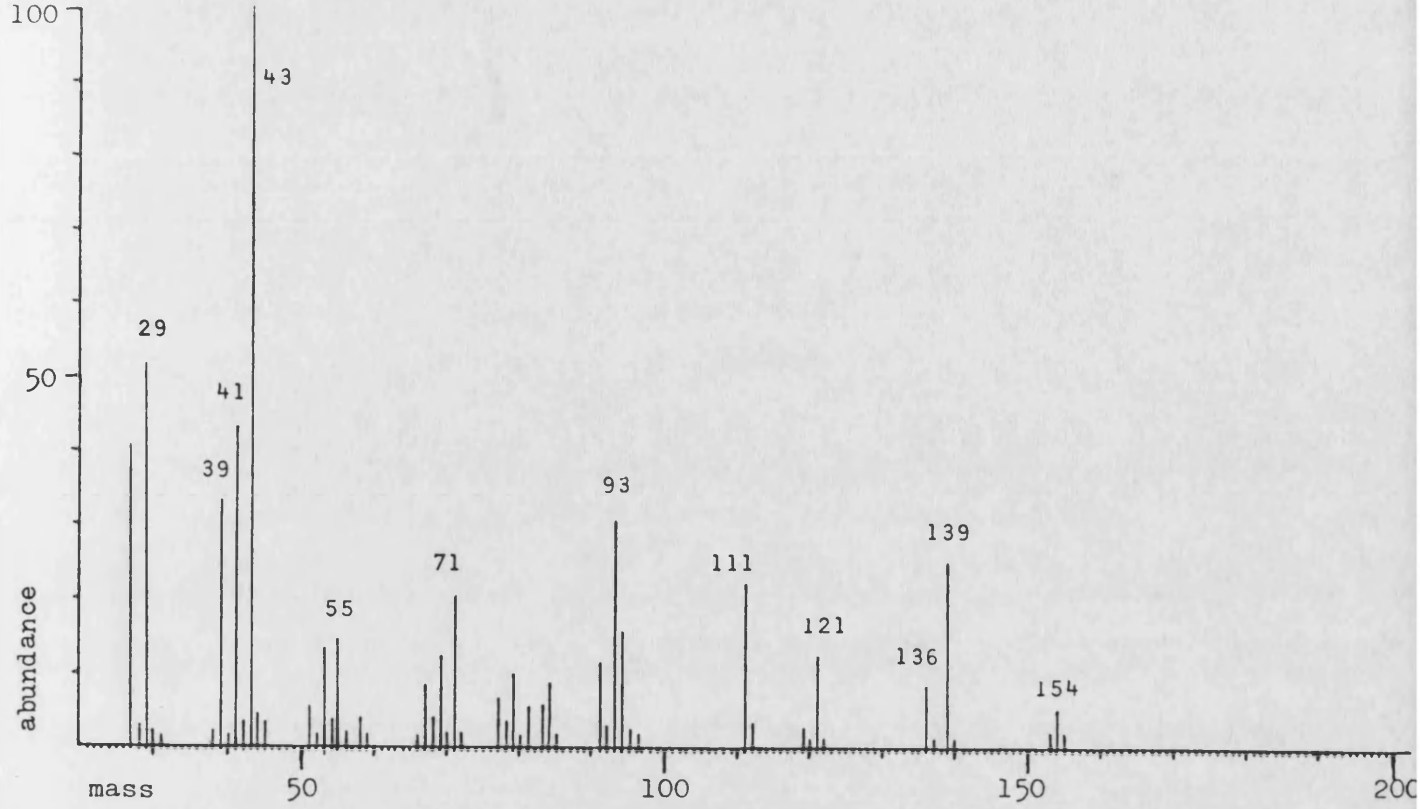
calcium nitrate, $Ca(NO_3)_2.4H_2O$	290 mg
magnesium sulphate, $MgSO_4.7H_2O$	730 mg
potassium chloride, KCl	65 mg
potassium nitrate, KNO_3	80 mg
sodium sulphate, $Na_2SO_4.10H_2O$	450 mg
sodium dihydrogen phosphate, $NaH_2PO_4.2H_2O$	22 mg
boric acid, H_3BO_3	1.5 mg
copper sulphate, $CuSO_4.5H_2O$	0.02 mg
manganous chloride, $MnCl_2.4H_2O$	6.0 mg
molybdic acid, H_2MoO_4	0.0017 mg
potassium iodide, KI	0.75 mg
zinc sulphate, $ZnSO_4$	2.6 mg

Iron source:

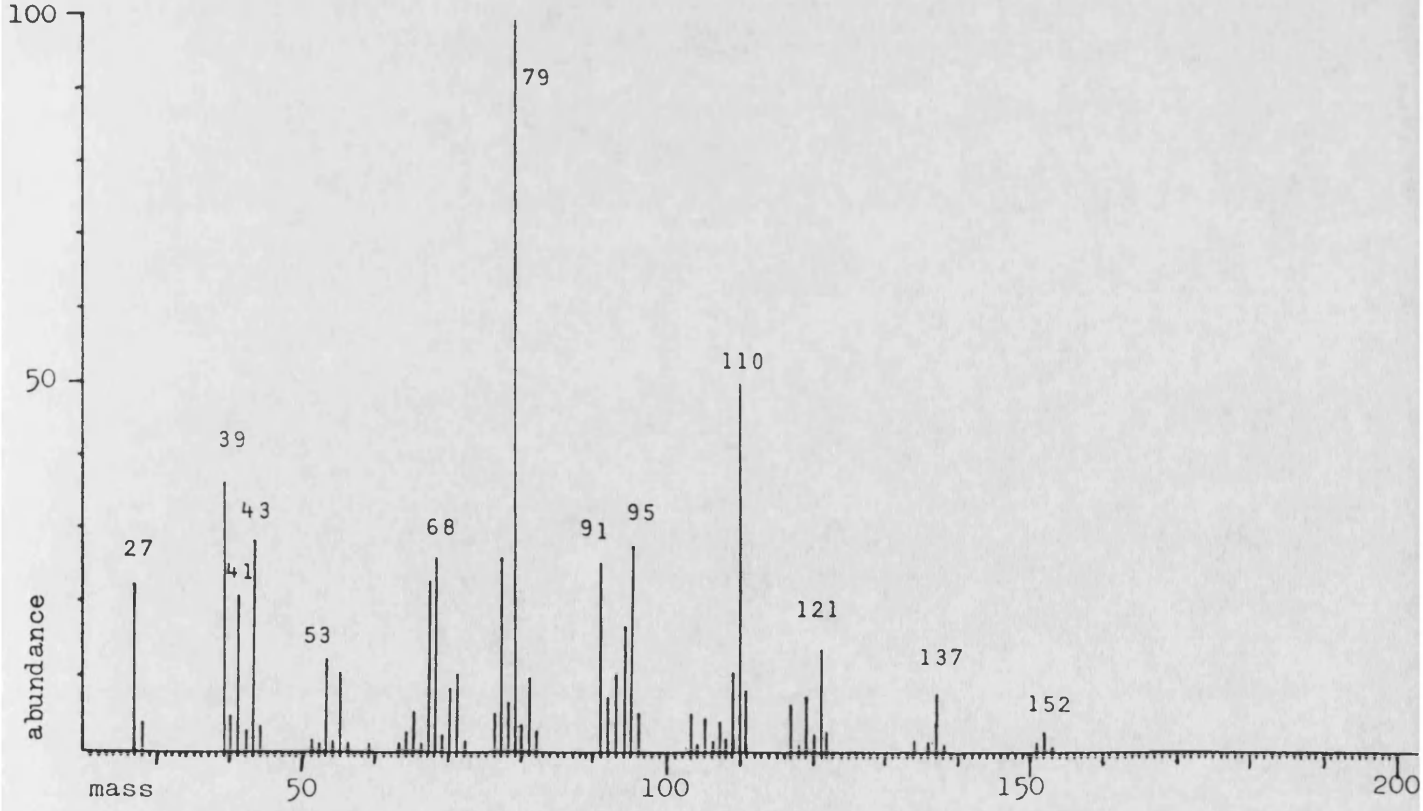
ferric chloride, $FeCl_3.6H_2O$	3.1 mg
sodium ethylenediaminetetra-acetate, EDTA	8.0 mg

Vitamins and other additives:		
aneurine hydrochloride (thiamine HCl)	0.1	mg
pyridoxine hydrochloride	0.1	mg
nicotinic acid	0.5	mg
glycine	3.0	mg
Carbon source:		
glucose	20	g
Glass distilled water to	1000	cm^3

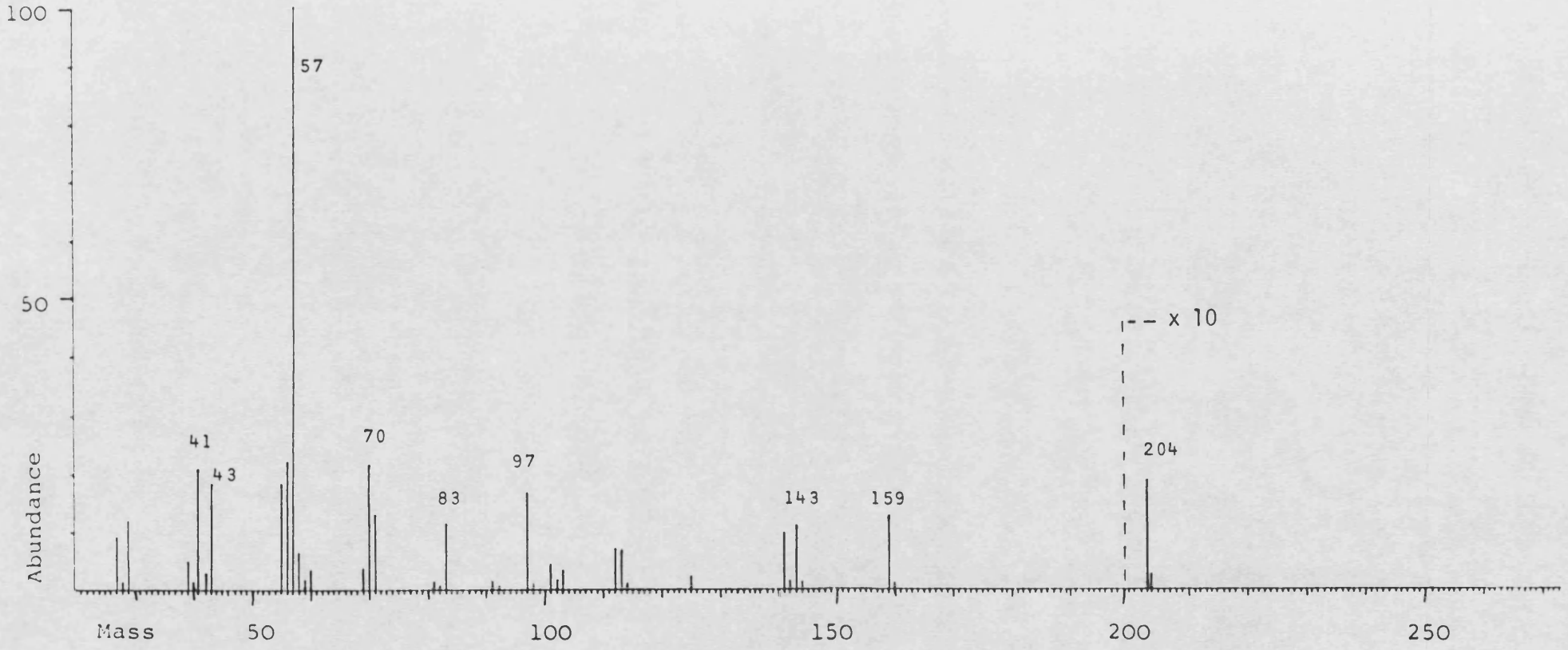
Appendix 2



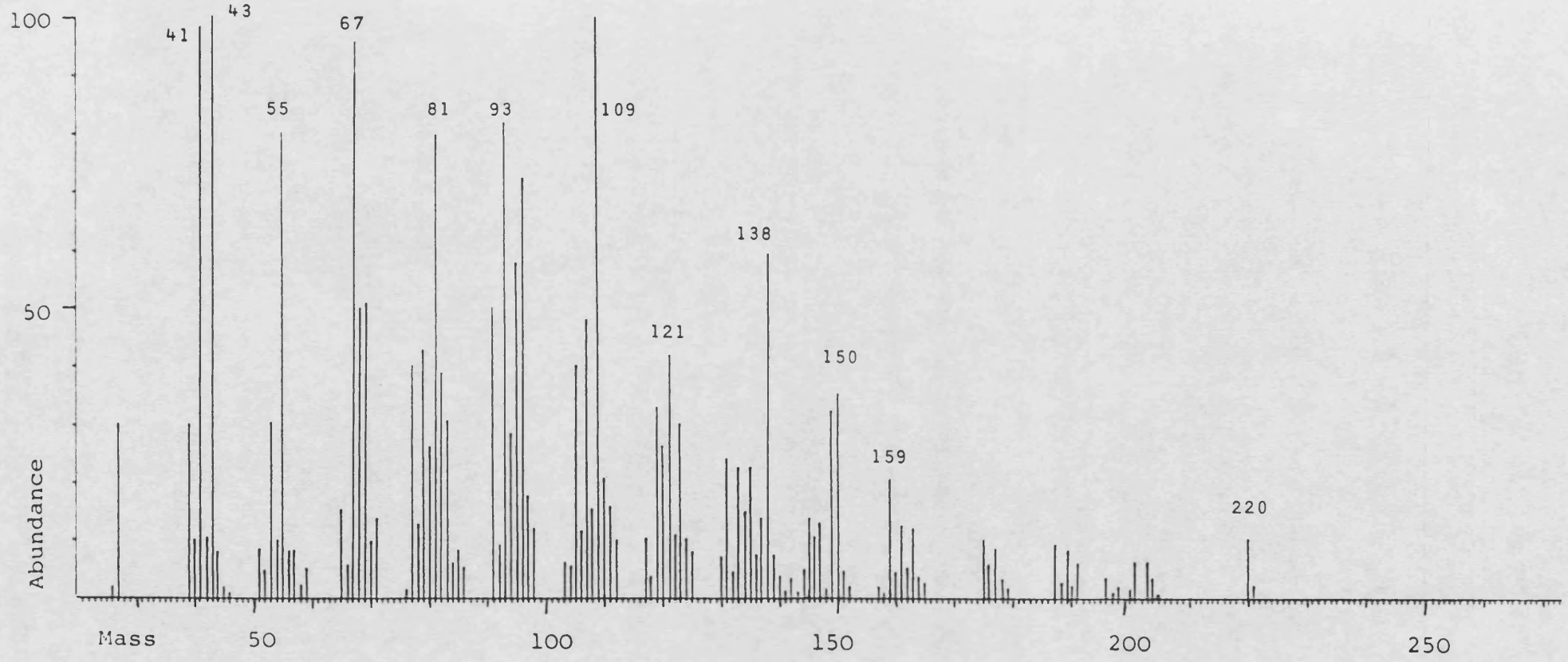
Mass spectrum of unknown compound 1



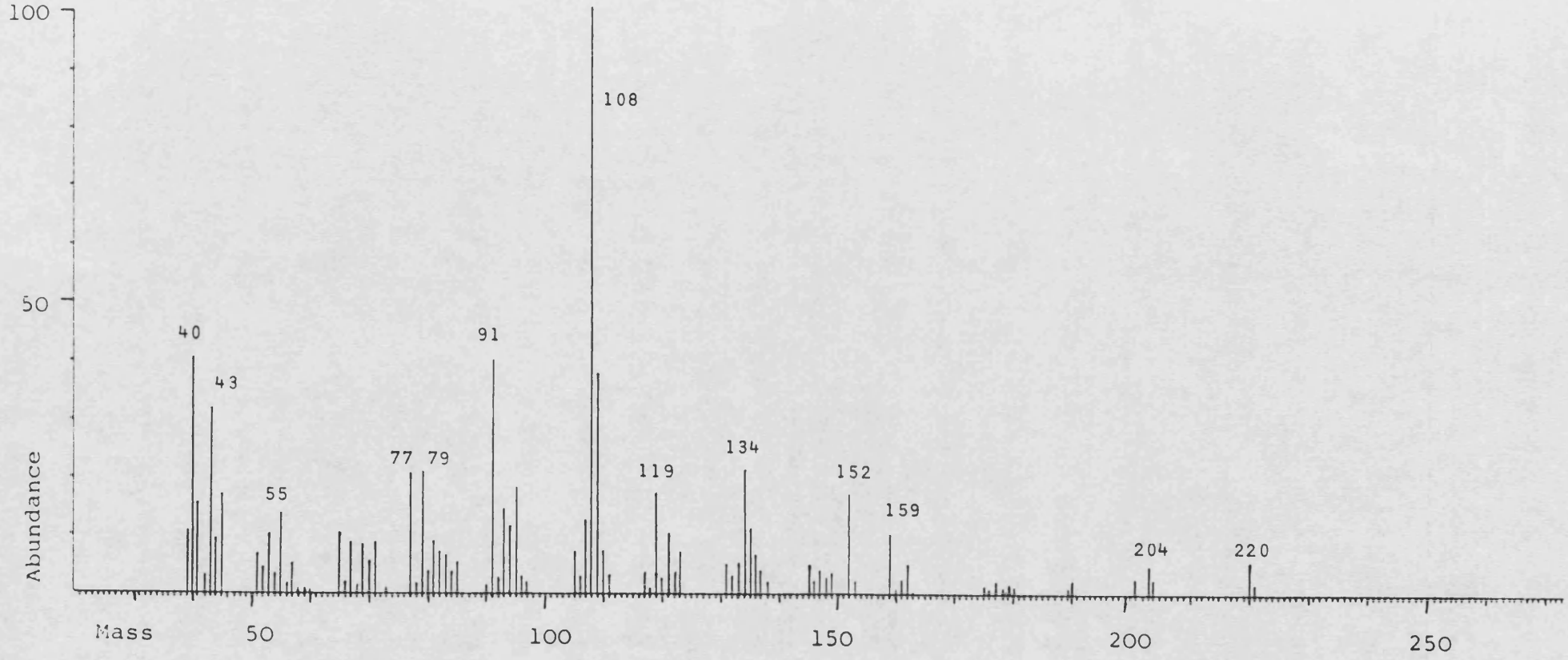
Mass spectrum of unknown compound 2



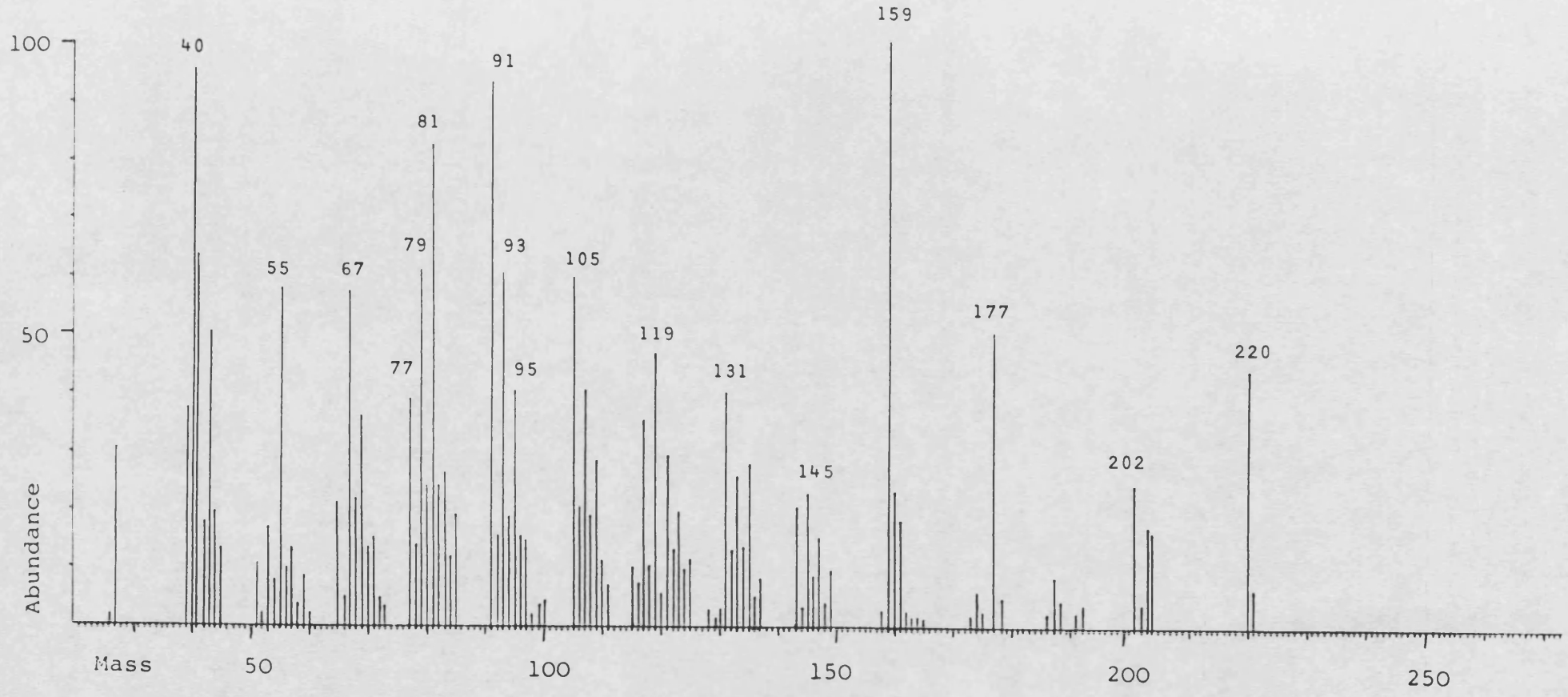
Mass spectrum of unknown compound 3



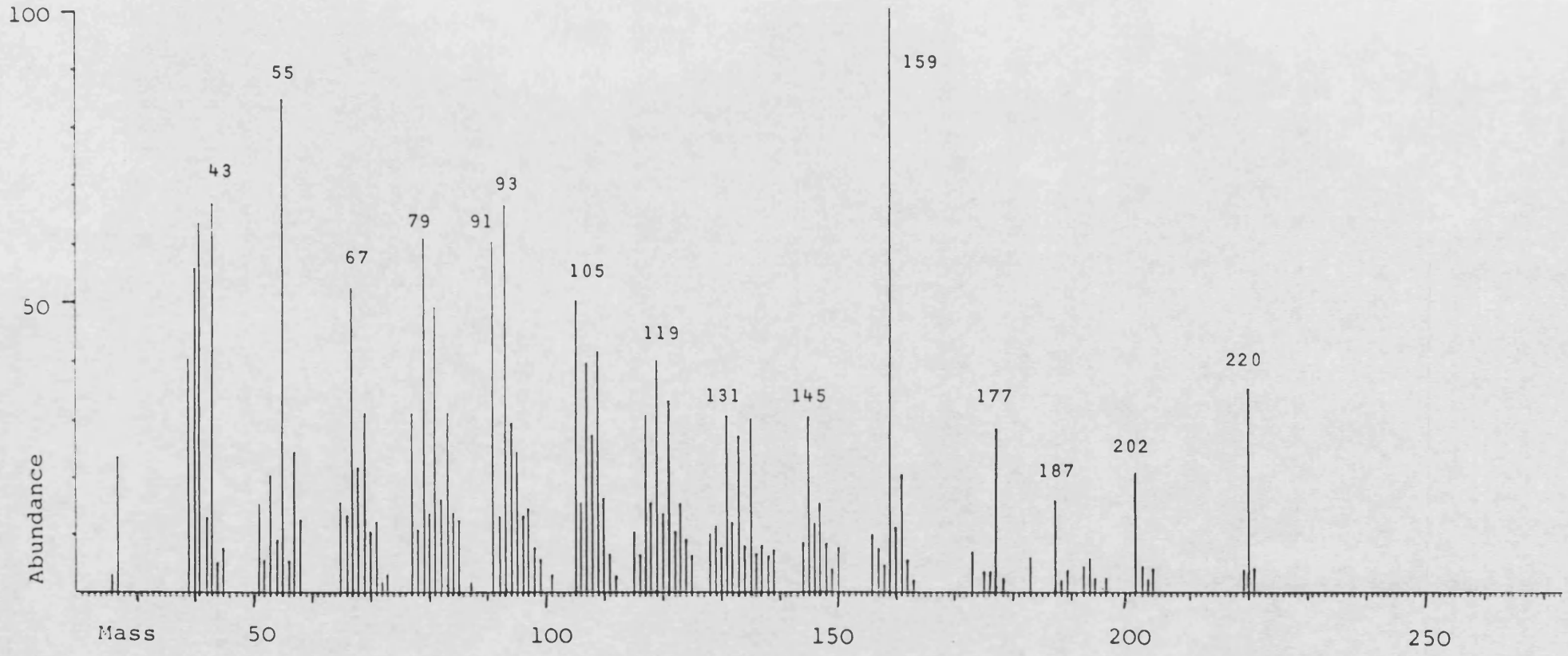
Mass spectrum of unknown compound 4



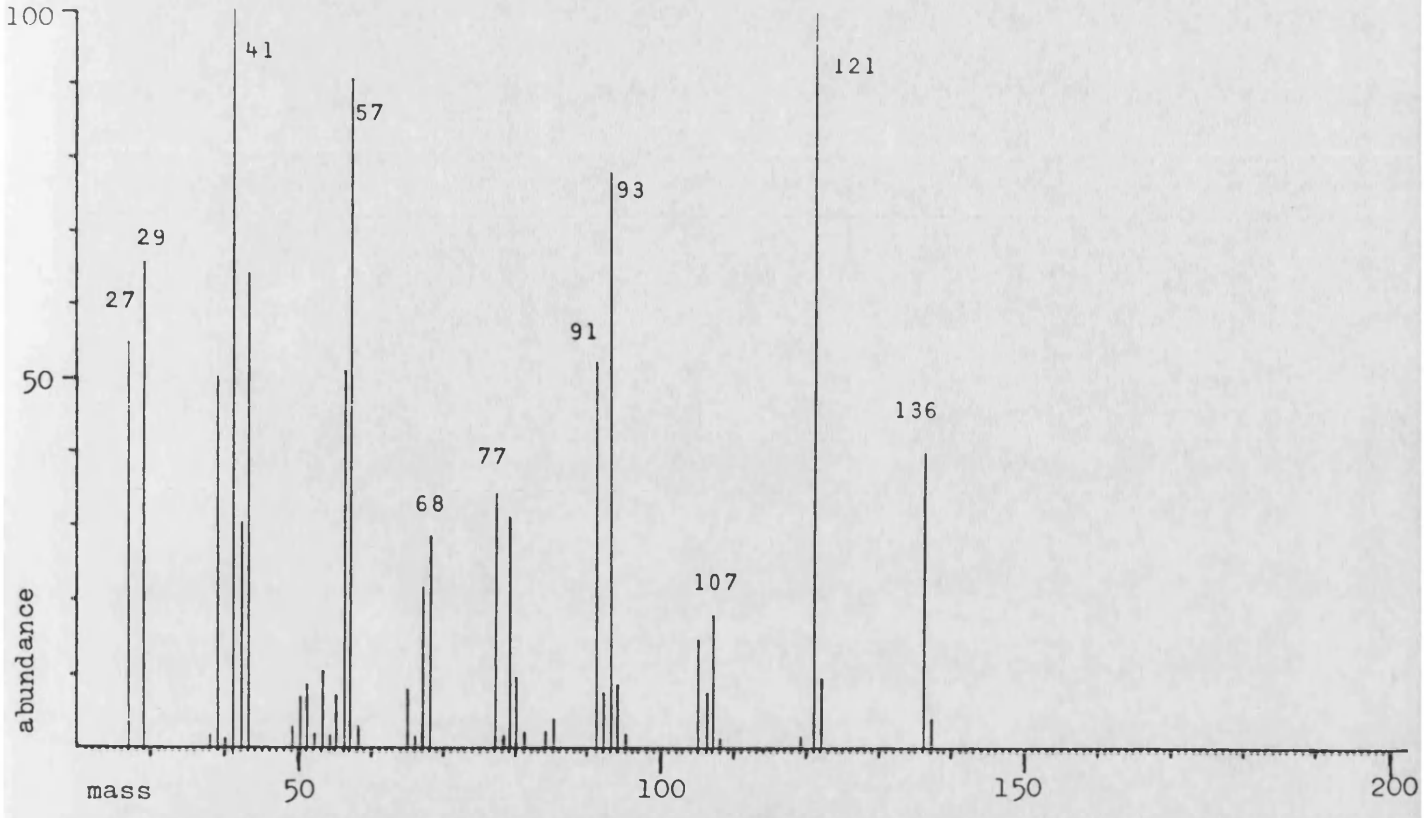
Mass spectrum of unknown compound 5



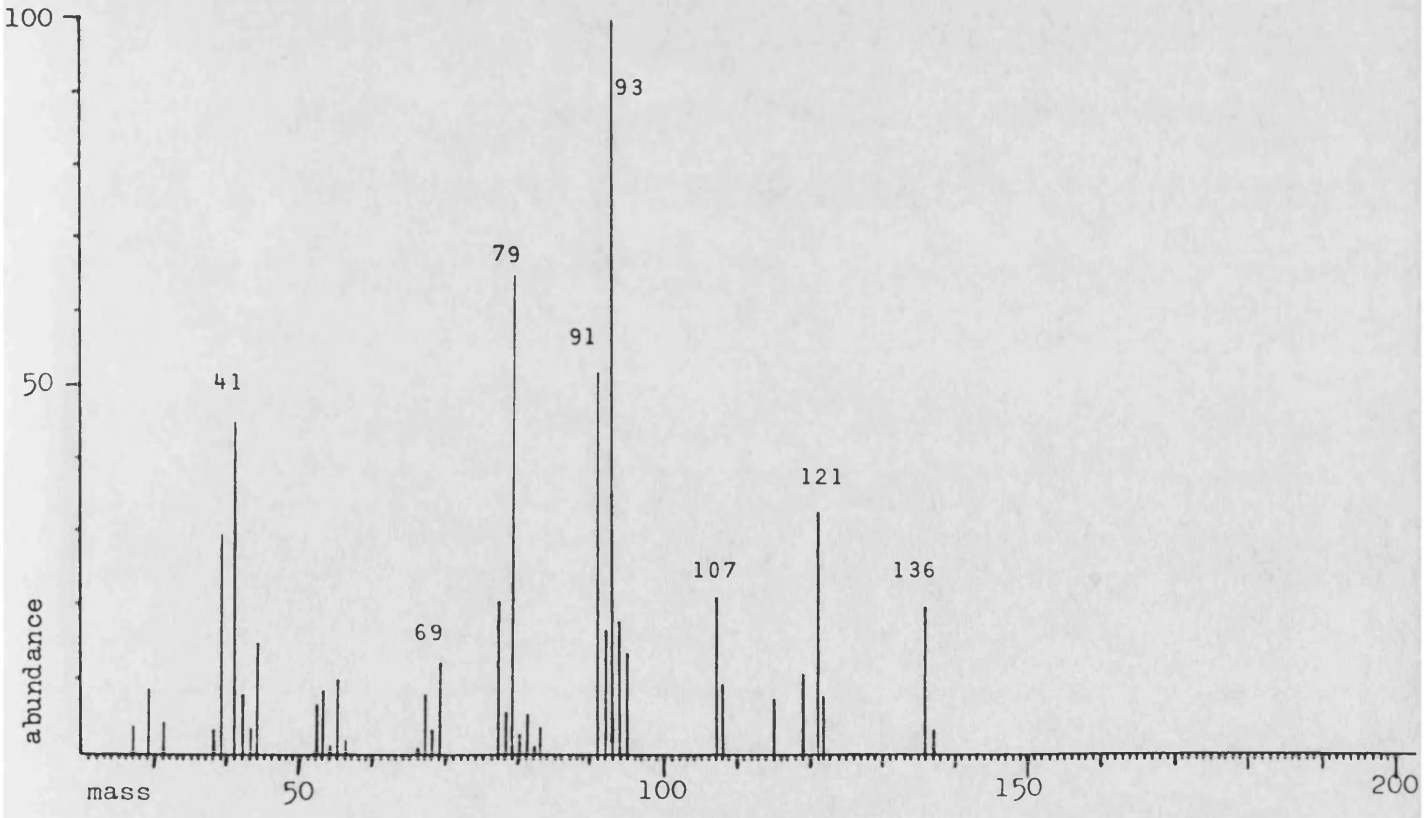
Mass spectrum of unknown compound 6



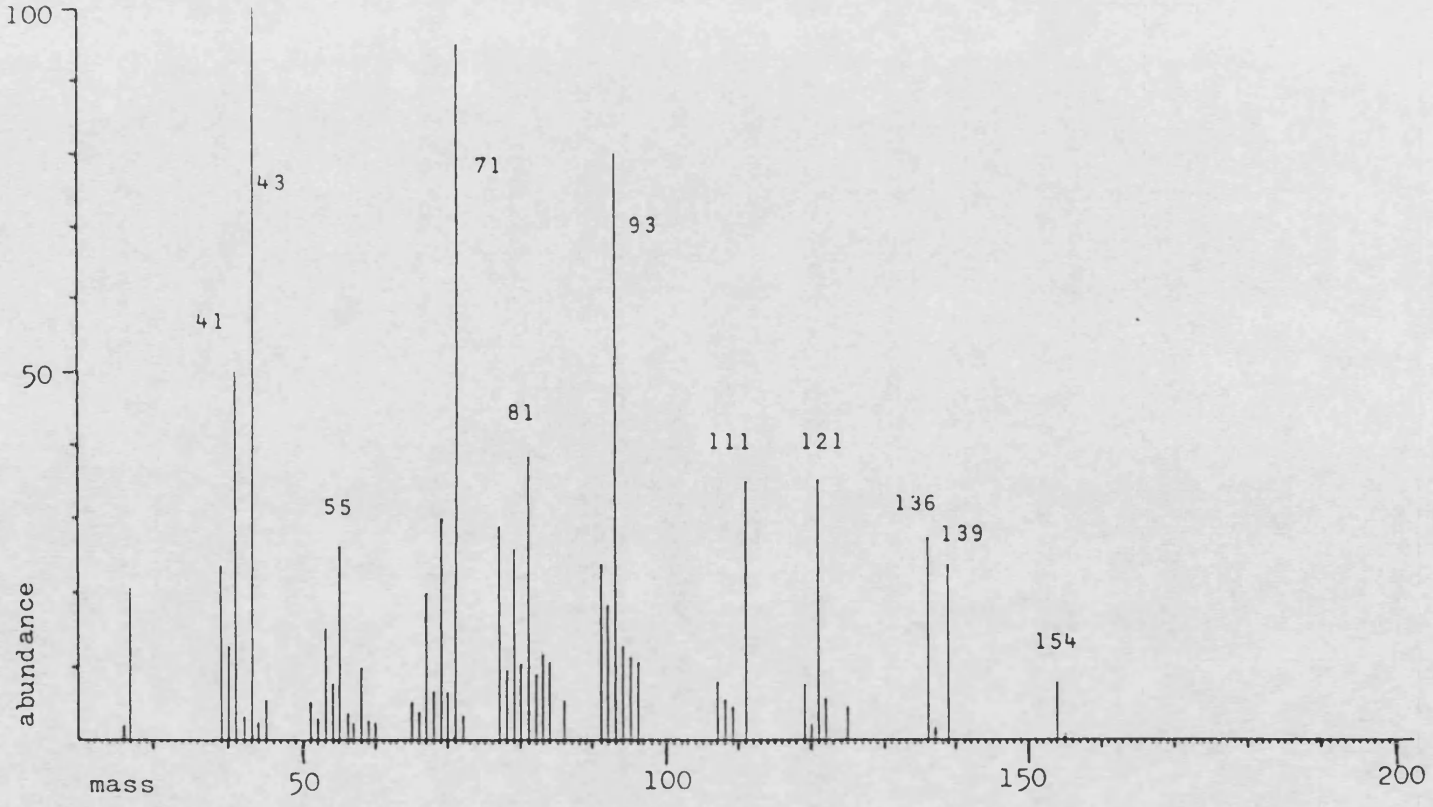
Mass spectrum of unknown compound 7



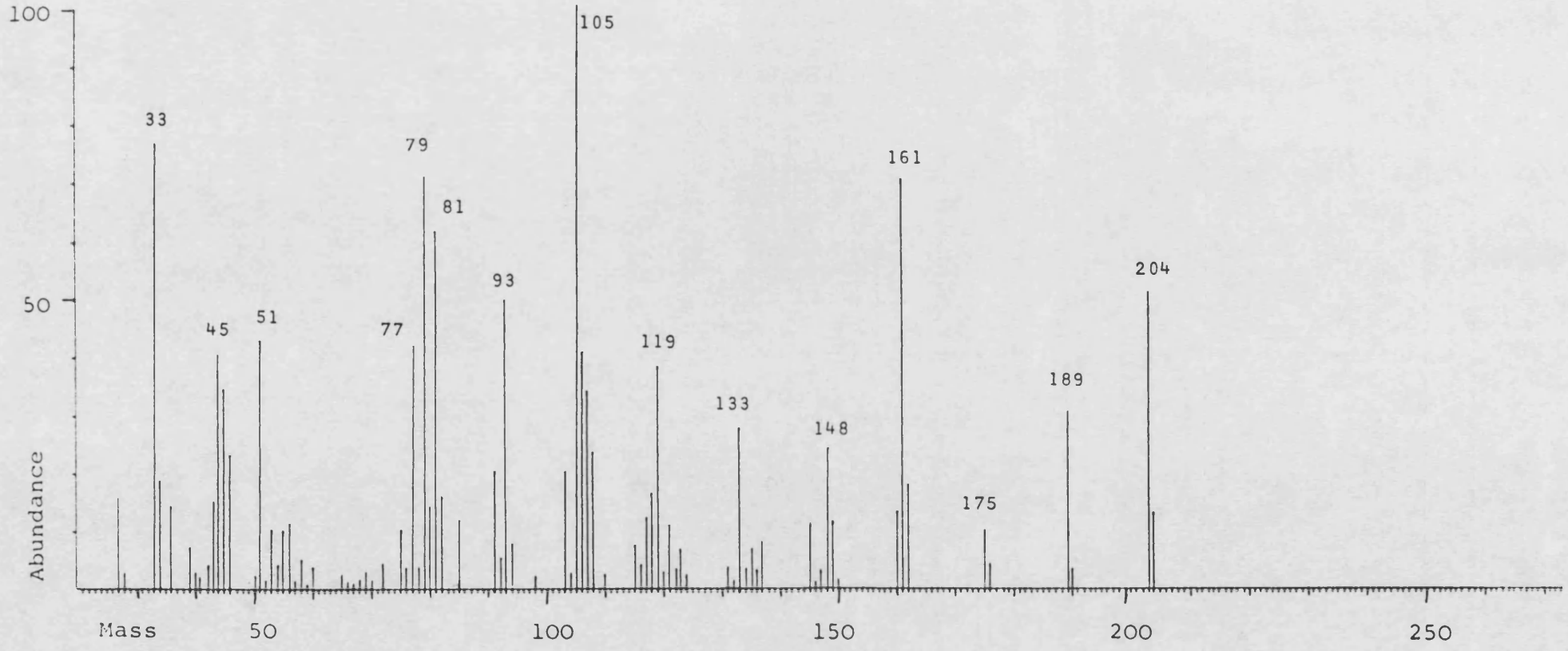
Mass spectrum of unknown compound 8



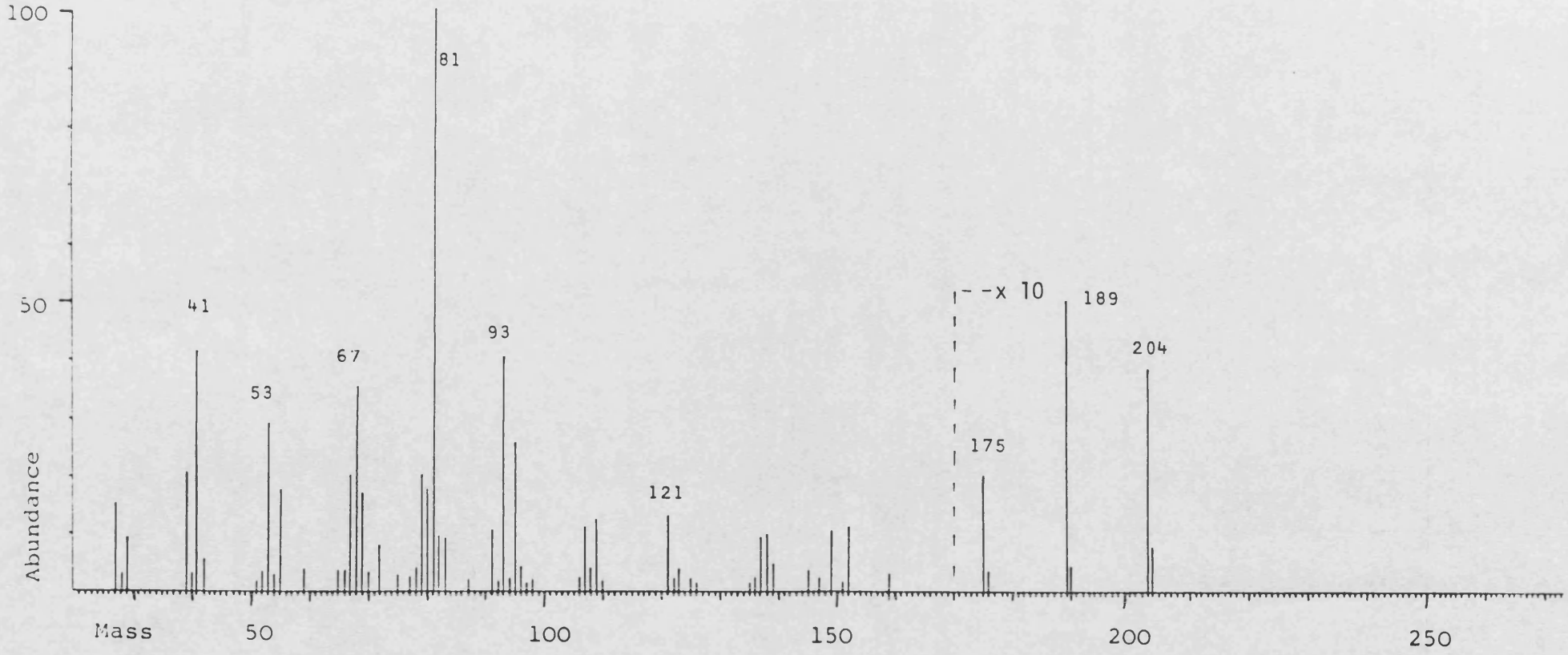
Mass spectrum of unknown compound 9



Mass spectrum of unknown compound 10



Mass spectrum of unknown compound 11



Mass spectrum of unknown compound 12