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# MICROANALYTICAL METHODS IN THE STRUCTURE ELUCIDATION OF SEX-SPECIFIC COMPONENTS IN THE LARGE PINE WEEVIL, *HYLOBIUS ABIETIS* L. (*COLEOPTERA, CURCULIONIDAE*)

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## SUMMARY

The sex-specific components isolated from ovaries of the large pine weevil, *Hylobius abietis* L., were studied by microchemical methods in combination with gas chromatography and mass spectrometry. A boric acid reaction loop prepared in a glass capillary was used for detection of alcohols in the hydrolysate of the sex-specific components. Gas chromatographic retention data and electron impact and chemical ionization mass spectra were used in the identification of the sex-specific components and their hydrolysis products. Double bond positions were determined and the absence of chain branching verified by recording the electron impact mass spectra of nicotinic acid esters of the hydrolysed sex-specific components. With this method, the double bond positions and chain branching could be determined from a sample of a few tens of nanograms. The female-specific components identified were hexadecyl acetate, octadecyl acetate, Z-9-octadecenyl acetate and eicosyl acetate.

## INTRODUCTION

Several microanalytical methods have been developed for structure elucidation of insect pheromones, chemical messenger compounds present in low concentrations. Microchemical methods commonly used during the past two decades include carbon skeleton chromatography (hydrogenation over palladium catalyst)[1], the detection of functional groups by reaction gas chromatography (GC)[2], and the location of double bonds by micro-ozonolysis[3] or mass spectral analysis of appropriate derivatives[4,5]. The methods are applicable for samples of 10–500 ng. The identification by spectrometric methods (IR, NMR, MS) can be done with sophisticated instruments from a few micrograms of isolated component. Pheromones are more often multicomponent mixtures than single compounds, and for them microchemical methods in combination with GC and MS are superior to the pure spectrometric methods, because reliable identification is possible without isolation of components and from a smaller sample. In addition there is less need for sophisticated instruments.

Kalo and Nederström[6] have found female-specific components in the ovaries of the large pine weevil, *Hylobius abietis* L. This paper reports on the use of micro-

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chemical methods in combination with GC and MS for the identification of these components.

## EXPERIMENTAL

### *Isolation of the mixture of sex-specific components*

For removal of fats, the hexane extract of ovaries was steam-distilled under nitrogen in a Clevenger apparatus for 1.5 h. The distillate was collected in the condensed hexane, and the hexane solution was dried with anhydrous sodium sulphate. The mixture of sex-specific components was isolated from fatty acids by micro-column chromatography as follows. A 25 × 1.5 mm I.D. Kieselgel 60 (70–230 mesh ASTM, Merck) column was prepared in a Pasteur pipette by the wet method with hexane as solvent. Before sample introduction the column was washed with 100 μl of dry methanol and 2 ml of hexane. The column was eluted successively with 300 μl of hexane and 200-μl portions of 20, 40 and 100% solutions of dichloromethane in hexane. The sex-specific components were eluted in fraction 2 with 20% dichloromethane in hexane.

### *Hydrolysis*

The solvent from a sample of sex-specific components (fraction 2) was evaporated in a 20 × 0.6 cm I.D. glass tube with a gentle stream of nitrogen. Then 100 μl of 0.5 *M* methanolic sodium hydroxide solution were added, and the mixture was refluxed for 1 h in a water-bath. To the cold reaction mixture, 200 μl of hexane were added and the hexane solution was washed successively with water to remove methanol and sodium hydroxide, and dried with anhydrous sodium sulphate.

### *Acetylation*

The hexane solution of a sample of the hydrolysate was evaporated to dryness with a gentle stream of nitrogen in a 20 × 0.6 cm glass tube. Then 100 μl of redistilled acetic anhydride were added, and the tube was heated in a boiling water-bath for 1 h. Then 200 μl of hexane were added to the cold reaction mixture. The mixture was washed successively with water and dried with anhydrous sodium sulphate.

### *Gas chromatography*

A Carlo Erba Fractovap gas chromatograph with a Grob-type split-splitless injector and flame ionization detector was used. Hydrogen was the carrier gas. The analytical columns (all 0.32 mm I.D.) were a 50-m OV-101 glass capillary, a 15-m OV-1 silica capillary, a 15-m SE-30 silica capillary, a 25-m OV-351 silica capillary and a 25-m Silar 10 c glass capillary. The boric acid subtractor column was prepared as described elsewhere[7]. The splitless injection technique was used.

### *Detection of alcohols by reaction gas chromatography*

A 3-m length of subtractor column was connected through a low volume union to the back end of the analytical silica column (15-m OV-1). Before the subtraction experiments the columns were heated to 210°C for 20 min. The splitless injection technique was used. The sample of the hydrolysate, containing *ca.* 10 ng of each component, was injected at a column temperature of 60°C. After an isothermal period

of 1 min the temperature was raised to 150°C. After a second isothermal period of 3 min the temperature was raised 2°C/min to 210°C. The subtraction experiment was repeated with reference compounds.

*Mass spectrometry*

The electron impact (EI) mass spectra of the acetates were run with a Varian 112S mass spectrometer connected to a Varian 1400 gas chromatograph and SS166 data system. Helium was the carrier gas. An OV-1 or OV-351 column was coupled through an open split connection. The temperatures of the ion source and the injector were 220 and 250°C, respectively. The mass spectra were taken at 70 eV electron ionization energy and 1.5 mA emission current.

The chemical ionization (CI) mass spectra of the acetates and the EI mass spectra of the alcohols and nicotinates were run with a JEOL JMS-DX 300 mass spectrometer equipped with a Dani 3800 gas chromatograph. When the EI spectra of the alcohols and the CI spectra of the acetates were recorded, a 25-m SE-30 silica column was directly connected to the ion source. The temperature of the ion source and the injector was 250°C. Mass spectra were taken at 70 eV electron ionization energy and 300 $\mu$A ionization current. Methane was used as reactant gas when the CI spectra were run. For recording of the EI spectra of the nicotinates a 15-m OV-1 column was connected through a jet separator. The temperatures of the injector and the ion source were 290 and 280°C, respectively. The spectra were taken at 70 eV electron ionization energy and 100 $\mu$A ionization current.

*Preparation of Z- and E-9-octadecen-1-ols*

Methyl *Z*-9-octadecenoate and methyl *E*-9-octadecenoate (Nu-chek-prep.) were reduced with lithium aluminium hydride in dry diethyl ether. The configurations of reaction products were verified by IR spectra. The IR spectra of *Z*- and *E*-9-octadecen-1-ols had, as expected, absorptions due to out-of-plane C–H bending vibrations at 724 and 966 $cm^{-1}$, respectively.

*Preparation of Z- and E-9-octadecenyl acetates*

The respective alcohols were acetylated with acetic anhydride.

*Preparation of hexadecyl, octadecyl and eicosyl acetates*

The saturated alcohol (Fluka, puriss.) was acetylated with acetyl chloride.

*Preparation of nicotinates*

The nicotinates of 1-hexadecanol, 1-octadecanol, 1-eicosanol and *Z*- and *E*-9-octadecen-1-ol were prepared as described by Vetter and Meister[8]. The nicotinates of the hydrolysate were prepared according to the same method, as follows. A sample of hydrolysate in a screw-cap vial was evaporated to dryness in a gentle stream of nitrogen, and 100 $\mu$l of saturated solution of nicotinoyl chloride. HCl in dry pyridine were added. The mixture was heated at 100°C for 10 min. Hexane was added to the reaction mixture and the solution was washed successively with water and dried with anhydrous sodium sulphate.

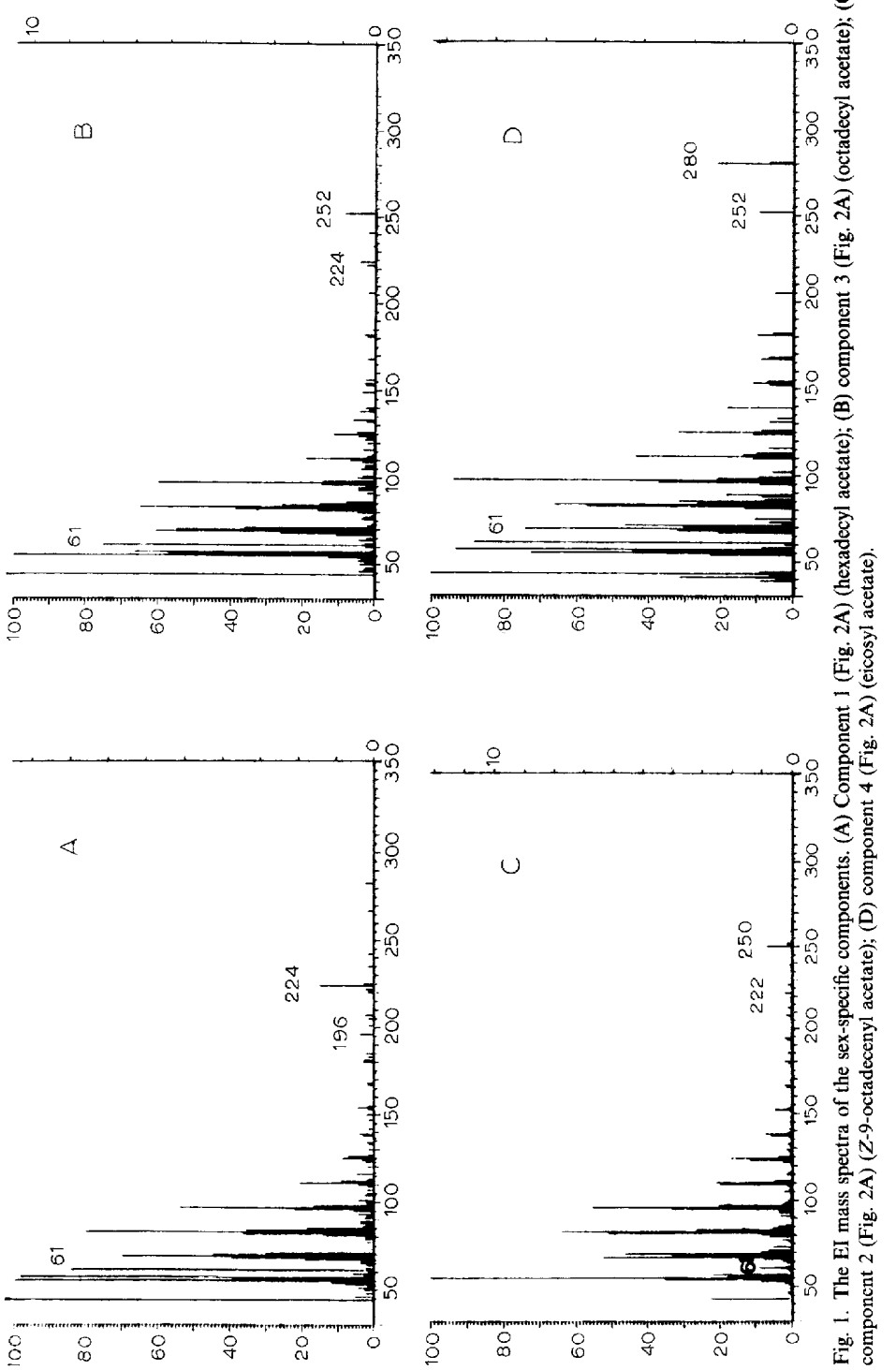


Fig. 1. The EI mass spectra of the sex-specific components. (A) Component 1 (Fig. 2A) (hexadecyl acetate); (B) component 3 (Fig. 2A) (octadecyl acetate); (C) component 2 (Fig. 2A) (Z-9-octadecenyl acetate); (D) component 4 (Fig. 2A) (eicosyl acetate).

## RESULTS AND DISCUSSION

In a GC study, Kalo and Nederström[6] found sex-specific peaks in the hexane extracts of pine weevil ovaries. The concentrations of these components were higher in spring than in autumn. In addition to the sex-specific components the hexane extracts also contained large amounts of free fatty acids and fats[6]. To isolate the mixture of sex-specific components in the present work, the hexane extract was steam-distilled under nitrogen in a Clevenger apparatus and the steam distillate was chromatographed on a silica micro-column. In the EI mass spectra of the sex-specific components (Fig. 1) the fragment *m/z* 61 (protonated acetic acid) and the $C_nH_{2n-1}$ fragments of series *m/z* 55, 69, 83, 97, ... indicate aliphatic acetic acid esters. The molecular ions reported for published spectra of acetates were lacking.

*Reaction gas chromatography*

Subtraction loops, which are short columns containing an appropriate reagent, have been developed to subtract aldehydes, ketones, alcohols, epoxides, nitrogen bases and carboxylic acids[2,7]. Reaction loops coated with *o*-dianisidine, FFAP, 2,4-dinitrophenylhydrazine and benzidine for subtraction of carbonyl compounds and a loop coated with boric acid for subtraction of alcohols have been prepared in a 0.35 mm I.D. glass capillary[7]. With these columns a functional group can be detected in a few nanograms of a component in a mixture.

The isolated mixture of sex-specific components was hydrolysed in methanolic sodium hydroxide solution. The hydrolysate, together with a mixture of $C_{16}$, $C_{18}$, $C_{20}$, $C_{22}$ and $C_{24}$ *n*-alkanes, was injected in one set of experiments into a column connected through the back end to a boric acid subtractor column (Fig. 2B) and in another set of experiments into a column used without the subtractor column (Fig. 2A). The hydrolysate injected contained *ca.* 10 ng of each component. Compounds 1, 2, 3 and 4 (Fig. 2) were subtracted at elution temperatures of 170, 183, 185 and 201°C, respectively.

*Mass spectral and retention data*

The EI mass spectra of components 1, 3 and 4 (Fig. 2A) showed the fragments $M-18$ ($M-H_2O$) and $M-46$ ($M-H_2O-C_2H_4$), the $C_nH_{2n-1}$ fragments *m/z* 55, 69, 83, ... and the $C_nH_{2n+1}$ fragments *m/z* 57, 71, 85, ..., which are consistent with *n*-alkanol structure. The spectra were identical with the spectra of 1-hexadecanol, 1-octadecanol and 1-eicosanol, respectively. The spectrum of component 2 (Fig. 2A) had in addition an abundant peak at *m/z* 96 and the spectrum was identical with the spectrum of *Z*-9-octadecen-1-ol.

The retention data of components 1, 2, 3 and 4 (Fig. 2) on the SE-30 column were identical with 1-hexadecanol, 9-octadecen-1-ol, 1-octadecanol and 1-eicosanol, respectively. Analysis of component 2 on an OV-1 silica capillary column, which separated *Z*- and *E*-9-octadecen-1-ols with resolution $R_s = 0.9$, showed it to be identical with *Z*-9-octadecen-1-ol. The acetates formed by acetylation of the hydrolysate with acetic anhydride had the same retention data on the OV-351 column as the isolated sex-specific components. The retention data of the sex-specific components were identical with those for hexadecyl acetate, octadecyl acetate, 9-octadecenyl acetate and eicosyl acetate on OV-101, OV-351 and Silar 10 c (Fig. 3) columns. The

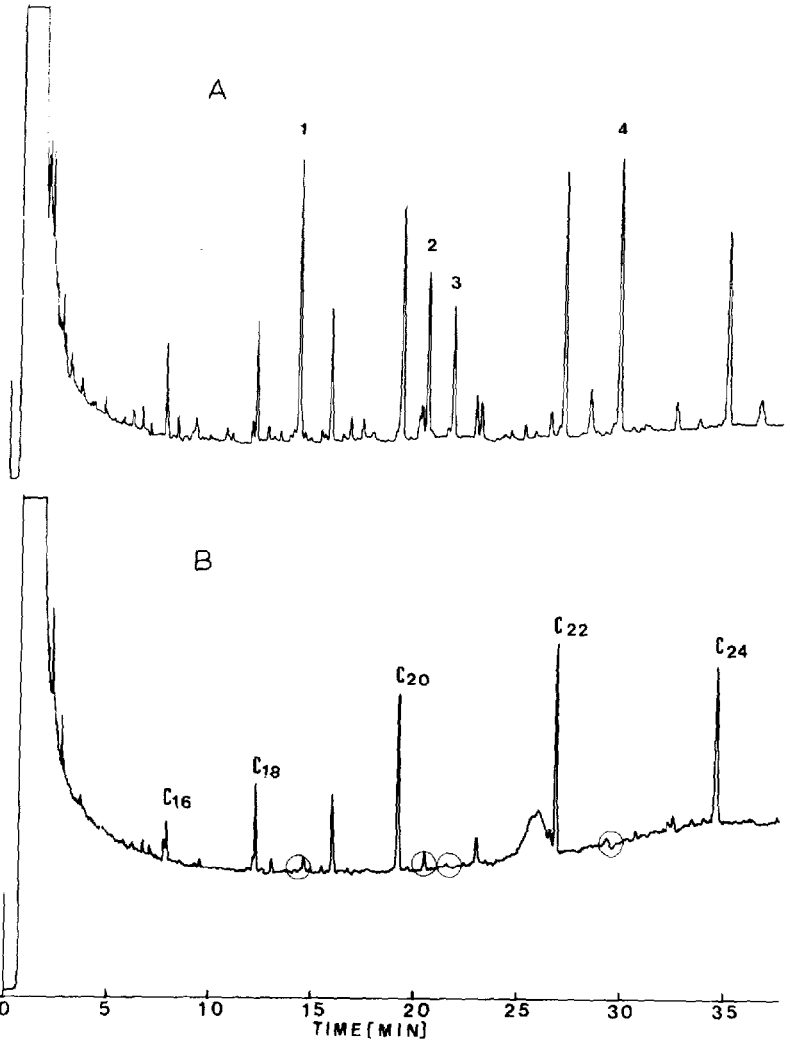


Fig. 2. Chromatograms of hydrolysed sex-specific compounds with $C_{16}$, $C_{18}$, $C_{20}$, $C_{22}$ and $C_{24}$ *n*-alkanes run on a SE-30 fused-silica column (15 m × 0.32 mm I.D.), with hydrogen as the carrier gas (0.4 kg/cm$^2$). Temperature programme: 1 min at 60°C, 3 min at 150°C and then 2°C/min to 210°C. (A) Chromatogram without subtractor column; (B) chromatogram run with boric acid subtractor column, with the same amount injected as in A. Peaks: 1 = 1-hexadecanol; 2 = *Z*-9-octadecen-1-ol; 3 = 1-octadecanol; 4 = 1-eicosanol.

acetates were prepared from the reference alcohols mentioned above. In the analysis with the Silar 10 c column, which gave a baseline separation between *Z*- and *E*-9-octadecenyl acetate, it was found that the *Z*:*E* isomeric ratio of 9-octadecenyl acetate in the sex-specific mixture varied in the different samples between 94:6 and 85:15.

The CI mass spectra of sex-specific components and reference compounds were then run, with methane as reactant gas. The CI mass spectra of sex-specific components and reference compounds were similar and showed the following diagnostic peaks:

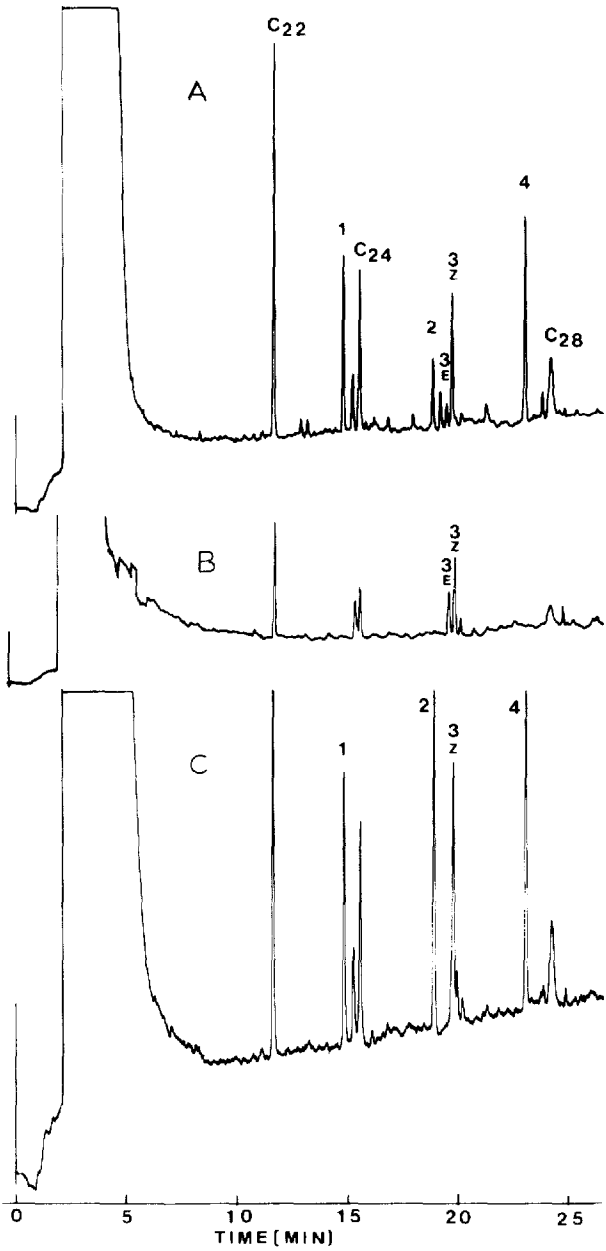


Fig. 3. Chromatograms of isolated sex-specific compounds and reference compounds run on a Silar 10 c glass capillary column (25 m × 0.32 mm I.D.), with hydrogen as the carrier gas (0.3 kg/cm$^2$). Temperature programme: 1 min at 60°C, 3 min at 150°C and then 3°C/min to 210°C. (A) The mixture of sex-specific components with $C_{22}$, $C_{24}$ and $C_{28}$ *n*-alkanes; (B) geometric isomers of 9-octadecenyl acetate with the same *n*-alkanes as in A; (C) reference mixture with the same *n*-alkanes as in A. Peaks: 1 = hexadecyl acetate; 2 = octadecyl acetate; 3E = *E*-9-octadecenyl acetate; 3Z = *Z*-9-octadecenyl acetate; 4 = eicosyl acetate.

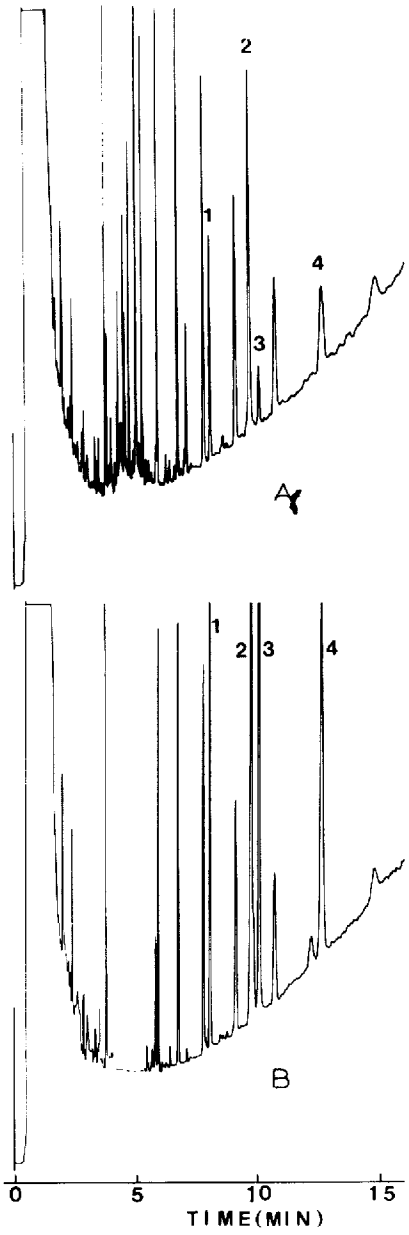


Fig. 4. Chromatograms of nicotinates run on a OV-1 silica capillary column (15 m × 0.32 mm I.D.), with hydrogen as the carrier gas (0.4 kg/cm$^2$). Temperature programme: 1 min at 60°C, 3 min at 250°C and then 3°C/min to 290°C. (A) The mixture of nicotinates prepared from hydrolysed sex-specific components; (B) the nicotinates prepared from reference alcohols. Peaks: 1 = hexadecyl nicotinate; 2 = *Z*-9-octadecenyl nicotinate; 3 = octadecyl nicotinate; 4 = eicosyl nicotinate.

| | M + 1 | (M + 1)−60 | $AcOH_2$ | $AcOH_2+CH_3$ | $AcOH_2+C_2H_5$ |
|---|---|---|---|---|---|
| 16:OAc | 285(43%) | 225(100%) | 61(83%) | 75(17%) | 89(64%) |
| 18:OAc | 313(45%) | 253(100%) | 61(77%) | 75(17%) | 89(72%) |
| *Z*-9-18:OAc | 311(12%) | 251( 23%) | 61(10%) | 75( 7%) | 89(36%) |
| 20:OAc | 341(40%) | 281(100%) | 61(93%) | 75(12%) | 89(65%) |

The intensities of ions M + 29 were low. In addition the spectra showed a series of hydrocarbon fragments *m/z* 69, 83, 97, 111, 125, ..., of which *m/z* 97 showed the

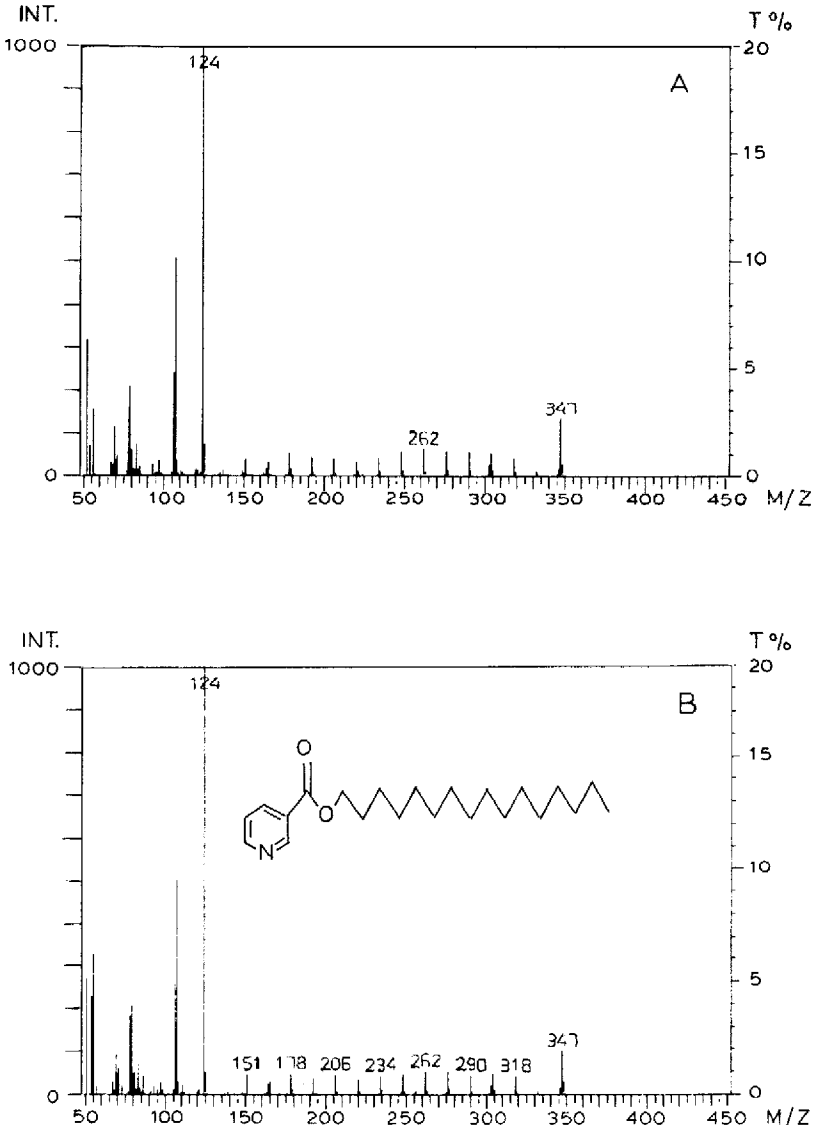


Fig. 5. The EI mass spectra of hexadecyl nicotinate. (A) The authentic nicotinate prepared from the hydrolysed sex-specific mixture; (B) the nicotinate prepared from hexadecanol.

most intense peak. Among the low-intensity fragments those of the series *m/z* 99, 113, 127, ..., were more abundant for the saturated acetates and those of the series *m/z* 95, 109, 123, ... more abundant for the *Z*-9-octadecenyl acetate.

The results presented above, based on mass spectral and retention data of the acetates and their hydrolysis products, indicate the presence of hexadecyl acetate, *Z*-9-octadecenyl acetate, octadecyl acetate and eicosyl acetate in the sex-specific mixture. Saturated acetates can have structural isomers due to chain branching. These can be separated by high resolution GC. The identification of saturated acetates by MS and high resolution GC on columns of different polarity is adequate. Unsaturated

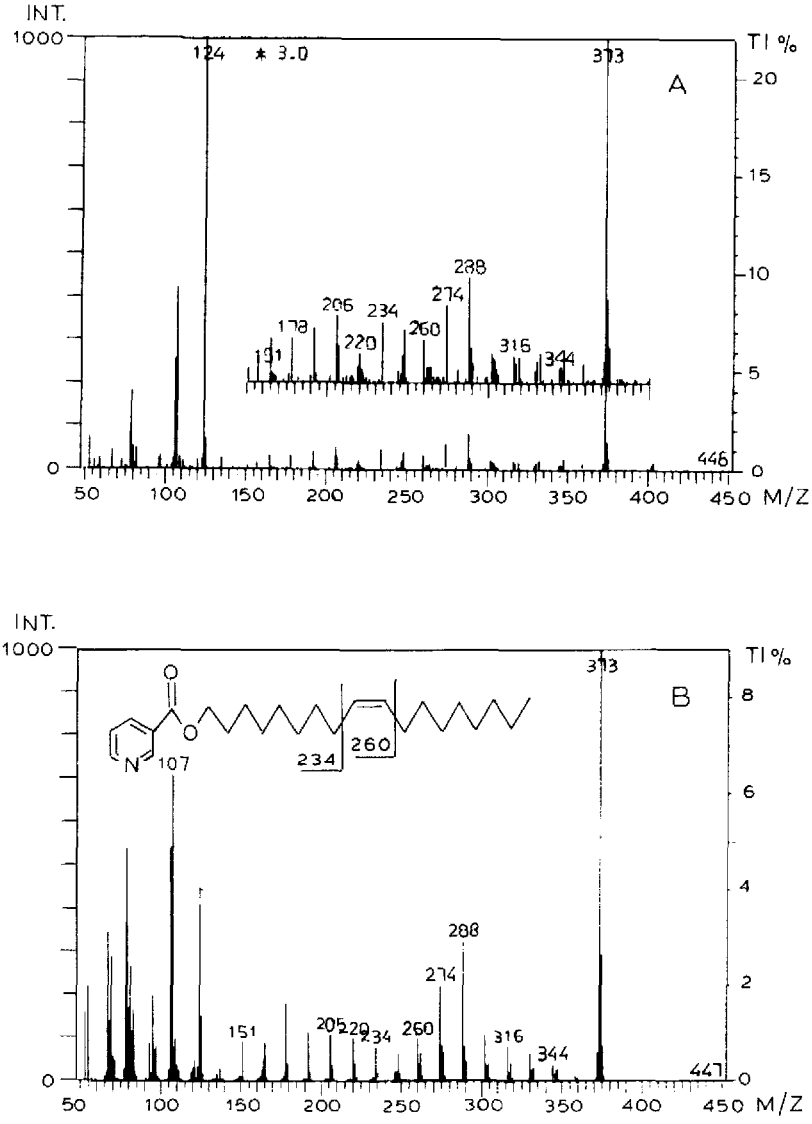


Fig. 6. The EI mass spectra of *Z*-9-octadecenyl nicotinate. (A) The authentic nicotinate prepared from the hydrolysed sex-specific mixture; (B) the nicotinate prepared from *Z*-9-octadecen-1-ol.

acetates can have structural isomers due to chain branching and different double bond positions, and also geometric isomers. Chain branching, double bond positions and *cis-trans*-isomerism cannot be deduced reliably from mass spectra of acetates. High resolution GC on polar and non-polar columns, on the other hand, allows resolution of structural isomers due to chain branching, resolution of double bond positional isomers and resolution of geometric isomers, but not the resolution of these three kinds of isomers from each other. The determination of double bond position is necessary for reliable identification.

*Location of double bonds*

Double bond positions can be determined by analysis of the EI or CI mass spectra of a number of derivatives of an unsaturated compound. Epoxides[4,5] and ozonolysis products[3] have been used for location of double bonds from submicrogram amounts of sample. The EI spectra of aliphatic nicotinic acid esters also reveal the chain branching[8]. Other common methods are methoxymercuration[9,10] and silylation[11,12].

For the determination of double bond positions and chain branching in the present acetates, nicotinic acid esters were prepared from the hydrolysate of the mixture of sex-specific components and from the reference alcohols by the method of Vetter and Meister[8]. Analysed on a 15-m OV-1 silica capillary column (Fig. 4), the authentic and reference nicotinates were found to have identical retention times. The authentic mixture contained *ca.* 30–150 ng of different nicotinates. The EI mass spectra of the authentic components 1 (Fig. 5A), 3 and 4 reveal unbranched structures[8] and are identical with the mass spectra of hexadecyl nicotinate (Fig. 5B), octadecyl nicotinate and eicosyl nicotinate, respectively. The EI mass spectrum of component 2 (Fig. 6A) shows the double bond position to be 9 by change of sequence between the peaks $m/z$ 234 and $m/z$ 260, which are the vinylic positions. The spectrum is identical with the spectrum of *Z*-9-octadecenyl nicotinate (Fig. 6B).

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