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IDENTIFICATION AND QUANTIFICATION OF INSECT JUVENILE HORMONES AS THEIR 10-HEPTAFLUOROBUTYRYLOXY-11-METHOXY DERIVATIVES BY COMBINATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTRON CAPTURE GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A convenient procedure is described for the isolation and quantitative determination of juvenile hormones from biological material by their conversion into the 10-heptafluorobutyryloxy-11-methoxy derivatives. After precipitation at low temperature, the lipid is separated by thin-layer chromatography (TLC), and the isolated hormone fraction is converted into the 10-hydroxy-11-methoxy derivatives which are purified by high-performance liquid chromatography (HPLC). These derivatives are treated with heptafluorobutyrylimidazole and the resulting derivatives are finally separated and quantified by electron capture gas–liquid chromatography. Overall yields are 20–50% with 1–4 g insects extracted. Marker substances used are triphenylmethanol for HPLC, and bis(ethylhexyl) phthalate and dibutyl phthalate for TLC and HPLC.

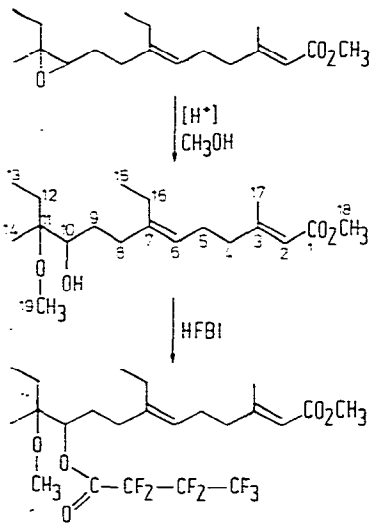
INTRODUCTION

At present, none of the available methods of analysis is sufficiently specific and sensitive for a direct quantitative estimation of the juvenile hormones (JH) contained in biological samples in the picomole range. The method developed by Trautmann *et al.*[1], which employs capillary gas chromatography, is sensitive only in the range of 0.15 μg JH contained in the final extract, and hence requires the extraction of relatively large amounts of insect material. With the aid of mass spectrometry and chemical ionization, Lanzrein *et al.*[2] were able to detect and quantify the hormones in nanogram amounts.

The sensitivity of detection can be drastically increased by the use of juvenile hormone derivatives. Thus, Broekhoven *et al.*[3] converted juvenile hormone III (JH-III) into its dihydric alcohol, esterified it to the bis(heptafluorobutyrate) and were able to detect this JH derivative in picogram quantities by gas–liquid chromatography with electron capture detection (GLC–ECD). However, this method is only applicable to JH III. Bergot *et al.*[4] and Peter *et al.*[5] prepared a 10-hydroxy-11-methoxy derivative

by methanol addition to the epoxide group of JH. The 10-hydroxy group of this derivative could then be esterified either with pentafluorophenoxyacetyl chloride[4] or with 2,4-dichlorobenzoyl chloride[5]. With this method all the three naturally occurring juvenile hormones could be separated and quantified in the picogram range.

A convenient method will now be described which allows the isolation and quantitative determination of all juvenile hormones from biological material by conversion into their 10-heptafluorobutyryloxy-11-methoxy derivatives according to the following scheme:



MATERIALS AND METHODS

Analytical grade solvents and perchloric acid (70%) were purchased from E. Merck (Darmstadt, G.F.R.). For high-performance liquid chromatography (HPLC), in some cases the hexane used was distilled through a packed column over Na, Pb alloy or $LiAlH_4$. Other *n*-hexane samples were from Carlo Erba (Milan, Italy), Mallinckrodt (Wesel, G.F.R.), J. T. Baker (Phillipsburg, N.J., U.S.A.) and Riedel de Haën (Seelze, G.F.R.). N-Heptafluorobutyrylimidazole was a product of Pierce (Rockford, Ill., U.S.A.). Juvenile hormones I and II (JH-I and -II) were gifts from Procida (Puteaux, France) and Zoecon Corp. (Palo Alto, Calif., U.S.A.) respectively. JH-III was synthesized according to Anderson *et al.*[6]. [^{3}H-C_{10}]JH-I, activity 13.5 Ci/mmol, was purchased from NEN (Dreieichenhain, G.F.R.).

Pre-coated silica gel 60 F_{254} plates (E. Merck) of different thickness were used for thin-layer chromatography (TLC). HPLC separations were performed with a modified Siemens S200 chromatograph (detector wavelength, 218 nm; slit width, 20 nm). A Valveseal Septumless Injector was used for sample injection with a high-pressure 212B syringe (Precision Sampling, Baton Rouge, La., U.S.A.). The heptafluorobyturyloxy derivatives were measured and quantified by the use of a Model 5730 Hewlett-Packard gas chromatograph with ^{63}Ni-ECD. Mass spectra were re-

corded on a CH7 A-Varian-MAT mass spectrometer (70 eV, emission 1 mA, ion source temperature 250°) and a SS-100 MAT computer. NMR spectra were obtained with a Bruker WH90 Fourier instrument; solvent, C^2HCl_3–^2H_2O; tetramethylsilane (TMS) as internal standard.

Reaction mixtures

For reactions on the microgram scale, the juvenile hormone was dissolved in *n*-hexane. A known volume of this solution was transferred to the reaction vessel (Serva, Heidelberg, G.F.R.) and the solvent was removed by a gentle stream of nitrogen. For preparation of the methanol solution containing $HClO_4$, 10 μl of 70% $HClO_4$ was dissolved in 50 ml of absolute methanol. This solution, if kept in a refrigerator, can be stored for 4 weeks.

Preparation of juvenile hormone derivatives

10-Hydroxy-11-methoxy derivatives

Macro scale. In a dry 6-ml reaction vessel, 1–5 mg juvenile hormone were dissolved in 0.3 ml methanol–perchloric acid; after at least 30 min at room temperature, 3 ml of 2% NaCl in water and then 0.3 ml ethyl acetate were added. After removal of the solvent, the extraction was repeated five times each with 0.1 ml ethyl acetate. The solvent was finally removed under a stream of nitrogen. The total yield of the derivative was measured as follows. About 20 μg JH-I and 10^4 cpm [^{3}H-C_{10}]JH-I were derivatized and the product purified by TLC as described in the next section. The R_F values for the derivatives were: JH-I, 0.31; JH-II, 0.30; JH-III, 0.27. The derivatives are visible as dark spots under a UV lamp (240 nm) if their concentration is in the microgram range or higher. An aliquot of the isolated derivative was taken for scintillation counting. The rest of the material was fractionated by HPLC, the eluent was collected in the counting vessel, removed under a stream of nitrogen and the fractions were counted. Yields were 83–91% after TLC, and 76–85% after HPLC. NMR (TMS): JH-I, δ 5.68 ppm (H-2, s), 5.08 (H-6, m), 2.37 (C_{10}-OH, s), 0.84 (H-13, t), 1.03 (H-14, s), 0.96 (H-15, t), 2.16 (H-17, s), 3.68 (H-18, s), 3.19 (H-19, s). Mass spectrum: JH-I, *m/e* 326 (M^+, 0.1%), 294 (0.5%), 239 (25%), 207 (23%), 87 (100%).

Micro scale, biological material. A biological sample of less than 1 mg, or juvenile hormone in the micro- or nanogram range, was transferred to a 3-ml reaction vessel, 0.05 ml methanol–$HClO_4$ was added and the substance was completely dissolved. After 30 min, 0.5 ml of 2% NaCl in water and 0.05 ml ethyl acetate were added. This extraction was repeated four times with the same volume of ethyl acetate, and the solvent was removed under nitrogen. The residue was dissolved in a small volume of ethyl acetate and placed on a TLC plate (0.25 mm) as a thin line. The vessel was carefully rinsed with more solvent to transfer the material quantitatively. The plate was developed with hexane–ethyl acetate (65:35) and air-dried. Dibutyl phthalate, R_F 0.51, was used as an external standard. The area containing the labelled JH-I was localized with a TLC Scanner (Berthold, Wildbad, G.F.R.). The area containing the derivatives of JH-I to JH-III (R_F 0.21–0.40) was scraped off, and the silica gel was carefully extracted with ethyl acetate on a sintered glass funnel. After the solvent had been removed in a nitrogen stream, the residue was kept under nitrogen in a refrigerator.

A prepacked silica gel column (Whatman, Ferriers, France; Partisil 5 μm, 4.3

mm I.D., 250 mm long) with 1% methanol in hexane as eluent was used for HPLC. For calibration, triphenylmethanol was co-chromatographed with the methoxy adducts. After calibration, the whole system was carefully rinsed by pumping through it *ca.* 500 ml of eluent. For a quantitative injection of biological material, the sample was dissolved in 10–20 μl of solvent, taken up with a 50-μl syringe which already contained 5–10 μl of eluent and the vessel was rinsed twice with the solvent which, after concentration under nitrogen, was also sucked into the same syringe. After application of the material to the column, the total loss including substance retained in the syringe was usually 1–5%. The HPLC eluate was concentrated to dryness under nitrogen in a reaction vessel and kept for subsequent derivation.

Esterification with heptafluorobutyrylimidazole

Macro scale. About 1 mg of the JH hydroxy methoxy derivative was warmed in the reaction vessel to 40°, 50–100 μg heptafluorobutyrylimidazole were added and the sealed vessel was kept at 60° for 30 min. Volatile material was then removed with a stream of nitrogen and the residue extracted with diethyl ether. The extract was immediately placed on a TLC plate which was developed in hexane–ethyl acetate (65:35). The heptafluorobutyryloxy (HFB) derivative was extracted from the silica gel with diethyl ether and the solvent was removed in a stream of nitrogen. For HPLC, a column (300 × 3 mm) was packed with LiChrosorb Si 60 (5 μm, E. Merck), as described by Asshauer and Halász[7], and hexane–diethyl ether (6 or 8%) was used as eluent. The yield was measured as described under *10-Hydroxy-11-methoxy derivatives* and was 79–85% after TLC, and 75–85% after HPLC. NMR (TMS): JH-I, δ 5.67 (H-1, s), 5.15 (H-6, m), 5.0 (H-10, m), 0.94 (H-13, t), 1.12 (H-14, s), 0.88 (H-15, t), 2.17 (H-17, s), 3.68 (H-18, s), 3.16 (H-19, s). Mass spectrum: JH-I, *m/e* 522 (M^+, 2.4%), 276 (9.4%), 87 (100%).

Micro scale, biological material. A dry sample was warmed to 40° in a reaction vessel, dried for a few seconds with a stream of nitrogen and then dissolved in 10–15 μl heptafluorobutyrylimidazole. The sealed vessel was kept at 60° for 30 min and then flushed with nitrogen to remove volatile material. The residue was extracted six times, each with 25 μl diethyl ether, and the extracts were immediately placed on a 0.25-mm TLC plate. The residue was finally dissolved in 100 μl water and twice extracted with 25 μl diethyl ether which was also applied to the TLC plate. Dibutyl phthalate, which has a similar R_F to the HFB derivatives, was used as external reference. The plate was developed in hexane–ethyl acetate (65:35), the area which contained the JH derivatives was scraped off, the silica gel was extracted with diethyl ether and the solvent was removed in a stream of nitrogen. For HPLC, the procedure described under *Macro scale* was followed. Either dibutyl phthalate or bis(2-ethylhexyl) phthalate was used as internal reference. The V_R value of the first reference substance is between that of the JH-II and JH-III derivatives, whereas that for the second reference is less than that of the JH-I derivatives. Using these two makers, which are common in biological extracts, the HPLC eluate can be easily fractionated. In addition, each of the fractions can also be checked for the presence of the JH derivatives by GLC. The total eluent, containing a JH derivative, was concentrated at 40–50° under nitrogen and transferred to graduated tubes having a capacity of 100–150 μl. The solvent was completely removed in a stream of nitrogen and the residue was dissolved in 50 or 100 μl hexane. One aliquot was counted, and a second was used in GLC

for quantitation of the yield and JH contents of the sample. Hexane, which is the main solvent for HPLC, often contains impurities which interfere with the detection of a JH derivative. In order to check the purity, 6 ml of hexane was removed with nitrogen and the vessel was rinsed with 50 μl of the same solvent; 4 μl of this concentrated material was injected into the gas chromatograph using the same attenuation as for the JH run. If a signal is found to interfere with the peak of the heptafluorobutyryloxy methoxy derivative another sample of hexane must be chosen. Sometimes one can avoid this interference if HPLC fractions are collected at 20-sec intervals and an aliquot is checked for the JH content.

Argon–methane (5%) or nitrogen was used as carrier gas for the detection of the HFB derivatives by GLC–ECD. The sensitivity of the detector is five times higher with nitrogen than with argon–methane. Columns were filled with SE-30, OV-1, OV-17 or OV-225, each 3% on Gas-Chrom Q. The carrier gas flow-rate, in all separations, was 23–30 ml/min and the separation was carried out isothermally at 195°. The injector and detector temperatures were 200° and 300°, respectively. For the calibration curve, the heptafluorobutyryloxy methoxy derivative of JH-III (3.5 mg) was synthesized as described earlier and was purified by HPLC. A solution of this material was diluted in steps down to a final concentration of 0.1 pmol/μl. After injection of 0.1 μl of this solution, 0.01 pmol (5 pg) were visible as a distinct GLC peak. The peaks were quantified according to their retention times and peak areas by use of an integrator (Minigrator or System I, Spectra Physics).

Determination of juvenile hormones in biological material

The amount of JH in biological samples was determined principally as described for the synthetic compounds by use of the isotope dilution technique. A defined amount (usually 2.9 ng) of radioactive JH-I ($1.5 \cdot 10^5$ cpm) was placed in a glass homogenizer (Braun, Melsungen, G.F.R.), made up to 5 ml with methanol–ethyl acetate (1:1) and 1–4 g of insects were added. The mixture was homogenized by use of a PTFE piston, cooled in ice and centrifuged. The procedure was repeated 3–4 times, each with 5 ml of the solvent. The pooled extracts were finally concentrated to a volume of 15 ml in a rotary evaporator. To this concentrated extract were added 20 ml of 1.7% NaCl solution and the lipids were extracted first with 20 ml hexane and then with five equal portions (10 ml) of hexane. After drying with Na_2SO_4, filtration, concentration and transfer of the hexane to a small centrifuge tube, the solvent was removed in a stream of nitrogen and the residue dissolved in 6 ml methanol–diethyl ether (1:1). The solution was kept in solid carbon dioxide for 30 min, then packed in solid carbon dioxide and centrifuged. The precipitate was washed three times, each with 4 ml of cold methanol–diethyl ether (1:1). The pooled extracts were concentrated to dryness in a stream of nitrogen, redissolved in ethyl acetate, applied to a 0.5-mm silica gel TLC plate and developed in hexane–ethyl acetate (65:35). Dibutyl phthalate was used as an external reference. The silica gel was scraped off between R_F 0.38 and 0.57, extracted with ethyl acetate and the extract was used for derivatization. In some cases, primarily with adult insects, it may be necessary to repeat the purification, using chloroform–carbon tetrachloride (1:1) for developing the TLC plate.

The total yield of purified JH derivatives varied between 0 and 50%. Only extracts having yields between 20 and 50% were used for calculation of the JH titre. The radioactivity which has been added for isotope dilution was recovered, as calcu-

lated from the residual radioactivity and from the ECD signal. The difference in results obtained from the labelling experiments and from measurements of the GLC peaks was found to be less than 20% with respect to the amount of radioactive hormone added to the crude extract.

RESULTS AND DISCUSSION

Thin-layer chromatography

Juvenile hormone I and III can be completely separated from each other by the system hexane–ethyl acetate (65:35), as shown in Table I. The migration of tritaited JH-I is slightly retarded (R_F 0.50) compared to the unlabelled hormone. A complete separation of JH-II from each of its two homologues is not possible.

TABLE I

R_F VALUES OF JUVENILE HORMONES AND THEIR 10-HYDROXY-11-METHOXY AND 10-HFB-11-METHOXY DERIVATIVES

TLC plates, silica gel 60 (Merck); solvent, hexane–ethyl acetate (65:35).

	Hormone	*10-OH-11-OCH$_3$*	*10-HFB-11-OCH$_3$*
JH-I	0.51	0.31	0.58
JH-II	0.49	0.30	0.55
JH-III	0.47	0.27	0.51

For the examination of biological samples it is therefore reasonable to extract the whole area in which the hormones are contained. An ideal reference substance is dibutyl phthalate which has the same R_F value (0.51) as JH-I. After derivatization with methanol, the R_F values are reduced, whereas after the subsequent esterification the less polar 10-heptafluorobutyryloxy-11-methoxy derivatives have higher R_F values than the starting hormone. The HFB derivative of JH-III has the same R_F as dibutyl phthalate.

High-performance liquid chromatography

The hydroxy methoxy derivatives of the juvenile hormone can be completely separated from each other by the system hexane–methanol (1%). The retention volumes with a column of length 25 cm are 9.15 ml for JH-I, 10.4 ml for JH-II and 12.3 ml for the JH-III derivative. Triphenyl methanol is a suitable reference in this system with a V_R of 10.9 ml (Fig. 1A). The HFB esters are completely separated from each other (Fig. 1B) with retention volumes of 8.0 ml for the JH-I, 9.3 ml for the JH-II and 13.4 ml for the JH-III HFB derivative. The retention volumes for the reference substances bis(2-ethylhexyl) phthalate and dibutyl phthalate are 5.4 and 10.5 ml, respectively. Since both of these compounds are present in biological material, they are ideal as markers for the chromatographic system because the juvenile hormone derivatives are not usually visible due to their low concentration (Fig. 1C).

Gas chromatography

One of the main problems in a quantification of the juvenile hormone HFB esters is interference by impurities in the hexane solvent with the GLC signals of the hormone derivatives after concentration of the solvent. Typical examples of the chromatogram of such impurities are shown in Fig. 2 where three different hexane

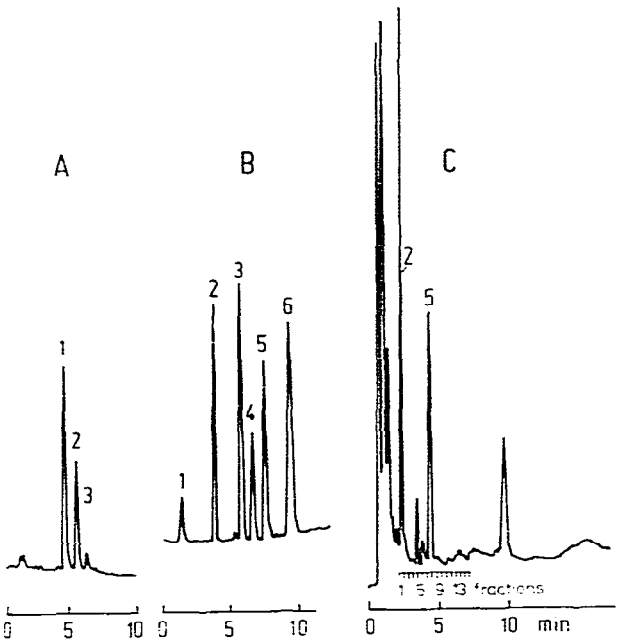


Fig. 1. HPLC chromatograms of juvenile hormone derivatives. (A) Chromatogram of hydroxy methoxy derivatives and marker substance. Column, 250 × 4.3 mm I.D. Eluent, 1% methanol in *n*-hexane; flow-rate, 1.88 ml/min. Peaks: 1 = hydroxy methoxy-JH-I; 2 = triphenylmethanol; 3 = hydroxy methoxy-JH-III. (B) Chromatogram of the HFB esters and the marker substances. Column, 300 × 3 mm I.D. Eluent, 6% diethyl ether in *n*-hexane; flow-rate, 1.45 ml/min. Peaks: 1 = solvent front; 2 = bis(2-ethylhexyl) phthalate; 3 = JH-I HFB ester; 4 = JH-II HFB ester; 5 = dibutyl phthalate; 6 = JH-III HFB ester. (C) Chromatogram and fractionation of a biological sample which was derivatized to the HFB esters. Column as in B. Eluent, 8% diethyl ether in *n*-hexane; flow-rate, 1.6 ml/min. Peaks: 2, 5 = identical with those in B.

samples (nanograde purity, for residual analysis, *pro analysi* grade) were injected after concentration. None of the hexane samples screened was completely free from disturbing compounds. Two extreme examples are shown in Fig. 2A and 2B: both are *pro analysi* hexanes obtained from the same company. Solvents such as those shown in Fig. 2B or 2D contain an impurity which severely interferes with the signal of the 11-heptafluorobutyryloxy-10-methoxy derivative of JH-III, whereas a solvent such as that in Fig. 2C can be used with some reservations.

Under constant conditions of gas chromatography, the retention time of the HFB derivatives is highly reproducible with a maximum deviation of 2 sec, if the computer is started at the time of appearance of the solvent front signal. For peaks which are separated from each other, a quantitative calculation of the juvenile hormone contents is possible from the retention time and integral of the peak area. In Fig. 3A, for example, the JH-III derivative has a retention time of 165 sec with an area integral of 47.518 units which corresponds to 0.9 pmole of hormone.

When interfering impurities are present in the hexane, the HPLC chromatogram has to be dissected into 20-sec fractions (see Fig. 1C) each of which is analyzed by GLC-ECD. A typical example is shown in Fig. 3B–E. After the HPLC signal for ethylhexyl phthalate, in fraction 4 the JH-I derivative is expected. The signal at 226 sec (Fig. 3B) corresponds to 0.038 pmole JH-I. No such signal is present in fraction 5.

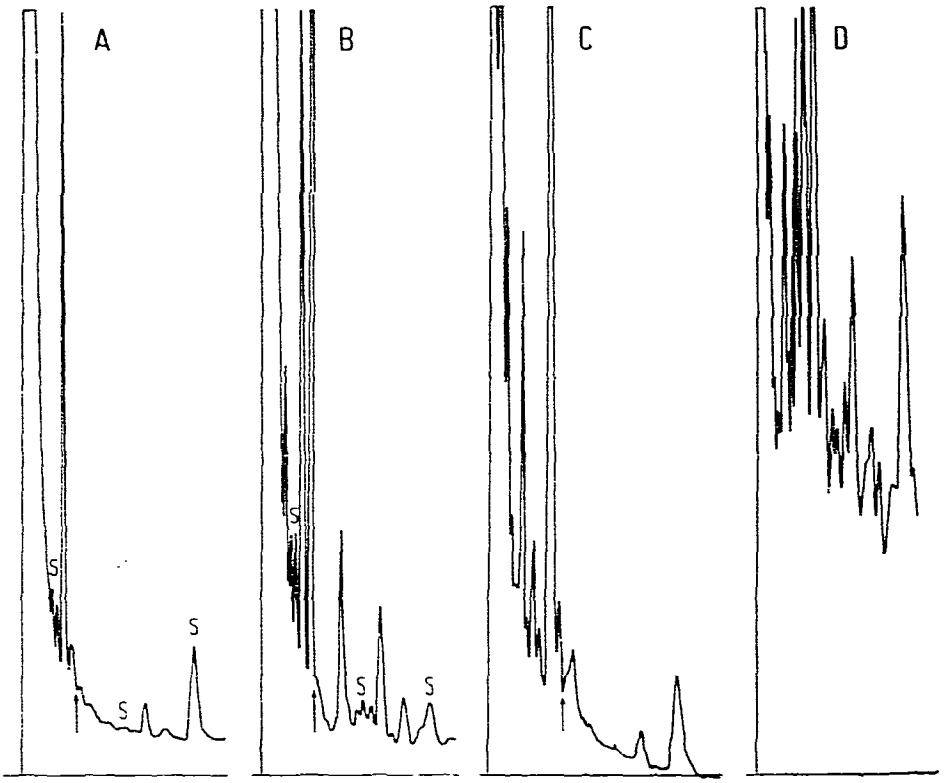


Fig. 2. Gas chromatograms of concentrated (1:120) *n*-hexane samples. A, B, D, *n*-Hexane, *pro analysi*. C, *n*-Hexane, nanograde (adequate to Resi Analyzed). Column, 200 mm × 2 mm I.D.; 3% SE-30 on Gas-Chrom Q. Carrier gas, 5% methane in argon. The arrow marks the point at which the JH-III HFB derivative is to be expected. Conditions: A and B, temperatures, oven 200°, injector 250°, detector 300°; gas flow-rate 32 ml/min; attenuation 16; C, temperatures, oven 190°, injector 250°, detector 300°; flow-rate 28 ml/min; attenuation 16; D, temperatures, oven 195°, injector 250°, detector 300°; flow-rate 30 ml/min; attenuation 32. S = Septum material.

In an aliquot of fraction 4 the radioactivity indicated that 54% of labelled JH-I was present: the remaining 46% of the ECD signal could be due to natural juvenile hormone. Fraction 6 of the HPLC should contain JH-II which, with a low probability, could be represented by the peak maximum at 184 sec in Fig. 3C, whereas JH-II should appear after 188 sec. Chromatography on the two other GLC columns definitively showed that JH-II is absent from this biological sample. In fraction 10 (Fig. 3D), a clear signal equivalent to 0.055 pmole JH-III is obtained, whereas fraction 11 (Fig. 3E) shows the appearance of an impurity peak together with some of the JH-III derivative, the amount of which can be calculated as 0.034 pmoles. In neither fraction 9 nor 12 is there a signal due to more of the JH-III derivative.

Two methods have been described which make use of a 10-ester 11-methyl ether derivative of the there juvenile hormones for their quantification in biological material[4,5]. Although the cost of the derivatization procedure is rather high, the method has many advantages when compared with the isolation of the unaltered hormones. One is that the three chemical species, hormone to hydroxymethoxy derivatives to 10-ester 11-methyl ether differ in polarity to such an extent that a good

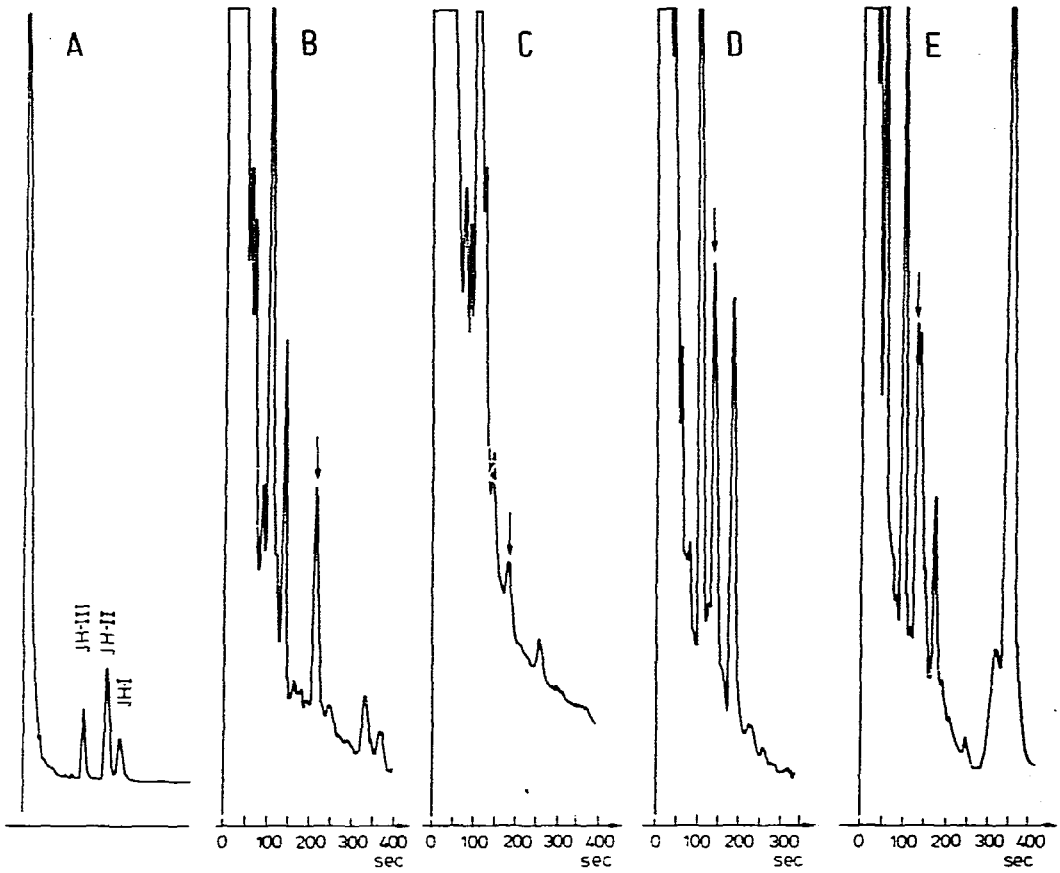


Fig. 3. Gas chromatograms of the JH HFB esters (A) and of HPLC fractions from a biological sample (B–E). B, Fraction 4 of the biological sample (see Fig. 1C), containing the JH-I derivative. C, Fraction 6, containing the JH-II eluate from HPLC. D, Fraction 10, containing the JH-III derivative. E, Fraction 11, containing the JH-III derivative accompanied by an interfering peak. Column, 200 × 2 mm I.D.; nitrogen as carrier gas. The arrows indicate the retention time for the corresponding JH HFB esters if present in the biological sample. A, Chromatogram of the three JH derivatives on OV-225 (3% on Gas-Chrom Q); temperatures, oven 200°, injector 200°, detector 300°; gas flow-rate 28 ml/min; attenuation 512. B–E, 3% SE-30 on Gas-Chrom Q; temperatures, oven 195°, injector 200°, detector 300°; gas flow-rate 23 ml/min; attenuation 16.

separation from by-products is achieved in each derivatization and purification step. On the basis of the available data, the limitations of this procedure are the variable yields and, due to the high GLC column temperature, a rapid contamination of the ECD. Other problems arise from the availability and purity of the acid chlorides used for esterification of the hydroxy methoxy derivatives. We therefore decided to use heptafluorobutyrylimidazole as the acid component since this compound is commercially available in sufficient purity. For a convenient and reproducible isolation procedure, marker compounds for both HPLC separations were found which, in combination with tracer techniques, allow good overall yields even when only small amounts of insects are available. A prerequisite for this highly sensitive method is that any contamination by the synthetic hormone is carefully avoided and that solvents

are employed in the final purification which cannot interfere with the ECD signals of the JH derivatives. The isolation procedure described allowed the quantitative estimation of 0.5 ng JH-III isolated from *ca.* 1 g of honey bee larvae[8].

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