

Gas Chromatographic Determination of Subnanogram Amounts of the Juvenile Hormone Methyl (2E, 6E)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate (JH III) in Insect Material by Electron Capture Detection

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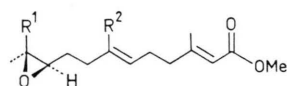
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Quantitative Determination, Electron Capture Detection, Insects, Juvenile Hormone

Using an electron capture detector, a sensitive gas chromatographic method has been developed for the determination of the juvenile hormone methyl (2E, 6E)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate (JH III) in insect material. The sensitivity in electron capture detection of the bisheptafluorobutyrate was determined. The work-up procedure was checked by adding known amounts of JH III to insect material. The method seems equally applicable to all three juvenile hormones.

During the last years a few analytical methods were developed for the determination of the three naturally occurring juvenile hormones (JH I, JH II and JH III).



JH I : $R^1 = R^2 = \text{ethyl}$
JH II : $R^1 = \text{ethyl}$, $R^2 = \text{methyl}$
JH III : $R^1 = R^2 = \text{methyl}$

Fig. 1. Structure of JH I, JH II and JH III.

Bieber *et al.*¹ described a method using combined gas chromatography-mass spectrometry with an accelerating voltage alternator. (Detection limit 200 ng.)

More recently Trautmann² published a method based on radioactive dilution analysis. With this method it was possible to detect JH III in several insect orders^{2,3}. (Detection limit 0.1 ng/g of the whole insect.)

A method using a gas chromatographic determination with an electron capture detector was only mentioned briefly by Judy *et al.*⁴.

Ajami⁵ described a method for the determination of JH I as the bistrifluoroacetate by electron capture detection. The sensitivity was 4 ng hormone per μl .

We would like to report on a sensitive method, which allows both qualitative and quantitative determination of JH III after a relatively rapid and

simple procedure and for which only low amounts of insect material are necessary. The method was tested on insect material, to which known amounts of JH III were added. Preliminary results show, that the method allows a simultaneous determination of JH I, II and III.

Materials and Methods

Solvents

Special treatment of all solvents and adsorbents used was required in order to avoid obscuring artefacts and contaminants during the sensitive detections.

Ether used for the extraction and work-up of juvenile hormone containing material was obtained from Mallinckrodt (Anhydrous, AR). Otherwise ether, which was passed through an alumina column (Woelm, neutral, activity I) and distilled twice thereafter, was used. It was stored in dark brown bottles.

Hexane was treated similarly.

Methanol was refluxed over and distilled from sodium hydroxide and zinc powder.

Pyridine was refluxed over potassium hydroxide for four hours and subsequently distilled. The fraction boiling at 115 °C was collected and stored over calciumoxide.

Tetrahydrofuran (THF) was refluxed for three hours over potassium hydroxide and next distilled from potassium hydroxide. Only freshly distilled THF was used for the experiments.

All other solvents were Baker analyzed quality.

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Heptafluorobutyric anhydride obtained from several commercial sources was refluxed for several hours with a large excess of P_2O_5 . Next, it was distilled from fresh P_2O_5 and stored at room temperature in a desiccator over P_2O_5 .

Silicagel (Merck, G) for thin-layer chromatography was washed three times with boiling methanol. After drying at 120 °C, the adsorbent was suitable for application.

Glass-ware was cleaned before use by washing it once with acetone and hexane respectively.

Insects

Colorado beetles (*Leptinotarsa decemlineata* Say) were reared on potato plants and stored before use at -25 °C in a refrigerator.

Apparatus

A Becker 409 gas chromatograph equipped with a ^{63}Ni electron capture detector and a 2 m glass coiled column, 4 mm in diameter (2.5% OV-17 on chromosorb W-AW HMDS 80/100 Mesh) was used for gas chromatographic analysis. The carrier gas (10% methane in argon) flow rate was 60 ml/min. Injector temperature 200 °C, oven temperature 170 °C and detector temperature 300 °C. The detector was pulsed with a pulse period of 1000 μ sec and a pulse width of 1 μ sec.

Samples were introduced into the column with a 10 μ l Hamilton syringe. The same gas chromatograph and a similar column was used for analyses with FID.

Methyl(2E, 6E)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate (JH III)

The JH III was synthesized according to the method of Anderson *et al.*⁶. The reaction of (E)-geranylacetone with trimethylphosphonoacetate provided (2Z/E, 6E)-methyl farnesoate. After separation of both isomers by chromatography (SiO_2 , toluene) JH III was formed from the (2E, 6E)-isomer *via* the 10,11-bromohydrin.

Methyl(2E, 6E)-10,11-dihydroxy-3,7,11-trimethyl dodecadienoate

The diol was prepared by treatment of JH III with a mixture of THF and 0.125 N H_2SO_4 (3:2). The diol was purified by chromatography (SiO_2 , chloroform/acetone = 9/1).

JH III-bisheptafluorobutyrate (JH III-bisHFB)

40 mg of the diol was dissolved in 10 ml hexane, 0.5 ml benzene - 5% pyridine and 0.5 ml HFBA

were added and the mixture was allowed to stand at room temperature for about one hour. TLC and GLC showed no detectable amount of the diol to be present. Subsequently the mixture was washed with water, 10% $NaHCO_3$ -solution and water again. After drying over $MgSO_4$ solvents were evaporated. Repeated column chromatography (SiO_2 , chloroform/acetone = 19/1), provided 70 mg of the bis-heptafluorobutyryl derivative

IR: γ^{CCl_4} : No OH vibrations; 1780 cm^{-1} (carbo-

O
||
nyl: O - C - C₃F₇) 1725 and 1650 cm^{-1}
(resp. carbonyl and double band of α ,
 β unsaturated-ester).

60 MHz NMR: $\delta_{TMS}^{CCl_4}$: 1.6 (9H, s, two C₁₁-CH₃, one C₇-CH₃); 1.9-2.1 (11H, C₃-CH₃, -CH₂-); 3.6 (3H, s, O-CH₃); 5.1 (1H, br s, C-6 vinyl H); 5.25 (1H, tr, C-10H); 5.55 (1H, br s, C-2 vinyl H).

MS: *m/e* 676, 11% (M⁺); 617, 6%; 248, 13%; 189, 12%; 135, 33%; 119, 9%; 114, 80%; 81, 100%.

The material was also checked for purity and thermostability by GCMS.

Work-up procedure

After clipping off the head of the Colorado beetles the haemolymph material was collected with glass-capillaries (5.3 g haemolymph from 300 insects). The haemolymph was chilled in ice and treated with a small amount of reduced glutathione in order to inhibit tyrosinase activity⁷.

The material was extracted with ether/ethanol (6/1) and ether according to the method described by De Wilde *et al.*⁷. The extracts were evaporated to dryness and chromatographed on thin-layer plates (0.30 mm, SiO_2 -G, 20 \times 20 cm, $CHCl_3$ /methanol = 9/1).

The JH-zone was scraped off the plate and the adsorbed material was extracted with ether.

The solvent was evaporated and 0.5 ml of a mixture of THF and 0.125 N H_2SO_4 (3:2) was added. After four hours a saturated NaCl-solution (0.5 ml) was added and the mixture was extracted five times with 1 ml ether. After evaporation of the solvents the material was brought onto a TLC plate (0.30 mm, SiO_2 -G, 20 \times 20 cm). The plate was developed in $CHCl_3$ /EtOAc = 2/1 and the JH-diol zone was scraped off. After extraction with ether the extract was mixed with 1 ml hexane, 50 μ l benzene - 5% pyridine and 50 μ l HFBA in a glass stoppered tube. After one hour the mixture was

evaporated in a N_2 -stream and the remaining material was put on a TLC plate (0.30 mm, SiO_2-G , 5×20 cm). The plate was developed in $CHCl_3$ /acetone = 9/1. The JH-bisHFB-zone was scraped off, the adsorbent extracted with ether and dried under N_2 . After adding a known amount of hexane the material was ready for GLC analysis.

At each TLC separation the 2,4-dinitro-phenylhydrazine of nonanal was used as marker. The position of the zones were calculated in relation to the R_F -value of the marker.

Results and Discussion

First, the linearity of the quantitative response of the electron capture detector towards JH III-bisHFB was determined by measurement of the peak areas after triplicate injections of the derivative in varying amounts from 25 pg to 5 ng. As shown in Fig. 2 the response was not linear over a large range.

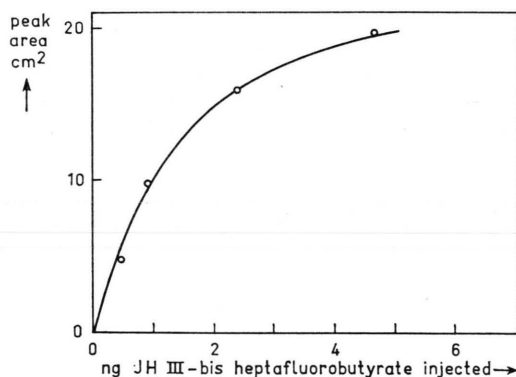


Fig. 2. Response of JH III-bisHFB toward ECD in the range 0–5 ng. The derivative was injected onto the column in 1 μ l aliquots. Att.=4.

However, the smaller region between 0 and 100 pg showed satisfactory linearity (Fig. 3).

Under these conditions as little as 20 pg of the JH III-bisHFB (*i.e.* 10 pg of the hormone) can be measured after chromatographic separation. Considering the amounts recovered following the complete procedure and the interference of the impurities (introduced by the blank) – which allows only the injection of 1/100 of the material at one time – the sensitivity of the method may be estimated at 1 ng/g haemolymph material (it should be noted, that 1 g haemolymph or less is sufficient for the determination). Measurements with pure JH III-diol show about 50% recovery after esterifi-

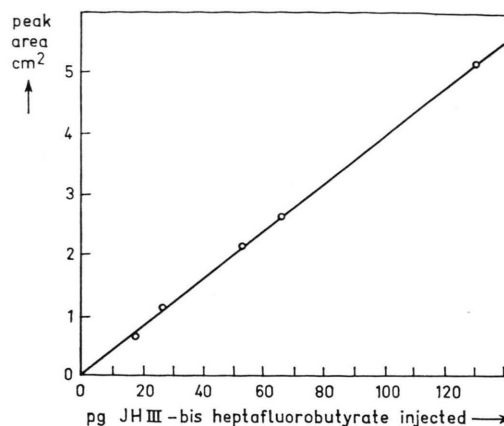


Fig. 3. Response of JH III-bisHFB toward ECD in the range 0–100 pg. The derivative was injected onto the column in 1 μ l aliquots. Att.=2.

cation and TLC, when working with submicrogram amounts of the diol. When adding a known amount of JH III (50 ng) to the haemolymph (1 g) and running through the complete procedure still a recovery of more than 40% was achieved. Fig. 4 shows the results of the procedure after adding a known amount (0–25 ng) of JH III to 0.6 g amounts of haemolymph material of the Colorado beetle.

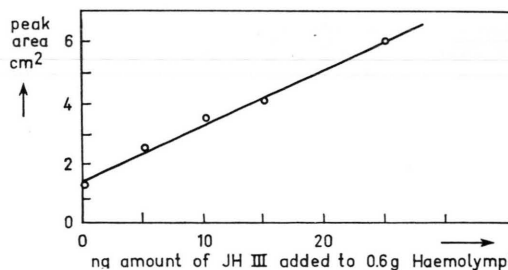


Fig. 4. Results after working up haemolymph material of the Colorado beetle with known amounts of JH III added. Samples of the resulted JH III-bisHFB (in 0.1 ml hexane) were injected in 1 μ l aliquots onto the column. Att.=2.

The above method allows a quantitative determination of JH III in biological material with an accuracy of 10%.

The amount of JH III present in the used haemolymph may be calculated as 17 ng/g haemolymph.

Experiments with mixtures of JH I, II and III under these conditions show an excellent separation of the three derivatives*.

* This work was performed by Miss L. Minderhoud, in our Department.

Thus, this method seems also suitable for the simultaneous identification and determination of the individual three known JH's in one sample.

The method described has several advantages as compared to previous methods: it is highly sensitive and rapid and needs relatively simple instrumentation. The method shall be further extended towards simultaneous detection of all three JH's. The possible presence of JH I, II and III in different de-

velopmental stages shall be subject of forthcoming papers.

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