Journal of Chromatography, 196 (1980) 425–434
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,889

GLASS CAPILLARY COLUMN GAS CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS QUANTITATIVE DETERMINATION OF INSECT JUVENILE HORMONES AT THE PICOGRAM LEVEL: A COMPARITIVE STUDY OF VARIOUS HALOGENATED DERIVATIVES

L. HUIBREGTSE-MINDERHOUT, A. C. VAN DER KERK-VAN HOOF, P. WIJKENS, H. W. A. BIESSELS and C. A. SALEMINK*

Laboratory of Organic Chemistry, State University, P.O. Box 5055, 3502 JB Utrecht (The Netherlands)

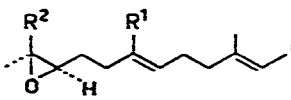
(Received April 15th, 1980)

SUMMARY

A gas chromatographic method has been developed for the simultaneous quantitative determination of the juvenile hormones I, II and III (JH-I, JH-II and JH-III) in insects. After extraction and partial purification the hormones are converted into their 10-heptafluorobutyryl-11-methoxy derivatives and analysed as such by gas chromatography. The gas chromatographic system consists of an all-glass moving needle injector, a glass capillary column and an electron-capture detector. JH-I, JH-II and JH-III can be determined simultaneously in amounts as low as 0.5, 0.3 and 0.2 ng, respectively, per sample. The 10-heptafluorobutyryl-11-methoxy derivatives were selected after an extensive comparative study of potentially suitable halogenated derivatives. Geometrical stereoisomers of the juvenile hormones are used as internal standards.

INTRODUCTION

At present three homologous juvenile hormones, *viz.*, JH-I, JH-II and JH-III (Fig. 1), are known from insects. Derivatization and subsequent gas chromatographic (GC) analysis using an electron-capture detector (ECD) has become a well known technique for determining the JHs qualitatively and quantitatively. Judy *et al.*[1] reported the analysis of JH-III after derivatization to the 10,11-ditrifluoroacetyl derivative. In our laboratory Van Broekhoven *et al.*[2] developed a method for determining JH-III using the 10,11-diheptafluorobutyryl derivative. The 10-(2,4-dichloro-



JH-I $R^1 = C_2H_5$; $R^2 = C_2H_5$
JH-II $R^1 = CH_3$; $R^2 = C_2H_5$
JH-III $R^1 = CH_3$; $R^2 = CH_3$

Fig. 1. Structural formulae of natural juvenile hormones.

0021-9673/80/0000–0000/$02.25

benzoyl)-11-methoxy derivatives were used by Peter *et al.*[3] in their non-simultaneous determination of JHs in *Manduca sexta*. Recently, Hagenguth and Rembold[4] presented a method incorporating the derivative that showed optimal characteristics in our comparative study, *viz.*, the 10-heptafluorobutyryl-11-methoxy product (preliminary communication by Huibregtse-Minderhoud *et al.*[5]). Earlier, Bergot and Schooley[6] reported the only method as yet using GC–ECD to determine JHs both quantitatively and simultaneously; they made use of the 10-pentafluorophenoxyacetyl-11-methoxy derivatives.

In all of the methods mentioned packed columns were used for GC analysis. In this work we used capillary columns. Two liquid stationary phases were used separately, making the method very reliable for quantitative analysis.

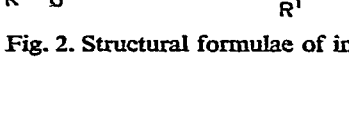
EXPERIMENTAL

Materials

Benzene, ethyl acetate, isopropanol, absolute methanol (all p.a. grade; Merck, Darmstadt, G.F.R.), toluene (p.a. grade; UCB, Brussels, Belgium), ethanol 96% and chloroform (analysed reagent; Baker, Phillipsburgh, NJ, U.S.A.) were used without further purification. Pyridine (p.a. grade; Merck) was distilled over potassium hydroxide before use. Diethyl ether (analytical reagent; Mallinckrodt, St. Louis, MO, U.S.A.) was used for treatment of samples containing JHs and/or their isomers. For all other purposes fresh diethyl ether (commercial quality) was passed through an alumina column (Woelm, Eschwege, G.F.R., neutral, activity 1) and distilled. Commercial-quality *n*-hexane was passed through a column containing one part of silica gel (Baker analysed reagent, 0.4–0.1 mm) and four parts of alumina (Woelm, neutral, activity 1) and distilled (distillation was stopped after 60% had been distilled). Acetone (commercial quality) and water were both distilled once.

Reagents for derivatization (p.a. grade or purity better than 97%) were perchloric acid (Merck), trifluoroacetic anhydride (Fluka, Buchs, Switzerland), pentafluoropropionic anhydride (ICN, Irwine, CA, U.S.A.), heptafluorobutyric anhydride (Macherey, Nagel & Co., Düren, G.F.R.; ampoules of 1 ml), pentadecafluorooctanoylchloride (ICN), chloroacetyl chloride (BDH, Poole, Great Britain), monochlorodifluoroacetic anhydride (ICN), monobromoacetic anhydride (prepared according to Vogel[7]), pentafluorobenzoyl chloride (Aldrich, Milwaukee, WI, U.S.A.), pentafluorophenoxyacetyl chloride (Aldrich; distilled once) and bromomethyldimethylchlorosilane (prepared according to Speier[8]).

(±)-JH-I and JH-II were synthesized according to Loew *et al.*[9] and (±)-JH-III according to Anderson *et al.*[10]. The (±)-10-*trans*-isomer of JH-II [Fig. 2 (4)] was obtained as a side-product from the Loew synthesis. The ethyl ester of the (±)-2*Z*-isomer of JH-III [Fig. 2 (5)] was synthesized by transesterification of (±)-2*Z*-JH-III, obtained as a side-product from the Anderson synthesis. Stock solutions were made in diethyl ether and in diethyl ether–*n*-hexane (1:1).



(4) $R^1 = H$; $R^2 = COOCH_3$; $R^3 = CH_3$; $R^4 = C_2H_5$

(5) $R^1 = COOC_2H_5$; $R^2 = H$; $R^3 = CH_3$; $R^4 = CH_3$

Fig. 2. Structural formulae of internal standards in JH analysis.

Instrumentation

Gas chromatography was performed on a Packard Becker 417 gas chromatograph equipped with a ^{63}Ni electron-capture detector (714), a "pulsed power supply" unit (734), an all-glass moving needle injector (Koppen, Best, The Netherlands) and a glass capillary column. Two columns were used separately: a 25 m × 0.25 mm I.D. wall-coated open-tubular (WCOT) SE-30 column, film thickness 0.4 μm (Chrompack, Vlissingen, The Netherlands), and a 40 m × 0.5 mm I.D. WCOT OV-17 column, film thickness 0.2 μm (manufactured by Lic. S. Räisänen, University of Helsinki, Finland). The column was connected to the injector by two layers of shrinkable PTFE. Connection with the detector was made with a platinum–iridium capillary, by which the effluent was lead just below the ^{63}Ni foil, minimizing the dead volume. Nitrogen (Hoekloos, Schiedam, The Netherlands, greenband quality) as carrier and make-up gas was passed through a molecular sieve filter and an oxygen trap. During analysis the injector, detector and column were kept at temperatures of 195, 300 and 216°C (SE-30) or 212°C (OV-17), respectively.

Gas chromatography with flame-ionization detection was carried out on a 2.0 m × 4 mm I.D. column packed with 3% SE-30 on Chrompack SA (80–100 mesh). Nitrogen was used as carrier and make-up gas. During analysis the injector, detector and column were kept at temperatures of 230, 350 and 230°C, respectively.

Liquid chromatography at atmospheric pressure was performed on silica gel columns (Baker analysed reagent, 0.4–0.1 mm). High-performance liquid chromatography (HPLC) was carried out on a Varian-4100 liquid chromatograph, equipped with a 0.30 m × 2.2 mm I.D. Micropak column (Varian, Palo Alto, CA, U.S.A.) and a 0.25 m × 6.0 mm I.D. Microporasil column (Waters Assoc., Milford, MA, U.S.A.) coupled in series.

Thin-layer chromatography (TLC) on the milligram scale was carried out with pre-coated silica gel 60 F_{254} plates (Merck). Chromatography of biological and pilot samples was carried out on home-made plates, coated with silica gel (Merck, TLC). Before application this silica gel had been washed five times with boiling methanol, dried for 15 h at 120°C, washed five times with diethyl ether and dried.

IR spectra were recorded with a Perkin-Elmer 283 infrared spectrometer (as a solution in carbon tetrachloride or as a liquid film, and NMR spectra were recorded with a Varian EM-390 NMR spectrometer (as a solution in carbon tetrachloride with tetramethylsilane as internal standard). Mass spectra were obtained with a Jeol JMS 07 mass spectrometer, which was coupled to a Jeol 1100 gas chromatograph by a double jet-separator.

All glassware was cleaned thoroughly with water, rinsed twice with acetone and *n*-hexane, dried in an oven and rinsed twice with diethyl ether just before use.

Insects

Locusts (*Locusta migratoria*) were reared under crowded conditions at 30°C, 40% relative humidity and a photoperiod of 12 h. Insects were fed with reed, supplemented with rolled oats.

Potato beetles (*Leptinotarsa decemlineata* Say) were reared on fresh potato leaves at 25°C and a photoperiod of 18 h, or were collected in the field in August (estimated photoperiod 14 h).

Wax moths (*Galleria mellonella*) were reared at 30°C, 60% relative humidity

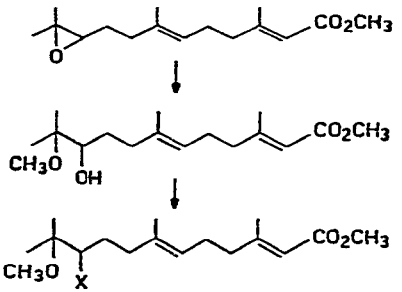
in the dark, on a diet containing honey, glycerol, flour of wheat, yeast flakes, beeswax and honeycomb.

Collection of haemolymph

After anaesthetizing the insects with carbon dioxide, samples of haemolymph were taken by puncturing the ventral membrane between the head and the thorax with a Hamilton microsyringe (locusts), by clipping off a hind leg (beetles) or by a puncture in the abdomen (moths). Haemolymph was kept at 0°C for 10 min at most. Some crystals of reduced glutathione were added to inhibit tyrosinase activity. After sampling, haemolymph was kept frozen at —30°C until extraction. Haemolymph samples were first extracted three times with diethyl ether–ethanol (6:1), then three times with diethyl ether, according to De Wilde *et al.*[11]. After combination of the organic phases two internal standards (I.S.) were added.

Preparation of derivatives on the milligram scale

The derivatization procedure consists in two successive steps (Fig. 3): firstly the formation of the 10-hydroxy-11-methoxy-JH (10-OH-11-MeO-JH) and secondly acylation of the hydroxyl function to introduce halogen atoms into the molecule, leading to the 10-X-11-MeO-JH (X contains one or more halogens).



Structure of X	Abbreviation	
CF_3CO_2-	TFA	(7)
$C_2F_5CO_2-$	PFP	(8)
$C_3F_7CO_2-$	HFB	(9)
$C_7F_{15}CO_2-$	PFO	(10)
CH_2ClCO_2-	MClA	(11)
$CClF_2CO_2-$	CldFA	(12)
CH_2BrCO_2-	MBrA	(13)
$C_6F_5CO_2-$	PFB	(14)
$C_6F_5OCH_2COO-$	PFPA	(15)
$CH_2Br(CH_3)_2SiO-$	BrMdMSi	(16)

Fig. 3. Synthesis of the halogenated derivatives of JH-III.

10-Hydroxy-11-methoxy derivatives. A solution of 220 mg of (±)-JH-III in 15 ml of 0.014% perchloric acid in methanol was stirred for 90 min at room temperature. Saturated sodium chloride solution was added and the mixture was extracted four times with ethyl acetate. The combined organic phases were washed once with 4% sodium hydrogen carbonate solution and once with water and dried over mag-

nesium sulphate. Liquid chromatography over silica (column 0.3 m × 20 mm I.D.) with toluene–ethyl acetate (1:1) yielded 206 mg (83%) of (6). Derivatives of JH-I and JH-II and of the isomers were prepared similarly, starting from about 15 mg. All products were characterized by IR and NMR spectroscopy as described by Huibregtse-Minderhoud[12]. Stock solutions were prepared in *n*-hexane–ethyl acetate (1:1).

Halogenated derivatives of JH-III. All derivatization reactions were performed in a nitrogen atmosphere for 1 h at room temperature, unless stated otherwise.

10-TFA-11-MeO-JH-III (7). One millilitre of benzene–pyridine (95:5), 1.5 ml of trifluoroacetic anhydride and a solution of 15 mg of (6) in 10 ml of *n*-hexane were allowed to react. After extraction four times with *n*-hexane–water the combined organic phases were washed with 4% sodium hydrogen carbonate solution, then with water and dried over magnesium sulphate. By purification of the residue by TLC (toluene–ethyl acetate, 4:1), (7) was obtained in 85% yield. IR: 1785 (α-F–C=O), 1720 (C=C–C=O). MS: 394 (2; M^+); 280 (0.7; $M^+ - CF_3CO_2H$); 248 (7; $M^+ - CH_3OH - CF_3CO_2H$); 73 (100). NMR in accordance with (9).

10-PFP-11-MeO-JH-III (8). (8) was synthesized in a similar manner to (7), using pentafluoropropionic anhydride, in 72% yield. IR: 1782 (α-F-C=O), 1720 (C=C–C=O). MS: 444 (1; M^+); 280 (5; $M^+ C_2F_5CO_2H$); 248 (4; $M^+ - CH_3OH - C_2F_5CO_2H$); 73 (100). NMR in accordance with (9).

10-HFB-11-MeO-JH-III (9). (9) was synthesized in a similar manner to (7), using heptafluorobutyric anhydride, in 94% yield. Spectral data have been described[5].

10-PFO-11-MeO-JH-III (10). (10) was synthesized in a similar manner to (7), using benzene–pyridine (95:5) as a solvent and perfluorooctanoyl chloride. The reaction mixture was extracted with ethyl acetate–water. The yield was 65%. IR: 1785 (α-F–C=O), 1725 (C=C–C=O). MS: 694 (2; M^+); 280 (0.5; $M^+ - C_7F_{15}CO_2H$); 248 (6; $M^+ - CH_3OH - C_7F_{15}CO_2H$); 73 (100). NMR in accordance with (9).

10-MClA-11-MeO-JH-III (11). (11) was prepared in 80% yield in a similar manner to (7), using chloroacetyl chloride. IR: 1768 (α-Cl–C=O), 1725 (C=C–C=O). MS: 374 and 376 (0.3 and 0.2; M^+); 280 (0.2; $M^+ - CH_2ClCO_2H$); 248 (2; $M^+ - CH_3OH - CH_2ClCO_2H$); 73 (100). NMR in accordance with (9); additional signal, δ 4.00 (s, 2H, CH_2Cl).

10-CldFA-11-MeO-JH-III (12). (12) was obtained in 99% yield in a similar manner to (7), using monochlorodifluoroacetic anhydride. IR: 1783 (α-halogen–C=O); 1725 (C=C–C=O). MS: 410 and 412 (1 and 0.4, M^+); 280 (0.3; $M^+ - CClF_2CO_2H$); 248 (4; $M^+ - CH_3OH - CClF_2CO_2H$); 73 (100). NMR in accordance with (9).

10-MBrA-11-MeO-JH-III (13). (13) was obtained in 78% yield in a similar manner to (7), after reaction of (6) with monobromoacetic anhydride for 2 h in benzene–pyridine (9:1). IR: 1735 (α-Br–C=O), 1718 (C=C–C=O). MS: 418 and 420 (0.2 and 0.2; M^+); 280 (0.3; $M^+ - CH_2BrCO_2H$); 248 (2; $M^+ - CH_3OH - CH_2BrCO_2H$); 73 (100). NMR in accordance with (9); additional signal, δ 3.75 (s, 2H, CH_2Br).

10-PFB-11-MeO-JH-III (14). (14) was prepared in 55% yield in a similar manner to (7), using pentafluorobenzoyl chloride and a reaction time of 2 h. IR: 1741 (O–C=O), 1723 (C=C–C=O). MS: 492 (0.6; M^+); 280 (0.2; $M^+ - C_6F_5CO_2H$); 248 (3; $M^+ - CH_2OH - C_6F_5CO_2H$); 73 (100). NMR in accordance with (9).

10-PFPA-11-MeO-JH-III (15). (15) was prepared in 84% yield in a 15-min reaction at 70°C using pentafluorophenoxyacetyl chloride. IR: 1756 (O–C=O); 1718 (C=C–C=O). MS: 522 (0.3; M^+), 280 (0.3; $M^+-C_6F_5OCH_2CO_2H$); 248 (3; $M^+-CH_3OH-C_6F_5OCH_2CO_2H$); 73 (100). NMR in accordance with (9); additional signal, δ 4.78 (br.s, 2H, OCH_2CO_2).

10-BrMdMSi-11-MeO-JH-III (16). (16) was prepared in benzene–pyridine–bromomethyldimethylchlorosilane (2:1:2) for 2 h. The reaction product was purified by liquid chromatography (silica gel, toluene). The yield was 76%. IR: 1720 (C=C–C=O). MS: 433 and 435 (0.2 and 0.2; M^+-CH_3); 375 and 377 [3 and 3; $M^+-C(CH_3)_2OCH_3$]. NMR in accordance with (9), but δ 1.01 (s, 6H, 2× CH_3C_{11}) and δ 3.39 (m, 1H, HC_{10}); additional signals, δ 0.23 [s, 6H, $Si(CH_3)_2$] and δ 2.42 (s, 2H, CH_2Br).

10-HFB-11-MeO-derivative of JH-II, of JH-I, of (4) and of (5). Amounts of 10–15 mg of the 10-OH-11-MeO derivatives were converted into their 10-HFB-11-MeO derivatives according to the preparation of (7) in 80–95% yield. Spectral data have been described[5,12].

Pre-treatment of haemolymph extracts

Solvent was removed in a stream of nitrogen. The residue was dissolved in a small volume of diethyl ether and subjected to two successive TLC separations. In the first separation with chloroform–methanol (9:1) the 2,4-dinitrophenylhydrazone (DNPH) of nonanal was used as a marker substance (R_F 0.81) and the zone with R_F value 0.63–0.87 was scraped off and extracted with diethyl ether. After removing the solvent with nitrogen the residue was subjected to TLC with *n*-hexane–chloroform–ethyl acetate (7:7:1) and the 2,4-DNPH of ethanal as the marker substance (R_F 0.43). The zone with R_F value 0.29–0.54 was scraped off and extracted with diethyl ether. The extract was stored at −30°C until derivatization.

Derivatization and analysis of purified haemolymph extracts

The purified extract was freed from solvent with nitrogen and to the residue 0.5 ml 0.014% perchloric acid in methanol was added. The mixture was allowed to stand at 0°C and occasionally stirred with a vortex mixer. After 45 min, 0.5 ml of saturated sodium chloride solution was added and the mixture extracted five times with diethyl ether. The organic phases were combined, the solvents removed in a stream of nitrogen and the resulting residue was subjected to TLC. After development with toluene–ethyl acetate (9:1) and nonanal DNPH as marker substance (R_F 0.68), the zone with R_F value 0.03–0.23 was scraped off and extracted with diethyl ether.

The residue of the diethyl ether extract was dissolved in 0.2 ml of *n*-hexane, 10 μl of benzene–pyridine (95:5) and 10 μl of HFBA were added, and the reaction mixture was allowed to stand at room temperature, with occasional stirring with a vortex mixer. After 1 h, 0.5 ml of water was added to convert the excess of HFBA into the acid (according to Lawrence *et al.*[13]), 0.8 ml of *n*-hexane was added and the two phases were mixed thoroughly. After 5 min the upper layer was removed and the lower layer extracted three times with *n*-hexane. The residue of the combined organic phases was subjected to TLC. After development with toluene–ethyl acetate (9:1, v/v), with cyclopentanone DNPH as marker (R_F 0.53), the zone with R_F value 0.38–0.61 was scraped off and eluted with diethyl ether. A further HPLC sample clean-up

was necessary for samples containing less than 5 ng of JH. With *n*-hexane–ethyl acetate–isopropanol (970:30:0.25) as a solvent the fraction of volume 15 ml eluting between 7.00 and 15.00 min after injection was collected. After concentration to 50–500 μl (depending on the amounts of JHs present), 2 μl were injected on to the capillary column with a 10-μl Hamilton syringe by means of the all-glass moving needle injector.

Each series of samples was accompanied by a blank (starting with an amount of solvent necessary for extraction of haemolymph) and a pilot sample (fraction with known amounts of all hormones and I.S.s added to a hormone-free haemolymph extract of *Galleria mellonella* pupae or to a volume of solvent). Each sample was analysed three times by GC. The amount of hormone was calculated from the peak heights by comparison with those of authentic JH derivatives and internal standards.

RESULTS AND DISCUSSION

Comparative gas chromatographic study of halogenated derivatives

Table I gives the GC data obtained after testing the synthesized derivatives on an SE-30 packed column with FID and on an SE-30 capillary column with ECD. In no instance did the results of the packed column GC show adsorption or decomposition of the injected JH derivatives, although the retention times of the MBrA, PFB, PFPA and BrMdMSi derivatives were rather long. However, analysis on packed columns showed a serious drawback: blank samples analysed in this way with ECD produced gas chromatograms with numerous interfering peaks that could not be resolved from signals due to JH derivatives by varying the temperature or column conditions. The much better separating power of capillary columns resolved this problem almost completely, but the retention times increased considerably on the SE-30 capillary column. It was impossible to shorten the retention times by increasing the column temperature above 230°C because of rapid column deterioration.

Another possibility for decreasing the retention times would be to use a thin liquid film capillary column. However, this would give unreliable results at picogram levels and was not tried. Besides, we already had experience[5] that the di-HFB deriva-

TABLE I

GAS CHROMATOGRAPHIC DATA FOR THE 10-X-11-MeO-JH-III DERIVATIVES (REFERENCE, 10-HFB-11-MeO-JH-III)

Derivative	*Relative retention time, SE-30-phase*		*Relative response capillary column ECD*
	Packed column FID	*Capillary column ECD*	
TFA	0.89	0.91	0.03
PFP	0.91	0.93	0.73
HFB	1.00 (= 2.5 min)*	1.00 (= 10 min*)	1.00
PFO	1.55	1.55	1.70
MClA	2.80	2.60	—
CldFA	1.40	1.49	0.80
MBrA	3.60	—	—
PFB	4.80	2.90	—
PFPA	7.30	—	—
BrMdMSi	3.50	—	—

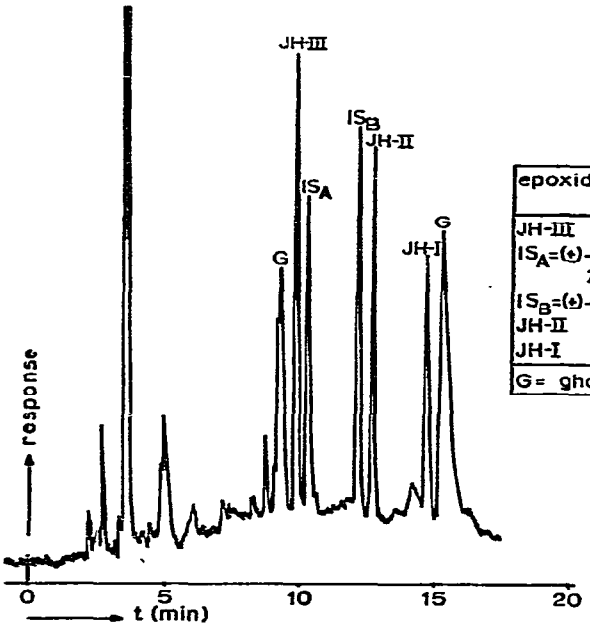
* Absolute retention times.

tives of JH-I and JH-II decomposed under the capillary GC conditions necessary to elute them within a reasonable time, whereas they were stable under packed column conditions. As it turned out, MBrA, PFPA and BrMDMSi derivatives did not produce any signal on injection of 2000 pg on to the capillary column, possibly because of adsorption in the GC system. The MClA and PFB derivatives showed considerable deterioration, excluding them from being useful in JH analysis under these circumstances. The other derivatives tested showed good chromatographic properties but the response of TFA derivative was extremely poor. Eklund *et al.*[14] have found that TFA derivatives of sugars could be used in sugar analysis only after extensive and careful pre-treatment of the capillary column.

Of the remaining derivatives, the PFO derivative is the most promising, having the highest ECD response and eluting within a reasonable time. In our capillary system, however, two interfering peaks are still present, almost certainly due to the use of the all-glass moving needle injector at room temperature. The measures taken by Franken[15] and Evrard *et al.*[16] might give a solution to this. One of these peaks appears almost simultaneously with that due to 10-PFO-11-MeO-JH-III. The same holds for the corresponding CldF derivative. For these reasons we decided to investigate the 10-HFB derivatives of JH-I, JH-II and JH-III more closely for possible use in JH analysis.

Gas chromatography of the 10-HFB-11-MeO derivatives

Under the circumstances mentioned the 10-HFB-11-MeO derivatives were completely separated on an SE-30 (see Fig. 4) and an OV-17 column. On OV-17 no



epoxide	10-HFB-11-MeO-product (pg)
JH-III	118
IS$_A$=(±)-ethyl-2Z-JH-III	90
IS$_B$=(±)-10-trans-JH-II	106
JH-II	117
JH-I	110
G= ghost peak	

Fig. 4. Gas chromatogram after injection of a standard solution. Conditions: 25 m × 0.25 mm I.D. SE-30 column, ECD with moving needle injector; T_{col}, T_{inj} and T_{det} 216, 195 and 300°C, respectively.

baseline separation of the JH-I derivative and one of the interfering peaks could be achieved, making this column unsuitable for JH-I analysis.

Quantitative determination

The accuracy of the measurements, defined as the deviation from the true value at the level of 5 ng per sample ($n = 10$), was found to be +8, +2 and +2% for JH-I, JH-II and JH-III, respectively. The precision, expressed as the standard deviation, was 12, 8 and 8%, respectively.

Limits of detection

Injection of standard solutions showed that 2.5 pg of HFB derivatives can be detected with a signal-to-noise ratio of 10:1. From this value a detection limit of about 0.01 ng of JH per sample is calculated. For biological samples, however, the lowest amounts of JH-I, JH-II and JH-III that can be detected are 0.5, 0.3 and 0.2 ng per sample, respectively.

Applications

The method was applied to two insect species. *Leptinotarsa decemlineata* (adult males and females, pre-diapause) was found to contain 0.48 ng of JH-III per animal, and no JH-I and JH-II could be detected. This is in good agreement with literature values[17]. In haemolymph of *L. decemlineata* (males and females, post-diapause, long-day conditions) JH-I, JH-II and JH-III were present in amounts of 0.45, 0.52 and 32 ng/g, respectively.

In *Locusta migratoria* (female adults of different ages) neither JH-I nor JH-II could be detected. The JH-III titre increased gradually from 4.0 ng/g haemolymph at the second day of the adult stage to of 60–90 ng/g when the insects were 15–23 days old. The method has also been used[18] to determine JH titres in haemolymph of 5th-stage larvae of *L. migratoria*, after implantation of active corpora allata.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. H. Schooneveld, Dr. C. A. D. de Kort and Prof. Dr. J. de Wilde of the Laboratory of Entomology, Agricultural University, Wageningen, The Netherlands, and to Drs. M. A. M. van den Hondel-Franken, Dr. D. J. van den Horst and Prof. Dr. A. M. Th. Beenakkers of the Laboratory of Chemical Animal Physiology, State University of Utrecht, Utrecht, The Netherlands, for their gifts of haemolymph of *Leptinotarsa decemlineata* and *Locusta migratoria*, respectively. They express their gratitude to Lic. S. Räisänen, Instrument Centre for Helsinki, University of Helsinki, Helsinki, Finland, for his gift of an OV-17 capillary column.

REFERENCES

1 K. J. Judy, D. A. Schooley, L. L. Dunham, M. S. Hall, B. J. Bergot and J. B. Siddall, *Proc. Nat. Acad. Sci. U.S.*, 70 (1973) 1509.
2 L. W. van Broekhoven, A. C. van der Kerk-van Hoof and C. A. Salemink, *Z. Naturforsch., C.*, 30 (1975) 726.
3 M. G. Peter, K. H. Dahm and H. Röller, *Z. Naturforsch. C.*, 31 (1976) 129.

4 H. Hagenguth and H. Rembold, *J. Chromatogr.*, 170 (1979) 175.
5 L. Huibregtse-Minderhoud, A. C. van der Kerk-van Hoof, P. Wijkens, H. W. A. Biessels and C. A. Salemink, *Rec. Trav. Chim. Pays-Bas*, 98 (1979) 421.
6 B. J. Bergot and D. A. Schooley, *Life Sci.*, 18 (1976) 811.
7 A. I. Vogel, *A Textbook of Practical Organic Chemistry Including Qualitive Organic Analysis*, Longmans, London, 1970, p. 428.
8 J. L. Speier, *J. Amer. Chem. Soc.*, 73 (1961) 826.
9 P. Loew, J. B. Siddall, V. L. Spain and L. Werthemann, *Proc. Nat. Acad. Sci. U.S.*, 67 (1970) 1824.
10 R. J. Anderson, G. A. Henrich, J. B. Siddall and R. Zurflüh, *J. Amer. Chem. Soc.*, 94 (1972) 5379.
11 J. de Wilde, G. B. Staal, C. A. D. de Kort, A. de Loof and G. Baard, *Proc. Kon. Ned. Akad. Wetensch., Ser. C*, 71 (1968) 321.
12 L. Huibregtse-Minderhoud, *Simultane Kwantitatieve Bepaling van de Juveniel Hormonen I, II en III in Insekten, Thesis*, Utrecht, 1979.
13 J. F. Lawrence, D. A. Lewis and H. A. Mcleod, *J. Chromatogr.*, 138 (1977) 143.
14 G. Eklund, B. Josefsson and C. Roos, *J. Chromatogr.*, 142 (1977) 575.
15 J. J. Franken, personal communication, 1977.
16 E. Evrard, C. Razzouk, M. Roberfroid, M. Mercier and M. Bal, *J. Chromatogr.*, 161 (1978) 97.
17 17 M. G. Peter, H. W. A. Biessels, K. R. Seshan, H. Röller, G. Bhaskaran and K. H. Dahm, *Abstr. Pap., 172nd Amer. Chem. Soc. Mtg. Pest. Div.*, No. 040 (1976).
18 L. Huibregtse-Minderhoud, M. A. M. van den Hondel-Franken, A. C. van der Kerk-van Hoof, H. W. A. Biessels, C. A. Salemink, D. J. van der Horst and A. M. Th. Beenakkers, *Insect Biochem.*, in press.