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METHOD FOR QUANTITATIVE DETERMINATION OF THE FOUR KNOWN JUVENILE HORMONES IN INSECT TISSUE USING GAS CHROMATOGRAPHY–MASS SPECTROSCOPY

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SUMMARY

A rapid, specific, and sensitive method is described for the extraction, purification, and quantitative determination of the three known juvenile hormones in insect tissue, using combined gas chromatography–mass spectroscopy (GC–MS) in the electron impact mode with selected ion-monitoring (SIM) for detection. A fourth, new, naturally occurring juvenile hormone was detected (and subsequently identified by other means) in this way. After initial extraction of biological material, a fast and efficient purification sequence utilizing non-aqueous reversed-phase and alumina mini-column chromatography achieves quickly a 10^3–10^4 purification factor. Derivatization for analysis by acid-catalyzed methan-d_3-olysis of the 10,11-epoxide moiety, followed by liquid chromatography separation of the resultant 11-methoxy-d_3-10-hydroxy (methoxyhydrin) derivatives, completes the sample workup. The methoxyhydrins are then directly analyzed by GC–MS (SIM). A tritium-labeled analogue is added during the extraction as an internal standard, which allows determination of recovery for each sample and simultaneously assures quantitative accuracy. Recoveries are 75–80%, and detection limits of 10–40 pg per gram of tissue are normally achievable.

INTRODUCTION

Several chemical or spectral methods currently exist for the isolation and simultaneous quantification of the insect juvenile hormones (JH) 2–4. Using gas chromatography–electron capture detection (GC–ECD) Bergot *et al.*[1] and Peter *et al.*[2] were able to identify the JHs from insect tissue after appropriate derivatization to electrophoric aromatic esters. More recently, Hagenguth and Rembold[3], and Huibregtse-Minderhoud *et al.*[4] employed much the same methodology, using a heptafluorobutyryl derivative for ECD. Earlier, Van Broekhoven *et al.*[5] developed an ECD method, applicable to JH III only, employing a bis-heptafluorobutyryl ester derivative of the JH diol for detection. Although these methods offer excellent absolute sensitivities, sample preparation is lengthy and tedious, with moderately low

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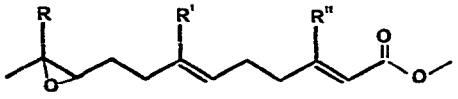
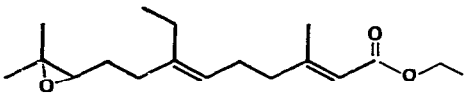


1 $R = R' = R'' = C_2H_5$ (JH 0)
2 $R = R' = C_2H_5$, $R'' = CH_3$ (JH I)
3 $R = C_2H_5$, $R' = R'' = CH_3$ (JH II)
4 $R = R' = R'' = CH_3$ (JH III)



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recoveries. More importantly, the lack of sufficient specificity of detection inherent in electrophoric derivatization schemes necessitates analysis on several packed (or capillary) columns before a final quantitative and qualitative determination of JH can be made with any degree of confidence.

JH determination by GC–MS, on the other hand, offers better selectivity of detection and requires less derivatization and cleanup. The first such method reported by Bieber *et al.*[6] suffered from low sensitivity and was applicable to JH I only. Subsequently, Trautmann *et al.*[7] developed a method of analysis for JH I, II, and III with a good limit of detection (0.1 ng/g), but requiring kilogram samples of insect tissue. Recently Mauchamp *et al.*[8] described a method of analysis for JH I with good sensitivity, but requiring selected ion-monitoring (SIM) of three ions for JH I due to a relatively low degree of sample cleanup. Lanzrein *et al.*[9] reported an isolation procedure for JH I–III from cockroach hemolymph using chemical ionization (CI) GC–MS for qualitative identification. Recently Rembold *et al.*[10] reported a procedure using glass capillary GC–MS to detect JH derivatives formed by solvolysis of the epoxide moieties with nonafluorohexanol; the procedure affords excellent sensitivities with small samples and apparently requires rather few purification steps, though full experimental details were absent. We now wish to report full procedural details of an improved assay using GC–MS, offering a high degree of specificity and sensitivity, for the simultaneous identification and quantification of JH I, II, III, and the recently discovered[11] JH 0, on the sub-picomole level. We require a derivatization step which converts JHs to their 11-methoxy-d_3-10-hydroxy (MH) derivatives in good yield. Final analysis of the purified MHs uses a GC–quadrupole mass spectrometer in the SIM mode. We add a tritiated analogue of JH on tissue extraction for use as an internal standard for quantitative purposes, and for monitoring recoveries at any stage of the workup. Because of sensitivities attained, only gram-quantities of insect tissue are required for the assay.

This method has now been tested with hundreds of samples from several insect species, and has proven both remarkably specific for JH detection and general in its applicability. The versatility of this method is most vividly illustrated by our ability to detect, then subsequently identify by other means, the new, naturally occurring juvenile hormone, JH 0, the details of which are reported elsewhere[11].

MATERIALS AND METHODS

Reagents

Acetonitrile was "pesticide grade, distilled-in-glass" (Burdick & Jackson Labs., Muskegon, MI, U.S.A.); pentane (technical, Phillips Petroleum, Bartlesville, OK, U.S.A.) and diethyl ether (analytical reagent, Mallinckrodt, St. Louis, MO, U.S.A.) were distilled through a glass helix-packed column before use. Dichloromethane (analytical reagent, Mallinckrodt) was purified as previously described[1]. Methanol-d_4 (99.9%) and trifluoroacetic acid (Aldrich, Milwaukee, WI, U.S.A.) were used as received. Neutral alumina (Woelm, Eschwege, G.F.R.) was heated to 600°C overnight, then supplied with 6% of its weight in water to bring the absorbent to activity III. Sodium chloride and Celite (Johns-Manville, Denver, CO, U.S.A.) were heated as above before use. All glassware was rinsed with deionized water, and heated at 500°C for 8–16 h before using for trace analysis.

Thin-layer chromatography

All thin-layer chromatographic (TLC) separations were performed on 5 × 20 cm, 0.5 mm thick, pre-coated plates of silica gel GF (Analtech, Newark, DE, U.S.A.). Plates were pre-washed by development in methanol and air-dried before use.

Liquid chromatography

Liquid chromatography (LC) was performed on a modular unit as previously described[1]. The column was prepacked silica gel (μPorasil, 30 × 0.4 cm I.D., Waters Assoc., Milford, MA, U.S.A.)*, and the eluting solvent was 3.4% diethyl ether in dichloromethane, 50% water-saturated; flow-rates were 1.5 ml/min at 24 atm, with peak detection by UV at 254 nm and 0.16–0.32 a.u.f.s.

Gas chromatography–mass spectroscopy

GC–MS was carried out on a Hewlett-Packard Model 5985 quadrupole mass spectrometer–computer system (electron impact, 70 eV, ion source temperature 200°C, emission current 300 μA, electron multiplier potential 2400–3000 V), using the appropriate SIM program. Other instrument operating parameters were optimized using perfluorotributylamine (PFTBA) standard. GC columns were 1.8 m × 2 mm I.D. packed with either 3% OV-225 Chromosorb W AW DMCS or Ultra-Bond II. (Supelco, Bellefonte, PA, U.S.A.). Separations were performed using a programmed temperature mode (190–230°C, Ultra-Bond II, 215–240°C, OV-225) at 4°C/min. Total time for elution was 5–6 min. Earlier a Hewlett-Packard Model 5984 GC–MS–data system unit was employed for titer determinations. Serious problems were encountered with peak tailing and inadequate precision and reproducibility, attributed to an inherently reactive interface between the chromatograph and the spectrometer, as well as inadequate ion source temperature control.

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* We find that a LiChrosorb SI 100 column (25 × 0.46 cm, Brownlee Labs., Santa Clara, CA, U.S.A.) is directly interchangeable with μPorasil. No modification of mobile phase or marker compounds is required, and higher column efficiency is obtained.

Standards

JH 0, I, II, and III were obtained from the Zoecon Chemical Research group and purified by micro-preparative LC. Methyl (2*E*)-6,7-epoxy-3,7-dimethyl-oct-2-enoate (epoxy methyl geranoate, EMG) was prepared by epoxidation with *m*-chloroperbenzoic acid of methyl (2*E*,*Z*)-geranoate (gift of Dr. R. J. Anderson, Zoecon), followed by preparative LC to remove the (2*Z*)-isomer.

N,N-Diethyl-N′-phenylurea and N-*sec.*-butyl-N′-phenylurea, the LC marker compounds, were obtained by reaction of the appropriate amine with phenylisocyanate, and recrystallization of the crude product.

Internal standard

Both tritiated and non-labeled samples of the internal standard were prepared from a common intermediate using a scheme adopted from a stereoselective synthesis of JH I (ref. 12). 2,2-Dimethoxy-3-chloro-3-methylbutane, prepared as described[13], was reacted with 0.1 equivalent of methyl (2*E*)-6-hydroxy-3-methyl-7-methylene-2-nonenoate (gift of Dr. R. J. Anderson, intermediate 3 in ref. 12) with acidic catalysis, in refluxing toluene[12]. The product, methyl (2*E*,6*E*)-11-chloro-3,11-dimethyl-7-ethyl-10-oxo-2,6-tridecadienoate, was purified first on a silica open column eluted with 10% diethyl ether in hexane, and then rigorously purified by preparative LC (Zorbax SIL 22 × 0.79 cm, eluted with 3% diethyl ether in pentane at 9.8 ml/min). A portion of this pure chloroketone was reduced with excess $NaBH_4$ in methanol to a chlorohydrin, which was isolated and treated subsequently with dipotassium carbonate in methanol[12] to give the desired epoxide, methyl (2*E*,6*E*)-10,11-epoxy-3,11-dimethyl-7-ethyl-2,6-dodecadienoate, an isomer of JH II ("iso-JH II").

To prepare the labeled sample of iso-JH II, the above chloroketone was reduced with NaB^3H_4 (performed by New England Nuclear, Boston, MA, U.S.A.). The labeled chlorohydrin spontaneously cyclized during the reduction (apparently due to contamination of the NaB^3H_4 with base) to give the [10-^{3}H]epoxide directly. The crude labeled epoxide was purified by TLC (15% ethyl acetate in hexane, single development) to remove polar radio-labeled impurities.

Samples of nonlabeled and [10-^{3}H]iso-JH II were transesterified by treatment with dry ethanol in the presence of sodium cyanide according to Mori *et al.*[14] to afford the internal standard (IS, 5), iso-JH II ethyl ester. Each sample was first purified by TLC (as above), then by LC (Zorbax SIL, 22 × 0.46 cm, eluted with 7% diethyl ether in pentane). Final chemical and radiochemical purities were ⩾99%, as determined by LC using the above conditions.

The specific activity of [10-^{3}H]IS was determined by methane CI mass spectroscopy in the SIM mode, monitoring ions at *m/z* 323 $(M + C_2H_5)^+$, *m/z* 249 $(M + H - C_2H_5OH)^+$, and their corresponding isotopic ions at *m/z* 325 and 251. The ratio of ^3H:^1H was determined as 0.234, corresponding to a specific activity of 6.78 Ci/mmol (51,000 dpm/ng).

Preparation of methoxy-d_3-hydrin standards

Milligram amounts of JHs and IS were converted to the respective MH derivatives by stirring in 0.08 *M* d_4-methanolic trifluoroacetic acid (1.5 h, 20°C), quenching with 10 μl of 2 *M* aqueous potassium hydrogen carbonate, and purifying

by TLC. Standard solutions, in acetonitrile, were then obtained by serial dilution of stock preparations, and stored over sodium hydrogen carbonate at −20°C.

Insects

Colonies of the tobacco hornworm, *Manduca sexta*, and the tobacco budworm, *Heliothis virescens*, were reared on artificial diets for many generations. The diet for the former was prepared according to Yamamoto[15], while the diet for the latter was based on lima beans and split peas. Specimens from carefully synchronized stages were selected for workup and chemical assay.

Extraction, purification, derivatization and analysis of JH from insect tissue

Hemolymph processing. A volume of 3–6 ml of hemolymph is collected in increments in a chilled 13-ml centrifuge tube containing ≈5 ml acetonitrile and the requisite amount of [^{3}H]IS. The tube is agitated on a Vortex mixer between incremental additions of hemolymph, then briefly centrifuged to sediment precipitated solids. The supernatant is transferred to a separatory funnel containing 40 ml of pentane and 150 ml of 4% aqueous sodium chloride, and the tube contents then extracted three times with 5 ml of acetonitrile. The combined supernatants are added to the separatory funnel, shaken for a few minutes, the aqueous layer removed, and the organic phase washed with a 50-ml portion of brine. Upon solvent removal of the separated organic layer, an oily pentane-soluble residue (2–30 mg) is obtained, which is transferred in pentane to a 1-ml conical bottom vial (Microflex tube, Kontes, Vineland, NJ, U.S.A.). After solvent removal, the contents are vigorously agitated with 5 × 100 μl of acetonitrile, and each portion of acetonitrile is pipetted onto a pre-washed (5 ml acetonitrile) C_{18} Sep-Pak cartridge (Waters Assoc.). After the cartridge is loaded, 2.2 ml of acetonitrile is pumped through by means of a 2500-μl gas-tight syringe (Hamilton Reno, NV, U.S.A.). The eluent, containing the JH fraction (≤1 mg), is concentrated to dryness, then redissolved with a small (200 μl) volume of pentane.

The pentane solution is carefully transferred —with rinses— to a small (6 × 40 mm) glass column fashioned from a Pasteur pipet which is packed with 1.0 g alumina (Woelm, activity grade III), previously washed with ether. A 2.0-ml portion of diethyl ether is then percolated through the column, and the eluent, containing the JHs and IS is bulked and reserved for subsequent methanolysis.

The residue is supplied with ≈20 μg of EMG diluent, and the derivatization carried out in 150 μl of 0.08 *M* d_3-methanolic trifluoroacetic acid. Reaction conditions are as described for *Preparation of methoxy-d_3-hydrin standards*, exept after quenching all solvent is evaporated under vacuum. Ether is added and evaporated (to remove residual methanol), the residue is rinsed several times with ether, and the washings (filtered if necessary through a pre-washed plug of cotton) are evaporated with a stream of nitrogen, then supplied with 1–2 μg of the phenylurea markers, redissolved in dichloromethane and chromatographed on LC as described. The MH zone is collected, concentrated, and the MHs are transferred to 1-ml Microflex tubes by acetonitrile rinsing for GC–MS analysis. Usually 10–20% of a 40 μl solution is then injected on-column.

Whole-body processing. An amount of 5–15 g of insects, anesthetized with carbon dioxide if necessary, is placed in a 100-ml blending flask containing ≈40 ml acetonitrile and an amount of Celite equal to ≈1/3 of the sample weight of insects.

The IS is added, the glass homogenizer cup chilled in an ice bath, and the contents then homogenized using a high-speed blender (VirTis Model 23) for 3–5 min. The homogenate is filtered through a Buchner-type fritted glass funnel with 14/20 ground joint (Ace Glass 9439-08), and the cake is returned to the cup for a second homogenization. The combined filtrates are evaporated *in vacuo* (cautiously to avoid bumping or foaming) to ≈20 ml, then decanted into a separatory funnel for the pentane-brine partition. Further processing of the recovered lipoidal mass (usually 20–150 mg) is as described under *Hemolymph processing*.

Quantification. Titer data were derived from peak area values using an internal standard technique. A response factor (K), reflecting differences in ionization efficiencies among the various species of interest, was calculated for each JH from the following formula: $K = A_{JH}/A_{IS} \times W_{IS}/W_{JH}$, where A is the peak area and W a known mass of the MH derivative of the JH and the internal standard, respectively. Titer levels were subsequently calculated from the relationship $[C_{JH}] = A_{JH}/A_{IS} \times [C_{IS}]/K$, where $[C]$ is the concentration expressed in ng/g or ng/ml, derived from the known amount of IS added to the original mass (or volume) of insect tissue.

RESULTS

Isolation and purification

Because of the inherent specificity of the ion-monitoring detection method, we were able to develop a substantially shortened extraction and purification protocol essentially based on that utilized in our earlier GLC–ECD procedure[1]. Fig. 1 depicts the flow scheme for tissue processing that we ultimately have refined. The excellent

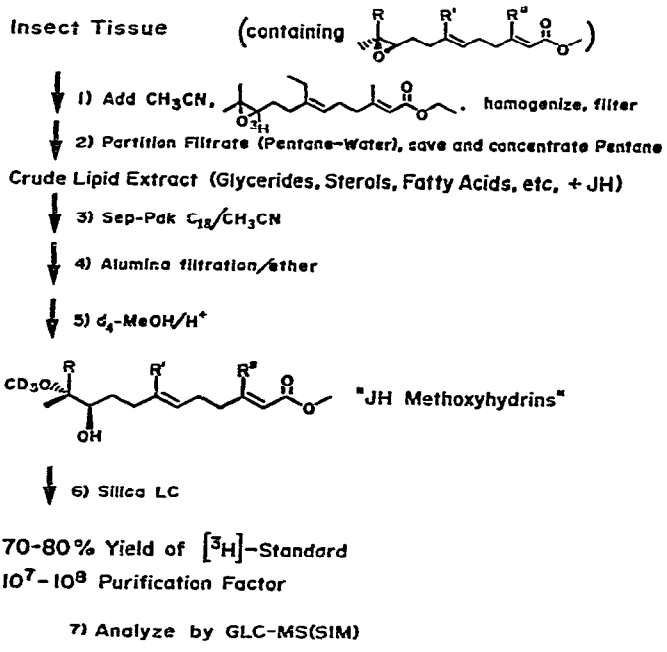

Fig. 1. Flow diagram for JH analysis by GC–MS (SIM) of insect tissue.

sensitivity of the method permits small sample sizes, minimizing the amounts of extractable material one must process.

We found that the combination of acetonitrile extraction of the crude lipoid fraction, followed by C_{18} non-aqueous reversed-phase chromatography quite efficiently separates JHs, fatty acids, and materials of similar molecular weight from the bulk of the sterols and di- and triglycerides which usually comprise the major portion of the pentane-soluble extract. It was necessary to include a final alumina filtration of the underivatized JHs in order to remove fatty acids. Certain insect samples were analyzed without the latter step; some gave apparently reliable results while others showed severe interferences. These interferences with one sample were found to be due to a 5- or 6-hydroxy fatty acid which was partly converted to a d_3-methyl ester during methan-d_3-olysis. The alumina step easily removed these impurities.

Finally, excellent selectivity of separation was achieved via LC using the dichloromethane-based system. Fig. 2a illustrates the LC profile of JH-MH standards and phenylurea markers, while Fig. 2b shows a typical chromatogram of a crude MH preparative cleanup from a hemolymph extract. Note the plethora of UV-absorbing substances eluting before the JH-MH zone. The fast-eluting region (forerun) contains even more extraneous material from whole-body workups, and the elution profile is indicative of the chromatographic selectivity obtained *vis-a-vis* the desired compounds of interest.

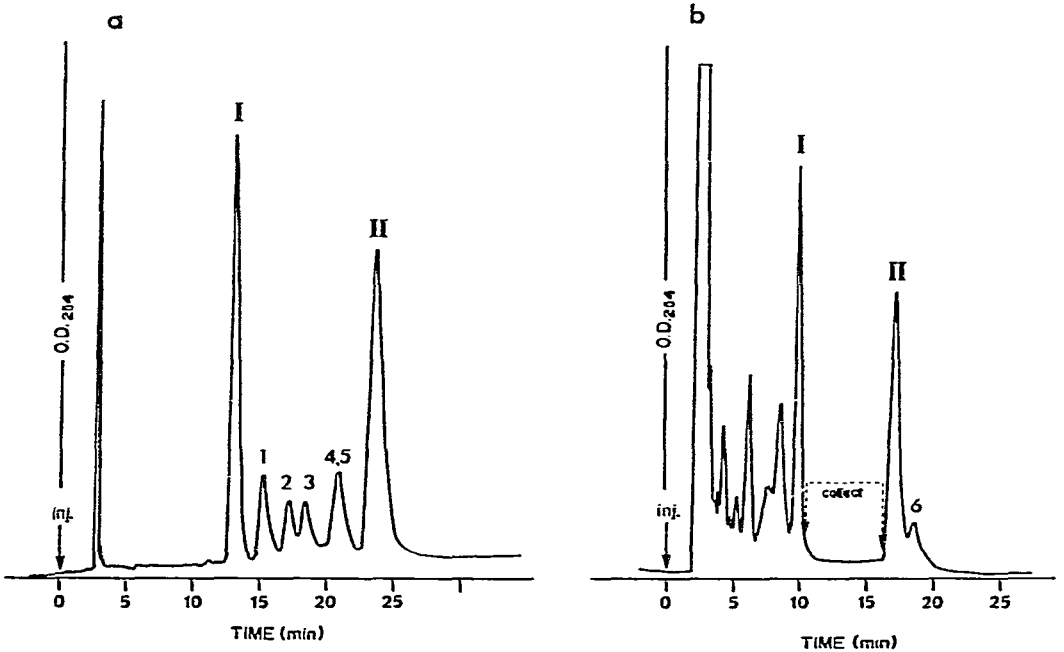

Fig. 2. LC chromatograms of (a) phenylurea markers and JH methoxyhydrin (MH) derivatives, and (b) derivatized JH extract from *Manduca sexta* Vth instar larval hemolymph. Peaks: I = N,N-diethyl-N′-phenylurea; II = N-*sec.*-butyl-N′-phenylurea; 1 = JH 0 MH; 2 = IS MH; 3 = JH I MH; 4, 5 = JH II and JH III MH; 6 = methyl geranoate MH. Conditions: column and solvent system as described in Materials and methods; flow-rate (a) = 1.3 ml/min; (b) = 1.9 ml/min; detector (254 nm) attenuation = 0.16 a.u.f.s.

Mass spectroscopy

Fig. 3 shows the electron impact mass spectrum of the IS methoxy-d_3-hydrin. The base peak, *m/z* 76, results from scission at C-10, producing the fragment $CD_3O^+C(CH_3)_2$. The latter carries 32% of the total ion current (TIC). An even higher value is obtained for the base peak of JH III MH (38%) while the analogous base peak of the MH derivatives of JH 0, I and II, *m/z* 90 [$CD_3O^+C(CH_3)(C_2H_5)$] carries 37, 47 and 47%, respectively, of the TIC. The substantial intensity of these fragments allows full exploitation of the inherent sensitivity of the SIM technique, in contrast to attempts to apply the SIM mode to electron impact analysis of underivatized JH[6].

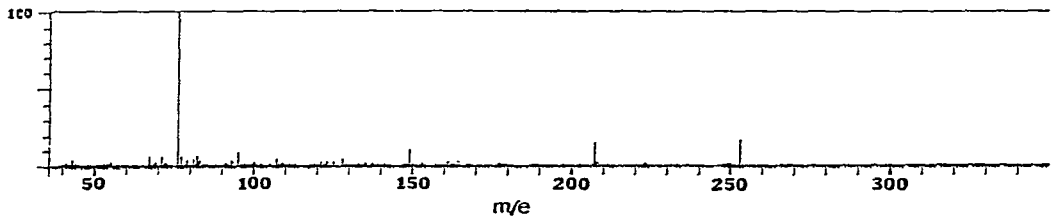

Fig. 3. Reference electron impact mass spectrum of internal standard (5) MH derivative.

A response factor *K* must be applied to reflect discrepancies between the base peak current for each of the JH–MH fragments. In turn, *K* is a direct function of instrumental operating parameters, and —for such reason— is calculated anew for each day's series of titer analyses. The basic instrument tuning parameters were obtained through the use of the H–P AUTOTUNE program; the sensitivity was further enhanced by appropriate adjustments of voltage parameters affecting the low mass end of the spectrum, namely, monitoring PFTBA fragments at *m/e* 69, 100 and 131 while optimizing their relative abundances. This technique biases the instrument's mass sensitivity toward the low mass end (within which the JH–MH peaks lie) to the detriment of higher masses, producing a gain in sensitivity of 3–4×.

Accuracy and precision of the method

Mass spectroscopic linearity studies were performed using standard solutions of the MHs, comprising a constant weight of IS (0.65 ng/ml) admixed with JHs whose weight ratios *vs.* the IS ranged from 1:50 to ≈ 40:1 (*i.e.*, a range of 2000-fold). Fig. 4 shows the excellent fit obtained for JH 0, as an example. Identical linearity characteristics were found for the other substrates, though not shown on the graph.

The accuracy of the method was determined by adding known amounts of JH standards to acetonitrile solutions containing 2.4 ng (≈0.48 ng/ml-equivalents) of IS, and working up as in Fig. 1. Table I illustrates the results obtained for levels equivalent to ≈ 6, 0.6 and 0.06 ng/ml. Acceptable correlations were found, though there was significant deviation from theoretical values in JH 0 at the 0.05 ng/ml level.

The precision of the method was checked further in the following manner: pools of insect hemolymph or whole bodies (*Manduca sexta*) were gathered from stages in which the JH titers were previously determined to be "high" (0.5–1.5 ng/ml or g)[16], and from stages having "low" titers (≤0.05 ng/ml)[16]. These were each divided into three equal portions, separately carried though the workup, and analyzed by

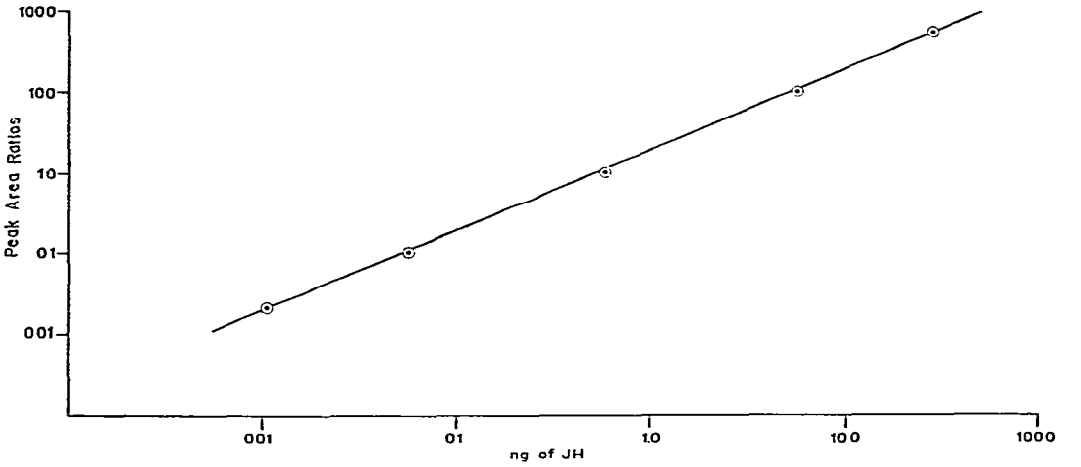

Fig. 4. Mass spectroscopic linearity-of-response curve for MH derivatives of JH 0 (0.011–28.5 ng) *vs.* IS (0.65 ng). Each point represents a minimum of three determinations.

TABLE I

ACCURACY OF METHOD

Data are expressed in ng/ml. Values are the mean of six determinations, except for JH 0 (values are the mean of three determinations).

	JH I			*JH II*		
Added	0.058	0.58	5.80	0.060	0.60	6.00
Found	0.067	0.64	5.84	0.065	0.62	5.90
Difference (%)	15.5	10.3	0.7	8.3	3.3	1.7
	JH III			*JH 0*		
Added	0.062	0.62	6.20	0.065	0.54	5.40
Found	0.066	0.61	5.95	0.086	0.64	5.67
Difference (%)	6.5	1.6	4.0	59.2	18.5	5.0

GC–MS (Table II). The data show reasonably good coefficient of variation (95% confidence limit) values for the "high level" replications (4.4–11.8%), while much higher values were obtained for the "low levels" (27–115%). However, it must be noted that the latter titer values are only 2–3× above our limits of detectability, and at such low levels (≈0.02 ng/ml) one would anticipate substantial inter-sample variation.

Recoveries

Through the aid of the tritiated IS, we were able to determine recoveries at any point in the workup scheme. Overall recoveries were virtually identical for "high level" *vs.* "low level" analyses: the recovery of IS added at 0.7 ng/ml (4.2 ng total) averaged 76%, while that at 0.043 ng/ml (0.26 ng total) averaged 78.5%. The latter data, in concert with standard recovery determinations, fully support our method of calculation of titers, which assumes identical losses in workup for endogenous JH and IS. For all samples, our recoveries were in the range of 75–80%.

TABLE II

PRECISION OF ANALYSIS AT VARIOUS TITER LEVELS, DETERMINED BY TRIPLICATE ANALYSIS OF EXTRACTS FROM THREE DIFFERENT PHYSIOLOGICAL STAGES (A, B, AND C) OF *MANDUCA SEXTA*

	Assay	*Mean (ng/g, ml)*	*Mean std. deviation (S_x)*	*Coefficient of variation**
JH I	A	0.021	0.024	115
	B	0.17	0.020	11.8
	C	0.50	0.026	5.2
JH II	A	0.012	0.012	100
	C	0.71	0.031	4.4
	B	1.12	0.085	7.6
JH III	A	Undetectable		
	B	0.03	0.017	56.7
	C	0.043	0.012	27.2

* 95% confidence limit.

Biological applications

Hemolymph and/or whole animal samples from several insect species were processed to determine the general applicability of the method (Fig. 5a–c). Attention is drawn to Fig. 5b, wherein barely detectable levels of JH were found. Previous titer data reported for *M. sexta* mid-Vth instar larvae by bioassay[17] suggest JH levels at or below 0.05 ng/ml hemolymph; our data seem to confirm these observations. Fig. 5c shows SIM analysis of tissue extracts from an insect species (*Heliothis virescens* adult females) which contains "high" levels (1–2 ng/g) of JH I and II, and undetectable levels ($\leqslant$0.03 ng/g) of JH III. The *Heliothis* titer data confirms earlier *in vitro* studies by a Zoecon group[18], who found essentially the same qualitative ratio of JHs based on LC analysis of radiolabeled products from extracts of culture medium containing adult female *Heliothis* corpora allata incubated in the presence of [methyl-^{14}C]-methionine.

Procedural blanks are normally run concurrently with a series of 5–6 samples; background levels are determined at retention times corresponding to JH 0, I, II, and III derivatives, and these values are then factored into the computation program (see Discussion).

DISCUSSION

A particular shortcoming of all GLC–ECD assays[1–5] for JHs has been the need for two derivatization steps (to provide electron-capturing functionality), followed by extensive chromatographic purification in order to remove a host of both endogenous and introduced electron-capturing impurities. Specificity of detection, however, is still low even after rigorous cleanup. The specificity of detection inherent in MS, though, has enabled us to: (a) simplify and shorten heretofore cumbersome and time-consuming purification procedures; (b) use small sample sizes, thereby "miniaturizing" workup procedures, and sparing insect colonies of severe depletion due to assay requirements; (c) achieve desired sensitivities for low level analysis [at

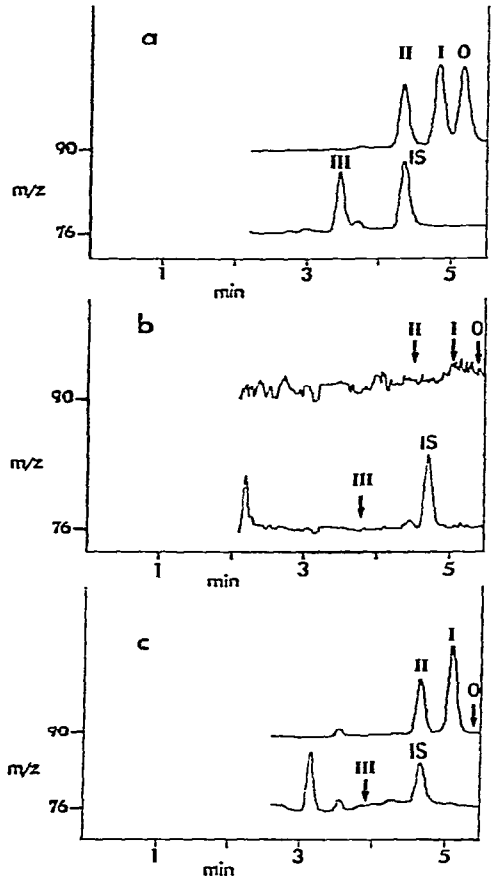

Fig. 5. Selected-ion chromatograms of (a) JH and IS MH standards (upper trace = m/z 90: JH II, I and 0; lower trace = m/z 76: JH III and IS), (b) analysis of *Manduca sexta* Vth instar larval hemolymph extract. The IS is present at 0.5 ng/ml of hemolymph, and no JH can be detected above $\approx$0.01 ng/ml, and (c) analysis of *Heliothis virescens* 0–24-h old adult female whole-body extract. IS = 0.76 ng/g; JH II = 1.4 ng/g; JH I = 2.1 ng/g; JH 0, JH III $\leqslant$0.02 ng/g. Analytical conditions: 2 m × 2 mm 3% OV-225 GC column programmed from (a) 215 to 250°C, or (b, c) 220 to 260°C, at 4°C/min, helium carrier at 40 ml/min, with a MS detector actuation delay of 2–2.4 min.

or below 0.01 ng/g (ml) (0.01 ppb, or $4 \cdot 10^{-11}$ M)] by "eliminating" interfering impurities through mass detection selectivity; and (d) obtain titer data of high confidence rapidly and accurately. Crucial to the success of this method is our development of a simple, streamlined cleanup procedure that does not require time-consuming low-temperature precipitation, molecular distillation, or preparative TLC purifications previously used for delipidation of JH-containing extracts. This has been achieved through a highly efficient and selective combination of acetonitrile extraction, nonaqueous reversed-phase filtration chromatography, and alumina mini-column chromatography. These rapid methods afford complete removal of glycerides, sterols, fatty acids, and hydrocarbons. The only additional purification after methan-d_3-olysis is an experimentally facile LC separation, wherein a broad zone of eluate is collected between the limits defined by two UV-active marker compounds.

In principle one could further extend the specificity of detection by monitoring a second or third ion fragment (*e.g.*, *m/z* 253 and 207 for IS–MH, Fig. 3); however, this significantly reduces overall sensitivity of itself, and one would at one point have to monitor more than four ions simultaneously, which is beyond the capability of our data system. Indeed, some methods[8] require monitoring several ions simultaneously for each JH, to compensate for a substantially lower degree of purification than that achieved in our assay.

We also evaluated CI mass spectroscopy in the SIM mode for detection, utilizing methane as reagent gas. However the base peak in the CI mass spectra of MH derivatives bears only about 20% of the TIC (about half the value found in the EI spectra). Also, a separate ion must be monitored for each JH (total of four); we encountered severe baseline difficulties due to column bleed in the 250–350 a.m.u. range, in which lie the ions of interest. These factors combine to reduce sensitivity and signal-to-noise ratio. Further it is generally found that protracted operation in the CI mode increases the rate of contamination of the ion source, thereby reducing sensitivity. Because of these considerations, we have not employed CI. In a different procedure, Mauchamp *et al.*[8] used CI with ammonia reactant gas for analysis of JH I as the underivatized epoxide.

We chose deuterated methanol as our derivatizing reagent, in preference to methanol, in order to raise the base peak of the analytes by 3 a.m.u. from *m/z* 73 and 87 to *m/z* 76 and 90. This avoids a need to monitor the troublesome *m/z* 73 ion, which is a high background ion from silicone stationary phases (trimethylsilyl ion). Monitoring an even mass ion is also advantageous due to their lower abundance in most spectra. We examined the suitability of perdeutero-ethanol to give a higher mass ion where background is lower still, and could obtain the analogous ethoxy-d_5-hydrin upon acid-catalyzed solvolysis of the juvenile hormones (ethoxy-d_5-hydrin base peaks at *m/z* 92 and 106). However, the ethanolysis was slower and very susceptible to "poisoning" by the presence of water, producing the diol competitively. Also, the selectivity of separation on LC was somewhat compromised by the decrease in polarity of the ethoxyhydrins *vs.* fast-eluting impurities.

The most appreciable loss in recovery occurs during methanolysis. The capricious nature of mineral acid-catalyzed solvolysis of the JH epoxide moiety on a micro scale is well documented[19,20]. With picomoles of substrate the acid is not a "catalyst", but actually an excess reagent. Difficulties with this hydration reaction provoked the development of enzymatic hydration[21] and weak acid-catalyzed hydration[20] procedures. We have examined the trifluoroacetic acid-catalyzed methanolysis carefully with the aid of the radiolabeled IS and in the presence of a carrier, EMG, and find consistently a 5–8% loss of material as polar by-products, and 2–6% as non-polar products. Without using EMG carrier, these losses vary greatly according to the particular sample. The amount of EMG added is typically at least 1000-fold the quantity of endogenous JH or IS. At such levels, impurities in the EMG are potential interferences, necessitating its rigorous purification before use. The MH derivative of EMG is selectively removed during the LC purification. Our use of a tritiated internal standard has been of key importance in ascertaining sample-to-sample reliability of the derivatization procedure. In our experience, methods for JH analysis[10] which require prior solvolysis of the epoxide group may not be reliable unless they include use of a JH-like carrier and a labeled internal standard.

The methanolysis is a highly regioselective reaction, affording a 97:3 ratio of the 11-methoxy-10-hydroxy derivative to the (analytically useless) 11-hydroxy-10-methoxy isomer. In contrast, solvolysis with nonafluorohexanol is reported to give about an 80:20 mixture of the corresponding alkoxy hydroxy isomers[10].

Excellent linearity of response for the MHs is observed over a >500:1 range; quite adequate accuracy and precision values (especially for levels of JH greater than 0.06 ng/g) allow confident data acquisition. As some insects have been reported to have two or more JHs present at a particular developmental state[9], one or more of which occurs at a substantially higher (or lower) level than the other(s), it is essential that the linearity encompass at least two orders of magnitude.

Having examined a host of biological samples, including a comprehensive study on *Manduca sexta* JH production over the complete life cycle[16], we routinely use only a single GC column per analysis. Our earlier work using GC–ECD[1] and that of Hagenguth and Rembold[3] and Peter *et al.*[2] employ a minimum of three columns of varying selectivity per analysis, in order to shift around interfering substances (unavoidably present by virtue of the methods) and to confirm retention time data for suspected JHs. Exceptions to our single-column analysis by GC–MS are sometimes made for first-time samples of an unusual hormone composition, or for mass spectral structure confirmation of JH present in unusually high quantities. This, however, is prudent analytical practice.

SIM analyses of "blanks" rarely show backgrounds exceeding 0.005–0.02 ng/ml for JH I and II, or 0.01–0.03 ng/ml for JH III. Currently, the only extraneous substance detected with regularity is a fast-eluting peak attributable to a phthalate ester (base peak *m/z* 149), that produces a minor fragment detectable at *m/z* 76. As this peak does not interfere with quantification, its presence is ignored. However, precautions are taken to minimize use of plastic or rubber apparatus throughout sample processing; in particular, nitrogen lines are of PTFE or metal to reduce possible phthalate contamination.

A dramatic illustration of the versatility of our method was recently furnished by the discovery of the fourth naturally-occurring juvenile hormone, JH 0 (ref. 11). We noticed the presence of this JH in extracts from eggs of *M. sexta* during routine SIM analysis, when a second component, monitored at *m/z* 90 and eluting slightly after JH I, was detected. Subsequent mass spectral analysis of the purified parent epoxide confirmed our findings and we now are examining other insect sources for presence of this hitherto unreported JH.

In conclusion, analysis to date of literally hundreds of hemolymph and whole-body extracts from diverse insect species for JH titers has convinced us that this assay procedure with SIM detection offers an excellent combination of specificity and sensitivity. The procedure as outlined allows an experienced technician to process 15–20 samples per week, substantially improving through-put when compared to many existing chemical assays. In most cases one will find that procurement and synchronization of specimen colonies is more time-consuming than the subsequent chemical processing and analysis.

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