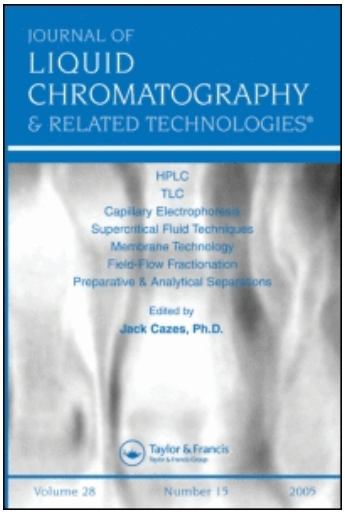

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Boll Weevil: Determination of Ecdysteroids and Juvenile Hormones with High Pressure Liquid Chromatography

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BOLL WEEVIL: DETERMINATION OF ECDYSTEROIDS AND JUVENILE HORMONES WITH HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

High pressure liquid chromatographic methods were developed to separate 20-hydroxyecdysone and juvenile hormone I in the pupal and adult stages of the boll weevil. Minimum detectable levels were 4 ng for 20-hydroxyecdysone and 4 ng for juvenile hormone. No juvenile hormones were detected in male boll weevils, whereas titers were readily determined in female boll weevils. The ecdysteroid, 20-hydroxyecdysone was present in pupae (not sexed), in adult males from 3 to 6 days of age, and in adult females from days 2 through 6.

INTRODUCTION

Methods currently available for quantification of ecdysteroids in insects include bioassays (1), chromatography (2), radioimmunoassay (3), and high pressure liquid chromatography (HPLC) (4). Ecdysteroids have been identified from hemolymph and ovaries of insects (5,6) and are involved in an interaction with juvenile hormones (JH) in reproductive cycles of insects. The JHs may regulate the synthesis of the vitellogenic protein present in developing eggs (7) and ecdysteroids may inhibit JH synthesis. A

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highly sensitive and specific assay for JHs is the radioimmunoassay technique (8); another method is gas chromatography-mass spectrometry (9). HPLC has not been utilized extensively for determination of ecdysteroids or JHs because of a lack of sensitivity. We report herein the determination of ecdysteroids in pupae and JHs in adults of the boll weevil, Anthonomus grandis Boheman, with HPLC methodology.

MATERIALS AND METHODS

Ecdysone Titers in Pupae

Larvae, pupae and prepupae were washed off the surface of larval diet trays with warm water at 13 days after egg implantation. Pupal age then was synchronized by separating and removing only prepupae. The prepupae were held in total darkness at 50% RH and 30°C.

Juvenile Hormone and 20-hydroxyecdysone Titers in Adults

Newly emerged adult weevils were obtained from the Gast Rearing Laboratory (10) and held at 50% RH and 30°C in a 16 h light:8 h dark regime. One hundred male and 100 female weevils were sexed and collected each day for assay. They were frozen or assayed on the day of collection.

Apparatus and Chemicals

A high performance liquid chromatography system from Waters Associates, Inc. (Milford, Mass.), model M-6000 A solvent pump, a model 6UK injector coupled to a Waters C_{18} Bondapack (10 μ silica) column (3.9 mm x 300 mm) and a model 440 fixed-wavelength detector @ 254 nm was used for ecdysteroid determinations. All HPLC solvents were obtained from Burdick and Jackson Laboratories (Muskegon, Mich.). A Hewlett-Packard 5985A GC-mass spectrometer was used to confirm peak identities. Standards of ecdysone and 20-hydroxyecdysone (Fig. 1) were obtained from Sigma Chemical Co.

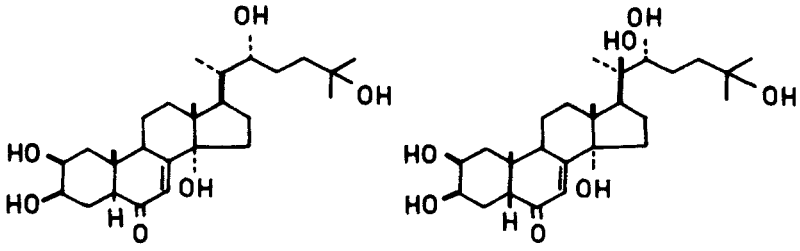


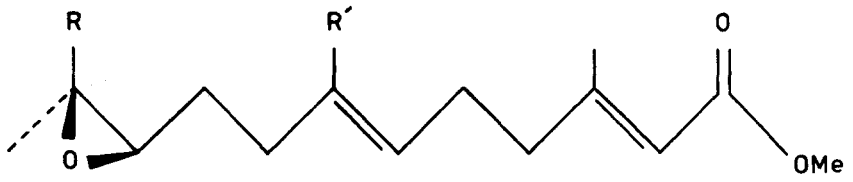
FIGURE 1. The Chemical Structures of Ecdysone and 20-hydroxyecdysone.

(St. Louis, Mo). The juvenile hormones I, II, and III (Fig. 2) were obtained in ampules from Calbiochem (San Diego, CA). An ES Industries (Marlton, NJ) 10 μ MC-18 Chromegabond column (30 cm X 4.6 mm) also was used for ecdysteroids. For JH determinations the HPLC column used was an RCM-100 column system with an 8 mm X 10 cm silica (10 μ).

EXTRACTION PROCEDURES

Extraction of Ecdysone

Assays were conducted at 8 hr intervals on 400-700 weevils with the beginning of the prepupal stage as the starting time (0 hour). At hours 40 to 56 (post prepupal stage) samples were assayed at 4 hour intervals. Weevils were ground in 50 ml acetonitrile with a Willems Polytron homogenizer. Samples were filtered with a Buchner funnel and qualitative filter. The funnel and retained material were rinsed three times with 10 ml acetonitrile, and the filtrate was evaporated to dryness at 60°C under reduced pressure in a rotary evaporator. The sample was taken up in 20 ml of acetonitrile and then was divided into two 10 ml aliquots. One of the aliquots was spiked with 25 ng ecdysone and 25 ng 20-hydroxyecdysone. The two samples were partitioned



JH I: R=R'Et
JH II: R=Et R'=Me
JH III: R=R'=Me

FIGURE 2. The Structure of the Juvenile Hormones.

three times with 20 ml hexane in a separatory funnel to remove nonpolar lipid. The acetonitrile layer was removed and evaporated to dryness at 60°C under reduced pressure in a rotary evaporator. The dry samples were taken up in 1 ml methanol and filtered with a Swinney adaptor through a 0.45 μ filter.

Extraction of Juvenile Hormones

One hundred male or female boll weevils were homogenized in 40 ml acetonitrile. The samples were filtered with a Buchner funnel and the funnel was rinsed with acetonitrile to give a volume of 50 ml. Distilled water (25 ml) was added to make the final volume 75 ml. Juvenile hormones were extracted by partitioning with 25-30 ml pentane in a separatory funnel three times. The pentane fractions were combined and evaporated to dryness under reduced pressure in a rotary evaporator at 30°C. The samples were taken up in 2 ml hexane for injection on the HPLC.

Injections

Ten microliters of the ecdysteroid samples were injected into the chromatograph. The eluting solvent was 18% acetonitrile in water and the flow rate was 2 ml/min. Quantitation was achieved by comparison of peak heights of samples to peak heights of known

quantities of standards. Ecdysone was used as an internal standard.

Collected peaks of 20-hydroxyecdysone were evaporated to dryness and taken up in methanol to be placed on the solid probe of the mass spectrometer for confirmation of peak identity. The spectra of samples were compared to spectra of standards prepared under the same conditions.

One hundred microliters of samples for JH analysis were injected in the HPLC assays. The flow rate was 3 ml/min. and the eluting solvent was 3% tetrahydrofuran in hexane.

Peak areas were obtained and quantitation for JHs and ecdysteroids was made by comparison of sample peak areas to the areas of standards. Hewlett-Packard integrators (3390A) were used to plot peaks and give data reduction. Figure 3 shows chromatograms of standards for JHs and ecdysteroids and of samples.

RESULTS

Ecdysteroids

Figure 4 shows the titers of 20-hydroxyecdysone from 24 through 64 hours after pupation. In the prepupal stages, no 20-hydroxyecdysone was detected. A titer of 20-hydroxyecdysone was found beginning at 24 hours after pupation. The quantity increased to the highest level determined 48 hours after pupation. The 20-hydroxyecdysone was also found in hemolymph at this time. At 48 hours, the quantity of 20-hydroxyecdysone present was 600 $\pm$ 40 ng/g. After 48 hours the quantity dropped rapidly and was not detectable at 64 hours. Ecdysone was not found in any of these samples.

On day 2, the adult female boll weevils produced a peak of 20-hydroxyecdysone (11 ng/weevil). The level of 20-hydroxyecdysone decreased through day 5 (7 ng/weevil) and ended on day 6. The adult male boll weevils on days 3-6 produced small amounts (4 ng/weevil) of 20-hydroxyecdysone (Figure 7).

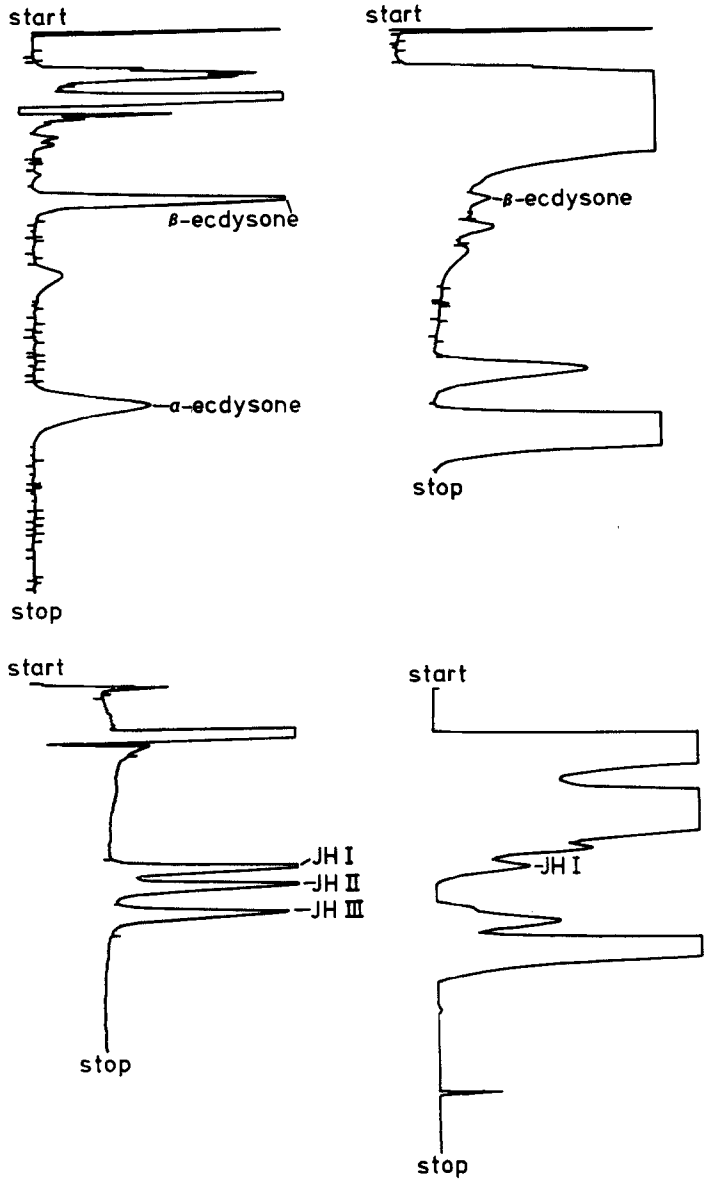


FIGURE 3. Chromatograms of Standards and Samples for Juvenile Hormones and Ecdysteroids.

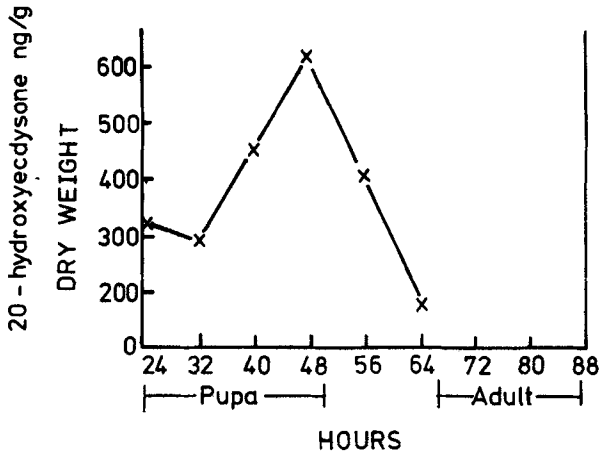


FIGURE 4. Titers of 20-hydroxyecdysone in Boll Weevil Pupae.

The time required to assay one sample for ecdysone (α) and 20-hydroxyecdysone (β) was ca. 20 minutes. The minimum detectable quantity of ecdysone or 20-hydroxyecdysone was 4 ng. Each of the samples tested for recovery averaged 95%.

The standard curve for 20-hydroxyecdysone and ecdysone is in Figure 5. The ratio of area counts obtained from the Hewlett-Packard integrator for concentration of ecdysteroid was linear over a wide range.

Juvenile hormones were not found in male boll weevils in these studies. Figure 6 also shows the pattern of JH I production by the female boll weevil for 10 days. Each point represents 4 replicates. Titers were low until the peak on day 4 (55 ng/weevil) which then decreased until day 9. No JH II or III was found in the samples.

The LDC 214 nm detector had a detection limit of 4 ng for each JH. The assay time on the HPLC was 6 minutes due to the high flow rate and resolution of the RCM-100 silica cartridge system. Figure 6 is the standard curve for the juvenile hormones I, II, and III. The ratio of area counts measured by the Hewlett-Packard

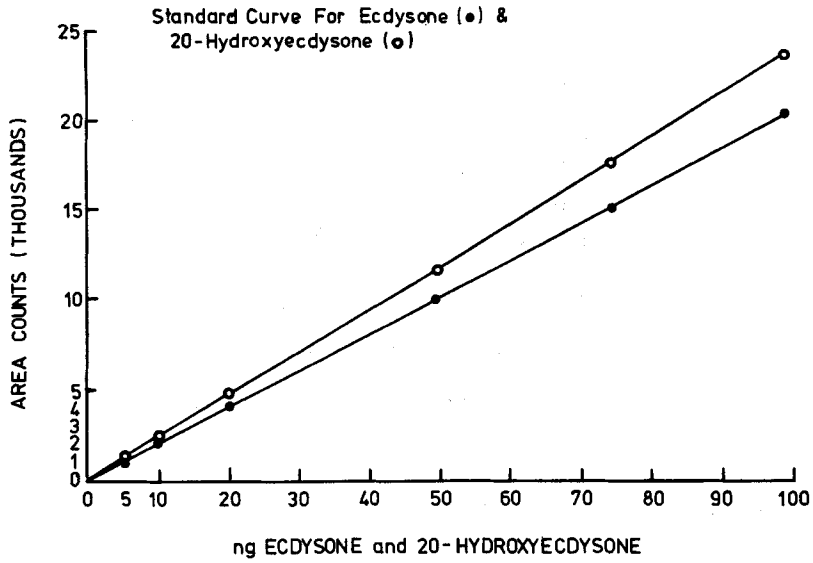


FIGURE 5. Standard Curve for Ecdysone and 20-hydroxyecdysone.

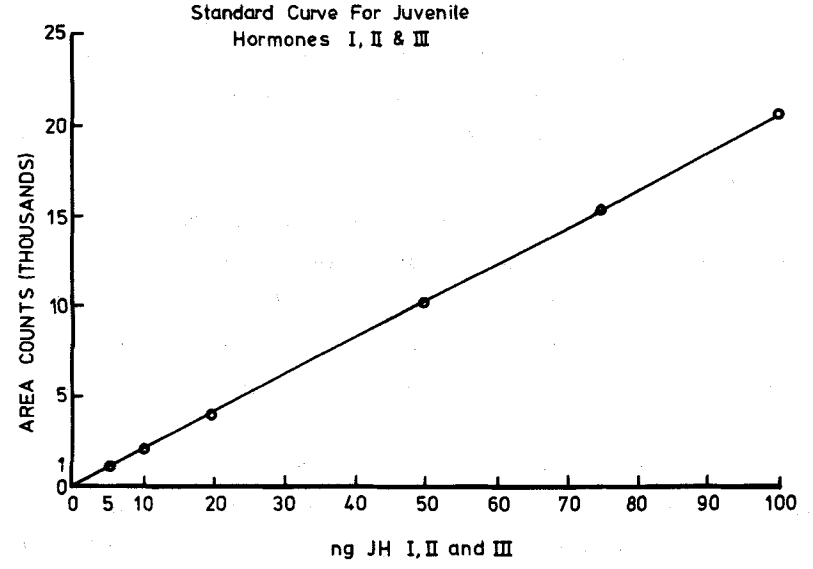


FIGURE 6. Standard Curve for Juvenile Hormones I, II, and III.

integrator to the concentration of JH was linear. All three hormones showed an absorption curve that increased in the UV as the wavelength decreased.

DISCUSSION

To study the hormonal influence on reproduction in the boll weevil, it was necessary to define methodology for definition of the ecdysteroid and juvenile hormones. The extraction methods were compatible for injection and subsequent separation and quantification by the methods developed in this study for these compounds. The sensitivity for the JH was less than GC-election capture technique of Hagenguth and Rembold (11) but this HPLC method was faster with less complications caused by impurities. The RIA method for ecdysteroid quantification is more sensitive but lacks specificity (12).

Our results demonstrate the presence of one ecdysteriod (20-hydroxyecdysone) and JH I were in the boll weevil. The JH found in most Coleopterans is JH III whereas JH I is found primarily in Lepidoptera (13). Henson et al. (14) reported that 20-hydroxyecdysone was found in boll weevil pupae and used a bioassay to estimate 17-35 µg/kg wet weight. Otherwise, little information is available or known concerning the presence or changes in ecdysteroid and JH titers in the boll weevil.

Embryogenesis begins in the pupal stage in this insect and our results indicate that 20-hydroxyecdysone is present in the early prepupal stages (mixed sexes) and decreases below detection levels as the pharate adult stage appears. For days 1 and 2 after adult emergence neither JH I nor 20-hydroxyecdysone was detected but the ecdysteroid, in females, reached a high titer 1 day prior to that of JH I and remained so until day 6. The JH remained at a detectable level throughout day 9 (Fig. 7). Bownes (15) reported that this ecdysteroid must be continuously present in Drosophila for fat body synthesis of yolk polypeptides in females. No JH was

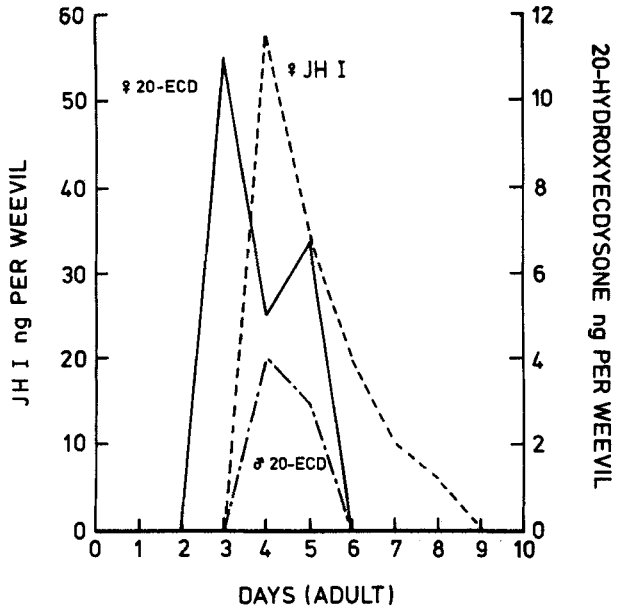


FIGURE 7. 20-hydroxyecdysone and Juvenile Hormone Titers in Adult Male and Female Boll Weevils.

detected in the males and 20-hydroxyecdysone was found in adult males at 3 to 6 days of age. Its role in males is yet unknown.

The HPLC method developed and the results obtained in this study provide an immediate, rapid, and sensitive system for determining the levels of 20-hydroxyecdysone and JH I in the boll weevil at given times on an immediate basis. These determinations can be performed on whole body tissues, hemolymph, or fractions thereof.

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