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HPLC METHOD FOR ANALYSIS OF RED DYE FROM MARKED *PECTINOPHORA GOSSYPIELLA* (LEPIDOPTERA: GELECHIIDAE)

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ABSTRACT

We describe an HPLC analytical technique for detection of minute amounts of oil-based Red 26 dye used for marking insects. Chromatographic separation was achieved by an isocratic elution on an Inertsil 100A, 5µ silica column using hexane: isopropyl alcohol (98:2) as the mobile phase and with absorption detection at 520 nm. This newly developed technique is fast and very sensitive, with a detection threshold at 0.1 ng and quantitation down to 0.3 ng per pink bollworm adult. Using the newly developed technique we were able to correlate the titer of dye extracted from adult moths to the amount of dye included in the larval diet. This method is currently accepted by the U.S. cotton industry as a preferred technique for distinguishing artificial diet-reared sterile from wild pink bollworm moths.

Key Words: sterile insect technique (SIT), pink bollworm, rearing, chromatography, cotton

RESUMEN

Se describe una técnica de HPLC analítica para la detección de pequeñas cantidades de tinte Rojo 26 de base de aceite utilizado para marcar los insectos. Se logró la separación cromatográfica mediante una elución isocrática en una columna Inertsil 100A de sílice de 5µ usando hexano: alcohol isopropílico (98:2) como fase móvil y con la detección de la absorción a 520 nm. Esta técnica recientemente desarrollada es rápida y muy sensible, con un umbral de detección a 0.1 ng y una cuantificación hasta 0.3 ng por los adultos del gusano rosado del algodón. Utilizando esta nueva técnica pudimos correlacionar el título de la tinta extraída de las polillas adultas a la cantidad de tinta incluida en la dieta de las larvas. Este método es aceptado actualmente por la industria del algodón de EEUU como una técnica preferida para distinguir las polillas estériles del gusano rosado alimentadas con una dieta artificial, de las polillas salvajes.

Palabras Clave: técnica del insecto estéril (TIE), gusano rosado del algodón, crianza, cromatografía, algodón

Significant reduction of the pink bollworm (PBW) population was achieved with a multi-tactical approach including the sterile insect technique (SIT) implemented in an area-wide eradication program in the southwestern USA and the northern States of Mexico. Verification of population reduction was obtained by detecting a decrease in capture rates of wild PBW in Delta traps (Flint & Merkle 1983). Trapping and monitoring is an inherent element of an area-wide PBW control program to show status of field populations. Capturing of PBW with Delta traps is based on pheromone lures that are attractive to wild and sterile released male moths, alike. Sterile moths are released in large numbers and trapped alongside wild moths; hence, the ability to distinguish between wild PBW and sterile moths captured in the trap is important. Sterile PBW are marked by an oil-soluble internal red dye that is incorporated into the larval diet (Graham & Mangum 1971), and in the great majority of cases it clearly labels diet-reared moths. In a small percentage of cases, however, trapped moths exhibit extremely low amounts of red dye, which can be easily overlooked by identifiers and could result in costly, and unnecessary, control measures. To overcome this problem, a simple technique, based on paper chromatography, was developed (Smith 2007) and later modified (Liesner 2011).

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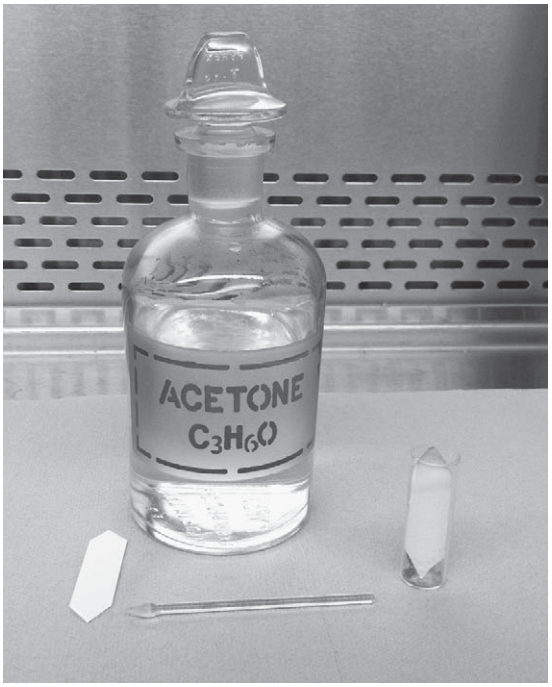


Fig. 1. Strip test: The moth is placed into the vial and covered with 0.5 mL of acetone. The moth is then crushed with a glass rod to allow dye to dissolve in the acetone. The filter paper is then inserted into the vial. (The filter paper must first be cut into a strip with points so that it fits inside the glass vial (15 × 45 mm) and that the upper tip of the paper rests just below the rim of the vial.) The dissolved contents in the acetone will travel up the filter paper where it will concentrate at the tip.

Ingenious in its simplicity this technique uses inexpensive materials (Fig. 1) and achieves a relatively good sensitivity at 5 ng. The paper chromatography technique was routinely used for detection of diet-reared insects that contain marginal amounts of dye; thus allowing them to be differentiated from wild PBW. Unfortunately, this method was based on visual assessments that are subjective and carry a high risk of misidentification. At the late stages of this eradication process, when the wild population is strongly reduced, false positives carry a high cost associated with increased control measures. For that reason accurate sterile moth identification is of critical importance. This issue was resolved by development of a sensitive analytical method based on high performance liquid chromatography (HPLC) that we present here. A previous analytical technique for detection of Red 26 dye was based on absorption spectroscopy (California Environmental Protection Agency 2006). This is an excellent technique for analysis of red dye, but is not practical for analyzing minute samples such as those obtained from individual PBW adults that have too little average mass (8 mg or less). HPLC analysis and detection of these small quantities easily resolves this problem.

Materials and Methods

Pink bollworm eggs from moths fed standard diet (Stewart 1984) were obtained from the PBW rearing facility in Phoenix, Arizona. We raised insects on diets with 5 different red dye C.I. Solvent Red 26, "(red dye)" (Color and Chemical Co.) dilutions and on the diet without red dye. The highest level of dye in the diet was 0.395 g dye/kg dry diet ingredients, the concentration used in PBW production facility for marking sterile insects for field release; the other 4 dilutions were fractions of that value: ¼, ⅛, 1/16, 1/32 plus zero dye.

One sixteenth of an egg ring (~1,500 eggs) was placed in each of 6 thermoformed plastic cells, each filled with ~250 grams diet. Rearing units were held in the Methods Development rearing rooms of the rearing facility at 29 °C, 50% RH and darkness for the first wk, then with a moon-glow light cycle of 12:12 h L:D. Pupae were collected and placed individually in vials after 20 days. After eclosion, samples from each treatment were collected, killed by freezing and kept at -20 °C until analysis.

Sample Preparation

Dye levels were determined by placing a single pink bollworm adult in a 13 × 100 mm test tube with 1 mL of hexane, followed by sonication for 1 min using a 0.25 in. (0.635 cm) microtip horn (Branson Co., Danbury, Connecticut). This extract, along with an additional 0.5mL hexane used to rinse the test tube, was clarified through a 13 mm 0.45 µ nylon syringe filter (Pall) into a 2.5 mL Reacti-vial (Pierce). The hexane was removed under nitrogen at 60 °C, with the residue dissolved in 50 µL hexane and transferred to a 350 µL micro autosampler vial (National).

Analysis

A stock solution of 1.000 mg/mL standard red dye in hexane was diluted 8-fold to obtain a solution of 125.0 µg/mL dye in hexane that translated into an HPLC peak with a height of just under 1.00 absorbance units, full scale, at a detection wavelength of 520 nm. This solution was diluted by half 13 times to obtain red dye standard solutions ranging from 125.0 to 0.0153 µg/mL, or 2500 to 0.305 ng per 20 µL injection. The 14 standard solutions were analyzed in triplicate by HPLC to obtain a calibration curve correlating red dye in 20 µL to HPLC peak area

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generated with detection at 520 nm. Standards and pink bollworm extracts were analyzed for red dye using an HPLC system consisting of a degasser, autosampler, quaternary pump, and diode array detector (Agilent), with injection volumes of 20 µL on a 4.6 × 250 mm Inertsil 100A, 5 µ silica column (GL Sciences) at a flow of 1.0 mL/min using an isocratic mobile phase of hexane: isopropyl alcohol (98:2) and detection at 520 nm. Red dye had a retention time of 3.8 min., with a total run time of 5.0 min.

Analytical data were quantified using Agilent ChemStation software. For statistical analysis the SPSS-14 package was used.

Results

The sensitivity of this newly developed HPLC technique is 0.1 ng per moth, which is about 50 times more sensitive than any previously used technique. This sensitivity reflects limit of detection, however limit of quantification is slightly higher at 0.305ng per 20µl injection. The extraction method based on hexane gave samples that rendered a clean peak at 3.8 min, which spectrally corresponds to the standard red dye and that from single moth (Figs. 2A and 2B).

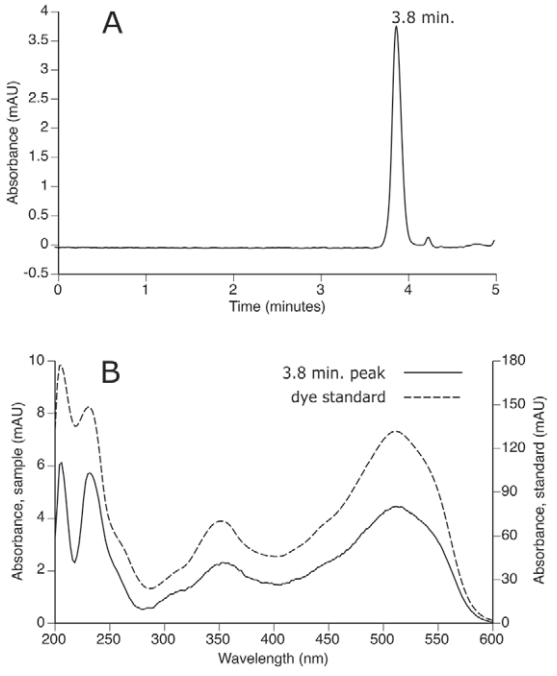


Fig. 2. Detectability of C.I. Solvent Red 26 in an individual pink bollworm adult: a) Identification of the separated compound was obtained by matching UV-visible spectrum to standard dye solution. b) The peak corresponds to dye extracted and separated from moth fed diet with the lowest tested dye concentration.

The experimental testing effects of dye concentration in the diet on titer of dye in PBW moth bodies showed a positive linear correlation. The regressions for both sexes were highly significant for estimating dye titer based on 6 treatments. For females F = 890.3 at 1 and 62 df; $P < 0.001$, and for males F =485.005 at 1 and 76 df; $P < 0.001$. The predictive values R^2 were high for both sexes (0.935 for females and 0.865 for males). At the great dilution range the HPLC method was able to separate well between treatments (Fig. 3), and recorded fine concentration differences between treatments. Dye was also detected in two moths out of 20 analyzed specimens that were reared on control diet containing no red dye, most likely due to cross-contamination somewhere in the rearing process. This was confirmed by analysis of moths reared on okra. Moths reared on a plant did not have any traces of red dye (unpublished data), showing that the developed method distinguishes between wild and reared moths, despite some background levels found in our dye free diet-reared controls.

Discussion

The primary objective of our study was to find a technique that is reliable for detecting minute amounts of red dye in insects, in the case where less sensitive techniques are not powerful enough to distinguish between diet-reared sterile PBW and wild moths. The initial phase of our study involved development of proper analytical methods for extraction and detection of red dye in PBW, because no appropriate literature sources were available. Our approach was based on HPLC detection, primarily because this method is highly sensitive and it can utilize small samples, i.e., those from an individual insect. Sample size is a major issue in analyzing individual chemical compounds from insect bodies, and this is especially true for compounds that are present in minute amounts. Chromatographic methods also provide separation of red dye from other pigments with similar coloration that can be present naturally in insect bodies and may produce background noise masking the desired signal when absorption spectroscopy is used.

This newly developed analytical method for dye extraction and detection in PBW moth was further tested for its reliability and sensitivity by analyzing moths reared on diets with different levels of red dye. In PBW red dye accumulates in fat bodies, although small amounts can be found in all body parts. Titer of red dye extracted from insect bodies was highly correlated to amount of dye in the rearing media (Fig. 3 and Fig. 4). Curve linearity was affected by variability which increases with dose. Solvent Red 26 is oil soluble and light sensitive compound, therefore source of

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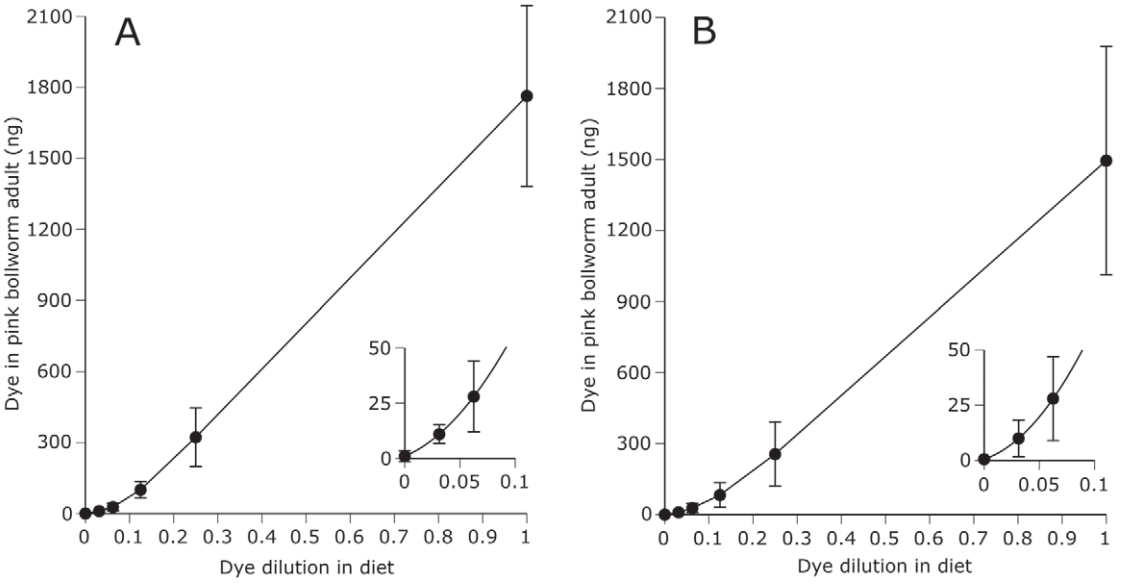


Fig. 3. Amount of red dye detected in pink bollworm adults reared on diets containing 5 different concentrations of red dye and a dye-free control. Dye was detectable at all tested concentrations. Inserts in the lower left corner of the graphs are enlarged segments of the curve representing control and treatments at the lowest 2 concentrations. Female (A) and male (B) moths retained very similar levels of dye at the lowest 2 concentrations.

variability can be associated with insect size and or size of fat bodies.

It has been shown that different insect species can tolerate C.I. Solvent Red 26 that produces long lasting color without significant adverse effects (Hagler & Jackson 2001). Boll weevil adults, *Anthonomus grandis* Boheman (Cuculionidae), readily retain red dye from red diet rendering red larvae and pupae. Newly emerged marked adults are capable of depositing marked eggs that further hatch into marked first instars, but after the first instar the amount of color decreases and color becomes too faint to be visually distinguished (Gast & Landin 1966). With this highly sensitive HPLC technique, it may be possible to observe the F1 generation. It has been shown that different economically significant species like *Heliothis virescens* (F.) (Noctuidae) (Hendricks & Graham 1970) *Eldana saccharina* Walker (Pyralidae) (Walton & Conlong 2008), *Epiphys postvittana* (Walker) (Tortricidae) and *Lobesia botrana* (Denis & Schiffermüller) (Tortricidae) can be marked with red dye. The presented HPLC technique opens new possibilities for identifying sterile insects used in area-wide eradication programs, and for using marked insects in other population, behavioral and ecological studies.

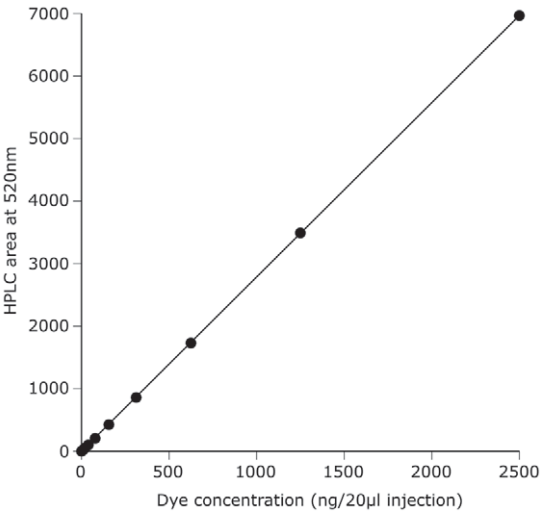


Fig. 4. Standard curve relating the areas under the HPLC peaks to various concentrations of red dye C.I. Solvent Red 26, "(red dye)" ranging from 0.305 ng to 2,500 ng per 20µL injection.

Acknowledgments

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