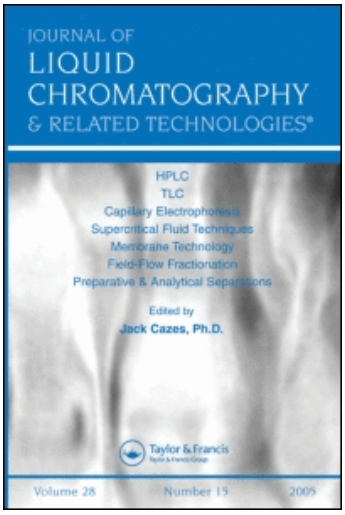

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Wright, James E. and Thomas, B. R.(1981) 'Determination of the Components of the Boll Weevil Pheromone with a High Pressure Liquid Chromotographic Method', Journal of Liquid Chromatography & Related Technologies, 4: 8, 1409 — 1416
To link to this Article: DOI: 10.1080/01483918108059618
URL: http://dx.doi.org/10.1080/01483918108059618

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(8), 1409-1416 (1981)

DETERMINATION OF THE COMPONENTS OF THE BOLL WEEVIL PHEROMONE WITH A HIGH PRESSURE LIQUID CHROMOTOGRAPHIC METHOD

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ABSTRACT

A high pressure liquid chromatographic method was developed to separate the 4 components of the pheromone of the boll weevil. Minimum detectable amounts with 3.5% tetrahydrofuran in hexane was 10 ng for (+)-cis-2-isopropenyl-1-methyl-cyclobutaneethanol, 1 ng for (Z)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethenol, and 2.5 ng each for (Z)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde and (E)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde at 214 nm.

INTRODUCTION

The pheromone produced by the male boll weevil, Anthonomus grandis Boheman, is found in the frass of the feeding male and is attractive to females.

Tumlinson et al. (1) first described the pheromone of the boll weevil as 4 components; compound I is (+)-cis-2-isopropenyl-1-methyl-cyclobutaneethanol; II, (Z)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; III, (Z)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; and IV, (E)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde. Several studies have since used GLC analysis of the pheromone from the frass of the male boll weevil. The limit of detection in these studies was about 20 ng of each compound by GLC.

The clean-up procedures used for the GLC method consisted of steam-distillation or extraction with microsoxhlets, which are quite tedious and time consuming. Because of our interest in the pheromone as an indicator of the quality of mass-reared insects, we needed a quick method for analysis with a sensitivity at least equivalent to that of the GLC method. We report in this paper the development of a sensitive high pressure liquid chromatographic procedure that requires a minimum of clean-up preparation and also permits the analysis of the four components of the boll weevil pheromone with a single injection by use of dual wavelength detection at 214 and 254 nm.

APPARATUS

A high performance liquid chromatographic system (Waters Associates, Inc., Milford, Mass.) was used with an M6000-A solvent pump, a U6K injector coupled through a column to a Waters model 440 fixed-wavelength detector @ 254 nm and a Laboratory Data Control (Riviera Beach, Fla.) fixed-wavelength detector @ 214 nm. For preliminary investigations, a Waters model 450 variable-wavelenth detector @ 214 nm and a Waters model R401 refractive index detector were used. A Texas Instruments Omniscribe (Houston, Tex.) dual pen chart recorder was used. A Waters micro-porasil column (3.9 mm ID x 300 mm L.) with a 10μ particle size was used in the system. Hamilton (Reno, Nev.) 10 and 25 μℓ syringes (#701 and 702) were used for injections.

Puffer-Hubbard Calumet (Grand Haven, Mich.) incubators with envirotrol microcomputer programmers were used to hold the boll weevils at 55 ± 5% relative humidity, 29 ± 1°C; and were programmed for the 16L:8D, 2L:22D and 0L:24D photoperiods used. A Vortex Jr. Mixer from Scientific Industries (Queens Village, N.Y.) was used to stir mixtures in the test tubes.

CHEMICALS

All HPLC solvents were obtained from Burdick and Jackson Laboratories (Muskegon, Mich.). The tetrahydrofuran was held in containers flushed with N_2 gas and kept in the dark to prevent peroxide formation. Solvents for extraction of the samples were obtained from Fisher Scientific Co. (Norcross, Ga.). The pheromone standards were obtained from Chem-samp Co., Inc. (Columbus, Ohio) and had a ratio of 30:40:15:15 of compounds I:II:III:IV. Individual standards were available for compounds I and II but not III and IV which were mixed.

METHODS

All analyses were conducted at 25°C. The eluting solvent consisted of 3.5% tetrahydrofuran in hexane degassed under reduced pressure with stirring. Samples were isocratically eluted from the microporasil at a flow rate of 2 ml/min. We used a dual detection system consisting of single wavelength detectors at 214 nm and 254 nm. The microporasil column was washed weekly with 100 ml methanol, 100 ml methylene chloride, and then 500 ml hexane.

Frass (feces) was collected from 100 male boll weevils. The newly emerged adults were fed for 5 days on slabs of artificial rearing diet (3) in plastic trays held under three different photoperiods: 16L:8D, 2L:22D and 0L:24D (total darkness). After the five day feeding on slabs, all weevils were fed cotton squares (flower buds) and held in a photoperiod of 16:8 for 3 days. This procedure is similar to that used for mass-reared weevils to be released in the field. The frass was collected on day 3 and stored below 0°C in a closed container.

Samples of boll weevil frass were placed in graduated test tubes, hexane or pentane (5 ml) added, and then the tubes were stoppered and vortexed vigorously for 30 seconds. The suspension

was allowed to settle for about five min, and then a 10-to 25-µℓ aliquot of the clear supernatant was injected into the chromatograph.

RESULTS

The final choice of solvent for the assay was 3.5% tetrahydrofuran in hexane after methylene chloride and ethyl acetate were found in preliminary experiments to be inappropriate. A Waters refractive index detector was sensitive to µg quantities, but a Waters variable wavelength detector set at 214 nm would not maintain a stable baseline and was an order of magnitude less sensitive than the single-wavelength 214 nm detector chosen for the assay. Detection at 254 nm was also used, and although it did not detect compounds I and II, it was nearly as sensitive as the 214 nm detector for compounds III and IV. The advantages of dual detection were the ability to determine the pheromone peaks III and IV when obscuring peaks were present and the accurate quantitation of compounds III and IV relative to the differences in sensitivity at the two wavelengths. The isocratic 2 ml/min. flow rate was chosen because it was simple and because gradient methods with either solvent concentration or flow rate did not increase resolution.

A separation of standards with 3.5% tetrahydrofuran in hexane demonstrated the minimum detectable amounts of the pheromone compounds to be 10 ng of compound I, 1 ng of compound II, and 2.5 ng each of compounds III and IV at 214 nm. At 254 nm, 5 ng of compounds III and IV were detectable. In comparison, Bull et al. (3) stated that in their GLC method 20 ng of each component standard was needed for accurate quantitation, and McKibben et al. (4) found that 1 µg of total pheromone was necessary for quantitation because of interfering peaks in actual frass samples.

Figure I is a chromatograph of a typical frass sample. Elution time for good resolution was approximately 20 min. with 3.5% tetrahydrofuran in hexane. The α-terpineol used in GLC assays as

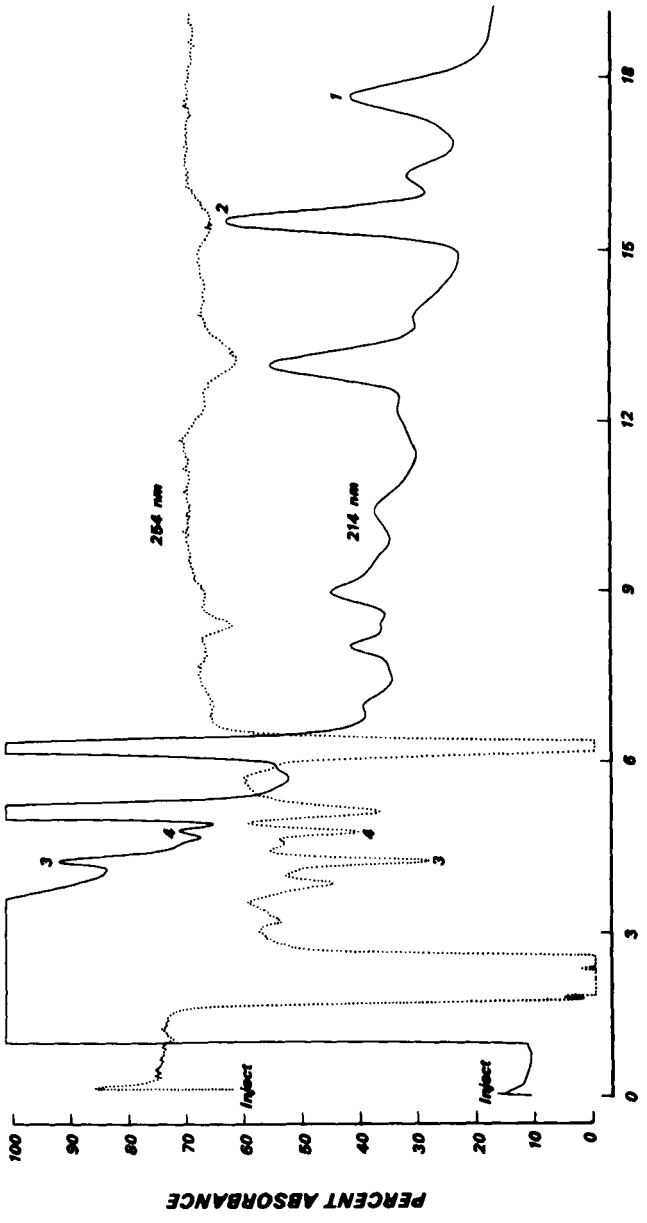

FIGURE I. Typical HPLC chromatographic separation of 4 components of the boll weevil pheromone from the frass of males with 3.5% tetrahydrofuran in hexane.

an internal standard was found to elute before compound II and was impure, as 3 peaks were identified on the HPLC, although only one peak was found on the GLC. Therefore, we did not use α-terpineol as an internal standard for quantitation. Other peaks were present in the analysis besides those of the pheromone components; in most cases, these did not interfere with analysis. The relatively simple and quick extraction method used gave 100% recovery from frass spiked with pheromone. The extraction method for GLC required 1 hour or more for a single sample, whereas 5 min. or less was required for 10 samples with our method.

The results in Table I were from 3 separate experiments with 100 male boll weevils each. Three injections were made of each sample from each experiment. While the weevils were fed on diet slabs no pheromone was found in their frass, but when they were later fed squares in the 16L:8D photoperiod pheromone was produced. The weevils held in the dark and then fed squares in 16L:8D produced more pheromone than the weevils maintained in the other photoperiods. This was significant since the 24L:0D photoperiod, not total darkness, is used in mass rearing the boll weevil. Gueldner and Wiygul (9) found low levels of pheromone in frass of weevils fed on squares and reared in total darkness, but

TABLE I

Effects of Photoperiod on Pheromone Production.

Photoperiod	Pheromone Compounds (ng/Weevil ± SD)			
(light:dark)	I	II	III+IV	Total[a/]
16:8	14± 8	10± 8	8± 9	32 a
2:22	26±25	11± 7	20±10	51 a
0:24	96±22	56±17	69±22	187 b

a/ Means not followed by a common letter differ significantly at the 0.01 level of probability according to Duncan's multiple range test.

because they did not later feed these weevils in the 16L:8D photoperiod a direct comparison is not possible. Our weevils fed only in darkness on slabs of diet produced no pheromone. The weevils in the dark produced a pheromone compound ratio of approximately 2:1:1 (I:II:III+IV).

McGovern et al. (6) reported that with the GLC method the ratio of components was 40:18.5:23:18.5, and Hedin et al. (7) reported a ratio of 40:40:14:6 Hardee et al. (8) used a formulation in the field based on the ratio 23:17:30:30.

DISCUSSION

The HPLC method presented in this paper is more sensitive, i.e., 10 ng I, 1 ng II, 2.5 ng III, and 2.5 ng IV, than the reported GLC methods that require about 20 ng for each of the compounds. The methods of McKibben et al. (3), Bull et al. (2), Hedin et al. (6), and Gueldner and Wiygul (9) depend on crucial sample preparation and have not been extended to assay the pheromone produced by an individual weevil as this HPLC method has. Boll weevils are reared in large numbers (millions) for release as sterile insects, and it is necessary to quantitate pheromone production as a measure of the quality of the released adults. This HPLC method now gives us a rapid and sensitive assay in a minimum of time.

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