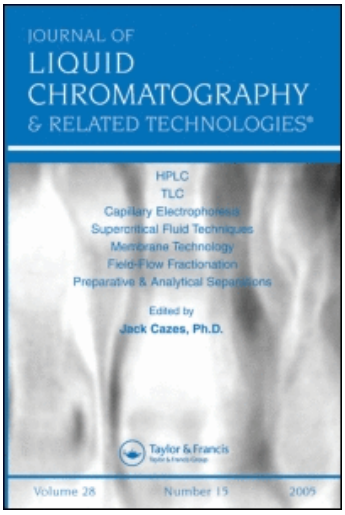

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LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ANTHRAQUINONE RESIDUES IN WEATHERED AND UNWEATHERED FORMULATED RICE SEED AND SURFACE WATER IN RICE FIELDS

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LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ANTHRAQUINONE RESIDUES IN WEATHERED AND UNWEATHERED FORMULATED RICE SEED AND SURFACE WATER IN RICE FIELDS

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ABSTRACT

Anthraquinone was extracted from weathered and non-weathered formulated rice seed and analyzed by reversed-phase high performance liquid chromatography. Anthraquinone was quantified by UV absorbance at 325 nm. Recovery data were determined by analyzing anthraquinone fortified control rice seed. The mean recovery of anthraquinone in rice seed was 99.3 ± 0.5% for the range of 0.050 to 1.00% anthraquinone. Anthraquinone residues in surface water samples from rice fields were determined by direct injection of filtered samples into a reversed-phase

high performance liquid chromatograph and quantified by UV absorbance at 254 nm. Recovery data were produced by analyzing anthraquinone fortified control surface water samples from rice fields. The mean recovery of anthraquinone in surface water samples was 97.0 ± 2.4% for the range of 8.0 to 400 ng/mL anthraquinone.

INTRODUCTION

Bird damage to seeded rice is a persistent problem in the southern United States that costs producers millions of dollars annually.[1,2] Currently, a commercially available effective bird repellent registered for rice seed is not available. Anthraquinone (9,10-Anthracenedione), a naturally occurring chemical found in many species of plants[3] is used as a precursor in the dye industry and as a pulping catalyst in the paper industry[4]. Anthraquinone has been shown to be an effective bird repellent[5] when applied to rice seed in cage and pen trials.[6] An analytical method was needed to verify anthraquinone concentrations on formulated rice seed and to determine residues on rice seed samples collected during field experiments. This methodology will also be utilized for additional studies to determine the effectiveness of anthraquinone as an avian repellent when applied to ripening rice. Additionally, a need to monitor anthraquinone residues in surface waters may be required for environmental concerns.

For the paper industry, instrumental methods for monitoring anthraquinone residue levels in wood pulps, pulping liquors, and condensates based on gas chromatography/mass spectrometry,[4] gas chromatography with electron capture detection,[7,8] differential pulse polarography,[9] ac polarography,[10] spectrophotometry,[11] and high performance liquid chromatography with ultra-violet[12-15] detection have been reported. Two simple and reproducible analyses utilizing high performance liquid chromatography (HPLC) were developed.

EXPERIMENTAL

Samples

The samples consisted of rice formulated with a proprietary anthraquinone formulation and a food grade adhesive. This treated rice seed was analyzed to confirm the anthraquinone concentration in various batches prior to use in field studies. Control and treated rice seed samples were collected during the field efficacy portion of the study on day 0, 1, 3, and 5. Lafitte rice seed was utilized for all field and laboratory experiments.

Apparatus

The high performance liquid chromatography (HPLC) system consisted of a Hewlett-Packard 1090 liquid chromatograph (Palo Alto, CA) equipped with a diode array detector. Additionally, a Hewlett-Packard 1050 ultra-violet/visible variable wavelength detector was used for additional sensitivity. For rice seed samples, 10 µL of methanolic extracts were injected automatically by the pneumatically controlled injector valve. The anthraquinone was separated on a 25-cm x 0.46-cm i.d. stainless steel analytical column packed with 5-µm Keystone ODS/Hypersil at 35°C. To prolong column lifetime, a 1.5-cm x 0.46-cm i.d. Keystone ODS/H (Bellefonte, PA) guard column was used. The samples were chromatographed with a methanol:water (75:25) mobile phase at 1.00 mL/min. The chromatographic response was recorded at 325 nm. For water samples, aliquots of 100 µL were injected into the chromatographic system. The chromatographic response was recorded at 254 nm. The anthraquinone peak was identified by comparison with the retention time of a standard. A Hewlett-Packard Vectra computer work station with an Epson printer was used to collect, process, store, and print the chromatographic data.

Reagents

Methanol (Fisher Scientific, Denver, CO) was liquid chromatography grade. Chloroform (Baxter, Denver, CO) was high purity solvent grade. Deionized water was purified using a Milli-Q water purification system (Millipore, Bedford, MA). The solvents were degassed by bubbling helium through the solvents.

Anthraquinone with a purity of 99.0% was obtained from Chem Service (West Chester, PA). Concentrated fortification stock solutions of anthraquinone were prepared from the commercial products, without further purification, by dissolving 50 mg in 10 mL of chloroform. Working solutions were prepared weekly by dilution with mobile phase. All standard solutions were stored in the dark at 5°C.

Calibration Curve

For rice seed analysis five anthraquinone working solutions (1.01 µg/mL to 151 µg/mL) were prepared and analyzed by HPLC in duplicate at 325 nm. For the analysis of surface water samples from rice fields, five anthraquinone working solutions (5.2 ng/mL to 500 ng/mL) were prepared and analyzed by HPLC in duplicate at 254 nm. A plot was constructed of anthraquinone chromatographic peak response (y-axis) vs. anthraquinone concentration (x-axis) for each wavelength. Linear regressions were performed on the data (SAS, Kroy, NC).

Determination of Anthraquinone Residue on Formulated Rice Seed

For avian repellent efficacy studies, the anthraquinone fortified rice seed was applied to flooded test fields located in southwestern Louisiana, by aerial seeding. To simplify sampling of fortified and control rice seed for residue analysis, approximately 50 g of anthraquinone fortified seed was placed in small sacks of porous cloth and placed randomly in the flooded rice fields. The control field, with rice seed containing no active ingredient, was adjacent to the treated test fields with at least a 45 meter buffer and levies separating the treated and control fields. The 50 g sacks of rice seed were collected for residue analyses from the treated and control fields on post-treatment days 0, 1, 3, and 5. The rice fields were drained after 5 days. The rice seed samples were placed in plastic bags, sealed, and refrigerated at 4°C for approximately 4 hours after collection in a cooler. The samples were transferred to a freezer at -10°C, then shipped overnight, in coolers with ice packs, at the end of the field study. Upon receipt, the samples were stored in a freezer at -20°C until analyzed. To correct for moisture differences in study samples, a 10 gram portion of each sample was pre-weighed into an open container and freeze dried (Labconco, Inc. Model #77545) for 20 hours at -20°C, followed by 4 hours at -10°C. The samples were re-weighed after freeze drying. Prior to extraction, each sample was ground with a hand held coffee mill (Black and Decker, Model#CBM-1, Type 2) for approximately three minutes to homogenize the samples. The samples were assayed in triplicate. Sub-samples of approximately 0.25 grams of rice seed were weighed into 50-mL glass screw cap test tubes. A 25.0 mL aliquot of methanol was added to each sample and the tubes were capped. The samples were vortexed, mechanically shaken (Eberbach Inc., Ann Arbor, MI) for 15 minutes, and sonicated twice for 20 minutes. The sample tubes were centrifuged (VWR, Denver, CO) for five minutes at approximately 2500 rpm to separate the suspended solids of the ground rice seed from the methanol extract. Aliquots were transferred into sample vials, capped, and the anthraquinone concentrations quantified by HPLC.

To determine recovery efficiencies, anthraquinone fortified control rice seed samples were prepared by fortifying 0.250 gram portions of homogenized adhesive coated control rice seed with an aliquot of a concentrated anthraquinone stock solution in chloroform, as specified in Table 1. The chloroform was evaporated by blowing a gentle stream of nitrogen over the fortified sample in a fume hood. Fortified control samples were prepared at 1.0%, 0.50%, 0.10%, and 0.05% anthraquinone. The fortified samples were analyzed with the same procedure as stated above.

Determination of Anthraquinone Residue on Ripening Rice

Ripening rice was sprayed with a proprietary formulation containing anthraquinone. Rice grains were hand picked with tweezers from the stalks of

Table 1

Fortification of Control Rice Seed Samples

Level	Conc. of Stock Sol. (μg/mL)	Aliquot (mL)	Mass of Rice Seed (g)	% (wt/wt) Anthraquinone
0.05%	1000	0.125	0.25	0.050
0.10%	5000	0.050	0.25	0.100
0.50%	5000	0.250	0.25	0.500
1.00%	5000	0.500	0.25	1.00

plants sent from the field. Approximately 10 grams of rice grain was collected for each sample. The rice grain was then ground with a hand-held coffee grinder into a powder and transferred to a storage container. Each sample was assayed in triplicate, with a sample size approximately of 0.250 grams, used for extraction. The extraction procedure was the same as reported in the previous anthraquinone assay on formulated rice seed. Control rice was fortified at 10 and 100 ppm to assess the recovery of the assay.

Determination of Anthraquinone Residue in Water Samples

Water samples were filtered with a 0.45 μm Teflon syringe filter and injected into the HPLC system. Recovery of anthraquinone in surface water in rice field samples was assessed by fortifying control surface water from rice fields (Vermilion Parish and LSU-Rice Research Station) with a fortification standard of anthraquinone in methanol. Fortified control samples were prepared at 8, 30, 100, and 400 ng/mL.

RESULTS AND DISCUSSION

Instrument Limit of Detection

Two useful absorbance maxima were observed at 254 and 325 nm. Previous HPLC methods[12-15] utilized 254 nm as the analytical wavelength. While this was the most sensitive wavelength, the selectivity of anthraquinone compared to co-extracted matrix constituents was greater at 325 nm. Each of these wavelengths were acceptable for analysis of anthraquinone by HPLC with a UV/visible detector. Using the criterion of three times the peak-to-peak noise of the baseline, the instrument limit of detection for the variable wavelength detector for both analytical wavelengths was estimated. At 254 and 325 nm the

instrument limit of detection was 0.020 and 0.11 μg/mL for a 10 μL injection volume and 0.003 and 0.013 μg/mL for a 100 μL injection volume, respectively. For most rice seed extracts, a late eluting compound was observed at 254 nm which was not detected at 325 nm, therefore, quantification was completed at 325 nm. For the rice seed samples, quantitation of anthraquinone at 325 nm afforded adequate sensitivity and a shorter runtime then was possible at 254 nm. For analysis of surface water in rice field samples, the sensitivity afforded by detection at 254 nm was desirable but not as selective as 325 nm.

Linear Regression Data

The correlation coefficients of peak response versus concentration for 1.01 to 151 μg/mL was equal to 0.9997 and the log-log plot had a correlation coefficient of 0.9999 and a slope of 1.0079, which demonstrates that peak response was linear and proportional to concentration over the range of concentrations used for the analysis of treated rice seed at 325 nm. The correlation coefficient of peak response versus concentration for 5.2 to 500 ng/mL was equal to 0.9995, and the log-log plot had a correlation coefficient of 0.9997 and a slope of 0.9979, which demonstrates that peak response was also linear and proportional to concentration over the concentration range used to quantify anthraquinone in surface water from rice field samples at 254 nm.

Residue on Rice Seed

The recoveries of anthraquinone from fortified control rice seed samples obtained by the HPLC method are listed in Table 2. The recoveries ranged from 98.3 to 100%. With the variable wavelength detector at 254 and 325 nm, 0.05% fortified control samples were used to determine the method limit of detection of 2.0 and 11 μg/g (0.00020 and 0.0011%), respectively. The concentration of anthraquinone in the formulated rice seed prepared in the field ranged from 0.669 to 0.819% as shown in Table 3.

Example chromatograms of a day 5 control sample extract and a treated sample extract are shown in Figure 1. The residue results from the field test are shown in Figure 2. The mean anthraquinone residues on rice seed decreased between day 0 and day 1; between day 1 and day 5 there appears to be no significant decrease. The formulated rice seed samples exposed to field conditions were very wet compared to the un-weathered formulated rice seed samples. Rice samples collected from flooded rice fields contained varying proportions of water. The anthraquinone concentration appears to be reduced by approximately 20% after 1 day in the field (Figure 2). The field samples mass increased by 15 to 25% due to the absorption of water. In addition to anthraquinone on rice seed, the rice seed was being screened for degradates which may be thermally labile. Therefore, to compensate for this increase in

Table 2

Validation Results for the Determination of Anthraquinone Residues in Fortified Rice Seed

	Fortification Levels and Percent Recovery			
Replicate	**0.050%**	**0.10%**	**0.50%**	**1.0%**
1	99.1	98.5	99.6	100
2	98.3	99.8	99.4	99.9
3	99.5	99.3	99.3	99.7
4	99.6	98.3	99.2	99.5
Mean	99.1	99.0	99.4	99.8
SD	0.6	0.7	0.2	0.2
CV	0.6%	0.7%	0.2%	0.2%

water mass, a portion of the samples were freeze dried, ground, and analyzed. The freeze dried field samples exhibit only a 10% loss of anthraquinone as shown in Table 4. Therefore, the absorption of water for field samples gives the appearance of decreased anthraquinone concentrations. Rice samples not exposed to field conditions contain approximately 4 to 5% water. Anthraquinone concentrations for freeze dried field samples were corrected by 4.5% for the loss of naturally occurring water. No degradates were observed in the chromatograms collected at 254 and 325 nm.

Table 3

Anthraquinone Concentration in Formulated Rice Seed

Sample Description	**Date of Preparation**	**%(wt/wt) Anthraquinone**	**%(wt/wt) Anthraquinone w/Freeze Drying**
Fort. Rice Seed	12/12/96	0.819	----
Fort. Rice Seed	3/11/97	0.746	----
Fort. Rice Seed	3/18/97	0.740	----
Fort. Rice Seed	3/18/97	0.669	----
Fort. Rice Seed	3/18/97	0.752	----
Control	3/22/97	<MLOD	<MLOD

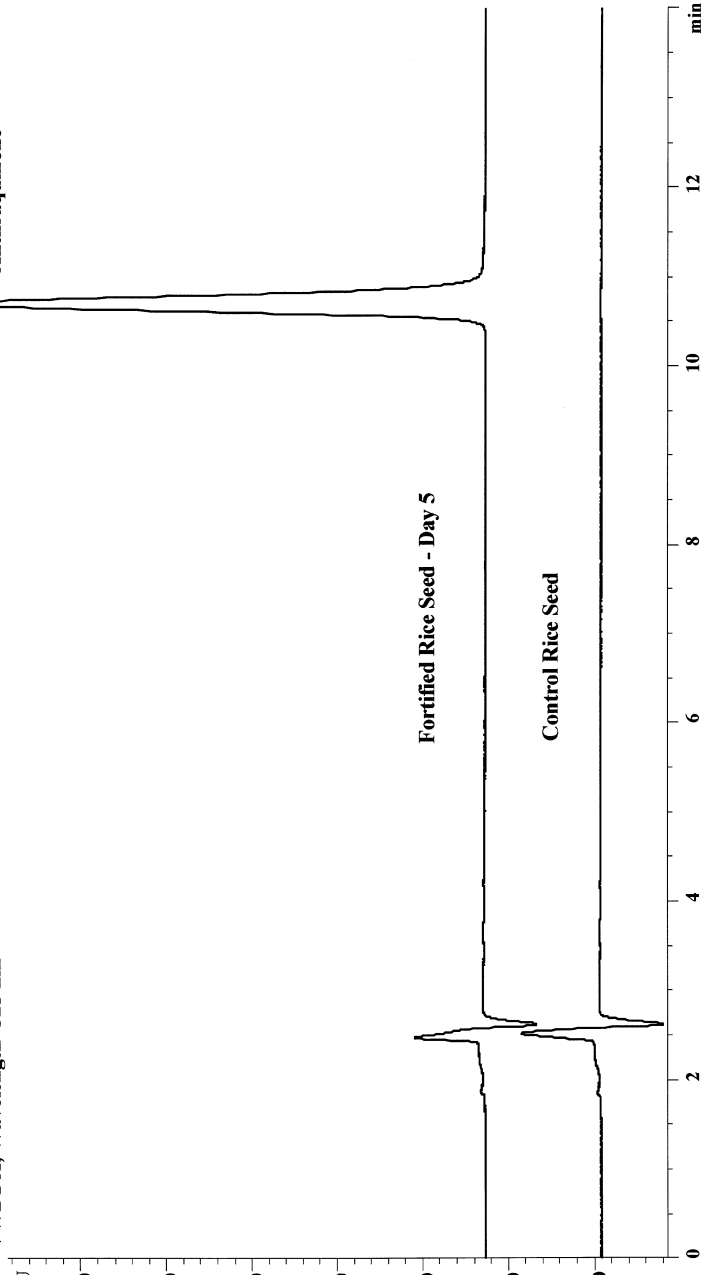

Figure 1. Chromatogram of a control rice seed sample and a formulated rice seed sample exposed to field conditions for 5 days.

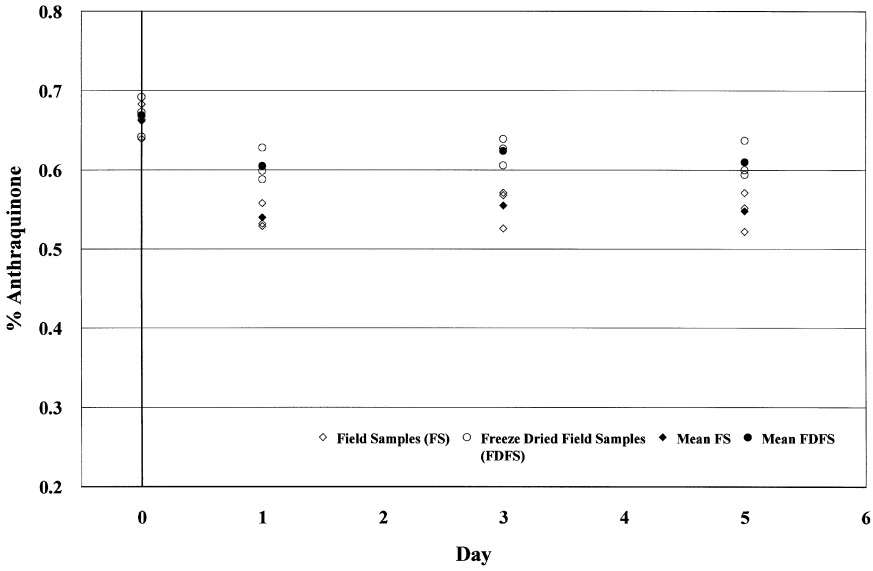

Figure 2. Anthraquinone residue results on formulated rice seed from a field test site in Louisiana over a 5 day period.

The recoveries of anthraquinone from fortified control ripening rice samples at 10 and 100 ppm, obtained by the HPLC method are listed in Table 5. The recoveries ranged from 94.3 to 99.1%, with a mean of 97.1 ± 1.9%. With the variable wavelength detector at 254 nm, the 10 ppm fortified control samples were used to determine the method limit of detection of 2.0 ppm. The

Table 4

Anthraquinone Concentration in Weathered Formulated Rice Seed

Sample Description	Date of Preparation	%(wt/wt) Anthraquinone	Anthraquinone w/Freeze Drying
Day 0	3/22/97	0.662	0.669
Day 1	3/23/97	0.540	0.605
Day 3	3/25/97	0.555	0.624
Day 5	3/27/97	0.548	0.610

Table 5

Percent Recovery Data for the Determination of Anthraquinone Residues in Fortified Ripening Rice

Replicate #	10 ppm	100 ppm
1	98.6	95.4
2	97.1	97.9
3	94.3	99.1
4	95.2	98.9
Mean	96.3	97.8
SD	1.9	1.7
CV	2.0%	1.7%

anthraquinone residues on samples of ripening rice sprayed with anthraquinone formulations for two sites was 80.2 ± 2.3 ppm and 56.3 ± 1.5 ppm.

Residues in Surface Water Samples from Rice Fields

The recoveries of anthraquinone from fortified control surface water in rice field samples obtained by the HPLC method are listed in Table 6. The recoveries ranged from 93.2 to 101%, with a mean of 97.0 ± 2.4%. With the

Table 6

Percent Recovery Results for the Determination of Anthraquinone Residues in Fortified Surface Water from Rice Fields

	Fortification Levels and Percent Recovery			
Replicate #	8.0 ppb	40 ppb	100 ppb	400 ppb
1	101	95.4	96.6	99.9
2	96.0	101	100	97.3
3	94.6	93.2	94.9	96.9
4	95.0	98.3	95.2	96.5
Mean	96.9	97.0	96.7	97.6
SD	3.5	3.4	2.3	1.5
CV	3.6%	3.5%	2.4%	1.5%

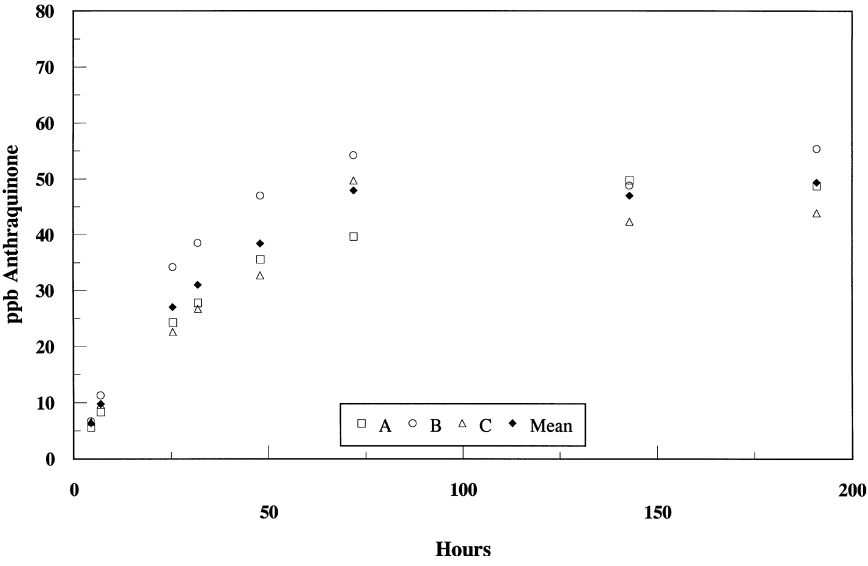

Figure 3. Anthraquinone residues in rice field surface water treated with formulated rice seed over an 8 day period.

variable wavelength detector at 254 nm, 8.0 ppb fortified control samples were used to determine the method limit of detection of 2.0 ppb. To demonstrate the usefulness of the method, anthraquinone formulated rice seed prepared in the field, were placed in simulated rice fields at a seeding rate of 150 lbs of rice seed per acre at approximately 25°C. The pH of the water was measured at 6.5. Water samples were collected at 0, 4, 7, 24, 30, 48, 72, 144, and 192 hours. Each sample was assayed according to the method. The results are shown graphically in Figure 3. The concentration of anthraquinone in the simulated rice fields samples over an eight day period ranged from <MLOD to 56 ppb.

CONCLUSION

This method for the determination of anthraquinone in the un-weathered and weathered rice seed was simple and efficient. The mean recovery for the determination of anthraquinone in the rice seed was 99.3 ± 0.5% for the range of 1.0 to 0.05% anthraquinone. This methodology can be utilized for additional studies to determine factors contributing to anthraquinone instability, leading to the improved effectiveness of anthraquinone as an avian repellent, as well as, to evaluate the concentration of anthraquinone applied to ripening rice. The

method for the determination of anthraquinone residues in water can help to assess the impact on aquatic organisms in the environment. The mean recovery for the determination of anthraquinone in surface water was 97.0 ± 2.4% for the range of 8.0 to 400 ng/mL anthraquinone.

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