High-Pressure Liquid Chromatographic Analysis of Hexazinone in Alfalfa Tissue (*Medicago sativa* L.)

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A procedure is described for the extraction of hexazinone [3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1*H*,3*H*)-dione] from alfalfa (*Medicago sativa* L.) foliage and its analysis by reversed-phase high-pressure liquid chromatography (HPLC) with monuron [3-(*p*-chlorophenyl)-1,1-dimethylurea] as an internal standard during extraction for quantification. Hexazinone was extracted from plant samples by homogenizing and sonicating the foliage tissue with an extractant. The filtrate was recovered by suction filtration, and the solvent was evaporated. The hexazinone was dissolved in water, washed with hexane, and partitioned into chloroform. The solvent was evaporated at reduced pressure, and the hexazinone was dissolved into water and passed through a C-18 minifilter in preparation for HPLC analysis. The extraction efficiency was 81 ± 2%, and much of this loss could be accounted for by using the internal standard. The detector responded linearly to hexazinone and monuron concentration with high precision (r = 0.95).

Hexazinone [3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1*H*,3*H*)-dione] is the active ingredient in the formulated herbicide Velpar (E. I. du Pont de Nemours & Co., Inc., Wilmington, DE). It is registered in the United States for nonselective weed control in noncropland areas, for general weed control in alfalfa (*Medicago sativa* L.), and for the control of woody plants. Hexazinone can be classified as an *s*-triazine and reportedly inhibits photosynthesis (Hatzios and Howe, 1982). Hexazinone is absorbed through plant roots and foliage and is translocated primarily through the apoplast to the site of action in the leaf mesophyll chloroplasts (Baron and Monaco, 1986; McNeil et al., 1984).

Several analytical procedures have been described for the isolation and quantification of hexazinone in plant foliage tissue (Holt, 1981; McIntosh et al., 1984; Pease and Holt, 1971). However, the analytical instrumentation utilized in these procedures was nitrogen-selective gas chromatography of extracts from fresh or freeze-dried plant tissue and high-performance liquid chromatographic methods (HPLC) of standard aqueous solutions of hexazinone. This paper describes methodology for the extraction of hexazinone from freeze-dried plant material and the quantification of hexazinone by reversed-phase HPLC.

EXPERIMENTAL SECTION

Apparatus, Chemicals, and Reagents. Analytical standard-grade hexazinone and monuron [3-(*p*-chlorophenyl)-1,1-dimethylurea] were obtained from Du Pont. All solvents were reagent grade, and the water and methanol were glass-distilled before use. The mobile phase (water–methanol, 50:50) was determined, in preliminary studies, to be most effective for the separation of hexazinone and monuron using the available HPLC system under the prevailing laboratory conditions and will be referred to as solvent.

The HPLC system consisted of the following components: (1) Micromeritics (Micromeritics Inc., Norcross, GA) autoinjector 725 equipped with a 50-μL injector loop; (2) Beckman (Beckman Instrument Co., Fullerton, CA) 110A solvent delivery system operated at a flow rate of 2.00 mL/min; (3) precolumn (25 × 4.6 mm) packed with CO:Pell ODS-C18 (Whatman, Inc., Clifton, NJ); (4) column (250 × 4.6 mm) packed with 10-μm R sil C-18 (Alltech Associates, Inc., Deerfield, IL); (5) Micromeritics 786 variable-wavelength detector operated at 254 nm and 0.05 AUFS; (6) Hewlett-Packard 3390A integrator/recorder (Hewlett Packard Co., Atlanta, GA) operated at a chart speed 0.3 cm/min, threshold setting 4, peak area rejection 20K, signal voltage output +0.1–0.4, and attenuation 3. The HPLC system was operated at ambient conditions in the laboratory (ca. 22 ± 2 °C).

The previously freeze-dried and ground (to pass a 1-mm screen) alfalfa foliage samples were homogenized with the extractant in a Kinematica (Brinkman Instruments, Westbury, NY) homogenizer and sonicated in a ultrasonic Cavitator (Mettler Electronics Corp., Anaheim, CA). Methanol, chloroform, methylene chloride, 20% methanolic chloroform, and 20% methanolic methylene chloride were tested for efficiency of extracting hexazinone. All extractants were evaporated (60 °C) at reduced pressure on a Yamato rotary evaporator (Yamato Scientific Ltd., Tokyo, Japan).

Sample Preparation for HPLC. Monuron was selected as the internal standard (IS) for this procedure. The relative retention time (RRT) and relative concentration (μg/mL) response (RCR) of hexazinone compared to those of monuron were determined with standard solvent solutions of these compounds individually and combined using hexazinone and monuron concentrations adjusted across a range from 1.0 to 10 μg/mL. In experiments to determine hexazinone recovery during extraction and loss at each stage of the cleanup procedure, the monuron concentration was adjusted to give a final concentration of 1.0 μg/mL in the final solvent. Hexazinone losses during the extraction procedure were determined by introducing known concentrations at each step and analyzing the recovery following each step in the procedure. Losses to filtration were calculated by subtraction. In these experiments each hexazinone concentration was analyzed in three replicates, and the experiments were conducted two times for appropriate statistical analysis.

One-milliliter aliquots of aqueous hexazinone solutions were added to 2- or 5-g plant samples, resulting in plant samples containing 10, 25, 50, 75, and 100 μg of hexazinone. Three replicates of the treated plant samples were thoroughly mixed, frozen in closed containers for 72 h, and freeze-dried in preparation for extraction. The IS (20 μg) in 1 mL of methanol was added to each plant sample, and the plant material was thoroughly mixed. The samples were extracted twice with 30 mL of each of the extractants

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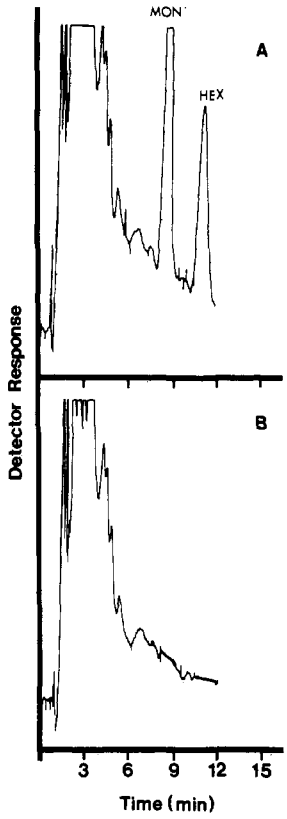

Figure 1. Typical HPLC chromatograms of (A) monuron (MON) and hexazinone (HEX) extracted from 2.5 g of alfalfa tissue spiked with 20 μg of hexazinone and monuron and (B) processed extract of alfalfa tissue not spiked with either herbicide.

by homogenizing the mixture for 5 min and sonicating the homogenate for 10 min. Following each extraction, the filtrate was collected by suction filtration through Whatman No. 1 filter paper and combined. The combined extracts were evaporated to dryness at reduced pressure. The hexazinone and monuron were dissolved in 40 mL of water, and the aqueous solution was partitioned twice with 40 mL of hexane to remove nonpolar contaminants. The compounds were partitioned from the aqueous solution into chloroform by partitioning twice with 40 mL of chloroform. The chloroform was evaporated to dryness at reduced pressure, and the compounds were dissolved in 10 mL of water. A 2-mL aliquot of the aqueous solution was filtered through a Supelclean (Supelco, Inc., Bellefonte, PA) C-18 minifilter that had been preconditioned with methanol at reduced pressure. An additional 2 mL of methanol was filtered through the minifilter. The flow rate of the liquids through the minifilter was controlled to 4 mL/min at 60-kPa pressure. The hexazinone in the aqueous methanol solvent was analyzed by HPLC following filtration through a 45-μm Millipore filter.

RESULTS AND DISCUSSION

Monuron was selected for use as the IS for hexazinone analyses because of the similarities of the two compounds to light absorption spectra, retention time on the HPLC column, solubility in the solvent, and partition efficiency into chloroform. Both compounds have an absorption peak at 254 nm. The RRT for hexazinone is 1.29 compared to that for monuron, and both herbicides chromatograph at a retention time where the chromatogram is void of interfering peaks from alfalfa foliage contaminants (Figure 1). The RCR for hexazinone compared to that for monuron was 0.63. The detector response to hexazinone concentration was linear over the concentration range examined (Figure 2) and maintained a high correlation coefficient.

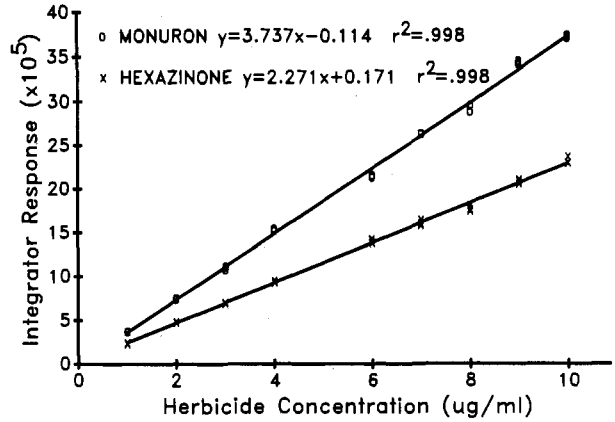

Figure 2. Regression equations and best-fit curves for integrator responses to monuron and hexazinone concentration.

Table I. Recovery of Hexazinone from 2- and 5-g Plant Material Samples Treated with 10, 25, 50, 75, and 100 μg of Hexazinone[a]

		recovery %		
treatment, μg	sample size, g	M	MC	ME
10	2.0	97 (2)[c]	93 (2)	92 (2)
	5.0	98 (2)	93 (2)	96 (2)
25	2.0	98 (2)	95 (2)	94 (3)
	5.0	96 (3)	92 (3)	92 (2)
50	2.0	98 (2)	94 (2)	90 (2)
	5.0	97 (3)	93 (2)	92 (3)
75	2.0	94 (2)	94 (3)	93 (2)
	5.0	97 (3)	98 (3)	93 (3)
100	2.0	94 (2)	96 (3)	95 (3)
	5.0	95 (3)	94 (3)	95 (3)
LSD (0.05)[d]		4	4	5

[a] Data are recovery percentages for the extractants methanol (M) and 20% methanolic chloroform (MC) and methylene chloride (ME). The IS (20 μg) was added to the plant material preceding extraction. [b] Mean of six analyses. [c] Standard deviation in parentheses. [d] Least significant difference at a probability level of 5%.

Table II. Loss of Hexazinone during Each Stage of the Extraction and Cleanup Procedure (Addition of IS Succeeded Filtration of Extract through the C-18 Minifilter)

extraction step	hexazinone loss, %
plant material extraction	
methanol	6 ± 3[a]
chloroform	100
methylene chloride	100
20% methanolic chloroform	8 ± 3
20% methanolic methylene chloride	10 ± 3
methanol evaporation, water dissolution	4 ± 2
water to hexane partitioning	0
water to chloroform partitioning	3 ± 2
chloroform evaporation, water dissolution	4 ± 2
C-18 minifilter filtration	2 ± 2

[a] Mean ± SD of six samples.

The extraction procedure utilizing methanol or 20% methanolic chloroform or methylene chloride was determined to be efficient for the 2- and 5-g sample sizes containing the concentration range used in this procedure (Table I). Chloroform and methylene chloride were not effective extractants of hexazinone from freeze-dried plant material (Table II). It was noted that chloroform and methylene chloride are effective extractants of hexazinone from plant material containing water. However, the resulting emulsion became a major problem during extrac-

tion even when sodium sulfate was included during extraction (data not included). Water was an efficient extractant of hexazinone from the plant material. However, certain pigments were extracted with all solvents more polar than methanol, and aqueous solutions of nonpolar solvents that could not be easily removed by the cleanup process utilized in this procedure. It was determined that aqueous methanol solutions higher than 10% resulted in interfering peaks on the chromatogram, and less than 10% aqueous methanol did not improve extraction efficiency for hexazinone (data not included).

Generally, the total extraction procedure, utilizing methanol, consistently accounted for 81 ± 2% of the hexazinone added to the alfalfa foliage samples. Analysis of data from hexazinone lost at each step in the procedure indicated that much of this loss is during stages subsequent to the initial extraction stage (Table II) and this loss is adjusted when using the IS to quantify hexazinone in plant tissue samples (Table I). Also, use of an IS adjusts for variations in extraction efficiency and instrument sensitivity.

This procedure of quantifying hexazinone was determined to be simple, inexpensive, accurate, and reproducible. Also, the sensitivity range of this method is adequate for most research with hexazinone in plant tissue. The choice of extractants will depend on pigments in the plant material to be extracted. Methanol was effective for alfalfa tissue; however, other species may contain interfering pigments that are extracted with methanol, and methanolic solutions of nonpolar solvents could be more effective extractants.

Registry No. Hexazinone, 51235-04-2.

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Rapid Acid Hydrolysis of Plant Cell Wall Polysaccharides and Simplified Quantitative Determination of Their Neutral Monosaccharides by Gas–Liquid Chromatography

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A rapid method for the determination of plant cell wall neutral polysaccharides is described. A two-step acid hydrolysis procedure, suitable for a wide range of plant cell wall materials, has been developed. The effect of several parameters (substrate particle size, reaction time, temperature) on the hydrolysis rate of various substrates (microcrystalline cellulose, wheat straw, beet pulp, soybean hull, sunflower husk) has been studied. Among tested parameters, sample particle size and primary hydrolysis temperature predominantly affect acid degradation of plant cell wall polysaccharides. Maximal substrate hydrolysis rate is obtained with finely ground materials (average particle size 80×10^{-3} mm) submitted to a 30-min primary hydrolysis carried out at 25 °C in 72% sulfuric acid, followed by a 120-min secondary hydrolysis in boiling 2 N sulfuric acid. Neutral sugars released by hydrolysis are quantitatively determined, after reduction and acetylation, by gas–liquid chromatography. In the optimized procedure, alditol acetates are totally recovered and reagents interfering in chromatographic separation are eliminated. The validity of the proposed procedure has been tested with various plant materials.

Several sophisticated analytical methods for quantitative determination of cell wall polysaccharides and their neutral sugars have been proposed. They usually involve sugars release by acid hydrolysis and their separation and determination by gas–liquid chromatography (Dutton, 1973). Current procedures for preparing volatile derivates of sugars are not suitable for routine analysis in nutrition investigation. They have therefore not been frequently applied to digestion studies of food polysaccharides (Graham et al., 1986; Nyman and Asp, 1982). Rapid analysis of cell wall sugars, applicable to various substrates and to digesta samples, is necessary in nutritional experiments. The present work describes several adaptations, with the aim of obtaining the most accurate method of determination of nonstarch polysaccharides in various substrates. At first, the possibility of defining a standardized hydrolysis procedure using finely ground materials was investigated; in the second part, a method for alditol acetate preparation (Blakeney et al., 1983; Harris et al., 1988) has been shortened and improved: In the modified procedure, alditol acetates are completely extracted and,

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