Determination of Herban Residues

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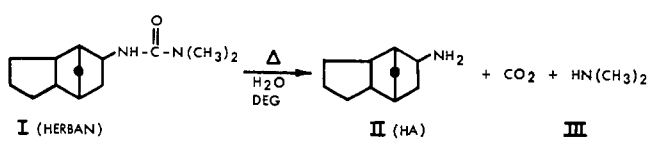
A quantitative residue procedure has been developed for 3-(3a,4,5,6,7,7a-hexahydro-4,7-methancindan-5-yl)-1,1-dimethylurea, a new herbicide commonly known as Herban. The method is capable of determining as little as 0.1 p.p.m. with good accuracy and precision. It has been successfully applied to the determination of residues on a wide variety of crops, including cotton, potatoes, sorghum, soybeans, and sugar cane. Elaborate cleanup procedures are not required. Herban is first rapidly hydrolyzed in potassium hydroxide–diethylene glycol. The resulting amines, dimethylamine and hexahydro-4,7-methanoindanamine (HA), are steam-distilled and then derivatized with 1-fluoro-2,4-dinitrobenzene. After separation, the color of the HA derivative is measured spectrophotometrically in alkaline dimethylformamide. The method is specific for Herban. Other amines, which are derived from the aromatic substituted urea herbicides, are separated from the HA derivatives by column chromatography on alumina.

HERBAN, 3-(3a,4,5,6,7,7a-hexahydro-4,7 - methanoindan - 5 - yl)-1,1-dimethylurea, is a selective herbicide for use on cotton, potatoes, soybeans, sorghum, and other agricultural commodities. As Herban is registered with the U.S. Department of Agriculture for pre-emergence use on cotton on a no-residue basis, a sensitive residue procedure is required for its determination. The method developed is capable of determining Herban at the 0.1-p.p.m. level, based on a 100-gram sample.

With Herban, as with the aromatic class of substituted urea herbicides (*2, 3, 4, 7, 12*), caustic hydrolysis to hexahydro - 4,7 - methanoindanamine (HA) provided the most promising approach to a residue procedure. Unlike members of the aromatic series, HA cannot be diazotized to form a colored dye. However, the reaction of 1-fluoro-2,4-dinitrobenzene (FDNB) with aliphatic amines is well known (*5, 6, 8, 10, 11*), and the recent publication of two papers, dealing with separation (*1*) and spectrophotometric measurements of FDNB derivatives of amines in alkaline solution (*9*), provided a sensitive, specific approach to the determination of Herban.

The method developed is a five-step process: caustic hydrolysis in diethylene glycol (DEG),



I (HERBAN) II (HA) III

steam distillation of II and III from the reaction mixture, reaction of the amines with FDNB, chromatographic separation of the amine derivatives on alumina, and spectrophotometric determination in alkaline dimethylformamide (DMF).

The chromatographic step provides the necessary specificity. The FDNB derivatives of the anilines from the aromatic-substituted urea herbicides as well as the derivative of III, are separated from the FDNB derivative of HA (HA–DNP) by this step, and do not interfere.

The other approaches to a residue procedure included infrared and gas chromatography. Infrared was neither sensitive nor specific enough for our purposes. As Herban could not be gas-chromatographed under any of the conditions tried, another approach is needed. Currently, a method based on the gas chromatography of HA is under investigation.

Apparatus

Six-place hot plate, E. H. Sargent and Co., Catalog No. 541315.

Steam-distillation apparatus, Micro-Kjeldahl distilling apparatus, A. H. Thomas Co., Catalog No. 7497.

Spectrophotometer, Beckman Model B or equivalent, equipped with 1-cm. cells.

Reflux condensers, Allihn, bulb-type, 200-mm., with standard taper 19/38 male joint, A. H. Thomas Co., Catalog No. 3908A.

Hydrolysis flasks, 50-ml. Erlenmeyer flasks with 19/38 female joint.

Separatory funnels, 60-ml., borosilicate glass, Corning Glass Works, Corning, N. Y., Catalog No. 6400.

Chromatographic tubes, 11-mm. o.d. (9-mm. i.d.) × 13 cm. (Ŧ 10/18), Scientific Glass Apparatus Co., Bloomfield, N. J., Catalog No. JC-2400.

Boiling chips, silicon carbide grain, Grit No. 10, Carborundum Co., Niagara Falls, N. Y.

Reagents

Potassium hydroxide–diethylene glycol solution, 10%. Prepare fresh daily by adding 10 grams of Mallinckrodt analytical reagent grade potassium hydroxide to 90 grams of commercial grade diethylene glycol in an Erlenmeyer flask. Stopper and stir until the potassium hydroxide is dissolved (about 1 hour).

Saturated boric acid (reagent grade) solution in distilled water.

Acetonitrile (commercial grade). To determine if the following purification is necessary, run a blank of 10 ml. of the solvent, beginning at the derivative preparation step in the general procedure. If necessary, purify as follows: Add 20 grams of P_2O_5 and 1 ml. of 85% phosphoric acid to 2 liters of acetonitrile. Distill and collect the fraction boiling at 80–82° C.

1-Fluoro-2,4-dinitrobenzene, Eastman Organic Chemicals, Catalog No. 6587. Prepare a 1% (v./v.) solution in acetonitrile.

Alumina, Woelm, basic (cationotropic), Alupharm Chemicals, P.O. Box 755, New Orleans, La. Add 2% (v./w.) distilled water to the alumina and tumble for several hours to equilibrate. Store in and dispense from containers carefully protected from atmospheric water vapor.

Tetramethylammonium hydroxide (TMAH), 10% aqueous solution, Distillation Products Industries, Catalog No. 1515.

Technical Herban for preparation of calibration curves. Recrystallized from acetone and/or ethyl acetate for use as a standard (m.p. 168–69° C.). Technical Herban is available from the Hercules Powder Co., Wilmington, Del.

n-Hexane, Skellysolve B, Skelly Oil Co.

Dow Corning Antifoam B, 10% (v./v.) solution in distilled water.

All other reagents are commercial or reagent grade materials.

Preparation of Standard Curves

STOCK SOLUTION. Weigh accurately 1.00 gram of recrystallized Herban, transfer to a 1000-ml. volumetric flask, and make to volume with isopropyl alcohol (IPA).

HIGH RANGE STANDARD. Pipet 50.00 ml. of stock solution into a 1000-ml. volumetric flask and make to volume with IPA. Herban concentration, 50 μg. per ml.

LOW RANGE STANDARD. Pipet 5.00 ml. of stock solution into a 1000-ml. volumetric flask and make to volume with IPA. Herban concentration, 5 μg. per ml.

Prepare two calibration curves covering the 5- to 40-μg. range in a 5.0-ml. final volume and the 50- to 500-μg. range in a 25.0-ml. final volume. Determine each point on the curves by measurements in triplicate. For the high range calibration, pipet 1.00-, 2.00-, 5.00-, and 10.00-ml. aliquots of high range standard into the 50-ml. Erlenmeyer flasks. For the low range, pipet 1.00-, 2.00-, 5.00- and 8.00-ml. aliquots of low range standard into the same type of flask. Follow the procedure given below. A plot of absorbance at 443 mμ *vs.* micrograms of Herban should yield a straight line.

General Procedure

Carefully evaporate the solvent in the Erlenmeyer flasks using a 60° C. water bath and a gentle stream of dry air. When dry, cool and add 10 ml. of the 10% potassium hydroxide–diethylene glycol mixture and several silicon carbide boiling stones. Attach to the reflux condensers and heat the flasks at high heat until reflux begins; at this point, switch to low heat, and allow the samples to reflux for 2 minutes. After this time raise the flasks from the hot plate and support them on an asbestos platform, while they are still attached to the condensers. Allow the samples to cool for a few minutes, then immerse the flasks in ice water to hasten the cooling. When the samples are cool, rinse down each reflux condenser with two 1.0-ml. portions of methanol so that the entire inner surface of the condenser is wet with each wash. Allow the methanol to drain between washes. While the samples are cooling, have the steam-distillation apparatus preheated and steamed out. Remove the cooled flasks, one at a time, from the condensers, and rinse the condenser joints with a few milliliters of distilled water, catching the wash in the flasks. Transfer each hydrolyzate to the Kjeldahl steam-distillation apparatus, using three 5-ml. washes of distilled water to clean the flask. Add 2.0 ml. of the 10% Antifoam B solution. Finally, rinse with another small portion of distilled water to clean the inlet system of the steam-distillation apparatus.

Add 10 ml. of the saturated boric acid solution to a 50-ml. beaker and place this under the tip of the condenser of the distillation apparatus so that the end of the condenser is submerged below the surface of the boric acid solution. Collect a total of 25 ml. of distillate from the still. After completing the distillation, remove the beaker and rinse the tip of the condenser with distilled water. Add 5 ml. of redistilled acetonitrile as cosolvent, and 0.5 ml. of the 1% 1-fluoro-2,4-dinitrobenzene reagent to the distillate in the 50-ml. beaker. Slowly add 2.0 ml. of 2*N* NaOH while rapidly stirring the solution with a short glass rod. Place the beaker and contents in a 60° C. water bath for 5 minutes, remove, and cool for 10 to 15 minutes.

Transfer each sample to a 60-ml. separatory funnel, and rinse the beaker with distilled water, adding the washings to the funnel. Extract the aqueous phase with three 10-ml. portions of chloroform, using the first portion of chloroform to wash the beaker which previously contained the sample. Collect the chloroform washes in a 50-ml. beaker and evaporate to dryness in a 60° C. water bath. (This is a convenient stopping point in the procedure.)

Pack a chromatographic tube to a height of 4 inches with basic alumina containing 2% (v./w.) added water. Pack the column by applying suction (water aspirator) to the exit end of the column while adding the adsorbant. With the suction still on, tap the column sides with a wooden dowel so that the column is uniformly packed. Turn off the suction. With a medicine dropper, transfer the dry residue from the chloroform extraction with three successive 1.0-ml. portions of *n*-hexane. Allow each *n*-hexane wash to flow onto the column. To be certain all material is transferred, rinse the beaker with three 1.00-ml. washes of the column eluent—5% (v./v.) acetone in *n*-hexane. For samples which are particularly difficult to transfer, warming the beaker containing these washes may be advisable. If heating fails to dissolve the residue, use 1 to 2 drops of straight acetone to aid in the transfer. Then dilute this acetone with several milliliters of *n*-hexane and transfer to the chromatographic column. Elute the column with the 5% acetone in *n*-hexane eluent at a maximum pressure of 2 p.s.i., collecting only 25.0-ml. total volume. This eluent includes the solvent from the transfer operations which may be in excess of a column volume and should contain only HA-DNP. Remove the solvent by evaporation in a 60° C. water bath; make certain all solvent is removed at this point. Traces of acetone will cause abnormal color development. Make sure that all glassware used from this point on, including spectrophotometer cells, is acetone-free.

Dissolve the dried residue obtained from the alumina column in a minimum volume of DMF and transfer to either a 5.0- or a 25.0-ml. volumetric flask, depending on the amount of material to be measured.

If the final measurement is to be made in 5.0 ml., add 2 drops of TMAH solution and 0.2 ml. of distilled water; if in 25.0 ml., add 6 drops of TMAH and 1.0 ml. of distilled water. Dilute to the proper volume with DMF. Add the TMAH just prior to spectrophotometric measurement. Make the solution to near volume, add the base, make the sample to volume and mix well, and measure the absorbance within 1 minute at 443 mμ in a 1-cm. cell. Use a 1-cm. cell filled with water to zero the instrument. For highest accuracy, develop the color of only one sample at a time, if a series is being run, because color sometimes fades.

Extraction and Cleanup of Crops

Two general extraction and cleanup procedures are used: one for oil-producing crops and one for vegetable and grain crops.

The validity of the Soxhlet extraction procedure for oil-producing crops was demonstrated by extracting lint-bearing cottonseed with two different solvents: *n*-hexane and isopropyl alcohol (IPA). The solubility of Herban in *n*-hexane at 65° C. (the approximate extraction temperature) is 6 grams per liter; in IPA it is 89 grams per liter at 23° C. and at 85° C. is approximately 375 grams per liter.

The data in Table I, A, represent the extraction and analysis of two samples of lint-bearing cottonseed, each treated at 6 pounds per acre pre-emergence. Each sample was subdivided into two 300-gram portions; one subsample was extracted with *n*-hexane and the other with IPA. The *n*-hexane extracts were obtained from 10 cycles of the Soxhlet, while the IPA extracts were taken from five-cycle runs. The IPA removed more extraneous material from the seed than did *n*-hexane; the buildup of material in the extraction pot resulted in severe bumping and necessitated a shorter extraction time. On cooling the IPA extract, much material precipitated; this was filtered off before proceeding.

No significant difference was observed between the results for the two solvent extractions and the control samples (Table I, A). Herban is soluble in oil, and *n*-hexane is an excellent oil solvent. Hence, *n*-hexane was chosen as the extraction solvent.

For residue samples a thorough 2-hour Soxhlet extraction is used. To demonstrate that Herban can be extracted for the cotton plant, if it is present, cotton seedlings which had been hydroponically treated in water containing 250 p.p.m. of Herban (saturated) were extracted

by homogenizing the plant with IPA. The purée was centrifuged and the supernatant liquid removed. The marc was then extracted twice more with small portions of IPA. The washes were combined and made to volume before analysis. The results are shown in Table I,B.

For vegetable and grain crops, IPA is the solvent of choice, since it is compatible with water. Actually, the composition of the solvent after extraction is approximately 70% IPA–30% water. At 23° C., the solubility of Herban in this mixture is 50 grams per liter. Even in 1 to 1 IPA–water mixtures, the solubility is 22 grams of Herban per liter.

Herban labeled with C^{14} on the dimethylamine portion of the molecule was used to demonstrate the validity of the two cleanup procedures described below. Lint-bearing cottonseed and potato extracts were selected as representative of the widely different crops encountered. Recovery data for the two crops are given in Table II.

Table I. Effect of Solvent on Extraction of Herban from Lint-Bearing Cottonseed and Cotton Plants

Sample No.	Treatment Lb./Acre	Extraction Solvent	Gross P.P.M. Herban
	A. Field-Treated Samples		
1A	6	*n*-Hexane	0.04
1B	6	IPA	0.06
2A	6	*n*-Hexane	0.03
2B	6	IPA	0.06
3A	Control	*n*-Hexane	0.04
3B	Control	*n*-Hexane	0.03
3C	Control	IPA	0.03
3D	Control	IPA	0.05

B. Hydroponically Treated Cotton Seedlings

Exposure Interval, Hr.	Sample Size, Grams	No. of Detns.	Gross Herban Found, μg.	Gross Herban Found, P.p.m.
Control, 24	1.0	1	9.8	9.8
Treat, 24	3.0	4	301 ± 8	100 ± 3
Control, 48	1.0	2	10.1	10.1
Treat, 48	2.1	4	435 ± 20	207 ± 8

Table II. Recoveries of C^{14}-Labeled Herban from Cottonseed and Potato Extracts

Lint-Bearing Seed, G.	No. of 50-Ml. Acetonitrile Extractions	No. of 50-Ml. *n*-Hexane Backwashes	Herban, P.P.M. Added	Herban, P.P.M. Found	% Recovered
A. Extraction of Herban from *n*-Hexane Solutions of Cottonseed Oil					
100	1	...	2.0	1.52	76.1
	2	...	2.0	1.86	92.8
	3	...	2.0	2.13	106.8
100	3	1	0	0	0
		1	0.1	0.11	110
		1	0.1	0.11	110

B. Extraction of Herban from Aqueous Solutions of Isopropyl Alcohol Extracts

Potatoes, G.	No. of 50-ml. Chloroform Extractions			
90	3	0	0	...
		0.11	0.12	109
		0.11	0.13	113
89	3	0	0	...
		1.12	1.16	103.2
		2.24	2.10	93.5

Oil-Producing Crops

Weigh approximately a 300-gram subsample of oil-bearing seed and grind to a fine meal with a coffee grinder. Catch the meal in a tared container, so that the final sample weight can be determined. Pack the meal into a 1-liter Soxhlet extractor, add 1200 ml. of *n*-hexane, and extract continuously until at least 10 cycles (about $2^1/_2$ hours) have been completed. After extraction drain the Soxhlet and cool the extract to room temperature. Measure the extract and calculate the ratio of grams of oil-bearing seed per milliliter of extract recovered.

To clean up the oil-bearing seed extract, aliquot a portion equivalent to 100 grams of seed and concentrate to about 50 ml. Transfer the concentrate to a 500-ml. separatory funnel with *n*-hexane, and adjust the final volume in the funnel back to 100 ml. with additional *n*-hexane. Wash the extract with three 50-ml. portions of redistilled acetonitrile by shaking the funnel 30 seconds for each wash. Combine the acetonitrile washes in a second 500-ml. funnel and backwash with one 50-ml. portion of *n*-hexane. Discard the *n*-hexane phase. Collect the acetonitrile phase in a 250-ml. beaker and carefully evaporate it on a 60° C. water bath with a gentle air stream. Transfer the residue to a 50-ml. hydrolysis flask with chloroform, remove the solvent by evaporation, and determine Herban according to the general procedure.

Vegetable and Grain Crops

Weigh a 1000- to 1500-gram subsample and finely grind or chop it into a tared 1-gallon paint can. Add 1 or 2 ml. of IPA per gram of sample, depending on the sample type. Seal the lid and place the can on a tumbler. After a 1-hour extraction, decant the extract through a wire screen. When considerable fines are present, allow these to settle overnight and then filter the extract through a porous filter paper. Measure the extract volume and determine the ratio of grams of sample per milliliter of extract.

For cleanup, pipet an aliquot of the extract equivalent to 100 grams of crop into a 2-liter separatory funnel containing 1500 ml. of distilled water. Wash the aqueous layer with three 50-ml. portions of chloroform by vigorously shaking the funnel 30 seconds for each wash. Allow any emulsion formed to break as much as possible before adding a few crystals of anhydrous sodium sulfate to beak the remainder. Combine the chloroform extract in a 250-ml. beaker and carefully evaporate on a 60° C. water bath with a gentle stream of air. Transfer the residue to a 50-ml. hydrolysis flask with chloroform, evaporate the solvent, and proceed with the Herban determination according to the general procedure.

Discussion

The determination of Herban at the residue level has been described as essentially a five-step process. These steps outlined above, warrant some individual discussion.

The first step, the hydrolysis of Herban, is extremely temperature-dependent, and, unlike the aromatic class of substituted urea herbicides, Herban can not be hydrolyzed to a great extent in aqueous acidic or caustic media (*7*). Only 2% of the Herban was recovered after a 30-minute reflux in 1 to 1 aqueous sulfuric acid and only 17% was recovered from a 1-hour reflux in 20% aqueous potassium hydroxide. Initially, ethylene glycol was chosen as the hydrolytic solvent because of its high boiling point and its ability to dissolve Herban and a fairly high concentration of potassium hydroxide.

Figure 1 shows the dependency of Herban recoveries on temperature with various solvents. Recoveries are based on a calibration curve prepared with purified HA–DNP (fine yellow needles from ethyl acetate, m.p. 172.1° C.)

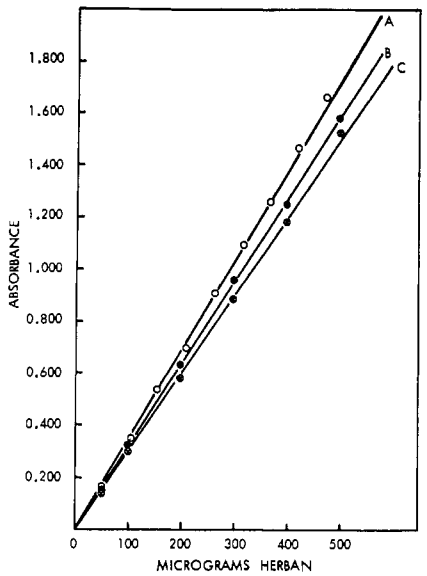


Figure 1. Effect of hydrolysis temperature on recovery of Herban

A. Purified HA-DNP calculated as Herban
B. Hydrolysis of Herban in KOH–diethylene glycol. Pot temperature, 180°C.
C. Hydrolysis of Herban in KOH–ethylene glycol. Pot temperature, 170°C.

(Figure 1, *A*). In ethylene glycol, approximately a 5% increase in hydrolytic action is observed with each 10° increase in oil-bath temperature from 160° to 190° C. At an oil-bath temperature of 190° C. (approximately 170° C. in the reaction flask), Herban is only 88% recovered (Figure 1, *C*). If, however, diethylene glycol is used as solvent, the pot temperature rises to 180° C. (oil bath still 190° C.) and again there is a 5% rise in recovery to a value of 93% (Figure 1, *B*). The latter value was consistent with recoveries obtained for HA alone, when it was subjected to the entire procedure. At this point, the hydrolysis was considered satisfactory and a calibration curve based on Herban could be prepared.

Table III. Relative Elution Values (R_e)[a] for FDNB Derivatives on Various Amines on 4-Inch No. 1 Columns of Alumina[b]

Amine Tested[c]	Column Type 1: Neutral Al_2O_3, 3% acetone in n-hexane	2: Basic Al_2O_3, 3% acetone in n-hexane	3: Neutral Al_2O_3, 5% acetone in n-hexane	4: Basic Al_2O_3, 5% acetone in n-hexane
Aniline	0.97	1.4	1.1	1.2
p-Chloroaniline	1.50 (NE)[d]	2.2 (NE)	1.4	1.4
3,4-Dichloroaniline	[e]	2.4 (NE)	2.1	2.6
Ammonia	[e]	[e]	[e]	[e]
Dimethylamine	[e]	[e]	4.7 (NE)	3.2
Benzylamine	[e]	[e]	4.2 (NE)	3.5
HA	1.0	1.0	1.0	1.0

[a] R_e = volume (ml.) at which test compound begins to elute / volume (ml.) at which HA-DNP begins to elute
[b] 2% (v./w.) added water.
[c] Column loaded with 100 μg. of each test amine derivative.
[d] NE = not entirely eluted in 100 ml.
[e] Did not begin to come off column in 100 ml.

The steam-distillation step is straightforward. Minimum time should be taken for removing the hydrolysis flask from the reflux condenser and introducing the sample into the steam-distillation apparatus. The reaction flask should be thoroughly cooled before the transfer, as HA has a considerable vapor pressure.

It is possible that HA might be extracted directly into another solvent at this point, and the HA content measured by gas chromatography. However, steam distillation provides a means of cleanup which might not be accomplished by an extraction step.

The third step, formation of the FDNP amine derivatives, can be accomplished in a number of organic solvents—e.g., acetone, dioxane, *n*-hexane, pyridine, and acetonitrile. Because of the steam-distillation step and the apparent volatility of HA, it must be absorbed in an aqueous acidic medium. The derivative is then prepared by merely adding cosolvent, 1-fluoro-2,4-dinitrobenzene, adjusting the pH to 9.3, and heating for 5 minutes at 60° C.

At room temperature, with no special conditions other than proper pH adjustment, the reaction is more than 90% complete in 30 minutes. Addition of acetonitrile cosolvent increases the recovery from an average of 93% to 99%, presumably through better solvation of the FDNB reagent in the aqueous medium. In addition, elevation of the temperature not only provides for a more rapid reaction rate, but also helps to increase over-all recoveries.

Destruction of excess 1-fluoro-2,4-dinitrobenzene is unnecessary. The reagent hydrolyzes slowly to 2,4-dinitro-

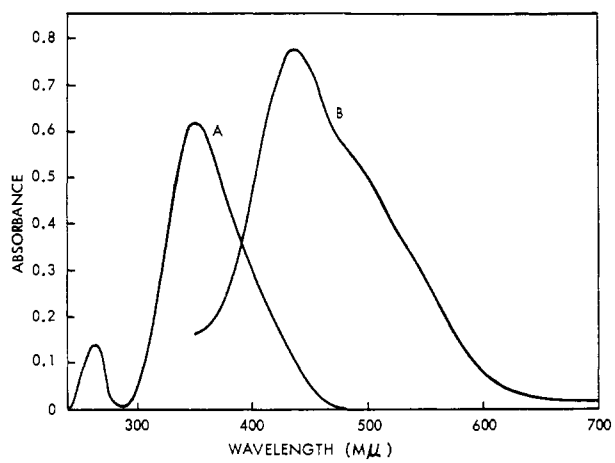


Figure 2. Visible and ultraviolet spectra

A. Visible and ultraviolet spectra of HA-DNP in ethyl alcohol
1-cm. cell
[HA-DNP], 10 μg./ml.
Vol., 25.0 ml.
Beckmon DK-2 spectrophotometer
B. Visible spectrum of HA-DNP in alkaline dimethylformamide
1-cm. cell
[HA-DNP], 10 μg./ml.
Vol., 25.0 ml.
Beckman DK-2 spectrophotometer

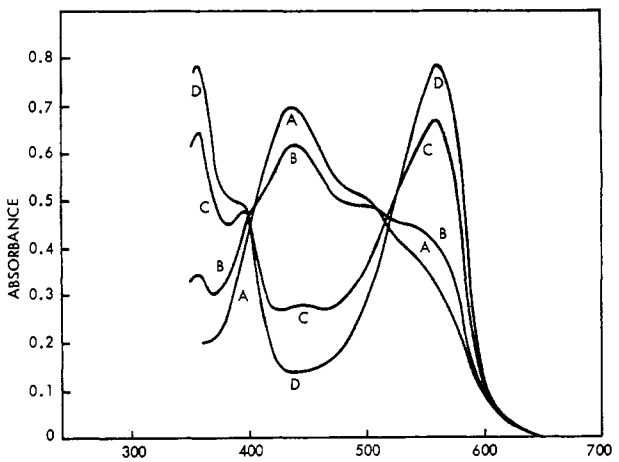


Figure 3. The effect of acetone on color of HA-DNP in alkaline dimethylformamide

A. 0.15 ml. acetone
B. 0.3 ml. acetone
C. 0.5 ml. acetone
D. 1.0 ml. acetone
1-cm. cells
[HA-DNP], 15 μg./ml.
Vol., 25.0-ml.
Beckman DK-2 spectrophotometer

phenol under the conditions of derivative formation and both excess reagent and phenol are removed by the column chromatographic step.

The chromatographic step in the procedure provides the method with its specificity. All of the FDNB derivatives of both aliphatic and aromatic amines examined were yellow or orange-yellow compounds. All had similar ultraviolet or visible spectra in solvents such as acetone, ethanol, dimethylformamide, and alkaline dimethylformamide and hence interfered with the determination of HA-DNP.

Experiments with various types of alumina (acid, basic, and neutral) provided a column system whereby HA-DNP could be readily resolved from other possible interfering amine-FDNB derivatives. In Table III, this column (No. 4) is compared with the others examined. Although column 2 appears to be more satisfactory with respect to resolution of HA-DNP, much more band spreading occurs with this system. When column 4 is used, HA-DNP is eluted rapidly (within 25 ml.) and with a clean separation.

To reproduce conditions of column separation and obtain adequate separations with a minimum of band spreading, close attention must be given to the manner in which the column is packed and the derivatives are transferred to the column. Especially critical is the step which involves rinsing the beaker with acetone and transferring this wash to the column. Too little acetone will not completely transfer the residue. Too much acetone will cause streaking, resulting in band overlap. Transferring the derivatives as described in the procedure has proved most reliable.

The visible and ultraviolet spectra of HA-DNP in ethyl alcohol are shown in Figure 2*A*. The maximum at 350 mμ was unsatisfactory because of the limited capacity of the Beckman Model B spectrophotometer in this spectral region.

Recently (*9*), it was shown that amine-FDNB derivatives produced different colors in alkaline solution. The visible spectrum of HA-DNP in alkaline dimethylformamide is represented by Figure 2*B*. At 443 mμ (the peak maximum on our spectrophotometer) conformity to Beer's law was observed over the range of 5 to 500 μg. of Herban (7 to 700 μg. of HA-DNP).

The orange-red color of the derivative in dimethylformamide forms immediately upon addition of TMAH, and is insensitive to at least a threefold change in base concentration. Water decreases the color intensity. A 4% decrease in absorbance was observed over a fourfold increase in water concentration. As water must be added to dissolve the TMAH, care is taken in its addition.

In experiments performed with purified HA-DNP, color stability was not a serious problem. The color was stable for at least 30 minutes, with serious decay being observed only after 2 hours. When actual residue samples are run, color intensity often decreases. Hence, the normal procedure is to prepare the final solution to near volume, add the base, make final volume adjustments, and read the color within 1 minute. Apparently some interference which is carried through from the crop being analyzed produces color instability.

Aside from other possible amine–FDNB derivatives, the only other serious interference encountered was from acetone. HA-DNP in acetone and TMAH produces a violet color (λ_{max} = 560 mμ) of much greater intensity than a solution of HA-DNP in alkaline dimethylformamide. However, very rapid color fading was observed and no way was found to stabilize the system. With the dimethylformamide–TMAH solvent, traces of acetone cause major color suppression and fading (Figure 3). One milliliter of acetone in 25 ml. of dimethylformamide completely washes out the 443-mμ maximum. Therefore, as noted in the procedure, acetone used in the chromatographic step must be rigorously excluded during color measurement.

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