inhibitory activity (*1*). A disadvantage of the inhibition methods is the lack of specificity to distinguish among different types of organophosphorus compounds.

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FUNGICIDE RESIDUES

Colorimetric Determination of Dexon Residues in Crops

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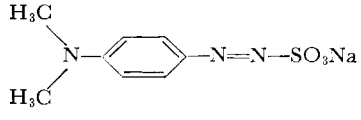
A colorimetric method has been developed for the determination of Dexon residues in plant material. The method is based on the light-catalyzed coupling of the compound with resorcinol in alkaline solution. The yellow product is extracted into benzene and measured in a spectrophotometer at 450 mμ.

DEXON (trademark, Farbenfabriken Bayer), *p*-dimethylaminobenzenediazo sodium sulfonate, is a nonmercurial fungicide for the protection of germinating seed and seedlings. It is particularly effective against those damping-off fungi in the genera *Pythium*, *Aphanomyces*, and *Phytophthora*. In the literature, this material has been referred to as Bayer 22555. Its structural formula is:

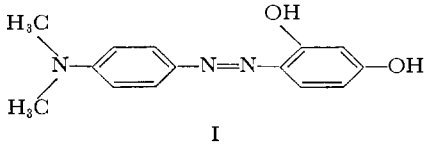


Dexon is a yellow-brown powder which dissolves in water to the extent of 2 to 3% at 25° C. to form an intensely orange-colored solution. The compound is soluble in highly polar solvents, such as dimethylformamide, but is insoluble in most organic solvents.

As is the case with many diazo compounds (*2*), Dexon is extremely sensitive to light. Dilute aqueous solutions of the compound are completely decolorized in 30 minutes or less when exposed to ordinary laboratory light conditions. However, solutions, including plant extracts, may be stabilized by the addition of sodium sulfite. Therefore, a 1% solution of sodium sulfite is used for the initial extraction of plant material.

The analytical method developed depends upon the light-catalyzed coupling of Dexon with resorcinol in alkaline solution. Light energy converts the stabilized diazo sulfonate into a labile form of the compound which then couples with the resorcinol.

The colored reaction product has been reported (*1*) to be:



I

The product (I) is yellow in acid solution, from which it may be quantitatively extracted with a nonpolar solvent such as benzene. The product (I) is stable in benzene for days.

The absorption spectrum of the yellow product (I) is shown in Figure 1. Maximum absorption occurs at 450 mμ. The molar absorptivity for the product (I) is approximately 26,800. The molar absorptivity of Dexon is approximately 16,100 with maximum absorption at 420 mμ.

As indicated above, Dexon is water-soluble and, as such, should be dialyzable. Experiments were undertaken to determine the practicability of dialysis as a process for the separation and clean-up of Dexon residues found in plant extracts. Seamless cellulose dialyzer tubing (The Visking Corp.) was selected as the dialysis membrane.

Dialysis Conditions

A study was made to determine the effect of time, temperature, and agitation on the rate of dialysis. Dexon, ring-labelled with C^{14}, was used for this purpose. One hundred fifty milliliters of sodium sulfite (1%) containing 200 to 300 μg. of radioactive Dexon were added to 100 grams of plant material (fresh corn kernels) or 100 ml. of distilled water, and blended for 2 minutes. The mixture was introduced into the dialysis tubing and dialyzed against 600 ml. of sodium sulfite (1%). The dialysis system was contained in a 2-quart, screw-capped jar covered with aluminum foil. Dialyses were conducted either in the cold (5° C.) or at room temperature (25° C.). Half of the samples for each temperature were placed on reciprocal shakers and agitated slowly during the dialysis period (120 to 140 cycles per minute).

The other half of the samples were not shaken. Aliquots (2 to 3 ml.) of the diffusate were removed at various times, and the radioactivity present was measured in a liquid scintillation spectrometer manufactured by Packard Instrument Co., La Grange, Ill. The radioassay procedure was similar to that proposed by Steinberg (*3*). The extent to which equilibrium had been reached after each interval of time was determined by comparing the radioactivity of the aliquots from the diffusate with the radioactivity of similar size aliquots from a standard solution. The standard solution was prepared by diluting a quantity of radioactive Dexon equivalent to that used for each dialysis to 850 ml. with sodium sulfite solution (1%). Thus, at equilibrium, the radioactivity of the aliquot from the diffusate should be equal to the radioactivity of the aliquot from the standard solution, and the ratio of the radioactivity of the diffusate aliquot to the radioactivity of the standard solution aliquot, reported

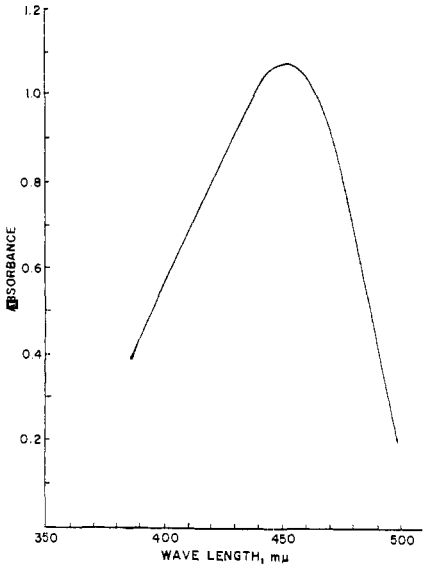


Figure 1. Absorption spectrum of Dexon-resorcinol reaction product

Table I. Effect of Dialysis Time, Temperature, Agitation, and Plant Material on Recovery of Dexon by Dialysis

Dialysis Period, Hours	Equilibrium Obtained (Recovery), %: Without Plant Material, without Agitation, at 5° C.	Without Plant Material, without Agitation, at 25° C.	With Plant Material, With Agitation, at 5° C.	With Plant Material, With Agitation, at 25° C.	With Plant Material, Without Agitation, at 5° C.	With Plant Material, Without Agitation, at 25° C.
1	47	36	58	46	25	20
2	62	53	73	61	39	32
4	77	74	79	74	54	49
6	..	84	83	83	..	57
6.5	83	..	..	..	68	..
20	94	..	..	..	79	..
24	..	96	..	..	82[a]	84
48	..	..	..	..	80[a]	89
72	..	..	..	..	87[a]	..

[a] Values determined by analytical procedure (colorimetrically). Other values determined by radioactivity measurements.

as percent, should represent the extent to which equilibrium has been reached. The data which were obtained are presented in Table I.

The data in Table I show that the presence of plant material inhibits the rate of dialysis of Dexon significantly during static dialysis. However, the concentration of Dexon reaches 80% of the equilibrium value if the dialysis proceeds for at least 20 hours. The data indicate that the effect of dialysis temperature on recovery of Dexon is negligible between 5° and 25° C.

The dialysis system used is a good medium for the growth of microorganisms and even though four or five drops of toluene are routinely added to the receiving solvent, prior to dialysis, microbial growth has been observed in some samples. The fact that the recovery of Dexon at 25° C. was as good as that at 5° C., in spite of the much greater microbial growth at the higher temperature, indicates that Dexon is stable toward biochemical attack. However, the presence of this microbial growth does appear to increase the amount of emulsion formed during the initial benzene extraction, and therefore the addition of a few drops of toluene is recommended.

The data in Table I indicate that agitation increases the rate of dialysis significantly. The system approaches equilibrium in 6 hours with agitation, while 16 to 20 hours are required for comparable recovery during static dialysis. However, in the selection of the type of dialysis to be employed routinely, other factors were considered, namely the number of samples which could be conveniently analyzed simultaneously, based on the available equipment, and the time element involved as it fits established work schedules. Therefore, even though agitation increases the rate of dialysis significantly, other considerations prompted the selection of overnight static dialysis as the procedure of choice for routine analysis.

Conditions for Color Development

In the development of the method, a study was made of the optimum conditions necessary for the coupling reaction. To determine the effect of the concentration of base at the time of irradiation, aliquots of a $4N$ solution of potassium hydroxide were mixed together with 10 ml. of $0.5M$ sodium sulfite, 1 ml. of an aqueous solution of Dexon (50 μg. per ml.), and 5 ml. of $4M$ resorcinol, and diluted to 220 ml. The final concentration of base varied between $0.05N$ and $0.25N$. After a 10-minute irradiation period, an amount of acid sufficient to neutralize the base and give an excess of 10 meq. was added to each sample. All samples were extracted with 50 ml. of benzene, and the amount of color present in each was determined. No differences were observed in any of the samples. All subsequent irradiations were carried out in $0.15N$ potassium hydroxide solution.

The effect of varying the resorcinol concentration was likewise investigated. Varying amounts of resorcinol were added to 200-ml. portions of $0.15N$ potassium hydroxide containing 10 ml. of $0.5M$ sodium sulfite. The resorcinol concentrations ranged from $0.0005M$ to $0.1M$. One milliliter of an aqueous solution of Dexon (50 μg./ml.) was added to each sample. The samples were irradiated for 10 minutes and then acidified and extracted with benzene as described above. The results were the same at all resorcinol concentrations. All subsequent irradiations were carried out on solutions having a resorcinol concentration of $0.1M$. The rate of coupling was determined by irradiating a series of samples for periods ranging up to 60 minutes. Coupling was found to be complete in 2 to 3 minutes in the absence of plant material but took 15 to 20 minutes in the presence of tissue extract. A 30-minute irradiation period was selected for routine use and gave very reproducible results even in the presence of plant extracts.

Variation of the concentration of sodium sulfite between $0.01M$ and $0.5M$ during irradiation had no effect upon the amount of color obtained. A concentration of 1% (approximately $0.1M$) was selected for routine use.

At the level of sodium sulfite concentration adopted for routine use, it was observed that the pH following acidification of the irradiated samples was 1.5 or lower. Under these conditions, a single extraction with benzene did not remove the colored complex completely. Therefore, it was decided to neutralize and buffer the system with potassium dihydrogen phosphate to a pH of 5.5. At this pH, extraction was found to be complete. This was accomplished by the addition of 20 meq. of excess acid and an amount of the buffer sufficient to give a final buffer concentration of $0.05M$.

Reagents and Special Apparatus

Dexon, recrystallized (Chemagro Corp., Kansas City, Mo.).

Dialysis tubing, seamless cellulose tubing manufactured by the Visking Corp., Chicago, Ill.

Refrigerator trays, borosilicate glass, 6 × 10 × 2 inches deep.

Spectrophotometer, Beckman Model DU or equivalent, equipped with 10.0-cm. microcuvettes (6-ml. volume).

Spotlights, Eldecor projector flood or equivalent, 150 watts.

Sample Preparation

Crops other than Cottonseed. The wide variation in the physical nature and moisture content of the samples necessitated the use of several different blend-

ing procedures. In all cases, 100-gram portions of the sample were analyzed. Fresh corn kernels and sugar cane juice are blended with 150 ml. of 1.0% sodium sulfite solution. Pineapple is ground in a food chopper prior to blending with the same amount of sulfite solution. Sorghum seed is ground dry in a blender at high speed for 1 minute before blending with 250 ml. of the sulfite solution. Dried corn kernels are blended directly with 250 ml. of the sulfite solution. Sugar cane is cut into small pieces, ground dry in a blender at high speed for 1 minute, and then blended with 300 ml. of sulfite solution. In all cases, the final blending is at high speed for a period of 2 minutes.

Cottonseed. The high oil content of cottonseed indicated that an entirely aqueous system would not be practical for the dialysis of Dexon residues which might be present in or on the cottonseed. However, it was found that a mixture of aqueous sodium sulfite and benzene would give a satisfactory extraction of ground cottonseed. In the procedure finally adopted, a 25-gram portion of cottonseed is blended for 2 minutes at high speed with a mixture of 100 ml. of benzene and 200 ml. of the sulfite solution. Dexon can be removed from the resulting mixture by dialysis.

Table II. Recovery of Dexon from Plant Material

Plant Material	Control Values, P.P.M.	Dexon Added, P.P.M.	Dexon Found,[a] P.P.M.	Mean Recovery, %
Corn	0.015, <0.015, <0.015 <0.015, <0.015	0.050	0.039	78
		0.100	0.078 ± 0.004(3)	78
		0.250	0.220	88
Pineapple	<0.015, <0.015, <0.015 <0.015, <0.015, <0.015 0.016	0.050	0.051 ± 0.003(2)	102
		0.100	0.087 ± 0.019(4)	87
		0.200	0.226 ± 0.012(2)	113
Sorghum seed	<0.015, <0.015, <0.015 <0.015, <0.015, <0.015 0.023	0.050	0.041 ± 0.005(4)	82
		0.100	0.077 ± 0.001(4)	77
		0.200	0.13 ± 0.018(2)	65
Sugar cane	<0.020, <0.020, <0.020 <0.020, 0.037	0.050	0.068 ± 0.001(2)	136
		0.100	0.105 ± 0.019(4)	105
		0.200	0.172	86
Sugar cane juice	<0.015, <0.015, <0.015 <0.015	0.100	0.083 ± 0.002(3)	83
		0.200	0.168	84
Cottonseed	<0.060, <0.060, <0.060 0.108, 0.084	0.100	0.100 ± 0.015(2)	100
		0.200	0.190	95
		0.400	0.352	88

[a] Numbers in parentheses refer to number of determinations involved.

Dialysis of the Sample Macerate

The slurry obtained above is transferred quantitatively from the blender into a seamless cellulose dialysis tube, previously double knotted at one end. (Note: All dialysis tubing should be soaked a minimum of 5 minutes in the 1.0% sodium sulfite solution prior to use. The tubing should be twice the length required to contain the sample.) Collapse the tubing above the sample to remove the air, and double knot the open end of the tube. Place the tube in a 2-quart, screw-capped glass bottle containing 600 ml. of 1.0% sodium sulfite. Five drops of toluene are added as a preservative. Dialyze the sample in the dark for 20 hours at room temperature. Pour off the diffusate into a 1000-ml. graduated cylinder and measure the volume.

Coupling Reaction

Transfer the diffusate into a shallow borosilicate-glass refrigerator tray. [In the case of cottonseed, transfer the diffusate to a 1-liter separatory funnel and add 100 ml. of benzene. Shake the mixture vigorously for 30 seconds. Allow the phases to separate and then slowly draw off the lower aqueous phase into a 1-liter beaker. Discard the upper (benzene) phase. Transfer the aqueous phase to a shallow, borosilicate-glass tray and proceed as with the other crops.] Place the tray in a larger tray containing crushed ice. With stirring, add 15 ml. of 4.0*M* resorcinol to the sample followed by 25 ml. of 4.0*N* potassium hydroxide. Irradiate the sample for 30 minutes with two spotlights (150 watts) held 7 to 8 inches above the surface of the liquid. Turn off the spotlights and pour the sample into a 2-liter beaker. Add 60 ml. of 0.6*M* potassium dihydrogen phosphate buffer solution and then 30 ml. of 4.0*N* hydrochloric acid to the sample in the beaker. Immediately transfer the sample to a 1-liter separatory funnel and add 100 ml. of benzene. Shake the mixture vigorously for 60 seconds. Allow the phases to separate and then slowly draw off and discard the lower (aqueous) phase. Drain the benzene layer into a 100 ml. graduate and measure the volume. Following irradiation, the analysis may be interrupted only when the coupled product is in benzene.

Final Cleanup and Concentration of the Sample

Transfer 90 ml. of the benzene fraction into a 250-ml. separatory funnel. If less than 90 ml. of the benzene fraction are obtained, record the volume and apply an appropriate correction to the calculations. Add 25 ml. of 1.0*N* potassium hydroxide to the separatory funnel and shake the mixture vigorously for 30 seconds. Allow the phases to separate and then slowly drain the lower (aqueous) phase into a second 250-ml. separatory funnel. Repeat the extraction with a second 25-ml. portion of the alkali solution.

Add 25 ml. of the buffer solution (0.6*M* potassium dihydrogen phosphate) to the separatory funnel containing the combined alkali extracts. Then add 25 ml. of 2.0*N* hydrochloric acid and 10 ml. of benzene and shake the mixture vigorously for 30 seconds. Allow the phases to separate and then slowly draw off and discard the lower (aqueous) phase. Drain the benzene extract into a 25-ml. graduated cylinder containing 2 or 3 grams of anhydrous sodium sulfate.

Transfer the dry benzene extract into a 10-cm. microcuvette (6-ml. volume) and read the absorbance in the spectrophotometer at 450 mμ against a reagent blank prepared as described above beginning with the irradiation step.

Calculation of Dexon Concentration

For purposes of calculation, it is arbitrarily assumed that dry samples, such as dried corn, sorghum seed, and cottonseed, are absolutely dry although the moisture content may be as high as 20%, and the other plant materials—i.e., fresh corn, pineapple, sugar cane, and sugar cane juice—are entirely composed of water although the moisture content may be as low as 80%. Although these assumptions may introduce an error of 20 ml. or so in the measurement of total volume, this would represent a gross error of only 2 or 3% in the final result. This is considered to be within the normal experimental error and therefore may be disregarded.

Let A = Volume of diffusate
W = Weight of sample in grams
T = Total volume of liquid in the dialysis system, assumed to be 1000 ml. for

sugar cane, 800 ml. for cottonseed, and 850 ml. for other plant materials

C = Dexon concentration in μg. per ml. as read from the standard curve

A standard curve is obtained by adding known amounts of Dexon to 600 ml. of 1.0% sodium sulfite solution, processing as a sample beginning with the coupling reaction, and plotting optical density versus micrograms of Dexon present per milliliter. The curve obeys Beer's law. A concentration of 1 μg. per ml. in the final solution has an absorbance of 1.09.

P.P.M. Dexon in sample =

$$\frac{(10)\,(C)\,(100)\,(T)}{(W)\,(90)\,(A)}$$

This determination should also be carried out on untreated control samples. In most cases, the value obtained on these is negligible. In the event that significant values are obtained from control samples, they must be subtracted from the value observed for the treated samples in order to obtain the true values for the residues of Dexon.

Discussion

Recovery Experiments. Recovery experiments were conducted on each of the plant materials listed in Table II, in which known amounts of Dexon were added just prior to dialysis. While such experiments do not indicate the efficiency of the initial extraction system, they do show whether or not the material is lost in the steps subsequent to extraction.

The data presented in Table II indicate that satisfactory recovery of Dexon may be achieved by the procedure described. Where appropriate, the reported values are followed by the average deviation from the mean and, in brackets, by the number of independent determinations carried out.

Precision and Sensitivity. The data in Table II show the precision of results obtainable by this method. In the range up to 0.2 p.p.m., the average deviation from the mean is approximately 10% of the measured value. The precision is considered satisfactory for the low concentrations being measured. The instrumental limit of sensitivity is approximately 0.06 p.p.m. for cottonseed, 0.02 p.p.m. for sugar cane, and 0.015 p.p.m. for the other crops since these residue levels are necessary for an absorbance of 0.1. For all crops other than cottonseed, control values are well below these values so that the sensitivity of the method may be considered to be equivalent to the instrumental limit of sensitivity. Cottonseed control values ranged from <0.060 p.p.m. to 0.108 p.p.m.; therefore, the sensitivity for this crop is approximately 0.10 p.p.m.

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FUNGICIDE-MITICIDE RESIDUES

Extraction and Determination of Karathane Residues in Fruits

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A procedure is described for the extraction and determination of Karathane residues in apples, grapes, and strawberries. The residue is suitably extracted with mixed hexanes and purified either by column chromatography or by washing with sulfuric acid. Following solvent evaporation, the residue is dissolved in *N,N*-dimethylformamide for color development. The color which forms is measured at 444 mμ and is stable for at least 60 minutes. Recovery experiments and residue data are also presented.

KARATHANE (Rohm and Haas Co., trade name), 2-(1-methylheptyl)-4,6-dinitrophenyl crotonate, is used on a variety of crops for the control of powdery mildew diseases and certain species of mites. The method commonly used for the determination of Karathane residues (*3*) requires careful evaporation of the extracting solvent, steam distillation, extraction of the distillate, evaporation, and finally color development with a pyridine-water reagent. Recently, a much shorter procedure (*4*) has been described which utilizes ethanolic tetraethylammonium hydroxide as the color developing reagent.

Recent studies in this laboratory have revealed that Karathane forms an intense yellow color when dissolved in *N,N*-dimethylformamide without the addition of alkali required for color development with most mono- and dinitro compounds (*2*). A similar observation (*1*) has been made with Nitrosal, a feed medicament. Since apparently few nitro compounds form colors under these conditions, this color formation combined with a relatively simple cleanup procedure served as the basis for development of a new method for the determination of Karathane residues in fruits. In the present study, a short and sensitive procedure is described for the extraction and determination of Karathane residues in apples, grapes, and strawberries. In general, the residue is extracted with mixed hexanes and purified by column chromatography (or washed with sulfuric acid), the solvent is removed under reduced pressure, and the residual Karathane is dissolved in *N,N*-dimethylformamide for color development.

Reagents

N,N-dimethylformamide, spectro grade.