Use of 70 to 100 ml. of 10% ethyl acetate for elution, while complete, eluted a portion of the extractives which interfered in the colorimetry portion of the procedure.

Recoveries. Natural Waters. Table II presents the recovery and background values from control and fortified natural stream water accrued by the method. Background values averaged 5 p.p.b., and recoveries averaged 81%. In addition, background values from water taken from several locations in California and New Jersey ranged from 2 to 11 p.p.b. (7).

Mud. In Table II are collated the recovery and background values from control and fortified stream bed mud found by the method. Background values, not corrected for reagent blanks, averaged 0.07 p.p.m., and recoveries averaged 79%. In addition, background values from mud taken from various locations in California and New Jersey were less than 0.1 p.p.m. (7).

Oysters. Table II shows the recovery and background values from control and fortified oysters purchased in local markets. Background values, not corrected for reagent blanks, averaged 0.07 p.p.m., and recoveries averaged 72%. In addition, background values from Florida oysters ranged from 0.05 to 0.14 p.p.m. (7).

Rice Grain and Foliage. Table II also presents the recovery and background values from control and fortified whole rice grain and rice foliage. Background values, not corrected for reagent blanks, averaged 0.06 p.p.m. for whole rice grain and 0.06 p.p.m. for rice foliage; recoveries for whole rice grain averaged 67% and for rice foilage averaged 71%. In addition, background values for rice grain and foliage from California ranged from less than 0.01 to 0.07 p.p.m. and less than 0.01 p.p.m., respectively (7).

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ANALYTICAL METHODOLOGY

Colorimetric Determination of Potasan in Coumaphos

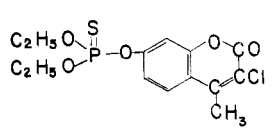
D. M. WASLESKI

Research Department, Chemagro Corp., Kansas City, Mo.

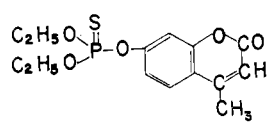
Potasan is separated from coumaphos by thin-layer chromatography on silica gel with chloroform. The Potasan spot, detected under ultraviolet light, is excised under vacuum and eluted from the silica gel with methanol. The residue after stripping is hydrolyzed with sodium carbonate and coupled with diazotized *p*-nitroaniline giving an intensely colored dye (λ_{max}. 500 mμ) which is compared with pure Potasan subjected to the same procedure.

Coumaphos, *O,O* - diethyl *O* - 3- (chloro - 4 - methyl - 7 - coumarinyl) phosphorothioate, is the product of the reaction of diethyl thiophosphoryl chloride and 3-chloro-4-methyl-7-hydroxycoumarin. Coumaphos has been formulated as a 25% wettable powder, Co-Ral, for control of animal parasites. Technical 3-chloro-4-methyl-7-hydroxycoumarin contains 3 to 5% of the unchlorinated analog, 4-methyl-7-hydroxycoumarin, as an impurity which reacts with diethyl phosphoryl chloride to form Potasan.

The structures of coumaphos and Potasan are similar, the only difference being the 3-chloro substituent:



Coumaphos



Potasan

Coumaphos is analyzed by measuring the ultraviolet absorption in methanol at 290 mμ (1), but Potasan, which has an ultraviolet maximum at 277 mμ, interferes. An accurate analysis for coumaphos requires a correction for the Potasan present. Also, as Potasan exhibits insecticidal properties, it is desirable to know the amount present. The ultraviolet and infrared spectra of these compounds do not show sufficient differences to make the determination of Potasan in coumaphos at the 3 to 5% level practical. Therefore, thin-layer chromatography was investigated.

Potasan can be separated cleanly from coumaphos by thin-layer chromatography using silica gel with chloroform as the solvent. Visual determinations on the thin-layer plate, after treatment with various color reagents, gave poor accuracy and reproducibility, so a method was developed involving separation, detection, extraction, and colorimetric determination (2) of the Potasan.

Experimental

Preparation of Standards. All standards were prepared from the purest Potasan—prepared by the reaction of diethyl thiophosphoryl chloride and 4-methyl-7-hydroxycoumarin and recrystallized from methanol—(m.p. 37–38° C.; one spot by TLC) and coumaphos (m.p. 94.5–95.0° C., two spots by TLC: coumaphos plus a trace of Potasan) (available from Chemagro Corp.). Solution A was a 5.00% w./v. solution of coumaphos in acetone. Solution B was a 0.500% w./v. solution of Potasan in acetone. Aliquots of 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 ml. of Solution B were

pipetted into 25-ml. volumetric flasks. Ten milliliters of Solution A was added to each flask and diluted to volume with acetone. These are equivalent to 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0% Potasan in coumaphos. The final solutions were 2% with respect to coumaphos.

Procedure. APPARATUS. Black Box equipped with short wavelength ultraviolet lamp.

Cell, absorption, 1 cm.

Cotton, absorbent.

Filter paper, Whatman No. 3, 9 cm. and No. 541, 3 cm.

Pipets, diSPo, 5 inch, capillary tips (Scientific Products Co., Evanston, Ill.).

Silica gel G (with 2% finely ground uranyl acetate), TLC plates, 0.5 mm., 8 × 8 inches.

Spectrophotometer, colorimeter.

Tank, chromatography, 8 × 8 inches.

Water bath, 100° C.

REAGENTS. Chloroform, reagent grade.

Hydrochloric acid, reagent grade.

Methanol, reagent grade.

p-Nitroaniline hydrochloride solution. Weigh 0.70 gram of *p*-nitroaniline into a 100-ml. volumetric flask and add 9.0 ml. of concentrated hydrochloric acid and about 10 ml. of water. Swirl the mixture until complete solution occurs, and dilute to the mark with water. Mix thoroughly. Cool the solution to 0° C. (It may be kept at this temperature for several weeks.)

Diazotized *p*-nitroaniline. Pipet into a 100-ml. volumetric flask in an ice bath 10.0 ml. of the acidified *p*-nitroaniline solution and cool to 0° C. Add 10 ml. of chilled 5% sodium nitrite with swirling. Keep the solution at 0° C. for 10 minutes, then dilute to volume with water and allow to come to room temperature. Do not use before 20 minutes or after 2 hours.

Sodium carbonate, 5%, reagent grade.

Sodium nitrite, 5%, reagent grade.

Uranyl acetate, reagent grade.

METHOD. The equivalent of 2.00 grams of coumaphos is weighed into a 100-ml. volumetric flask, about 50 ml. of acetone is added, and the flask is swirled for about 1 minute. It is diluted to volume with acetone, stoppered, and inverted 30 times. For formulated material a small portion is centrifuged at this point. About 50 µl. of the sample and the standards are spotted on a uranyl acetate treated 0.5-mm. silica gel plate. Adjacent spots are at least 1/2 inch apart, and they are made as small as possible without allowing the coumaphos to cake. Note: After the syringe is embedded in the silica gel layer, all loose silica gel is blown away before any solution is allowed to flow into the layer. The plate is developed by ascending chromatography using chloroform as a solvent. After the solvent has ascended the full length of the plate, it is removed and allowed to air dry.

The Potasan spots are located at R_f 0.4 (coumaphos R_f 0.6) as quenching spots against a green fluorescent background under short wavelength ultraviolet light. The green fluorescence is caused by the presence of uranyl acetate on the plate. The locations are marked with a sharp, pointed instrument. Each spot is vacuumed off the plate by attaching a vacuum line to the capillary end of a disposable pipet which has been plugged with cotton and applying the open end to the spot. A "blank" spot is also removed from below the origin with another plugged pipet. Each pipet is placed in a holder with the capillary end down in a 160 × 20 mm. test tube. Each pipet is filled with methanol and allowed to drain and this is repeated four more times. Alundum boiling chips are added, test tubes are placed on a steam bath, and all tubes are shaken vigorously until boiling begins. All tubes are permitted to evaporate to dryness.

Five milliliters of 5.0% sodium carbonate is pipetted into each tube by rinsing the sides, and they are heated at 100° C. for 15 minutes. The solutions are allowed to cool to room temperature and 3 ml. of diazotized *p*-nitroaniline are pipeted into each flask with vigorous shaking. After 10 minutes, the solutions are filtered through a Whatman 9-cm. diameter No. 3 filter paper backed with a 3-cm. diameter Whatman No. 541 filter paper. The solutions are read in 1-cm. absorption cells immediately on a spectrophotometer at 500 mµ after adjusting the blank to 100% transmission ($A = 0$) before each reading. All samples should be read within a 10-minute period of each other. The absorbance of the 0% Potasan is subtracted from each standard reading. The absorbance of standard *vs.* % Potasan is plotted, and the % Potasan in the sample is read directly from the graph.

Discussion

The system 0.5-mm. silica gel G and chloroform gave good separation of coumaphos and Potasan (Figure 1), but a detection system was needed so that the components would be visible as quenching zones under ultraviolet light. Various compounds were added to the silica gel G to render the thin layer fluorescent. These included eosine, fluorescein, chromotropic acid, Eosine Y, disodium 4,5-dihydroxy-2,7-naphthalenedisulfonate, Acridine Orange, and uranyl acetate. All except uranyl acetate either did not fluoresce sufficiently or interfered with the colorimetric procedure. Therefore, uranyl acetate was adopted. The Potasan spot was clearly defined as a quenching spot against a green fluorescent background.

After the spot was removed from the plate, the diSPo pipet was inverted and rinsed with methanol. The pipet was filled almost to the top with methanol and allowed to drain into a 20 × 160 mm. tube. This process was repeated nine more times. Over 98% of the Potasan was eluted in four extractions (Figure 2). Five extractions were used for the procedure.

A variation on the colorimetric diazo procedure described by Snell and Snell (*2*) for the determination of coumarin was investigated. To ascertain the hydrolysis rate of Potasan, the methanol was evaporated from six different extracts of 5% standards, 5 ml. of 5% sodium carbonate was pipetted into each, and they were heated at 100° C. (steam bath) for 0, 5, 10, 15, 20, and 25 minutes, respectively. After cooling to room temperature, 3.0 ml. of a diazotized solution of *p*-nitroaniline was pipetted into each with shaking. The color formed gave a maximum absorbance at 500 mµ. At this wavelength, maximum color development occurred after hydrolyzing for 10 minutes at 100° C. Further heating did not affect the amount of color. Fifteen minutes' heating at 100° C. for hydrolysis was used (Figure 3).

The diazonium salt gave a re-

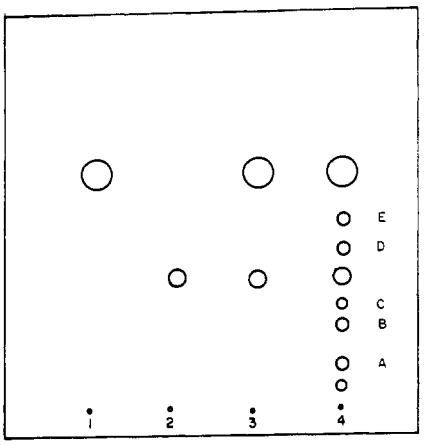


Figure 1. Thin-layer chromatogram of coumaphos and Potasan

Silica gel G 0.5 mm.–chloroform
1. Coumaphos
2. Potasan
3. Potasan plus coumaphos
4. Technical coumaphos

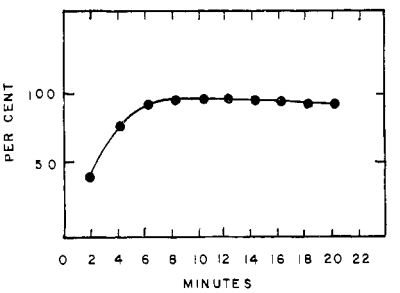


Figure 2. Extraction of Potasan from silica gel G

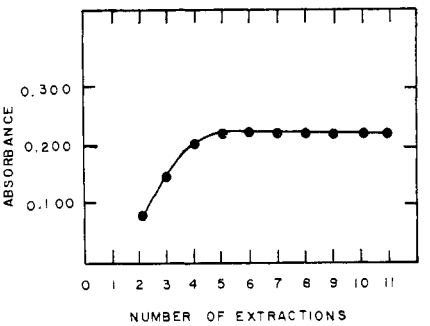


Figure 3. Hydrolysis of Potasan with sodium carbonate at 100° C.

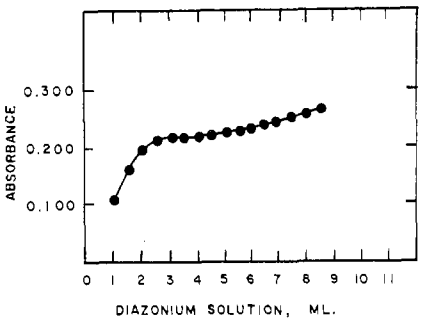


Figure 4. Effect of concentration of diazonium salt

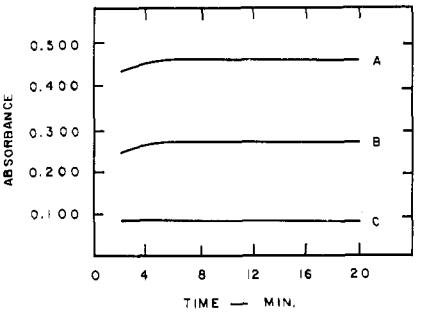


Figure 5. Color development of Potasan with time

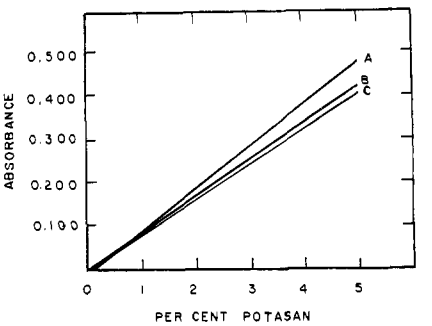


Figure 6. Standard curves and recovery

A. Potasan solution
B. Potasan extracted from silica gel G, not chromatographed
C. Potasan extracted from silica gel G after chromatographing

producible color reaction only if it was used between 20 and 120 minutes after preparation. The optimum amount of diazonium salt needed was determined by adding various aliquots to equivalent but different solutions of hydrolyzed Potasan. When absorbance was plotted against milliliters of salt solution, a plateau was obtained between 2.5 and 4.0 ml. of solution (Figure 4). Therefore, a 3-ml. aliquot of this solution was used.

Color development time was investigated by carrying all standards through the procedure employing the optimum concentrations found above. After adding the diazonium salt, a moderate increase in absorbance was observed from 1 to 5 minutes and a very slight increase from 5 to 20 minutes (Figure 5). Ten minutes was the standard reading time.

Three sets of standards were run. These were prepared as described under Preparation of Standards. Set No. 1 consisted of 50-μl. aliquots color-developed directly. Set No. 2 was a series of 50-μl. aliquots applied to silica gel but not chromatographed, then extracted and color developed. Set No. 3 was the same as set No. 2, but chromatographed, then extracted and color developed.

All sets gave straight lines when per cent Potasan *vs.* absorbance was plotted. Set No. 2 curve passed through the origin but sets No. 1 and No. 3 curves passed slightly below (Figure 6). Sets Nos. 2 and 3 gave values about 10% lower than set No. 1, showing that some Potasan could not be removed from the silica gel. This indicated the need to chromatograph the standards along with the sample to compensate for incomplete extraction.

The need to run a 0% Potasan standard arises only when the coumaphos standard used is suspected to contain small amounts of Potasan. Otherwise no interference occurs, and the absorbance of 0% Potasan may be taken as zero.

To evaluate interferences from adjacent spots, those marked *C* and *D* (Figure 1) were removed and gave absorbances of 0.078 and 0.007, respectively. The 1 and 5% Potasan standards run at the same time gave 0.078 and 0.428, respectively.

The determination of 1% Potasan in coumaphos is possible with the colorimetric method and solutions obey Beer's law. It is estimated that the maximum amount of adjacent spots *C* and *D* that might be removed accidentally along with the Potasan would be less than 10% considering the good resolution of the chromatogram. This would contribute a maximum of 0.1% absolute to the actual Potasan values.

A standard curve, passing through the origin, was verified by a large number of runs in the control laboratory. Samples and standards must be run together to eliminate differences due to variations in TLC plates, operator error, and incomplete recovery (Figure 6). Although this is more time-consuming, it provides a higher degree of reliability.

Reproducibility studies show the standard deviation on technical coumaphos to be ± 0.16 for 2.7% Potasan content ($2\sigma = \pm 0.32$, 35 degrees of freedom).

Conclusions

Addition of uranyl acetate to the silica gel permits easy detection of Potasan under ultraviolet light.

The method is designed for maximum sensitivity and is considered to be specific for Potasan. Since a larger sample cannot be chromatographed with good resolution and the dye solution cannot be further concentrated, the practical lower limit of detection of Potasan by this procedure is 10 p.p.m.

Acknowledgment

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