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Determination of Zinc Phosphide Residues in Corn (*Zea mays*) Grain, Fodder, and Forage

M. J. Goodall, S. A. Volz, J. J. Johnston, D. B. Hurlbut, R. E. Mauldin, D. L. Griffin, E. E. Petty

USDA/APHIS/National Wildlife Research Center, Analytical Chemistry Project, 3350 Eastbrook Drive, Fort Collins, CO 80525, USA

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Zinc phosphide (ZN_3P_2) is an acute rodenticide. One of its current uses employs application as a grain bait or pellet at the time of planting to reduce agricultural losses by field rodents. To mitigate rodent induced losses to corn, we hoped to expand the approved uses of zinc phosphide to include corn applications. After determining acceptable efficacy, we conducted a study to determine if potentially hazardous zinc phosphide residues in corn would result from this use. A field study was conducted in 5 corn growing states to provide samples for residue analysis of corn treated with zinc phosphide.

Corn is used both as a food and feed commodity. Therefore, the plant was harvested and subsequently analyzed as forage/silage, the immature green plant at the earliest state used for grazing (milk-dough stage). It was also harvested and analyzed at maturity as fodder, the plant material remaining after harvest of the grain/ears, and lastly as grain.

Development of analytical methodology was required to permit the quantification of zinc phosphide residues on corn. Previous methods quantified zinc phosphide residues in sugar cane (Hilton and Robinson 1972), range vegetation (Okuno et al. 1975), sugar beets (University of California 1989), and potato tubers (University of Idaho 1995) by hydrolyzing zinc phosphide in an acid solution to produce phosphine gas, which was subsequently quantified by headspace sampling and gas chromatography-flame photometric detection. Unfortunately, phosphine gas is highly reactive and appears to react with plant constituents, resulting in limited and variable recoveries ranging from 33 · 84 percent (Hilton and Robinson 1972, Okuno et al. 1975, University of California 1989, University of Idaho 1995, Berk 1968, Berk and Gunther 1979).

Contributing to this variation is the insolubility of zinc phosphide in a solvent for the preparation of standards. Hilton and Robinson (1972) prepared serial dilutions of zinc phosphide in glucose to achieve a 33% mean recovery for fortified sugar cane. Okuno et al. (1975) prepared suspensions of zinc phosphide in water to fortify range grass. Recoveries ranged from 56 to 107 percent.

Our target performance criteria was the development of methodology to quantify residues at levels as low as 10 ppb zinc phosphide on forage/silage, fodder and grain with recoveries of 70% or better. This was accomplished only after the development of unique fortification

Correspondence to: .J. J. Johnston

and quality control procedures for the preparation of fortified samples for method development, method validation and quality control purposes.

MATERIALS AND METHODS

Zinc phosphide bait formulated at 2% active ingredient (EPA Registration No. 2393-185) and zinc phosphide pellets formulated at 2% active ingredient (EPA Registration No. 2393-521) were provided by Hacco, Inc. (Madison, WI) for use in this study. Both the bait and pellets were analyzed by Hacco and found to contain 2.09% and 2.11% zinc phosphide respectively (Leppert 1996).

Test sites were selected in five states. These states were representative of commercial corn production areas of the United States that are susceptible to ground rodent deprivation. They also reflected seasonal variation and cultural practices associated with the production of corn. The raw agricultural commodity used in this study was field corn (Zea mays). Each field test site consisted of one control and three or four treated plots of approximately 100 m^2. None of the field sites had been treated with zinc phosphide during the three years preceding this study. Maintenance pesticides used during the course of the study were recorded and were not expected to interfere with the analyses. Thirty cm soil cores were collected at each site for complete characterization.

A single application of zinc phosphide bait was made by either in-furrow, planter-slot or mechanical broadcast at planting with a formulation consistent with proposed labeling for the test substance. Untreated control plots were located upslope and upwind from treated plots and at a minimal distance of 30 m to prevent possible contamination by drift or erosion. A minimal buffer zone of 15 m existed between treated plots. All baits were applied using properly calibrated applicator equipment to assure uniform application of the bait or pellets.

Daily rainfall or irrigation were measured and recorded for each site from planting until the last day of sampling. Daily maximum/minimum air temperatures were recorded either on-site or obtained from the nearest NOAA or similar weather station for the same period. Historical weather data for the various areas going back 10 or more years were also recorded.

Forage (silage) samples were collected at the late dough/early dent stage (also called R4 to R5 stage) which occurred 80-113 days after application. Grain and fodder samples were collected at normal crop maturity which occurred at 117-155 days after application. The ears of corn were removed from the stalks and the grain removed from the cob. Control and treated plots were harvested on the same day, with the control plots harvested first to prevent possible cross-contamination. Samples were taken from at least 12 separate areas within a plot and combined to form one sample of at least 1.1 kg. Two samples were collected per plot. Forage and fodder stalks were collected by hand and cut into pieces. Ears of corn were harvested by hand, threshed and cornposited. The samples were kept cool until they could be frozen. All samples were frozen within 5 hours of collection and kept frozen until analysis.

Samples were homogenized using a Hobart food processor (Troy, OH) and high speed industrial Waring blenders (New Hartford, CT). The samples were mixed with dry ice during homogenization to maintain their frozen condition. The ground samples were then transferred to labeled sample containers and stored frozen until analysis.

The headspace volume occupied by the sample was calculated from its density. The density of each of the three matrices was determined by weighing 5 x 15.0 g of control grain or forage matrix into 100-ml graduated cylinders fitted with ground glass stoppers. Fifty ml of 10% sulfuric acid solution were added to each cylinder and the mixture was shaken by hand for one minute and then allowed to stand for 30 minutes. Any material adhering to the inner walls of the cylinder was washed into the solution using 25.0 ml of the acid solution. The volume of acid used was subtracted from the total volume occupied by the matrix and solution. The density was calculated by dividing the weight of the matrix by the difference in volume. The same procedure was used for determining the density of the fodder matrix using a 1% phosphoric acid solution and 5.0 g of sample.

To prepare ground grain or forage for analysis, 15 grams (g) of sample were weighed into a volume-calibrated 1000-ml Erlenmeyer flask. Three hundred ml of an aqueous sulfuric acid solution (10% v/v) were added to each flask to hydrolyze the zinc phosphide to phosphine. The flask was capped with a rubber sleeve stopper, placed on an Eberbach horizontal mechanical shaker (Ann Arbor, MI) and agitated at low speed for 30 minutes. The phosphine in the headspace was quantified by gas chromatography/flame photometric detection.

To prepare ground fodder for analysis, 7.5 g of sample were weighed into a volume calibrated 500-ml Erlenmeyer flask. One hundred fifty ml of an aqueous phosphoric acid solution (1% v/v) were added to each flask. The flask was then stoppered and treated as above.

Due to the apparent insolubility of zinc phosphide in any solvent, a standard suspension in propylene glycol was prepared for each analysis by placing 150 ml of propylene glycol in a tall 200 ml beaker. The propylene glycol was stirred with a stir bar on a stir plate set at the highest mixing speed. Additionally, a Virtis Model 45 homogenizer (Precision Co. Racine, WI) with its blades positioned about 3 cm over the stir bar, stirred the propylene glycol suspension in the opposite direction. Weighed aliquots of 50 - 100 µg Zn_3P_2 were added to the stirring propylene glycol. After mixing for 15 minutes, measured aliquots of this zinc phosphide/propylene glycol suspension were transferred to volume calibrated Erlenmeyer flasks. The zinc phosphide was hydrolyzed by the subsequent addition of acid and analyzed as previously described.

To assess the effects of storage stability, six 15-g samples of the ground control grain and forage matrices were weighed directly into 500-ml wide-mouth glass screw top jars with Teflon® lined plastic caps. Samples were fortified at 50 ppb from a prepared suspension of zinc phosphide in propylene glycol, immediately capped and transferred to the freezer. Six 7.5-g samples of the ground fodder matrix were fortified in the same manner from a prepared suspension. Samples were stored at -20 ± 5°C for a comparable or longer length of time as field samples were in frozen storage prior to analysis.

On the day of analysis, the stored samples to be assayed were removed from the freezer. The corn grain and forage samples were transferred to volume-calibrated 1000-ml flasks using a wide-mouth funnel. The glass storage container was then rinsed with 150 ml of deionized water and the washings were added to the flask. Lastly, 150 ml of 20% sulfuric acid solution (v/v) were added to the flask. The flask was immediately stoppered and placed on a mechanical shaker for 30 minutes. Seventy-five ml of deionized water and 75 ml of 2% phosphoric acid solution (v/v) were used in the same manner to transfer the corn fodder samples to 500-ml volume-calibrated flasks.

In addition, three individual 15-g samples of the control grain and forage matrices or 7.5 g of the control fodder were weighed directly into 500-ml wide-mouth glass screw top jars w/Teflon® lined plastic caps. A freshly prepared suspension of zinc phosphide in propylene glycol was used to fortify each sample at 50 ppb. The fresh fortifications were then transferred in the same manner as the stored samples and placed on a mechanical shaker for 30 minutes.

A Hewlett-Packard 5890 Series II gas chromatograph (Walbronn, Germany) equipped with a flame photometric detector fitted with a phosphorus-specific filter (526 nm) was used for all analyses. The GC conditions were as follows: injector temperature, 70°C; detector temperature, 200°C; oven temperature, 50°C, isothermal; headspace volume injected, 100 µL, splitless; split vent flow, 16 mL/min; purge vent flow, 4 mL/min; carrier gas, helium, at 32 mL/min; detector gases: auxiliary gas, nitrogen, 115 mL/min; oxygen, 25 mL/min; hydrogen, 75 mL/min; analytical column, GS-Q Megabore (J&W Scientific, Folsom, CA) 30 m x 0.54 mm i.d., 0.25-µm film thickness.

The GC was equipped with a pneumatically actuated six-port gas sampling valve (Valco Instrument Company, Inc., Houston, TX; model DC6WP) and a mechanical vacuum pump (Edwards high vacuum, Crawley, Sussex, England; Model E2M-1). The gas sampling system and sampling procedure have been described previously (Mauldin et al. 1996).

For the determination of linearity, two zinc phosphide/propylene glycol suspensions were prepared and used to evaluate response linearity. At each of six zinc phosphide concentration levels, ranging from 9.52×10^{-5} to 4.35×10^{-3} µg/ml for the grain and silage samples and from 9.52×10^{-5} to 2.99×10^{-3} µg/mL for the fodder samples, four aliquots were removed from each suspension. These concentrations were equivalent to 5-225 ppb in the grain/silage samples and 5-150 ppb in the fodder samples. The glycol samples were prepared and analyzed as previously stated. Headspace samples were injected into the GC. The phosphine chromatographic peak area response (y-axis) was plotted as a function of the zinc phosphide head space concentration (x-axis). Linear regression analyses were performed on the data set.

To confirm the absence of matrix interferrants in the chromatograms (selectivity) seven control samples of ground corn grain, forage and fodder were prepared and analyzed by the procedures described previously.

The method limit of detection (MLOD) was defined as the concentration of zinc phosphide in ground corn grain or forage required to produce a chromatographic response equal to three

times the baseline noise. The MLOD was determined using the mean chromatographic response from seven samples fortified with an aliquot of propylene glycol suspension containing 150 ng (75 ng for fodder) of zinc phosphide (10 ppb) and the baseline noise observed from seven control samples of each matrix.

Fifteen-g grain and silage samples and 7.5-g fodder samples were fortified with known aliquots of the zinc phosphide suspension to give final concentrations of 10, 50 and 100 or 200 ppb. Fourteen fortifications were evaluated at the 10 ppb level and seven at both 50 and 200 ppb for grain and forage. Fourteen fortifications were evaluated at the 10 ppb level and seven at the 50 and 100 ppb level for fodder. (Table 1)

Response factors for standard suspensions were calculated as the headspace concentration of the sample divided by its peak area response. The concentration of the standard suspension was verified by comparing the mean response factor from three aliquots of the suspension containing approximately 5 - 10 μg zinc phosphide to the mean response factor from 3 weighed 10 μg aliquots of the zinc phosphide standard. The mean response factors had to agree +\- 20% for the analyses to proceed. If this criterion was not achieved, then 3 additional aliquots of zinc phosphide standard were weighed, analyzed and the mean response factor calculated. If this mean matched +\- 20% the mean response factor for the suspension, then sample analysis proceeded. If the suspension and technical mean response values did not agree, and the two mean technical response factors matched within +\- 20%, then the concentration of the suspension was calculated from the mean of the six technical aliquots. The mean response factor from the suspension aliquots was then used to calculate ZN_3P_2 sample residues. One of the standards used to verify the concentration of the suspension was also analyzed after approximately every 6 samples. The peak area of this standard could not change by more than 15% throughout the run for the accompanying sample data to be acceptable.

The continuity of method performance was monitored on each day of analysis by comparing the mean response factor of the suspension to the mean response factor from the latest standard curve used to demonstrate linearity. The response factors needed to agree within ± 25% for the sample data to be acceptable. At least one control sample fortified at 10 ppb and three control samples fortified at 50 ppb zinc phosphide were analyzed with each set of forage or grain samples. Six control samples fortified at 10 ppb and three control samples fortified at 50 ppb zinc phosphide were analyzed with each set of fodder samples. For corn grain and forage, the mean recovery was within 70-120% of target. The mean fortification recovery for fodder was required to be within 2 standard deviations of the mean recovery established during method validation. If the mean fortification recovery was outside the acceptable range, the set of samples were reanalyzed.

RESULTS AND DISCUSSION

As zinc phosphide proved to be virtually insoluble in any solvents at levels acceptable for fortification of standards and control matrices for method development and quality control, the major obstacle in developing the required analytical methodology was to develop an acceptable fortification procedure. Dilutions of the solid zinc phosphide technical material with a variety of solids including silica, barium sulfate, starch, manganese oxide and sugar

resulted in unacceptably high variation. Suspension of the ground technical material in a variety of liquids such as water, oils, and organic solvents indicated that propylene glycol was the most promising carrier. Slow dual mixing of the zinc phosphide/propylene glycol suspension with a stir bar and a homogenizer stirring in opposite directions gave acceptable precision as the standard deviation of 5 aliquots containing 5 µg each analyzed during method validation was 3.6%. The zinc phosphide concentrations of the suspensions were confirmed by comparing the response factors from the hydrolysis/headspace gas chromatography analysis of aliquots of the propylene glycol suspension with the response factor of comparable aliquots of the technical zinc phosphide.

Linear regression analysis of the linearity data yielded a r^2 of 0.9985 for the corn/ silage method and a r^2 of 0.9963 for the fodder method. Additionally, a log versus log regression of the same data yielded a slope of 1.02 for corn/silage and 0.969 for fodder, indicating a linear, directly proportional response. Response factors (zinc phosphide headspace concentration /response) were virtually identical across the entire concentration range with a mean value of 2.937×10^{-9} with a coefficient of variation of 10.8% for corn/silage. The mean value for fodder was 2.877×10^{-9} with a coefficient of variation of 10.6%.

During methods development, a small peak at the retention time of phosphine was occasionally observed in the control matrices. A rigorous glassware preparation regimen was introduced. The Erlenmeyer flasks were washed with a phosphate-free detergent (Baxter S/P Brand Micro All Purpose Liquid Cleaner), soaked for a minimum of 8 hours in a 10% sulfuric acid solution (v/v), rinsed thoroughly with deionized water and oven dried prior to the addition of sample. These procedures greatly reduced such occurrences. During method validation, no chromatographic interferences at the retention time of phosphine were observed in the control samples of each of the three matrices.

The MLOD was determined to be 7 ng (0.5 ppb) in ground grain, 10 ng (0.7 ppb) for ground silage, and 6 ng (0.8 ppb) in ground fodder. Densities were determined to be 1.35 g/ml in grain, 0.92 g/ml in forage, and 0.64 g/ml in fodder.

The mean fortification recoveries reported during method validation for the three sample matrices are shown in Table 1. Recoveries of > 70% were observed for 10-200 parts per billion (ppb) zinc phosphide fortifications in corn forage and grain, and 10-100 ppb zinc phosphide fortifications in fodder.

Method performance, as evaluated from fortification recoveries from each day's analyses, was comparable to that reported during method validation. At the 50 ppb fortification level, recoveries of 80.6 ± 6.1% (n=12), 77.9 ± 6.5% (n=12), and 80.9 ± 13% (n=12) were observed for grain, forage and fodder respectively. At the 10 ppb fortification level, recoveries of 86.8 ± 13% (n=4), 77.3 ± 12% (n=5) and 83.6 ± 23% (n=23) were observed for the same three matrices. While recoveries at the 10 ppb fortification level for fodder were more variable, the average recovery of all fortifications on each day of analysis was greater than 70% (Table 2).

The within-run standard did not vary more than 3.4% during any of the 12 days of analyses. The standard and suspension response factor match averaged 106 ± 6.2% over

the same 12 days. The suspension and linearity curve response factor match averaged 109 ± 6.9% over the same period. These data indicate that method performance and instrument sensitivity was consistent across analysis days.

Table 1. Validation Results

Matrix	GRAIN			FORAGE			FODDER		
Fortif. Level (ppb)	10	50	200	10	50	200	10	50	100
Sample Number	14	7	7	14	7	7	14	7	7
$\bar{x}$ Percent Recovery	109	89.7	92.0	75.1	77.6	84.5	78.6	71.3	70.2
Standard Deviation	23	2.2	5.3	17	7.7	6.0	10	4.5	3.8

Table 2. Quality Control Recoveries During Sample Analyses

Matrix	GRAIN		FORAGE		FODDER	
Fortif. Level (ppb)	10	50	10	50	10	50
Sample Number	4	12	5	12	23	12
Analysis Days	4	4	4	4	4	4
$\bar{x}$ Percent Recovery	86.8	80.6	77.3	77.9	83.6	80.9
Standard Deviation	13	6.1	12	6.5	23	13

Soil characteristics and rainfall were typical of the variety of corn growing regions of the U.S. Soil textures among the study sites were classified as silt loam, loam, clay loam, and sandy loam. The pH of the soils ranged between 4.7 and 5.9 and organic matter ranged from 0.4 to 3.3%. Rainfall ranged from 74-121% of normal for this time period. Average air temperatures from the month of planting to the last sampling date were comparable to published norms at three sites and higher than normal at two sites. Irrigation water was not provided at any of the sites (Leppert 1996).

The 50 ppb fortified grain, silage, and fodder were stored for 112, 162 and 140 days, respectively. Analysis after storage indicated losses of 15.6% for grain, 44.8% for silage and 32.1% for fodder.

The analytical methodology was used to determine zinc phosphide residues in 132 field samples. Residues were detected in only one treated sample at a level approximately 2X the MLOD. A duplicate sample, simultaneously harvested from the same plot contained no detectable zinc phosphide residues.

The resulting method proved to be sufficiently rugged to support a major field study with multiple sites and three different matrices. Though field and environmental conditions were variable, they had no impact on the outcome of the study. The resulting data indicated that the use of zinc phosphide to control rodent pests in corn fields poses little threat of contamination of corn products ultimately intended for animal or human consumption.

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REFERENCES

Berk B.J. (1968). Sorption of phosphine by cereal products. J Agric Food Chem, 16:419-425.

Berk B, Gunther FA (1970). Rapid determination of sorption affinity of phosphine by fumigation within a gas chromatographic column. J Agric Food Chem, 18: 148-153.

Hilton HW, Mee JML (1972). Studies with radioactive phosphine ^{32}P in sugar cane. J Agric Food Chem, 20:1209-1213.

Hilton HW, Robinson WH (1972). Fate of zinc phosphide and phosphine in soil-water environment. J. Agic. Food Chem., 20:1209-1213.

Leppert BC (1996). Magnitude of Zinc Phosphide Residues in No-Till Corn. Stewart Agricultural Research Services, Inc. Protocol Number: SARS-95-60. (unpublished Report, DWRC Archives: (QA-428) 222 pp.)

Mauldin RE, Goldade DA, Engeman RM, Goodall MJ, Craver RK, Johnston JJ (1996) Determination of Zinc Phosphide Residues in the California Ground Squirrel (Spermophilus beecheyi) by Gas Chromatography-Flame Photometric Detection. J Agric Food Chem, 44: 189-194.

Okuno I, Wilson RA, White RE (1975). Determination of zinc phosphide in range vegetation by gas chromatography. Bull Environ Contam Toxicol, 13:392-396.

University of California, Davis (1989). Zinc Phosphide: Magnitude of residue on sugar beet. Interreg. Res. Proj. 4. Davis, California: University of California.

University of Idaho (1995). Analytical Sciences Laboratory, Standard Methods Manual, Section 60.300.60. Moscow, Idaho, Regents of the University of Idaho.