Zinc Phosphide Residue Determination in Alfalfa (*Medicago sativa*)

Richard E. Mauldin,* Margaret J. Goodall, Stephanie A. Volz, Doreen L. Griffin, Eric J. Petty, and John J. Johnston

Animal and Plant Health Inspection Service, U.S. Department of Agriculture, National Wildlife Research Center, 3350 Eastbrook Drive, Fort Collins, Colorado 80525

An analytical method for the determination of zinc phosphide (Zn_3P_2) residues in alfalfa ranging from 10 $\mu g\ g^{-1}$ to 100 mg g^{-1} is described. Using a suspension of Zn_3P_2 in propylene glycol, Zn_3P_2 standards were prepared and midharvest alfalfa and dried hay control samples fortified to assess method recovery. Addition of 1% phosphoric acid to Zn_3P_2-containing samples produced phosphine (PH_3) gas for headspace gas chromatography/flame photometric detection analysis. Response linearity was assessed for (8.9×10^{-5})–0.027 μg of Zn_3P_2 mL^{-1} headspace ($r^2 = 0.9914$) and 0.027–2.02 μg of Zn_3P_2 mL^{-1} headspace ($r^2 = 0.9998$) concentration ranges. Analyte recovery exceeded 81% for Zn_3P_2 fortification levels ranging from 0.010 to 100 $\mu g\ g^{-1}$, and the method limit of detection was 2.1×10^{-3} $\mu g\ g^{-1}$ in midharvest alfalfa and 3.9×10^{-3} $\mu g\ g^{-1}$ in dried hay.

Keywords: *Zinc phosphide; phosphine; residues; Medicago sativa; alfalfa; gas chromatography/flame photometric detection*

INTRODUCTION

In California, alfalfa (*Medicago sativa*) is grown on nearly 1 million acres and is the most important forage crop (University of California—Davis, 1995). Cultivated alfalfa fields represent optimal habitat for meadow voles (*Microtus* spp.), which can cause severe damage to crops (Lewis and O'Brien, 1990). Because such damage results in significant economic loss for producers, effective control of vole populations is necessary.

Zinc phosphide (Zn_3P_2) has long been used as an acute rodenticide (Chitty and Southern, 1954). It is frequently applied in various grain-based matrices as a bait which, when consumed, releases highly toxic phosphine (PH_3) gas through Zn_3P_2 hydrolysis by stomach acid. Zn_3P_2 is currently the only rodenticide registered for broadcast use (Johnson and Fagerstone, 1994) and is an effective, low-cost, and environmentally safe rodent control agent. Additionally, Zn_3P_2 residue levels in bait-killed voles pose little secondary hazard to predators and scavengers (Tkadlec and Rychnovsky, 1990; Sterner and Mauldin, 1994).

Currently, a 2% Zn_3P_2 crimped oat bait is registered with the U.S. Environmental Protection Agency (U.S. EPA) for local use in overwintered sugarbeet fields, orchards, and rangeland in California. Extending the use of this bait to alfalfa fields under current U.S. EPA registration required setting a tolerance level for Zn_3P_2 residues in postharvest alfalfa. To assist the California Department of Food and Agriculture in achieving this goal, researchers at the National Wildlife Research Center (NWRC) conducted a field study evaluating Zn_3P_2 residue levels following bait application at various stages of growth. This required development and validation of an analytical method for the determination of 0.010 $\mu g\ g^{-1}$ Zn_3P_2 residues in samples of green forage (25 day midharvest) and postharvest alfalfa (dried hay).

Zinc phosphide residues have been analyzed in sugarcane (Robison and Hilton, 1971), range vegetation (Okuno et al., 1975), alfalfa (Rutgers University, 1985), sugarbeet tops and roots (University of California—Davis, 1989), and potato tubers (University of Idaho, 1995). In these studies, Zn_3P_2 levels were determined by hydrolyzing standards and samples with acid (HCl or H_2SO_4) and sampling (by syringe) the evolved PH_3 from either a layer of toluene or the headspace of a sealed reaction vessel, followed by analysis using gas chromatography with flame photometric detection. These methods present two concerns. First, PH_3 is highly reactive and adsorbs or reacts with various plant matrices (Berck, 1968; Berck and Gunther, 1970; Robison and Hilton, 1971; Hilton and Mee, 1972), thereby reducing its recovery. The second problem in submicrogram per gram level analysis is the difficulty in preparing analytical standards when Zn_3P_2 amounts cannot be weighed directly. Usually, reference standards are dissolved and/or diluted in a suitable solvent. While Zn_3P_2 is reputedly soluble in benzene and carbon disulfide (CS_2) (*Merck Index*, 1996), studies in our laboratory have shown that such solutions have inadequate concentrations and exhibit poor sampling repeatability.

Robison and Hilton (1971) made a 3.795% Zn_3P_2 (1.00% available PH_3) standard mixture by mixing Zn_3P_2 with <100 mesh ground D-glucose, followed by preparation of 3.795, 3.795×10^{-1}, and 3.795×10^{-2} mg g^{-1} Zn_3P_2 (0.10, 0.01, and 0.001% available PH_3, respectively) standards by serial dilution with glucose. A 50 mg aliquot of each mixture was then used to produce a standard curve or to fortify a 50 g sugarcane sample, producing a final Zn_3P_2 sample concentration range of (3.79×10^{-2})–37.9 $\mu g\ g^{-1}$. The mean recovery for all sample fortification levels was 33%, assessed once at each level. Detector response for PH_3 standards was linear, but no data were provided. Using this method for range vegetation samples, Okuno et al. (1976) were unable to prepare homogeneous 1 $\mu g\ g^{-1}$ mixtures of Zn_3P_2 in glucose that yielded reliable recovery data. Alternatively, they suspended Zn_3P_2 in water by vigorous shaking. The suspension was filtered and the filtrate concentration determined by chromatographic signal comparison with a primary PH_3 gas standard. Analysis of replicate aliquots (0.1–1.0 mL, 0.01–3.0 μg of Zn_3P_2) from seven suspensions yielded coefficients of

* Author to whom correspondence should be addressed [fax (970) 225-1854].

S0021-8561(96)00689-9

Published 1997 by the American Chemical Society

variation (CV) ranging from 4 to 23%, with a mean CV of 13%. Using these suspensions, range vegetation control samples were fortified with 0.01−1.6 μg of Zn_3P_2 (0.010−1.6 μg g^{-1}) and recoveries assessed by comparing the fortified sample response with the response of a paired standard receiving an identical suspension volume aliquot. Recoveries ranged from 56 to 107%, with CV ranging from 12 to 20%.

Residues of Zn_3P_2 in 2 g samples of freshly cut alfalfa were assayed using standards dissolved in CS_2 (Rutgers University, 1985). Recoveries from single samples fortified at the 0.05, 0.1, and 0.25 μg g^{-1} levels were 60, 70, and 84%; repeatability and linearity statistics were not given. This method was also used on fresh and dried alfalfa (1 g) fortified at the 0.05, 0.10, and 0.25 μg g^{-1} levels, with recoveries of 100, 90, and 92%, respectively, for fresh alfalfa and 100, 85, and 92% for dried alfalfa. Again, precision and linearity data were not given. The same method was used to determine Zn_3P_2 residues in sugarbeet roots/tops (University of California—Davis, 1989). A single sample of each sample matrix was fortified at 0.01, 0.025, and 0.05 μg g^{-1}; recoveries for roots were 100, 96, and 100%, and for tops, 100, 92, and 98%, respectively. The method limit of detection (MLOD) was 0.01 μg of Zn_3P_2 (0.01 μg g^{-1} sample equivalent), indicating sample quantification at the limit of detection. In all uses of this methodology, precision could not be evaluated because only one sample was fortified at each level, and standard linear characteristics were never reported.

To quantify Zn_3P_2 residues in potato tubers (University of Idaho, 1995), reference grade Zn_3P_2 was serially diluted with 200−425 mesh silica gel to give standard concentrations of 1, 10, and 100 μg mL^{-1} (fairly consistent results were also obtained using a 0.7 μg mL^{-1} mixture). Between 100 and 500 mg of the standard mixture was used to fortify control samples at 0.05, 0.10, and 1 μg g^{-1} levels and to produce standard curves ranging from 0.05 to 1 μg g^{-1}. The homogeneity of Zn_3P_2 dispersion decreased in mixture aliquots of <100 mg. Correlation coefficients from this method ranged from 0.96 to 0.99, with variability attributed to several causes including the solid dilution standards. Recovery of Zn_3P_2 in fortified control samples ranged from 70 to 120%. The limit of quantitation was 0.05 μg g^{-1}.

While useful, these methods lacked the necessary level of detection or were either too unrepeatable or unreliable to achieve the quantification levels required for residue analysis in alfalfa. As stated previously, production of homogeneous submicrogram per gram level Zn_3P_2 standards and enhancement of PH_3 recovery in plant tissues presented the greatest problems for method development. This paper summarizes (1) the production and use of homogeneous suspensions of Zn_3P_2 in propylene glycol as analytical standards to reliably and repeatably produce standard curves and fortified alfalfa samples in the 0.010−100 μg g^{-1} range and (2) modifications made to a previously described Zn_3P_2 analytical method (Mauldin et al., 1996) to increase PH_3 recovery and reliably quantify Zn_3P_2 residues in samples of midharvest alfalfa and dried alfalfa hay.

EXPERIMENTAL METHODS

Sample Collection and Preparation. Samples of green, field-grown alfalfa were collected in San Joaquin County, California, 25 days (midharvest) after a previous harvest and were obtained by clipping plants with hand-held grass shears 5 cm above the ground. About 500 g of alfalfa was placed in a plastic bag, sealed, and labeled. Bags were put in a portable ice chest containing dry ice immediately after transport from the field and shipped overnight to the NWRC, where they were stored at −20 ± 5 °C. After harvest, alfalfa (hay) was allowed to field-dry to a moisture content of 15−20%, and then sampled, stored, and shipped as described for midharvest samples. To process, the alfalfa was partially thawed and ground in a large food processor (Model RSI 6V, Robot Coupe U.S.A., Inc., Ridgeland, MS) or blender (Waring, New Hartford, CT), placed in a plastic bag, and refrozen until needed.

Standard Preparation. *Zn_3P_2.* Using a mortar and pestle, Zn_3P_2 (H. R. Harkins, purity 98.8 ± 1.7%) was ground to reduce particle size and transferred to a 25 mL glass screw cap vial. The vial was capped, shaken, and rolled to coat the inner walls with Zn_3P_2 and then turned upright and gently tapped to dislodge any large particles or groups of particles adhering to the tube walls. This provided a coating of microfine Zn_3P_2 particles, which were then removed for weighing by lightly scraping the tube walls with a metal micro spatula. About 52 μg of Zn_3P_2 was weighed into an aluminum pan using a microbalance (Model C-31, Cahn Instruments, Inc., Cerritos, CA).

Zn_3P_2/Propylene Glycol Suspension. Zn_3P_2 standard suspensions were prepared according to a modification of the method used by Mauldin and Mishalanie (1997), by weighing 155.4 g of propylene glycol (150.0 mL at 20 °C) into a tared 200 mL tall beaker. A triangular stir bar with a length slightly shorter than the internal diameter of the beaker was carefully added and adherent air bubbles were removed. The beaker contents were then stirred continuously.

A high-speed homogenizer (Model 45, The Virtis Co., Inc., Gardiner, NY) was fitted with a 12.7 cm long "Macro" shaft containing two 35 mm long blades oriented perpendicular to each other and spaced 13 mm apart. The homogenizer was positioned above the beaker, centered, and lowered to immerse the lower of the two blades to a depth of ≥3 cm above the stir bar. The homogenizer was slowly brought to speed until a vortex formed extending ≈1.3 cm into the propylene glycol. Excessive shaft speeds caused dispersion of tiny air bubbles and made quantitative sampling difficult.

The aliquot of Zn_3P_2 was quantitatively transferred into the propylene glycol, which was stirred for at least 15 min. The concentration of the resulting suspension standard was 0.343 μg of Zn_3P_2 mL^{-1} of glycol. All suspensions were prepared fresh daily.

Suspension Sampling. Suspension aliquots calculated to deliver the desired amount of Zn_3P_2 were removed using fixed and variable volume (100−1000 μL) pipetters, the only devices of several tested which were able to provide the necessary sampling precision. The pipetting technique also significantly decreased sampling variability. The pipet tip was placed in the suspension, the plunger quickly released, and the sample withdrawn at its own rate. The tip was removed and wiped, and both tip and contents were transferred to a volume-calibrated 500 mL narrow-mouth Erlenmeyer flask. Phosphoric acid (150 mL, 1% v/v) was added to the flask, which was then quickly sealed by inserting a No. 15 rubber sleeve stopper (Fisher Scientific) and pulling the stopper sleeves tightly around the flask mouth. The flask was then shaken in a mechanical shaker (Equalpoise, Model 6550, Eberbach, Ann Arbor, MI) for 30 min at a speed of ≈175 strokes min^{-1} (low speed).

Alfalfa Sample Preparation. About 7.5 g of thawed, ground sample was accurately weighed into a tared, volume-calibrated 500 mL narrow-mouth Erlenmeyer flask, followed by the addition of 150 mL of a 1% v/v phosphoric acid solution. The flask was quickly sealed and shaken in a mechanical shaker for 30 min at low speed.

Alfalfa Density. To ensure the accurate determination of headspace volume, it was necessary to account for the sample volume, which was calculated using sample density. The density of midharvest alfalfa was determined by weighing five replicates of 7.5 g of ground sample into separate 100 mL graduated cylinders, each fitted with a ground glass stopper. Fifty milliliters of 1% v/v phosphoric acid was added to each

cylinder, which was then stoppered, shaken by hand for 1 min, and then allowed to sit for 30 min. Any material adhering to the inner walls of the cylinder was washed into the solution with 25.0 mL of additional acid and allowed to settle. The sample volume was determined by subtracting 75.0 mL from the total volume occupied by the mixture. Sample density was calculated by dividing alfalfa weight by sample volume. For ground dried hay, density was determined by weighing five replicates (5.0 g) and proceeding as previously described.

Chromatography. A Hewlett-Packard 5890 Series II gas chromatograph (Walbronn, Germany) equipped with a flame photometric detector with a phosphorus-specific filter (526 nm) was used. The GC was equipped with a pneumatic sampling system (Mauldin et al., 1996) modified by the addition of a minivalve into the 1 m tefzel transfer line to increase sample transfer line pressure. Separate sets of GC conditions were established for submicrogram per gram and microgram per gram samples as follows:

For Submicrogram per Gram Concentrations: column, GS-Q Megabore (J&W Scientific, Folsom, CA), 30 m × 0.53 mm i.d., 0.25 μm film thickness; injection port, 70 °C; oven, 40 °C isothermal; pneumatic valve, 50 °C; detector, 200 °C; gas flows (instrument), carrier (helium), 32 mL min^{-1}; split vent, 16 mL min^{-1}; purge vent, 4 mL min^{-1}, (detector) auxiliary (nitrogen), 115 mL min^{-1}; oxygen, 25 mL min^{-1}; hydrogen, 75 mL min^{-1}; mode, splitless; injection volume, 100 μL; run duration, 2.00 min.

For Microgram per Gram Concentrations: Column, temperature, detector gas flows, and injection mode parameters are described under submicrogram per gram concentrations. Gas flows (instrument): carrier (helium) 4.5 mL min^{-1}; split vent, 50 mL min^{-1}, purge vent, 4 mL min^{-1}; mode, split with a 2 mm i.d. open liner; run duration, 5.00 min.

Flask headspace was sampled as described by Mauldin et al. (1996), with the following exception. The gas sampling valve was loaded for only 0.01 min before the valve was switched and the sample swept onto the column.

Linearity. To cover the entire range of anticipated Zn_3P_2 residue concentrations from alfalfa field samples, linearity was separately assessed for submicrogram per gram and microgram per gram ranges.

For the Submicrogram per Gram Range: Two suspensions with concentrations of ≈0.359 μg of Zn_3P_2 mL^{-1} of glycol were prepared by weighing ≈56 μg of Zn_3P_2 and proceeding with suspension preparation as previously described. Four replicate aliquots ranging from 0.100 to 12.887 mL (≈0.0359 to ≈5 μg of Zn_3P_2; equivalent to ≈5 to ≈670 μg g^{-1} sample) were removed from each suspension and transferred to individual 500 mL flasks to produce Zn_3P_2 flask headspace concentrations ranging from 8.9×10^{-5} to 0.0137 μg mL^{-1}. An additional standard level (≈0.027 μg mL^{-1} headspace, $\approx 1.3 \times 10^{-3}$ μg g^{-1} sample) was prepared by weighing duplicate ≈10 μg of Zn_3P_2 standard aliquots into each of two flasks. Standards were then processed and sampled singly as described.

For the Microgram per Gram Range: Five Zn_3P_2 standard aliquots ranging in weight from 12 to 827 μg (equivalent to ≈1.6 to ≈110 μg g^{-1} sample) were weighed in duplicate, transferred to individual 500 mL flasks, and prepared as previously described. These resulted in Zn_3P_2 headspace concentrations ranging from ≈0.027 to ≈2.02 μg mL^{-1}. Each standard was analyzed twice.

Linear regression analysis of chromatographic response area (y-axis) versus Zn_3P_2 concentration (x-axis) from standard samples was performed using the SAS PROC REG program (version 6.04, SAS, Inc., 1989), to determine r^2, linear model fit, y-intercept, slope, and response factors (Zn_3P_2 concentration $response^{-1}$). As an additional test of direct proportionality between Zn_3P_2 concentration and chromatographic response, a $\log x$ versus $\log y$ regression was also performed on the same standard data.

Suspension Concentration Confirmation. To assure suspension accuracy and day-to-day repeatability, two checks were performed on each newly made suspension. First, the mean response factor (MRF) of triplicate samples of the largest suspension aliquot (12.887 mL, ≈5 μg of Zn_3P_2) was compared with the MRF of three accurately weighed ≈10 μg aliquots of Zn_3P_2 standard, prepared as previously described. The suspension was accepted if the MRF ratio was 100 ± 20%. If this criterion was not met, three new 10 μg aliquots were weighed, processed, and compared. If the criterion was not met a second time, the concentration of the suspension was calculated using the mean MRF of the six 100 μg Zn_3P_2 weighings. As a second test, the suspension aliquot MRF was compared to the overall MRF from the most recent submicrogram per gram standard curve. A 100 ± 25% match criterion was applied and a new suspension prepared if exceeded. This test was omitted for suspensions prepared for standard linearity validation.

Bias and Repeatability. Samples of ground control midharvest alfalfa were weighed (7.5 g) and fortified at 0.010, 0.050, and 0.10 μg g^{-1} levels using a standard suspension. Additional midharvest samples were fortified at the 10, 50, and 100 μg g^{-1} levels with weighed aliquots of Zn_3P_2 standard added directly to the tissue. Thirteen sample replicates were used at the 0.010 μg g^{-1} level, with seven replicates used for all other levels. Samples of ground, dried hay (7.5 g) were fortified at the 0.010, 0.050, and 0.100 μg g^{-1} levels using a standard suspension. Following fortification, samples were prepared and analyzed as previously described. Recovery data from each fortification level were tested for normal distribution using the SAS PROC UNIVARIATE option of SAS PROC REG.

Matrix Interference: Method Limit of Detection. Seven control samples of ground midharvest alfalfa and ground dry hay were prepared and analyzed to assess matrix interferences. The MLOD was defined as the amount of Zn_3P_2 in a 7.5 g sample of ground alfalfa required to a produce a PH_3 chromatographic response equal to 3 times the baseline noise found at the retention time of PH_3 in the control samples. A suspension aliquot of known Zn_3P_2 concentration was used to fortify the midharvest alfalfa and dried hay.

RESULTS AND DISCUSSION

Method Development. During method development, obtaining acceptable PH_3 recovery (>70%) from Zn_3P_2-fortified alfalfa matrices using the method of Mauldin et al. (1996) proved difficult. Extensive experimentation with sample/acid volume ratios, flask sizes, shake time/speed, and sample handling techniques was performed.

Decreased PH_3 recovery in the presence of plant products and soils has been previously reported. This loss was attributed to irreversible PH_3 sorption to protein or mineral surface reactive sites (Berck, 1968; Berck and Gunther, 1970), and matrix-catalyzed PH_3 oxidation to form phosphates (Hilton and Robison, 1972), or soluble phosphorus oxyacids/insoluble metal oxyacid salts (Robison and Hilton, 1971; Hilton and Mee, 1972). Assuming such processes were responsible for the initially low PH_3 recoveries from alfalfa, addition of various chemical compounds to inhibit interfering matrix reactions was investigated. Because PH_3 reacts with various metals (Van Wazer, 1958), chelating agents (EDTA, citric acid) were added to reduce metal availability. Antioxidants (ascorbic acid, propyl gallate) were used to minimize direct PH_3 oxidation, and phosphorus (sodium phosphite, dibasic potassium phosphate) was added to (1) compete for active site availability or (2) provide excess reaction end-product. Additionally, H_2SO_4 was replaced by other inorganic and organic acids at various strengths to assess effects on PH_3 recovery.

The substitution of H_2SO_4 by H_3PO_4 was the only modification that significantly enhanced PH_3 recovery. Further recovery improvement was achieved by performing the hydrolysis step using 7.5 g of tissue in a 500 mL narrow-mouth Erlenmeyer flask, followed by shaking for 30 min.

To produce submicrogram per gram Zn_3P_2 standards, several solid diluents were evaluated. Confectioner's

Table 1. Zn_3P_2 Recovery Comparisons between Method Validation (V) and Field Sample (F) Analysis in Fortified Midharvest Alfalfa and Dried Hay

alfalfa type:	midharvest						dried hay			
Zn_3P_2 spike level:	0.01 μg g^{-1}		0.050 μg g^{-1}		50 μg g^{-1}		0.10 μg g^{-1}		0.050 μg g^{-1}	
sample type:	V	F	V	F	V	F	V	F	V	F
mean recovery (%)	108.3	84.7	93.7	93.3	92.5	90.6	96.4	92.5	82.4	90.3
SD	16.0	10.7	7.2	12.3	1.8	1.8	11.5	18.2	7.0	12.7
CV (%)	14.8	12.6	7.7	13.2	1.9	2.0	11.9	19.7	8.5	14.1
n	13	20	7	53	7	9	7	4	7	15
P ($\alpha = 0.05$)	0.0001		0.94		0.05		0.67		0.15	

sugar, silica, starch, barium sulfate, and manganese oxide were mixed with Zn_3P_2 by both manual and mechanical shaking; none were sufficiently homogeneous. Water, methanol, acetonitrile, and propylene glycol were also investigated for the preparation of homogeneous liquid suspensions. While barium sulfate provided the most homogeneous solid/solid mixtures, these were inferior to liquid suspensions using rapidly stirred propylene glycol.

The double-mixing action provided by the stir bar/homogenizer combination was necessary to achieve the desired suspension homogeneity, which could not be accomplished with either device separately. Additionally, the two devices stirred in opposite directions, slowing the rotation rate of the suspension surface and allowing greater homogenizer speeds while delaying vortex formation. The use of microfine Zn_3P_2 particles was also found to be critical to homogeneous suspension preparation.

Matrix Density. The mean densities for seven replicates of midharvest alfalfa and dried hay were 0.87 ± 0.090 and 0.49 ± 0.21 g mL^{-1}, respectively. As expected, sample density decreased by almost 44% in dried hay, primarily due to water loss.

Response Linearity. *Submicrogram per Gram Standards:* Linear regression analysis of the submicrogram per gram (8.9×10^{-5})–0.027 μg mL^{-1} headspace) standards generated an r^2 of 0.9914, a significant linear model fit ($p < 0.0001$), a y-intercept of -5.51×10^5 mAU, and a slope of 2.68×10^8. The y-intercept was not statistically different from 0 ($p = 0.72$). Response factors were similar throughout the Zn_3P_2 concentration range, with a mean of 4.00×10^{-9} and a CV of 16%. No nonlinear trends were observed. The slope of the log x versus log y plot was 0.9790 and was not significantly different from 1.0 ($p > 0.05$). These data supported the use of a single-point standard calibration during actual sample analysis. The concentrations of the two suspensions used in linearity validation were confirmed by response factor comparison with weighed ≈10 μg Zn_3P_2 standards. Both suspensions were acceptable, with response factor matches of 120 and 105%.

Microgram per Gram Standards: Regression analysis of the microgram per gram range standards (0.027–2.02 μg mL^{-1}) generated an r^2 of 0.9998, a significant linear model fit ($p < 0.0001$), a y-intercept of -2.91×10^5 mAU, and a slope of 2.35×10^7 . The mean response factor was 4.465×10^{-8}, with a CV of 6%. While response factors were essentially uniform throughout the Zn_3P_2 concentration range, the y-intercept was different from 0 ($p = 0.0016$). The slope of the log x versus log y plot of the same data was 1.02, but was different from 1.0 ($p = 0.014$). This indicated that, while linear, the Zn_3P_2 concentration/response ratio was not proportional, eliminating the use of single-point calibration. During analysis of relevant field samples, a three-point standard curve was prepared daily by weighing out ≈10, 100, and 700 μg of Zn_3P_2 into separate 500 mL flasks.

Bias and Repeatability. Recovery data for all fortification levels in both midharvest and dried hay were normally distributed ($\alpha = 0.05$). Mean percent recoveries for the 0.010, 0.05, 0.10, 10, 50, and 100 μg g^{-1} levels of Zn_3P_2-fortified ground midharvest alfalfa were 108 (±16), 93.7 (±7.2), 95.1 (±5.7), 104 (±2.7), 92.5 (±1.8), and 92.8 (±1.5), respectively. CV ranged from 15% (0.010 μg g^{-1}) to 1.6% (100 μg g^{-1}).

Mean percent recoveries for the 0.010, 0.050, and 0.10 μg g^{-1} levels of Zn_3P_2-fortified dried hay were 96.4 (±12), 82.4 (±7.0), and 81.2 (±7.5), respectively. Contrary to the findings of Mauldin et al. (1996), who observed that Zn_3P_2 recoveries generally increased with increasing concentrations of Zn_3P_2 in the stomach and intestinal contents of the California ground squirrel (*Spermophilus beecheyi*), recoveries in dried hay decreased with increased Zn_3P_2 concentration. A similar trend was observed in recoveries from midharvest alfalfa.

Recoveries at all Zn_3P_2 levels in both matrices exceeded 81%, with most levels exhibiting excellent repeatability. The sample fortification validation process required preparation of five separate suspensions. For each suspension, concentration was confirmed by MRF ratio matches between the 5 μg suspension samples from that suspension and the replicate 10 μg samples weighed that day. These ratio matches ranged from 101 to 111%, with a mean of 105.4 ± 3.6%, indicating good repeatability between suspensions. The suspension versus standard curve MRF match was not performed.

Matrix Interferences/Method Limit of Detection. No chromatographic interferences were observed at the retention time of PH_3 (0.80 min) in the midharvest alfalfa, and the MLOD was 2.1×10^{-3} μg g^{-1} for that tissue. A very small peak was found to elute at the retention time of PH_3 in the control dried hay. To account for the presence of this peak, its mean height ($n = 7$) was defined as the baseline noise and the MLOD calculated to be 3.9×10^{-3} μg g^{-1}.

Field Sample Quality Control Fortifications. Over a 2 month period, alfalfa field samples were analyzed and each day's analysis included suspension concentration confirmation and quality control fortification of the alfalfa type being analyzed. The mean suspension confirmation match for the entire period was 106.7 ± 5.7% ($n = 23$), with an associated mean suspension/standard curve match of 102.2 ± 8.4% ($n = 23$). Control midharvest alfalfa was fortified at the 0.010 and 0.050 μg g^{-1} levels and at the 50 μg g^{-1} concentration. Control dried hay was fortified at the 0.010 and 0.050 μg g^{-1} levels. These data are summarized in Table 1 and were compared by t test with recoveries from the same matrix fortifications performed during method validation. At the midharvest 0.010 μg g^{-1} level, a $p < 0.0001$ indicated a decrease in recovery between validation (108 ± 16%) and field sample analysis (84.7 ± 10.7%). This difference may have been due to the use of a fixed-volume 100 μL pipet during field sample analysis, in contrast to the variable-volume

Table 2. Zn_3P_2 Recoveries from 0.010, 0.050, and 0.10 mg g^{-1} Suspension Aliquots

	Zn_3P_2 spike level		
	0.010 $\mu g\ g^{-1}$	0.050 $\mu g\ g^{-1}$	0.10 $\mu g\ g^{-1}$
mean recovery (%)	94.5	96.5	100.4
SD	13	11	7
CV (%)	14	11	7

pipet used during validation. While mean Zn_3P_2 recovery decreased, sampling repeatability improved greatly.

During field sample analysis, an additional suspension quality control measure was employed. Single aliquots having Zn_3P_2 concentrations equal to 0.010, 0.050, and 0.10 $\mu g\ g^{-1}$ were treated as standards, prepared, and analyzed ($n = 23$). Using the determined suspension concentration, the theoretical mass of Zn_3P_2 delivered in each aliquot was calculated. Actual recoveries were determined by comparing sample response with the mean response from the ≈ 5 μg suspension confirmation samples and had to match theoretical recoveries within $\pm 20\%$ for the 0.010 $\mu g\ g^{-1}$ aliquot and $\pm 15\%$ for the 0.050 and 0.10 $\mu g\ g^{-1}$ aliquots for that day's analysis to be accepted. In addition to increasing the number of daily suspension checks, these data were indicative of suspension preparation and sampling technique uniformity throughout the analysis period. Mean recoveries, standard deviations, and CV for the 0.010, 0.050, and 0.10 $\mu g\ g^{-1}$ suspension samples are summarized in Table 2. Mean recoveries exceeded 94% in all cases.

Conclusion. The combination of suspension preparation techniques and sample treatment procedures resulted in a reliable, repeatable, analytical method with PH_3 recoveries >80% for Zn_3P_2 residue concentrations ranging from 0.010 to 100 $\mu g\ g^{-1}$ in alfalfa. Suspension quality control checks assured that the data generated by the method conformed to EPA Good Laboratory Practices (*Fed. Regist.*, 1991).

ACKNOWLEDGMENT

We thank Ron Timm, Ray Sterner, Kathleen Fagerstone, and the California Department of Food and Agriculture for their assistance and support. We also thank Dan Hurlbut for his efforts in the validation of this method and David Goldade and Rori Craver for their help in sample preparation and analysis.

LITERATURE CITED

Berck, B. J. Sorption of phosphine by cereal products. *J. Agric. Food Chem.* **1968**, *16*, 419–425.

Berck, B.; Gunther, F. A. Rapid determination of sorption affinity of phosphine by fumigation within a gas chromatographic column. *J. Agric. Food Chem.* **1970**, *18*, 148–153.

Chitty, D.; Southern, H. N. Properties of the poisons used in rodent control. In *Control of Rats and Mice*; Chitty, D., Ed.; Oxford University Press: New York, 1954; Vol. 1, pp 102, 106–108.

Fed. Regist. **1991**, *40*, 1989.

Hilton, H. W.; Mee, J. M. L. Studies with radioactive phosphine ^{32}P in sugarcane. *J. Agric. Food Chem.* **1972**, *20* (2), 334–336.

Hilton, W. H.; Robison, W. H. Fate of zinc phosphide and phosphine in the soil-water environment. *J. Agric. Food Chem.* **1972**, *20*, 1209–1213.

Johnson, G. D.; Fagerstone, K. A. *Primary and Secondary Hazards of Zinc Phosphide to Nontarget Wildlife—A Review of the Literature*; U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Denver Wildlife Research Center Research Report 11-55-005; U.S. Government Printing Office: Washington, DC, 1994.

Lewis, S. R.; O'Brien, J. M. Survey of rodent and rabbit damage to alfalfa hay in Nevada. *Proc. Vertebr. Pest Conf.* **1990**, *14*, 116–119.

Mauldin, R. E.; Mishalanie, E. A. The determination of zinc phosphide in technical grade materials, rodenticidal baits, and propylene glycol formulations by gas chromatography-flame photometric detection. *J. Assoc. Off. Anal. Chem.* **1997**, submitted for publication.

Mauldin, R. E.; Goldade, D. A.; Engeman, R. M.; Goodall, M. J.; Craver, R. K.; Johnston, J. J. Determination of zinc phosphide residues in the California ground squirrel (*Spermophilus beecheyi*) by gas chromatography-flame photometric detection. *J. Agric. Food Chem.* **1996**, *44*, 189–194.

Merck Index, 12th ed.; Budavari, S., Ed.; Merck & Co., Inc.: Rahway, NJ, 1996; p 1735.

Okuno, I.; Wilson, R. A.; White, R. E. Determination of zinc phosphide in range vegetation by gas chromatography. *Bull. Environ. Contam. Toxicol.* **1975**, *13* (4), 392–396.

Robison, W. H.; Hilton, H. W. Gas chromatography of phosphine derived from zinc phosphide in sugarcane. *J. Agric. Food Chem.* **1971**, *19*, 875–878.

Rutgers University. Petition proposing a tolerance for zinc phosphide for use in alfalfa production. Interreg. Res. Proj. 4.; Rutgers University: New Brunswick, NJ, 1985; 37 pp.

Sterner, R. A.; Mauldin, R. E. Regressors of whole-carcass zinc phosphide/phosphine residues in voles: indirect evidence of low hazards to predators/scavengers. *Arch. Environ. Contam. Toxicol.* **1995**, *28*, 519–523.

Tkadlec, E.; Rychnovsky, B. Residues of Zn_3P_2 in the common vole (*Microtus arvalis*) and secondary poisoning hazards to predators. *Folia Zool.* **1990**, *39* (2), 147–156.

University of California—Davis (UC-D). Zinc phosphide: magnitude of residue on sugar beet. Interreg. Res. Proj. 4; University of California: Davis, CA, 1989.

University of California—Davis (UC-D). *Intermountain Alfalfa Management*; Publication 3366; Orloff, S. B., Ed.; Division of Agriculture and Natural Resources, University of California—Davis: Oakland, CA, 1995; 138 pp.

University of Idaho. *Analytical Sciences Laboratory, Standard Methods Manual*; Regents of the University of Idaho: Moscow, ID; 1995; Section 60.300.60.

Van Wazer, J. R. *Phosphorous and Its Compounds*; Interscience: New York, 1958; Vol. 1, pp 93–177.

Received for review September 12, 1996. Revised manuscript received February 28, 1997. Accepted March 12, 1997.[⊗]

JF960689K

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.