Zinc Phosphide Analysis in Voles: Revisiting an Old Technique

Philippe Berny
Vetagro-Sup, Toxicology Laboratory TOXLAB, Marcy l'Etoile, France
Olivier Cardoso
French Agency for Biodiversity (OFB), SAGIR network
Mickaël Cœurdassier
Université Franche-Comté - UMR Chrono Environnement, Besançon, France
Geoffroy Couval, **Aurélien Levret**, and **Manon Pizoli**
FREDON Bourgogne-Franche-Comté, Beaune, France
Léa Rouzoul, and **Danièle Vey**
Vetagro-Sup, Toxicology Laboratory TOXLAB, Marcy l'Etoile, France
Joël Caspar
French Ministry of Agriculture, DRAAF Grand Est, France

ABSTRACT: Zinc phosphide has been recently approved in Europe as a vole control product. Currently, only one formulation (lentils/pellets) is marketed with 0.8% Zn_3P_2. It is applied with a delivery device for burrow baiting. In many instances, zinc phosphide poisoning has been confirmed in non-target species (primary poisoning). In order to be prepared for potential non-target poisoning incidents in wildlife, the SAGIR network, FREDON Franche-Comté, and University of Franche-Comté conducted a field study on common voles to test the sampling method and storage impact under realistic field conditions on the detection of zinc phosphide. The toxicology laboratory of Vetagro Sup, member of SAGIR, worked on the improvement of the World Health Organization WHO technique in order to lower the Limit of Quantification (LOQ) and to validate the technique for the correct identification of field cases. The specificity was tested on 20 gastric content samples (100%), and the LOQ was established at 0.01 g/l (i.e., a 100-fold decrease as compared with the 1995 WHO technique). Zinc content was measured by Flame Atomic Absorption Spectroscopy and non-poisoned animals were tested to check baseline values and to estimate recovery of spiked samples (94-102%). Quantification of zinc in the liver of poisoned versus control animals was also performed. A total of 30 voles were collected in treated and control fields and submitted for analysis. Technicians were not aware of the poisoning status of the animals when performing analytical investigations. Twenty-one individuals were trapped in the control area and nine in the treated fields. Phosphine and high levels of Zn could be detected in eight of the nine intoxicated individuals. Phosphine was not detected in the control voles. Zinc concentrations in the gastric content were significantly different between negative control and exposed animals, but liver concentrations of zinc were similar.

KEY WORDS: analytical technique, field trial, *Microtus ochrogaster*, poisoning, rodenticide, vole, zinc phosphide

Proceedings, 29th Vertebrate Pest Conference (D. M. Woods, Ed.)
Paper No. 54. Published December 10, 2020. 5 pp.

INTRODUCTION

Zinc phosphide (Zn_3P_2) was authorized at the E.U. level in 2010 as a vole-control product (European Commission 2010). It is marketed as two different formulations: wheat bait (Ratron®GW, 25 $g.kg^{-1}$ active substance) and granules (Ratron®GL, 8$g.kg^{-1}$ active substance). Only Ratron®GL is currently (since 2017) marketed in France to control vole populations (E-Phy 2017). It is only available for professional users and a maximum quantity of 2.5 kg bait can be applied per ha, directly in the burrows of rodents. When zinc phosphide is ingested, low pH of the gastric content will hydrolyze the product and release a very toxic gas: phosphine.

Zinc phosphide poisoning is commonly described in domestic and wild animals after direct consumption of the bait. Recent papers in Europe indicate that zinc phosphide poisoning occurs in dogs (Eleni et al. 2014, De Roma et al. 2018), sometimes as a result of malicious poisoning (Nagy et al. 2015). There is also evidence of accidental poisoning of various farm animal species; free-ranging chickens were accidentally exposed to contaminated feed (Muraina et al. 2018). In wild species, published evidence has been gathered by the U.S. Environmental Protection Agency (USEPA) (Erickson and Urban 2002). Wild Canada geese (*Branta canadensis*) are highly susceptible and may be affected in epizootic toxicosis phenomena (Bildfell et al. 2013). Indeed, most species display an oral LD_{50} of 21-60 mg per kg body weight, with ruminants and carnivores apparently less susceptible, but Canada geese have an LD_{50} between 7 and 12 mg per kg body weight. At present, zinc phosphide is considered as a primary toxicant with very limited evidence of secondary poisoning. The National Pesticide Information Center (NPIC) reported some mild clinical signs in Great horned owls (*Bubo virginianus*) fed poisoned prairie voles (*Microtus ochrogaster*) for three consecutive days; they were reluctant to move but no mortality was noted (Gervais et al. 2010). It is generally agreed that zinc phosphide does not accumulate in body fluids or tissues and is not transferred along the food web (Crowell et al. 2013).

Diagnosis of zinc phosphide poisoning is based on strong evidence from clinical signs, appropriate sampling, and analytical investigations (Bildfell et al. 2013, Muraina et al. 2018). Several methods have been published to investigate suspected cases of zinc phosphide poisoning in animals. Several techniques based on gas chromatography are available. Some use mass spectrometry to identify phosphine (Norman and Leonard 2000), with or without headspace detection (Perz et al. 2014). A specific technique using an arsine/phosphine detector has been

suggested by Agilent (Santa Clara, CA) (Smith-Henry and Quimby 2016). More recently, phosphine determination in cereals has been developed using Gas Chromatography-Nitrogen Phosphorus (GC-NPD) detection (Tsiantas et al. 2018). Unfortunately, due to limitations in available material in the lab, but also to sample preparation techniques, none of these techniques appeared suitable for the investigation of field samples.

The World Health Organization (WHO) published general recommendations for the rapid determination of toxicants in biological specimens (usually qualitative techniques) based on very standard material. The WHO document (Flanaghan et al. 1995) recommends the use of two colorimetric tests to detect zinc phosphide in biological specimens. The first test is based on silver nitrate (to detect phosphine and sulfides) and the second test is based on lead acetate as a confirmatory analysis (detection of sulfides only). This approach has been successfully used in several instances, including animal poisoning cases (Muraina et al. 2018).

The purpose of this study was to 1) develop/refine a sensitive and robust method to identify Zn_3P_2 poisoning in wildlife, based on the WHO method; 2) quantify zinc in gastric content as a confirmatory analysis and semi quantitative approach; and 3) apply the technique to field cases.

METHODS

Field Trials and Vole Collection

Assessments of field exposure were conducted in March 2019 in the Jura mountains (eastern French border). Four fields were selected: three with zinc phosphide application, one without zinc phosphide application. All fields were separated in order to avoid movements of rodents from one field to another.

Bait application was part of an existing rodenticide bait application program to control common vole (*M. arvalis*) populations. A total number of 91 baiting points (one baiting point = one shot with the special baiting device in one burrow) were established and baits were applied in the burrows according to label directions (i.e., using the baiting system to deliver a few grains directly in the burrow, approximately 10 cm underground). Each baiting area was covered with nets to protect the baiting point from non-target consumption as well as to protect voles from predation or scavenging. In the control field, rodents were trapped using Sherman traps and immediately euthanized with carbon dioxide overdose. Search for dead rodents was conducted in/around burrows and baiting points several times up to 24 hours post application.

In order to mimic field collection of carcasses for post-mortem investigations, dead rodents were immediately sampled: stomach was collected and ligated at both ends to avoid spillage of its content. The liver was also collected, and all biological specimens were kept in freezing boxes to be frozen as soon as possible back at the office (the same day). All samples were kept at -20°C until analysis. A total of 30 voles were trapped or found dead and the laboratory technicians did not know the exposure status of the rodents. Nine rodents were collected in the Zn_3P_2 fields, and 21 were trapped in the control field.

Sample Preparation

In tube #1: 1.5 mL H_2O_2 30% and 5mL HNO3 65% were added and the tube was placed in a microwave (180°C) for 30 min. After cooling, it was completed to 20 mL with distilled water. The analytical conditions are described in Table 1. For liver samples, 1g of liver was weighed and prepared with H_2O_2 and HNO_3 as described above. Wheat baits were also tested similarly. Recovery was determined with control samples spiked with zinc solution and, later on, with zinc phosphide solution.

Table 1. Analytical conditions for zinc determination by Flame Atomic Absorption Spectroscopy.

Gas	Air-C2H2
Gas flow	0.9 L/min
Burner height	7 cm
Baseline correction	D2 lamp
Number of repeated measures	3
Wave length (absorption)	213.9 nm
Lamp current	>75%
Band length	0.5 nm

For all zinc analysis, the validation process included the following steps: 1) Daily 6-point calibration curve (3 days); 2) five replicates of spiked samples (gastric content, liver) at a high dose (1g/L for gastric content, 100 mg/kg for liver samples); 3) five replicates of samples spiked at the lowest calibration point (LOQ); and 4) acceptability was based on a Coefficient of Variation <30% for the LOQ, and CV < 20% for all other data points.

Sensitivity of the diagnostic test was defined as the proportion of true positive individuals that were correctly identified by the test. Specificity of the diagnostic test was defined as the proportion of true negative individuals that were correctly identified by the test.

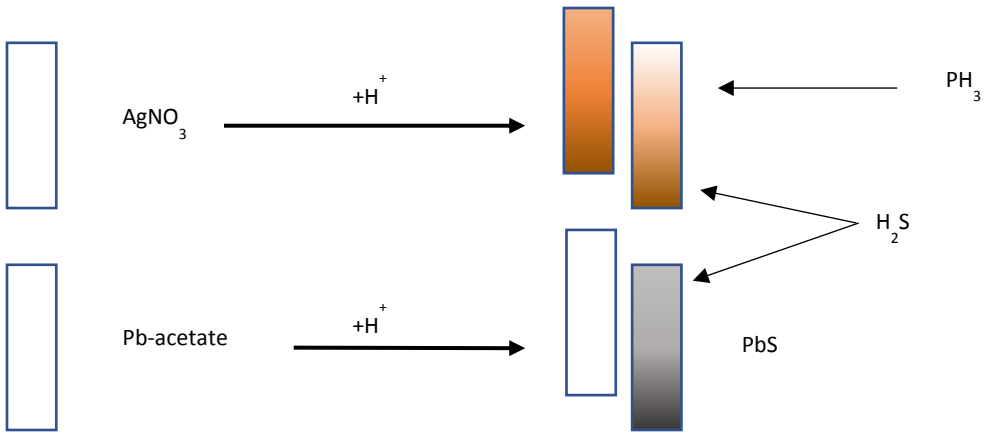
Analyses

Zinc phosphide analysis was conducted based on the WHO method (Flanaghan et al. 1995). Several changes were made to adapt the method to our field work; the method outlined below is the result of this preliminary work (Figure 1):

Two 1-g gastric content samples were weighed and placed in two tubes. Five mL of distilled water were added in each tube, vortexed for 5 min. The first tube was acidified with three droplets of nitric acid (65%) (silver nitrate test). The second tube was kept for the lead acetate test. Silver nitrate solution was prepared by dissolving 2.5 g $AgNO_3$ in 50mL 99% methanol, kept protected from light with an aluminum foil. In tube #1, the silver nitrate paper (filter paper impregnated 1 min with saturated silver nitrate solution and dried at 45°C for 30 minutes) was placed, and the tube sealed and heated at 60°C for 15 min. In the tube #2, a commercial lead acetate paper was placed, and the tube sealed and heated at 60°C for 15 minutes.

Negative control and spiked samples were prepared from commercial cat food. In order to check the specificity of the method, we used 10 samples of gastric content from different species known to be not exposed to Zn_3P_2: dog (*Canis familiaris*), lynx (*Lynx lynx*), cow (*Bos taurus*), pigeon (*Columba palumbus*), and donkey (*Equus africanus*). Also, different samples of rodenticide baits

a)



b)

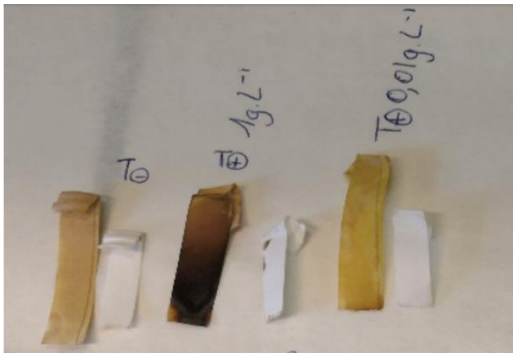


Figure 1. Colorimetric determination of phosphine with silver nitrate ($AgNO_3$) and lead acetate (Pb-acetate) papers. (a) Reaction and (b) test pictures. T- : neg control, T+ : positive control 1g/L and 0.01 g/L. Note that all lead acetate papers remain white, and we observe large/small brownish spot on the silver nitrate paper depending on the concentration of the tested sample.

were tested. Repeatability of the technique was determined on five replicates of the same sample spiked at the limit of detection or at a high dose (1 g/L).

Zinc analysis was performed by Flame Atomic Absorption Spectroscopy (FAAS). All standards and samples were diluted in 0.5% acid solution (2.5 mL HNO_3 completed to 500 mL with distilled water). The calibration curve was based on a 6-point curve from 0.25 to 2 mg/L zinc.

Statistical analyses were non-parametric Kruskall-Wallis or Mann-Whitney-Wilcoxon rank tests at a significance level of $p < 0.05$.

RESULTS

Analyses

The WHO technique (Flanaghan et al. 1995) reports a LOD of 1g/L. Under our conditions, we could lower this detection limit to 0.01 g/L (Table 2). All tests conducted on negative animals were negative and this held true for other rodenticide baits. Five different anticoagulant rodenticides were tested: bromadiolone, difenacoum, brodifacoum, difethialone, and flocoumafen; one non-anticoagulant was used (alpha-chloralose). Each gastric content was tested five times (i.e., 40 tests were run) and the result was negative in 39/40. The last test was inconclusive, but it was suspected that the filter paper touched the gastric content. Spiked samples (at the LOD) were positive in all tests conducted (i.e., 40/40). The zinc determination method is linear, repeatable, reproducible, and recovery was between 94 and 102% (between 0.25 and 2 mg/L).

Table 2. Determination of the limit of detection of phosphine by the colorimetric method.

Zinc (g/L)	0.005	0.01	0.02	0.1	0.2	0.5	1
Test	±	+	+	+	+	+	+
Conclusion	NEG	LOD	POS	POS	POS	POS	POS

NEG = negative, LOD = limit of detection, POS = positive

Field Trials

Analyses were conducted six months after collection in the field. The phosphine detection method was positive for eight of the nine animals from the treated field, and all controls tested negative for phosphine exposure. We noted an inconclusive phosphine test for one exposed individual. The lead acetate paper remained uncolored for all tested animals. Based on this analysis, the field sensitivity of the test would be 88%.

Zinc determination gave very interesting results (Table 3, Figure 2). The median zinc concentration in the gastric

content was 15.8 µg/g in control animals, and 351.2 µg/g in exposed animals ($p < 0.05$). Liver residues were not significantly different between groups (34 µg/g in control animals, 26.1 µg/g in exposed individuals). The individual found to be inconclusive at the phosphine test had "normal" (background) levels of zinc in the gastric content, suggesting a negative individual, but because of the inconclusive results, it was excluded from the statistical analyses. It is also important to highlight that there was no overlap of the range of zinc in the gastric content between exposed and non-exposed voles. Based on the field observations and using both phosphine determination and zinc measurement, the field-sensitivity of the test reached 100%.

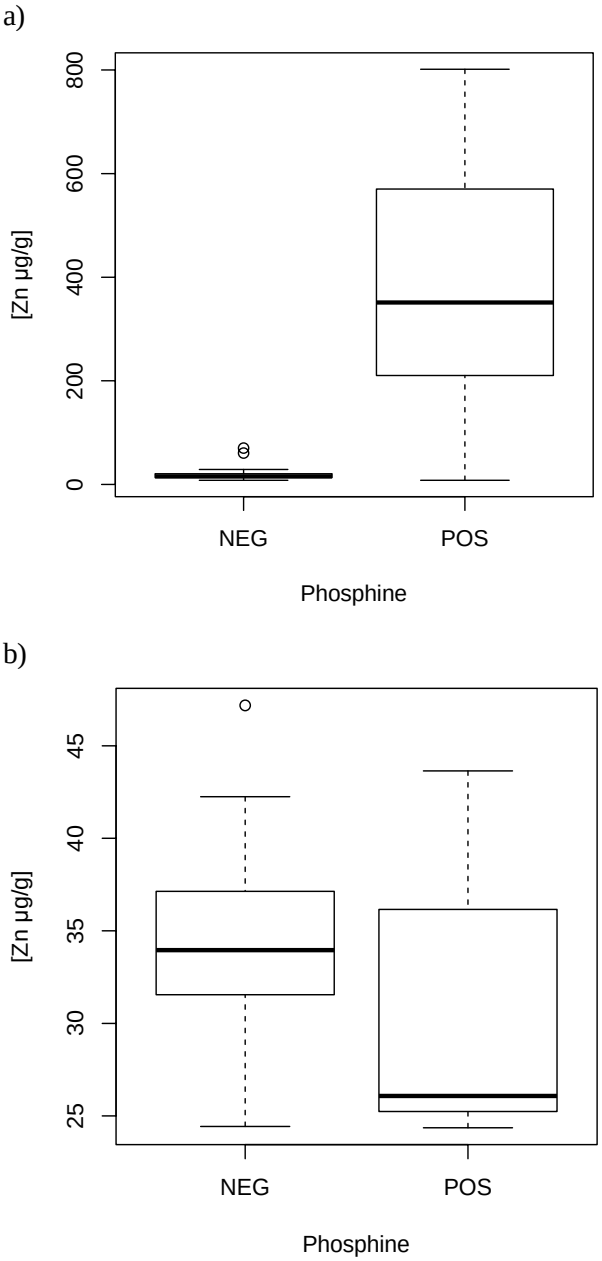


Figure 2. Distribution of zinc concentration in (a) gastric content and (b) liver of contaminated (Phosphine-POS) or negative control (Phosphine-NEG) voles.

Table 3. Zinc concentration in gastric content or liver samples from negative control (not exposed-) or exposed voles.

Group	Not Exposed	Exposed
N	21	8
[Zinc] Gastric content – median - µg.g-1	15.79	351.18*
[Zinc] Gastric content – mean - µg.g-1	20.78	384.13*
[Zinc] Gastric content – range - µg.g-1	8.07-4-70.40	153.82-801.36
[Zinc] Liver – median - µg.g-1	33.96	26.08
[Zinc] Liver – mean - µg.g-1	34.13	30.37
[Zinc] Liver – range - µg.g-1	24.43-47.19	24.36-43.64

DISCUSSION

Diagnostic testing for zinc phosphide poisoning may be difficult and expensive because it usually requires expensive material and skilled technicians to perform investigation using Gas Chromatography coupled with Mass Spectrometry (GC-MS) or GC with ECD detector (GC-ECD) with special conditions for phosphine extraction/ recovery (Norman and Leonard 2000, Braselton and Johnson 2003, Tiwary 2005, Perz et al. 2014). In contrast, the WHO test is a quick and easy technique, but it is only qualitative and with a poor sensitivity (1g/L) (Flanaghan et al. 1995). Our standardization of the analytical conditions for the colorimetric test, including the use of sealed tubes, and standard preparation of test reagents enabled us to increase the sensitivity of the analysis by 100-fold. This makes the colorimetric method potentially usable for diagnostic testing in field cases.

The optimization and validation of zinc determination in the gastric content also improved the specificity of the analysis. Using both tests at the same time, it is possible to conclude with a high probability on the exposure of an animal to zinc phosphide (Bildfell et al. 2013). Both techniques are easy to use, fairly standard in an analytical laboratory, and robust. It appears therefore quite reasonable to recommend doing both the colorimetric determination and zinc analysis on the gastric content (or bait) when zinc phosphide poisoning is suspected. At that stage, we have not concluded the quantitative analysis of zinc phosphide: determination of digestive levels of zinc may be of interest but this would need an experimental approach.

Despite the potential degradation of zinc phosphide in the stomach and production of phosphine gas (i.e., sample manipulation and storage need serious precautions to protect manipulators), it appears that immediate freezing of stomach content can keep samples in a good condition for phosphine detection for up to six months. Zinc concentrations in the liver of voles were similar, which would support the idea that poisoning is too fast to result in increased zinc concentration in the liver of exposed animals, despite the high concentrations in the gastric content. Liver analysis for zinc is therefore not interesting in diagnostic testing of zinc phosphide poisoning.

Some limitations of our work should be mentioned: 1) Animals were grouped according to the collection site (i.e.,

an individual found dead in a treated field was considered "positive"), even though we did not have necropsy data or monitoring data to demonstrate actual exposure of the vole. 2) The analytical investigations were conducted six months after the exposure trial, and some phosphine may have been released and eliminated from the samples. If it happened, this had no impact on the detection of exposed individuals. We suggest that storage at -20°C is sufficient to keep the biological samples in good conditions for further analysis, though within a few weeks preferably. 3) Because our samples were collected fresh and frozen rapidly, we did not experience any of the difficulties sometimes mentioned with the colorimetric determination of phosphine in decayed samples giving rise to false positive results (Tiwary et al. 2005), but it must be said that the use of two different techniques (especially zinc determination) can overcome this issue. Finally, 4) this study was conducted on vole samples, and we confirmed that our detection limit was sufficient to identify ingestion of a single grain bait by a vole. We may need to work on cases of larger animals (either by spiking negative gastric content or on real field cases when they occur) to determine the field sensitivity and it's applicability to accidental poisoning, although we have evidence that the colorimetric method alone is usually effective to diagnose those cases (Tiwary et al. 2005, Nagy et al. 2015, Muraina et al. 2018).

In order to have a definite diagnosis, veterinary toxicologists will always rely on clinical data, and when available, necropsy findings including histopathology (at least lung examination in this case) and toxicological investigations. Only when all findings are consistent can a positive diagnosis of zinc phosphide poisoning be confirmed (Nagy et al. 2015, Muraina et al. 2018). All other situations give reasonable evidence of poisoning, but not conclusive evidence. By revisiting this old technique and improving it, we consider that we can provide scientifically sound analytical support to our game and wildlife agency whenever a suspected case of zinc phosphide exposure arises.

LITERATURE CITED

Bildfell, R. J., W. K. Rumbeiha, L. K. Schuler, C. U. Meteyer, P. L. Wolff, and C. M. Gillin. 2013. A review of episodes of zinc phosphide toxicosis in wild geese (*Branta* spp.) in Oregon (2004-2011). Journal of Veterinary Diagnostic Investigation 25(1):162-167.

Braselton, W., and M. Johnson. 2003. Thin layer chromatography convulsant screen extended by gas chromatography-mass spectrometry. Journal of Veterinary Diagnostic Investigation 15(1):42-45.

Crowell, M. D., K. G. Broome, C. T. Eason, A. A. C. Fairweather, S. Ogilvie, and E. C. Murphy. 2013. How long do vertebrate pesticides persist in living mammals? DOC Research and Development Series 331 New Zealand Department of Conservation, Wellington, New Zealand.

De Roma, A., G. Miletti, N. D'Alessio, L. Marigliano, T. Bruno, P. Gallo, G. Binato, and M. Esposito. 2018. Inspective and toxicological survey of the poisoned baits and bites. Forensic Science International 287:108-112.

Eleni, C., F. Scholl, and P. Scaramozzino. 2014. Causes of death in dogs in the province of Rome (Italy). Veterinaria Italiana 50(2):137-143.

Erickson, W., and D. Urban. 2002. Comparative rodenticide risk assessment. U.S. Environmental Protection Agency, Washington, D.C.

E-Phy. 2017. Product information for Ratron®GL. https://ephy.anses.fr/ppp/ratron-gl

European Commission. 2010. Commission Directive 2010/85/EU. Official Journal of the European Union. https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:317:0032:0035:EN:PDF

Flanagan, R. J., R. A. Braithwaite, S. S. Brown, B. Widdop, and F. A. de Wolff. 1995. Basic analytical toxicology: phosphine. World Health Organization, Geneva, Switzerland.

Gervais, J. A., B. Luukinen, K. Buhl, and D. Stone. 2010. General fact sheet: zinc phosphide/phosphine. National Pesticide Information Center, Oregon State University Extension Services, Corvallis, OR.

Muraina, I. A., O. O. Oladipo, O. B. Akanbi, J. J. Shallmizhili, M. D. Gyang, G. O. Ijale, F. P. Govwang, and A. A. Atiku. 2018. Phosphine poisoning in free-range local chickens: a case report. Veterinary Medicine and Science 4(3):263-268.

Nagy, A.-L., P. Bolfa, M. Mihaiu, C. Catoi, A. Oros, M. Taulescu, and F. Tabaran. 2015. Intentional fatal metallic phosphide poisoning in a dog: a case report. BMC Veterinary Research 11(158):1-6.

Norman, K. N. T., and K. Leonard. 2000. Gas chromatography-mass spectrometry determination of phosphine residues in stored products and processed foods. Journal of Agriculture and Food Chemistry 48:4066-4070.

Perz, R., A. Bankenstein, H. Kobler, E. Scherbaum, D. Kohl, A. Barth, and M. Anastassiades. 2014. Analysis of phosphine in dried foodstuffs via Headspace-GC-MSD. Aspects of Food Control and Animal Heath 2:17.

Smith-Henry, A., and B. Quimby. 2016. Analysis of arsine and phosphine in ethylene and propylene using the Agilent arsine phosphine GC/MS analyzer with high efficiency source. Application Note. Agilent Technologies, Inc., Wilmington, DE.

Tiwary, A. K., B. Puschner, B. R. Charlton, and M. S. Filigenzi. 2005. Diagnosis of zinc phosphide poisoning in chickens. Avian Disease 49(2):288-291.

Tsiantas, P., G. Miliadis, G. Siragakis, and A. D. Lampidonis. 2018. Phosphine determination in cereals with NPD gas chromatography. Unpublished report. Abstract *in* 7o Metrology Conference, May 2018. https://www.researchgate.net/publication/325301587_Phosphine_determination_in_cereals_with_NPD_gas_chromatography