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Penetration Studies of Propoxur and Phoxim from Eggshell into Whole Egg after Experimental Exposure and Application in Henhouses

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The penetration of propoxur and phoxim from eggshell into whole egg was investigated in vitro by spraying eggs directly and in vivo after application of the compounds in henhouses. Although mean concentrations of the compounds on eggshells were up to 23000 μg kg^{-1}, mean residue concentrations in whole eggs were far below the current maximum residue levels (50 μg kg^{-1} for propoxur and 60 μg kg^{-1} for phoxim). These results provide the first evidence that propoxur and phoxim do not penetrate from eggshell into whole egg under experimental and field conditions. Subsequently, residue carry-over after egg cracking in households and during a worst-case situation in an egg-cracking plant was investigated. However, when eggs were cracked manually, a negligible contamination of whole egg values occurred. If, in an automated process, eggshells accidentally come into close contact with whole egg, very high residue levels of propoxur and phoxim may be generated time dependently. These results suggest that eggshell contact with whole egg during egg cracking must be avoided to prevent pesticide carry-over.

KEYWORDS: Phoxim; propoxur; HPLC; egg; laying hen; penetration

INTRODUCTION

The poultry red mite *Dermanyssus gallinae* is the most important ectoparasite of egg layers across Germany and Europe (*1*). It is an obligatory blood-feeding parasite of birds that attacks the resting hens, mainly during the night, for a short blood meal. After feeding, the mites hide in cracks and crevices away from daylight, where they mate and lay eggs. This behavior of the parasite makes its control very difficult (*2*). Another great problem in the fight against *D. gallinae* is still the lack of safe substances that may be used as veterinary drugs to directly treat hens.

Propoxur (2-isopropoxyphenyl-*N*-methylcarbamate) and phoxim (phenylgloxylonitrileoxime *o*,*o*-diethyl phosphothionate) are carbamate and organophosphorous insecticides that are effective against *D. gallinae*. The chemical structures of these compounds are shown in **Figure 1**. Within the European Union, the maximum residue limit (MRL) for phoxim in eggs is set at 60 μg kg^{-1}. In a German regulation for propoxur residues in whole eggs, a MRL value of 50 μg kg^{-1} can be found (*3*, *4*).

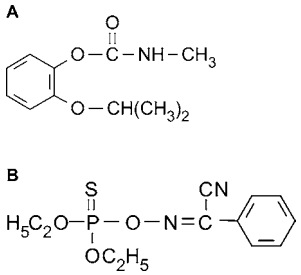

Figure 1. Molecular structure of propoxur (**A**) and phoxim (**B**).

The manufacturers of propoxur and phoxim emphasize the need to spray all crevices in the poultry house with solutions containing 1% propoxur or 0.2% phoxim and to prevent the hens from coming into direct contact with the spray solutions. As recently shown, laying hens staying in the cages during treatment are exposed to the active compounds and phoxim and propoxur residues occurring in eggs. Mean residue levels were in general lower than the current MRLs for both compounds (*5*, *6*).

Only a few studies have been undertaken to demonstrate that pesticides may penetrate the eggshell after external exposure and contaminate the whole egg (*7*, *8*). To our best knowledge, no investigations have been performed comparing residue levels on the eggshells and in the whole egg.

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After spraying phoxim and propoxur solutions in henhouses, the active compounds may be absorbed through the skin or inhaled by the hens and subsequently deposited in the body, including the ovary. Furthermore, it also seems possible that the compounds may be directly transferred from dust particles or from hen feathers to freshly laid eggs.

Therefore, we have now undertaken detailed experimental and field studies to investigate the penetration of propoxur and phoxim residues from the eggshell into the whole egg. We investigated accidental contamination in the whole egg during egg cracking as well.

MATERIALS AND METHODS

Chemicals and Apparatus. Propoxur and phoxim reference standards were obtained from Promochem (Wesel, Germany). Water with a resistance of >18.0 MΩ/cm was prepared in-house by a Milli-Q-System (Millipore, Eschborn, Germany). Acetonitrile, dichloromethane, and *n*-hexane were high-performance liquid chromatography (HPLC) grade solvents from J.T. Baker (Griesheim, Germany) and Sigma (Munich, Germany). Silica gel (35−70 mesh) was obtained from Fluka (Munich, Germany). HPLC solvent A was 100% water, and solvent B was 100% acetonitrile. A high-speed blender (IKA Ultra-Turrax, Germany), a stirrer (IKA RCT basic, Germany), a vacuum rotary evaporator with water bath (Laborata 4002 Heidolph, Germany), a centrifuge and 200 mL centrifuge tubes (Beckman CS-15R, Germany), chromatographic glass columns (10 mm × 300 mm, LAT, Germany), an ultrasonic bath (Bandelin, Germany), and a vortex mixer were used.

Chromatography. The HPLC system used for analysis of propoxur and phoxim consisted of a Shimadzu 10 VP system equipped with two LC 10 AD VP pumps, a SIL 10 AD VP autoinjector, a CTO 10 A VP oven, a SPD M 10 A VP diode array detector (DAD), a GT-154 degasser, a SCL 10 AVP system controller, and an analytical C 18 column (Lichrospher reversed phase C 18, 5 μm, 250 mm × 4 mm, VDS Optilab, Germany). Methods for propoxur and phoxim analysis were recently developed in our laboratory (*5*, *6*). Briefly, the injection volume was 20 μL and the compounds were separated by gradient elution using water (solvent A) and acetonitrile (solvent B). Eggshell samples containing propoxur or phoxim in amounts outside the linear calibration range were reinjected in volumes as low as 2 μL. For propoxur analysis, 20% B for 1 min and then a linear gradient of 20−45% in 14 min was performed. After propoxur eluted, the column was rinsed for 2 min with 99% B. For phoxim analysis, we started with 40% B, then a linear gradient of 40−75% B in 15 min, kept at 75% for 2 min. After elution of phoxim, the column was rinsed with 99% B for 2 min. The flow rate was 1 mL min^{-1}. The detection wavelength was performed at 220 and 281 nm for propoxur and phoxim, respectively. The oven temperature was 40 °C, and the DAD recorded UV spectra ranging from 200 to 350 nm. The peak purity was electronically checked by employing Class VP software (Version 5.03). Briefly, a "3 peak point purity" was calculated by comparing spectra at the beginning and at the end within a chromatographic peak to the spectrum at the peak apex. From these spectral data, a similarity factor was calculated. A factor higher than 0.90 was set for sufficient peak purity for confirmation purposes for both compounds under investigation.

Extraction and Cleanup. Again, the extraction procedures for propoxur and phoxim of whole egg samples were performed as already described in detail by Hamscher et al. (*5*, *6*). Briefly, samples were extracted with acetonitrile and defatted with hexane, followed by a cleanup with silica gel column chromatography. The limits of detection (LODs) and quantification (LOQs) were 2 and 5 μg kg^{-1} for both compounds. The mean recovery value for propoxur was 88.4%, and for phoxim, it was 89.2%. All data were corrected for mean recoveries.

For eggshell extraction, 5 g of eggshell was crumbled and extracted with 50 mL of acetonitrile in a 250 mL centrifuge tube for 5 min employing a magnetic stirrer. The tube was centrifuged at 5200*g* for 5 min. The supernatant was transferred into a 250 mL round-bottomed flask, and then, the residue was re-extracted with another 50 mL of acetonitrile. The combined acetonitrile extracts were evaporated to dryness (40 °C). The dried sample was reconstituted with 1.0 mL of acetonitrile under sonification, transferred with a glass Pasteur pipet into a 1.5 mL plastic tube (Eppendorf, Germany), and centrifuged for 1 min at 20000*g*. The supernatant extract was transferred into an autosampler vial and stored at −18 °C until analysis. All data were corrected for mean recoveries.

To evaluate the accuracy and precision of the method, fortified eggshell samples at five concentration levels (10, 50, 250, 1000, and 5000 μg kg^{-1}) were prepared by spiking 5 g of blank of eggshell with propoxur and phoxim standard. Triplicate samples at each level were prepared for phoxim, and at least duplicate samples were prepared for propoxur. The extraction procedure was performed after 10 min of equilibration at room temperature.

Experimental Studies. *Experimental Study One: Penetration of Propoxur and Phoxim from Eggshell into Whole Egg.* The application of the pesticides was accomplished in a handmade rectangular spray chamber (40 cm × 30 cm × 30 cm). The distance from the spray nozzle to the egg was approximately 20 cm, and the volume of the sprayed pesticide solution was approximately 20 mL. Six eggs were sprayed with 1% CBM 8 (Interhygiene, Cuxhaven, Germany; active ingredient is propoxur) and another six eggs with 0.2% Sebacil 50 (Bayer AG, Leverkusen, Germany; active ingredient is phoxim) according to the recommendations of the manufacturers. After air drying for 1 h, the whole egg was immediately separated from the shell, weighed, and stored in individual polyethylene tubes with caps (84 mm × 30 mm, Sarstedt, Nümbrecht, Germany) and frozen at −18 °C until they were analyzed for pesticide residues.

Experimental Study Two: Effect of Egg Cracking on Propoxur and Phoxim Contaminations in Whole Eggs. Six eggs of each treatment were sprayed with 1% CBM 8 and 0.2% Sebacil 50 as described above. After air drying for 1 h, eggs were manually cracked, and for each compound, two subgroups of three eggs were set up. In the first group, whole eggs were mixed with complete eggshells and incubated at room temperature, whereas in the other group three whole eggs were stored without eggshells. Ten milliliters of whole egg samples was collected after 1, 2, and 4 h of incubation. All samples were immediately stored at −18 °C prior to analysis.

Field Studies. *Field Study One: Penetration of Propoxur from Eggshell into Whole Egg.* This study was conducted in a research henhouse at the field station Ruthe at the University of Veterinary Medicine Hannover. Three different henhouse systems (an aviary system and the furnished cage systems Eurovent and Aviplus) located in one building were sprayed with 1% CBM 8 on days 0, 5, and 11 of the studies. Hens were kept in these systems, and six eggs of each investigated system were collected on days 0, 1, 5, 6, 11, and 12 after the first application. Please note that the collection of eggs on days 0, 5, and 11 was performed prior to spraying. Again, the whole egg was separated from the shell, weighed, and stored in individual polyethylene tubes with caps (84 mm × 30 mm, Sarstedt, Nümbrecht, Germany) and frozen at −18 °C until they were analyzed for pesticide residues.

Field Study Two: Penetration of Phoxim from Eggshell into Whole Egg. This study was conducted in a conventional chicken house stocked with layers. The house was naturally infested with *D. gallinae*. The henhouse was sprayed with Sebacil EC 50 mixed with water containing phoxim in a final concentration of 0.2% following the recommendations of the manufacturers on day 0 and day 7 of the study. The hens were not sprayed directly. Twelve eggs were collected on days 1, 2, and 3 after the second application of Sebacil 50. Six eggs from each day were immediately divided into whole eggs and eggshells after sampling, weighed and stored in individual polyethylene tubes with caps, and frozen at −18 °C until they were analyzed for phoxim residues. Another six eggs were kept in the dark at 4 °C for 14 days and then separated into whole eggs and eggshells and further processed as described above.

RESULTS AND DISCUSSION

Accuracy, Precision, Quantification, and Detection Limits for Propoxur and Phoxim in Eggshells. The mean recovery of fortified samples in the 10−5000 μg kg^{-1} range was 72.1% with a relative standard deviation from 1.6 to 10.6% for phoxim and 71.0% with a relative standard deviation from 3.7 to 19.3%

Table 1. Mean Values and Range of Propoxur and Phoxim Concentrations (in μg kg^{-1}) in Eggshells ($n = 6$) and Whole Eggs ($n = 6$) after Artificial Contamination of the Eggs[a]

	propoxur mean ± SD (range)	phoxim mean ± SD (range)
eggshell	20225 ± 2601 (16803–24610)	23732 ± 3768 (19310–30208)
whole egg	6.5 ± 3.1 (3.7–12.5)	<2.0 (ND–3.2)

[a] ND, not detected (LOD = 2 μg kg^{-1}).

for propoxur. The linearity of the extraction procedure for both compounds was excellent with $r^2 \geq 0.9994$ and 0.9988 for phoxim and propoxur, respectively.

From our recovery data, a LOQ of 10 μg kg^{-1} was established for both compounds. The smallest amount that could be detected (LOD) based on a signal-to-noise ratio greater than 3 was approximately 5 μg kg^{-1}.

Experimental Study One: Penetration of Propoxur and Phoxim into Whole Egg. The concentrations of propoxur and phoxim in eggshell and whole egg after treatment with 1% CBM 8 and 0.2% Sebacil 50 via spraying are shown in **Table 1**. These data clearly show that our experimental design was suitable for obtaining very high pesticide residues from the eggshells with average concentrations >20 mg kg^{-1} for both compounds under investigation. In the corresponding whole egg samples, only traces of propoxur (<7 μg kg^{-1}) and phoxim (<2 μg kg^{-1}) were found, which represent carry-over rates of approximately 0.03% for propoxur and <0.01% for phoxim. Although the eggshells were artificially contaminated with high concentrations of propoxur and phoxim, no substantial penetration into whole eggs was observed. Furthermore, an accidential contamination during separation of eggshell from whole egg in this study cannot be ruled out completely.

Experimental Study Two: Carry-over of Propoxur and Phoxim Residues in Whole Egg via Egg-Cracking. In this set of experiments, two completely different procedures were investigated. While eggs are generally used without eggshells in households, there may only be a low risk of contamination when a whole egg comes into contact with parts of the eggshell during cracking. The second experiment was carried out with the purpose of simulating a worst-case situation, which might occur when eggs are mechanically broken in food companies and substantial amounts of eggshells come into contact or are mixed with whole eggs for longer periods. The residue profiles during incubation for up to 4 h are shown in **Figure 2**. For egg cracking in households, all samples were far below the current MRL values and the residue levels did not increase during incubation as one might have expected.

When eggshells are incubated in whole eggs, the residue levels are immediately higher than the MRLs (propoxur, 777 μg kg^{-1}; phoxim, 109 μg kg^{-1}). After 4 h of incubation, both compounds were detected at similarly high levels (5928 μg kg^{-1} for propoxur and 4694 μg kg^{-1} for phoxim). The higher initial concentration of propoxur may be in part explained by its lower octanol/water partition coefficient (log K_{ow}) of 1.52 as compared to phoxim with a log K_{ow} of 4.39 (*9*). With increasing incubation time, the more lipophilic compound phoxim may be solubilized by egg yolk components.

These preliminary results from a worst-case experiment suggest that eggshell contact with whole egg during egg cracking must be avoided whenever possible to prevent pesticide carry-over. One should take into consideration that when eggs are processed in food-producing companies a washing step is usually performed prior to cracking. This should help to reduce pesticide

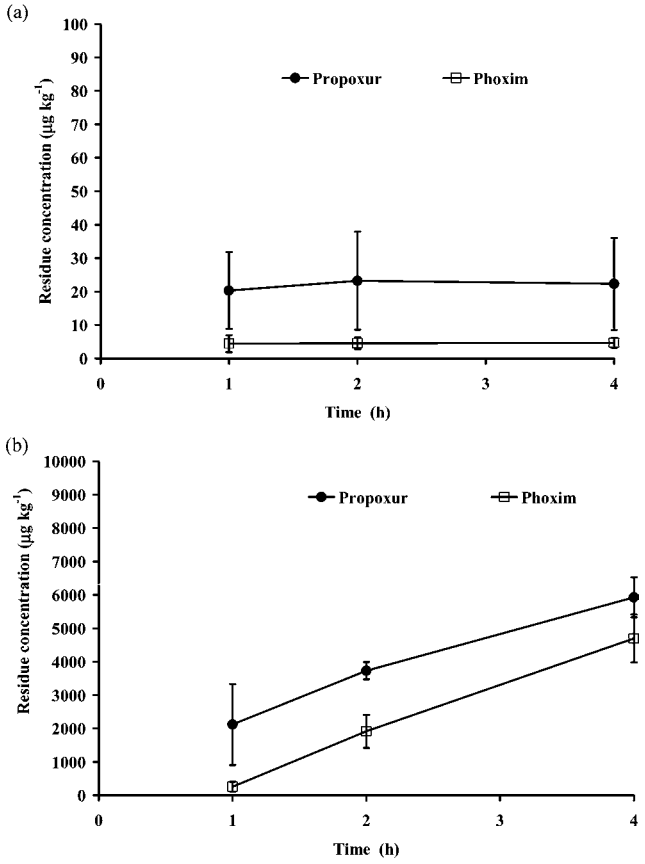

Figure 2. Pesticide residue profiles during whole egg incubation for 4 h. After artificial contamination of the eggshell with high amounts of propoxur ($n = 3$) or phoxim ($n = 3$), the eggs were manually cracked under household conditions (**a**). In a second experiment, a worst-case situation in a food producing company was simulated: Whole eggs were mixed with eggshells (**b**, $n = 3$ for each compound).

levels on the eggshell and lower the carry-over risk. However, especially phoxim has a comparatively low water solubility of 4.1 mg/L (*9*) and the efficiency of the washing procedure has not been evaluated so far.

Field Study One: Penetration of Propoxur from Eggshell into Whole Egg. Propoxur residues in eggshells and corresponding whole eggs obtained from different henhouse systems are shown in **Table 2**. Propoxur residues were also found in eggshells of control eggs collected on day 0. This finding indicated that the henhouse was contaminated with propoxur before the study. Indeed, this henhouse had been regularly treated with propoxur before our study as recently reported (*5*). However, after spraying with CBM 8, no eggshell contained propoxur residues higher than 4.42 mg kg^{-1}. For whole egg, the highest concentration was found with 11.9 μg kg^{-1} on day 1 of the study. No substantial differences in levels were obtained from the different henhouse systems of laying hens. When compared to our experimental study, similar residue levels in whole eggs were obtained although the concentrations on the eggshells were approximately four-fold lower. These data may indirectly support our observation that penetration of propoxur from eggshell into whole egg is not a major route for carry-over.

Field Study Two: Penetration of Phoxim from Eggshell into Whole Egg. Figure 3 shows the residue data after spraying 0.2% Sebacil 50 in a conventional battery cage layer unit. In this study, phoxim residues were generally found below 1 mg kg^{-1} on eggshells and above 10.3 μg kg^{-1} in whole eggs. When

Table 2. Mean Values and Standard Deviations (SD) and Range of Propoxur Residues (in μg kg^{-1}) in Eggshell ($n = 6$) and Whole Egg ($n = 6$) after Application of 1% CBM 8 on Days 0, 5, and 11 in Different Henhouse Systems[a]

	aviary cages		Eurovent		Aviplus	
day	eggshell	whole egg	eggshell	whole egg	eggshell	whole egg
0	12.6 ± 5.1 (9.0–16.2)	ND	7.6 ± 2.5 (5.9–9.4)	ND	308.7 ± 276.2 (113.4–504.0)	ND
1	726.1 ± 300.2 (384.2–1096.8)	3.1 ± 3.4 (ND–6.4)	1524.7 ± 1468.9 (540.3–4415.9)	7.4 ± 4.0 (ND–11.9)	819.4 ± 485.3 (432.2–1464.4)	8.4 ± 2.2 (6.7–11.3)
5	427.5 ± 126.4 (338.1–516.8)	ND	338.5 ± 117.6 (255.3–421.6)	2.9 ± 4.0 (ND–1.0)	146.1 ± 24.2 (129.0–163.2)	ND
6	930.1 ± 1061.9 (98.4–2486.9)	3.1 ± 3.4 (ND–6.3)	339.8 ± 98.4 (174.7–440.2)	ND	274.7 ± 372.6 (41.4–1026.3)	2.1 ± 3.3 (ND–7.0)
11	287.0 ± 311.1 (67.1–507.0)	ND	458.4 ± 220.3 (641.1–329.6)	ND	341.9 ± 2.6 (340.1–343.8)	ND
12	56.1 ± 23.3 (21.6–83.5)	ND	65.4 ± 25.2 (41.8–102.0)	ND	106.8 ± 175.2 (8.8–461.6)	ND

[a] Please note that the different henhouse systems were located in the same building and that the collection of eggs on days 0, 5, and 11 was performed prior to spraying. ND, not detected (LOD = 2 μg kg^{-1}).

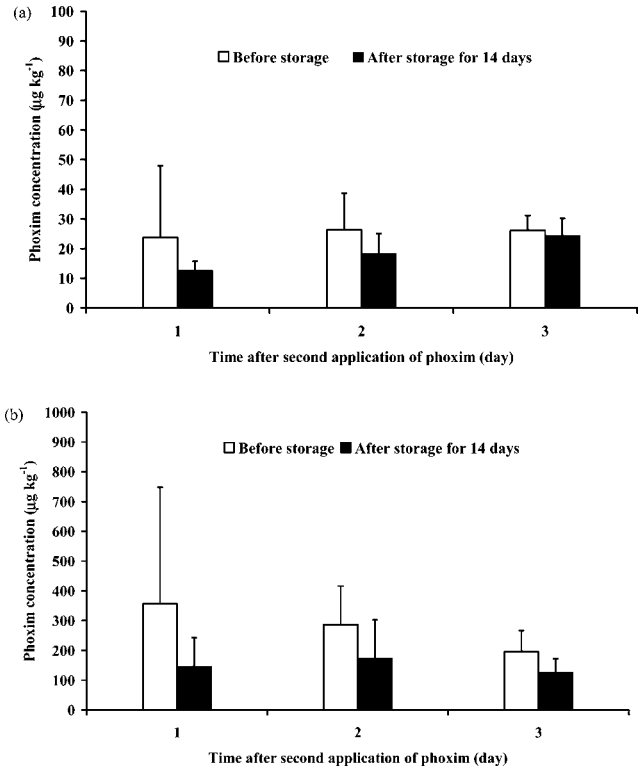

Figure 3. Phoxim concentrations (μg kg^{-1}) in whole eggs (**a**, $n = 6$) and in eggshells (**b**, $n = 6$) before and after storage at 4 °C for 14 days. Eggs were collected on days 1, 2, and 3 after the second application of Sebacil 50.

comparing findings with those of the experimental study, we found an approximately 25-fold lower concentration of phoxim in egg shells but an approximately 10-fold higher average phoxim concentration in whole eggs. Again, this may also indirectly support our observation that penetration of phoxim from eggshell into whole egg is not a major route for carry-over.

Another interesting point note in this study is that average phoxim residue concentrations are higher than propoxur residues although the spraying solution contained only 0.2% phoxim. One explanation for this may be the different log K_{ow} values of phoxim leading to a higher accumulation rate in fat-rich compartments. Another reason may be that carbamates degradate more rapidly in the environment and in the body than organophosphates.

Figure 3 summarizes average phoxim residue concentrations on different days of the study and the effects of storage. Although only a limited number of eggs were analyzed, storage of the eggs for 14 days at 4 °C had no substantial effect on the residue concentrations. Furthermore, the phoxim concentrations obtained in whole eggs are very similar to those recently reported by Hamscher et al. (*6*). These data support our recent findings, namely, that it is virtually impossible to avoid contamination of hens during spraying in battery cages (*5*, *6*).

In conclusion, we have demonstrated with various experimental and field studies that propoxur and phoxim residues on eggshells do not penetrate into whole eggs even when eggshells are highly contaminated. However, one risk for pesticide carry-over may exist when eggs are mechanically cracked and substantial amounts of contaminated eggshells come into close contact with whole egg for longer periods.

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