Evaluation of Brine Shrimp (Artemia salina) Larvae as a Bioassay for Mycotoxins in Animal Feedstuffs

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ABSTRACT

Brine shrimp larvae were tested as a possible simple biological screening system to identify specimens of animal feedstuffs that should be examined further by chemical analytical procedures for mycotoxins. All extracts of the control, nonmouldy feedstuffs increased larval mortality, this being most marked in the case of silage. Chemical and biological testing of diagnostic specimens indicated that the bioassay identified two of four chemically positive specimens and 59 of 135 chemically negative specimens. Unidentified larvicidal compounds present in normal feedstuffs gave a high percentage (56%) of false-positive bioassay results when compared to the results of chemical analyses for three mycotoxins. The use of brine shrimp larvae did not materially reduce the necessity of conducting chemical analyses for mycotoxins.

RÉSUMÉ

Cette expérience visait à vérifier la possibilité d'utiliser des larves de crevettes d'eau salée comme système de tamisage biologique simple des échantillons d'ingrédients alimentaires destinés aux animaux, qu'il faudrait ensuite soumettre à des analyses chimiques plus poussées et aptes à déceler les mycotoxines. Tous les extraits des échantillons témoins et exempts de moisissures, en particulier ceux d'ensilage, augmentèrent la mortalité des larves. L'épreuve biologique et l'analyse chimique des échantillons suspects révélèrent que l'épreuve biologique permit d'identifier seulement deux des quatre échantillons trouvés positifs, par l'analyse chimique, et 59 des 135 que cette dernière permit de déclarer négatifs. La comparaison des résultats de l'épreuve biologique avec ceux de l'analyse chimique, relativement à trois mycotoxines, révéla que 56% des résultats positifs erronés et obtenus par l'épreuve biologique, étaient attribuables à la présence de substances larvicides non identifiées, dans les ingrédients normaux. L'utilisation de larves de crevettes d'eau salée ne réduisit pas de façon satisfaisante la nécessité de recourir à des analyses chimiques, pour déceler les mycotoxines.

INTRODUCTION

Evaluation of the toxicity of fungal metabolites requires a biological monitoring system. Use of higher animals to determine mortality or pathological lesions is laborious and severely restricts the number of isolates that can be tested. Chick embryos (2,3), *Bacillus megaterium* (4) and *Bacillus stearothermophilus* (11) are among the biological organisms used instead of the higher animals. Harwig and Scott (7) described the use of brine shrimp (*Artemia salina*) larvae as a screening system for toxic fungi and investigated their sensitivity to 17 known mycotoxins. They showed that brine shrimp were sensitive to aflatoxins B and G, diacetoxyscirpenol, gliotoxin, ochratoxin A, sterigmatocystin, rubratoxin B, stemphone, T-2 toxin and kojic acid. Brine shrimp larvae have been used as a biological confirmation for detection of toxic 12,13-epoxy-$\triangle^9$ trichothecenes (6).

In our laboratory brine shrimp larvae have been tested as a possible simple biological screening system to identify those specimens of animal feedstuffs that should be examined further by chemical

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analytical procedures for the presence of mycotoxins. There are three naturally occurring mycotoxins either found or suspected in Western Canada, namely aflatoxin B_1 (AFB), ochratoxin A (OA) and T-2 toxin (9,10,12). The brine shrimp bioassay was applied to animal feedstuffs as a biological screening system for these three mycotoxins.

MATERIALS AND METHODS

The feedstuffs consisted of grains (wheat, barley, oats), hay, commercial feeds and silage. Two groups of feedstuffs were examined. The first group of 102 samples was not associated with animal disease, visible mouldiness or chemically detectable levels of AFB, OA or T-2 toxin. The second group comprised 139 diagnostic specimens (grains, hay, commercial feeds) submitted for mycotoxin analysis. Both groups of feedstuffs were examined chemically and biologically. Dose response curves for the lethality of AFB, OA and T-2 toxin for brine shrimp larvae were established using methanolic solutions of a series of known concentrations of authentic mycotoxins.

Chemical analyses for ABF (1), OA (8) and T-2 toxin (10) were conducted on all feedstuffs. Detection limits were determined by bioassay and chemical analysis for the three mycotoxins, using spiked nonmouldy feedstuffs. For the bioassay 50 g of the feedstuffs were extracted with chloroform using a soxhlet apparatus, reduced to dryness under nitrogen and redissolved in 250 µL chloroform immediately prior to test.

Suspensions of brine shrimp larvae were prepared as follows. Plastic Petri dishes (100 x 16 mm) were modified by the insertion of a perforated divider (6) and brine shrimp medium (BSM) (pH 6.5) was modified after (7). A modified Petri dish was filled with BSM to a level above the holes in the divider and 100 to 200 mg brine shrimp eggs added to one side of the divider. After masking the side of the Petri dish containing the eggs, the dish was incubated at room temperature for 30 hours, with a 100 W incandescent lamp positioned directly above the dish during incubation. Larvae swam from hatched eggs to the lighted half of the dish. Concentration was adjusted to 40-50 larvae per mL.

For the bioassay aliquots of 50 µL of either the methanolic mycotoxin solution or the redissolved feedstuff extract were placed in each of four beakers and the methanol or chloroform allowed to evaporate. To each beaker was added 4.5 mL BSM and 0.5 mL hatched larvae. The pH was adjusted to 6.5. Controls consisted of larval suspensions with the addition of 50 µL chloroform. Incubation was at room temperature for 16 hours. At the end of incubation the live and dead larvae were counted using a stereoscopic microscope (7). Control mortality varied between zero and three percent.

RESULTS

The relative sensitivity of brine shrimp larvae for the three mycotoxins is summarized in Table I. The larvae were most sensitive to T-2 toxin and least sensitive to OA, as indicated by the concentrations killing 30% (LC_{30}) and 50% (LC_{50}) of the larvae.

The effects of extracts of 102 nonmouldy feedstuffs on brine shrimp larvae are summarized in Table II. All extracts increased larval mortality, the increase being most marked in the case of silage. This group of feedstuffs contained no chemically detectable levels of AFB, OA or T-2 toxin. The results of chemical and biological testing of 139 diagnostic specimens submitted to the laboratory for mycotoxin analysis are summarized in Table III. The bioassay identified two of the four chemically positive specimens and 61 of the 139 as having larval mortalities of less than 30%. Two of these 61 were found to contain mycotoxins by chemical analysis.

DISCUSSION

The brine shrimp bioassay is very easy to perform and it was thought that its use might reduce the number of more costly and time-consuming chemical analyses. However, thre are several disadvantages. The hatchability of stored

TABLE I. Mean Lethal Concentrations and Detection Limits of Three Mycotoxins by Bioassay and Chemical Analysis

Toxin	Mean Lethal Concentration ± SD		Detection Limits	
	LC_{30}[a] µg/mL	LC_{50}[b] µg/mL	Biossay ppm[c]	Chemical ppm
Aflatoxin B_1	0.4 ± 0.2	1.5 ± 0.3	0.7	0.1
Ochratoxin A	2.0 ± 0.8	10 ± 2.3	2.0	0.1
T-2 toxin	0.1 ± 0.01	0.4 ± 0.1	0.2	0.2

[a]LC_{30} - that concentration of toxin killing 30% of brine shrimp
[b]LC_{50} -that concentration of toxin killing 50% of brine shrimp
[c] -at 30% mortality, data obtained from spiked, nonmouldy feedstuffs

TABLE II. Mortality of Brine Shrimp Larvae in the Presence of Extracts of 102 Nonmouldy Animal Feedstuffs

Feedstuff	Number Tested	Percent Mortality Mean ± SD	No. of Extracts with >30% Mortality
Grain (wheat, barley, oats)	52	22.4 ± 2.2	19
Hay	12	21.0 ± 6.4	4
Commercial feeds	28	33.9 ± 3.9	16
Silage	10	77.2 ± 11.9	8
Control (methanol)	102	1.2 ± 0.1	0

TABLE III. Results of Parallel Testing of 139 Diagnostic Specimens by both Chemical Analysis and Bioassay

Results		No. of Specimens		
Chemical Analysis	Brine Shrimp Bioassay	Hay	Feeds	Grains
ND	Negative[a]	8	18	33
ND	Positive[b]	17	22	37
Positive	Negative	1	0	1
Positive	Positive	0	0	2
Total no. of specimens		26	40	73

ND = Not detected
[a]Negative = mortality < 30%
[b]Positive = mortality > 30%

brine shrimp eggs appeared to decrease with time and increased quantities of eggs were required to obtain satisfactory hatches. It was necessary to separate viable and nonviable eggs by flotation using a concentrated salt solution. The basal survival rate with normal feedstuffs was markedly lower than control values (Table II). The low survival rate observed with silage occurred despite attempts to maintain pH 6.5 and may be due to the presence of long chain fatty acids, which are lethal to brine shrimp (5). The presence of unidentified larvicidal compounds excluded the use of mortality rates less than 30% as the identifier of samples that might contain one of the three mycotoxins. The unidentified larvicidal compounds might include inorganic or organic compounds, or mycotoxins other than AFB, OA or T-2 toxin. Their presence raised the detection limits of the bioassay as compared to chemical procedures (Table I).

Dilution of the extracts of control and spiked samples to a concentration at which larval mortality was insignificant further reduced the sensitivity of the bioassay to the three mycotoxins. Chemical clean-up procedures were not conducted in a previous study (7) and were not performed in the present study because of the unknown nature of the larvicidal compounds. The addition of fur-

ther steps in the bioassay procedure further reduced the time saving factor of the bioassay as compared to a chemical multimycotoxin procedure (13).

Although AFB, OA and T-2 toxin are lethal for brine shrimp larvae, unknown compounds in normal feedstuffs gave rise to a large number of false-positive bioassay results when compared to the results of chemical analyses (Table II). This is confirmed by similar findings with the diagnostic specimens (Table III). The brine shrimp bioassay, as described, is not suitable as a biological screening system for the presence of mycotoxins in animal feedstuffs.

REFERENCES

1. **ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS.** Official Methods of Analysis. 11th Ed. E. Horwitz, Editor. Washington D.C.: Association of Official Analytical Chemists. 1970.
2. **BROWN, R. F.** Some bioassay methods for mycotoxins. In Proc. 1st U.S.-Japan Conf. on Toxic Micro-organisms. pp. 12-18. M. Herzborg, Editor. 1970.
3. **CHOUDHURY, H. and C. W. CARLSON.** Lethal dose of ochratoxin for chick embryos. Poult. Sci. **52**: 1202-1203. 1973.
4. **CLEMENTS, N. L.** Mycotoxin inhibition of **Bacillus megaterium**. In Abst. Am. Ass. Cereal Chem. — Am. Oil Chem. Soc. Joint Meeting No. 54. Washington, D.C. March 31 - April 6, 1968.
5. **CURTIS, R. F., D. T. COXON and G. LEVELT.** Toxicity of fatty acids in assays for mycotoxins using brine shrimp (**Artemia salina**). Fd Cosmet. Tox. **12**: 233-235. 1974.
6. **EPPLEY, R. M.** Sensitivity of brine shrimp (**Artemia salina**) to trichothecenes. J. Ass. off. analyt. Chem. **57**: 618-620. 1974.
7. **HARWIG, J. and P. M. SCOTT.** Brine shrimp (**Artemia salina**) larvae as a screening system for fungal toxins. Appl. Microbiol. **21**: 1011-1016. 1971.
8. **NESHEIM, S., N. F. HARDIN, O. J. FRANCIS and W. S. LANGHAM.** Analysis of ochratoxins A and B and their esters in barley, using partition and thin layer chromatography. 1. Development of the method. J. Ass. off. analyt. Chem. **56**: 817-821. 1973.
9. **PRIOR, M. G.** Mycotoxin determinations on animal feedstuffs and tissues in Western Canada. Can. J. comp. Med. **40**: 75-79. 1976.
10. **PULS, R. and J. A. GREENWAY.** Fusariotoxicosis from barley in British Columbia II. Analysis and toxicity of suspected barley. Can. J. comp. Med. **40**: 16-19. 1976.
11. **REISS, J.** Mycotoxin bioassay using **Bacillus stearothermophilus**. J. Ass. off. analyt. Chem. **58**: 624-625. 1975.
12. **SCOTT, P. M., W. VAN WALBEEK, J. HARWIG and D. I. FENNELL.** Occurrence of a mycotoxin, ochratoxin A, in wheat and isolation of ochratoxin A and citrinin producing strains of **Penicillium viridicatum**. Can. J. Plant Sci. **50**: 583-585. 1970.
13. **STOLOFF, L., S. NESHEIM, L. YIN, J. V. RODERICKS, M. STACK and A. D. CAMPBELL.** A multimycotoxin detection method for aflatoxins, ochratoxins, zearalenone, sterigmatocystin and patulin. J. Ass. off. analyt. Chem. **54**: 91-97. 1971.