

LITERATURE CITED

- Asao, T.; Büchi, G.; Abdel-Kader, M. M.; Chang, S. B.; Wick, E. L.; Wogan, G. N. *J. Am. Chem. Soc.* **1965**, *87*, 882.
- Bergot, B. J.; Stanley, W. L.; Masri, M. S. *J. Agric. Food Chem.* **1977**, *25*, 965.
- Brekke, O. L.; Peplinski, A. J.; Lancaster, E. B. *Trans. ASAE* **1977a**, *20*, 1160.
- Brekke, O. L.; Peplinski, A. J.; Nofsinger, G. W.; Conway, H. F.; Stringfellow, A. C.; Montgomery, R. R.; Silman, R. W.; Sohns, V. E.; Bagley, E. B. *Trans. ASAE* **1979**, *22*, 425.
- Brekke, O. L.; Sinhuber, R. O.; Peplinski, A. J.; Wales, J. H.; Putnam, G. B.; Lee, D. J.; Ciegler, A. *Appl. Environ. Microbiol.* **1977b**, *34*, 34.
- Burton, J. S.; Stevens, R. *J. Chem. Soc.* **1964**, 952.
- Cucullu, A. F.; Lee, L. S.; Pons, W. A., Jr.; Stanley, J. B. *J. Agric. Food Chem.* **1976**, *24*, 408.
- Koetz, R.; Neukom, H. *Carbohydr. Res.* **1975**, *42*, 365.
- Lee, L. S.; Conkerton, E. J.; Ory, R. L.; Bennett, J. W. *J. Agric. Food Chem.* **1979**, *27*, 598.
- Lee, L. S.; Cucullu, A. F. *J. Agric. Food Chem.* **1978**, *26*, 881.
- Lee, L. S.; Stanley, J. B.; Cucullu, A. F.; Pons, W. A., Jr.; Goldblatt, L. A. *J. Assoc. Off. Anal. Chem.* **1974**, *57*, 626.
- Norred, W. P. *Toxicol. Appl. Pharmacol.* **1979**, *51*, 411.
- Southern, L. L.; Clawson, A. J. *J. Anim. Sci.* **1980**, *50*, 459.
- Vesonder, R. F.; Beckwith, A. C.; Ciegler, A.; Dimier, R. J. *J. Agric. Food Chem.* **1975**, *23*, 242.
- Wogan, G. N.; Edwards, G. S.; Newberne, P. M. *Cancer Res.* **1971**, *31*, 1936.
- Yamada, Y.; Hagiwara, K.; Iguchi, K.; Takahashi, Y. *Chem. Lett.* **1978**, 319.

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Procedure for Minimizing Losses in Sample Processing and Assay of Rubratoxin B from Mixed Feed

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A study was conducted on some factors that affect recovery of rubratoxin B from mixed feed. Results are presented that show that storage temperature, feed moisture content, and atmosphere have a marked effect on the deterioration rate of rubratoxin B as measured by its recovery from mixed feed. The method of sample processing and extraction that evolved from this study included brief storage at freezer temperature (-20°C) and a 5-min Waring blender extraction with 5% acetic acid in ethyl acetate at 6°C . The high-pressure liquid chromatography assay system included a regular-phase column and the ethyl acetate-chloroform-acetic acid (80:20:1 v/v/v) solvent system. Retention time of rubratoxin B was 7.9 min, k' was 1.53, and percent recovery was 79% for the first extraction and an additional 5-10% for the second extraction. This system provided excellent resolution and detected as little as 5 ng of rubratoxin B.

Rubratoxin B is produced by *Penicillium rubrum* Stoll and *Penicillium purpurogenum* Stoll when grown on various agricultural commodities and laboratory media (Hayes et al., 1970; Emeh and Marth, 1976). *P. rubrum* Stoll was initially described as a toxigenic organism following isolation from moldy corn (Burnside et al., 1957). *P. rubrum* and *Aspergillus flavus* Link were the only fungi of 13 isolated by Burnside et al. (1957) from that moldy corn that caused illness and death when fed to swine, horses, and mice. Oral administration of artificially contaminated feedstuffs resulted in congestion and hemorrhages in several organs, especially the liver and kidney (Forgacs et al., 1958).

Rubratoxin B was extracted and partially purified by Wilson and Wilson (1962) and purified and characterized by Townsend et al. (1966), and the chemical structure was determined by Moss et al. (1967, 1968, 1969) and Büchi et al. (1970). Rubratoxin B decomposed on melting and pyrolytic decomposition resulted in the loss of a molecule of carbon dioxide. When a UV spectrum was done in a hydroxylic solvent, the strong absorption maximum at 250 nm produced by the disubstituted maleic anhydride function decreased in intensity. This decrease demonstrated the ease with which the anhydride rings equili-

brated with the open form (acid) (Moss, 1971). Moss et al. (1971) have reported that rubratoxin B acts as an acid in aqueous solutions.

Methods for extraction and purification of rubratoxin B have been reported for cultures grown on liquid media, corn, rice, and soybeans (Wilson and Wilson, 1962; Hayes and Wilson, 1968; Natori et al., 1970; Hayes and McCain, 1975; Emeh and Marth, 1977). Recently, Whidden et al. (1980) published a method for detection of rubratoxin B and seven other mycotoxins from corn. Although 67% of the rubratoxin B was extracted, only 31% was recovered at the end of the assay. They suggested that the difference was due to the length and complexity of the multimycotoxin analysis and the instability of rubratoxin B.

This paper defines some factors responsible for the deterioration of rubratoxin B and describes an improved method of sample handling, extraction, and assay of rubratoxin B from mixed feed.

EXPERIMENTAL SECTION

Materials. Crude rubratoxin B was extracted from the liquid of surface cultures of *P. rubrum* Stoll and purified by the method of Hayes and Wilson (1968). This preparation was further purified by chromatography on organic Sephadex LH-20 (100×2.4 cm) in acetonitrile. Purity of the rubratoxin B standard was determined by high-pressure liquid chromatography (HPLC) in three nonaqueous solvent systems: (a) ethyl acetate-chloroform-butanol-acetic acid, 40:55:4:1 v/v/v/v; (b) ethyl acetate-chloro-

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form-acetic acid, 55:45:1 v/v/v; (c) ethyl acetate-chloroform-acetic acid, 80:20:1 v/v/v. The standard was stored in stoppered vials or flasks in the cold room (6 °C) protected from air, light, and humidity. Ten milligrams of purified rubratoxin B (2 mg/mL in ethyl acetate) was added to individual feed samples (artificial contamination) and mixed thoroughly by shaking.

Mixed feed was prepared by United Suppliers of Eldora, IA. The ingredients included 45% wheat middlings, 19.9% ground oat hulls, 10% finely ground corn, 7.2% soybean meal, 5% dehydrated alfalfa, 3% hominy feed, 2.5% molasses, 2.3% dried whey, 2.2% meat meal, 0.5% animal fat, 2.2% minerals, and 0.1% vitamins. Solvents used for HPLC were from Burdick and Jackson, Muskegon, MI. Mycotoxins and sources were as follows: aflatoxins B₁, B₂, G₁, and G₂ (Calbiochem, LaJolla, CA), patulin and penicillic acid (Alex Ciegler, Northern Regional Research Center, Peoria, IL), zearalenone (C. J. Mirocha, University of Minnesota, St. Paul, MN), and sterigmatocystin (Sigma Chemical Co., St. Louis, MO).

Methods. Pelleted mixed feed was ground in a Wiley mill to reduce the particle size prior to use in the various experiments, and 50-g samples were transferred to 500-mL Erlenmeyer flasks. Mixed feed samples were stored at 23 °C (room temperature), 6 °C (cold room), or -20 °C (freezer) prior to the addition of rubratoxin B. The mixed feed samples were extracted with 300 mL of 5% acetic acid in ethyl acetate at 6 °C for 5 min with a Waring blender at high speed (stainless steel jar). After extraction, samples were gravity filtered through Whatman No. 1 filter paper, the volume was adjusted to 250 mL, and the samples were transferred to glass-stoppered sample bottles, and stored at 6 °C until quantified by HPLC. Extraction of the individual ingredients of mixed feed was done in the same way as the mixed feed samples. In other experiments, mixed feed samples were heated in an electric oven at 50 or 85 °C for 24 h prior to addition and two others for 2-24 h after addition of rubratoxin B. Water was added to one sample to increase the moisture content prior to addition of rubratoxin B. Rubratoxin B was added to three other samples at room temperature and placed in a vacuum chamber or stored under nitrogen.

High-Pressure Liquid Chromatography Assay. The HPLC assays were conducted with a Model ALC 502/401 liquid chromatograph equipped with a Model 440 UV detector, M6000 pump, U6K septumless injector, and normal-phase μ Porasil column from Waters Associates, Inc., Milford, MA. The solvent mixture was ethyl acetate-chloroform-acetic acid (80:20:1 v/v/v; flow rate of 1 mL/min). Chromatograms were recorded on a Linear Instruments Model 281 recorder (Costa Mesa, CA).

RESULTS AND DISCUSSION

In preliminary experiments, recovery of rubratoxin B from mixed feed was found to be much less than that reported by Hayes and McCain (1975) for cracked corn (90% recovery). Townsend et al. (1966) and Hayes and McCain (1975) used ethyl acetate as the solvent for extraction of rubratoxin B from an aqueous culture medium and cracked corn, respectively. We also found that ethyl acetate was the best solvent for extraction of rubratoxin B based on amount recovered from mixed feed. In related experiments, the addition of 5% acetic acid to ethyl acetate for extraction improved the recovery of rubratoxin B an average of 16%. This more efficient extraction solvent was used in all subsequent experiments.

We developed a normal-phase HPLC solvent system for use in these studies (ethyl acetate-chloroform-acetic acid, 80:20:1 v/v/v). An example of a chromatogram of a mixed

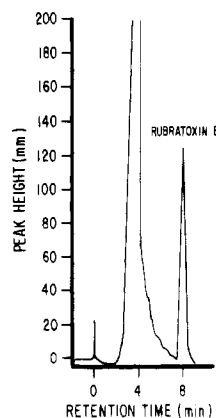


Figure 1. HPLC chromatogram of a mixed feed extract supplemented with rubratoxin B. The solvent system was ethyl acetate-chloroform-acetic acid (80:20:1 v/v/v) with a normal-phase column and a flow rate of 1.0 mL/min. The UV detector had a 254-nm filter and a sensitivity setting of 0.005 AUFS. The k' for rubratoxin B was 1.53 and its retention time was 7.9 min.

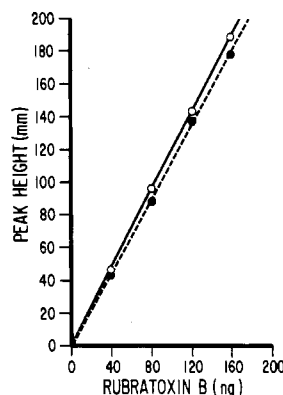


Figure 2. The standard curve was constructed from peak heights obtained by HPLC of a rubratoxin B standard solution (10.0 mg/250 mL; solid line) and from identical samples that had been Waring blended at high speed for 5 min in the cold room (6 °C) and filtered and the volume had been adjusted to 250 mL (dashed line).

feed extract artificially contaminated with rubratoxin B (Figure 1) illustrated the fact that no UV-absorbing component was eluted at a point in the chromatogram that interfered with the resolution of rubratoxin B. The k' for rubratoxin B in this system was 1.53 and its retention time was 7.9 min at a flow rate of 1 mL/min.

A standard curve was constructed from peak height values obtained by HPLC assay of 10.0-mg samples of rubratoxin B diluted to 250 mL with 5% acetic acid in ethyl acetate (solid line, Figure 2). A second curve was prepared from identical rubratoxin B samples that had been submitted to a 5-min treatment in the Waring blender at high speed and filtered prior to HPLC assay (dashed line, Figure 2). This treatment was essentially the same as the feed extraction procedure described under Methods. Mixed feed samples stored at -20 °C were artificially contaminated with rubratoxin B and extracted within 10 min; the extracts gave an average peak height of 139 mm/4- μ L injection (range of six samples = 126-150 mm). This calculated to be 7.9 mg total or 79% recovery of rubratoxin B from mixed feed. Recovery was increased 5-10% with a second extraction.

Hayes and McCain (1975) have reported that rubratoxin B was stable to heat up to 65 °C for 2 h but was rapidly destroyed at higher temperatures. In order to determine whether this was true with our mixed feed samples, we

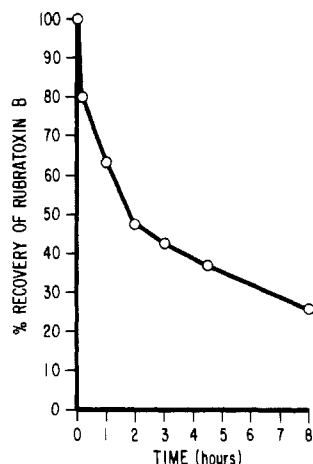


Figure 3. Rate of deterioration of rubratoxin B in mixed feed at room temperature (23 °C). Fifty-gram samples were supplemented with 5.0 mL (10.0 mg) of a rubratoxin B standard in 5% acetic acid in ethyl acetate. Extraction was with 300 mL of 5% acetic acid in ethyl acetate in a Waring blender at high speed for 5 min at 6 °C. Each extract was analyzed in the HPLC assay a minimum of 3 times and the average determined.

Table I. Deterioration of Rubratoxin B during Storage at Three Different Temperatures^{a, b}

storage time	rubratoxin recovered, mg		
	23 °C	6 °C	-20 °C
10 min	6.52 (2) ^c (6.37-6.67)	7.03 (2) ^c (6.81-7.25)	8.04 (2) ^c (7.90-8.18)
32 h	1.11 (2) (1.06-1.16)	3.65 (2) (3.40-3.90)	7.22 (2) (6.78-7.65)
5 days	0.58 (2) (0.55-0.61)	3.12 (3) (2.87-3.30)	6.78 (5) (6.59-7.10)
3 weeks	0.28 (2) (0.26-0.29)	2.54 (3) (2.39-2.68)	6.01 (3) (5.07-6.74)

^a Extraction was with 300 mL of 5% acetic acid in ethyl acetate in a Waring blender at high speed for 5 min at 6 °C. ^b Fifty-gram samples were supplemented with 5.0 mL (10.0 mg) of a rubratoxin B standard in 5% acetic acid in ethyl acetate. Each extract was analyzed in the HPLC assay a minimum of 3 times and the average was determined. UV detector had a 254-nm filter and a sensitivity setting of 0.005 AUFS. ^c Number of samples.

added 10 mg of rubratoxin B to a 50-g sample, placed it in an oven at 50 °C for 24 h, allowed it to cool at room temperature, and extracted it. The extract did not contain enough rubratoxin B to be detectable on the chromatogram compared to the control (nonheated). This result provided another demonstration of the lability of rubratoxin B to heat.

Experiments were performed to test the effect of time between artificial contamination and extraction of the feed and the effect of temperature on recovery of rubratoxin B. Feed samples were placed in open 500-mL Erlenmeyer flasks at room temperature (23 °C) and exposed to laboratory conditions of air, temperature, light, and humidity for various periods of time (10 min-8 h). The results presented in Figure 3 clearly showed the rapid rate of deterioration of rubratoxin B in mixed feed at room temperature (23 °C). For investigation of the effect of time and temperature on the assay system, feed samples were stored in stoppered flasks at 23, 6, and -20 °C for periods of time from 10 min to 3 weeks. The results presented in Table I demonstrated the importance of storage temperature on the rate of deterioration of rubratoxin B in mixed feed. Although the recovery of rubratoxin B from mixed feed stored at 6 °C was much better than that stored at 23 °C, it was not nearly as good as the recovery from mixed

Table II. Effect of the Atmosphere on Recovery of Rubratoxin B from Mixed Feed^{a, b}

storage atmosphere	time	rubratoxin B recovered, mg
standard laboratory ^c	10 min	6.52
standard laboratory	32 h	1.11
nitrogen	24 h	3.55
vacuum	3 days	5.29
vacuum	5 days	4.57
standard laboratory	5 days	0.58

^a Extraction was with 300 mL of 5% acetic acid in ethyl acetate in a Waring blender at high speed for 5 min at 6 °C. ^b Fifty-gram samples were supplemented with 5.0 mL (10.0 mg) of a rubratoxin B standard in 5% acetic acid in ethyl acetate. Each extract was analyzed in the HPLC assay a minimum of 3 times and the average was determined. UV detector had a 254-nm filter and a sensitivity setting of 0.005 AUFS. ^c Standard laboratory temperature was 23 °C and the humidity varied between 50 and 60%.

Table III. Effect of Addition and Removal of Water on Recovery of Rubratoxin B from Mixed Feed^{a, b}

pretreatment	time and temperature between addition of rubratoxin B and its extraction	rubratoxin B recovered, mg
23 °C	10 min (23 °C)	6.52
23 °C	2 h (23 °C)	4.78
23 °C + water ^c	2 h (23 °C)	2.03
50 °C for 24 h	2 h (50 °C)	2.90
50 °C for 24 h	24 h (23 °C)	6.67
85 °C for 24 h	24 h (23 °C)	7.97
23 °C	24 h (50 °C)	0

^a Extraction was with 300 mL of 5% acetic acid in ethyl acetate in a Waring blender at high speed for 5 min at 6 °C. ^b Fifty-gram samples were supplemented with 5.0 mL (10.0 mg) of a rubratoxin standard in 5% acetic acid in ethyl acetate. Each extract was analyzed in the HPLC assay a minimum of 3 times and the average was determined. UV detector had a 254-nm filter and a sensitivity setting of 0.005 AUFS. ^c Five milliliters of water was added to a 50-g sample of mixed feed and thoroughly mixed before addition of the rubratoxin B.

feed stored at freezer temperature (-20 °C), especially for the longer storage times. Despite the obvious advantage of storing rubratoxin B contaminated feed samples at -20 °C, losses still occurred over a period of time.

When mixed feed samples supplemented with rubratoxin B were stored under vacuum or nitrogen or dried for 24 h at 50 or 85 °C and cooled before addition of rubratoxin B, recovery of rubratoxin B was greatly increased (Tables II and III). When water was added to mixed feed (5.0 mL/50 g) before rubratoxin B was added, recovery of rubratoxin B was reduced relative to that of the control (Table III). These results demonstrated the importance and effect of atmosphere and feed moisture content on the recovery of rubratoxin B from mixed feed. They do not, however, rule out the possibility that some of the deterioration of rubratoxin B may be due to oxidation resulting from exposure to air.

In other experiments, 50-g samples of individual mixed feed ingredients were artificially contaminated with rubratoxin B, exposed to laboratory atmosphere for 2 h, and extracted. The lowest recovery was obtained from wheat middlings, which was the major constituent of mixed feed (Table IV). Recovery of rubratoxin B from animal fat, whey, and meat meal was equal to or better than that from corn or the mixed feed control (Table IV). To determine if the lower recovery from wheat middlings was due to a relatively higher moisture content, we heated a 50-g sample

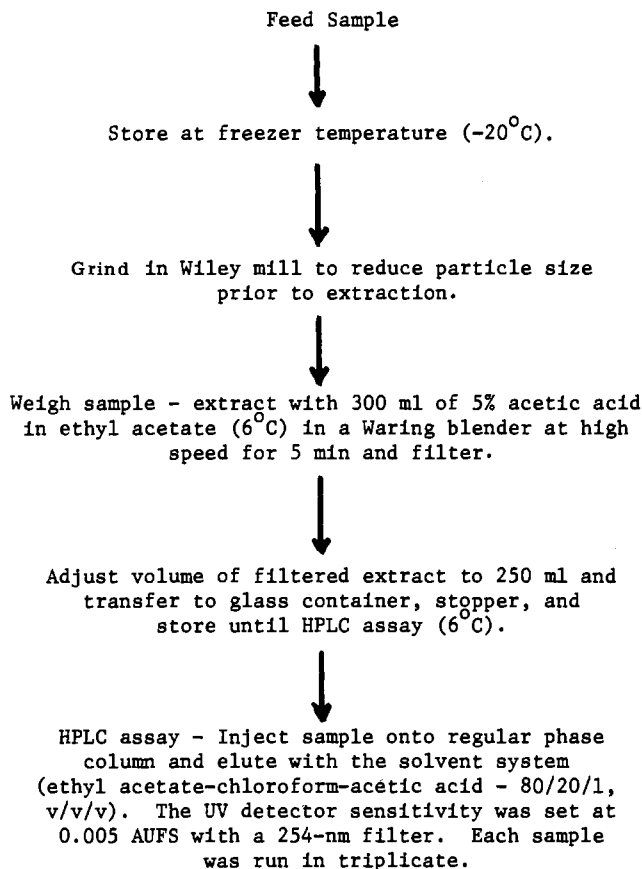


Figure 4. Flow diagram which presents our procedure for sample processing, extraction, and assay of rubratoxin B from mixed feed.

of wheat middlings for 24 h at 50 °C, cooled, it, added rubratoxin B, and extracted the sample. The amount of rubratoxin B found in this extract was comparable to that obtained when samples of mixed feed were treated in the same way (Table III). This result supported the conclusion that the feed moisture content was a very important factor in the deterioration of rubratoxin B in mixed feed.

Papers have been published that report the instability of rubratoxin B to atmosphere (Hayes and Wilson, 1968; Moss and Hill, 1970), temperature and relative humidity (Moss, 1971), and water in a HPLC solvent system (Engstrom et al., 1977). Our results presented in this report demonstrated that deterioration of rubratoxin B in mixed feed was due in part to heat (Table I), atmosphere (moisture) (Table II), and feed moisture content (Table III). The variation found in recovery of rubratoxin B from individual mixed feed ingredients (Table IV) also appeared to be due to variation in their moisture content.

Our demonstration of the lability of rubratoxin B to water in mixed feed and in the atmosphere and the effect of temperature on the rate of its deterioration provides an explanation as to why natural contamination of various agricultural commodities by rubratoxin B has not been reported very often. This would be particularly true if samples were stored at room temperature for a short time or in a cold room for longer periods before extraction and assay. Rubratoxin B was much more stable in the extraction solvent than it was in the feed even when stored at -20 °C. Thus, it is very important to process the feed sample through the solvent extraction step immediately.

In conclusion, our method for sample processing and assay of rubratoxin B from mixed feed reported here provides excellent resolution, recovery (80–90%), detect-

Table IV. Recovery of Rubratoxin B from Single Ingredients of Mixed Feed^a

feed ingredient ^b	time between addition of rubratoxin B and extraction	rubratoxin B recovered, mg
mixed feed	10 min ^c	7.81
animal fat	2 h	6.85
whey	2 h	6.24
meat meal	2 h	5.56
corn (fine)	2 h	5.28
soybean meal	2 h	4.44
dehydrated alfalfa	2 h	4.16
wheat middlings	2 h	2.98
mixed feed	2 h	1.97

^a Extraction was with 300 mL of 5% acetic acid in ethyl acetate in a Waring blender at high speed for 5 min at 6 °C.

^b Fifty-gram samples were supplemented with 5.0 mL (10.0 mg) of a rubratoxin standard in 5% acetic acid in ethyl acetate. Each extract was analyzed in the HPLC assay a minimum of 3 times and the average was determined. UV detector had a 254-nm filter and a sensitivity setting of 0.005 AUFS. ^c Samples were at 23 °C prior to and during storage.

ability (5 ng), and sensitivity (20 ppm) and requires only a relatively small amount of time (2 h). The individual steps involved in our method are presented as a flow diagram in Figure 4.

LITERATURE CITED

- Büchi, G.; Snader, K. M.; White, J. D. *J. Am. Chem. Soc.* **1970**, *92*, 6638.
- Burnside, J. E.; Sippel, W. L.; Forgacs, J.; Carll, W. T.; Atwood, M. B.; Doll, E. R. *Am. J. Vet. Res.* **1957**, *18*, 817.
- Emeh, C. O.; Marth, E. H. *J. Milk Food Technol.* **1976**, *39*, 95.
- Emeh, C. O.; Marth, E. H. *Z. Lebensm.-Unters. -Forsch.* **1977**, *163*, 115.
- Engstrom, G. W.; Richard, J. L.; Cysewski, S. J. *J. Agric. Food Chem.* **1977**, *25*, 833.
- Forgacs, J.; Koch, H.; Carll, W. T.; White-Stevens, R. M. *Am. J. Vet. Res.* **1958**, *19*, 744.
- Hayes, A. W.; McCain, H. W. *Food Cosmet. Toxicol.* **1975**, *13*, 221.
- Hayes, A. W.; Wilson, B. J. *Appl. Microbiol.* **1968**, *16*, 1163.
- Hayes, A. W.; Wyatt, E. P.; King, P. A. *Appl. Microbiol.* **1970**, *20*, 469.
- Moss, M. O. "Microbial Toxins"; Ciegler, A.; Kadis, S.; Ajl, S. L., Eds.; Academic Press: New York, 1971; Vol. VI, pp 381–407.
- Moss, M. O.; Hill, I. W. *Mycopath. Mycol. Appl.* **1970**, *40*, 81.
- Moss, M. O.; Robinson, F. V.; Wood, A. B. *Chem. Ind. (London)* **1968**, 587.
- Moss, M. O.; Robinson, F. V.; Wood, A. B. *J. Chem. Soc. C* **1971**, 619.
- Moss, M. O.; Robinson, F. V.; Wood, A. B.; Morrison, A. *Chem. Ind. (London)* **1967**, 755.
- Moss, M. O.; Wood, A. B.; Robinson, F. V. *Tetrahedron Lett.* **1969**, *5*, 367.
- Natori, S.; Sakaki, S.; Kurata, H.; Udagawa, S.; Ichinoe, M.; Saito, M.; Umeda, M.; Ohtsubo, K. *Appl. Microbiol.* **1970**, *19*, 613.
- Townsend, R. J.; Moss, M. O.; Peck, H. M. *J. Pharm. Pharmacol.* **1966**, *18*, 471.
- Whidden, M. P.; Davis, N. D.; Diener, U. L. *J. Agric. Food Chem.* **1980**, *28*, 784.
- Wilson, A. W.; Wilson, B. J. *J. Bacteriol.* **1962**, *84*, 283.

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