in bacterial protein production resulted in 100% inhibition of cellulose digestion. Bacterial protein production is a measure of growth and the amount of reduction in growth required to completely inhibit cellulose digestion from rumen inoculum is much less than the relationship between perloline concentrations and growth of cellulolytic bacteria observed in pure cultures (Bush et al., 1972). Perloline concentrations that inhibited growth in pure cultures of cellulolytic bacteria from 9 to 24% inhibited cellulose digestion 30%. Perloline levels that inhibited cellulose digestion 100% inhibited growth of the cellulolytic bacteria from 44 to 80%. The association between in vitro rumen cellulose digestion and inhibition of bacterial protein production supports our earlier observations with perloline and growth of pure cultures of cellulolytic bacteria. The differences in response to perloline treatments cannot be explained with present data, but in vitro rumen cellulose digestion occurs as a result of the interaction of many organisms and not just cellulolytic bacteria. The production of VFA was probably a consequence of bacterial protein production and inhibition of cellulose digestion. The increased VFA content with 0.04 and 0.08 mM perloline corresponded with an enhanced bacterial protein production and cellulose digestion (Table II and Figure 3A). Bacterial protein production and VFA content were increased at 0.1 mM phenanthridine, but cellulose digestion was not increased. Composition of the VFA in response to perloline and related substances appears to be variable. In this study no change occurred with the addition of many of the materials, whereas in others the percentage of propionic acid decreased. Acetic acid percentage decreased and propionic acid percentage increased with addition of 4-(2′-amino)phenyl-2-pyridone to the fermentation tubes. Previous in vitro experiments reported no change in acetic acid percentage and a decrease in propionic acid with increased perloline levels (Bush et al., 1972). However, in vivo experiments with lambs showed that propionic acid increased with addition of perloline to the diet and that acetic acid decreased or did not change with perloline added to the diet (Boling et al., 1975).

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Analysis of T-2 Toxin (and HT-2 Toxin) by Mass Fragmentography

Stephen R. Pareles,[1] George J. Collins, and Joseph D. Rosen*

A method for the rapid screening of T-2 toxin in milk is presented. At the 1.25-ppm level, recovery is about 70%. The lower limit of detectability is estimated at 300 ppb. Although more research involving the use of deuterated T-2 toxin as an internal standard is needed before the method can be used as a routine quantitative procedure, an acceptable alternative to the rabbit-skin assay confirmation procedure is provided for use with both milk and corn. Furthermore, the proposed method can be used for the simultaneous semiquantitation and confirmation of both T-2 and HT-2 toxins.

Several outbreaks of toxicoses associated with moldy corn and other grains have been observed in recent years (Smalley et al., 1970; Hsu et al., 1972). One of the mycotoxins implicated in these episodes is the highly toxic 3-hydroxy-4,15-diacetoxy-8-(3-methylbutyrloxy)-12,13-epoxy-Δ^9-trichothecene (T-2 toxin). These outbreaks generally occur after cold, late harvest seasons and after the corn is stored during the following winter. It has been shown that 8 °C is the ideal temperature for T-2 toxin formation (Bamburg et al., 1968). Because of the possibility that dairy animals ingesting this corn (or other infected grains) may produce milk containing T-2 toxin, and there are no methods presently available for the analysis of this mycotoxin in milk, an attempt was made to devise a method for the analysis of T-2 toxin in milk.

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EXPERIMENTAL SECTION

Instrumentation. A DuPont 21-490 mass spectrometer equipped with a digital mass marker and interfaced to a Varian Model 2740 gas chromatograph (equipped with flame ionization detector) via a glass, single-stage jet separator was used. A mass fragmentography accessory of our own design (Pareles and Rosen, 1974) was set to detect only those gas chromatograph effluents whose mass spectra exhibited ions at m/e 436 or 350 for determination

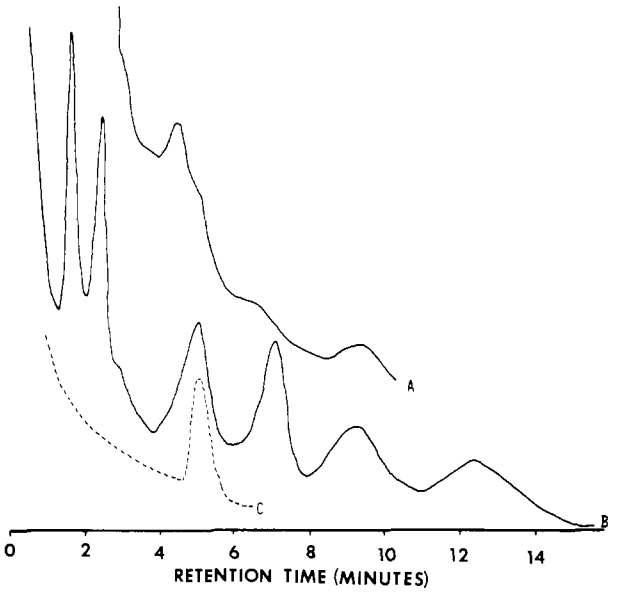

Figure 1. Gas chromatograms of trimethylsilylated (A) corn blank; (B) milk blank; (C) T-2 toxin; chart speed, 0.5 in./min.

of T-2 toxin. For detection of both HT-2 and T-2 toxins, the accessory was set to simultaneously monitor the respective M – 102 fragments of the silylated toxins, *m/e* 466 and 436.

GC–MS Conditions. Separations were performed with a 3 ft × $^1/_8$ in. i.d. glass column containing 1% OV-17 on High Performance Chromosorb W, 80–100 mesh. Column, injector, and detector temperatures were 230, 230, and 255 °C, respectively. Carrier gas flow (99.9999% helium) was 30 cm^3/min with the vent to the mass spectrometer closed. The flow rate increased to 39–40 cm^3 after the vent to the mass spectrometer was opened. Mass spectrometer source, jet separator, and glass transfer (between gas chromatograph and mass spectrometer) line temperatures were 270, 250, and 260 °C, respectively. Under these conditions, T-2 toxin had a retention time of 5.1 min with the vent closed and 4.5 min when the vent was opened 3 min after injection. For HT-2 toxin, the values were 3.8 and 3.5 min, respectively.

Flame ionization detection response was recorded on a 1 mV potentiometric recorder utilizing a chart speed of 0.5 in./min (Figure 1). The mass fragmentogram response was recorded on a 50-mV recorder operated at a chart speed of 0.25 in./min (Figures 2 and 3).

Preparation of Calibration Standards and Spiked Samples. A stock solution (1 mg/ml) of T-2 toxin in ethyl acetate was serially diluted to give solutions with concentrations of 0.5, 0.25, and 0.125 mg/ml. One milliliter of each of these solutions was placed in a vial, 0.5 ml of bis(trimethylsilyl)acetamide (BSA) was added, and the vials were tightly sealed. After heating on a steam bath for 1 h, four calibration standards with T-2 toxin equivalent concentrations of 0.667, 0.338, 0.169, and 0.085 mg/ml were obtained. No detectable changes in concentration over a 1-month period were observed if the solutions were stored in a freezer. For spiking purposes, 1 ml each of the unsilylated solutions was added to 100 g of milk in duplicate resulting in two sets of milk samples having T-2 toxin concentrations of 10, 5, 2.5, and 1.25 ppm.

Extraction from Milk. The method used was essentially that of Corley et al. (1974) for analysis of an insecticide in milk. Granular sodium sulfate (250 g) was added to 100 g of milk to form a friable mass. This was followed by extracting three times with 200 ml of ethyl

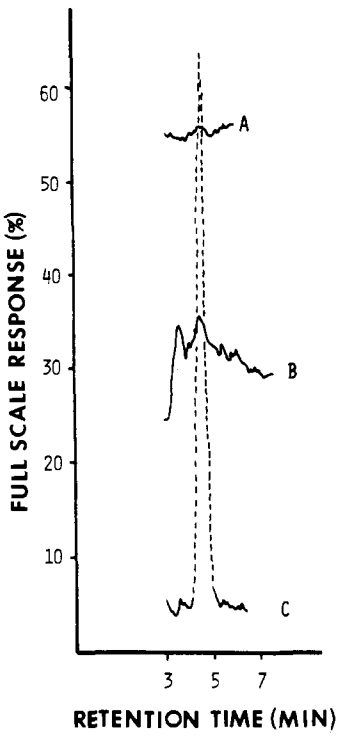

Figure 2. Mass fragmentograms (*m/e* 436) of trimethylsilylated (A) milk blank; (B) corn blank; (C) 2.5 ppm of T-2 toxin equivalent (vent to mass spectrometer opened 3 min after injection); chart speed, 0.25 in./min.

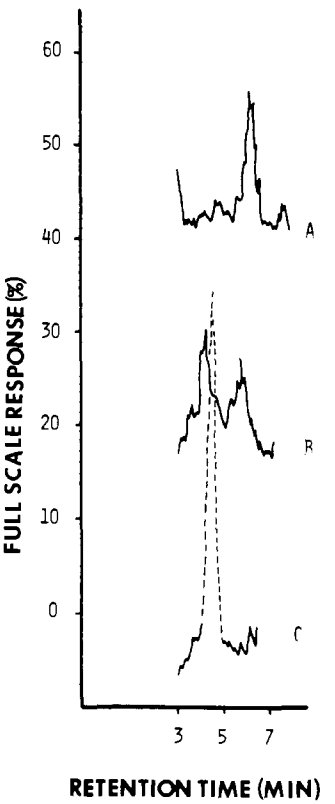

Figure 3. Mass fragmentograms (*m/e* 350) of trimethylsilylated (A) milk blank; (B) corn blank; (C) 2.5 ppm of T-2 toxin equivalent (vent to mass spectrometer opened 3 min after injection); chart speed, 0.25 in./min.

acetate. The combined ethyl acetate extracts were evaporated to dryness on a rotary evaporator and the oily residue was dissolved in 50 ml of acetonitrile. The fat was

then extracted with three 50-ml portions of hexane and the acetonitrile layer was evaporated to dryness. The residue was dissolved in ca. 1 ml of ethyl acetate and transferred to a 4-dram vial with several rinsings. After evaporation of the solvent under a stream of nitrogen, 1 ml of ethyl acetate was added and the solution was derivatized in a manner identical with the calibration standards. A 3-μl injection was made in all analyses.

For determination of the minimum detectable amount, 100 g of milk was processed as above, except that just before derivatization 1 ml of ethyl acetate containing 31.3 μg of T-2 toxin was added to the milk residue.

Preparation of Spiked Corn. A 100-g sample of powdered field corn was extracted with ethyl acetate for 2.5 h in a Soxhlet extractor. After evaporation of the solvent, the corn residue was treated exactly as the milk residue. A "spiked sample" corresponding to 2.5 ppm of T-2 toxin in corn was created by adding 1 ml of ethyl acetate containing 0.25 mg of T-2 toxin to the corn residue before derivatization.

RESULTS AND DISCUSSION

Ikediobi et al. (1971) have demonstrated the feasibility of determining trichothecenes (as their trimethylsilyl esters) by gas–liquid chromatography and Hsu et al. (1972) have shown that the mass spectrum of the trimethylsilyl ester of T-2 toxin exhibits peaks at m/e 436 and 350, among others. Because mass fragmentography (Hammer et al., 1968; Gordon and Frigerio, 1972) is much more sensitive and specific than flame ionization detection, the technique offers an approach to solution of difficult analytical problems. Figure 1 compares the gas chromatograms of blank milk and corn with that of T-2 toxin and shows that at isothermal conditions, there is interference with T-2 toxin determination for both commodities. Figure 2 compares the mass fragmentograms (monitored at m/e 436) obtained from the same solutions with the mass fragmentogram of a solution containing an amount of T-2 toxin which would be present (assuming 100% recovery) after extraction of a milk or corn sample contaminated at the 2.5-ppm level. Figure 3 shows mass fragmentograms of the same solutions discussed in Figure 2, but monitored at m/e 350. Comparison of the gas chromatograms (Figure 1) with the mass fragmatograms monitored at m/e 436 indicates that interferences from milk and corn are essentially eliminated. It is also quite obvious that T-2 toxin in both milk and corn could easily be determined at below the 1-ppm level with a method that requires essentially very little "cleanup" time. The present method for T-2 toxin in corn, based on thin-layer chromatography, is sensitive to only 1 ppm (Eppley et al., 1974), requires extensive cleanup, and is not confirmative. The rabbit-skin assay (Chung et al., 1974), while more sensitive, cannot distinguish between T-2 toxin and other skin irritants (Eppley et al., 1974). There are, at present, no published methods for T-2 toxin in milk to compare with. Figure 3 indicates that further confirmation of identity could be obtained by monitoring milk samples at m/e 350 but not corn.

A serious reproducibility problem was encountered during the recovery studies. Although succeeding injections of calibration standards gave essentially reproducible peak heights (points A through E, Figure 4), injection of a spiked sample caused a much enhanced response from the succeeding injection of the same calibration standard (compare points E and G, Figure 4). Further complicating the situation was the fact that succeeding injections of the calibration standard 7 min apart resulted in continued decreases in response from the high response level (points G through J, Figure 4). A wait of 14 min before injecting the next calibration standard, however, lowered the response to the previously established level (compare point K with points A–E, Figure 4). It was also found that a 14-min interval between injection of calibration standard and spiked sample resulted in only an 8.6% increase in response for the calibration standard (points K and M, Figure 4). Subsequent experiments indicated that 18 min was needed to elapse between injections of calibration standard and spiked sample and 7 min had to elapse between injection of spiked sample and calibration standard in order to obtain reproducible results. Therefore, the following protocol had to be followed for determination of the amounts recovered (Table I): injection of calibration standard at time 0, injection of spiked sample no. 1 at 0 + 7 min, injection of calibration standard at 0 + 25 min, and injection of spiked sample no. 2 at 0 + 32 min.

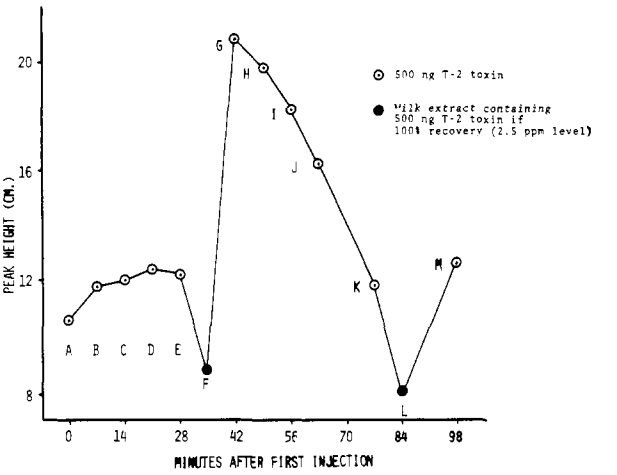

Figure 4. The effect of injection time interval on response.

Table I. Recovery Data; Milk Spiked with T-2 Toxin

Amount added, ppm	% recovered
0	0
1.25	70.3, 72.1
2.5	63.8, 66.9
5.0	75.7, 82.9
10.0	83.8, 93.8

These manipulations were performed *only* to obtain an idea of the sensitivity (ca. 300 ppb) and the recoveries obtainable by the proposed method. Obviously, the strict timing control makes the method at its present stage of development too inconvenient for quantitative analysis of more than just a few samples. However, in spite of its drawbacks, it is very attractive as a rapid screening method for T-2 toxin because of its speed, comparative sensitivity, and specificity. The method also offers an attractive alternative to the rabbit-skin assay for confirmation of T-2 toxin.

For development into a more acceptable quantitative procedure, a deuterated trimethylsilyl derivative of T-2 toxin must be used as an internal standard. This is a common technique (Sweeley et al., 1966; Gordon and Frigerio, 1972) used in mass fragmentography and offers the added advantage of enhancing sensitivity because the internal standard acts as a carrier for the undeuterated material (Axen et al., 1971).

The increased response phenomenon may be due to the silylation of the active sites on the glass transfer line

and/or the glass jet separator by the silylated milk constituents. Increased response due to saturation of the active sites on the chromatography column as originally thought (Rosen and Pareles, 1974) has been ruled out as the flame ionization detector response remained essentially constant. In support of our present thoughts, MacLeod and Nagy (1968) showed that in situ silylation of their jet separator with bis(trimethylsilyl)acetamide decreased the minimum amount necessary for the GC–MS determination of some polar compounds from 1 μg to 1 ng.

Since T-2 toxin formation in moldy grains would probably be accompanied by formation of other trichothecenes and zearalenones, it is of some importance to speculate as to which of these materials, if any, could interfere with the analysis of T-2 toxin. Pohland and Sphon (1975) have published the mass spectra of the trimethylsilyl derivatives of zearalenone, monomethoxyzearalenone, F53, and F54. None of these exhibit fragments at m/e 436, and only the second and the fourth exhibit small peaks at m/e 350. From the retention time data published by Ikediobi et al. (1971) for the trimethylsilyl derivatives of 13 trichothecenes and the fact that 3,4,8,15-tetrahydroxy-12,13-epoxy-Δ^9-trichothecene (T-2 tetraol) exhibited three peaks with a retention time of 1.5 min for the slowest of the three under our gas chromatographic conditions, it was concluded that the only trichothecene that could possibly interfere was 15-acetoxy-3,4-dihydroxy-8-(3-methylbutyryloxy)-12,13-epoxy-Δ^9-trichothecene (HT-2 toxin). However, under the GLC conditions described, the trimethylsilyl derivatives of HT-2 toxin and T-2 toxin are easily separated. At the retention time of HT-2 toxin there are no milk interferences (either FID or MF) and it is possible to determine both HT-2 toxin and T-2 toxin by this procedure by scanning m/e 466 for the former and m/e 436 for the latter simultaneously. This is of some importance in that in vitro studies with beef liver have indicated that HT-2 toxin is a major metabolite of T-2 toxin (Ellison and Kotsonis, 1974). Unfortunately, not enough HT-2 toxin could be obtained in order to conduct recovery or minimum detectability studies. Similarities in the chromatographic behavior of T-2 and HT-2 toxins and the relative intensities of their respective M – 102 (m/e 436, 466) fragments strongly suggest that similar sensitivities will be obtained.

NOTE ADDED IN PROOF

Changes in experimental procedure have eliminated the reproducibility problem and improved the sensitivity of the method to 100 ppb. After addition of 0.5 ml of BSA to the ethyl acetate solution of milk extract, the mixture is evaporated to dryness under a stream of dry nitrogen. One milliliter of trimethylsilyl-d_9 T-2 toxin (made by reaction of T-2 toxin with bis(trimethylsilyl)acetamide-d_{18}, available from Supelco Co.) in ethyl acetate is then added before analysis. Both m/e 436 (from trimethylsilyl T-2 toxin) and m/e 445 (from trimethylsilyl-d_9 T-2 toxin) are monitored. Using this modification, recoveries from three samples spiked at the 200-ppb level were 65%. Further details will follow in a subsequent publication.

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