Long-Term Storage Stability of Deoxynivalenol Standard Reference Solutions

Martin J. Shepherd*[1] and John Gilbert

The stability of the trichothecene mycotoxin deoxynivalenol during prolonged storage was studied in ethyl acetate, acetonitrile, and methanol/chloroform solution (5:95) at several storage temperatures. Solutions containing paracetamol and benzamide as internal standards were sealed into glass vials and held with exclusion of light at −18, +4, ambient temperature, and +37 °C. Samples were analyzed by HPLC at 0, 1, 4, 7, and 27 months. Results show that ethyl acetate is the preferred solvent, with no losses seen after 27 months at any of the four storage temperatures. Decomposition of deoxynivalenol was evident in acetonitrile solution after 7 months of storage at ambient temperature and in methanol/chloroform after 7 months of storage at +4 °C. However, storage in either of these solvents for up to 27 months at −18 °C was acceptable.

Deoxynivalenol (DON) is a member of the trichothecene family of mycotoxins elaborated by several *Fusarium* species, principally *Fusarium graminearum* and *Fusarium culmorum*, commonly found on cereal and other crops (Ueno, 1983; Marasas et al., 1984). The discovery of high concentrations of DON in spring wheat from the 1980 harvest in Ontario, Canada (Trenholm et al., 1981), prompted renewed interest in DON and other *Fusarium* toxins. Since then, DON has been found to occur in cereals from many other countries (Gilbert et al., 1983; Ueno et al., 1986; Tanaka et al., 1985; Pettersson et al., 1986) and is now regarded as a worldwide fungal contaminant of grain.

Research into various aspects of the contamination of foods with DON has been hindered by a number of factors, including the uncertain stability of solutions of the toxin. It is not convenient to prepare standards by weight from the crystalline compound at frequent intervals because of the high cost of purified DON. Preparation of standard solutions using the UV extinction coefficient for DON in methanol of 6384 at 216 nm (Scott et al., 1984) is less reliable than the widely accepted procedure for aflatoxins, because of the risks of interferences at the low wavelength employed, coupled with uncertainty over the purity of commercially available crystalline material. No published data are available on the stability of DON in solution, although it was shown that T-2 toxin gave rise to decomposition in aqueous media (Trusal, 1985) and eight trichothecenes containing acetoxy groups underwent transformation to several products on storage in methanol (Wei and Chu, 1986). The European Community Bureau of Reference (BCR) provides and certifies a wide range of reference materials, including currently aflatoxin M_1 in milk powder (Wagstaffe, 1987), and has commissioned this study of the stability of DON standard solutions as part of a larger project aimed at the production of reference DON-contaminated cereal standards. The objective of the current work was to establish conditions under which solutions of DON could be held for extended periods without decomposition in order that standard solutions of DON of certified purity and concentration may be made available through the BCR to all analysts.

Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Haldin House, Queen Street, Norwich NR2 4SX, U.K.

[1]Present address: Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Colney Lane, Norwich NR4 7UA, U.K.

MATERIALS AND METHODS

Materials. DON was prepared in this laboratory by alkaline hydrolysis of 3-acetyl-DON purchased from Research Foods (Downsview, Canada). The product was standardized by HPLC against crystalline material obtained from Myco-Lab (Chesterfield, MO), shown to be pure both by chromatography and by UV spectroscopy at 220 nm. Benzamide (BA) was from Aldrich (Gillingham, Dorset, U.K.) and paracetamol (PC, 4-acetamidophenol) from BDH (Poole, U.K.). All solvents were HPLC grade, from Rathburn Chemicals (Walkerburn, U.K.). Acetonitrile was "S" quality, having high transparency at 200 nm. Chloroform/methanol (95:5) was produced by weighing 19.79 g (25.0 mL) of methanol into a 500-mL volumetric flask and making up to volume with chloroform. Water for preparation of the HPLC mobile phase was purified in a Millipore (Bedford, MA) Milli-Q system.

Preparation of Samples. Mixed solutions of DON, PC, and BA were made up in each of the three selected solvents to contain toxin, PC, and BA at concentrations close to 100, 40, and 25 μg/mL, respectively. Aliquots (0.5 mL) of each bulk solution were dispensed by microsyringe into glass ampules that were immediately cooled in liquid nitrogen and sealed using a gas burner. When cold, the ampules were checked for leaks and packed in beakers wrapped in aluminum foil to exclude light.

Four storage temperatures were employed, nominally −36, +4, ambient temperature, and +37 °C. One set of ampules was held in an incubator at 37 ± 1 °C throughout the study. Ambient storage was at 18–22 °C. Samples stored nominally at +4 °C were kept in a refrigerator at 4 ± 1 °C for the first 18 months; it was later found that for the remainder of the study the temperature had been lowered to −10 °C. The remaining set was kept at −18 °C for 1 month and then maintained at −36 °C up to 18 months after preparation. Subsequently these ampules were stored again at −18 °C.

When required, ampules were opened and solvent was removed by evaporation under a stream of oxygen-free nitrogen. The residue was redissolved in 1.00 mL of HPLC mobile phase and this solution transferred into autosampler vials, which were immediately sealed for analysis.

HPLC Analysis. The system employed for measurement at 0, 1, 4, and 7 months consisted of a Waters Model 6000A pump (Millipore) and a UV detector (Pye-Unicam, Cambridge, U.K.) set at 220 nm and having an 8-μL flow cell. The Hypersil 5-μm ODS column (250 × 4.9 mm) was

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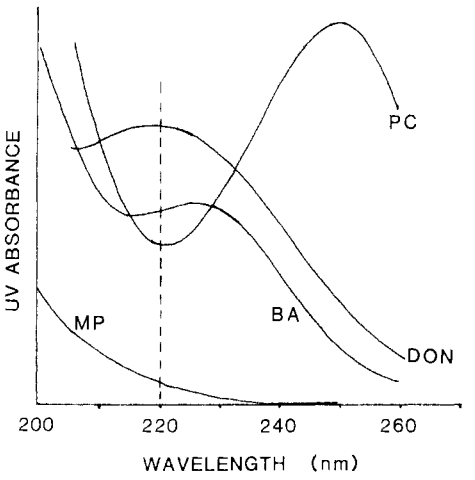

Figure 1. UV spectra of 4-deoxynivalenol (DON), paracetamol (PC), benzamide (BA), and HPLC mobile phase (MP). Spectra were obtained by stopped-flow scanning of chromatographic peaks in the HPLC UV detector.

thermostated at 35 °C and eluted at 1.0 mL/min with 88:12 v/v water/acetonitrile, prepared by adding 94.3 g of acetonitrile to 880 g of HPLC-grade water. Injections were made with a Waters WISP autoinjector and peak areas measured with a Spectra-Physics SP4270 integrator. For the analysis after 27 months, the same column and mobile phase were used with a Varian 5500 chromatograph and UV detector and a Perkin-Elmer ISS-100 autosampler. Integration was with a Trivector Trilab 2000 data system.

RESULTS AND DISCUSSION

Choice of Conditions. The three solvents selected [ethyl acetate, acetonitrile, and chloroform/methanol (95:5)] were chosen on the basis both of current analytical practice and of desirable solvent properties including volatility. Acetonitrile was selected because of its water miscibility and its UV transparency at 220 nm, the wavelength at which DON exhibits an absorption maximum. An internal standard was used to correct for possible instrumental contributions to the standard deviation of replicate analyses performed at different times.

Choice of Internal Standard. Choice of an appropriate internal standard was dependent upon the initial choice of analytical technique. HPLC analysis of DON has not been widely used primarily because of the interferences likely to be experienced with food extracts when utilizing UV detection at 220 nm. However, for this analysis HPLC was preferred because sensitivity was adequate without derivatization, thus placing fewer constraints on the chemical structure of internal standards and also considerably reducing the analysis time.

A preliminary screening of potential internal standards, in terms of their chromatographic and spectroscopic properties suggested that polar aromatics including di- and trihydroxybenzenes, PC, and BA should be considered. Further investigation indicated that the polyhydroxybenzenes were not sufficiently stable to fulfill the requirements of an internal standard. Thus, it was decided to employ both benzamide and paracetamol in case either one should prove unsuitable during the course of the study. Both gave retention times similar to that of DON. Internal standards should preferably exhibit UV absorption maxima or minima near 220 nm, in order to avoid making measurements on a steep portion of the adsorption curve. Although it was not entirely possible to achieve this because of the other constraints on their properties, both BA and PC approximate well to this condition. Figure 1 shows

Table I. Normalized Ratio of Peak Areas of Deoxynivalenol to Benzamide (Mean ± SD on Six Measurements)[a]

time, months	temp, °C	ethyl acetate	acetonitrile	chloroform/ methanol
0		100.0 ± 1.7	100.0 ± 0.6	100.0 ± 1.3
1	−18	94.5 ± 2.8	98.4 ± 0.7	97.4 ± 1.7
	Am	98.4 ± 5.2	104.0 ± 3.4	94.8 ± 4.9?
	+37	98.4 ± 2.7	95.0 ± 1.3	87.7 ± 6.1*
4	−18 to −36	94.9 ± 0.5	97.0 ± 0.7	95.4 ± 1.6
	+4	94.9 ± 0.2	94.1 ± 2.3	91.3 ± 3.5
	Am	95.5 ± 0.6	94.2 ± 1.8	92.2 ± 1.3?
	+37	95.9 ± 0.2	93.4 ± 1.2?	85.6 ± 8.7*
7	−18 to −36	99.7 ± 0.9	102.0 ± 0.4	101.7 ± 1.0
	+4	98.4 ± 0.4	100.9 ± 1.1	74.3 ± 20.6*
	Am	98.7 ± 0.6	86.9 ± 3.6*	82.6 ± 13.3*
	+37	100.2 ± 0.5	75.6 ± 25.8*	72.7 ± 24.9*
27	−18 to −36	98.7 ± 0.8	99.3 ± 1.9	101.6 ± 1.1
	−10 to +4	101.7 ± 1.2	97.4 ± 2.0	84.1 ± 1.3*
	Am	101.0 ± 1.2	81.9 ± 6.2*	87.2 ± 4.0*
	+37	102.9 ± 1.9	87.9 ± 1.3*	35.4 ± 23.2*
Renormalized Data from Measurement after 4 Months				
4	−36 to −18	100.0 ± 0.5	100.0 ± 0.7	100.0 ± 1.7
	+4	100.0 ± 0.2	97.0 ± 2.4	95.7 ± 3.7
	Am	100.6 ± 0.6	97.1 ± 1.9	96.6 ± 1.4
	+37	101.1 ± 0.2	96.3 ± 1.2	89.7 ± 9.1

[a] Key: ?, marginal deoxynivalenol instability (see Discussion); *, definite deoxynivalenol instability (see Discussion).

spectra of the mobile phase and also DON, PC, and BA in mobile phase, plotted following manual scanning after stopped-flow trapping of HPLC peaks or base line in the UV detector cell. The manufacturer's specification for the HPLC detector wavelength setting accuracy and repeatability is ±1 nm, which (Figure 1) would imply an error of less than 1% in the DON to BA ratio and a negligible effect on the DON to PC ratio.

Precision of Measurements. HPLC analyses were performed under isocratic conditions, with the mobile phase premixed, since delivering the individual components of the eluent from separate pumps gave rise to greater variability both in retention times and in peak areas. This was attributed to pump pulsing, resulting in momentary fluctuations in mobile phase composition that were significant due to the high water content of the solvent system required to displace DON from the column. This effect was much less evident with the Varian chromatograph, which employs a single pump and proportioning valves with subsequently a much greater dead volume for mixing.

The retention times observed during analysis at any one storage period were found to be reproducible to within ±0.4% over the course of about 100 injections. The actual retention times are crucially dependent upon the composition of the mobile phase, and although the method of preparation by weight is appreciably more accurate than with volumetric glassware, small differences between lots must be expected. However, HPLC retention times varied between periods by more than anticipated. Thus for DON the values at 4 and 7 months of storage were 7.21 and 7.58 min, respectively. Previous analyses had been carried out with an apparently duplicate HPLC column, and retention times fell within this range, but peak dispersions for the two columns were slightly different. For this and other instrumentation reasons, the absolute peak areas were not reproducible between the different analysis times, and consequently evaluation of the data has been carried out predominantly using the ratios of DON to internal standards. Each ratio reported is the average of measurements on three replicate ampules, two analyses per ampule. The results are given in Tables I and II. The

Table II. Normalized Ratio of Peak Areas of Deoxynivalenol to Paracetamol (Mean ± SD on Six Measurements)

time, months	temp, °C	ethyl acetate	acetonitrile	chloroform/ methanol
0		100.0 ± 1.6	100.0 ± 0.2	100.0 ± 4.3
1	−18	93.0 ± 1.0	97.7 ± 0.3	106.3 ± 1.6
	Am	89.6 ± 1.6	95.5 ± 2.1	91.9 ± 4.5
	+37	92.3 ± 1.4	95.5 ± 1.3	89.8 ± 6.4
4	−36 to −18	94.0 ± 0.3	96.0 ± 0.1	102.7 ± 0.9
	+4	93.7 ± 0.1	95.3 ± 1.8	98.9 ± 3.6
	Am	94.3 ± 0.2	94.2 ± 2.1	102.4 ± 4.1
	+37	94.6 ± 0.2	90.5 ± 1.7	112.9 ± 24.0
7	−36 to −18	95.7 ± 1.3	98.0 ± 0.6	103.8 ± 1.6
	+4	95.8 ± 0.6	97.0 ± 1.2	*a*
	Am	96.6 ± 0.5	86.0 ± 18.0	91.5 ± 18.3
	+37	95.6 ± 0.6	85.6 ± 46.7	95.5 ± 45.5
27	−36 to −18	114.4 ± 1.4	121.1 ± 4.0	122.4 ± 1.8
	−10 to +4	111.9 ± 0.8	118.7 ± 2.0	105.2 ± 13.3
	Am	110.9 ± 0.8	109.2 ± 8.0	108.8 ± 5.2
	+37	114.7 ± 1.8	105.1 ± 1.7	64.1 ± 50.9

[a] Incomplete data.

Table III. Integrated Peak Areas at 27 Months of Storage Time (Mean ± SD on Six Measurements)

solvent[a]	temp, °C	deoxy-nivalenol	paracetamol	benzamide
EA	−36 to −18	247.1 ± 1.4	127.5 ± 1.1	195.9 ± 2.0
	−10 to +4	263.1 ± 7.7	138.8 ± 3.9	202.4 ± 7.2
	Am	242.8 ± 20.7	129.3 ± 10.8	188.1 ± 16.3
	+37	258.8 ± 1.6	133.3 ± 2.3	197.0 ± 3.8
A	−36 to −18	243.8 ± 6.9	123.7 ± 1.9	200.9 ± 2.5
	−10 to +4	237.1 ± 3.3	122.7 ± 1.0	199.4 ± 1.4
	Am	223.8 ± 14.9	126.0 ± 1.8	200.8 ± 3.3
	+37	216.0 ± 7.2	126.3 ± 4.5	201.4 ± 7.9
C/M	−36 to −18	256.4 ± 3.7	137.7 ± 2.1	198.2 ± 1.7
	−10 to +4	203.5 ± 27.5	127.0 ± 1.9	189.7 ± 4.4
	Am	214.2 ± 13.9	129.3 ± 5.3	192.7 ± 5.8
	+37	83.4 ± 57.2	91.7 ± 10.2	180.6 ± 8.5

[a] Key: EA, ethyl acetate; A, acetonitrile; C/M, chloroform/ methanol.

standard deviations given for the results at any given storage time are calculated on all six measurements but do not include the error on the initial samples.

Stability of the Internal Standard. Assessment of the stability of the internal standards requires evaluation of peak area data. The results obtained at 27 months are presented in Table III. There is no evidence for instability of either internal standard in ethyl acetate or acetonitrile, but PC was labile in chloroform/methanol at 37 °C. Consideration of the data for ethyl acetate and acetonitrile indicates that only for PC in ethyl acetate at −18 °C is there a greater than 95% probability that an individual internal standard peak area diverges from the mean of the results for all temperatures. Statistics on what are essentially only three independent measurements are however subject to large errors. Comparison of Tables I and II with Table III demonstrates the marked reduction in standard deviations afforded by the use of internal standards.

Thus, except for paracetamol in chloroform/methanol at 37 °C, the ratios of DON/internal standard appear to reflect the decomposition of the mycotoxin. No significant additional peaks were observed with any solvent/temperature combination except for chloroform/methanol at 37 °C where another component was found to elute after benzamide, presumably arising from the decomposition of paracetamol. Where degradation of DON or PC occurred, it was found to progress at different rates in replicate vials as indicated by high coefficients of variation in Tables I and II. No explanation can be offered for this observation.

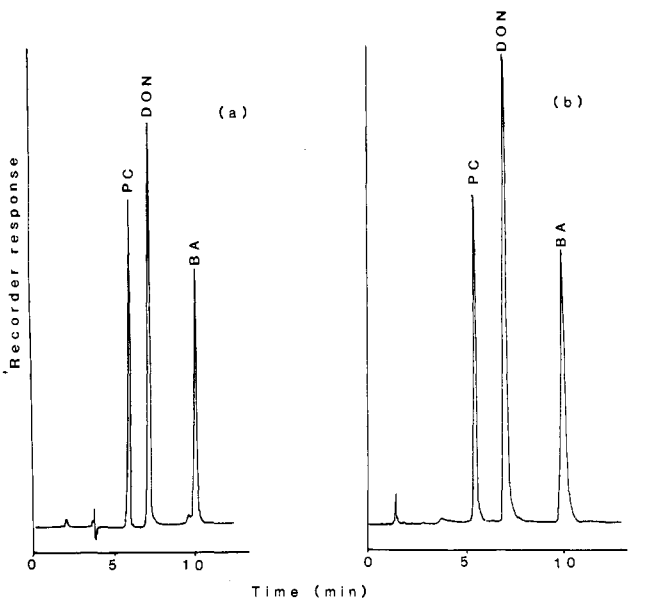

Figure 2. Typical HPLC chromatograms of stored solutions: (a) sample after storage in acetonitrile at 4 °C for 4 months; (b) sample after storage in ethyl acetate at −18 °C for 27 months.

Variation between replicate injections from the same vial was typically less than 2% and often less than 1%.

Within-Sample Reproducibility. This was additionally estimated from the results of a series of 15 replicate injections of a single sample. The coefficients of variations for the PC, DON, and BA peak areas were found to be 0.53%, 0.13%, and 0.36%, respectively. Because of the use of an autosampler, a time delay of up to 15 h between sample preparation and analysis was occasionally experienced, although the average hold time was under 4 h. In order to evaluate the effects of this upon the reproducibility of results, a group of 12 samples was analyzed twice with an approximately 6-h interval between injections. The averages and standard deviations found for the first and second injections were 1.601 ± 0.009 and 1.606 ± 0.004, and 1.203 ± 0.004 and 1.201 ± 0.004, respectively, for DON/PC and DON/BA. These differences were not statistically significant.

Typical chromatograms at 7 and 27 months are shown in Figure 2. The small late-eluting shoulder on the deoxynivalenol peak was present in the original material and could not be resolved from DON under isocratic conditions although it could just be separated when using a gradient. It was, however, felt that the use of gradient elution would significantly reduce the accuracy of peak area measurements. This shoulder was not skimmed from the DON peak by integration using parameters dictated by the detector output, and it did not alter in apparent proportion over the whole period of the study.

Reproducibility of peak area ratios of the −36 °C samples throughout this study was generally good with the exception of DON/PC in all three solvents at 27 months. Normalization of the data (as shown in Table II) indicated an instrumental origin for this discrepancy, as did a comparison with the DON/BA results (Table I). The chromatograph and integrator were changed for this last measurement point. Many of the 4-month ratios are slightly more than 2 standard deviations lower than those of the corresponding initial sample, but consideration of the complete data set suggests that this is a systematic error for all measurements made at this time. Thus, before the data were analyzed as described below, these ratios were renormalized with the −36 °C sample values for each solvent taken as 100%.

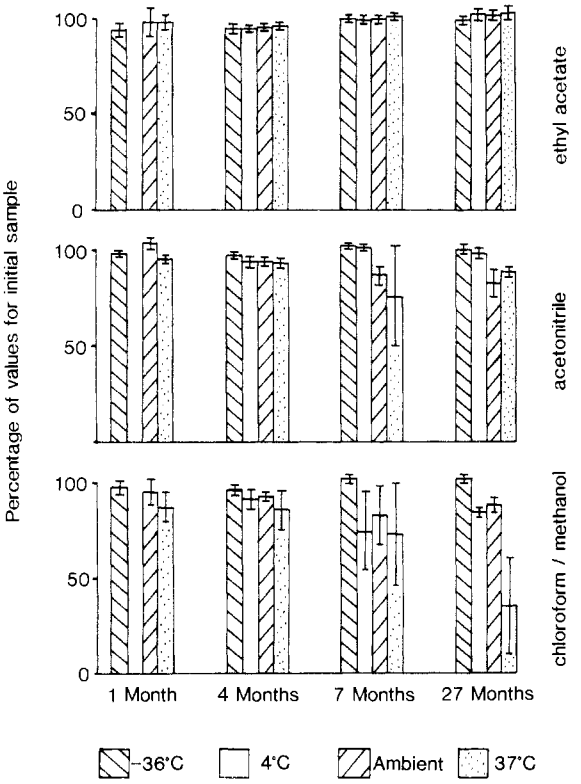

Figure 3. Normalized deoxynivalenol to benzamide peak area ratio (see Table I).

Assessment of DON Stability. Decomposition of DON was deemed to have occurred in a sample when inspection of the mean ratios of DON/BA showed either or both (a) a difference of 2 standard deviations or more from the initial sample or (b) an overall standard deviation in excess of 5%.

Samples where these criteria were met are indicated in Table I by an asterisk and, if borderline, with a question mark. Inevitably, there is some degree of subjectivity about this assessment, and the value of statistical interpretation on such a limited data set is questionable, but the conditions chosen are a realistic reflection of the reproducibility of HPLC analysis. This interpretation is supported by inspection of the ratios of DON/PC. The anomalously high standard deviation for DON/BA in ethyl acetate after 1 month of storage at ambient temperature cannot be explained. Table I is shown in histogram form in Figure 3.

The results of the study indicate that DON was stable in ethyl acetate under all the conditions tested. Acetonitrile caused decomposition of the toxin progressively with increasing time and temperature, but storage for up to 27 months at temperatures of +4 °C, or below, is acceptable. Refrigeration at +4 °C is adequate for periods of at least 7 months, and this solvent may be preferred where water miscibility is an advantage. Furthermore, standardization by UV extinction coefficient is feasible in this solvent but not in ethyl acetate. Chloroform/methanol solutions were stable in the freezer for the duration of the study, but decomposition at higher temperatures was evident as early as 1 month. Given the considerable variation in ratios of DON/internal standard between replicate vials found with chloroform/methanol, this solvent combination is not recommended.

Conclusions. Solutions of deoxynivalenol at 100 μg/mL in ethyl acetate are stable at temperatures of up to 37 °C for at least 27 months. If a water-miscible solvent is preferred, then 100 μg/mL acetonitrile solutions of the toxin may be stored at −18 °C for a minimum of 27 months. These results were obtained with the exclusion of light from the samples. Chloroform/methanol, although acceptable under certain conditions, is best avoided.

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Registry No. DON, 51481-10-8; ethyl acetate, 141-78-6; acetonitrile, 75-05-8; methanol, 67-56-1; chloroform, 67-66-3.

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