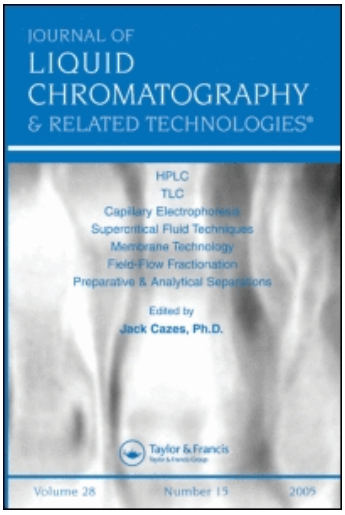

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ANALYSIS OF TRACE LEVELS OF DEOXYNIVALENOL IN COW'S MILK BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

An HPLC method using UV detection for the analysis of trace levels of the mycotoxin deoxynivalenol (DON) in milk is described. For a small sample size of 3.0 ml, the cleanup method included precipitation of milk protein with acetic acid, followed by the additional removal of protein and fat by filtration. The resulting filtrate was eluted through an Extrelut column, followed by a second elution through a charcoal-alumina column. HPLC separation of the eluent was accomplished on a C18 reversed phase column and 4% acetonitrile (ACN) in water as the mobile phase. Acceptable detection limit of 5 ng/ml milk and very consistent recoveries of 57.1±1.2% were obtained with milk spiked in the range 25-200 ng/ml. This method is superior to the existing methods that are tedious and/or required expensive GC/MS instrumentation.

INTRODUCTION

Deoxynivalenol (DON, vomitoxin) is a fungal metabolite, produced by various species of Fusarium, most commonly present in cereals and feed (Vesonder et al. 1973). In swine exposure of feed contaminated with DON has been reported to cause feed refusal, reduced weight gain and in case of acute exposure, emesis (Forsyth

et al. 1977, Friend et al. 1982 & 1983, Schuch et al. 1982, Trenholm et al. 1984, Vesonder 1980/81). However, dairy cattle, sheep and poultry do not display any apparent sign of toxicity on exposure to DON at the levels typically found in naturally contaminated grain and feedstuffs (Trenholm et al. 1984, Hamilton et al. 1985). While dairy cows have been shown to transmit negligible levels of DON or its metabolites (Cote et al. 1986) into milk on exposure to DON after a single dose (Prelusky et al. 1984), exposure over an extended period of five days to three cows at a very high dose of DON led to noticeable quantity of DOM-1, a metabolite of DON, in one cow. However the exposure of DON contaminated feed over a longer period and to a larger number of cows needed to be carried out to fully evaluate the accumulation of DON or DOM-1 into its milk in free form and/or as glucronide conjugate. For this type of study a simple method was required that could be applied to a large number of samples at the same time.

Although there are several methods reported in the literature for the analysis of DON in cereal grain and mixed feed (e.g., Bennett et al. 1983, Chang et al. 1984, Cohen and Lapointe 1982, Ehrlich et al. 1983, Scott et al. 1981, Visconti and Bottalico 1983), the methods reported for its analysis in milk are limited. Swanson et al. (1986) used 40 ml of milk and the procedure is tedious and time consuming. The method described by Prelusky et al. (1984) although comparably simple required the selectivity of GC-selective ion monitoring (SIM)/MS. The purpose of the present study was not only to develop a method that was relatively simple, rapid and sensitive but also that used small sample size and did not require GC/MS instrumentation.

EXPERIMENTAL

Chemicals and Materials

Sterile 0.45 μm Millex-HA filters were purchased from Millipore Products Division, Bedford, MA, USA. Extrelut columns, 3 ml capacity, manufactured by EM Science (A division of EM Industries Inc., Gibbstown, NJ, USA) were used for liquid/liquid extraction. The charcoal/alumina (1.5 g/1.4 g) columns used were packed in-house by using 10 ml (8 mm id) borosilicate glass pipets (Fisher Scientific, Pittsburgh, PA, USA), activated charcoal (Darco G-60, Baker Chemical co., Phillipsburg, NJ, USA), neutral alumina (70-230 mesh, Merck M 01077-36, BDH Chemicals, Toronto, ON, Canada) and glass wool, according to the method reported by Trenholm et al.(1985). Water used was of NANOpure quality and all the solvents were of HPLC grade (BDH Chemicals). ß-Glucuronidase (500,000 units in 4.0 ml) and deoxynivalenol were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Standard Solutions

DON was dissolved in 4% acetonitrile (ACN) in water to give a stock concentration of 1 μg/ml. Recoveries were calculated by comparing peak-heights of DON from milk extracts with standards. Clean milk was obtained from cows fed with standard feeding ration and housed at the CFAR green-belt farm, Nepean, Ontario. Milk samples were stored frozen prior to use.

Sample Preparation

Three ml milk samples, spiked with DON (0-200 ng/ml), were incubated at 50°C for 15 min with 300 μl of 5% acetic acid to

precipitate protein, followed by centrifugation at 3500 rpm for 10 min. The supernatant was filtered through a 0.45 μm millipore filter to remove any remaining protein and fat. The filtrate was made basic (pH 7-8) with the addition of solid sodium bicarbonate (100 mg), which was then transferred to the Extrelut column. The sample was allowed to be absorbed on the column matrix over a period of 15 minutes. DON was then eluted from the column with 7 x 3 ml of 6% methanol in ethyl acetate and the eluent (approximately 20 ml) collected in a 25 ml glass tube. The eluent was evaporated to dryness under a stream of nitrogen at 50°C, redissolved in 4 ml of ACN:water (21:4), and loaded onto a charcoal/alumina column that was pre-washed with 15 ml of ACN:water (21:4). The sample tube was washed with 4+2 ml of ACN:water (21:4) and the washings were added on to the charcoal/alumina column. The column was eluted with an additional 20 ml of ACN:water (21:4). All eluted fractions (~30 ml) from the charcoal/alumina column were collected, evaporated to dryness (N_2, 50°C) and redissolved in 600 μl of 4% ACN in water. A sample of 100 μl (equivalent to 0.5 ml milk) was then injected onto HPLC for analysis.

Instrumentation

The HPLC analysis of DON was performed with a Spectra-Physics SP 8800 autosampler, SP 8810 pump, SP 4400 integrator and 783 A programmable absorbance detector from Applied Biosystems Inc. A reversed-phase 25 X 0.46 cm column packed with C18 packing material of 5 μm particle size (CSC-Nucl. 120A/ODS, Chromatographic Sciences Company Inc., Montreal, QC, Canada) was used. The guard column used was ADSORBOSPHERE C18 with 5 μm packing (Alltech Associates Inc., Applied Sciences Labs, Deerfield, Il, USA).

The mobile phase consisted of 4% ACN in water, pumped at a flow rate of 1.0 ml/min. The UV detector was set at a wavelength of 220 nm, range 0.001 AUF and rise time of 5 sec; and integrator was used at attenuation of 8-32 and chart speed of 0.25 cm/min. A 100 µl sample solution was injected on full-loop injection mode in the sequence: standard, 2 samples and standard.

Enzymatic Hydrolysis

A 3 ml milk sample was incubated with 1.5 ml of 0.1 M phosphate buffer (pH 6.8) containing 3000 units of ß-glucuronidase (0.4 ml of ß-glucuronidase was dissolved in phosphate buffer to give a total volume of 25 ml; 2000 units/ml) for 16 h at 37°C. Following hydrolysis milk contents were then prepared for HPLC analysis as described under sample preparation.

RESULT AND DISCUSSION

Due to the complexity of the milk matrix with components of a wide range of polarity, it was challenging to get extracts purified sufficiently to be analyzed for trace levels of DON at an UV-wavelength of 220 nm by HPLC. To achieve this, it was necessary that the interfering fat and proteins present in milk be adequately removed. Attempted initially was the addition of ammonium sulfate solid to precipitate out the protein, but the resulting product obtained after cleanup remained unsuitable for analysis. Subsequently, a weak acid such as 5% acetic acid was found to be a better choice to precipitate the protein. Although protein and fat tend to aggregate on addition of acid to warm milk (50°C), filtration was done with 0.45 µm filter to get a clear

filtrate from the whey. This process removed most of the fat globules that have diameter of 1-8 μm (Mulder and Walstra 1974). Increasing the pH of the filtrate with sodium bicarbonate to 7-8 was necessary to obtain a sufficiently clean chromatogram. Without this step the extract was not suitable for analysis of DON due to interfering compounds carried over during the cleanup.

Because the Extrelut columns supplied by the company were found to introduce contaminants into the analyses, it was necessary that columns were washed with 3 x 5 ml of ethyl acetate and dried overnight under vacuum prior to use. The alkaline filtrate (equivalent to 3 ml of milk) was loaded onto these pre-cleaned Extrelut columns. For elution, 7 x 3 ml of 6% methanol in ethyl acetate was found to be appropriate for optimum recovery of DON. At any higher proportion of methanol, although recoveries may have improved slightly, the columns bled unwanted contaminants. The eluent was dried at 50°C under a stream of nitrogen. The residue, however, was found not suitable at this point for direct analysis on HPLC and required further cleanup.

Standard charcoal/alumina columns (0.75 g/0.7 g) were tried as a second step of cleanup, but did not give sufficiently clean extracts. However, samples obtained by using twice the amount of charcoal and alumina (i.e., 1.5 g of charcoal and 1.4 g of alumina) were found to be suitable for analysis. Chromatograms of milk extracts did not show any interfering peaks at the retention time of DON, when sample extracts equivalent to 0.5 ml of milk were injected.

The hydrolysis with ß-glucronidase was carried out to free any DON or DOM-1 glucronide conjugate that may be present in the milk of cows fed contaminated feed. It was done to ensure that the

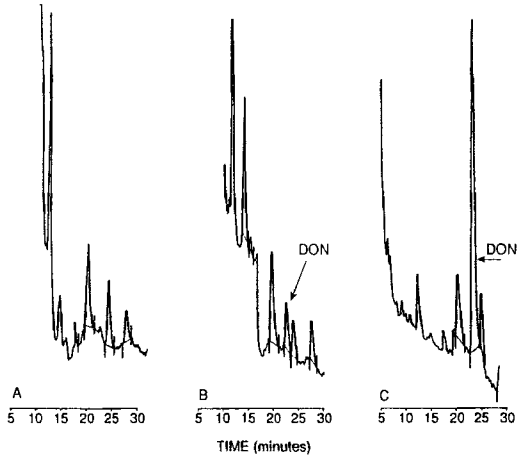

Figure 1: High pressure liquid chromatograms of (a) extract of clean milk; (b) milk spiked with 10 ng/ml of DON; and (c) milk spiked with 100 ng/ml of DON. Equivalent of 0.5 ml milk was injected for each of these chromatograms.

addition and hydrolysis of spiked samples with ß-glucronidase does not introduce any interference with the DON analysis.

Recoveries of DON from Milk

Three ml of clean milk, free of DON, was spiked with various amounts of DON to determine the recoveries and limit of detection. To establish the detection limit milk spiked with 5, 10, 15, 20 and 25 ng/ml of DON was used and for recoveries milk spiked at 25, 50, 75, 100, 125, 150, 175 and 200 ng/ml of DON was used. The recoveries for 25-200 ng/ml were consistently in the range of 55-59% regardless of concentration (shown in Table 1).

A number of factors might have contributed to the lower recovery of DON from milk. First, the concentration of DON used

Table 1: Recoveries of DON from Milk

Conc. of DON (ng/ml of milk)	% Recovery[1]
25	57.2±6.4
50	56.3±9.7
75	56.2±5.6
100	58.4±9.1
125	57.9±4.3
150	55.4±8.2
175	58.7±3.4
200	56.3±4.6

1. average ± standard deviation (n=6)

was very low. The precipitation of protein followed by filtration to remove protein and fat could be the major factor. It is very difficult to get all the liquid from the protein precipitate by filtration. The residual liquid in the protein precipitate and the protein itself might have retained some DON. The two sequential cleanups through Extrelut column and charcoal/alumina column could also have made some contribution to the lower recovery.

Linearity and Sensitivity

The relationship between the peak height and concentration of DON was linear over the range of 25-200 ng/ml of milk (r^2=0.98). Although DON could be detected in spiked milk at a minimal concentration of 5 ng/ml (based on 3 X baseline noise), the response varied below 25 ng/ml. It could be quantified readily over the range of 25-200 ng/ml.

Precision

Since equivalent to 0.5 ml of milk was injected on column, the system precision of the assay was determined by making multiple injections from a set of standard solutions with known DON contents of 10-100 ng on column. System precision, using the equipment described here, was 2.3% relative standard deviation (RSD) at 10 ng on column (n=6) and 1.6% at 100 ng on column (n=6).

CONCLUSIONS

A novel HPLC method for the analysis of trace levels (5-200 ng/ml) of DON in small milk volumes is described. This method is well suited to monitor trace residues of DON in milk after exposure to DON contaminated feed. Although recovery of DON was less than ideal, it remained very consistent (57.1±1.2%) in the 25-200 ng/ml range.

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