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Studies on the Proteins from Safflower Seed (*Carthamus tinctorius* L)

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Safflower seed (*Carthamus tinctorius*) was analyzed for moisture, protein, ether extractives, ash, total sugars, and crude fiber. The total protein in the seed was extracted at pH 7.5 by low ionic strength buffers in the presence of sodium chloride and at different pHs. The extracted total protein was analyzed by using the techniques of sedimentation velocity, polyacrylamide gel electrophoresis, ion-exchange chromatography, and gel filtration. It consists of predominantly one major fraction of sedimentation value 12S and three other components of sedimentation values 2S, 7S, and 17S. The protein has an absorption coefficient, $E^{1\%,1cm}_{280nm}$, of 17.3 and an absorption maximum at 279–280 nm in the ultraviolet region. The presence or absence of sodium chloride during extraction has a profound effect on the extractability of the 12S component of the total protein.

Safflower (*Carthamus tinctorius* L.) seed is primarily grown for its oil. The seed contains nearly 35–40% oil, 15–20% protein, and 35–45% hull fraction (Betschart et al., 1975). The proteins from the safflower seeds are of good nutritional quality (Betschart et al., 1979; Kohler et al., 1966). The ultilization of the safflower proteins and protein isolates for food and feed purposes has been attempted (Betschart et al., 1975). However, utilization of safflower protein concentrate in food has been limited, because of color and bitter principles and also the high content of crude fiber (Lyon et al., 1979). Attempts have been made to obtain a protein concentrate low in crude fiber content (Betschart et al., 1975; Kohler, 1966).

However, much information is not available on the nature of the proteins of safflower. In the present investigation, total proteins have been isolated and analyzed by using various physicochemical techniques.

MATERIALS AND METHODS

Preparation of Defatted Safflower Meal. Safflower seeds of the variety A-1 were obtained from the Karnataka State Seed Corp., Ltd., Bangalore, India. They were flaked and dried in a cabinet air dryer at 60 °C for 3 h. The dried flakes were defatted with *n*-hexane and were air-dried to remove the residual solvent. The hulls were separated by differential sieving. The hull-free meal was passed through a plate mill and then passed through a 60-mesh sieve. The resultant flour had less than 1% fat.

Proximate Composition. Moisture, protein ($N \times 6.25$), ether extractives, ash, and crude fiber were determined by the AOAC (1980) method. Total sugars were determined by the phenol–sulfuric acid procedure (Montgomery, 1961).

Nitrogen Extractability. Two grams of the safflower flour was mixed with 20 mL of the solvent; the pH was adjusted to the desired value by 5 N HCl or 5 N NaOH. The suspension was shaken in a rotary shaker for 1 h at room temperature (~27 °C). The slurry was centrifuged at 6000*g* for 20 min in a Sorvall RC-5B refrigerated centrifuged at 20 °C. The pH of the supernatant was read in a Toshniwal pH meter, type CL 41. Aliquots (5 mL) were used for nitrogen determination by the Kjeldahl method (AOAC, 1980). The influence of parameters such as extraction time, solute to solvent ratio, pH, and various solvents for extraction of the protein was investigated by using the defatted flour.

For extractability studies the suspension pH was measured at intervals of 5 min after the slurry is made, and the pH was adjusted to a constant value within the first 15–20 min. The magnitude of the drift was of the order of 0.3 unit at extreme pH values and about 0.1 unit at neutral pH values. The pH of the slurry after the extraction time, i.e., 1 h was again measured. This pH value was used for plotting the data.

Extraction of Total Proteins. The total proteins from the safflower meal were extracted in 0.01 M phosphate buffer of pH 7.5, containing 1 M NaCl (hereafter referred to as PS buffer), by using a meal concentration of 10%. The slurry was centrifuged at 6000*g* at 20 °C. The supernatant was dialyzed against the same buffer and used for various experimentations after determining the concentration.

Protein Concentration. The protein concentration was routinely determined by using a value of $E^{1\%,1cm}_{280nm} = 17.3$ for the total protein. This was determined by measuring the absorbance of protein solutions of known concentration.

Gel Filtration. Sepharose 6B-100 (medium) gel in PS buffer was packed into a column, 1.8 × 100 cm. Approximately 40 mg of the protein in the above buffer was loaded onto the column and was eluted with the same buffer. Fractions (2.5 mL) were collected with an Emenvee au-

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tomatic fraction collector and the absorbance of the fractions at 280 and 320 nm read in a Shimadzu UV 150–02 double-beam spectrophotometer.

Ion-Exchange Chromatography. DEAE-cellulose (0.8 mequiv/g capacity) after regeneration by the procedure of Peterson (1970) was equilibrated with 0.025 M phosphate buffer of pH 7.5 and packed into a 1.8 × 20 cm column. Approximately 75 mg of the protein was loaded on the column and the elution was carried out using a continuous, linear gradient of NaCl from 0 to 0.8 M in the buffer, using a constant flow rate of 30 mL/h. Fractions were collected and the absorbance was monitored both at 280 and 320 nm. NaCl in the fractions was estimated by Volhard's method (Vogel, 1961).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried using a Toshniwal gel electrophoresis unit at 0.01 M phosphate buffer of pH 7.5 and using 7.5% acrylamide gel [with 0.4% methyline bis(acrylamide) as the cross-linking agent]. The gels were prerun in the same buffer. Protein sample (10–100 μg) containing nearly 10% sucrose and bromophenol blue as the indicator dye was loaded on the gel. The electrophoresis was performed at a constant current of 3 mA/gel for 90 min at which time the dye had almost moved to the bottom of the gel. The gels were stained in 0.5% Amido black in 7.5% (v/v) acetic acid solution and destained in 7.5% acetic acid by diffusion.

Sedimentation Velocity. Sedimentation velocity experiments were carried out with protein solutions in 0.01 M phosphate buffer of pH 7.5 with and without 1 M NaCl in a Spinco Model E analytical ultracentrifuge. A standard 12-mm single-sector Kel F centerpiece was used, and the experiments were carried out at room temperature (~27 °C) and at 56 100 rpm. The plates were read in a Abbe comparator adopted to read the ultracentrifuge plates and $s_{20,w}$ values calculated by standard procedure (Schachman, 1959). The percentage of the fractions was calculated by enlarged tracings of the centrifuge pattern with a Gaertner microcomparator.

Ultraviolet Absorption Spectrum. The ultraviolet absorption spectrum of the protein was recorded with a Perkin-Elmer 124 double-beam spectrophotometer in the range from 400 to 240 nm in a 1-cm quartz cell at room temperature (~27 °C).

Fluorescence. This was measured in a Perkin-Elmer fluorescence spectrophotometer, Model 203, at room temperature (~27 °C). The fluorescence emission spectrum was measured in the range 300–380 nm after exciting at 280 nm. The excitation spectrum was measured in the range 240–300 nm after fixing the emission maximum at 325 nm (Teale, 1960).

Analyses. Phosphorus was determined by the method of Tausky and Shorr (1953). The carbohydrate content of the protein was determined by the procedure of Montgomery (1961). Phytic acid was estimated by the procedure of Ellis et al. (1977). The presence of polyphenols as chlorogenic acid was checked by the procedure of Pomenta and Burns (1971). Proteolytic activity was determined by using 1% safflower protein solution in 25 mM citrate phosphate buffer at pH 5.7, by the method of Chrispeels and Boulter (1975). Casein was used as the substrate. Proteolytic activity was also determined at pH 8.0 by using papain as the standard in 0.05 M Tris-HCl buffer in presence of activators consisting of cysteine (0.005 M) and EDTA (0.002 M). Ribonuclease activity was determined by the method of Kalnitsky et al. (1959) using 1% ribonucleic acid in 0.1 M sodium acetate buffer of pH 5.0. Trypsin inhibitor activity was determined by the procedure of Kakade et al. (1969). Hemagglutinin activity was determined according to the method of Liener and Hill (1953). Urease activity was determined by incubating the appropriate volume of protein solution in 1 mL of 0.02% urea in pH 7.0 buffer at 50 °C (Varner, 1960).

Amino Acid Analysis. The amino acid analysis was carried out in a Durrum amino acid analyzer following the standard procedure of hydrolyzing the protein in 6 N HCl (Spackman et al., 1958).

Table I. Proximate Composition of Safflower Seed[a]

sample	moisture, %	protein ($N \times 6.25$), %	ether extracts, %	ash, %	total sugar, %	crude fiber,[b] %
seed	5.3	14.9	27.5	2.3	3.2	40.6
literature value for seed[c]		15–22	22–47	1–35		11–36
kernel	3.1	26.6	55.4	2.7	5.8	3.7
hull	6.7	2.3	0.7	0.9	1.4	61.1
flour	6.6	46.4	0.5	6.5	11.0	9.5

[a] Values are the average of two determinations. [b] AOAC method (AOAC, 1980); fat-free basis. [c] Values cited from Guggolz et al. (1968) and Betschart et al. (1975).

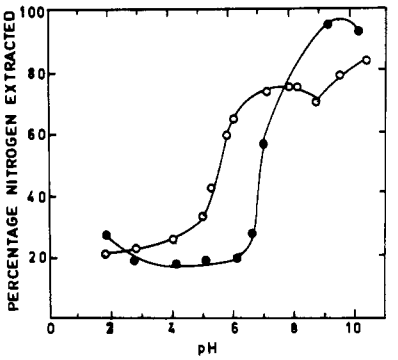

Figure 1. Nitrogen extractability of safflower seed total protein as a function of pH in (●) water and (○) 1 M NaCl.

RESULTS AND DISCUSSION

The proximate composition of the seeds, kernels, hulls, and the defatted flour is given in Table I. The seed contains nearly 15% protein and about 40% crude fiber. On the other hand, the kernel has nearly 27% protein and a low crude fiber of 3.7%. The defatted flour contains nearly 46% protein and about 10% crude fiber as shown in Table I. Guggolz et al. (1968) have compiled the composition of several types of safflower seeds. Our value for the protein content of the seed is slightly less than that of other varieties screened by Guggolz et al. (1968). However, the values of ash and ether extractives of the kernel and hull agree well with those of Guggolz et al., (1968). In general, there is considerable variation of protein in the flour in several varieties. This may be due to the variation in the extent of crude fiber present in the final flour, which decreases the net protein content of the flour.

The extractability of safflower meal nitrogen was examined in the range of pH 1.5–10.5. Figure 1 shows the extractability profile in different extractants. In water, the profile showed maximum extractability of 95% at pH 9.3 and minimum extractability of ~20% between pH 3 and pH 6. The extractability profile in 1 M NaCl was different from that in water (Figure 1). Maximum extractability of 80% occurs at pH 10 and minimum extractability of 20% between pH 2 and pH 4. Betschart (1975) has observed similar results with the nitrogen

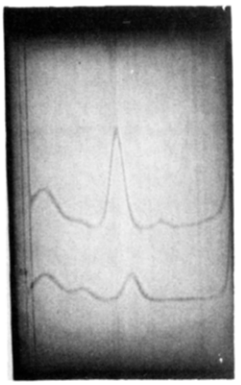

Figure 2. Velocity sedimentation pattern of safflower seed total proteins. (Upper) Extracted and dialyzed in 0.01 M phosphate buffer of pH 7.5 containing 1 M NaCl. (Lower) Extracted and dialyzed in 0.01 M phosphate buffer of pH 7.5 without NaCl.

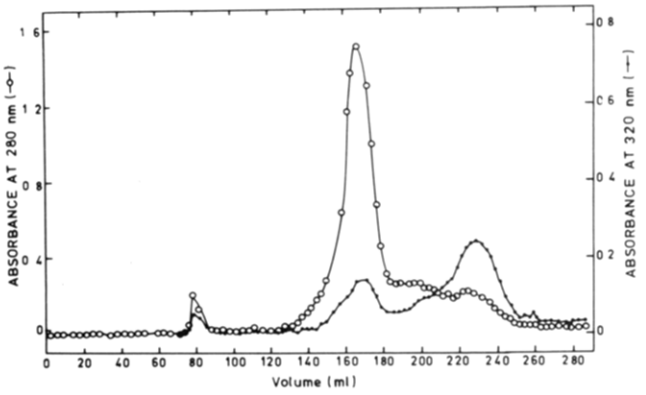

Figure 3. Gel filtration pattern of safflower seed total protein in Sepharose 6B-100 gel in 0.01 M phosphate buffer of pH 7.5 containing 1 M NaCl.

solubility of safflower seed with unheated meal, expeller pressed cake and desolventized meal. All of them show a minimum extractability in the region of pH 4 and 6. There appears also a second minimum around pH 8.8 (Figure 1). Thus, there was no sharp solubility minimum unlike in the case of other seed proteins such as groundnut, sesame, soybean, etc. (Prakash and Narasinga Rao, 1984). Such "minimum" near neutral or alkaline pH is also observed with other oilseed proteins, for example, mustard, cottonseed, and poppy seed (Prakash and Narasinga Rao, 1984). Such a second minimum has been shown to be due to the basic nature of some of the fractions of total proteins (Prakash and Narasinga Rao, 1984).

Repeated extractability of total proteins from safflower meal in 0.01 M phosphate buffer of pH 7.5 containing 1 M NaCl showed that the first extraction contained nearly 65–70% of the total nitrogen. The second extraction from the same residue contained nearly 15% of the total nitrogen, and the third extraction contained nearly 2.5% of the total nitrogen of the meal.

Sedimentation Velocity. The sedimentation velocity analysis of the total proteins indicated four components having sedimentation values of 2, 7, 12, and 17S constituting approximately 26%, 6%, 65%, and 3%, respectively, of the protein (Figure 2). The total protein pattern is similar to that of other oilseed proteins. They contain a high molecular weight and a low molecular weight protein in fair amounts; the 7S and 17S proteins are in low proportion (Prakash and Narasinga Rao, 1984).

Gel Filtration. The gel filtration of the total protein in Sepharose 6B-100 in PS buffer also indicated four peaks (Figure 3). The major peak eluting between 162 and 165 mL constituted nearly 65% of the total protein eluted.

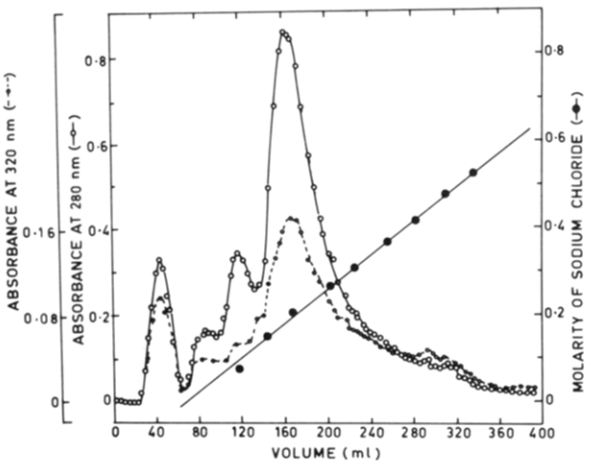

Figure 4. DEAE-cellulose chromatography of safflower seed total protein in 0.025 M phosphate buffer of pH 7.5.

Figure 5. Polyacrylamide gel electrophoresis pattern of safflower seed total protein in 0.01 M phosphate buffer of pH 7.5. The major and minor bands are indicated in the figure.

Ion-Exchange Chromatography. DEAE-cellulose chromatography in 0.025 M phosphate buffer of pH 7.5 showed four peaks, the major peak eluting at 0.19 M NaCl while the other three peaks eluted at 0, 0.5, and 0.11 M NaCl, respectively (Figure 4).

Polyacrylamide Gel Electrophoresis. The polyacrylamide gel electrophoresis in 0.01 M phosphate buffer of pH 7.5 also indicated a major component being present in the total protein, along with several slow- and fast-moving minor components (Figure 5). However, under the conditions of the experiment, no distinct four bands were observed.

Effect of Sodium Chloride. Since sodium chloride had a profound effect on the extractability of safflower seed proteins, it was of interest to investigate the effect of sodium chloride on the extent of solubility of the various components of the total protein using the sedimentation velocity technique. Figure 2 lower pattern shows the sedimentation velocity profile of total protein extracted in buffer alone without sodium chloride. Figure 2 (upper) as already mentioned is the profile of total protein extracted in the presence of sodium chloride. There is a marked decrease in the percentage of the 12S component in the absence of sodium chloride. This indicates that the high molecular weight fraction is solubilized by sodium chloride and hence is extracted more in the presence of salt [Figure 2 (upper)]. However, there was no significant change in the 2S and 7S components. Further, the 17S component is absent in the no-salt system [Figure 2 (lower)].

Physicochemical Properties. The different physicochemical properties of the total protein are listed in Table II. The total protein has an absorption coefficient $E_{280nm}^{1\%,1cm}$ = 17.3 and an ultraviolet absorption maximum at 279–280 nm. It has a nitrogen content of 14.2% and contains nearly 4% carbohydrate and 0.23% phosphorus. The total pro-

Table II. Chemical and Physicochemical Properties of Safflower Seed Protein

property	total protein
sedimentation coefficient (s), S	2, 7, 12, 17
absorption coefficient $E^{1\%,1cm}_{280nm}$	17.3
absorption maximum, nm	279–280
fluorescence emission maximum, nm	325
nitrogen content, %	14.2
protein content ($N \times 6.25$), %	88.8
phosphorus content, %	0.23
carbohydrate content, %	4.0
proteolytic activity, Kunitz units	22
phytic acid, %	0.01

Table III. Amino Acid Composition of Safflower Seed Protein[a]

amino acid	g/100 g of total protein[a]
aspartic acid	12.3
threonine	3.1
serine	4.0
glutamic acid	26.3
glycine	6.8
alanine	3.7
valine	7.1
methionine	3.5
isoleucine	3.4
leucine	6.8
tyrosine	3.0
phenylalanine	4.7
histidine	3.0
lysine	2.2

[a] Average of two analyses.

tein has proteolytic activity at pH 5.7 and no activity at pH 8.0. Nuclease activity, urease activity, the presence of trypsin inhibitor, hemagglutinin activity, and chlorogenic acid could not be detected. The phytate content was very low (0.01%) in the total protein.

Amino Acid Composition. The amino acid composition of the total protein is shown in Table III. The total protein contains high amount of aspartic acid, glutamic acid, valine, glycine, and phenylalanine. The amino acid composition of the protein isolate has been reported by Betschart et al. (1975) and Betschart and Saunders (1978). Their values agree fairly well with that reported by us for the extracted total protein. The high content of aspartic acid and glutamic acid is very similar to that of other oilseed proteins, indicating it to be acidic in nature (Prakash and Narasinga Rao, 1984).

Color/Ligand. Along with the total protein an yellow pigment is also extracted from the flour. This coloring principle, extracted in 50% aqueous ethanol (hereafter called ligand) has an absorption spectrum with maximum absorption at 270 and 320 nm in water as shown in Figure 6. It has also a very little absorption in the region of 400–550 nm with no specific peaks. In order to monitor the ligand, all the eluants in DEAE-cellulose chromatography and gel filtration were monitored at 320 nm also. In Figure 3 and 4 is shown the elution profile of ligand also along with that of the protein. In gel filtration one can see more ligand being eluted with the low molecular weight component and in DEAE-cellulose chromatography with the first and fourth peak (Figure 4).

In conclusion, the total proteins from safflower seed consist of predominantly one major fraction of sedimentation value 12 S and three other components of sedimentation values 2, 7, and 17 S. The presence or absence of sodium chloride during extraction has a profound effect on the extractability of the 12S component. Along with the protein, a ligand absorbing at 270 and 320 nm is also extracted. Further work is in progress in isolating the various fractions of the total protein and also studies are in progress with regard to the nature of the ligand.

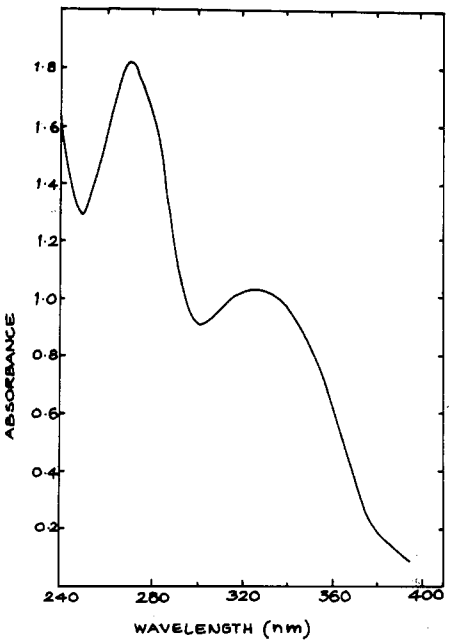

Figure 6. Ultraviolet absorption spectra of alcohol extracted color from safflower seed flour.

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Registry No. Phosphorus, 7723-14-0; nitrogen, 7727-37-9; phytic acid, 83-86-3.

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Synthesis, Characterization, and Occurrence in Bread and Cereal Products of an Isomer of 4-Deoxynivalenol (Vomitoxin)

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Acetylation of 4-deoxynivalenol (DON, vomitoxin) with acetic anhydride/pyridine gave mixtures of di- and triacetyl derivatives, which on further refluxing with acetic anhydride were transformed into an isomer. NMR, MS, IR, and UV spectral data of the latter indicated it to be 3,8,15-triacetoxy-12,13-epoxytrichothec-8-en-7-one. On hydrolysis, it gave a compound isomeric with 4-deoxynivalenol, to which the structure 3,8,15-trihydroxy-12,13-epoxytrichothec-8-en-7-one (isoDON) was assigned. Thermal decomposition of DON under nitrogen at 160 °C for 1 h resulted in approximately 65% decomposition and the formation of five products, the Me_3Si derivative of one having an MS identical with that of Me_3Si-isoDON. Samples of white and whole meal bread baked from fortified and naturally DON contaminated wheat when analyzed by GC/single ion monitoring showed that isoDON was formed to the extent of 3–13% of the DON present; levels were higher in the crust than in the crumbs. No isoDON was found in products made from uncontaminated wheat. IsoDON was also detected in processed wheat-based breakfast cereal made from grain, naturally contaminated with DON at levels ranging from 0.35 to 0.75 mg/kg DON.

Fungal infection of grains (i.e., barley, corn, and wheat) by mycotoxigenic *Fusarium* spp. is a common phenomenon in countries with temperate climates. Occasional, environmental factors favor epidemic development of the fungi, which may result in mycotoxin formation and contamination of food for human and animal consumption. In 1980, such an outbreak occurred in Canada in the Ontario soft white winter wheat crop. The predominant mycotoxin was identified as the trichothecene, 4-deoxynivalenol (DON, vomitoxin, 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one), present at levels averaging 1 mg/kg, with a maximum of 8.5 mg/kg (Trenholm et al., 1981). A maximum tolerance level of 0.3 mg/kg for uncleaned Ontario soft white winter wheat was introduced in Canada in 1982, but this has since been increased to 2 mg/kg based on estimated losses during further processing and revised toxicological data.

Milling studies with hard red spring wheat naturally contaminated with DON indicated that the mycotoxin was differentially distributed throughout the kernel, with relatively higher levels in the dockage, outer bran fractions, and shorts and lower levels in the inner flour fractions (Scott et al., 1983; Young et al., 1984). DON was not destroyed on processing naturally contaminated Quebec hard spring wheat into flour and baking into bread (El-Banna et al., 1983; Scott et al., 1983), although levels were slightly reduced. Similar results were reported with the milling of contaminated Ontario Soft white winter wheat; further processing of this wheat into a variety of baked products resulted in reductions of DON up to 35% (Young et al., 1984).

In the present baking study with DON-contaminated flour, the conversion of DON to an isomer is reported. This isomer is identical with a compound isolated during acetylation studies with DON based on spectral characteristics. A structure is assigned to the isomer of DON.

MATERIALS AND METHODS

Materials. 4-Deoxynivalenol (DON) was prepared biosynthetically from liquid cultures of *Fusarium roseum* (ATCC 28114) (Greenhalgh et al., 1984). The mixed silylating reagent (Trisil TBT) was purchased from Pierce Chemical Co., Rockford, IL. White winter wheat flour, naturally contaminated with DON (ca. 0.5 mg/kg), was supplied by Health and Welfare, Canada. DON-contaminated wheat samples together with corresponding samples of a wheat-based breakfast cereal were obtained from a Canadian breakfast cereal manufacturer.

Thermal Decomposition of DON. DON (0.5 mg) was sealed under nitrogen in a 1-mL glass vial and heated for 1 h at 160 or 200 °C. After cooling to room temperature, the contents of the vial were dissolved in 10% methanol in $CHCl_3$, and an aliquot was taken and subsequently derivatized.

Bread Manufacture. Loaves of bread were produced in the laboratory by using pilot-scale equipment and were prepared both from naturally DON contaminated flour

Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada (R.G. and B.A.B.), Ministry of Agriculture, Fisheries and Food, Haldin House, Old Bank of England Court, Queen Street, Norwich NR2 4SX, U.K. (J.G., J.R.S., and M.J.S.), and Research Station, Agriculture Canada, Fredericton, New Brunswick E3B 4Z7, Canada (R.R.K.).

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