Natural Occurrence of Moniliformin together with Deoxynivalenol and Zearalenone in Transkeian Corn

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Mycotoxicological investigations were carried out on a sample of moldy corn from Butterworth, Transkei, a district with a very high human esophageal cancer rate. Three species of *Fusarium*, i.e., *F. graminearum*, *F. sacchari* var. *subglutinans* (=*F. moniliforme* var. *subglutinans*), and *F. verticillioides* (=*F. moniliforme*), were isolated from the moldy corn kernels. Three *Fusarium* mycotoxins, i.e., moniliformin, deoxynivalenol, and zearalenone, were detected in the original sample of moldy kernels and at higher levels in a hand-selected subsample of visibly *Fusarium* infected kernels. This is the first indication of the natural occurrence of moniliformin. In culture, moniliformin was produced by isolates of *F. sacchari* var. *subglutinans* from the moldy corn, zearalenone by isolates of *F. graminearum*, and deoxynivalenol by an isolate of *F. graminearum*. Two isolates of *F. verticillioides* did not produce any of these three mycotoxins in culture.

Two mycotoxins produced by *Fusarium graminearum* Schwabe, i.e., deoxynivalenol and zearalenone (Figure 1), were recently reported to occur naturally in moldy corn of the 1977 crop produced in esophageal cancer areas in Transkei (Marasas et al., 1979c). In addition to *F. graminearum*, the Transkeian corn was also found to be infected by *F. verticillioides* (Sacc.) Nirenberg (=*F. moniliforme* Sheld) and by *F. sacchari* (Butl.) Gams var. *subglutinans* (Wr. & Rk.) Nirenberg (=*F. moniliforme* Sheld. var. *subglutinans* Wr. & Rk.). A Transkeian isolate of *F. sacchari* var. *subglutinans* has been shown to produce large quantities of moniliformin (Figure 1) in culture (Kriek et al., 1977), but this mycotoxin has not yet been demonstrated to occur naturally in corn.

A sample of moldy corn ears of the 1978 crop was obtained from a farm in the Butterworth District of Transkei during July 1978. Butterworth is the district with the highest esophageal cancer rate (57.9 per 100 000 per year) in Transkei (Rose and McGlashan, 1975). This sample was investigated mycologically and chemically in order to determine whether deoxynivalenol and zearalenone occurred naturally in corn produced in a high-incidence area of esophageal cancer in 1978. In addition, this sample was used to determine whether moniliformin occurs naturally in corn or not.

EXPERIMENTAL SECTION

General Instrumentation. A Micromeritics Model 7000 high-performance liquid chromatograph equipped with a Model 785 variable-wavelength detector was used. Paired ion chromatography separations were done on either a μBondapak C_{18} column (10 μm; 3.9 mm i.d. × 30 cm; Waters Associates) or a laboratory-packed Nucleosil 10 C_{18} column (10 μm; 5 mm i.d. × 25 cm; Macherey Nagel Co.). A laboratory-packed Partisil 10 SAX column (10 μm; 4.6 mm i.d. × 25 cm; Reeve Angel Co.) was used for anion-exchange chromatography.

Materials. A sample of moldy corn ears of the 1978 crop was obtained from a farm in the Butterworth District, Transkei, during July, 1978. Visibly *Fusarium* infected ears (=pink, red, or purple discolored ears) were selected from the original sample that also contained ears infected by *Diplodia maydis* (Berk) Sacc. and other fungi. These selected ears were shelled in a hand sheller, and the kernels divided into two groups. Half (3569 g) of the kernels obtained by shelling the ears showing signs of *Fusarium* infection (sample M-84) was retained as such, i.e., a mixture of *Fusarium*-infected kernels, healthy kernels, and kernels infected by other fungi. The other half was used for the selection of a subsample (M-84/F) of hand-selected, visibly *Fusarium* infected kernels (692.4 g, 17%).

Isolation and Culture of *Fusarium* Species. The incidence of *Fusarium* species in the two samples of moldy kernels were determined as described by Marasas et al. (1979a,c). Single-conidial isolates were made of representative cultures of the three *Fusarium* species encountered and maintained on potato dextrose agar slants. Conidial suspensions of each of two isolates of each species were used to inoculate autoclaved, yellow corn kernels (400 g of kernels in 400 mL of water in 2-L glass jars, autoclaved at 121 °C for 1 h on each of 2 consecutive days). Cultures on corn of each isolate were incubated either at 25 °C for 21 days or at 25 °C for 14 days followed by 14 days at 12 °C. After the required incubation period, the contents of the jars were harvested and dried at 50 °C for 24 h.

Chemical Analyses. The two samples of moldy corn kernels were ground in a coffee grinder. The dried culture material of the different *Fusarium* isolates were ground in a mortar and pestle.

(*a*) *Zearalenone Determination.* Zearalenone was analyzed in the meals prepared from the moldy kernels according to the method of Thomas et al. (1975). The presence of zearalenone in positive samples was further confirmed by intensification of its long-wave fluorescence by spraying with $AlCl_3$ (Roberts and Patterson, 1975). The culture material which was found to contain interfering substances was successfully cleaned up by the method of Mirocha et al. (1974) using the procedures described for batch extraction and base cleanup followed by a further cleanup on a Sephadex LH-20 column (Holder et al., 1977). Analytical thin-layer chromatography was carried out as described by Thomas et al. (1975) followed by spraying with $AlCl_3$. The limit of detection of zearalenone is ca. 50 μg/kg.

(*b*) *Trichothecene Analyses.* Deoxynivalenol, diacetoxyscirpenol, and T-2 toxin were analyzed in the meals prepared from the corn kernels as well as the culture material by the following modification of the method of Mirocha (1978).

The ground corn or culture material (50 g) was extracted with 200 mL of MeOH–1% NaCl (55:45) and 100 mL of petroleum ether (60–80°) by shaking for 30 min. The sample extract was filtered into a 500-mL Buchner flask through Whatman No. 1 filter paper by using water suc-

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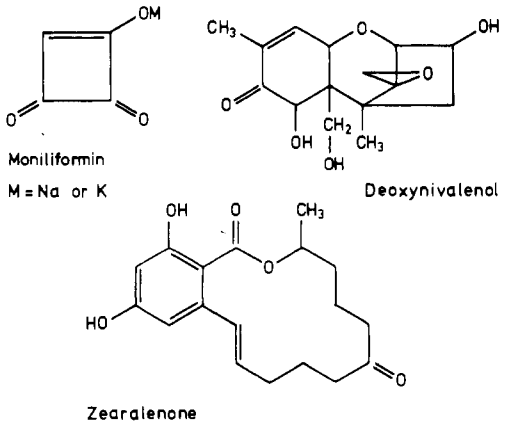

Figure 1. Chemical structures of moniliformin, zearalenone, and deoxynivalenol.

tion. The filtrate was transferred to a 500-mL separatory funnel, and the petroleum ether layer was discarded. The remaining aqueous MeOH layer was extracted with a further 60 mL of petroleum ether and again discarded followed by 3 × 50 mL portions of $CHCl_3$. The $CHCl_3$ extracts were combined in a 250-mL round-bottom flask and evaporated to dryness on a rotary evaporator. The flask was rinsed with up to 10 mL of acetone which was transferred to a small flask or vial and evaporated to dryness. The residue was reconstituted with 2–3 mL of MeOH–H_2O (2:3). A Sep Pak C_{18} (Waters) column was prepared by passing 2 mL of MeOH followed by 5 mL of distilled water through the column. The sample was filtered (Whatman No. 1) into a 10-mL syringe to remove any precipitate formed and pushed through the Sep Pak column into a vial. The flask was rinsed with a further 1–2 mL of MeOH–H_2O (2:3) solution and also pushed through the column. The contents of the vial was dried under a stream of nitrogen while gently heating with a hair dryer from time to time. The residue was dissolved in 200 μL of acetone, and the samples were analyzed by using silica gel thin-layer chromatography and the solvent system $CHCl_3$–MeOH (5:1) as the mobile phase.

The plates were air-dried and sprayed with *p*-anisaldehyde solution (7 mL of MeOH, 1 mL of glacial acetic acid, 0.5 mL of concentrated H_2SO_4, and 0.5 mL of *p*-anisaldehyde) followed by heating with a hair dryer to visualize the trichothecenes. All positive samples were confirmed by using three other developing solvent systems: (1) $CHCl_3$ followed by benzene–acetone (3:2), (2) $CHCl_3$–acetone (4:1) developed 3 times, and (3) toluene–ethyl acetate–acetone (3:2:1) developed twice followed by $CHCl_3$–acetone–propanol (85:10:5). The limit of detection for deoxynivalenol is ca. 250 μg/kg.

(c) *Moniliformin Analyses.* Moniliformin was determined on the two samples of moldy corn kernels and in the dried culture material of the different *Fusarium* isolates by high-performance liquid chromatography (HPLC). The results obtained by employing a strong anion-exchange column was substantiated by separation and quantification of moniliformin using a paired ion chromatography (PIC) technique on a reverse-phase column. HPLC analyses were carried out on both water extracts of the meal samples and water extracts prepurified on columns of DEAE-Sephadex.

Sample extracts were prepared by shaking 3 g of the dry meal with 40 mL of distilled water on a rotary shaker for 2 h at room temperature in centrifuge tubes. The contents were then centrifuged for 20 min at 10000 *g*, and the supernatant extracts were filtered through Millipore filters (0.45 μm) and retained for HPLC analyses or for prepurification on a DEAE-Sephadex column.

Table I. Incidence of *Fusarium* Species in Moldy Corn Kernels and in Hand-Selected Visibly *Fusarium* Infected Corn Kernels from Butterworth, Transkei

	% of kernels infected[a]	
Fusarium species	moldy corn kernels (sample M-84)	hand-selected visibly *Fusarium* infected corn kernels (sample M-84/F)
F. graminearum	19	21
F. sacchari var. *subglutinans*	30	43
F. verticillioides	37	35
total *Fusarium*	86	99

[a] Based on 100 surface-sterilized kernels of each sample. Some kernels were infected by more than one *Fusarium* species.

DEAE-Sephadex A-50 (Pharmacia Fine Chemicals) was equilibrated with 0.1 M ammonium acetate, and a column (1 cm i.d. × 10 cm) was packed under gravity. The water extract (10 mL) was applied to the column and was eluted with 0.1 M ammonium acetate at a flow rate of 0.5 mL/min. Moniliformin eluted between 60 and 120 mL after commencing sample application. This fraction of the eluate was lyophilized and the dried material dissolved in 1 mL of distilled water for HPLC analysis.

Ion-exchange chromatography separations were done on a strong anion-exchange column (Partisil 10 SAX) eluting at a flow rate of 1.0 mL/min with 0.01 M sodium dihydrogen phosphate (pH 5.0). The sample was applied by means of a loop injector (20 μL) and the moniliformin detected at 227 nm.

Paired ion chromatography (PIC) separations were done on either a μBondapak C_{18} (Waters Associates) or a laboratory-packed Nucleosil 10 C_{18} (Machery-Nagel Co.) column. The columns were eluted at 1.0 mL/min with either 0.1 M sodium phosphate buffer [pH 7.0; 0.005 M tetrabutylammonium hydrogen sulfate (TBAS); 8% methanol] or PIC-A reagent (Waters Associates) in 8 or 20% methanol. The sample was applied and the moniliformin monitored as in the ion-exchange procedure.

"Spiking" of extracts with a moniliformin standard was done by adding 10 μL of a moniliformin solution (10 mg/L) to 100 μL of the appropriate extracts.

RESULTS AND DISCUSSION

The levels of infection of the sample of moldy corn kernels (M-84) and the sample of hand-selected, visibly *Fusarium* infected corn kernels (M-84/F) by different *Fusarium* species are given in Table I. The same three species, i.e., *F. graminearum*, *F. sacchari* var. *subglutinans*, and *F. verticillioides* were isolated from both samples. The most prevalent species in the original sample of moldy kernels was *F. verticillioides* while *F. sacchari* var. *subglutinans* predominated in the sample of visibly *Fusarium* infected kernels.

Peaks coinciding perfectly with that of authentic moniliformin were shown to be present in chromatograms of prepurified water extracts of both samples M-84 and M-84/F when separation was achieved on the Nucleosil 10 C_{18} column with PIC-A reagent in 8% methanol as the mobile phase (Figure 2). This observation was substantiated by the demonstration of well-resolved peaks in the position of moniliformin in prepurified extracts of the two samples using the μBondapak C_{18} column with 0.1 M sodium phosphate buffer (pH 7.0; 0.005 M TBAS; 8%

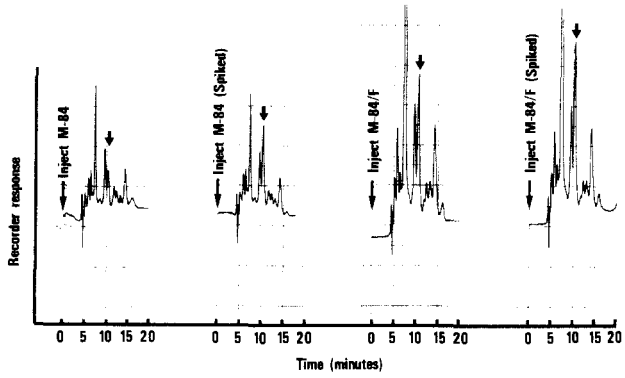

Figure 2. Separation of prepurified and spiked extracts of samples M-84 and M-84/F by paired ion chromatography. Column: Nucleosil 10 C_{18}. Solvent: Waters Associates PIC-A in 8% methanol. Chart speed: 0.1 in./min. AUFS: 0.05.

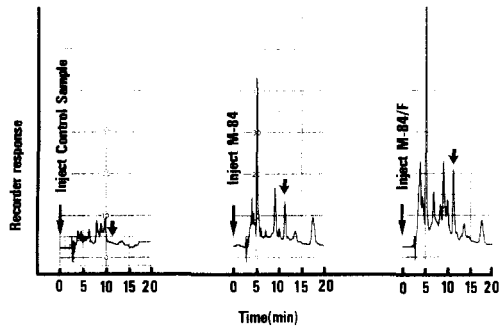

Figure 3. Separation of prepurified extracts of a control sample and samples M-84 and M-84/F by paired ion chromatography. Column: μBondapak C_{18}. Solvent: 0.1 M sodium phosphate buffer (pH 7.0; 0.005 M TBAS; 8% methanol). Chart speed: 0.1 in./min. AUFS: 0.1.

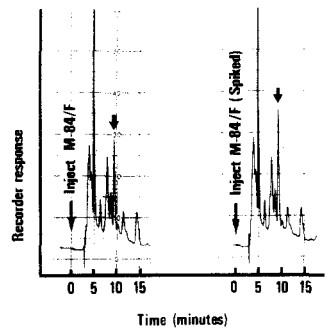

Figure 4. Separation of a prepurified and spiked extract of sample M-84/F by paired ion chromatography. Column: μBondapak C_{18}. Solvent: 0.1 M sodium phosphate buffer (pH 7.0; 0.005 TBAS; 8% methanol). Chart speed: 0.1 in./min. AUFS: 0.1.

methanol) as the mobile phase while an extract of uncontaminated corn meal showed no peak in this position (Fgure 3). Upon spiking the prepurified extract of sample M-84/F with authentic moniliformin, an increased single symmetrical peak was present in the position for moniliformin under chromatographic conditions similar to those in Figure 3 (Figure 4).

The recoveries of moniliformin in the prepurification step were low and varied considerably. Separations were therefore also done directly on the water extract without prepurification. The chromatograms obtained by separation of the water extracts and extracts spiked with authentic moniliformin on the Nucleosil 10 C_{18} column with PIC-A reagent in 8% methanol are given in Figure 5. Both samples M-84 and M-84/F contained a peak in the position of moniliformin. Although this peak was not well

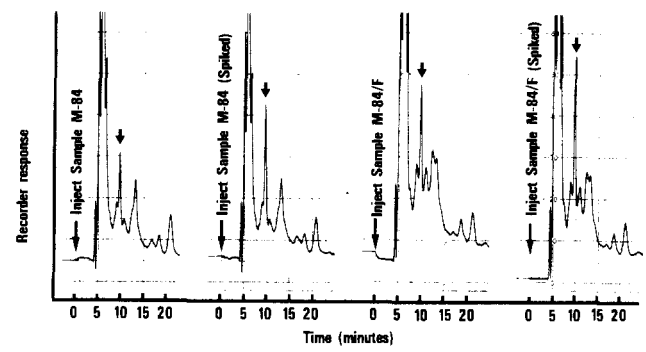

Figure 5. Separation of unpurified extracts and spiked extracts of samples M-84 and M-84/F by paired ion chromatography. Column: Nucleosil 10 C_{18}. Solvent: Waters Associates PIC-A in 8% methanol. Chart speed: 0.1 in./min. AUFS: 0.05.

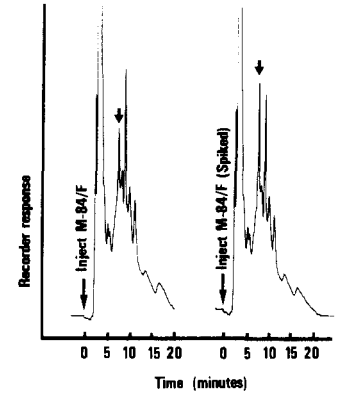

Figure 6. Separation of an unpurified and spiked extract of sample M-84/F by ion-exchange chromatography. Column: Partisil 10 SAX. Solvent: 0.1 M NaH_2PO_4 (pH 5.0). Chart speed: 0.1 in./min. AUFS: 0.05.

Table II. Levels of Moniliformin, Deoxynivalenol, and Zearalenone in Moldy Corn Kernels and in Hand-Selected, *Fusarium*-Infected Corn Kernels from Butterworth, Transkei

	Fusarium toxin content, mg/kg	
Fusarium toxin	moldy corn kernels (sample M-84)	hand-selected visibly *Fusarium* infected kernels (sample M-84/F)
moniliformin	16	25
deoxynivalenol	0.42	2.50
zearalenone	4.00	8.00

resolved from adjacent peaks, an increased single sharp peak was still present in the same position after spiking of the extracts with pure moniliformin. A peak in the moniliformin position was also present when the water extracts were chromatographed by ion-exchange chromatography on the Partisil 10 SAX column using 0.1 M sodium hydrogen phosphate (pH 5.0) as the mobile phase. An example of the analysis of sample M-84/F is given in Figure 6. Similar results were obtained for sample M-84 on the strong anion-exchange column.

In conclusion, it can be stated that the presence of moniliformin in both samples of moldy corn kernels seemed to be verified under different chromatographic conditions varying widely in the principles whereby separation was achieved. In all cases the "moniliformin peaks" coincided excellently with that of authentic moniliformin as demonstrated by a comparison of the chromatograms

Table III. Production of Moniliformin, Deoxynivalenol, and Zearalenone in Culture by *Fusarium* Species Isolated from Moldy Corn from Butterworth, Transkei[a]

Fusarium species	isolate no.	incubation regime[b]	*Fusarium* toxin content, mg/kg[c] monili-formin	deoxyni-valenol	zear-alenone
F. graminearum	MRC 1900	1	–	–	8.9
		2	–	–	13.7
F. graminearum	MRC 1901	1	–	3.75	26.1
		2	–	2.5	5.3
F. sacchari var. *subglutinans*	MRC 1898	1	67.2	–	–
		2	67.0	–	–
F. sacchari var. *subglutinans*	MRC 1899	1	86.7	–	–
		2	94.0	–	–
F. verticillioides	MRC 1896	1	–	–	–
		2	–	–	–
F. verticillioides	MRC 1897	1	–	–	–
		2	–	–	–

[a] Each isolate was cultured on moistened, autoclaved, yellow corn kernels. [b] Corn cultures were incubated either at 25 °C for 21 days (=1) or at 25 °C for 14 days followed by 12 °C for 14 days (=2). [c] A dash (-) indicates that a particular toxin was not chemically detectable. Detection limits: moniliformin = 1.00 mg/kg; deoxynivalenol = 0.25 mg/kg; zearalenone = 0.05 mg/kg. No T-2 toxin or diacetoxyscirpenol could be detected in any of the cultures by using the method described.

of spiked extracts with those of the extracts alone.

Apart from moniliformin, deoxynivalenol and zearalenone were detected chemically in both samples of moldy corn kernels (Table II). The sample of hand-selected, visibly *Fusarium* infected kernels contained higher levels of all three of *Fusarium* mycotoxins than the original sample of moldy corn kernels. Neither of the samples contained chemically detectable amounts of diacetoxyscirpenol or T-2 toxin when the method described was used.

In culture on autoclaved maize, moniliformin was produced by both isolates of *F. sacchari* var. *subglutinans* tested but by none of the isolates of either *F. graminearum* or *F. verticillioides* (Table III). Both isolates of *F. graminearum* produced zearalenone in culture but only one produced chemically detectable amounts of deoxynivalenol. Diacetoxyscirpenol and T-2 toxin were not detected in the culture material of any of the isolates tested. Two isolates of *F. verticillioides* did not produce any of the five *Fusarium* mycotoxins analyzed for.

It is concluded from these analyses that the moniliformin found to occur naturally in the two samples of moldy Transkeian corn was produced by *F. sacchari* var. *subglutinans* infecting the kernels and the deoxynivalenol and zearalenone by *F. graminearum*. It seems that *F. verticillioides* did not contribute to the presence of any of these three compounds in the moldy corn and if this fungus elaborated any mycotoxins in the naturally infected corn, they are unknown. Although three North American strains of *F. verticillioides* have been reported to produce moniliformin in culture (Burmeister et al., 1979; Cole et al., 1973; Springer et al., 1974), this mycotoxin was not produced by any of 14 toxic strains of this species isolated from South African corn (Marasas et al., 1979a). On the other hand, moniliformin was produced in culture at levels ranging from 120 to 1170 mg/kg by 16 of 23 toxic South African isolates of *F. sacchari* var. *subglutinans* (Marasas et al., 1979a), and a highly toxic isolate of this species from Transkeian corn has been found to produce up to 11.3 g/kg moniliformin in culture (Kriek et al., 1977). Although some strains of *F. verticillioides* have been reported to produce low levels of zearalenone in culture (Hacking et al., 1976; Kotsonis et al., 1975; Mirocha and Christensen, 1974; Mirocha et al., 1969), neither of the two Transkeian isolates produced chemically detectable levels of zearalenone in culture. In the present study zearlenone as well as deoxynivalenol were produced in culture only by *F. graminearum*, a species which is known to produce both of these mycotoxins (Lindenfelser et al., 1978; Marasas et al., 1977; Mirocha et al., 1976; Pathre and Mirocha, 1977, 1978).

The levels of deoxynivalenol and zearalenone found in the moldy corn kernels of the 1978 crop produced in Butterworth were within the same range as those detected in corn of the 1977 crop from the same area (Marasas et al., 1979c). The present finding that moniliformin also occurred naturally in the moldy corn kernels is of interest because moniliformin is a highly toxic compound (Cole et al., 1973) that causes remarkable pathological changes including myocardial degeneration and necrosis in experimental animals (Kriek et al., 1977) and the mechanism of action of which is a selective inhibition of mitochondrial pyruvate and α-ketoglutarate oxidation (Thiel, 1978). This is the first indication of the natural occurrence of moniliformin in corn. The fact that moniliformin is also produced in culture by several other *Fusarium* species, i.e., *F. fusarioides* (Frag. and Cif.) Booth (Rabie et al., 1978), *F. avenaceum* (Corda ex Fr.) Sacc. (Marasas et al., 1979b), and *F. oxysporum* Schlecht. (Marasas et al., 1979b), isolated from substrates such as barley, millet, and peanuts, raises the question of the possible natural occurrence of moniliformin in other agricultural products.

At present there is no evidence to implicate either moniliformin, deoxynivalenol, or zearalenone in the etiology of esophageal cancer in Transkei. However, the co-occurrence of these three compounds in corn, albeit hand-selected visibly *Fusarium* infected kernels, intended for human consumption, certainly warrants further investigation of the potential danger to human health of these and possibly other *Fusarium* mycotoxins.

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Composition of Peas (*Pisum sativum*) Varying Widely in Protein Content

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The composition of one variety of field peas (*Pisum sativum* L. cv. Trapper) containing 14.5–28.5% protein (dry, dehulled basis) was investigated. The variability in protein content did not appear to be related to the degree of maturation. Seventy-two percent of the difference in protein content between the highest and lowest protein content pea was accounted for by starch, 7.9% by lipid, 8.0% by neutral detergent fiber (NDF), 6.8% by sugars, and 3.6% by ash. Starch, lipid, NDF, sucrose, and nonprotein nitrogen content, the nitrogen solubility index, and zinc content were negatively correlated with pea protein content. In addition, the amino acids threonine, cystine, glycine, alanine, methionine, and lysine (expressed as milligrams of amino acid per gram of N) were similarly correlated. Total α-galactosides, verbascose, glutamic acid, arginine, total pigments, and carotenoid pigments were positively correlated with protein content. The amino acid score, an index of nutritional quality, was negatively correlated with protein content. Xanthophylls (monohydroxy type) accounted for most of the difference in carotenoid content between the high and low protein content peas.

The protein content of pea seeds appears to be highly variable and is influenced by both genetic and environmental factors. Slinkard (1972) analyzed 1452 pea varieties from the U.S. Department of Agriculture world collection grown in Saskatchewan. Protein contents ranged from 15.5 to 39.7%. Gottschalk et al. (1975) found that genetically identical pea plants grown in the same year on the same field produced seeds containing protein contents ranging from 19.3 to 25.2%. The 1970 field pea crop in Saskatchewan was seeded mostly to the Century variety and showed nitrogen contents of 2.43–4.06% (McLean, 1972). Since then a new variety (Trapper) has been introduced which presently accounts for over 75% of the acreage seeded to peas. A survey by the authors of farmers peas (n = 198) obtained in 1979 showed that these ranged from 13.3 to 27.1% protein on a dry, whole seed basis.

The environmental factors responsible for the very wide range in pea protein content are not fully understood. McLean et al. (1974) reported that pea protein contents were increased from 20 to 30% by application of nitrogen fertilizer. Holl and Vose (1980) found that seed protein was deposited early in the development of the seed but decreased (as a component of dry weight) with maturation. The largest decrease (27.5% protein at 15 days after anthesis to 20.7% at 80 days) was observed in the absence of added nitrogen fertilizer. Eppendorfer and Bille (1974) reported that increasing soil phosphorus and decreasing soil potassium increased pea nitrogen content from 3.5 to 4.9%. Robertson et al. (1962) analyzed pea seed protein contents up to the 30th day after flowering and found that protein synthesis was markedly delayed and reduced at low growth temperatures. Application of s-triazines to the leaves of young plants increased the protein content of bean and pea seeds (Singh et al., 1972).

The objective of this study was to determine the chemical composition of pea samples as a function of a very wide range in protein content (14.5–28.5% on a dry, dehulled seed basis). The components that make up the large difference in protein content and the correlations between protein content and chemical components were determined. Compositional data of this nature is important from the nutritional standpoint, since field peas are des-

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