Soybean Isoflavones. Characterization, Determination, and Antifungal Activity

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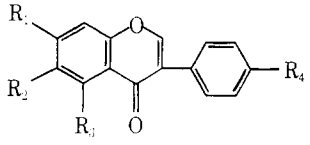
The isolation and separation of defatted soybean meal isoflavone glycosides by chelation with aluminum oxide and subsequent chromatography on polyamide columns have been described. A procedure for the quantitative determination *via* gas chromatography of isoflavones in soybean meal was elaborated. The resulting analytical data showed that the content of isoflavones in soybeans amounts to 0.25% and that 99% of the isoflavones are present as glycosides: 64% genistin, 23% daidzin, and 13% glycitein 7-*O*-β-glycoside. Soybean oil does not contain isoflavones. The growth depression of *Trichoderma lignorum, Rhizoctonia solani, Fusarium oxysporum, Pythium spp, Rhizopus spp,* and *Sclerotium rolfsii* exerted by soybean isoflavones is due mainly to the presence of free isoflavones in the growth medium, while the activity of the glycosides is in most instances negligible.

Soybeans have been known to contan the two isoflavone glycosides genistin and daidzin and their respective aglycones (Walz, 1931; Walter, 1941). Evidence has been provided recently on the presence of a third isoflavone, glycitein, the structure of which has been determined (Naim *et al.*, 1973).

The structures of the known soybean isoflavones are as follows.



genistein, R_1 = OH; R_2 = H; R_3 = OH; R_4 = OH
genistin, R_1 = *O*-glucosyl; R_2 = H; R_3 = OH; R_4 = OH
daidzein, R_1 = OH; R_2 = H; R_3 = H; R_4 = OH
daidzin, R_1 = *O*-glucosyl; R_2 = H; R_3 = H; R_4 = OH
glycitein, R_1 = OH; R_2 = OCH_3; R_3 = H; R_4 = OH
glycitein 7-β-*O*-glucoside, R_1 = *O*-glucosyl; R_2 = OCH_3; R_3 = H; R_4 = OH

The wide use of soybeans as a protein source for humans and farm animals implies the quantitative assessment and evaluation of minor constituents, such as isoflavones at the levels normally ingested. It has been the aim of the present study to isolate and separate the native free isoflavones and isoflavone glycosides in soybeans and to elaborate a reliable analytical method for their quantitative determination. In view of the presence of other glycosides of genistein in different plants (Zemplen *et al.*, 1943; Szabo *et al.*, 1958), it was of interest to establish the position of glycosidic linkages in soybean isoflavone glycosides.

Since antifungal factors in red clover have been identified as isoflavones (Virtanen and Hietala, 1958; Bredenberg, 1961), and isoflavonoids were recognized as phytoalexins in various plants (Kuc, 1972), and also in infected soya plants (Keen *et al.*, 1971), it has been attempted to study the effect of soybean isoflavones on pathogenic fungi and to relate fungistatic activity to their composition and structure.

EXPERIMENTAL SECTION

Isolation, Separation, and Identification of Isoflavone Glycosides. Isoflavone glycosides were extracted on a preparative scale from an aqueous syrup (Chayot Industries, Ashdod, Israel). The syrup, which contains about 50% dry matter, was obtained as a residue after extraction with 60% ethanol of commercially defatted soybean flakes and subsequent evaporation of the ethanol. Ten kilograms of soybean flakes yielded approximately 3 kg of syrup.

A portion of the syrup (3.5 l.) was stirred with two volumes of acetone for 2 hr at room temperature for extraction of the isoflavones. The isolation and separation of the isoflavone glycosides were carried out according to Naim *et al.* (1973). The glycosides were separated by chelation of those bearing an unsubstituted hydroxyl group at C_5 (such as genistin) with aluminum oxide G (Merck) (Wang, 1971). The nonchelated glycosides of daidzein and glycitein were washed out with 50% MeOH, filtered through Whatman paper 42, and separated by column chromatography as described later. The chelated glycoside (genistin) can be liberated from the cake by washing with 4% (w/v) HCl in methanol. Since the procedure did not enable full separation of genistin from accompanying aluminum oxide, pure genistin was obtained by recrystallization of the glycosides from 80% ethanol according to Walter (1941).

After evaporation of the solvent the nonchelated glycosides were separated by column chromatography on polyamide. Polyamide (Woelm) was mixed with an equal weight of Celite 545 and then equilibrated in ethyl acetate and poured into a 2.5 × 70 cm column. A 200-mg portion of the nonchelated glycosides was suspended in a small amount of methanol and applied to the column. Elution was started with ethyl acetate and followed by solutions of 8 and 10% methanol in ethyl acetate. Fractions of 10 ml were collected and their absorbancy was measured at 260 nm. The fractions were evaporated to dryness and analyzed for isoflavones as described later.

Separation and Identification of Free Isoflavones. Individual glycosides, obtained as described above, or mixtures of glycosides were subjected to acid hydrolysis according to Naim *et al.* (1973). For analyses of isoflavone aglycones, tlc and glc were used.

Tlc was performed on Kieselgel G (Merck) with the following solvent mixtures: (A) chloroform–methanol (9:1) (Beck, 1964); (B) benzene–ethyl acetate–petroleum ether (bp 40–60°)–methanol (6:4:3:1) (Barz, 1969); (C) ether–petroleum ether (bp 40–60°) (7:3) (Barz, 1969).

The isoflavones on the chromatoplates were detected either with the Folin–Ciocalteau reagent in an ammonia-saturated chamber (Beck, 1964) or with the aid of an ultraviolet lamp at 366 nm (Beck, 1964). R_f values were quoted relative to genistein. Solvent C was used for preparative purposes by double development in the same direction.

Glc was performed on trimethylsilyl derivatives using a 2 m × 0.52 cm column of 0.75% SE-30 on Gas Chrom Q at an operating temperature of 220°, as described by Lindner (1967). T_r values were quoted relative to formononetin.

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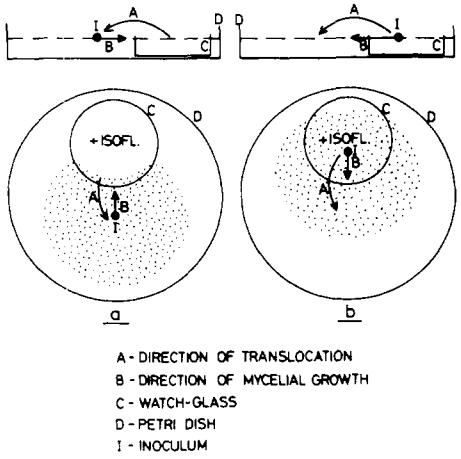

Figure 1. Schematic presentation of translocation of isoflavone aglycones in *R. solani*.

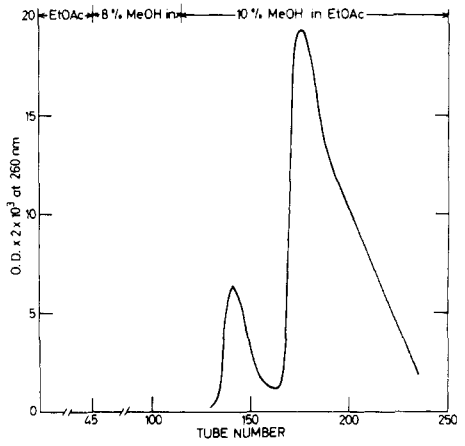

Figure 2. Separation of glycitein 7-*O*-β-glycoside and daidzin by chromatography on a polyamide column.

Characterization of Isoflavones. Uv spectra were determined in a Beckman DB-G spectrophotometer. Spectroscopic grade methanol was used as solvent. The effects of NaOMe, $AlCl_3$–HCl, NaOAc, and NaOAc–H_3BO_3 on the uv spectra were examined as described by Mabry *et al.* (1970).

Analysis of the Carbohydrate Moiety. The configuration and constitution of the carbohydrate moiety and its linking position to the aglycone were determined by methylation of separated isoflavone glycosides according to the procedure of Kuhn *et al.* (1955). After two successive methylations, methanolysis was carried out in 4% (w/v) methanol–HCl for 6 hr. Glc of the methylated sugars was performed on 2 m × 0.32 cm columns of Chromosorb W (AW), coated with 5% (w/w) neopentyl glycol adipate polyester at an operating temperature of 150° (Aspinall *et al.*, 1968). T_r values are quoted relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Quantitative Determination of Isoflavones in Soybeans. For this determination 250 g of the finely ground soybean flour (Wayne variety, 1969 crop) was exhaustively extracted first with ether and then with absolute methanol in a soxhlet apparatus. After evaporation of the ether, the free isoflavones present in the oil were extracted with three portions (100 ml each) of absolute methanol. The methanolic extracts of the oil as well as of the soybean flour were concentrated to a syrup. A 50-g portion of the soybean flour residue (after extraction with ether and methanol) was subjected to acid hydrolysis in 1 *N* H_2SO_4 in dioxane–water (1:3), for 10 hr and the liberated isoflavones were extracted with ether. The three extracts, *i.e.*, the methanol extracts of the oil, the methanol extract of the soybean flour, and the ether extract of the acid hydrolysate of the residual flour, were subjected to polyamide column chromatography. Elution was performed with ethyl acetate and then by solutions of 5, 7 (for aglycones), and 10% methanol (for glycosides) in ethyl acetate successively. Fractionation was followed up by measuring the absorbancy of the fractions at 260 nm, and the fractions were then evaporated to dryness. The fractions which contained isoflavones were analyzed by glc according to Lindner (1967). The free isoflavone content of the fractions which were high in lipids could not be determined by glc, since they contained impurities, which had retention times identical with those of isoflavones. The amount of free isoflavones was determined therefore by the tlc method of Beck (1964).

Effect of Isoflavones on Growth of Fungi. Isoflavone effect was determined by either of two methods. (*a*) *Determination of Dry Weight as a Measure of Growth. R. solani* was grown in 20 ml of growth medium which contained 0.5% yeast extract (Difco), 0.5% Bacto-peptone (Difco), and 2% glucose. After 48 hr, disks (5 mm diameter) of mycelium were cut from the margins of the colony and transferred to flasks containing 20 ml of yeast extract broth supplemented with 0.1% of soybean isoflavones. The flasks were incubated at 28° in a shaking water bath adjusted to 100 strokes/min. Mycelial growth was allowed to proceed for 72 hr, and flasks were withdrawn at 24-hr intervals (five replicates each time). The mycelia of each flask were separated from the growth medium by washing on a Buchner funnel, then transferred to previously dried and weighed petri dishes and dried at 90° until they reached constant weight.

(*b*) *Measurement of Mycelial Growth of Various Fungi.* The effect of isoflavones on mycelial growth was assayed on the following fungi: *Trichoderma lignorum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium spp.*, *Rhizopus, spp.*, and *Sclerotium rolfsii* (received from the Department of Microbiology and Phytopathology, the Faculty of Agriculture, Rehovot, Israel). The fungi were grown on potato dextrose agar (PDA), according to Johnson *et al.* (1959), in petri plates (85 mm in diameter) with 15 ml of medium. Disks (5 mm in diameter) of agar with mycelium were cut from the margins of 48-hr-old colonies with the aid of a sterile cork-borer and transferred to petri plates containing PDA supplemented with various amounts of isoflavones. Mycelial growth was allowed to proceed at 28° until the mycelium in the control reached the margin of the petri plate. Each experiment was carried out in five replicates, and mycelial growth of each fungus was determined by the area covered by the mycelium.

Liberation of aglycones from their glycosides into the growth medium was followed by tlc (Beck, 1964). The effect of methylated free isoflavones on *R. solani* was also examined. A mixture of methylated free isoflavones was prepared by treating the mixture of free isoflavones with CH_2N_2 in ether during 3 days. Absorption and translocation of free isoflavones by *R. solani* were studied by the "dish technique" as described by Schutte (1956) (Figure 1).

RESULTS AND DISCUSSION

Characterization of Soybean Isoflavone Glycosides. The results obtained by separation of the nonchelated glycosides on a polyamide column are shown in Figure 2. After acid hydrolysis of the fractions glc and tlc analyses of the aglycones revealed that 10% methanol in ethyl acetate elutes glycitein 7-*O*-β-glycoside which is followed by daidzin. When genistin is not removed by chelation prior to column chromatography, it is also eluted with the same solvent and overlaps the peak of glycitein 7-β-*O*-glucoside. A very good separation between the previously nonsepara-

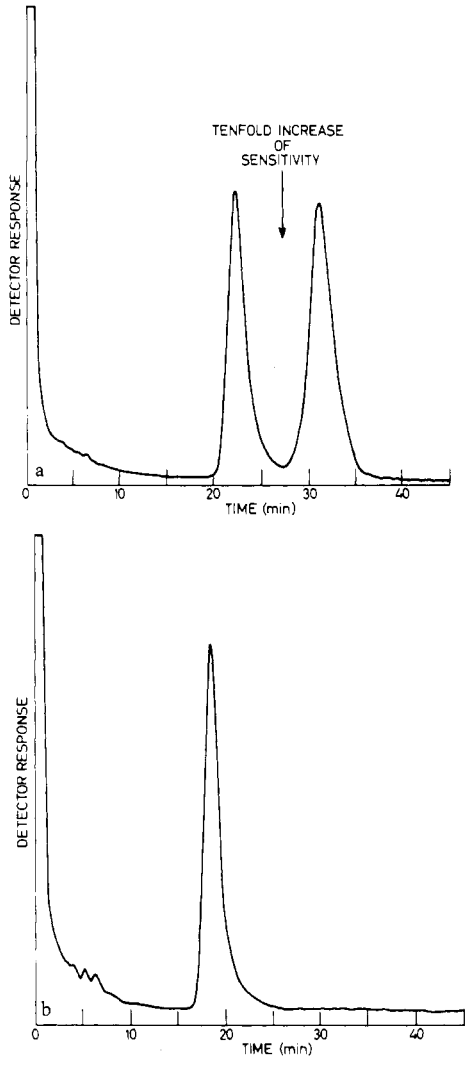

Figure 3. Gas–liquid chromatographic separation of free isoflavones, obtained by acid hydrolysis of their glycosides, eluted from a polyamide column with 10% MeOH in EtOAc: (a) first fraction; (b) second fraction.

ble genistin and daidzin could be achieved by this means (Figures 3a and b). Recovery tests, conducted with a mixture of known amounts of the glycosides, gave yields of 85–86% from the column.

The aglycones in the hydrolysates of mixtures of glycosides were identified by tlc and glc (Table I). The per cent composition of the mixtures was 87.5% genistein, 10% daidzein, and 2.5% glycitein. No additional isoflavones were present.

Gas chromatographic analysis of the separated methylated glycosides showed the presence of methyl 2,3,4,6-tetra-*O*-methylglucopyranoside as the only sugar derivative. This finding excludes the possibility of a carbohydrate chain attached to the aglycone, but it does not rule out the attachment of several glucose residues, each at another point, to the aglycone (Zemplen *et al.*, 1943; Szabo *et al.*, 1958). This possibility was, however, disproved by the following experimental results. Uv spectral analysis of the glycoside fraction, which contains genistein as its aglycone, did not show a bathochromic shift of band II after addition of NaOAc, but such a shift was found after acid hydrolysis, indicating that the OH group at C_7 provides a glycosidic linkage (Mabry *et al.*, 1970). Quantitative determination of the components of an acid hydrolysate of this fraction gave a 1:1.4 weight ratio between sugar and aglycone, corresponding to a 1:1 molar ratio, *i.e.* this fraction contains solely genistin. The conversion of

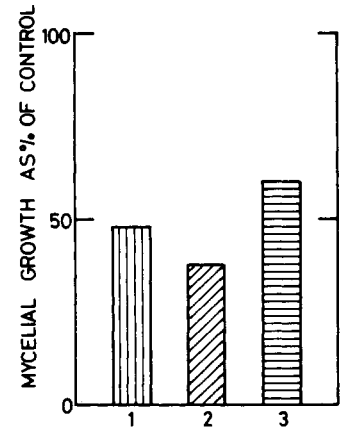

Figure 4. Effect of a mixture of isoflavone aglycones (0.1%) in growth medium on growth of *R. solani:* (1) native aglycones; (2) methylated aglycones (both measured by area of mycelium); (3) native aglycones (measured by dry weight).

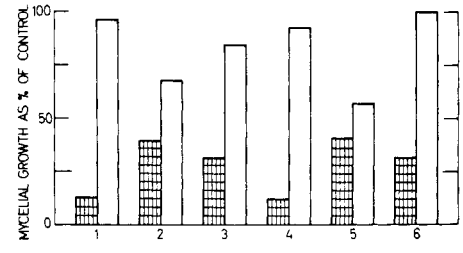

Figure 5. Effect of 0.1% of mixtures of isoflavone aglycones (▦) or isoflavone glycosides (□) in growth medium on mycelial growth of various fungi: (1) *Trichoderma lignorum;* (2) *Rhizoctonia solani;* (3) *Fusarium oxysporum;* (4) *Pythium spp;* (5) *Sclerotium rolfsii;* (6) *Rhizopus spp.*

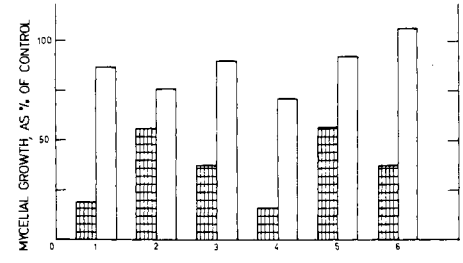

Figure 6. Effect of 0.005% of mixtures of isoflavone aglycones (▦) or isoflavone glycosides (□) in growth medium on mycelial growth of various fungi; see caption to Figure 5 for 1–6.

the glycoside of daidzein to formononetin (7-hydroxy-4′-methoxyisoflavone) or of the glycoside of glycitein to afrormosin (7-hydroxy-4′,6-dimethoxyisoflavone) could only have occurred when the sugar moiety was linked exclusively to the OH group at C_7 (Naim *et al.*, 1973). Thus it could be concluded that the carbohydrate moiety of each glycoside is composed only of one glucose residue, which is invariably linked to C_7 of the aglycone.

The uv spectrum of glycitein 7-*O*-β-glucoside had the following absorption: (MeOH) λ_{max} 318, 259, 228 (sh); (MeOH + NaOMe) 322 (sh), 272, 223; (MeOH + $AlCl_3$) 312, 264, 228 (sh); (MeOH + $AlCl_3$–HCl) 312, 263, 228 (sh); (MeOH + NaOAc) 316, 260, 229; (MeOH + NaOAc–H_3BO_3) 318, 260, 230.

Quantitative Determination of Isoflavones in Soybeans. The quantitative determination of isoflavones was performed in the ether extract of soybean meal, in the methanol extract of soybean meal, and in the acid hydrolysate of the residual soybean flour after etheric and methanolic extractions. These three extracts were subject-

Table I. Characterization of Soybean Meal Isoflavone Aglycones by Tlc and Glc[a]

Sample	Glc, 0.75% SE-30 on Gas Chrom Q	Tlc with solvent			Color appearance upon irrad. at 360 nm	Reaction with Folin–Ciocalteau reagent
		B	A	C		
Genistein	1.65	1	1	1	Deep purple	+
Daidzein	1.40	0.65	0.76	0.54	Fluorescent green	+
Glycitein	2.3	0.53	0.89	0.32	Fluorescent light blue	+

[a] R_f genistein values were used in tlc and T_R formononetin values were used in glc of soybean isoflavones.

Table II. Content of the Different Isoflavones Present in the Ether Extract of Whole Soybean Meal, in the Methanol Extract of Soybean Meal, and in the Acid Hydrolysate of the Residual Soybean Flour after Etheric and Methanolic Extractions

Fraction	mg/100 g of soybeans					
	Genistein	Genistin	Daidzein	Daidzin	Glycitein	Glycitein 7-*O*-β-glucoside
Ether	0.2		0.3		0.01	
Methanol	1.2	157.2	0.3	56.1	0.1	32.1
Residue		7.2		2.0		1.7
Total	1.4	164.4	0.6	58.1	0.11	33.8

ed to polyamide column chromatography of the nonchelated glycosides. Glc analyses of the emerging peaks showed that the peaks corresponding to the glycosides which resulted from the methanolic extract of soybean were free from interfering substances (Figures 3a and b). After hydrolysis the isoflavones could be determined by glc according to the gas chromatographic procedure of Lindner (1967). The transfer through the polyamide column and the hydrolytic treatment, which resulted in losses of 15 and 5%, respectively, were taken into account.

Since accompanying substances interfered with the quantitative gas chromatographic determination of free isoflavones in the etheric and methanolic extracts of soybean meal, their amount (traces) was determined by the method of Beck (1964). As shown in Table II, practically all the isoflavones in soybeans are present as glycosides, and the free isoflavones amount to less than 1% of the total. About 4% of the glycosides were still found in the residual soybean flour, obtained after etheric and methanolic extractions. The total percentage of isoflavones in soybeans is about 0.25%. The amount of genistin is considerably higher than of those of the two other isoflavones.

The finding that soybeans contain isoflavone aglycones which are soluble in ether raised the question of their possible presence in commercial soybean oil. Samples of 200 ml of commercial soybean oil (Shemen Co., Haifa, Israel) were subjected to the procedure of isolation of free isoflavones, including column chromatography on polyamide. No free isoflavone aglycones could be identified in the various fractions by tlc and glc. It may therefore be concluded that no detectable isoflavone aglycones are present in commercial soybean oil, probably since the commercial process uses petroleum ether for extraction.

Effect of Soybean Isoflavones on Growth of Fungi. Isoflavones exert pronounced fungistatic activity, as measured by the area of the mycelium as well as by the dry matter content (Figures 4–6).

It is remarkable that a significant fungistatic activity was noted at concentrations as low as 0.005% of free isoflavones (Figure 5) and it was not exceeded when the concentration was raised to 0.1% (Figure 6). The fungistatic potency of isoflavone glycosides was, however, very limited. A direct relationship was found between the extent of growth depression caused by isoflavone glycosides and the amount of free isoflavones released into the growth medium (unpublished results). It is assumed that fungal extracellular glycosidases enhance the fungistatic activity of isoflavone glycosides by releasing free isoflavones.

Experiments with *R. solani* showed that the different free isoflavones possess similar fungistatic potency. It was also found that methylation of hydroxyl groups increased significantly ($P < 0.05$) the fungistatic activity (Figure 4), probably by making the isoflavones more lipophilic and thus facilitating their passage through the cytoplasmatic membrane. These findings are consistent with previous results (Figures 5, 6), which showed a greater fungistatic activity of free isoflavones as compared to that of the glycosides. Increase of polarity of the inhibiting agent seems to reduce its activity.

In order to ascertain that isoflavones do indeed penetrate into the cytoplasm and can be translocated in the fungus, the "dish technique" of Schutte (Figure 1) was adopted. By using this method diffusion of the test material through the growth medium is prevented. If the examined isoflavones are found in mycelial parts, which had no direct contact with the growth medium containing the isoflavones, this can be taken as proof for their translocation by the fungus. Such translocation could be shown in the case of soybean isoflavones. It made no difference whether the mature point of the mycelium (the inoculum) or the younger one (the apex) was brought into contact with agar containing isoflavones; they could be identified by tlc in every part of the mycelium.

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Physical-Chemical Characteristics and Heavy Metal Content of Corn Grown on Sludge-Treated Strip-Mine Soil

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Corn was grown on strip-mine soil where anaerobically digested liquid sludge had been applied at a rate of 25 tons of sludge solids per acre. An adjacent plot of soil received no sludge. Corn grain grown on untreated strip-mine soil was characterized as immature and kernel size varied from small to intermediate, with about 20% of the kernels being diseased. In contrast, sludge-grown corn was well developed and corn yield increased fourfold over the untreated corn. Furthermore, a significant protein enhancement of 2.5 percentage points was also realized. Concentrations of seven heavy metals (Zn, Mn, Cu, Pb, Cr, Cd, Hg) increased in grain, cobs, and husks in that order. For corn grain grown on untreated and sludge-treated soils, essentially no significant differences were found in heavy metal content when compared to 11 other corn varieties grown normally. Heavy metal contents of both soil and sludge samples were also determined.

A preliminary investigation was initiated to study corn grown under unique conditions—on strip-mine land treated with anaerobically digested liquid sludge. The Metropolitan Sanitary District of Greater Chicago has instituted a project in Fulton County in Central Illinois encompassing two major objectives: (a) disposing of anaerobically digested sludge and (b) upgrading organically deficient strip mine soil to the point where it can be productive agriculturally. Corn, a product of major commerical importance in this area, was chosen as the demonstration crop to study the effects of land application of sludge under specific controlled conditions.

A commerical yellow seed corn was planted in a tilled field of strip-mine soil. For comparative purposes, one plot was treated with sludge representing an equivalent of 25 tons of solids per acre, and an adjacent plot was not treated with sludge. On October 28, 1971, corn samples representing sludge-treated and untreated conditions were collected.

We measured the major physical and chemical characteristics of the whole kernel corn produced and grown under these two conditions. In addition, we determined the heavy metal content for parts of the corn plant that were contiguous with the whole kernel to establish the uptake of these metals. We wanted to answer the important question—are heavy metals, which are present in the sludge in very small quantities, translocated by way of the soil to the various parts of the corn plant, particularly the edible parts? The metals studied included zinc, manganese, copper, lead, chromium, cadmium, and mercury.

Previously, Braids *et al.* (1970) studied the effect on crop yields of Reed Canary grass and sorghum grain but where digested sludge was applied to lysimeters. They also determined the elemental uptake of manganese, iron, copper, and zinc by corn grain and leaves.

MATERIALS AND METHODS

Sample Collection and Preparation. In each plot (untreated and sludge-treated), corn ears with husks intact were removed from the plants in the field and placed in large plastic-lined paper bags. Each bag contained ears representing many plants, and several bags were filled from each plot. The sealed bags were placed in cold storage at −29° until the samples were to be examined.

For analytical work, a composite whole grain corn sample representing each plot was prepared. For representative corn ear selection, all the bags were removed from cold storage and the contents were allowed to come to ambient laboratory temperature. All the ears of corn were separated into three relative sizes: large, medium, and small. An equal number of ears of each of the three sizes was then taken to start the formation of the composite grain sample. To avoid cross-contamination between untreated and sludge-treated samples, work on each lot was done on separate days.

The husks were removed and collectively gathered in a large plastic bag. The ears with the kernels intact were weighed and recorded. The kernels from each ear were then removed by hand and counted and both kernels and cob were weighed separately. To form a composite grain sample, all the kernels from each succeeding ear of corn were combined cumulatively until the total exceeded 10,000 kernels. The resultant composite samples required

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