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I am submitting herewith a dissertation written by Lakshma Oddiraju Rao entitled "Production of Aflatoxin and Citrinin in Soy Extended and Nonextended Thuringer Sausage as Influenced by Environmental Parameters." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Technology and Science.

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PRODUCTION OF AFLATOXIN AND CITRININ IN SOY EXTENDED AND NONEXTENDED THURINGER SAUSAGE AS INFLUENCED BY ENVIRONMENTAL PARAMETERS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Lakshma Oddiraju Rao

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DEDICATION

To the memory of my parents

Mr. and Mrs. Narasimha Rao

whose love and affection I

cherish.

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ABSTRACT

The objectives of this study were to develop a soy extended semidry Thuringer sausage and study its sensory characters to study its potential as a substrate for toxin production if contaminated by Aspergillus parasiticus or Penicillium citrinin.

Thuringer sausage was formulated by adding 0, 5, 10, 15 and 20% hydrated textured soy protein. The cooked and smoked products were subjected to sensory evaluation on an eight point hedonic scale. The addition of soy did not affect the pH of the final product. A linear relationship between the sensory score and soy level was observed. It was concluded that Thuringer sausage can be formulated by adding up to 12% hydrated soy protein without causing any adverse affect on the product. A reduction in premix time was achieved.

Slices of nonextended (NE), soy extended (SE) and commercial (CM) sausages were infected separately with 10^6 spores of A. parasiticus and P. citrinum and maintained at equilibrium relative humidities of 80% and 93% at 21°C and at 31°C. Samples were analyzed for aflatoxin B_1, aflatoxin G_1 and citrinin at 7, 14, 21, and 28 day intervals. A. parasiticus grew well on all three sausage samples. Toxin levels were detected as early as 7 days and remained detectable until 28 days. The effect of relative humidity (RH) and temperature on toxin production varied with each sample, but the incubation time did not show any effect. Overall, higher levels of aflatoxin B_1 were detected at 80% RH than at 93% RH and at 21°C than at 31°C. Higher

levels of B_1 were detected in CM samples than in NE and SE samples. Aflatoxin G_1 production followed the same patterns as those of aflatoxin B_1 in NE and SE samples, but in CM samples more G_1 was produced at 31°C than at 21°C. More G_1 was produced in CM and SE samples than in NE samples.

Citrinin production was sporadically detected in all three samples. The CM sausage samples appeared to be the best and SE to the worst substrates for citrinin production. Citrinin was detected more frequently at 93% RH than at 80%. All three sausage samples were equally hazardous if contaminated by toxigenic fungi, and addition of soy neither increased or decreased toxin production potential by toxigenic fungi.

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CHAPTER I

INTRODUCTION

Interest in the use of vegetable proteins as additives or components of foods has increased markedly in recent years. Proteins derived from soybeans constitute the major portion of plant proteins used as meat extenders. Cottonseed protein is the second most important source in the world, but its application in meat products has not been very successful. Other proteins being studied as potential extenders include peanut (Aguilera et al., 1980), tobacco leaf protein (Kung et al., 1980), dry beans (Chang and Satterlee, 1979), and unconventional cereals (Inglett, 1977). The rising cost of meat products has encouraged the development and utilization of new products derived form plants (Gallimore, 1974). The major objectives have been to extend meat products with less expensive materials that do not markedly decrease nutritional properties or flavor of the product. Thus far soy proteins have proved to be the most useful materials to meet the objectives (Siegel et al., 1979).

The role of soy proteins in the sausage industry is well known. Isolates of soy proteins are recognized for their rheological, gelling, emulsifying and stabilizing properties. They are established as functional ingredients in many meat, poultry and fish products (Kadane, 1979). The production of soy proteins has been steadily increasing. In 1976, 38,700 metric tons (MT) of textured soy protein (TSP) were produced. In 1985, the production of TSP is estimated to

be 77,000 M.T. (Minor and Gallimore, 1977; Aguilera et al., 1980). The continued increase in the consumption of soy proteins may be due to their use in USDA school lunch and other low cost institutional feeding programs (Campbell, 1981).

The functionality, economy, nutritional value, consistency, reduced cooking losses are some features for the increased popularity of soy proteins (Terrell and Staniec, 1975; Lyon et al., 1980; Fatima et al., 1982). Siegel et al. (1979) noted that research on soy proteins has resulted in the development of nonmeat proteins with specific physical properties for use as meat extenders. Different sausage products have been developed by adding varying levels of soy and other vegetable proteins. Acceptability of these products mainly depended upon the type of plant protein and the nature of the product. However, it is suggested that 8 to 10% of total meat can be substituted with soy proteins without affecting the characteristics of the products (Modic, 1979).

Addition of soy proteins to meat creates an environment which promotes the growth of spoilage bacteria (Draughon, 1980). Numerous reports suggest that the soy extended meat products undergo bacterial spoilage at a more rapid rate than nonextended ones (Stansbury, 1975: Craven and Mercuri, 1977; Bell and Shelef, 1978; Keeton and Melton, 1978; Draughon, 1980; Harrison, 1980). Besides bacterial spoilage, mold contamination of meat products is of serious concern.

Molds of the genus Penicillium and Aspergillus are widely distributed in nature and many produce secondary metabolites called

mycotoxins. Mycotoxins are proven to be carcinogenic and teratogenic to many animal species and humans. Presence of toxigenic fungi in meats and meat products has been reported by many researchers (Bullerman et al., 1969a, 1969b, Alperden et al., 1973a, 1973b; Ayers et al., 1974; Wu et al., 1974a, 1974b; Hadlok et al., 1976; Incze et al., 1976; Leistner et al., 1977). Production of mycotoxins has also been reported in soy beans (Davis and Diener, 1970; Gupta and Venkatasubramanian, 1975).

This research was undertaken based on the facts that (a) mycotoxins are hazardous to human health, (b) soy extended sausage products are feasible and economical, and (c) toxigenic fungi may grow and produce mycotoxins on sausage products. The objectives of the project were to develop a soy extended Thuringer sausage and study its potential as a substrate for growth and toxin production by Aspergillus parasiticus and Penicillium citrinum at varying levels of temperature and relative humidity, and over incubation times.

CHAPTER II

LITERATURE REVIEW

I. PLANT PROTEINS IN MEAT PRODUCTS

Soy and other plant proteins have been widely used as meat extenders in recent years (Maga, 1973; Pearson, 1973; Lin et al., 1975; Goltry et al., 1976; Sofos et al., 1977; Modic, 1979; Bushway et al., 1982). The use of soy isolates and concentrates in processed meats has been reviewed (Rakosky, 1974).

Common ingredients which are used in sausage manufacture include cereal flours, starch, corn syrup or corn solids (Forrest et al., 1975). Extensive research concerning incorporation of soy and other plant proteins in meat products has been reported (Anderson and Lind, 1975; Bowers and Engler, 1975; Drake et al., 1975; Fatima et al., 1982). However, reports related to use of soy proteins in coarse chopped sausage products have been very limited. Goltry et al. (1976) studied the use of textured vegetable protein in pork sausage. Soy and fish protein concentrates have been used with mechanically processed beef to prepare summer sausage (Drake et al., 1975; Berry et al., 1979). The acceptance of such products ranged from moderate to high depending upon the level of added plant protein. Comminuted meat products have been investigated for possible extension with plant proteins (Smith et al., 1973; Lin et al., 1975; Sofos and Allen, 1977; Bushway et al., 1982). Attempts with cotton seed protein did not produce desirable products (Smith et al.,

1973). Soy protein isolates and concentrates caused instability in products containing 30% fat and were less desirable than products with 8% fat levels (Sofos et al., 1977). Addition of 3% potato starch did not effect sensory and chemical properties of the product (Bushway et al. 1982). Sunflower concentrates were also added to weiners with moderate success (Lin et al., 1975). Wilding (1974) discussed the use of textured soy flour in meat blends for chili and pizza toppings.

II. HISTORICAL ASPECTS OF SOYBEANS

The soybean [Glycine max (L) Merr] probably first emerged as a domesticated species in the North China plains around the eleventh century B.C. and later was introduced to Manchuri. During the first three decades of the 20th century soybean production was largely confined to the Orient. However, by the late 40s and 50s, the United States production surpassed that of the Orient. In the Far East the soybean, sometimes called "The cow of China," was consumed in many different forms. In the seventeenth century, soybeans were known to Europe as an exotic food plant from the Orient. However, soybeans did not become popular in Europe at that time, possibly because of competition from milk products and the nutty taste of soy milk (Hymowitz, 1970). Recent developments in soybean processing offer a potential solution to protein needs of the rapidly growing world population (Meyer, 1967). Liener (1978) concludes that no other single source of plant protein could contribute so greatly and so promptly to the protein gap. Among oilseeds, the soybean assumes a

most prominant position; not only is its protein content high (30-56%) but this protein, when properly processed, is of good nutritional quality (Wilding, 1971). Ziemba (1975) predicted that the proteins from cereal grains and soybeans will ultimately fill the world protein gap.

The Nature of Soy Proteins

Soy proteins are used in various forms as extenders (Table 1). As a result of intensive industrial and academic research and wide exposure in the market place, soy proteins have become standard protein additives for the food industry. It is generally believed that the proteins in soy products are the primary determinants of the functional properties while the carbohydrate components play only a minor role (Satterlee, 1981). About 90% of the soy proteins are storage proteins; the remaining 10% are made up of intracellular enzymes, protein inhibitors, lectins and membrane proteins (Kinsella, 1979). Based on sedimentation rates, the soybean storage proteins are divided into four major groups. The molecular weight, total protein percentage and the principal component of each group are described in Table 2.

As can be seen in Table 2, the 7s and 11s fractions predominate in the soybean and are probably responsible for functionality. It is the ability of soy proteins to mimic the functional properties of many traditional proteins that makes this protein one of the most important plant proteins in our food systems (Satterlee, 1981). Soy proteins perform different functions in different food systems. Table 3 lists

Table 1. Protein Products from Soybeans.

Full fat flakes, grits and flour
Defatted flakes, meal, grits and flour
Defatted-toasted flakes, meal, grits and flour
Soy concentrate
Isoelectric soy protein isolate and soy proteinate
Textured vegetable protein (TVP), extended meal and grits
Textured soy concentrate
Spun soy isolate

Source: Satterlee, 1981.

Table 2. Chemical Characteristics of Soy Proteins.

Protein fraction by mean sedimentation coefficient	Molecular weight (daltons)	% of Total protein	Principal component
2S	8,000-50,000	22	Trypsin inhibitors, cytochrome C
7S	60,000-210,000	35	Lipoxygenase, globulin, lectins amylases
11S	350,000	33	Globulins
15S	600,000	10	Polymeric globulins

Source: Satterlee, 1981.

Table 3. Functional Properties of Soy Proteins.

Functional property	Mode of action	Food system	Preparation used[a]
Water absorption	Hydrogen bonding of water, entrapment, drip prevention	Meats, sausages bread, cakes	F, C
Gelation	Protein matrix formation and gelling	Meats, curd cheese	C, I
Cohesion-adhesion	Acts as adhesive material	Meats, sausage, baked goods, pasta products	F, C, I
ELasticity	Disulfide links in gels	Meats, bakery	I
Emulsification	Formation and stabilization of fat emulsions	Sausages, bologna, soups, cake	F, C, I
Fat Absorption	Binding of free fat	Meats, sausage, doughnuts	F, C, I
Flavor binding	Absorption, entrapment, release	Simulated meats bakery	C, I, H

[a]F = soy flour; C = soy concentrate; I = soy isolate; H = soy hydrolyzate.

Source: Kinsella, 1979.

various food systems in which soy proteins offer different functional properties.

III. MICROBIOLOGICAL ASPECTS OF SOY EXTENDED MEATS

Microbiological safety of soy extended meats is a subject of controversy. The reports published have conflicting opinions. However, a majority of researchers conclude that soy extended meat products have shorter shelf life and higher bacterial counts (Stansbury, 1975; Craven and Mercuri, 1977; Keeton and Melton, 1978; Bell and Shelef, 1978; Draughon et al., 1981; Harrison, 1981). Stansbury (1975) reported that ground beef containing 20% soy at 0°C had a shorter shelf life than nonextended samples. He also reported that aerobic mesophilic and psychrophilic counts were significantly higher in soy extended samples than in nonextended ones. Seideman et al. (1977) observed higher psychrotrophic counts in formulations containing soy and mechanically deboned beef. Higher total aerobic and coliforms counts in soy extended ground beef were reported (Craven and Mercuri, 1977; Keeton and Melton, 1978; Draughon et al., 1981; Harrison, 1981). Bell and Shelef (1978) reported that the addition of 25% textured soy protein (TSP) to ground beef reduced the shelf life by 2.2 days. All microbial counts except coliforms increased in beef soy blends upon storage at 4°C compared to controls. Sikes and Maxcy (1979) pointed out that combining plant and animal proteins may stimulate the growth of certain bacteria which may not otherwise grow on either medium alone. Observations made by Craven and Blankenship (1983) suggest a similar idea. They observed an increase in heat resistance in Salmonella in soy extended beef patties.

The microbiology of sausage products has been investigated (Joseph et al., 1978; Sofos et al., 1979; Craven and Blankenship 1983). Joseph et al. (1978) reported that substitutions of meat with 15-30% structured soy protein fiber in dry fermented salami did not create any microbiological problems. No effect on pH and lactic acid content was observed. Sofos et al. (1979) reported that production of botulinal toxin was equal or less rapid in soy extended frankfurters than in nonextended ones.

IV. MYCOTOXINS IN SOYBEANS

By 1974, it was evident that even in countries like the United States, where highest standards of harvesting and storage are practiced, crops were regularly contaminated with mycotoxins (Shotwell et al., 1969; Stoloff, 1977). Aflatoxin production occurs most readily in commodities with high fat content grown in warm and humid climates. It is perhaps due to this reason that corn is the cereal most likely to be contaminated. It is estimated that 80% of the Americans' exposure to aflatoxins in their diets can be attributed to corn products (Osborne, 1982). Because of the importance of the soybean crop and the frequent occurrence of mycotoxins in cereal grains; concern about potential mycotoxin production in soybeans has arisen. Dorworth and Christensen (1968) reported that soybeans held under controlled temperature and moisture were contaminated by molds such as Penicillium and Aspergillus species. Intact soybeans are generally regarded as resistent to mold invasion (Mislivec and Bruce, 1977).

The predominant contaminating species are considered to be the Aspergillus glaucus group. A total of 11 toxigenic species of Penicillium and Aspergillus were detected Mislivec and Bruce, 1977). Davis and Diener (1970) were able to obtain considerable amounts of toxin (48-138 μg/g) from unshelled soy beans incubated for 21 days. Sheretz et al. (1976) were also able to detect toxins in soybeans inoculated with Aspergillus isolates. Presence of aflatoxins in soy beans was also reported by other workers (Shotwell et al., 1969; Bean et al., 1972; Nagarajan et al., 1973). On the other hand some researchers failed to detect mycotoxins in soy beans (Chang et al., 1966; Hesseltine et al., 1966). They considered soy beans to be poor substrates for toxin production by toxigenic fungi. Factors such as humidity (Hesseltine et al., 1966) and zinc content are reported to play a role in toxin production. Gupta and Venkatasubramanyan (1975) were able to increase aflatoxin from 0.32 to 2.25 mg/100 g by addition of zinc. Toxin content was also reported to decrease with addition of phytates (O'Dell and Savage, 1960). They concluded that phytic substances tie up Zn which is an essential substance for aflatoxin production in soy beans. Autoclaving soy beans caused an increase in toxin production (Nagarajan et al., 1973; Gupta and Venkatasubramanyan 1975). Shotwell et al. (1978) concluded that the enzymes present in unsterilized beans may have an effect on toxin production, or the enzymes may degrade the toxin once it is produced. Strains that produced aflatoxin G_1 also produced aflatoxin B_1. More G_1 in relation to B_1 was produced in soybeans than in wheat grains

(Hesseltine et al., 1970). Aflatoxin is found to be produced by Aspergillus parasiticus during fermentation of soysauce; little aflatoxin was degraded in 6 weeks. Addition of lactic acid increased the degradation process (Maing et al., 1973).

V. MYCOTOXINS IN PROCESSED MEATS

Molds frequently occur on meats and are responsible for undesirable characteristics such as discoloration and off odors along with the potential risk of mycotoxin production. For some products such as fermented sausage of the Hungarian and Italian types, the presence of mold contributes to a characteristic appearance and flavor as well as to serve as preservatives (Leistner et al., 1977). During summer months, temperatures of 30-35°C usually prevail at which time heavy mold growth occurs on processed meats, particularly on the flesh side of country cured hams. Processors usually consider mold growth as a sign of proper aging (Ayers et al., 1974). The predominant fungal flora recovered from processed meats are Penicillium and Aspergillus (Strzelecki et al., 1968; Bullerman and Ayres, 1968; Bullerman et al., 1969a, 1969b; Sutic et al., 1972; Alperden et al., 1973a; Ayres et al., 1974; Hadlok et al., 1976). Penicillia prevail on refrigerated products with low water activity while aspergilli predominate on products with high water activity. Some of the penicillia are considered desirable while the aspergilli on meats are always considered undesirable (Leistner et al., 1977). Park et al. (1983) reported that summer sausage can support growth and toxin production by A. flavus while it is a poor substrate for A. parasiticus.

Aflatoxins are produced in large quantities in fermented sausages by toxigenic strains of A. flavus and A. parasiticus if the products are stored at 20° to 25°C (Bullerman et al., 1969a, 1969b; Alperden et al., 1973b; Ayres et al., 1974; Park et al., 1983). Besides the temperature factor, others such as water activity and purity of the starter culture are also considered crucial for toxin production (Incze and Frank, 1976). The toxicity of such toxins was tested in chicken embryos (Incze and Mihalyi, 1976). Sterilized beef infusions and beef pieces supported growth and toxin production by A. flavus. The detected range of toxin levels was 11-15 μg/g meat. No toxin was detected in raw meat, which may be due to competition from bacterial flora (Wildmann et al., 1967). Ceigler et al. (1972) suggested that amino acids in ripened sausages may react with mycotoxins, particularly penicilic acid, and convert them into nontoxic derivatives.

Mycotoxins such as sterigmatocystin and cyclopiazonic acid which are produced by A. versicolor are also of potential importance in processed meats (Leistner et al., 1977). A. versicolor is quite often recovered from meats, especially from products with relatively low water activity. A large number of Penicillium and Aspergillus isolated from processed meats were able to produce toxins in culture media (Alperden et al., 1973a, 1973b; Hall and Ayres, 1973). Country cured hams which were inoculated with toxigenic A. versicolor demonstrated the presence of sterigmatocystin in considerable amounts. The presence of other mycotoxins such as ochratoxin (Ayres et al., 1974), patulin (Alperden et al., 1973a), penicillic acid (Ceigler et al., 1972),

and citrinin (Wu et al., 1974a, 1974b) has been reported in processed meats. Toxicity of citrinin to chick embryos was confirmed (Wu et al., 1974b).

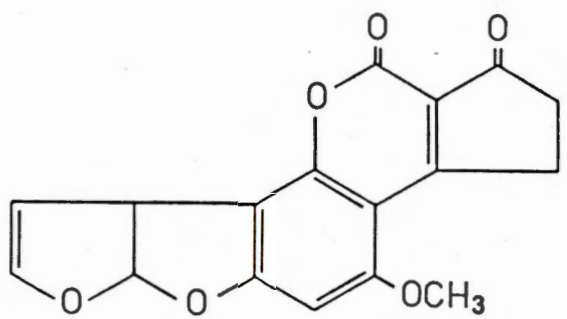
Based on information available thus far it is evident that microorganisms respond differently in combinations of soy proteins and meats when compared to either of the substrates. It is also evident that processed meats as well as soy proteins support toxigenic fungi and toxin production.

VI. AFLATOXINS

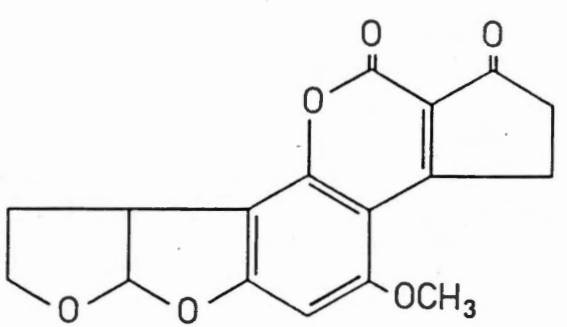
The existence of aflatoxins became recognized with the occurrence of Turkey "X" disease in England where 100,000 turkey poults died of a mysterious disease in 1960. Simultaneous investigations by Blount (1961) and Asplin and Carnaghan (1961) showed that the common factor was the presence of groundnut meal, contaminated with mold growth. Later Sargeant et al. (1961) showed that a metabolite of the common mold Aspergillus flavus was the responsible agent. Since that time, aflatoxins have become topics of extensive research and enormous amounts of scientific information have been published on this topic. Aflatoxins are produced primarily by some strains of Aspergillus flavus and most if not all strains of Aspergillus parasiticus (Diener and Davis, 1969).

Chemical Properties

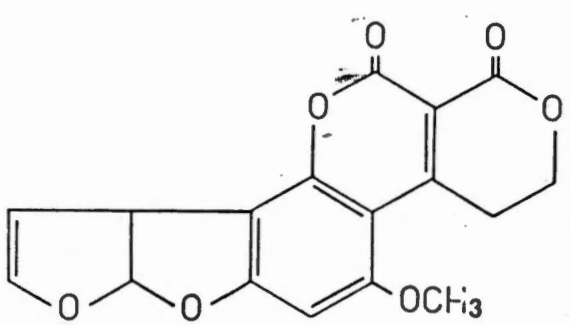
Aflatoxins are a large group of closely related compounds. The four major ones are aflatoxins B_1, B_2, G_1 and G_2 (Figure 1), of these



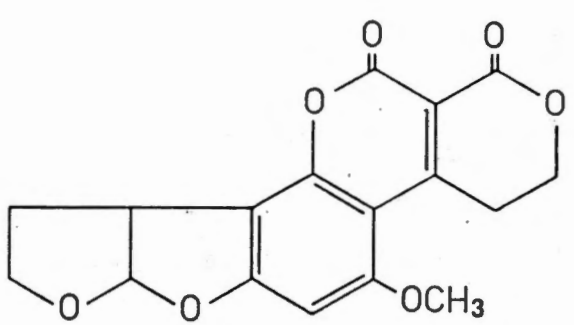
B_1



B_2



G_1



G_2

Figure 1. Four major aflatoxins.

B_1 and B_2 occur most frequently and in larger quantities (Bullerman, 1979). They were assigned the names aflatoxin B and aflatoxin G because they fluoresce blue and green, respectively, under ultraviolet light (Van Walbleek, 1973). The further designation of B_1, B_2, G_1, G_2 is based upon their separation into individual components when analyzed by thin layer chromatography (Mirocha and Christensen, 1976; Stoloff, 1977). The aflatoxin B group contains a coumarin molecule fused to a bifuran structure and a cyclopentenone ring which is replaced in G_1 by a six-membered lactone (Van Walbleek, 1973). Aflatoxins B_2 and G_2 lack a double bond in the bifuran structure and are designated as the dihydro derivatives of aflatoxin B_1 and G_1, respectively (Goldblatt, 1969). Aflatoxin B_1 has absorption maxima (ultraviolet) at 223, 265, and 362 nm. It is slightly soluble in water, insoluble in hexane and soluble in chloroform and alcohol (Pons and Goldblatt, 1969).

Occurrence

Aflatoxin-producing fungi are distributed throughout the world in air and soil. A. flavus is a seed-inhabiting fungus which is capable of invading a wide variety of food crops (Diener and Davis, 1969). They are naturally found in edible nuts such as peanuts, Brazil nuts, pistachio nuts, almonds; in cereal grains such as corn, rice, barley, oats, wheat; and oil seeds such as cottonseed and soybeans (Coker, 1979). Surveys reported by Hesseltine (1974) indicated that incidence and levels of aflatoxins in cereal grains and their products was low. However during 1977 aflatoxin contamination in corn was intense in

most Southeastern states of the United States. Half of the crop became unmarketable because the aflatoxin levels exceeded that of FDA's allowable limit (20 ppb) (Ayres, 1978). Because cereal grains are transported around the world, there are essentially no areas of the world that can be considered aflatoxin-free (Hesseltine, 1974).

Peanut and peanut products have a high risk of contamination by aflatoxins (Jones, 1975). In an analysis carried out in 1964 on farmers' stock peanuts from nine different areas of the United States, peanuts were found to contain up 95 μg/kg of toxin (Taber and Schroeder, 1967).

Aflatoxin M_2, a hydroxylated product of B_2, was found in cows' milk (Allcroft and Carnaghan, 1963). Consumption of aflatoxin contaminated feed resulted in the excretion of hydroxylated products of B_1 and B_2 (aflatoxin M_1 and M_2) (Allcroft, 1969). The levels of M_1 excreted were directly related to the B_1 levels in the feed (Purchase, 1973). Early studies (Allcroft and Carnaghan, 1963; Keyl et al., 1970) on aflatoxin residues in meat tissues of animals fed a contaminated diet indicated no sign of build up of toxins in meats. However, aflatoxins have been detected by more recent studies in the tissues of pigs (Krogh et al., 1973a), beef cattle (Purchase, 1973) and poultry (Van Zytveld et al., 1970) fed contaminated feed. Stubblefield et al. (1981) indicated that in steers fed with purified aflatoxin B_1, toxin was retained in heart, kidney, skeletal muscle and liver. The highest quantity was in the kidney (145.9 ng/g), while the lowest was in the muscle (12.9 ng/g). Aflatoxin residues have also been detected in milk and in human liver (Purchase, 1972; Campbell and Stoloff, 1974).

In samples collected from various parts of the world, particularly from Africa and Asia, aflatoxins were detected in biologically significant levels in a large number of commodities. Aflatoxin was detected in 41 of 74 samples of haricot beans and other pulses from the Sudan in concentrations ranging from 50-1000 µg/kg (Habish, 1972). Mungbeans and other beans from Thailand and Hong Kong and root crops such as sweet potatoes and casava were found to contain aflatoxins. Sun dried sweet potatoes in Taiwan had aflatoxins at levels of 10-180 µg/kg (Tung and Ling, 1968). Casava and potatoes from the Philippines were found to be highly contaminated with aflatoxin (Wogan, 1968).

Aflatoxin was also detected in spices such as cayenne peppers (Scott and Kennedy, 1973), Indian chili powder (Shank et al., 1972), black pepper (Suzuki et al., 1973), capsicum (Scott and Kennedy, 1973), and nutmeg (Suzuki et al., 1973; Beljaars et al., 1975). Other food products which were reported to be contaminated include dried fish, unrefined vegetable oil (Shank et al., 1972) sorghum (Tripathi, 1973) and pecans (Lillard et al., 1970).

Metabolism

The metabolism of aflatoxin, its effect on nucleic acids and protein, and the resulting pathophysiological effects have been thoroughly reviewed (Wogan, 1969; Patterson, 1977).

The aflatoxin molecule is biodegraded in at least six ways (Patterson, 1977) (Figure 2):

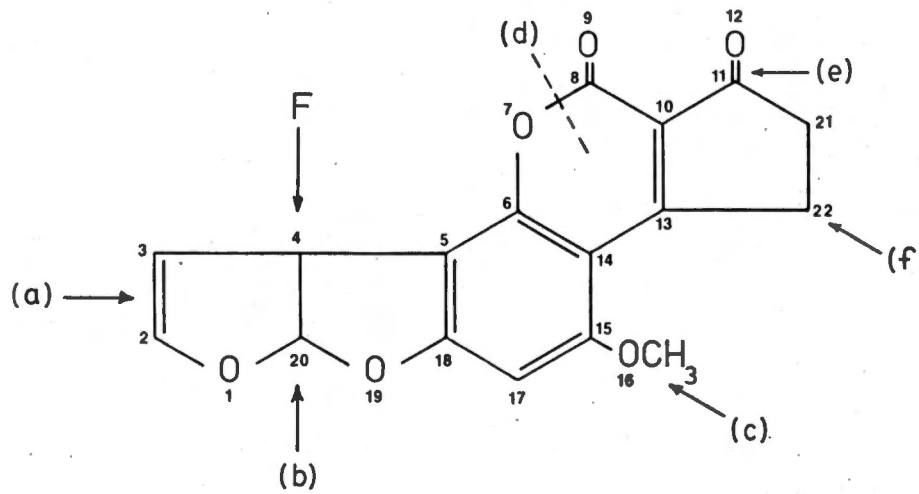


Figure 2. Routes of biodegradation of aflatoxin.

a. Attack by hydrogen or water molecule

b. Degradation of bisulfuranoid moiety

c. Demethylation of methoxycoumarin structure

d. Hydrolylic fission of the 7.8.C-O bond in the coumarin ring

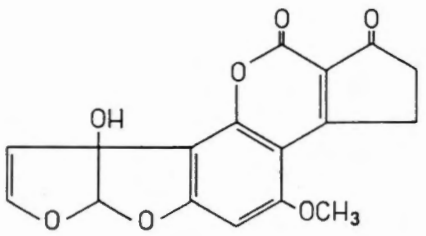
e. Reduction in the cyclopentanone moiety

f. Hydroxylation at one or more points prior to conjugation.

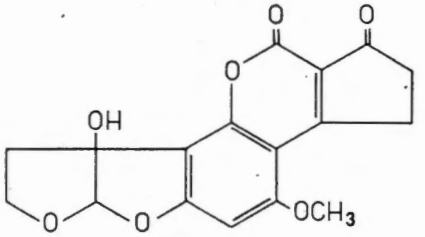
The formation of less toxic aflatoxin M_1 and M_2 from aflatoxin B_1 and B_2 is the result of 4-hydroxylation (Allcroft and Cornaghan, 1969). A nontoxic 2-hydroxylation product was shown in in vitro experiments with rat livers (Patterson et al., 1969). 0-demethylation products were obtained by Shank and Wogan (1965) in rats injected with 14C labeled aflatoxins. Bassir and Emafo (1970) also obtained similar products in vitro with liver slices from several animal species. The phenolic compound thus formed from aflatoxin B_1 in the liver is commonly referred to as aflatoxin P_1 (Dalezios et al., 1971). Hydrolytic fission of the coumarin structure can be achieved by simply boiling an aqueous solution of aflatoxin B_1 which also causes detoxification. A coumaric acid of the corresponding toxin will be formed, but such a metabolite has not been isolated from animal tissue (Coomes et al., 1966). The formation of a secondary alcohol by reduction of cycopentenone moiety of aflatoxin B_1 was first described by Detroy and Hesseltine (1968) as a product of in vitro transformation by the steroid reducing fungus Dactylium denobroides. This metabolite was found to be 18 times less toxic than aflatoxin B_1. Absidi repens and Mucor griseo-Cyanus can also convert aflatoxin into a similar product

(Detroy and Hesseltine, 1969), which was later named aflatoxicol. Its structure was confirmed in 1970 (Detroy and Hesseltine). The reduction of the 2-3 double bonds of aflatoxin which required NADH or NADPH as a cofactor was achieved in vitro with liver preparations of chick, duckling, guinea pig and mouse. The resulting products from aflatoxin B_1 and M_1 reduction were aflatoxin B_2 and M_2, respectively. They were considered to be less toxic than B_1 and M_1 (Patterson and Allcroft, 1970). Masri et al. (1974) described the formation of a 22-hydroxylation product of aflatoxin B_1, which they called aflatoxin Q_1. Aflatoxin Q_1 was produced in vitro using monkey liver microsomal preparations. Formation of the 2, 3-epoxides (8, 0-epoxoides according to IUPAC recommendation; Hsieh, 1979) may be a factor by which carcinogenic activity of aflatoxins can be explained (Patterson, 1977). Schoental (1970) proposed that aflatoxin epoxide might be the active form of the toxin, which is involved with chronic effects of the pathogenicity.

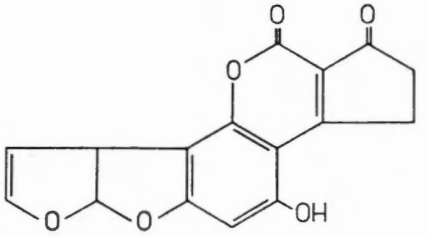
Aflatoxin is converted into one or more biologically active metabolites in the liver (Figure 3). The toxin, before entering the liver cell, must pass through the plasma membrane, a process which differs from species to species (Portman et al., 1970). Therefore, the susceptibility of animals to acute and chronic effects of toxin poisoning depends upon the fate of the toxin. The onset of toxicological effects depends upon the balance among hepatocellular uptake, rate and type of liver metabolism and the speed and the route of elimination by the body (Patterson, 1977). This is shown schematically in Figure 4.



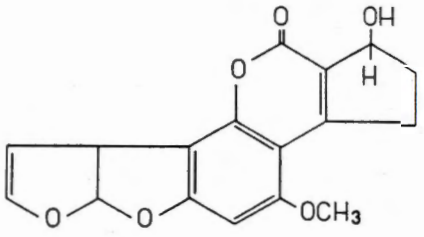
M_1



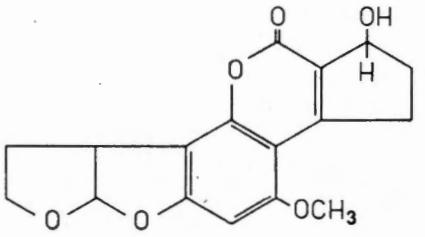
M_2



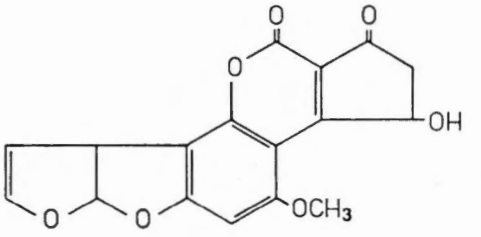
P_1



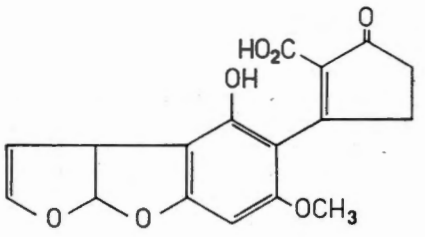
AFLATOXICOL



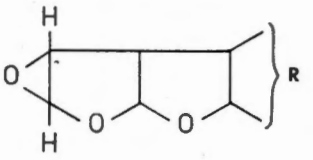
DIHYDROAFLATOXICOL



Q_1



B_1 COUMARIC ACID



2:3 EPOXIDE

Figure 3. Various metabolites of aflatoxin.

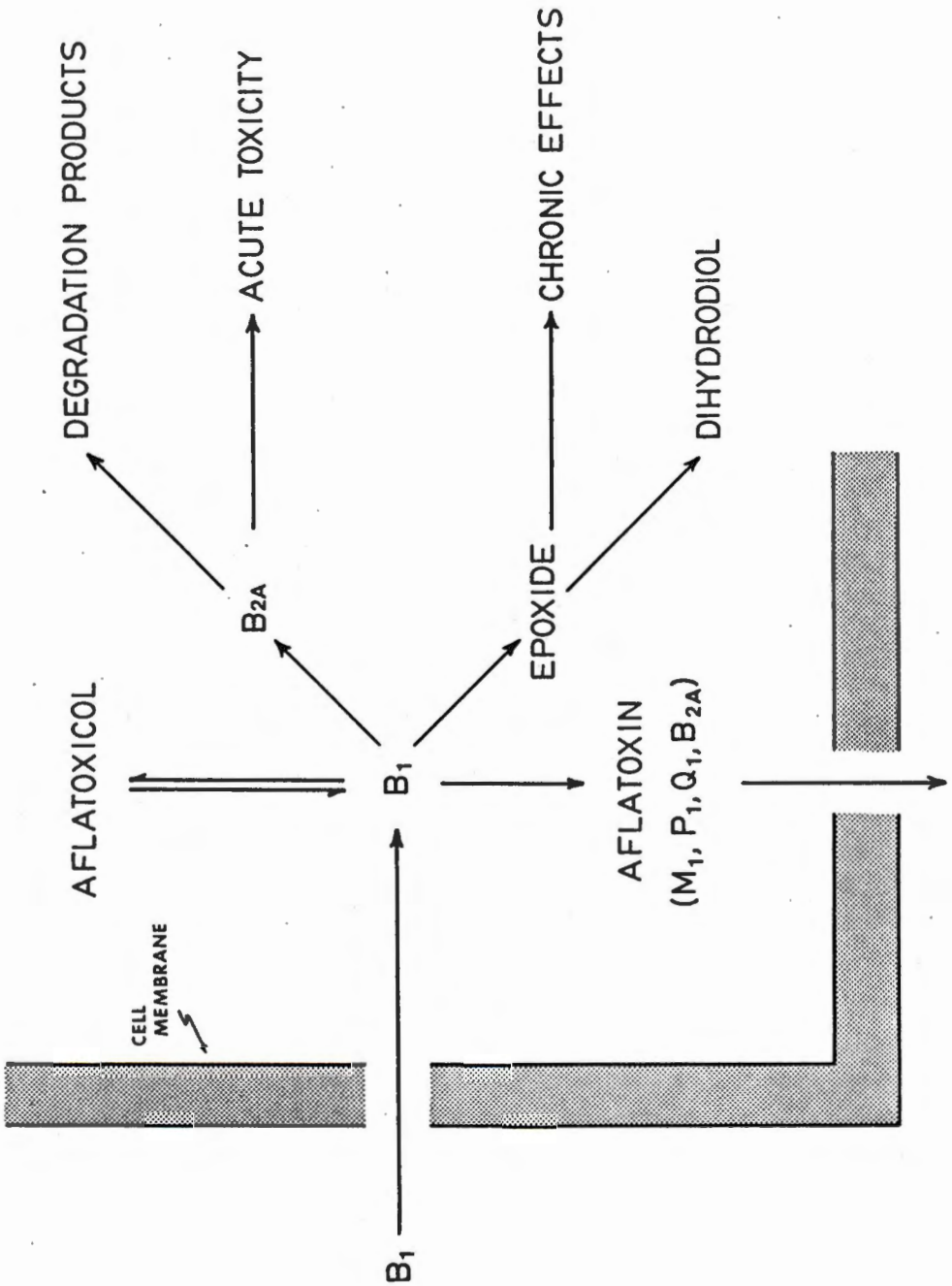


Figure 4. Routes of aflatoxin B_1 metabolism.

Biological Effects

Biological effects of aflatoxins have been reviewed by Goldblatt (1969), Detroy et al. (1971), monographed by Purchase (1974) and Wyllie and Morehouse (1978). The broad range of biological effects of aflatoxins probably relates to reactions with cell nucleoproteins and nucleic acids which ultimately affect protein synthesis and cellular integrity (Wogan, 1966). The liver is considered to be the primary target organ (Coker 1979). Among all the aflatoxins, B_1 is the most potent (Diener and Davis, 1969; Wogan, 1973) and toxicity studies have involved primarily aflatoxin B_1. The toxicity of aflatoxins depends upon the level of toxin in the feed and the susceptibility of the animal. The most susceptible animal known thus far is the rainbow trout, which shows pathological signs (or lesions) with a dose of ppb in the diet (Wogan, 1973); the mouse is considered to be the most resistant with a LD_{50} of 60 mg/kg body weight (Barnes et al., 1970). The LD_{50} values of various animal species are: rabbits 0.3, ducklings 0.34, pigs 0.62, dogs 1.0, turkey 1.4, and chick 6.5 mg/kg body weight (Patterson, 1977).

Depending upon toxin consumption and the animal's susceptibility, the toxic effects may occur as (a) an acute and clinically obvious disease (b) a chronic, less clinically apparent disease such as impairment of health and productivity, and (c) an impairment of resistance and immune response (Pier et al., 1977). Acute aflatoxin poisoning has been described in poultry, swine, cattle, dogs and several laboratory animals (Allcroft, 1969; Edds, 1973; Pier et al.,

1977; Carlton and Szezech, 1978). Impairment of liver function and fatty change occur within 3-6 hours of injection of aflatoxin B_1. These changes are followed by necrosis of hepatocytes (Cysewski et al., 1968; Carlton and Szezech, 1978). Necrosis of the kidney tubules has been reported in rats, guinea pigs and subhuman primates (Carlton and Szezech, 1978). Subacute aflatoxicosis resulting from low levels of toxin intake resulted in reduced weight gain and lowered productivity in poultry and livestock (Keyl and Booth, 1970; Neuberne, 1973; Pier et al., 1977).

Toxicity of aflatoxins to humans was confirmed by epidemiological studies. One of the recent episodes of acute aflatoxin poisoning was in 1974 involving 400 people with 106 fatalities. Contaminated corn was identified to be the source of the problem (Krishnamachari et al., 1975). In Uganda, where the incidence of human liver cancer is high, Alpert et al. (1971) established a correlation between dietary aflatoxin B_1 and incidence of the disease. Peers et al. (1976) suggested a correlation between dietary aflatoxin levels and incidence of hepatic carcinoma in Switzerland. Incidences of liver cancer in Mozambique (Van Rensburg et al., 1974) and in the Philippines (Campbell and Salamat, 1971) are related to aflatoxin contamination of the diet. In Thailand, Reye's syndrome was linked to aflatoxin poisoning (Shank 1978). In the United States Reye's syndrome is characterized as one of the possible causes of infant mortality (CAST, 1979). The human body converts most of the aflatoxin B_1 into less carcinogenic substances, but in the process some other toxic metabolities are formed

which is dependent upon the metabolic state and the resistance of the victim.

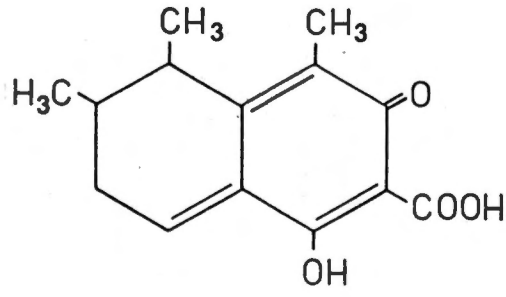
VII. CITRININ

Citrinin is a yellow crystaline metabolite that was first isolated as a pure compound in 1931 from Penicillium citrinum (Hetherington and Raistrick, 1931). The antibiotic properties of citrinin were described by Raistrick and Smith (1941), but it was too toxic to be used as antibiotic. Citrinin is also produced by thirteen other species of Penicillium and some aspergilli such as A. terreus (Raistorick and Smith, 1941), A. navious (Timonin and Rouatt, 1944), and A. candidus (Tandon et al., 1969). Occasionally, citrinin occurs with other mycotoxins such as patulin (Harwig et al., 1973; Ciegler et al., 1977) and ochratoxin (Scott et al., 1970).

Physical and Chemical Properties

Citrinin is a bicyclic compound with a free carboxylic acid group (Figure 5) (Harwig and Munroe, 1975). It forms yellow needle-like crystals when crystallized from cold ethanol. It has a molecular weight of 250 and its melting point ranges from 172 to 179°C depending upon the rate of heating (Cartwright et al., 1949). Ultraviolet absorption in 95% ethanol occurs at 222,253, and 319 nm. The flourescent excitation and emmission maxima in 95% ethanol are at 336 and 521 nm, respectively (Neely et al., 1972).

Citrinin is slightly soluble in water, but highly soluble in dilute sodium hydroxide and sodium carbonate or in sodium acetate and in



CITRININ

Figure 5. Structure of citrinin.

most organic solvents. Citrinin is slightly sensitive to heat or light; however, some decomposition takes place with flourescent light (Neely et al., 1972). It is thermolabile in acidic and alkaline conditions (Kawashiro et al., 1955). Color reactions with citrinin include orange color in alkaline solution, brown in $FeCl_3$ (Hetherington and Raistrick, 1931), and deep wine red with hydrogen peroxide followed by sodium hydroxide (Tauber et al., 1942). Brown et al. (1949) first proposed the structure of citrinin (Figure 5).

Occurrence

Citrinin has been detected in wheat, rye, oats and barley. In some of these grains citrinin was formed in combination with ochratoxin A. Penicillium viridicatum was isolated from the samples also (Scott et al., 1970). Citrinin, together with ochratoxin, was observed in barley and barley oat mixtures obtained from districts in Denmark, where the incidence of porcine nephropathy was high (Krogh et al., 1973b). Citrinin was also detected in Indian ground nuts infected with A. flavus, P. citrinum and A. terreus (Subramanyam and Rao, 1974). Citrinin was suspected to have been present in yellowed rice from Japan (Van Walbeek, 1973; Harwig and Munroe, 1975). Also the toxin has been detected in rotten apples (Harwig et al., 1973) and country ham (Wu et al., 1974a) P. citrinin has also been isolated from peanuts (Diener, 1960) and dent corn (Mislivic and Tuite, 1970).

Biological Effects

Porcine nephropathy in Danish swine was associated with the injestion of moldy rye and barley, and the incidence was often high

following harvesting periods (Krogh et al., 1973b). Similar nephropathy has been reported in Ireland, Norway and Sweden (Elling and Muller, 1973). Pathological lesions such as swelling of kidney and tubular necrosis were observed in rabbits and guinea pigs injected with acutely lethal doses of citrinin (25 mg/kg body weight) (Ambrose and DeEds, 1946). Such necrosis was also observed in rats and pigs 48 hours postmortem when the animals were given citrinin at levels of 48-100 mg/kg body weight for 2 days (Krogh et al., 1970). Chronic kidney degeneration was observed in rabbits injected with critinin (20 mg/kg body weight) intravenously (Ambrose and DeEds, 1946). Nephrotoxicity has also been reported in beagle dogs fed rice cultures of P. citrinum (Carlton et al., 1973). Besides nephrotoxicity, citrinin was also reported to cause vasodilation, bronchoconstriction and increased muscular tone in rabbits (Ambrose and DeEds, 1946). The LD_{50} values for rabbits were 67 mg/kg by subcutaneous and intraperitoneal injection. For guinea pigs the values were 37 mg/kg by subutaneous injection, and for rabbits the LD_{50} values were 19 mg/kg by intravenous injection. Effects of citrinin on humans included slight irritation of nasal passages of laboratory workers upon unintentional inhalation of citrinin (Ambrose and DeEds, 1946). However, intradermal injection of citrinin solution in man did not cause local irritation (Ambrose and DeEds, 1946; Chu, 1946).

Citrinin exhibits antibacterial effects against Gram positive organisms, particularly Bacillus subtilis (Timonin and Rouatt, 1944). It also prevents black rot of cabbage by inhibiting Xanthamonas

campestris (Rao and Tirumalachar, 1960). Citrinin is also a potent inhibitor of Saccharomyces cerevisiae and Candida albicans (Betina and Ruckova, 1971).

Metabolism

Citrinin was reported to be absorbed rapidly into the gastro-intestinal system (Ambrose and DeEds, 1945). Wang and Ting (1950) observed peak levels of blood citrinin (33-67 mg/ml) within 5 minutes after injecting a single dose of citrinin (24-44 mg/kg) in rabbits. The toxin remained in the blood for 24 hours. In blood, about 20% of the injected citrinin was bound to plasma proteins (Wang et al., 1950). Excretion studies carried out with dogs indicated that 72% of citrinin injected intravenously appeared in the urine within 48 hours (Wang and Ting, 1950). In rats, Chu (1946) detected citrinin in blood at levels of 3 to 250 μg/ml after intramuscular injection or introduction into ligated esophagus or intestine. Products of citrinin metabolism have not been investigated; however, dihydrocitrinins were suggested as metabolic products of citrinin (Wang and Ting, 1950).

CHAPTER III

DEVELOPMENT OF SOY EXTENDED THURINGER SAUSAGE

I. INTRODUCTION

Sausage research thus far has emphasized emulsion type sausages (Smith et al., 1973; Lin et al., 1975; Sofos and Allen, 1977; Sofos et al., 1977; Terrell et al., 1979; Bushway et al., 1982). Reports pertaining to coarse-ground sausages such as semidry and dry sausages have been very limited (Drake et al., 1975; Berry et al., 1977; Kadane, 1979; Modic, 1979). Modic (1979) reported that in dry sausage such as "Sremska" and "Tee," 8-10% total meat can be substituted with rehydrated textured soy protein (TSP) without negative effects on the finished product. Appearance of such products has been improved by not showing wrinkles and the processing time has been reported to be shortened with soy extended products (Kadane, 1979). Soy proteins are reported to have some antioxidant activity (Pratt, 1972; Sanger and Pratt, 1974) which would be a beneficial factor in sausage products. Addition of soy protein in emulsion type products was not as acceptable as in dry sausage products (Terrell et al., 1979). The objective of the research is to substitute meat with textured soy protein (TSP) in an intermediate moisture product such as semidry Thuringer sausage and to study the sensory properties.

II. MATERIALS AND METHODS

The Thuringer sausage was formulated according to the procedure suggested by Karmlich et al. (1973) with modifications in cooking

temperature, relative humidity and holding time. Components included: beef trim--6.81 kg; pork trim--4.54 kg; salt--226.80 g; dextrose--85.05 g; spice mix--113.4 g; sodium nitrite--1.7 g; and starter culture 42.5 g.

The spice mix was provided by Legg Packing Company, Inc., Birmingham, Alabama. Frozen starter culture, Lactacel MC (Microlife Techniques, Sarasota, Florida) containing Pediococcus cervesiae and Lactobacillus plantarum was diluted in water (1 part culture to 3 parts water (v/v)) before use.

Sausage Formulation

Beef and pork trim were ground through a 0.64 cm plate. The meat and other ingredients were mixed throughly and divided into five batches. Each batch was ground again through a 0.32 cm plate after the addition of 0, 5, 10, 15 or 20% hydrated (2 parts of water to 1 part of soy protein) soy protein (Bontrae Central Soy, Chem Way Div., Fort Wayne, Indiana) by weight. Soy protein was substituted for meat making certain that the beef/pork ratio remained the same. The ground sausage was stuffed into 5.08 cm by 40.64 cm fibrous cellulose casings. The stuffed sausages were rinsed with water at 82°C and hung for 15 hours in a chilled room at 0°C. The sausages were weighed and transferred to a smokehouse. The smokehouse was programmed for a cooking and smoking cycle of 43°C dry bulb and 29°C wet bulb with continuous smoke for 10 hours then cooked at 115°C for 1 hour so that the internal temperature of sausages reached 60°C. The sausages were cooked in the smokehouse

and washed with hot water, dried, weighed and transferred to a 4°C chilled room. The product was stored for 5 days at 4°C before evaluation by a sensory panel.

Microbiological Analysis

Eleven grams of each sausage sample were weighed aseptically into a sterile blender jar, 99 ml of sterile distilled water were added and the mixture was blended for 30 seconds. Serial dilutions were prepared for total bacterial count, coliform count and lactic acid bacteria count. Pour plates were prepared with Standard Methods agar for total bacterial counts, Violet Red Bile Agar (VRB) for coliform counts and All Purpose Tween (APT) for lactic counts, and they were incubated at 32°C. VRB plates were read after 24 hours incubation and the others after 48 hours incubation according the the guidelines of the American Public Health Association (APHA, 1972; Speck, 1976). Samples for bacterial counts were taken at two stages: immediately before stuffing and immediately prior to sensory evaluation.

Chemical Analysis

Measurements of pH were made prior to stuffing and before sensory evaluation. Samples weighing approximately 30 g were blended for 1 minute with 100 ml of distilled water. The mixture was filtered through Whatman No. 1 filter paper under vacuum. The pH of the filtrate was measured with a pH meter which was calibrated with commercial buffers of pH 7.0 and pH 4.0 (Haymon, 1979).

Proximate Analysis

Percent protein, fat, moisture and ash were determined on duplicate samples obtained from the final product according to AOAC (1980) methods.

Sensory Evaluation

Sausages were cut into slices (0.5 cm thickness) with a Hobart meat slicer. A three digit number was assigned to each level of soy extended sausage. Five sausage samples (0%, 5%, 10%, 15%, and 20%) were randomly arranged and served at one time on a plate accompanied by unsalted crackers to a sensory panel consisting of 25 persons. All samples were served under fluorescent light. The members of the panel were asked to rate the samples on an eight point hedonic scale (Larmond, 1977) where 1 = disliked extremely and 8 = liked extremely. The panelists were asked to comment on overall acceptability. Three replications were made and the data were analyzed by analysis of variance (Steel and Torrie, 1960). When significant, means were separated by Duncan's Multiple Range Test (Duncan, 1955).

III. RESULTS AND DISCUSSION

Total bacterial counts and lactic counts were approximately equal in unprocessed samples (Figure 6). Therefore, the lactic acid bacteria required for the fermentation were the predominant microflora. During the smoking and cooking process, the total counts were reduced by $\log_{10}$ 2.0/g and lactics were reduced by 3 $\log_{10}$ cycles (Figure 6). Coliform counts in unprocessed sausage were the same in all samples.

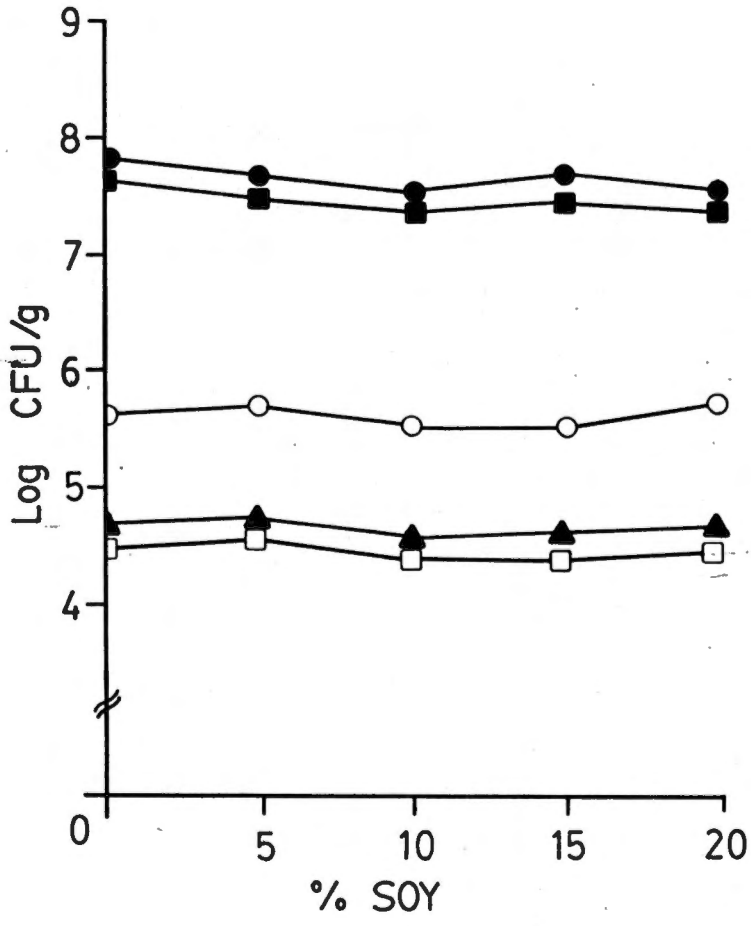


Figure 6. Bacterial counts of sausage samples before and after processing (● = total plant count before processing, ■ = total lactic count before processing, ▲ = coliform count before processing, ○ = total plate count after processing, □ = total lactic count after processing).

However, no coliforms were detected in samples obtained from the processed product at a dilution of 1:10.

The pH in the unprocessed products ranged from 5.78 to 6.13 (Table 4). There was a significant difference ($p < 0.05$) in pH of the nonextended and soy extended samples but not among various soy extended sausage samples. The pH of the final product ranged from 4.77 to 4.87 with no difference among samples ($p < 0.05$) A decrease in pH was apparent during processing. This decrease progressed to a common end range among all the treatments. The pH decrease with 0% soy was significantly different from that of samples containing 15 and 20% soy. This may be attributed to the increased availability of carbohydrate for lactic acid producing bacteria during fermentation. Similar results for soy extended ground beef were obtained by Keeton and Melton (1978). Although the pH of soy extended and nonextended sausage products was significantly different before processing, a reduction in pH was accomplished during processing by which products of uniform pH were obtained.

Table 4 also indicates the percentage moisture loss during processing. The nonextended sample lost 8.34% moisture, while the soy extended samples lost in the range of 6.3 to 7.4%. This can be attributed to the high water binding capacity of the textured soy protein. No relationship was demonstrated between the amount of soy and the percentage of moisture loss.

Table 5 presents the data from the proximate analysis. Moisture content of the samples varied with varying levels of soy ranging form

Table 4. Measurements of pH and Moisture Loss in Thuringer Sausage During Fermentation Processing.

% Hydrated soy	pH of Unprocessed sausage	pH of Processed sausage	pH Decrease during processing	Moisture loss during processing (%)
0%	5.78a	4.85a	0.94a	8.34a
5	5.90b	4.87a	1.03ab	6.32b
10	6.03b	4.80a	1.23bc	7.41b
15	6.05b	4.80a	1.27c	6.52b
20	6.13b	4.77a	1.37c	6.78b

[abc]Means within columns sharing the same letter are not significantly different ($p < 0.05$).

Table 5. Proximate Analysis of Processed Sausage Samples

% Hydrated soy	Moisture	Fat	Protein	Ash
0	51.2a	23.9a	16.7a	2.9a
5	51.4a	24.1ab	17.8a	3.0a
10	52.9ab	25.2abc	17.8a	2.9a
15	52.7ab	25.9bc	17.8a	3.0a
20	53.6b	26.2c	18.0a	3.2a

[abc]Means within treatments sharing the same letter are not significantly different ($p < 0.05$).

51.4 to 53.6% while the nonextended sausage sample had a moisture content of 51.2%. The relationship between increasing soy level and increasing moisture retention was observed by Goltry et al. (1976) in pork sausage, and our study partially substantiated such a relationship. The significant increase in moisture content was with the 20% soy extended sausage sample. Increased moisture retention in the 20% soy sausage may become an important factor in commercial operations.

Fat contents also followed similar patterns to moisture contents. The only difference was between nonextended and and 20% soy extended sausage samples. There was no difference in the protein content of sausage samples ($p < 0.05$), suggesting that the soy protein compensated for the meat protein in soy extended samples. The relationship between protein and moisture remained approximately at 1 to 3 ratio. This is a common range for sausage products (Terrell et al., 1977). Ash content was also not significantly different among the samples ($p < 0.05$).

A linear relationship ($r = 0.98$) between sensory scores and soy level was observed (Figure 7) in the sausage samples. An important observation was that none of the 25 panelists scored any sample less than 5 (5 = like slightly). The mean scores for sausage containing 0%, 5%, 10%, 15% and 20% soy were 6.4, 6.2, 6.1, 5.8 and 5.7 respectively thus showing a linear trend. Samples containing 15% and 20% soy received somewhat low scores. Using Figure 7 a sausage processor could determine the level of soy he might use and the type of

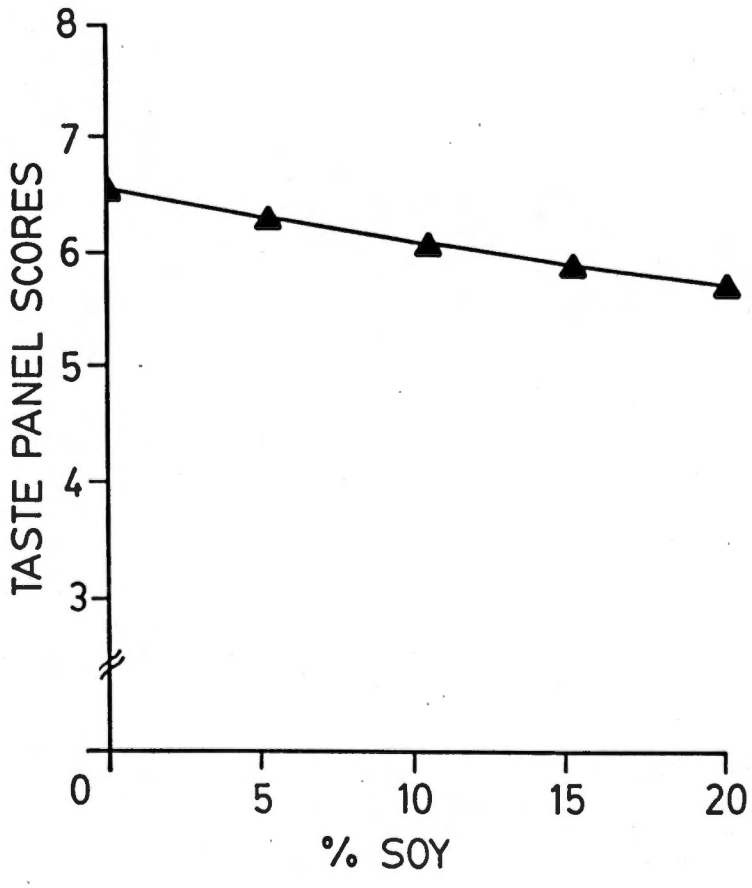


Figure 7. Relationship between taste panel scores and soy levels in fermented sausages.

consumer response he might expect and evaluate the most economical combination. For example, if a processor wants to make a product that would be liked moderately (panel score of 6) he can do so by adding 12% hydrated soy. A processor could add up to 20% soy and expect a mean panel score of 5.6 (midway between moderately and slightly liked). These results were similar to the results obtained by Baldwin et al. (1975) with soy extended turkey and beef patties and that observed by Goltry et al. (1976) in soy extended pork sausage. Berry et al. (1979) reported that there is a decrease in color intensity and an increase in toughness with increased soy content in fermented dry salami. Even though no attempts were made in this study to measure the color intensity or toughness objectively, no such observations were indicated by any panelists in the comments section of the sensory panel form. Soy extended samples agreed with all characteristics such as pH range, moisture, protein and fat levels suggested by Terrell et al. (1977) as suitable in sausage formulations.

Another important result was a reduction in processing time. The processing took 11 hours as compared to approximately 20 hours in the process described by Kramlich et al. (1973). This is approximately a 45% savings in processing time which would contribute to savings in cost. The shorter time arises from more rapid fermentation with soy present. Target pH was reached sooner.

Plant proteins play an important role in the world's food supply. By using plant proteins a substantial reduction in the prices of meat products can be achieved, especially when the meat prices are steadily

increasing and the soy prices are decreasing. Bird (1974), considering soy prices at the time, reported that 5 million dollars a year savings could be achieved by using soy extended meats in the nation's school lunch system. In 1971, the Food and Nutrition Service approved the use of up to 30% textured soy protein in the class A menu for National School Lunch Programs (USDA, 1971). Based on present meat and soy prices, the savings could be much higher than what is reported in 1974. Increasing meat prices affect the sausage industry and are passed on to the consumers. This type of formulation resulted in a product of acceptable quality and achieved a reduction in processing time, which would mean a substantial savings for processors and consumers.

CHAPTER IV

AFLATOXIN ANALYSIS

I. INTRODUCTION

Aflatoxins are proven to be potent carcinogens (Wogan and Newborne, 1967) and powerful hepatotoxins (Patterson, 1973). The analysis of aflatoxins in food products, particularly in animal tissue products which contain high levels of fat, is essential for regulatory, quality control and research purposes. Several methods of aflatoxin analysis are available both by thin layer chromatography (TLC) (Engebrecht et al., 1965) and by High Pressure Liquid Chromatography (HPLC) (Pons, 1976, Engelstrom et al., 1977). Sample purification is achieved by using silica gel columns (Pons, 1979). Sausage products provide good substrates for toxigenic fungi. Because of complexity the contaminated sausage products pose difficult problems in aflatoxin analysis.

II. MATERIALS AND METHODS

Microorganisms

Aspergillus parasiticus NRRL 3145 was obtained from the Northern Regional Research Laboratory Peoria, Illinois. It was maintained on 2%/20% yeast extract/sucrose agar and periodically transferred onto fresh medium.

Preparation of Spore Suspension

Five ml of sterile 0.005% Triton X was added to slants containing the fungus, shaken well and the spores were counted microscopically. The suspension was then diluted to obtain a solution containing one million spores per ml.

Preparation of Sausage Samples

Sausage samples containing 0 and 12.5% textured soy protein (rehydrated with 1:3 water v/v for 15 min) and a commercial brand Thuringer (obtained from local market) were sliced into 8 mm thickness slices weighing approximately 25 g. The sausage slices were hung on a looped thread and suspended above the salt solution in a wide mouth jar (Figure 8). The slices were first immersed in 1% NaOCl for 30 sec then rinsed with sterile water, blotted with sterile cotton gauze and prepared for inoculation (Wu et al., 1974).

Inoculation

One half ml of spore suspension was evenly sprayed on both sides of the slice with a tuberculin syringe. The inoculated slices were hung in two sets of wide mouth 946 ml Mason jars, one set containing saturated KNO_3 and the other set containing saturated $(NH_4)_2SO_4$ to obtain equilibrium relative humidities of 93% and 80% respectively (O'Brien, 1948, Bullerman et al., 1969b). The inoculated sausage slices were incubated at 31°C ±2 or 21°C ±2. Samples were pulled out at 1, 2, 3 and 4 week periods and stored in a freezer at -10°C until extraction.

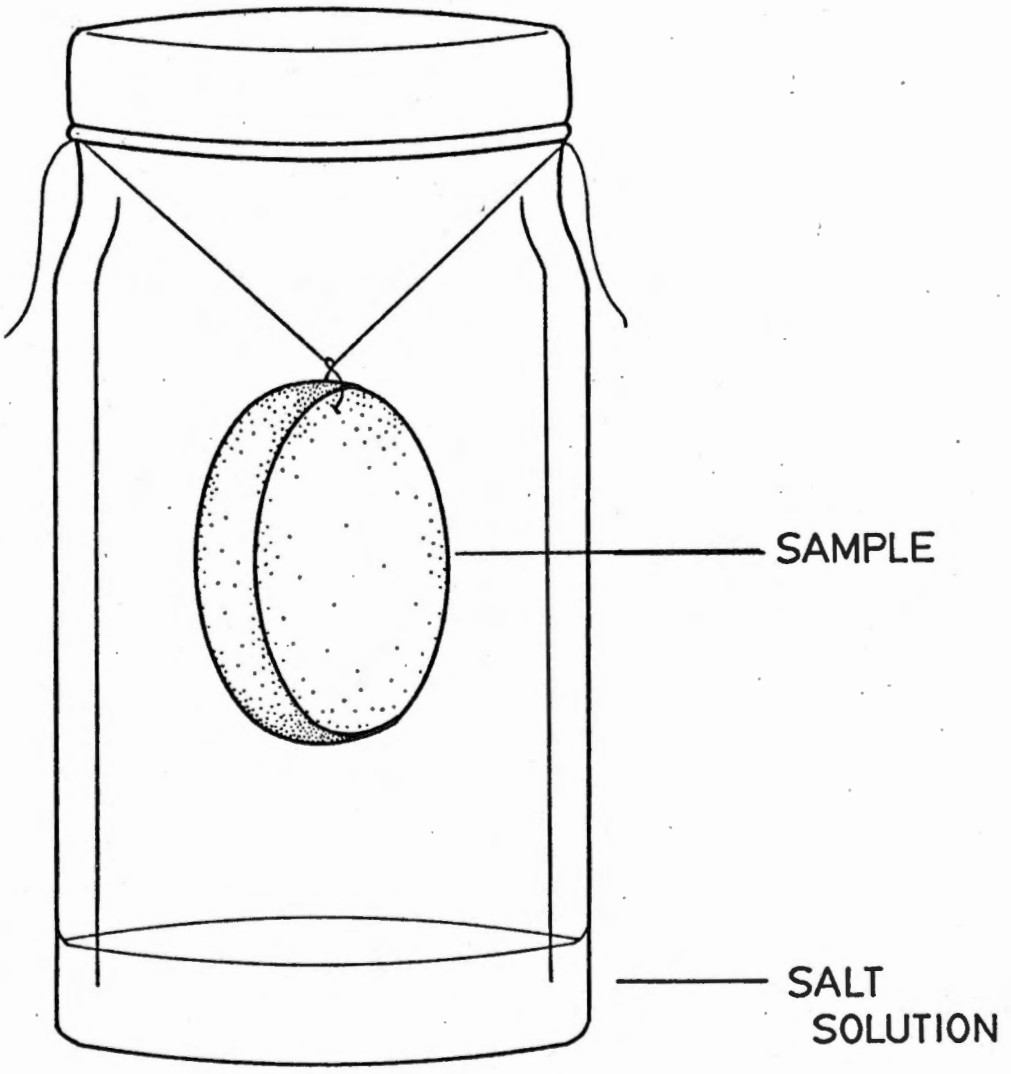


Figure 8. Mason jar containing sausage sample.

Extraction of Aflatoxin

Extractions were carried out by the Gregory and Manley (1981) procedure with some modifications in homogenization and column cleanup. The moldy sausages were thawed, the string and casing were removed. The slices were then weighed and cut into small pieces and placed in a Virtis homogenizer jar. Two g of citric acid and 8.4 ml saturated NaCl were added to the sample and homogenized for 1 min. The homogenized sample was then transferred into a 250 ml Erlenmeyer flask. Sixty ml acetone was added to the flask and it was shaken for 5 min on a Burrell Wrist Action shaker. Two g of celite was added to the flask and shaken again for 2 min. The sample was filtered with a Whatman no. 1 filter paper under vacuum. The filter cake was collected into a 250 ml flask and 40 ml of general extraction solution (GES) was added. GES was prepared by combining 169 ml H_2O, 101 ml saturated NaCl, 725 ml of acetone and 4.85 g citric acid. Samples were mixed again with the wrist action shaker for 5 min. The material was filtered through Whatman No. 1 filter paper under vacuum. The filtrates were pooled and diluted to 120 ml with GES. To 60 ml GES filtrate, 8 ml of 40% lead acetate soln, 30 ml H_2O and 2.8 g $(NH_4)_2\ SO_4$ were added and it was mixed on the wrist action shaker for 5 min. The material was filtered again through Whatman no. 1 filter paper under vacuum. The filtrate was transferred into a separatory funnel, and lipids were removed by extracting with 40 ml petroleum ether. The GES layer was extracted with 20 ml chloroform. The aqueous layer was extracted with 20 ml chloroform-acetone (50 +

50). The chloroform-acetone layer was collected and pooled with the chloroform layer and evaporated to dryness under vacuum in a rotary evaporator.

Sample Cleanup

Silica gel columns (Figure 9) were prepared by suspending silica gel (60-200 mesh) in chloroform (8 g in 40 ml $CHCl_3$). The slurry was poured into 60 cm long glass columns. Excess chloroform was drained and 1 g Na_2SO_4 was placed above the silica gel. Two ml of chloroform were added to the flask containing dried sample to suspend the material. Then the chloroform was poured into the silica gel column. The chloroform layer was drained through the column and discarded. Aflatoxins were collected with 50 ml chloroform: methanol (97:3) and evaporated to dryness. The dry flask was rinsed with 2 ml methylene chloride. The methylene chloride containing aflatoxins was filtered through a 0.5 µ microfilter into 3 ml vials and stored at -10°C.

Recovery of Aflatoxin: Part I

Experiments were conducted to determine if components in the sausages themselves influenced the fluorescence of aflatoxins B_1 and G_1. Extracts of fresh nonextended (NE), soy extended (SE) and commercial (CM) sausage samples were prepared according to the procedure described by Gregory and Manley (1981); 100 µl of extract from each sausage type was evaporated to remove methylene chloride and brought back to 100 µl with chloroform to which 20µl each of pure aflatoxin B_1 and G_1 were added. A standard was prepared with 20 µl

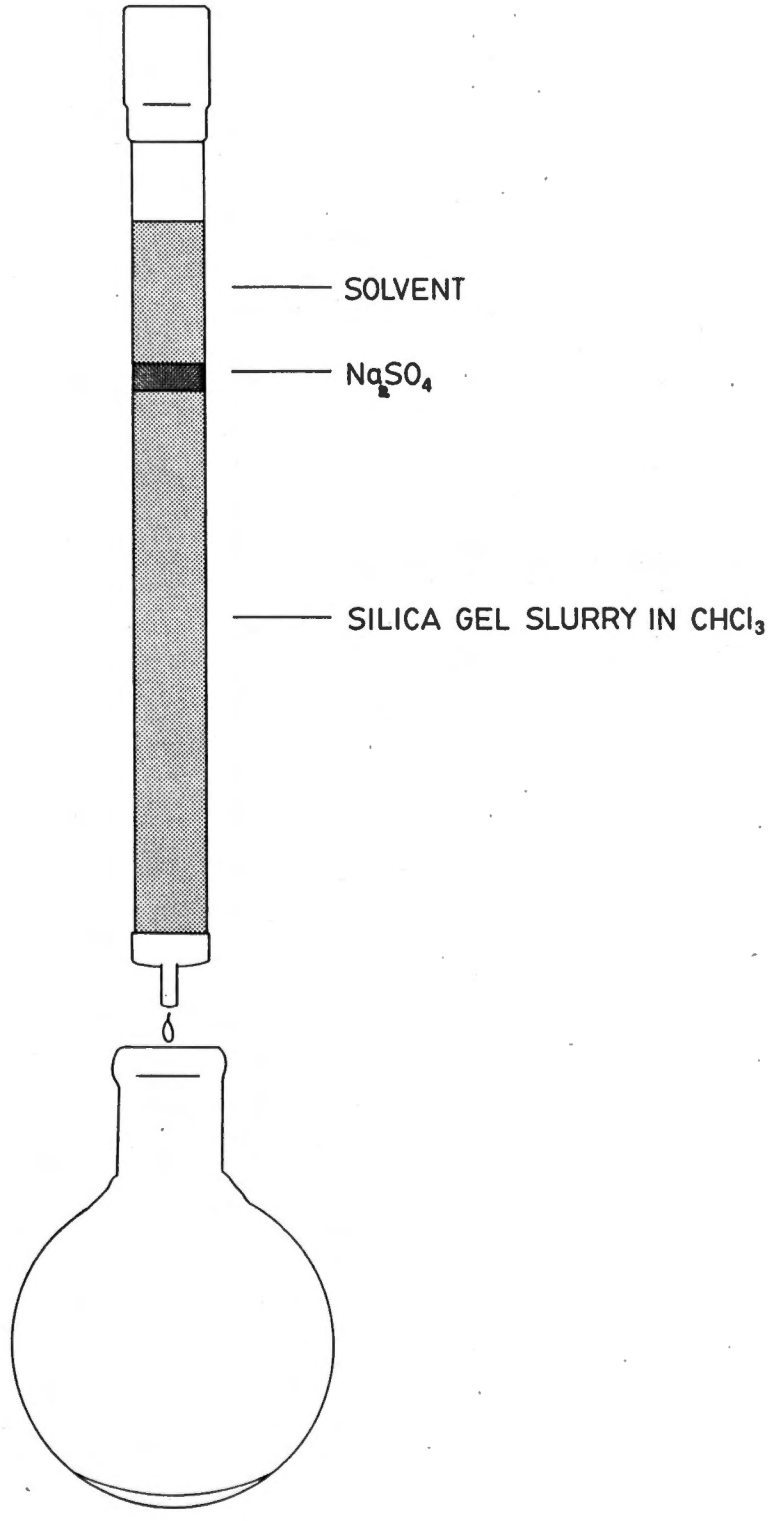


Figure 9. Silica gel cleanup column.

of pure aflatoxin B_1 and G_1 (1 µg/µl in $CHCl_3$) in 100 µl chloroform. Five µl of each sample and 5 µl of the standard were spotted on a linear k silica gel plate (LD6D .250 µ, Whatman, Clifton, NJ). Plates were developed in a chloroform: acetone (90:10) solvent system (Strzelecki et al., 1969).

The fluorescence was read using a SD 3000 spectrodensitometer (Schoeffel Instrument Co. Westwood, NJ). The instrument was adjusted for excitation of 365 nm and emission of 465 nm, slit width of 1.5 nm and band width of 1.5 nm. Chromatograms were obtained on the SDC 300 Density computer (Schoeffel Instrument Co. Westwood, NJ). Toxins were quantitated by measuring peak areas. Experiments were conducted in duplicate. Means and standard deviations were calculated.

Recovery of Aflatoxins: Part II

Experiments were conducted to determine recoveries of aflatoxins B_1 and G_1 from each sausage type. Levels of 10, 50 and 100 µg of pure aflatoxin (1 µg/µl in chloroform) were spiked into nonextended, soy extended and commercial sausage samples weighing approximately 25 g. The samples were held for 30 minutes to evaporate the solvent. The toxin extraction and clean up procedures were carried out according to the Gregory and Manley method (1981). Aflatoxin G_1 and B_1 were analyzed from the same sample. A 5 µl sample was spotted in a linear k silica gel plate along with four levels (2 µg, 5 µg, 10 µg and 20 µg) of pure standards of G_1 and B_1. The fluorescence was read as described in part I. Based on the fluorescence of standards,

a standard curve was developed, which was used to quantitate aflatoxin B_1 and G_1 in sausage samples. This experiment was performed in three replicates and each sample had a duplicate. In addition, each TLC plate was read twice. Means and standard deviations were calculated.

III. RESULTS AND DISCUSSION

Toxin Recovery: Part I

Recovery was measured in terms of increased or decreased fluorescence of pure aflatoxins added to sausage extracts compared with pure toxins in chloroform. Table 6 indicates the observed fluorescence of sausage extracts (in chloroform) with known quantities of aflatoxin B_1 and G_1 added compared to pure aflatoxin B_1 and G_1 in chloroform. The total toxin content of the control (5 µl) was measured to be 0.714 µg. Fluorescence levels were based on the assumption that 0.714 µg of pure aflatoxin B_1 and G_1 gave a 100% fluorescence and any variations in fluorescence are attributed to the dissolving medium (sausage extracts). The mean fluorescence of aflatoxin B_1 in sausage extracts was observed to be 108.0% while that of G_1 was 93.7%. The level of fluorescence was not different among the samples. The nonextended sausage extract had a B_1 fluorescence of 111.8% (±11.0) and a G_1 fluorescence of 97.9% (±15.6), the soy extended sausage extract showed a mean fluorescence of 105.4% (±5.4) and 89.7% (±6.1) for B_1 and G_1 respectively. Commercial sausage extract showed a mean fluorescence of 106.7% (±9.5) and 93.4% (±8.3) for B_1 and G_1

Table 6. Fluorescence Levels of Sausage Extracts Spiked with 20 µg Each of Aflatoxin B_1 and G_1.

Sample type	% Fluorescence[a]	
aflatoxin	B_1	G_1
Standard	100	100
Nonextended	111.8 (±11.0)	97.9 (±15.6)
Soy Extended	105.4 (±5.4)	89.7 (±6.1)
Commercial	106.7 (±9.5)	93.4 (±8.3)
Mean	108.0	93.7

[a]All data are the means and standard deviations of duplicate samples and duplicate runs.

respectively. The standard deviation for B_1 fluorescence ranged from 5.4 to 11.0% and G_1 fluorescence from 6.1 to 15.6%. Based on the observed fluorescence of B_1 and G_1 toxin in sausage extracts, the extracts appeared to have no significant effect on fluorescence of the toxins.

Toxin Recovery: Part II

This part of the experiment was conducted to test the efficiency of our extraction technique when a known quantity of toxin was added to sausage samples and the entire system was subjected to the extraction procedure. Sausage samples spiked with 10 µg of pure aflatoxin B_1 and G_1 did not show detectable amounts of fluorescence on the TLC plates and the peaks obtained on the chromatograms were not clear enough to calculate toxin levels. Due to these facts, only the samples spiked with 50 µg and 100 µg of aflatoxins B_1 and G_1 were used for toxin recovery. The sausage samples, spiked with 50 µg of aflatoxins had B_1 recoveries ranging from 35.0% to 41.5% (Table 7) with a mean of 38.7% while the G_1 recoveries ranged from 46.6% to 49.7% with a mean of 47.9%. The standard deviations for individual samples ranged from 3.5 to 10.8% for B_1 and 5.5 to 20.4% for G_1. This set of samples had higher recovery of G_1 than B_1. Recoveries of aflatoxin B_1 and G_1 were similar among the three types of sausage samples. Sausage samples spiked with 100 µg of toxin had different recovery patterns than the 50 µg spiked samples. The aflatoxin B_1 recovery levels ranged from 111.5% to 157.2% with an overall mean of 135.2%, while the G_1 recoveries ranged from 79.9% to 138.7% with an overall mean of

Table 7. Recoveries of Aflatoxin B_1 and G_1 from Three Types of Thuringer Sausage Spiked with 50 and 100 µg of Pure Aflatoxin in 25 g Sample.

Sample type	% Aflatoxin recovery[a]			
	50 µg spiked		100 µg spiked	
	B_1	G_1	B_1	G_1
Nonextended	41.5 (±10.8)	47.4 (±5.5)	157.2 (±48.6)	138.7 (±48.1)
Soy Extended	35.0 (±3.5)	49.7 (±20.4)	136.8 (±22.8)	79.9 (±20.1)
Commercial	39.5 (±5.7)	46.6 (±18.8)	111.5 (±15.7)	101.6 (±37.9)
Mean	38.7	47.9	135.2	106.7
S.D.	2.7	1.3	22.9	24.2

[a]All data are the means and standard deviations of three replicates each with a duplicate sample and duplicate TLC analysis.

106.7%. The standard deviations for B_1 ranged from 15.7 to 48.6% and for G_1 the standard deviations were between 20.1 and 48.1%. The type of sausage did not affect the recovery levels of either toxin. Higher percentages of recoveries were observed in sausage samples spiked with 100 µg toxins than with 50 µg spiked samples. This was indicated by the recovery levels of 38.7% for B_1, 47.9% for G_1, in samples spiked with 50 µg toxin and 135.2% for B_1, 106.7% for G_1 in the samples spiked with 100 µg of toxin. Loss of fluorescence (quenching or loss of toxin) was not observed in samples in part I of the experiment in which toxins were directly added to the sausage extracts. Therefore, the problem lies in the extraction procedure. The extraction medium consisted mainly of acetone, petroleum ether, lead acetate, NaCl and citric acid. Meat components which were removed during extraction process (i.e. fats, pigments) may have interfered with the detection of aflatoxins either by inactivating or by binding with them thus causing a loss of fluorescence. This interference was observed only in samples spiked with 50 µg aflatoxins possibly because 50 µg is close to the minimum level of detection. Higher levels of G_1 than B_1 were observed in 50 µg spiked samples, whereas higher levels of B_1 than G_1 were observed in samples spiked with 100 µg of toxin (Table 7). A conversion of B_1 to G_1 and vice versa may occur when both toxins are present if oxidative enzymes are present. These factors present a difficult problem in the quantitation of aflatoxins, in meat products.

Table 8 indicates the mean peak areas of various levels of aflatoxins B_1 and G_1 which were obtained to develop regression lines for

Table 8. Means and Standard Deviations of Peak Areas of Various Levels of Aflatoxin B_1 and G_1 Used to Develop Individual Daily Standard Curves.

Statistical description of peak areas	µg of Toxin spotted							
	2		5		10		20	
	B_1	G_1	B_1	G_1	B_1	G_1	B_1	G_1
Mean[a]	122.6	203.5	365.2	547.4	878.3	1200.9	1313.5	2053.8
std dev[a]	41.3	90.8	174.7	208.8	685.6	783.1	341.1	833.2

[a]Means and standard deviations of 10 observations each with duplicate runs.

quantitation of aflatoxins used in recovery experiments. Large standard deviations ranging from 41.3 to 208.8 µg (33.7 to 38.1%) for B_1 and 90.8 to 833.2 µg (40.6 to 44.6%) were observed with pure aflatoxins at various times. These large deviations, plus the problems with recoveries, made experiments difficult to duplicate with confidence. These problems also raise questions about the reliability of the published analytical procedures in fermented sausages. A better extraction system and a more suitable method of reading fluorescence is needed to study aflatoxin problems in meat products. Because of these problems, actual quantitation of aflatoxins was abandoned until future research can be conducted. Instead a format was developed to express the calculated toxin levels in terms of numerical values. These values, ranging from 1 to 5, covered a broad range of toxin values and are presented in Table 9.

Aflatoxin B_1 in Sausage Samples

Aspergillus grew well in sausage samples. Aflatoxin B_1 levels in nonextended (NE) sausage samples increased until week II and then stabilized during the remainder of the incubation period at 21°C. Aflatoxin B_1 reached a maximum at 1 week and then declined at 31°C (Table 10). Higher levels of toxin were observed at 80% RH than at 93%. This phenomenon was observed at both temperature levels. In nonextended sausage samples relative humidity (RH) was a dominant factor in aflatoxin B_1 production. However, no toxin was detected at week IV at 31°C and 93% RH. This could be an error in detection process or quantities might have been too low to detect. In soy

Table 9. Aflatoxin Ranges Found To Be Present in Sausage Samples versus Assigned Numerical Values.

Aflatoxin (mg/g.) sausage	Assigned numerical value
0.0001-0.0050	1
0.0051-0.1500	2
0.1501-1.0000	3
1.0001-2.0000	4
2.0001 and above	5

Table 10. Aflatoxin B_1 Distribution at Different Temperatures and Relative Humidities in Three Different Types of Thuringer Sausage Infected with Aspergillus parasiticus.[a]

	21°C								31°C							
	Week I		Week II		Week III		Week IV		Week I		Week II		Week III		Week IV	
	%Rh		%Rh		%Rh		%Rh		%Rh		%Rh		%Rh		%Rh	
Sample	80	93	80	93	80	93	80	93	80	93	80	93	80	93	80	93
Non-extended	2	1	3	2	3	2	3	1	3	2	3	2	2	2	2	0
Soy extended	3	2	2	2	3	3	2	3	3	1	2	2	2	1	3	1
Commercial	2	1	2	2	2	2	3	3	1	5	2	3	4	3	3	3

[a]Values are based on the means of two replicates each with a duplicate and duplicate run.

[b]For toxin levels represented by numerical values, see Table 9

extended (SE) examples the toxin production leveled off at week I without much change during the remaining period of incubation. More toxin was produced at 21°C than at 31°C. The levels of relative humidity did not affect toxin levels in SE samples at 21°C, however, at 31°C more toxin was observed at 80% RH than at 93%. In commercial sausage (CM) toxin production leveled off at week I and did not change during the rest of the incubation period. More toxin was produced at higher temperature (31°C) than at lower temperatures (21°C). Relative humidity variations did not affect toxin production at both temperature levels. The determining factor for maximum toxin production varied with each sample. In NE sausage the determining factor was the relative humidity (80%). In soy extended sausage the determining factor was the temperature (21°C), while in CM sausage the determining factor was the combination of 31°C temperature and incubation period. Variations in other environmental factors tested did not affect the toxin production. The product specificity and optimum conditions have been reviewed in the literature. The optimum conditions for toxin production in soy beans was 30°C at 99% RH (Davis and Diener, 1970), 28-32°C in rice (Sorensen et al., 1967) 24-32°C in synthetic medium at 90% RH (Northholt et al., 1976). Higher levels of toxin were detected at 20°C than at 30°C in stored meats (Bullerman et al., 1969a). Substrate variations may also have contributed to the toxin production in each type of sausage. SE sausage was different than NE and CM samples because of its soy protein content. The commercial sausage had different spice and meat combinations than soy

extended and nonextended sausage made in our laboratory. The complexity of meat-meat and meat-soy combinations are possibly responsible for variations in toxin levels. Although there were differences in toxin production in the NE and SE sausages, they followed no predictable trend.

Aflatoxin G_1 in Sausage Samples

Table 11 shows the aflatoxin G_1 production in sausage samples. In nonextended (NE) sausage, toxin production leveled off at week II without changing much during the remainder of the incubation period. Temperature variations showed little effect on toxin production, however, more toxin was generally produced at 80% than at 93% RH. The similar optimum conditions were observed for aflatoxin B_1 production. In soy extended (SE) samples, toxin levels remained approximately the same throughout the four week incubation period with a slight decrease in week II. More toxin was produced at 21^{o} than at 31^{o}. Relative humidity did not show any effect as in the case of aflatoxin B_1. In commercial sausage (CM) a steady increase in toxin production was observed at 21°C with increasing incubation time, however, no such effect was seen at 31°C. More toxin was produced at 21°C than at 31°C. RH had no effect at 21°C but at 31°C more toxin was produced at 93% RH than at 80% RH.

The overall production of aflatoxin B_1 and G_1 was approximately the same in nonextended sausage. However, more G_1 was produced than B_1 in soy extended and commercial sausage. Studies with aflatoxins B_1 and G_1 indicated that addition of textured soy protein (TSP)

Table 11. Aflatoxin G_1 Distribution at Different Temperatures and Relative Humidities in Three Different Types of Thuringer Sausage Infected with Aspergillus parasiticus.[a]

	21°C								31°C							
	Week I		Week II		Week III		Week IV		Week I		Week II		Week III		Week IV	
	%Rh		%Rh		%Rh		%Rh		%Rh		%Rh		%Rh		%Rh	
Sample	80	93	80	93	80	93	80	93	80	93	80	93	80	93	80	93
Non-extended	2	2	3	2	3	2	3	2	3	2	2	2	2	2	3	0
Soy extended	5	2	2	2	4	5	3	4	3	2	2	2	2	2	3	2
Commercial	2	1	2	3	4	3	4	5	2	5	3	3	3	4	2	4

[a]Values are based on the means of two replicates each with a duplicate and duplicate run.

to the substrate did not cause an increase in B_1 production but resulted in increased aflatoxin G_1 production (Patterson, 1977). Aflatoxin G_1 is a base catalyzed oxidation product of aflatoxin B_1, thus in soy extended and commercial sausage there may have been an increase in base catalyzed hydrolysis resulting in higher levels aflatoxin G_1. Lower quantities of aflatoxin B_1 can also be explained in terms of acid catalyzed conversion of aflatoxin B_1 to B_2 and B_{2a}, which is less fluorescent, harder to detect (Lindenfelser and Ciegler, 1970) and also 200 times less toxic than B_1 (Lillehoj and Ciegler, 1969). Availability of zinc has been stated as a required factor for aflatoxin production (Gupta and Venkatasubramanyan, 1975). Commercial sausage samples had higher levels of toxins than soy extended and nonextended samples. The soy extended samples may have contained phytates which affect Zn availability. However, because of similarities in toxin content of soy extended and nonextended samples the role of Zn appears unimportant in fermented sausage. Nitrate and pepper are suggested to reduce growth and toxin production while sucrose and garlic powder are found to stimulate the toxin production (Bullerman et al., 1969b). The composition of the mixture of spices used in the commercial sausage and in the spice mix used in preparation of Thuringer sausage are not known. The differences in spices may have some effect on higher levels of toxin in commercial sausage. It can be assumed that the spice combinations might cause a reduction in B_1 levels and an increase in G_1 levels either by influencing conversion of B_1 to G_1 or by inducing G_1 biosynthesis. Because of complexity of

meat and legume systems other factors not studied may have influenced aflatoxin G_1 levels in Thuringer sausage.

It is suggested that toxigenic strains produce mycotoxins only in stress conditions. Environmental and nutritional stress conditions are suggested to stimulate aflatoxin production (Osborne, 1982). The environmental stress causes an inhibition in the uptake of nutrients and prevents replicatory growth leading to a metabolic imbalance. Such metabolic imbalance triggers secondary metabolism by which secondary metabolites such as aflatoxins are synthesized (Bu'lock, 1965). High protein, low carbohydrate foods support the fungus growth but not toxin production under normal conditions (Bullerman, 1981). Carbohydrates are considered to be important for triggering secondary metabolism whereby acetyl CoA, acetates and other small molecules formed by primary metabolism are channeled into secondary metabolic products (Patterson, 1977). The stress factors may also vary with the substrate. Besides temperature, relative humidity and substrate composition. the strain of the fungus also influences the production of aflatoxins (Diener and Davis, 1967). Mycotoxins may not be present in foods unless they are subjected to some type of stress after being contaminated. Since sausage products are nutritious and provide a good substrate, one way of preventing mycotoxin contamination is to control the environmental conditions so that the fungi would not grow and liberate toxin. As far as textured soy protein (TSP) is concerned, it does not cause a substantial nutritional imbalance to initiate excessive aflatoxin B_1 production, but it does have a role in

increased aflatoxin G_1 production if B_1 is present. These results are in contrast to literature on bacterial quality of meat products, where soy extended products are reported to be spoiled sooner than non-extended ones. Since fermented sausages are more susceptible to mishandling in terms of storage temperatures, mold growth can take place fairly frequently. However, mold growth is customarily washed away without getting concerned about the possible mycotoxin contamination. A slight increase in temperature of meat display cases or home refrigerators may create suitable conditions for fungal growth and possible toxin production by toxigenic strains of Aspergillus flavus and A. parasiticus.

The procedures used for aflatoxin analysis (Gregory and Manley, 1981) were not highly successful for Thuringer sausage. The quantities of toxin produced may not have been large enough or the preformed toxins may have been inactivated or modified because of the interaction between the extraction solvents and the components of meat/meat and meat/soy combinations. The detection processes by thin layer chromatography (Strzelecki et al., 1969) were also not reproducible. Investigations cited in the literature did not focus on tightly controlled recovery experiments. Before we consider the soy extended meat products free of fungal contamination problems, suitable analytical procedures must be developed. It is important to establish the status of fungal contamination and toxin production in soy extended products. From our study, it is apparent that aflatoxins B_1 and G_1 can be produced in fermented sausages in both soy extended and nonextended varieties.

CHAPTER V

CITRININ ANALYSIS

I. INTRODUCTION

Citrinin is one of the earliest described toxins. Its toxicity is not as serious a health threat as that of aflatoxins. It is described as a nephrotoxin in various species of animals. The naturally occurring levels of citrinin were not significant enough to cause toxic effects in laboratory animals (Friis et al., 1969). Because of its less common occurrence and lower concentrations in food and feed stuffs, its contribution to pathogenicity might be limited. Presently there is no evidence of citrinin being a major cause of kidney disease in the United States (CAST, 1973). However citrinin is found to cause kidney damage, growth retardation and eventual death in laboratory animals (Ambrose and DeEds, 1946; Carlton and Tuite, 1970). Therefore, the presence of citrinin in foods may be hazardous to humans and animals (Wu et al., 1974a). Its synergistic effect must also be considered, particularly when foods are contaminated with several types of molds.

II. MATERIALS AND METHODS

Microorganisms

Penicillium citrinin NRRL 5927 was obtained from Northern Regional Research Laboratory, Peoria, Illinois. It was maintain on

2%/4% yeast extract/sucrose agar and transferred periodically onto a fresh medium.

Sample Preparation and Inoculation

The preparation of spore suspension, sample and inoculation of spores were carried out in the same way as described in Chapter IV for aflatoxin analysis.

Extraction of Citrinin

Citrinin was extracted by the procedure described by Jackson and Ciegler (1978) with some modifications. The sausage samples were thawed, thread and casing were removed and weighed. The samples were cut into small pieces, placed in a Virtis homogenizer jar and homogenized with 10 ml chloroform for 1 minute. The homogenized material was transferred into a 250 ml flask and another 60 ml chloroform was added to it. The material was shaken for 5 minutes on a Burrell wrist action shaker. After shaking was completed, 25 ml H_2O and 10 ml conc. H_2SO_4 were added to the sample and allowed to stand for 30 minutes. The sample was shaken again for 5 minutes and filtered through Whatman No. 1 filter paper under vacuum. The filtrate was transferred into a separatory funnel. The aqueous layer was washed with 25 ml water and extracted with 40 ml 0.1 M $NaHCO_3$. The chloroform layer was reextracted with another 40 ml of 0.1 M $NaHCO_3$. The $NaHCO_3$ layers were pooled and filtered with Whatman No. 1 filter paper under gravity. The amount of filtrate was measured and recorded, 10 ml of filtrate was saved for quantitation and the rest was discarded.

Quantitation of Citrinin

A citrinin standard curve was developed by the procedure described by Damodaran et al. (1973). Pure citrinin (1 µg/µl in chloroform) was dispensed into a series of 13 x 125 mm test tubes at the levels of 5, 10, 20, 50 and 100 µg. Tubes were allowed to sit undisturbed for 10 minutes to evaporate the chloroform. To each tube, 3 ml of sodium carbonate-bicarbonate buffer (pH 10.0), 1 ml of Folin's reagent and 1 ml of 15% Na_2CO_3 solutions were added. Tubes were shaken well and incubated at 37°C for 20 minutes. The blue color which developed was read with a double beam spectrophotometer (Shimadzu UV 190, Shimadzu Corp. Kyoto, Japan) at 640 nm, then absorbance and standard deviations were calculated (Table 12). Citrinin in extracts was calculated by the following regression equation:

$$y = 0.0065x - 0.032$$

A formula was generated using the regression equation as follows:

$$\text{Citrinin (µg/g sample)} = \left[\frac{y + 0.032}{0.0065} \times \frac{V\ ex}{(V\ sam)(SW)}\right] - CF$$

where

y = Absorbance

Vex = Total Volume of Sample Extract (ml)

Vsam = Volume of sample used in analysis (3 ml)

SW = sample weight (g)

CF = correction factor for each sausage type (Table 13)

Table 12. Absorbance of Citrinin at Various Levels Used in Colorimetric Reaction.

Vol µl	Absorbance mean[a]	Standard deviation
5	0.014	0.0015
10	0.032	0.004
20	0.085	0.004
50	0.287	0.028
100	0.620[b]	0.019
r^2 = 0.997; Intercept -0.032; slope 0.0065		

[a]Mean of three replicates.

[b]$y = 0.0065x + (-0.0032)$.

Table 13. Absorbance of Unspiked Sausage Extracts.

Sample type	Rep 1	Rep 2	Rep 3	Mean[a]	Standard deviation
Nonextended	0.210	0.179	0.157	0.182	0.022
Soy Extended	0.240	0.241	0.254	0.245	0.006
Commercial	0.030	0.152	0.022	0.068	0.059

[a]Correction factor (CF) for citrinin quantitation from sausage extracts.

Recovery of Citrinin from Sausage Samples: Part I

Experiments were conducted to determine if any interference by tyrosine was occurring in the detection process of the mycotoxin citrinin, because these two compounds react similarly with folin reagent and both result in a blue color which can be read at 640 nm. Sausage samples of approximately 20 g were extracted as described previously. Three ml of extracts was used for the colorimetric reaction (Damoderan et al., 1973). The blue color developed was read with a double beam spectrophotometer (Shimadzu UV 190, Shimadzu Corp. Kyoto, Japan) at 640 nm. Means and standard deviations were calculated from three replicates. Each observation was made in duplicate.

Recovery of Citrinin from Sausage Samples: Part II

Experiments were conducted to evaluate any interference in detection of citrinin by components of the sausage when known quantities of the toxin were carried through the actual extraction process. Levels of 50, 100, and 200 µl of pure citrinin (1 µg/µl in chloroform) were spiked into nonextended, soy extended and commercial sausage samples weighing approximately 20 g. The samples were held for 30 minutes to evaporate the solvent, the sausage extraction and quantitation were carried as previously described.

III. RESULTS AND DISCUSSION

Table 13 indicates the absorbance of sausage samples when extracts were subjected to the colorimetric reaction with folin reagent. One of the main reacting constituents which may mimic citrinin is tyrosine, thus tyrosine content interferes with citrinin quantitation.

Mean absorbance of soy extended sausage samples was higher than nonextended and commercial samples indicating the presence of free amino acids including tyrosine in large quantities. The large quantities may be the result of increased mechanical processing which the textured soy protein has undergone thus causing increased protein breakdown and the release of free amino acids. In addition, soy bean may itself have high tyrosine content. The commercial sample showed least absorbance indicating least interference if citrinin were to be present in the sample, however, a large standard deviation among replicates is evident. In estimating citrinin in high protein foods it is essential to study the background absorbance; this aspect was not looked into in the previous studies reported in the literature.

After considering background absorbance, the actual quantitation of recovery in sausage samples were carried out by using the difference in absorbance of spiked sample extracts and the unspiked sample of the same type of sausage. In some cases the soy extended samples showed lower absorbance levels for spiked samples than that of the unspiked sample, thus making it impossible to estimate the actual quantity of citrinin. In commercial and nonextended samples the citrinin recovery was calculated by using absorbance difference in the standard regression equation ($Y = 0.0065x - 0.032$, Table 12). Based on this method for nonextended samples showed recoveries of 274.9, 779.6, and 2379.8 µg of toxin for samples containing only 50, 100, 200 µg toxin. While the commercial samples showed 221.6, 339.3 and 381.6 µg in 50, 100, 200 µg spiked samples. These values are

corrected for background levels. These large quantities cannot be citrinin. Possibly some of the free amino acids were released during the extraction process and were reacting in the same way as citrinin and causing the blue color to develop. Because of these discrepancies actual quantitation of citrinin was abandoned. Instead a quantitative method was used in identifying sausage samples that showed the presence of citrinin. Citrinin levels detected in sausage inoculated with P. citrinum were very low. In most samples, none was detected after correction for background color caused by free amino acids. Where citrinin was detected certain trends were apparent.

Citrinin Distribution in Thuringer Sausage

Table 14 indicates the citrinin distribution in different types of Thuringer sausage samples maintained at 21°C after being inoculated with Penicillium citrinin spores. Even though the toxin distribution was sporadic certain patterns were observed. Citrinin was more frequently detected at a relative humidity of 93% than at 80%. Overall, 11 of the 48 samples (22.9%) showed a detectable level of toxin. Toxin detection was more frequent (48%) in commercial sausage samples than in nonextended and soy extended samples. The quantities detected in commercial samples ranged from 19.21 µg/g to 77.9 µg/g of sausage. Citrinin was least frequently detected (6.2%) in soy extended sausage samples. The detection levels on soy extended ranged from 7.95 µg/g to 12.49 µg/g sausage. Levels of toxin in nonextended sausage samples ranged from 13.23 to 67.45 µg/g of sample.

Table 14. Presence or Absence of Citrinin in Sausage Samples at 21°C and at Different Relative Humidity and Incubation Periods.

	Week I				Week II				Week III				Week IV			
	%Rh				%Rh				%Rh				%Rh			
	80		93		80		93		80		93		80		93	
	Rep		Rep		Rep		Rep		Rep		Rep		Rep		Rep	
Sample type	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Nonextended	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	+	ND
Soy extended	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
Commercial	ND	ND	ND	ND	ND	ND	+	ND	+	ND	+	+	+	ND	ND	ND

ND = Not Detectable.

+ = Toxin was detected.

Table 15 indicates the citrinin distribution in sausage samples incubated at 31°C. The relative humidity played a more prominent role at 31°C than at 21°C. None of the samples incubated at 80% RH showed detectable levels of citrinin. The duration of incubation from 1 week to 4 weeks did not show any affect on citrinin production or detection. The frequency of detection was higher (37.5%) in commercial sausage than in the other two types of samples. The quantity of citrinin detected ranged from 22.74 µg to 145.89 µg/g of sausage. Citrinin was least frequently detected in soy extended sausage samples (6.2%). Among the nonextended samples, 18.7% had a detectable level of citrinin. Quantities ranged from 13.23 µg to 26.20 µg/g of sausage.

A comparison of Tables 14 and 15 showed that the incubation temperature did not affect toxin distribution among the samples. The number of samples determined to contain citrinin remained approximately the same at both temperatures. Overall, the condition which favored toxin occurrence was the higher relative humidity (93%), and commercial sausage was the best substrate for toxin production.

Table 15. Presence or Absence of Citrinin in Sausage Samples at 31°C and at Different Relative Humidities and Incubation Periods.

	Week I				Week II				Week III				Week IV			
	%Rh				%Rh				%Rh				%Rh			
	80		93		80		93		80		93		80		93	
	Rep		Rep		Rep		Rep		Rep		Rep		Rep		Rep	
Sample type	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Nonextended	ND	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	+	ND	ND	+	+
Soy Extended	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	D	ND	ND	ND	ND
Commercial	ND	ND	+	ND	ND	ND	+	ND	ND	ND	+	+	ND	ND	+	+

ND = Not Detectable.

+ = Toxin was detected.

CHAPTER VI

SUMMARY

The use of soy and other vegetable proteins has become popular in recent years. Among plant proteins, soy proteins are the most common meat extenders in various products. The new environment created by meat soy combination is known to contribute favourably to bacterial growth. Toxigenic fungi have been identified in processed meats as well as in soy beans. Environmental factors are known to influence the toxin production in contaminated foods. The objectives of this study were to develop a soy extended semidry Thuringer sausage and study its sensory characters, and to study its potential for production of the mycotoxins citrinin and aflatoxin.

Thuringer sausage was formulated by adding 0, 5, 10, 15 and 20% textured soy protein. The products were cooked and smoked at appropriate temperatures and relative humidities. Samples were prepared in three replications and sensory evaluation was performed on an eight point hedonic scale. A relationship between pH level and soy content was observed among unprocessed samples. The pH ranged from 5.78 for 0% soy samples to 6.13 for 20% soy samples. The processed samples did not show any such relationship. The pH of processed samples ranged from 4.77 to 4.87 with no significant difference among samples ($p < 0.05$). A linear relationship was observed between soy content and taste panel scores. Based on the taste panel scores it can be concluded that soy extended Thuringer sausage can be

formulated by adding up to 12% hydrated soy protein without any adverse effect on the product. A reduction in processing time was achieved.

The toxin production potential of the sausage samples was investigated by inoculating 10^6 spores of Aspergillus parasiticus or Penicillium citrinum onto slices of nonextended (NE), soy extended (SE) and commercial (CM) sausage. The inoculated slices were hung in mason jars with a looped thread over saturated solutions of KNO_3 or $(NH_4)_2SO_4$ to obtain relative humidities of 93% and 80% respectively at 21°C and 31°C. Samples were analyzed in 7, 14, 21, and 28 day intervals for aflatoxin B_1, aflatoxin G_1 and citrinin. Two replications each containing a duplicate was performed. Recovery experiments for all three toxins were carried out using blank sausage extracts and spiked sausage samples. The identification and quantitation of toxins were performed by thin layer chromatography using regression equations developed with pure toxin standards.

The level of aflatoxin recovery depended upon the level of toxin present in the sample; 50 µg in 20 g sample appeared to be a detectable limit. The extraction process and the chemicals used in extraction caused a loss of fluorescence and/or loss of toxin. Components in sausage such as fats, pigments, and free amino acids which were removed during extraction appeared to interfere with the detection process. Recoveries could not be replicated with confidence. Samples with pure toxins showed standard deviations ranging from 38-44%. Because of interference and large standard deviations actual

quantitation of aflatoxins in sausage products was difficult to achieve, instead a quantitative method was used in identifying and describing aflatoxin B_1 and G_1 in sausage samples.

Aspergillus grew well in sausage samples. Toxin levels were detected in all three sausage types as early as 7 days and remained detectable until 28 days. Overall incubation time did not effect the toxin levels. The effect of environmental factors varied with different type of sausage. In nonextended (NE) sausage, higher aflatoxin B_1 was detected at 80% relative humidity (RH) than at 93% RH. The temperature variations did not show any influence on toxin levels. In soy extended (SE) sausage samples, more aflatoxin B_1 was produced at 21°C than at 31°C, more toxin was detected at 80% RH than at 93% RH. In commercial (CM) sausage, more toxin was produced at 31°C than at 21°C. Although there was a difference in toxin production in NE and SE samples, they followed no predictable trends. Higher levels of aflatoxin B_1 were detected in CM sausage than in NE and SE sausage types.

The levels of aflatoxin G_1 followed the same patterns as that of aflatoxin B_1 in nonextended and soy extended samples, showing higher levels at 80% RH than at 93% in nonextended samples and higher levels at 21°C than 31°C in soy extended samples. In commercial sausage samples, in contrast to B_1, more G_1 was produced at 31°C than at 21°C. At 31°C more toxin was produced at 93% RH than at 80% RH. Overall aflatoxin B_1 and G_1 levels were the same in nonextended samples, but in soy extended and commercial samples more G_1 was

detected than B_1, suggesting higher production of G_1 or acid base catalyzed conversion of B_1 to G_1.

Because of interference from fats, free amino acids and other components in sausage products, actual quantitation of citrinin could not be achieved. Instead a qualitative measure was used to identify toxin presence or absence. Detection of citrinin was sporadic among sausage samples. Citrinin was more frequently detected at 93% RH than at 80% RH. Overall 11 of the 48 samples (22.9%) showed a detectable level of citrinin. Toxin was more frequently detected in commercial sausage samples and less frequently detected in soy extended samples. The factor of relative humidity played a more prominent role at 31°C than at 21°C.

Even though no actual quantities of aflatoxins or citrinin were measured, mere presence of these toxins in Thuringer sausage is an important consideration for the safety of such products when contaminated. Addition of soy protein neither increased nor decreased the risk of potential for toxin production. Since temperature and relative humidity have proven to be important factors in toxin production, these products should be properly stored. An efficient method for toxin analysis and quantitation is an urgent necessity.

REFERENCES

REFERENCES

Aguilera, J. M., Rossi, F., Hiche, E. and Christer. 1980. Development and evaluation of extrusion-textured peanut protein. J. Food Sci. 45:246.

Allcroft, R. 1969. Aflatoxincosis in farm animals: Metabolism and excretion of alfatoxin. In "Aflatoxin. Scientific Background, Control and Implications" (Ed) Goldblatt, L. A. Academic Press, New York, N.Y.

Allcroft, R. and Carnaghan, R. B. A. 1963. Ground nut toxicity. An examination for toxin in human food products from animals fed toxic ground nut meal. Vet. Record 75:269.

Alperden, I., Mintzlaff, H. J., Tauchmann, F., and Leistner, L. 1973a. Formation of mycotoxin Patulin in dry sausage. Fleischwirtschaft 53(4):566.

Alperden, I., Mintzlaff, H. J., Tauchmann, F., and Liestner, L. 1973b. Formation of sterigmatocystin in bacteriological media and in dry sausage by Aspergillus versicolor. Fleischwirtschaft 53(5):707.

Alpert, M. E., Huff, M. S. R., Wogan, G. N., and Davidson, C. D. 1971. Association between aflatoxin content of food and hepatoma frequency in Uganda. Comcer 28:253.

Ambrose, A. M. and DeEds, F. 1945. Acute and subacute toxicity of pure citrinin. Proc. Soc. Exp. Biol. Med. 59:289.

Ambrose, A. M. and DeEds, F. 1946. Toxicological and pharmacological properties of citrinin. J. Pharmocol. Exp. Ther. 88:173.

Anderson, R. H. and Lind, K. D. 1975. Retention of water and fat in cooked patties of beef and beef extended with textured vegetable protein. Food Technol. 29(2):44.

AOAC. 1980. "Official Methods of Analysis," 13th ed. Association of Official Analytical Chemists, Washington, D.C.

APHA. 1972. "Standard Methods for the Examination of Dairy Products," 13th ed. American Public Health Association, Washington, D.C.

Asplin, F. D. and Carnaghan, R. B. A. 1961. The toxicity of certain ground nut meals for poultry with special reference to their effect on ducklings and chickens. Vet. Record 73:1215.

Ayerst, G. 1969. The effect of moisture and temperature on growth and spore germination in some fungi. J. Stored Prod. Res. 5:127.

Ayres, J. C. 1978. Significance of food mycology. An overview. Presented at the 3rd Ann. Assoc. Off. Anal. Chem. Meeting, May 1-3, 1978, Atlanta, GA.

Ayres, J. C. Leistner, L., Sutic, M., Koehler, P. E., Wu, M. T., Halls, N. A., Strzelecki, E. and Escher, F. 1974. Mold growth and mycotoxin production on aged hams and sausages. Proc. IV. Int. Congress Food Sci and Technol. Vol. III.

Baldwin, R. E., Korschgen, B. M., Vandepopuliere, J. M. and Russel, W. D. 1975. Palatability of ground turkey and beef containing soy. Poultry Sci. 54:110.

Barnes, J. M. 1970. Aflatoxins as a health hazard. J. Appl. Bacteriol. 33:235.

Bassir, O. and Emafo, P. O. 1970. Oxidative metabolism of aflatoxin B_1 by mammalian liver slices and microsomes. Biochem. Pharmaiol. 19:1681.

Bean, G. A., Schillinger, J. A. and Kicorman, W. L. 1972. Occurrence of aflatoxins and aflatoxin producting strains of Aspergillus spp. in soy beans. Appl. Microbiol. 24:437.

Beljaars, P. R., Schummans, D. H. M. and Koken, P. J. 1975. Quantitative fluorodensitometric determination and survey of aflatoxins in nutmeg. JAOAC. 58:263.

Bell, W. N. and Shelef, L. A. 1978. Availability and microbial stability of retail beef soy blends. J. Food Sci. 43:315.

Berry, B. W., Cross, H. R., Joseph, A. L., Wagner, S. B. and Maga, J. A. 1979. Sensory and physical measurments of dry fermented salami prepared with mechanically processed beef product and structured soy protein fiber. J. Food Sci 44:465.

Betina, V. and Ruckova, E. 1971. Growth inhibition of yeast and bacteria by citrinin. Biologia 26:463.

Bird, K. 1974. Plant proteins in USDA feeding program. Nat. Prov. 170:226.

Blount, W. P. 1961. Turkey "X" disease. J. Brit. Turkey Federation 9(2):52.

Bowers, J. A. and Engler, P. O. 1975. Freshly cooked and cooked frozen and reheated beef and beef soy patties. J. Food Sci. 40:624.

Brown, J. P., Robertson, A., Whalley, W. B. and Cartwright, N. J. 1949. Chemistry of fungi Part V: The constitution of the citrinin. J. Chem. Soc. 1949:867.

Bullerman, L. B. 1979. Significance of mycotoxins to food safety and human health. J. Food Prot. 42:65.

Bullerman, L. B. 1981. Public health significance of molds and mycotoxins in fermented dairy products. J. Dairy Sci. 64:2439.

Bullerman, L. B. and Ayres, J. C. 1968. Aflatoxin producing potential of fungi isolated from cured and aged meats. Appl. Microbiol. 16:1945.

Bullerman, L. B., Hartman, P. A. and Ayres, J. C. 1969a. Aflatoxin production in meats. I. Stored meats. Appl. Microbiol. 18:714.

Bullerman, L. B., Hartman, P. A. and Ayres, J. C. 1969b. Aflatoxin production in meats. II. Aged dry salamis. Appl. Microbiol. 18:718.

Bu'Lock, J. D. 1965. Aspects of secondary metabolism. In "Biogensis of Antibiotic Substances" (Ed) Vanek, Z and Hostalek, Z. Academic Press, New York, N.Y.

Bushway, A. A., Belyea, P. R., True, R. H., Work, T. M., Russell, D. D. and McGann, D. F. 1982. Potatoe starch and flour in frankfurters: Effect on chemical and sensory properties and total plate counts. J. Food Sci. 47:402.

Campbell, M. F. 1981. Processing and product characteristics for textured soy flours, concentrates and isolates. JAOCS. 58:336.

Campbell, T. C. and Salamat, L. 1971. Aflatoxin injection and excretion by humans. In "Mycotoxins" (Ed) Purchase, I.F.H. Elsevior Science Publishing Co., New York, N.Y.

Campbell, T. C. and Stoloff, L. 1974. Implication of mycotoxins for human health. J. Agric. Food Chem. 22:1006.

Carlton, W. W. and Szczech, G. M. 1978. Mycotoxicoses of laboratory animals. In "Mycotoxic Fungi, Mycotoxins. Mycotoxicoses," Vol. II (Ed) Wylie, T. D. and Moorehouse, L. G. Marcel Dekker, Inc., New York, N.Y.

Carlton, W. W., Szczech, G. M. and Tuite, J. 1973. Toxicosis produced in dogs by culture of Penicillium citrinum. Toxicol. Appl. Pharmacol. 25:457.

Carlton, W. W. and Tuite, J. 1970. Mycotoxicosis induced in guinea pigs and rats by corn cultures of Penicillium viridicatum. Toxicol. Appl. Pharmacol. 16:345.

Cartwright, N. J., Robertson, A. and Whalley, W. G. 1949. The chemistry of fungi. Part VII. Synthesis of citrinin and dihydorcitrinin. J. Chem. Soc. 1949:1563.

CAST. 1979. Aflatoxin and other mycotoxins: An agricultural perspective council for Agricultural Science and Technology. Report No. 80.

Chang, K. C. and Satterlee, L. D. 1979. Chemical nutritional and microbiological quality of a protein concentrate from culled dry beans. J. Food Sci. 44:1589.

Chang, Y. H., Beng, C. H., Ponnapalam, J. T. and Lim, R. K. H. 1966. Mouldy soybeans as potential source of aflatoxin contamination. Far East Med. J. 2:298.

Chu, W. C. 1946. Miscellaneous pharmacological actions of citrinin. J. Lab. Clin. Med. 31:72.

Ciegler, A., Besonder, R. F. and Jackson, L. D. 1977. Production and biological activity of Patulin and citrinin from Penicillium expansum. Appl. Environ. Microbiol. 33:1004.

Ciegler, A., Mintzlaff, H. J., Machnik, W. and Leistner, L. 1972. Investigations into the toxin forming ability of moulds of the genus Penicillium isolated from dry sausages. Fleischwirtchaft 52(10):1311.

Coker, R. D. 1979. Aflatoxin: Past present and future. Trop. Sci. 21:143.

Coomes, T. J., Crowther, P. C., Feuell, A. J. and Francis, B. J. 1966. Experimental detoxification of ground nut meals containing aflatoxin. Nature 209:409.

Craven, S. E. and Blankenship, L. C. 1983. Increased heat resistance of Salmonellae in beef with added soy proteins. J. Food. Prot. 46:380.

Craven, S. E. and Mercuri, A. J. 1977. Total aerobic and coliform counts in beef-soy and chicken-soy patties during refrigerated storage. J. Food Prot. 40:112.

Cysewski, S. J., Pier, A. C., Engstrom, G. W., Richard, J. L., Dougherty, R. W. and Thurston, J. R. 1968. Clinical pathologic features of acute aflatoxicosis in swine. Am. J. Vet. Res. 29:1577.

Dalezios, J., Wogan, G. N. and Wienreb, S. M. 1971. Aflatoxin P_1: A new aflatoxin metabolite in monkeys. Science 171:584.

Damodaran, D., Ramadoss, C. S. and Shanmugasundaram, E. R. B. 1973. A rapid procedure for the isolation, identification and estimation of citrinin. Anal. Biochem. 52:482.

Davis, N. D., Dalby, D. K., Diener, U. L. and Sansing, G. A. 1975. Medium scale production of citrinin by Penicillium citrinum in a semisynthetic medium. Appl. Microbiol. 29:118.

Davis, N. D. and Diener, U. L. 1970. Environmental factors affecting the production of aflatoxin. In "Proceedings of the first U.S.-Japan Conference on toxic microorganisms" (Ed) Herzberg, M. U.S. Government Printing Office, Washington, D.C.

Detroy, R. W. and Hesseltine, C. W. 1968. Isolation and biological activity of a microbial conversion product of aflatoxin B_1. Nature 219:967.

Detroy, R. W. and Hesseltine, C. W. 1969. Transformation of aflatoxin B_1 by steroid-hydroxylating fungi. Can. J. Microbiol. 15:495.

Detroy, R. W. and Hesseltine, C. W. 1970. Aflatoxicol structure of a new transformation product of aflatoxin B_1. Can. J. Biochem. 48:830.

Detroy, R. W. Lillehos, E. G. and Ciegler, A. 1971. Aspergillus toxins: Aflatoxin and related compounds. In "Microbial Toxins," Vol. VI. "Fungal toxins" (Ed) Ciegler, A., Kadis, S. and Ajl, S. J. Academic Press, New York, N.Y.

Diener, U. L. 1960. The microflora of peanuts in storage. Phytopathology 50:220.

Diener, U. L. and Davis, N. D. 1967. Limiting temperature and relative humidity for growth and production of aflatoxin and free fatty acids by Aspergillus flavus in sterile peanuts. J. Am. Oil Chem. Soc. 44:259.

Diener, U. L. and Davis, N. D. 1969. Aflatoxin formation by Aspergillus flavus. In "Aflatoxin: Scientific background and implications" (Ed) Glodblatt, L. A. Academic Press, New York, N.Y.

Dorworth, C. E. and Christensen, C. M. 1968. Influence of moisture content, temperature and storage time upon changes in fungus flora germinability and fatty acid values of soy beans. Phytopathology 58:1457.

Drake, S. R., Hinnergradt, L. C., Kluter, R. A. and Preli, P. A. 1975. Beef Patties: The effect of textured soy protein and fat levels of quality and acceptability. J. Food Sci. 40:1065.

Draughon, F. A. 1980. Effect of plant derived extenders on microbiological stability of foods. Food Technol. 34(10):69.

Draughon, F. A., Melton, C. C. and Stansbury, J. B. 1981. Growth of bacterial in soy extended ground beef stored at three temperatures. J. Food Prot. 45:699.

Duncan, D. B. 1955. New multiple F-tests. Biometrics 11:1.

Edds, G. I. 1973. Acute aflatoxicosis: A review. J. Am. Vet. Med. Assoc. 162:304.

Elling, F. and Muller, T. 1973. Mycotoxic nephropathy in pigs. Bull. Wld. Hlth O. G. 49:411.

Engebrecht, R.H., Ayres, J. L. and Sinnhuber, R. O. 1965. Isolation and determination of aflatoxin B_1 in cotton seed meal. JAOAC 48:815.

Engelstrom, G. W., Richard, J. L. and Cysewski, S. J. 1977. High pressure liquid chromatographic methods for detection and resolution of rubratoxin, aflatoxin and other mycotoxins. J. Agric. Food Chem. 25:833.

Fatima, S. A., Perry, A. K. and Van Duyne, F. O. 1982. Soybeans vs. textured soy proteins as meat extenders. J. Am. Dietet. Assoc. 81:439.

Forrest, J. C., Aberte, E. D., Hedrick, H. B., Judge, M. D. and Merkel, R. A. 1975. "Principles of meat science." W. H. Freeman and Co., San Francisco, CA.

Friis, P., Hassclager, E. and Krogh, P. 1969. Isolation of citrinin and oxalic acid from Penicillium virdicatum westling and their nephrotoxicity in rats and pigs. Acta. Pathol. Microbiol. Scand. 77:559.

Gallimore, W. 1974. Sales of soy-ground beef blends in selected stores. National Food situation Economic Research Service, USDA, NFS 147, February.

Goldblatt, L. A. 1969. "Aflatoxins. Scientific background, control and implications" (Ed) Goldblatt, L. A. Academic Press, New York, N.Y.

Goltry, S. J., Stringer, W. C. and Baldwin, R. E. 1976. Sensory evaluation of pork sausage containing textured vegetable protein. J. Food Sci. 41:973.

Gregory, J. F. and Manley, D. 1981. High pressure liquid chromatographic determination of aflatoxins in animal tissues and products. JAOAC 64:144.

Gupta, S. K. and Venkatasubramanyan, T. A. 1975. Production of aflatoxin on soy beans. Appl. Microbiol. 29:834.

Habish, H. A. 1972. Aflatoxin in harricot beans and other pulses. Exp. Agric. 8:135.

Hadlok, R., Samson, R. A., Stolk, A. C. and Schipper, M. A. A. 1976. Mould contamination of meat products. Fleischwirtschaft 56(3):374.

Halls, N. A. and Ayres, J. C. 1973. Potential production of sterigmatocystin on country cured ham. Appl. Microbiol. 26:636.

Harrison, M. A. 1981. Growth of spoilage bacteria in soy extended and nonextended ground beef with varying pH levels. Ph.D. dissertation, The University of Tennessee, Knoxville.

Harwig, J., Chen, Y. K., Kennedy, B. P. C. and Scott, P. M. 1973. Occurrence of patulin and patulin producing strains of Penicillium expansum in natural rots of apple in Canada. Can. Inst. Food Sci. Technol. J. 6:22.

Harwig, J., Munro, J. C. 1975. Mycotoxins of possible importance in disease of Canadian farm animals. Can. Vet. J. 16:125.

Haymon, W. L. 1979. Technical bulletin no. M12772. Lactacel M. C. Microlife Technics, Sarasota, Florida.

Hesseltine, C. W. 1974. Natural occurrence of mycotoxins in cereals. Mycopathol. Mycol. Appl. 53:141.

Hesseltine, C. W., Shotwell, O. L., Ellis, J. J. and Stubfield, B. D. 1966. Aflatoxin formation by Aspergillus falvus. Bacteriol. Rev. 30:795.

Hesseltine, C. W., Shotwell, O. L., Smith, M. L., Ellis, J. J., Vandergraft, E. E. and Shannon, G. M. 1970. Production of various aflatoxins by strains of the Aspergillus flavus series. In "Proceedings of the first U.S.-Japan Conference on toxic microorganisms" (Ed.) Herzberg, M. U.S. Government Printing Office.

Hetherington, A. C. and Raistrick, H. 1931. Studies in the biochemistry of microorganisms. Part XIV. On the production and chemical constitution of a new yellow coloring matter, citrinin produced from flucose by Penicillium citrinum thom. Phil. Trans. R. Soc. (London) Ser. B. 220:269.

Hsieh, D. P. H. 1979. Basic metabolic effects of mycotoxins. In "Interactions of mycotoxins in animal production." Am. Soc. Amin. Sci. Am. Dairy Sci. Assoc. Committee on Animal Nutrition, National Research Council Natl. Acad. Sci., Washington, D. C.

Hymowitz, T. 1970. On the domestication of soy bean. J. Econ. Botany 24:408.

Incze, K. and Frank, H. K. 1976. Is there a danger of mycotoxins in Hungarian salami? II. The importance of casing and smoke treatment. Fleischwirtschaft 56(6):1976.

Incze, K. and Mihalyi, V. 1976. Is there a danger of mycotoxins in Hungarian salami? III. Chemical and biological investigations with samples of slicable salami. Fleischwirtschaft 56(11):1616.

Inglett, G. E. 1977. Food proteins from unconventional cereals. Food Technol. 31(5):180.

Jackson, L. K. and Ciegler, A. 1978. Production and analysis of citrinin in corn. Appl. Env. Microbiol. 36:408.

Jones, B. D. 1975. Aflatoxins in feeding stuffs, its incidence, significance and control. In "Proceedings, conference on animals feeds of tropical and subtropical origin." Tropical Products Institute, London, U.K.

Joseph, A. L., Berry, B. W., Wagner, S. B. and Davis, L. A. 1978. Lactic acid, pH and bacterial values of dry fermented salami containing mechanically deboned beef and structured soy protein fiber. J. Food Prot. 41:881.

Kadane, V. V. 1979. Vegetable proteins in cooked and/or fermented sausage. JAOCS 56:330.

Kawashiro, I., Tanable, H., Takeuchi, H. and Nishumura, C. 1955. Determination of citrinin contained in rice by fluorometry. Bull. Natl. Hosp. Lab. Tokyo 73:191. In Chem. Abstr. 50:7229b (1956).

Keeton, J. T. and Melton, C. C. 1978. Factors associated with microbial growth in ground beef extended with varying levels of textured soy proteins. J. Food Sci. 43:1125.

Keyl, A. C., Booth, A. N., Masri, M. S., Gubman, M. R. and Gagne, W. E. 1970. Chronic effects of aflatoxin in farm animal feeding studies. In "Proceedings of the First U.S.-Japan Conference On Toxic Microorganisms" (Ed) Herzberg, M. U.S. Government Printing Office, Washington, D.C.

Kinsella, J. E. 1979. Functional properties of soy proteins. JAOCS. 56:242.

Kramlich, W. E., Pearson, A. M., Tauber, F. W. 1973. "Processed Meats." Avi Publishing Co., West Port, Conn.

Krishnamachari, K. A. V. R., Bhat, R. V., Nagarajan, V. and Tilak, T. G. B. 1975. Investigations into an outbreak of hepatitis in parts of Western India. Indian, J. Med. Res. 63:1036.

Krogh, P., Hald, B., Hesselager, E., Madsen, A., Mortensen, H. P., Larsen, A. E. and Campbell, A. D. 1973a. Aflatoxin residues in bacon pigs. Pure Appl. Chem. 35:275.

Krogh, P., Hald, B. and Pedersen, E. J. 1973b. Occurrence of Ochratoxin A and citrinin in cereals associated with mycotoxic porcine nehpropathy. Acta Pathol. Microbiol. Scand. Sect. B. 81:689.

Krogh, P., Hasselager, E. and Friis, P. 1970. Studies on fungal nephrotoxicity. 2. Isolation of two nephorotoxic compounds from Penicillium viridicatum westling: Citrinin and Oxalic acid. Acta Pathol. Microbiol. Scand. 78:401.

Kung, S. D., Sander, J. A., Tso, T. C., Vaughon, D. A., Womack, M., Staples, R. C. and Beecher, G. R. 1980. Tobacco as a potential food source and smoke material: Nutritional evaluation of tobacco leaf protein. J. Food Sci. 45:320.

Larmond, E. 1977. Laboratory methods for sensory evaluation of food. Research branch. Canada Dept. of Agriculture Publication No. 1637.

Leistner, L., Mintzlaff, H. J. and Maing, I. Y. 1977. Mycotoxin production in fermented sausage. Zeszyty Psoblemowe Postepow Nauk Rolniczych No. 189:69.

Liener, I. E. 1978. Nutritional value of food protein products. In. "Soy beans, chemistry and technology. Vol. I. Proteins" (Ed) Smith, A. K. and Circle, S. J. Avi Publishing Co., West Port, Conn.

Lillard, H. S., Hanlin, R. T., Lillard, D. A. 1970. Aflatoxin isolates of Aspergillus flavus from pecans. Appl. Microbiol. 19:128.

Lillehoj, E. B. and Ciegler, A. 1969. Biological activity of aflatoxin Bea. Appl. Microbiol. 17:516.

Lin, M. Y. J., Humbert, E. S., Sosulski, F. W. and Card, J. W. 1975. Quality of weiners supplimented with sunflower and soy products. J. Inst. Can. Sci. Technol. Aliment. 8(2):97.

Lindenfelser, L. A. and Ciegler, A. 1970. Studies on aflatoxin detoxification in shelled corn by ensiling. J. Agru. Food Chem. 18:640.

Lyon, C. E., Lyon, B. G. and Herdspeth, J. P. 1980. Effects of using mechanically deboned meat from broiler breast frames and isolated soy protein on objective and sensory characteristics of poultry rolls. J. Poultry Sci. 60:584.

Maga, J. A. 1973. A review of flavor investigations associated with soy products, raw soy beans, defatted flakes and flour isolates. J. Agru. Food Chem. 21:864.

Maing, I. Y., Ayres, J. C. and Kohler, P. E. 1974. Persistence of aflatoxin during fermentation of soy sauce. Appl. Microbiol. 25:1015.

Masri, M. S., Haddon, W. F., Jundin, R. E. and Hsieh, D. P. H. 1974. Aflatoxin Q_1: Newly identified major metabolite of aflatoxin B_1 in monkey liver. J. Agric. Food Chem. 22:512.

Meyer, E. W. 1967. Soy protein concentrates and isolates. In "Proceedings of International Conference On Soybean Protein Foods." USDA-ARS 71-35, Washington, D.C.

Minor, D. B. and Gallimore, W. W. 1977. Soy protein use can increase 70% by 1985. Reprint 4 Farmers Cooperative Service, USDA, Washington, D.C.

Mirocha, C. J. and Christensen, C. M. 1976. Mycotoxins and fungi that produce them. Proc. Am. Phyto Path. Soc. 3:110.

Mislivec, P. B. and Bruce, V. R. 1977. Incidence of toxic and other mold species and genera in soy beans. J. Food Prot. 40:309.

Mislivec, P. B. and Tuite, J. 1970. Species of Penicillium occuring in freshly harvested and in stored dent corn kernels. Mycologia 62:67.

Modic, P. 1979. From concept to market with a meat soy protein in dry sausage. JAOCS 56:414.

Nagarajan, V. R., Bhat, V. and Tulpule, P. G. 1973. Aflatoxin production in some varieties of soybeans (Glycine max L) Experientia 29:1302.

Neely, W. C., Ellis, S. P., Davis, N. D. and Diener, U. L. 1972. Spectroanalytical parameters of fungal metabolites. I. Citrinin. JAOAC 55:1122.

Neuberne, P. M. 1973. Chronic aflatoxicosis. J. Am. Vet. Med. Assoc. 163:1262.

Northholt, M. D., Verhulsdonk, C. A. H., Soentoro, P. S. S. and Paulsch, W. E. 1976. Effect of water activity and temperature on aflatoxin production by Aspergillus parasiticus. J. Milk Food Technol. 39:170.

O'Brien, F. E. M. 1948. The control of humidity by saturated salt solutions. J. Sci. Instrum. 25:73.

O'Dell, B. L. and Savage, J. E. 1960. Effect of phyhic acid on zinc availability. Proc. Soc. Exp. Bilo. Med. 103:304.

Osborne, B. G. 1982. Mycotoxins and the cereals industry--a review. J. Food Technol. 17:1.

Park, K. Y. and Bullerman, L. B. 1983. Effects of substrate and temperature on aflatoxin production by Aspergillus parasiticus and Aspergillus flavus. J. Food Prot. 46:178.

Patterson, D. S. P. 1973. Metabolism as a factor in determining toxic action of aflatoxins in different animal species. Food Cosmet. Toxicol. 11:287.

Patterson, D. S. P. 1977. Aflatoxin related compounds: Biochemistry and physiology. In "Mycotoxic Fungi, Mycotoxins, Mycotoxicoses. An Encyclopedic Handbook," Vol. I (Ed) Wyllie, T. D. and Morehouse, W. L. Marcel Dekker, Inc., New York, N.Y.

Patterson, D. S. P. and Allcroft, R. 1970. Metabolism of aflatoxin in susceptible and resistant animal species. Food Cosmet. Toxicol. 8:43.

Patterson, D. S. P., Roberts, B. A. and Allcorft, R. 1969. Aflatoxin metabolism. Food Cosmet. Toxicol. 7:277.

Pearson, A. M. 1973. What's new in research? Nat. Prov. 169:15.

Peers, F. G., Gilman, G. A. and Linsell, C. A. 1976. Dietary aflatoxins and human liver cancer. A study in Switzerland. Int. J. Cancer 17:167.

Pier, A. C., Cyjewski, S. J., Richard, J. L. and Thruston, J. L. 1977. Mycotoxins as a veterinary problem. In "Mycotoxins in Human and Animal Health" (Ed) Rodricks, J. V., Hesseltine, C. W. and Mehlman, M. A. Pathtox Publishers Inc., Park Forest South, Ill.

Pons, W. A. 1976. Resolution of aflatoxin B_1, B_2, G_1 and G_2 by high pressure liquid chromatography. JAOAC 59:101.

Pons, W. A. 1979. High Pressure Liquid Chromatographic determination of aflatoxin in corn. JAOAC 62:586.

Pons, W. A. and Goldblatt, L. A. 1969. Physio chemical assay of aflatoxins. In "Aflatoxin Scientific Background, Control, and Implications" (Ed) Goldblatt, L. A. Academic Press, New York, N.Y.

Portman, R. S., Plowman, K. M. and Campbell, T. C. 1970. On mechanisms affecting species susceptibility to aflatoxin. Biochem. Bio Phys. Acta. 208:487.

Purchase, I. F. H. 1972. Aflatoxin residues in food of animal origin. Food Cosmet. Toxicol. 10:531.

Purchase, I. F. H. 1973. The control of aflatoxin residues in food of animal origin. Food Cosmet. Toxicol. 35:283.

Purchase, I. F. H. 1974. "Mycotoxins." Elsevier Publishing Co., New York.

Pratt, D. E. 1972. Water soluble antioxident activity in soy beans. J. Food Sci. 37:322.

Raistrick, H. and Smith, G. 1941. Antibacterial substance from mould I. citrinin, a metabolic product of Penicillium citrinum Thom. Chem. Ind. 60:828.

Rakosky, J. 1974. Soy grits, flour, concentrates and isolates in meat products. JAOCS 51:123A.

Rao, K. R. and Thirumalachar, M. J. 1960. Control of blade rot of cabbage with citrinin. Hindustan Antibiot. Bull. 2:126.

Rodig, O. R., Ellis, L. C. and Glover, I. T. 1966. The biosynthesis of citrinin in Penicillium citrinum. I. Production and degradation of citrinin. Biochemistry 5:2451.

Sanger, M. R. and Pratt, D. E. 1974. Lipid oxidation and fatty acid chantes in beef combined with vegetable and textured vegetable protein. J. Am. Dietet. A. 64:268.

Sargeant, K., Sheridan, A., O'Kelly, J. and Carnaghan, A. B. A. 1961. Toxicity associated with certain samples of ground nuts. Nature 192:1096.

Satterlee, L. D. 1981. Proteins for use in foods. Food Technol. 35(6):53.

Schoental, R. 1970. Hepatotoxic activity of retrorsine, senkirkine, and hydroxy senkirkine in new born rats, and the role of epoxides in carcinogenesis by pyrrolizidine alkaloids and aflatoxins. Nature 227:401.

Scott, P. M. and Kennedy, B. P. C. 1973. Analysis and survey of ground black, white and capsicum peppers for aflatoxins. JAOAC 56:1452.

Scott, P. M., Van Walbeek, W., Harwig, J. and Fennem, D. I. 1970. Occurrence of mycotoxin ochratoxin A in wheat and isolation of ochratoxin and citrinin producing strains of Penicillium viridicatum Can. J. Plant Sci. 50:583.

Seideman, S. C., Smith, G. C. and Carpenter, Z. L. 1977. Addition of textured soy protein and mechanically deboned beef is ground beef formulations. J. Food Sci. 42:197.

Shank, R. C. 1978. Mycotoxicosis of man: Dietary and epidemiological considerations. In "Mycotoxic Fungi, Mycotoxins, Mycotoxicoses, An Encyclopedic Handbook," Vol. III (Ed) Wyllie, T. D. and Morehouse, L. G. Marcel Dekker, Inc., New York, N.Y.

Shank, R. C. and Wogan, G. N. 1965. Distribution and excretion of ^{14}C labled aflatoxin B_1 in the rat. Fed. Proc. 24:627.

Shank, R.C. Wogan, G. N., Gibson, J. B. and Nondasuta, A. 1972. Dietary aflatoxins and human liver cancer. II. Aflatoxins in market foods and food stuffs of Thailand and Hong Kong. Food Cosmet. Toxicol. 10:61.

Sheretz, P. C., Eadie, T., Young, J. W. and Llewellyn, G. C. 1976. Aflatoxin occurrence on raw and cooked york soy bens inocculated with three Aspergillic isolates. JAOAC 59:662.

Shotwell, O. L., Hesseltine, C. W., Burmeister, H. R., Kwolek, W. F., Shannon, G. M. and Hall, H. H. 1969. Survey of cereal grains and soy beans for presence of aflatoxin. II. Corn and soy beans. Cereal Chem. 46:454.

Shotwell, O. L., Vandergraft, E. E. and Hasseltine, C. W. 1978. Aflatoxin formation in sixteen soy bean varieties. JAOAC 61:574.

Siegel, D. G., Tuley, W. B., Norton, H. W. and Schimdt, G. R. 1979. Sensory textural and yield properites of a combination ham extended with isolated soy protein. J. Food Sci. 44:1049.

Sikes, A. and Maxcy, R. B. 1979. Differentiation of food spoilage bacteria on the basis of their ability to utilize different proteins. J. Food Sci. 44:1228.

Smith, G. C., Hyunil, J., Carpenter, Z. L., Mattil, K. F. and Cater, C. M. 1973. Efficiency of protein additives as emulsion stabilizers in frankfurters. J. Food Sci. 38:849.

Sofos, J. N. and Allen, C. E. 1977. Effects of lean meat source and levels of fat and soy protein on the properties of weiner type products. J. Food Sci. 42:875.

Sofos, J. N., Busta, F. F. and Allen, C. E. 1979. Effects of sodium nitrite on Clostridium botulinum toxin production in frankfurter emulsions formulated with meat and soy proteins. J. Food Sci. 44:1267.

Sofos, J. N., Izumi, N. and Allen C. E. 1977. Effects of soy proteins and their levels of incorporation on the properties of weiner type products. J. Food Sci. 42:879.

Sorensen, W. G., Hesseltine, C.W. and Shotwell, O. L. 1967. Effect of tempertaure on production of aflatoxin on rice by Aspergillus flavus. Mycopathologia, 33:49.

Speck, M. L. 1976. "Compendium of methods for the microbiological examination of foods." APHA. Washington, D.C.

Stansbury, J. B. 1975. Quantitative determination of selected bacteria in ground beef extended with varying levels of textured vegetable protein. Ph.D. dissertation, The University of Tennessee, Knoxville.

Steel, R. G. D. and Torrie, J. H. 1960. "Principles and procedures statistics." McGraw Hill Book Co., Inc., New York, N.Y.

Stoloff, L. 1977. Aflatoxins--an overview. In "Mycotoxins in Human and Animal Health" (Ed) Rodricks, J. V., Hesseltine, C. W. and Mehlman, M. A. Pathotox Publishers, Inc., Park Forest South, Ill.

Strzelecki, E. Lillard, H. S. and Ayres, J. C. 1968. Country cured ham as a possible source of aflatoxin. Appl. Microbiol. 18:938.

Stubblefield, R. D., Shotwell, O. L., Richard, J. L. and Pier, A. C. 1981. Transmission and distribution of aflatoxin in contaminated beef liver and other tissues. JAOCS. 58:1015A.

Subramanyam, P. and Rao, A. S. 1974. Occurrence of aflatoxins and citrinin in ground nut (Arachys hypogaea, L) at harvest in relation to pod condition and kernal moisture content. Curr. Sci. 43:707.

Sutic, M. Ayres, J. C. and Koehler, P. E. 1972. Identification and aflatoxin production of molds isolated from country cured hams. Appl. Microbiol. 23:656.

Suzuki, J. A., Dainius, B. and Kilbuck, J. H. 1973. A modified method for aflatoxin detection for spices. J. Food Sci. 38:949.

Taber, H. H. and Schroder, H. W. 1967. Aflatoxin producing potential of isolates of the Aspergillus flavus oryzae group from peanuts (Aveachys hypogeae). Applic. Microbiol. 15:140.

Tandon, R. C., Nigam, S. S. and Agarwal, P. N. 1969. Biosynthesis of citrinin by Aspergillus candidus link. Labdev. J. Sci. Technol. 7-B:263.

Tauber, H., Lamfer, S. and Goll, M. 1942. Color test for citrinin and method for its separation. J. Am. Chem. Soc. 64:2228.

Terrell, R. N., Brown, J. A., Carpenter, Z. L., Mallil, K. F. and Monagle, C. W. 1979. Effects of oil seed proteins at two replacement levels on chemical, sensory and physical properties of frankfurters. J. Food Sci. 44:865.

Terrell, R. N., Smith, H. C. and Carpenter, Z. L. 1977. Practial manufacturing techology for dry and semi-dry sausage. Proc. 30th Annual Recip. Meat. Conf. of the American Meat Sci. Assn. Auburn University, Auburn, Alabama.

Terrell, R. N. and Staniec, W. P. 1975. Comparative functionality of soy proteins used in commerical meat food products. JAOCS 52:263A.

Timonin, M. I. and Rouatt, J. W. 1944. Production of citrinin by Aspergillus sp. of the candidus group. Can. J. Publ. Health 35:80.

Tripathi, R. K. 1973. Aflatoxin in sorghum grains infected with head molds. Indian J. Exp. Biol. 11:361.

Tung, T. C. and Ling, K. H. 1968. Contamination of sweet potatoes with aflatoxin. J. Vitaminol. 14:48.

USDA. 1971. Food and Nutrition Service Notice 219 for the school lunch program. USDA, Washington, D.C.

Van Rensburg, S. J., Van DerWatt, J. J., Purchase, I. F. H., Coutinho, L. and Markham, R. 1974. Primary cancer rate and aflatoxin intake in a high cancer area. S. Afr. Med. J. 48:2508a.

Van Walbleek, W. 1973. Fungal toxins in foods. Can. Inst. Food Technol. J. 6:96.

Van Zytveld, W. A., Kehley, L. A. and Dennis, S. M. 1970. Aflatoxicosis: the presence of aflatoxins or their metabolites in livers and skeletal muscle of chickens. Poultry Sci. 49:1350.

Wang, Y. and Ting, H. S. 1950. Metabolism of citrinin II. Absorption, rentention and excretion of citrinin. Clin. J. Physiol. 17:271.

Wang, Y. and Ting, H. S. and Mann, W. 1950. Metabolism of citrinin I. Determination of free citrinin in blood. Clin. J. Physiol. 17:259.

Wilding, M. D. 1971. New products--the use of soy protein in the food service industry. Proc. Recip. Meat. Conf. of the American Meat Sci. Assn. University of Kentucky, Lexington, KY.

Wilding, M. D. 1974. Textured proteins in meats and meat like products. JAOCS 51:128A.

Wildman, J. D., Stolof, L. and Jacobs, R. 1967. Aflatoxin production by a potent Aspergillus flavus link isolates. Biotechnol. Bioengin. 9:429.

Wogan, G. N. 1966. Chemical nature and biological effects of the aflatoxins. Bacteriol. Rev. 30:460.

Wogan, G. N. 1968. Aflatoxin risks and control measures. Fed. Proc. 27:932.

Wogan, G. N. 1969. "Metabolism and biochemical effects of aflatoxins. I. Aflatoxin, scientific background, control and implication" (Ed) Goldblatt, L. Academic Press, New York.

Wogan, G. N. 1973. Aflatoxin carcinogenesis. Meth. Canc. Res. 7:309.

Wogan, G. N. and Newberne, P. M. 1967. Dose response characteristics of aflatoxin B_1 carcinogenesis in the rat. Cancer Res. 27:2370.

Wu, M. T., Ayres, J. C. and Koehler, P. E. 1974a. Production of citrinin by Penicillium viridicatum on country cured ham. Appl. Microbiol. 27:427.

Wu, M. T., Ayres, J. C. and Koehler, P. E. 1974b. Toxigenic aspergilli and penicillia isolates from aged cured meats. Appl. Microbiol. 28:1094.

Wyllie, J. 1944. A method of rapid production of citrinin. Canad. J. Pub. Health 35:80.

Wyllie, T. D. and Morehouse, L. G. (Ed). 1978. "Mycotoxic Fungi, mycotoxins and mycotoxicoses. An encyclopedic handbook." Vol. 2. Marcel Dekker, Inc., New York, N.Y.

Ziemba, M.D. 1975. Food Protein research accelerates--a look at R and D in the future. Food Processing 36(8):21.

APPENDIX

SENSORY EVALUATION SHEET

DATE _____ TASTER _____ PRODUCT _____

You will be receiving five samples of semi-dry (summer) sausage to evaluate for overall acceptability. While evaluating take into consideration flavor, texture, and color. Mark at the point that best describes your feeling about the sample. Please give a reason for your decision. Also indicate the sample which you prefer most in the space provided at the end of the sheet. An honest expression of your personal feeling will be valuable to this research.

	____	____	____	____	____
Like extremely	____	____	____	____	____
Like very much	____	____	____	____	____
Like moderately	____	____	____	____	____
Like slightly	____	____	____	____	____
Dislike slightly	____	____	____	____	____
Dislike moderately	____	____	____	____	____
Dislike very much	____	____	____	____	____
Dislike extremely	____	____	____	____	____

REASON	REASON	REASON	REASON	REASON

Which sample do you prefer? (please write in the code number) _____

VITA

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