Determination of Cyanide in Soybeans and Soybean Products

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Cyanide content was determined on distillates from soybean meal, various soybean fractions, and soybean products by a pyridine–barbituric acid colorimetric procedure. Values of 0.07–0.3 μg of hydrogen cyanide/g of sample in soy protein products and 1.24 μg/g in soybean hulls were obtained when browning was kept to a minimum. These results compare with values as high as 1 and 3 mg/g in cassava and certain varieties of lima beans, respectively, and with values of 0.001–0.45 μg/g reported in various cereal grains and cereal products. Much higher, more variable values resulted under severe browning to charring conditions. Addition of carbonyl compounds decreased recovery values of cyanide standards, and because these compounds occur naturally in soybeans, cyanide values for soybean samples may be low. Emulsion and a crude linamarase preparation were ineffective in releasing cyanide from soybean meal. Extracts of soybean meal were fractionated by column chromatography and tested for cyanide. No cyanogenic precursor was isolated and little concentration of cyanide was obtained. These studies indicate that cyanogenic compounds are present in soybean protein products in very small amounts and probably are of little nutritional significance.

Consumption of edible soy protein products in the United States is over 1 billion pounds annually (Food Protein Council, 1978), which is less than 3 g of protein per person per day. However, soy protein intake should increase, since rapid growth in consumption of traditional Oriental soy foods is predicted (Shurtleff and Aoyagi, 1979; Fukushima, 1981). In Japan, on the other hand, per capita intake of protein from soy is 10 g/day (Watanabe, 1978), and in some southeast Asia countries it is about 30 g daily (Protein Advisory Group of the United Nations, 1973). Where soy and other food legumes are a major item in the diet, minor constituents, such as cyanide, are of concern from a toxicological viewpoint.

Reviews on cyanogenic compounds (mainly glycosides), their physiological effects, and methods of characterization have been written by Liener (1973), Seigler (1977), Montgomery (1980), and Conn (1981). Cyanogenic glycosides are major constituents of some staple foods such as cassava and lima beans which contain mainly linamarin and lotaustralin. A potential of up to 35 mg of hydrogen cyanide or half the lethal dose is found in the individual daily diet of cassava consumers in Nigeria (Seigler, 1977). The legume family has long been recognized to contain cyanogenic genera (Montgomery, 1980; Seigler, 1977), but Montgomery (1964) and Katsuki et al. (1978) found no evidence of cyanogenesis in soybeans. Nevertheless, low levels of cyanide from sources other than cyanogenic glycosides or cyanogenic lipids have been reported for *Chlorella vulgaris* and New Zealand spinach by Gewitz et al. (1976). They also found that yields of cyanide were increased by the addition of certain amino acids to the *Chlorella* and spinach preparations. Lehmann et al. (1979) found from 0.001 to 0.45 μg/g hydrogen cyanide in a wide variety of cereal grains and their products. In addition to hydrolytic or enzymatic release of cyanide from plant materials, overheating of the sample during a cyanide determination can also lead to cyanide formation (Rosenthaler, 1932). Osborne et al. (1956) and Thomson and Anderson (1980) report on the formation of cyanide by pyrolysis of various materials. Cyanide is absorbed by humans from a number of sources and small amounts are found in most human blood; however, the cyanide is rapidly converted to thiocyanate or other compounds which must also be accounted for to determine the actual amount ingested (Pettigrew and Fell, 1973). Small amounts of cyanide may act as a brake on cellular oxidative processes; however, consumption of foods containing large amounts of cyanogenic glycosides has resulted in death or has been associated with chronic neurological effects (Montgomery, 1980). Factors that affect the potential toxicity of cyanogenic plants have been discussed by Conn (1979).

In view of the widespread use of soybean foods in countries to which we export soybeans and their increasing use in the American diet, we investigated various procedures to determine levels of hydrogen cyanide that can be released from soybean foods and to see if cyanogenic compounds can be isolated or identified.

EXPERIMENTAL PROCEDURES

Preparation of Samples. Dehulled, pentane–hexane defatted soy flakes were prepared from certified, seed-grade soybeans, 1976 crop, Amsoy 71 variety, according to previously described procedures (Sessa et al., 1969). Recovered soybean hulls were passed through an air classifier to remove fines and meal particles. Samples of hulls from TS-280 and Amsoy 71-1979 crop soybeans were hand sorted to remove other seed particles. Toasted, hexane–ethanol azeotrope extracted (HEAE) soy flour, toasted soy protein concentrate (SPC), and soy protein isolate were prepared from dehulled, defatted soy flakes as described by Honig et al. (1976). The soy isolate (pH 7, water extraction method), the residual soybean meal, and the whey from the isolate preparation were freeze-dried. Soy flakes, hulls, residue, and whole beans were ground in a Wiley mill through a 20- or 40-mesh screen. Whole soybeans were first cooled in liquid nitrogen before grinding. Soybeans and lima beans as well as a commercial raw, dehulled, defatted soy flour and a textured soy flour were purchased from various sources.

Enzyme Preparation. A crude preparation of lima bean linamarase was prepared according to the method of Wood (1965) used for the isolation of cassava linamarase.

Deionized, distilled water was used in all procedures. β-Glucosidase (emulsin) was purchased from Sigma Chemical Co., St. Louis, MO. Succinimide and *N*-

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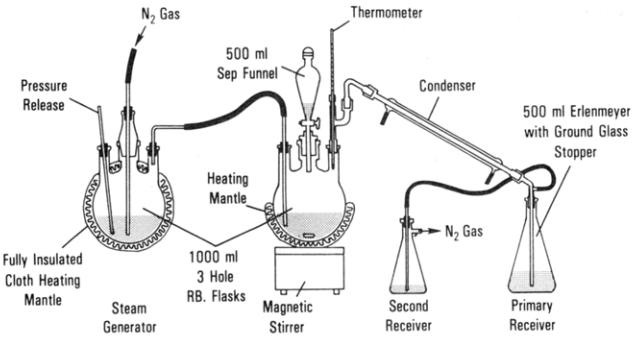

Figure 1. Cyanide distillation apparatus.

chlorosuccinimide (NCS) were obtained from Eastman Kodak Co., Rochester, NY, and barbituric acid was from Aldrich Chemical Co., Milwaukee, WI.

Standard Distillation Procedure for Hydrogen Cyanide Determination. The standard methods used for this study were adapted from those of Winkler (1958), the U.S. Environmental Protection Agency (USEPA, 1974), and Skaggs and Madl (1978). These methods involve acid hydrolysis of the sample with subsequent distillation and quantitation of the liberated hydrogen cyanide. The apparatus used is shown in Figure 1. Dry sample, 5–30 g, was suspended in 200 mL of water, and then 20 mL of a mixture of 10% lead nitrate and 10% tartaric acid (1:1), 0.1 mL of antifoam A, and 20 mL of 1 N H_2SO_4 were added. The final concentration of H_2SO_4 was about 0.1 N. The steam generator held about 500 mL of H_2O initially, and nitrogen was bubbled through the sample at a rate of two or three bubbles per second during distillation. The flask was heated with stirring at a rate such that 150–200 mL of water distilled over in 1.5–2 h from the start of heating. Distillates were collected in 30 mL of 0.25 N NaOH (primary receiver). A secondary receiver containing 10–30 mL of 0.25 N NaOH was used to trap any hydrogen cyanide which might escape the primary receiver. For collection of additional 150-mL fractions of distillate, the primary receiver was replaced with receivers containing 30 mL of 0.25 N NaOH.

Pyridine–Barbituric Acid Colorimetry. The NCS reagent was prepared as described in Lambert et al. (1975). The barbituric acid reagent was a mixture of 15 g of barbituric acid, 75 mL of pyridine, 15 mL of concentrated HCl, and 165 mL of H_2O similar to the USEPA procedure (1974). From 1 to 4 mL of distillate in triplicate was transferred to test tubes and adjusted to a total volume of 4 mL with 0.05 N NaOH. To this was added 1.5 mL of 1 N acetic acid–NCS reagent, 1:1, followed by 1 mL of barbituric acid reagent.

After being stirred, the tubes were allowed to stand 10 min and then were read at 580 nm on a Beckman B spectrophotometer along with a reagent blank and a cyanide standard. Potassium cyanide reference standards were prepared as in the USEPA procedure (1974). Distillations also were made of a reagent blank, a reference standard, and soy samples spiked with a cyanide standard to check for background, interferences, and cyanide recoveries.

Diffusion Method. A slurry of from 2 to 15 mL of the sample to be analyzed was added to the main compartment of a diffusion cell or Warburg flask, and from 1 to 4 mL of 0.05 N NaOH was placed in the center well. Acid or enzyme was then added to the sample. After the cell or flask was closed, the reaction mixture was kept at room temperature for 18–24 h to permit liberated cyanide to diffuse into the center well. The cyanide in the center well was then measured by colorimetry.

Other Methods. Picric acid colorimetry (Williams and Edwards, 1980), dinitrobenzene colorimetry (Guilbault and Kramer, 1966), and a cyanide electrode method (Orion Research, Inc., 1967) were also used to assay distillates or diffusates for cyanide. The dinitrobenzene method (Guilbault and Kramer, 1966) and Feigl-Anger test strips (Feigl and Anger, 1966) were also used as qualitative tests for the presence of cyanide.

Extraction and Column Fractionation. Three different extractions were used to try to evaluate recovery of cyanide and to isolate possible cyanogenic compounds from the soybean samples. Citrate buffer extraction as suggested by Hafner (1977) was used to prepare a deproteinized extract: The soy sample was extracted with a pH 5.9 citric acid buffer at a sample-to-buffer ratio of 1:10 and then brought to pH 8.4 with NaOH before adding 10% zinc acetate to precipitate protein. After centrifugation, the supernatant was adjusted to pH 5.5 and then subjected to distillation and colorimetry as in the direct cyanide determination in meal. Extracts were also made with boiling 80% ethanol for 1 h at a solvent-to-meal ratio of 10:1 and by Soxhlet extraction with hexane–ethanol azeotrope, 81:19 v/v. The extracts were concentrated on a rotary evaporator, freeze-dried, and then subjected to the distillation cyanide determination. Portions of the 80% ethanol and hexane–ethanol azeotrope extracts were also subjected to chloroform–methanol–water, 200:100:75, extraction to remove lipids and then fractionated on silicic acid or Sephadex G-10 columns. Silicic acid fractions were eluted with a chloroform–methanol gradient as in Honig et al. (1969).

RESULTS AND DISCUSSION

Enzymatic Determination of Hydrogen Cyanide. Defatted soybean meal, as well as 80% ethanol and hexane–ethanol azeotrope extracts of defatted soybean meal, was treated with linamarase and emulsin to determine if cyanogenic glycosides similar to those found in lima beans or other legumes might be present. No evidence for cyanide formation was found, although the enzymes were effective with lima bean samples. The possibility exists that linamarase and β-glucosidase were unable to hydrolyze the type of cyanogenic compound that may be present in soybeans. However, cyanogenic glycoside content was most likely below detectable levels.

Release of Hydrogen Cyanide by Acid Hydrolysis and Distillation. Cyanide recoveries from soybean products were greatly affected by the amount of acid used, rate and duration of distillation, concentration of sample, and rate of stirring.

Even under the standard conditions described for the distillation procedure under Experimental Procedures, there were some interferences with the cyanide analyses, as shown by the results in Table I. Recovery of a standard cyanide sample, which had been subjected to the distillation procedure with no soy sample present, was quantitative. In the presence of defatted soy flour, only 60–80% of the calculated cyanide present was recovered. This indicates that there is some reduction in cyanide values due to interference, but most of the cyanide released is being measured. Cyanide recoveries were lowered to 30 or 50% in the presence of 50 ppm of SO_2 or 56 ppm of malonaldehyde in the distillate. However, levels of malonaldehyde or SO_2 normally present in the distillates were well below these values. Other carbonyl or sulfur compounds may also be formed and interefere but were not investigated at this time. Winkler (1958) indicated that use of lead nitrate with tartaric acid reduced interferences

Table I. Yields of Hydrocyanic Acid in the Presence of Various Substances

substance	apparent cyanide present	cyanide added	cyanide found	recovery, %
reagents, 1.2 N H_2SO_4	none	150 μg	157.5 μg	105
30 g of commercial soy flour	2.4 μg	4 μg	5.15 μg	80
20 g of laboratory soy flour	1.6 μg	200 μg	114 μg	57
50 ppm of SO_2	none	4 μg	1.3 μg	32
56 ppm of malonaldehyde	none	250 ng	133 ng	47

Table II. Hydrocyanic Acid Released from Soybeans and Various Soybean Products under Mild Conditions[a]

description	μg/g (ppm)	determinations
whole soybean meal	0.26 ± 0.09	3
soybean hulls	1.24 ± 0.26	12
laboratory raw defatted soy flour	0.08 ± 0.02	3
commercial raw defatted soy flour	0.08 ± 0.02	12
toasted HEAE[b] soy flour	0.07	1
toasted soy protein concentrate	1.2	1
soy protein isolate	0.18 ± 0.04	2
soy protein residue	0.18 ± 0.001	2
soybean whey	0.09 ± 0.02	2
commercial textured soy flour	0.20 ± 0.02	2
lima bean meal	43 ± 11	6
HEA soybean meal extract	0.095 ± 0.005	2

[a] Hydrolysis in 0.1 N H_2SO_4; 8:1 water-to-meal ratio; magnetic stirring; distillation rate 150 mL/1.5 h from the start. [b] Hexane-ethanol azeotrope extracted (HEAE).

Table III. Hydrocyanic Acid Released from Various Soybean Fractions with Extended Distillation[a]

sample description	μg/g for distillate fraction 1	2	3	4
soy isolate residue	0.18	0.03	0.04	0.04
soy isolate	0.22	0.10	0.22	0.24
whole soybean meal	0.33	0.13	0.21	2.52
defatted soybean meal	0.08	0.04	0.04	0.07
Amsoy 71 soy hulls, 1976 crop	1.05	0.59		
TS-280 soy hulls[b]	0.84	5.68		
Amsoy 71 soy hulls,[b] 1979 crop	1.43	4.45		
Amsoy 71 soy hulls, 1976 crop and 1979 crop hand sorted	1.66	3.09	8.38	34.9
Amsoy 71 soy hulls[c]	0.57	0.16	0.18	0.29

[a] Conditions as for Table II except additional 150-mL fractions were collected. [b] Hand sorted. [c] Twice as much water was used in the steam generator and distillation flasks as was used for the other samples in this table.

in the analysis of cyanide in lima beans. With soybeans, cyanide values were more consistent in replication with lead nitrate and tartaric acid present but still remained well below lima bean values.

Hydrogen Cyanide Released under Standard Conditions. The most reliable and reproducible cyanide values are those listed in Table II. The lowest values for direct determination of cyanide in a meal sample were 0.08 μg/g of meal or 80 parts per billion (ppb) for raw, dehulled, defatted soy flour and 0.07 μg/g for a toasted, HEAE, dehulled, defatted soy flour. Very similar values were obtained with a laboratory and a commercially prepared defatted soy flour. Protein isolate, meal residue from isolate preparation, and whey samples yielded higher values than the defatted soybean meal from which they were prepared. The isolate and residue values were twice those of the original meal. The toasted soy protein concentrate yielded higher values than the raw soybean meal, also indicating that processing may increase amounts of cyanide that can be released. Yields from soybean hulls were about 10 times those of the raw dehulled soybean meal and account for most of the higher values in whole soybean meal. Cyanide content of the hulls may need further investigation.

Extended Distillation. The standard distillation procedure for soybean meal requires only a single collection of about 150 mL, since a second collection showed greatly reduced values. Because a second distillate fraction from soybean hulls showed a higher cyanide value, two to four successive fractions of about 150 mL each were collected from various soybean samples. By the time the fourth fraction had been collected, water levels had been lowered in the distillation flask, and a brown, nearly gelatinous mass was left and some dark brown baked material collected on the upper wall of the flask. As shown in Table III, as much as 35 μg/g was measured in the fourth fraction from a hull sample. Three different hull samples were analyzed. Especially large increases were found in the second fraction from the hand-sorted TS-280 and the 1979 crop Amsoy 71 hulls. Whole soybean meal also showed a major increase in cyanide in the fourth fraction, whereas defatted meal and its fractions showed little or no increase after collecting the first fraction. The 1976 crop Amsoy 71 hull sample was also subjected to an extended distillation with twice the amount of 0.1 N H_2SO_4 in the distillation flask and twice as much water in the steam generator. Under these conditions there was little browning and a decrease in the amount of cyanide was found as more fractions were collected. Lehmann and Zinsmeister (1979) report in their analysis of cereal grains that more cyanide was produced with extended acid treatment. They attributed this to formation of cyanide from nitrogeneous constituents and demonstrated formation of cyanide from carbohydrates and amino acids under similar conditions with oxygen present. Whether this explains the increased cyanide contents found in soybean hulls under our extended distillation conditions would require further study, especially since most oxygen should have been displaced by the nitrogen which was bubbled through. The experiment with the increased amount of water suggests, however, that most of the increase in cyanide measured after extended distillation of whole soybeans or soy hulls is due to artifact formation.

Harsh Distillation. A number of samples were also distilled under harsher conditions. The samples were stirred only by the nitrogen passing through; the acidity was 3–30% sulfuric; the water-to-meal ratio was reduced to 5 mL/g of sample, and the distillation rate was over 200 mL/h from the start of heating. Under these conditions, considerable charring took place and much of the sample was brown to black by the time distillation was ended.

Cyanide values for defatted soybean meal varied from 0.3 to as much as 61 μg/g. These high values appear to be due mainly to artifacts since they were difficult to reproduce; much lower values were obtained for defatted meal under the milder conditions, even with extended distillation. Strong acid conditions may also cause breakdown of cyanide and result in too low a value (Winkler, 1958).

Some of the distillates were subjected to diffusion or redistillation to remove more interferences. The cyanide was concentrated thereby and recovered in 75–80% of the original yields to confirm that the distillation values were representative of actual cyanide released. Picric acid, cyanide electrode, and *o*-dinitrobenzene detection also gave positive results with the distillates and some other samples, but these methods appeared to be more affected by interfering substances and are less reliable or sensitive than the pyridine–barbituric acid method.

Extraction and Chromatography of Cyanogenic Compounds. In an attempt to concentrate and purify cyanogenic compounds that might be present, some soybean samples were also subjected to the citrate extraction procedure of Hafner (1977). Extraction with hot 80% ethanol and with a hexane–ethanol azeotrope mixture was also used to extract possible cyanogenic compounds from dehulled, defatted soy flakes. The extracts, consisting essentially of the low molecular weight constituents, gave low yields of cyanide when subjected to acid hydrolysis and distillation. Overall yields from the extracts were lower than for the original samples assayed directly. There was likewise little concentration of cyanogen, as measured by liberation of cyanide, when extracts were subjected to chromatography on Sephadex G-10 and silicic acid. A diffusion procedure and detection with Feigl-Anger strips were also used to test for release of cyanide from soybean meal, as well as from extracts and their fractions, but were not sensitive enough for reliable results.

No reliable evidence for cyanogenic precursors was found in any soybean samples other than the low levels of cyanide detected. These cyanide levels were less than 0.1 ppm in raw soybean meal and less than 1.5 ppm in any sample under standard conditions. Higher levels of cyanide can be produced as artifacts under extreme conditions (Rosenthaler, 1932; Lehmann and Zinsmeister, 1979). Apparent cyanide content in soy protein products is far below the 10–20 mg/100 g or 100–200 ppm set as upper limits for cyanide from edible lima beans in the United States or several European countries (Montgomery, 1964). The soybean cyanide levels do not appear to be of nutritional significance.

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