The Protein Quality of Cottonseed Protein Concentrate Prepared by Two Different Industrial Processes

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The protein quality of two samples of cottonseed protein in the form of flour produced by two different technologies was evaluated in young and adult rats and in children, using growth and nitrogen balance techniques. One of the samples was produced by a modified prepress solvent method (B) and the other by a solvent extraction method, using hexane–acetone–water (CF-21). From growth and PER data, the CF-21 sample appeared slightly superior to sample B and both were inferior to casein. Both samples gave equal nitrogen retention values when fed to rats at different levels of protein intake. However, the digestibility of the CF-21 flour was superior to that of the B sample. Nitrogen retention values in children between the CF-21, B, and skim milk samples were not statistically significant at the 2-grams-of-protein-per-kg.-per-day level of intake. However, the values were lower than those from skim milk, at the lower level of dietary protein. Protein digestibility was greater in sample CF-21 than in sample B, and both were lower than milk.

One of the problems in the utilization of cottonseed protein concentrates as a food was, and still is to a limited extent, the lack of an appropriate process for its commercial preparation to meet the standards of quality for human use (*1*). Present technology is directed to the maximum extraction of the oil, giving little regard to the protein of the cake. The demand for this type of protein in both animal and human feeding stimulated research in this field, which resulted in the development of new technologies capable of producing a cottonseed protein concentrate in the form of flour, of an excellent quality. These methods have been described by several workers (*9, 10, 12, 13, 16*).

Studies carried out at INCAP (*5*) indicated, however, that it is also possible to produce high grade flours from cottonseed using ordinary techniques with some modifications. Thus, Bressani and coworkers (*5*) have described the changes to be made in a prepress solvent extraction process to make a cottonseed flour meeting the standards of quality for human use. These studies indicated that a human grade cottonseed flour can be prepared by:

Using dried seed with a low free fatty acid content

Allowing more than 10% seed coat with the kernel

Cooking the cracked kernels with moisture levels of 15 to 18% at temperatures of 180° to 200° F.

Working at 60 to 70% load capacity at the expeller level

Desolventizing at low temperatures

Grinding at low speed for lower temperatures

Screening for removal of husks to raise protein to a minimum of 50%.

On the other hand, the azeotropic solvent extraction method removes the oil and almost all gossypol at low temperatures from flaked cottonseed kernels. Some cooking prior to extraction appears to improve the nutritional quality of the flour. Since it is known that the azeotropic method using acetone–hexane–water (*9, 10, 12*) causes little damage to the protein, it was of interest to learn if there were any differences between cottonseed flour prepared by this process and that described by Bressani and coworkers (*5*) with respect to the protein quality of the products. If no differences are found, the older and more common prepress solvent process available in many areas of the world can be modified with little cost to produce protein of good quality, which is in great demand in the world today. The objective of this investigation was, therefore, to test the protein quality of two cottonseed flours produced by the two techniques, using both young and adult rats and children.

Materials and Methods

Cottonseed Protein Concentrates. Two cottonseed flours were used for the study. One of them, hereafter known as cottonseed flour B, is produced in a cottonseed oil mill in El Salvador, Central America, by a modified prepress solvent extraction method described by Bressani and coworkers (*5*). The CF-21 cottonseed flour was obtained from the USDA Southern Regional Research Laboratory and prepared by the mixed solvent method described by Frampton and coworkers (*9, 10, 12*), using hexane–acetone–water.

Chemical Analysis. Both cottonseed protein samples were analyzed for moisture, fat, crude fiber, ash, and nitrogen by the A.O.A.C. methods (*2*). Free and total gossypol content was also determined according to the methods of the A.O.C.S. (*1*). The free amino groups of lysine were determined by the method of Conkerton and Frampton (*8*) using the dinitrofluorobenzene reagent. The samples were also assayed for the essential amino acids using microbiological techniques as previously described (*7*). Each cottonseed flour sample was assayed three times.

Studies with Rats. Growth and Protein Efficiency Ratio (PER) Assays. For this experiment, a total of 144 weanling white rats of the Wistar strain from the INCAP colony were distributed according to weight into three groups of 48 rats per group. Each group was in turn distributed among six subgroups of eight rats each (four males and four females), with an average initial weight of 45 grams. The animals were placed in individual all wire screen cages with raised screen bottoms and fed water and food *ad libitum*. The changes in weight and the amount of food consumed were measured every seven days for a total of 28 days.

The animals were fed one of three rations: casein, cottonseed flour B, and cottonseed flour CF-21, added to six different diets each to give levels of protein from 6 to 45% in the diet. The partial composition of these diets is described under results. All diets were analyzed for nitrogen, and PER values

were calculated from food intake and weight gain data.

Nitrogen Balance in Adult Rats. For this study, 16 adult male rats were divided into two equal groups and each placed in stainless steel metabolism cages. One group was fed the diet containing flour B and the other the one with CF-21 flour. The amount of cottonseed protein in the diet decreased, and it was replaced by cornstarch. The levels of cottonseed flour used were 25, 20, 15, 10, 5, and 0% of each sample. The other components of the diet were: 4% mineral mixture (*11*), 5% refined cottonseed oil, 1% cod liver oil, 30% dextrose, and sufficient cornstarch to make up to 100%. Furthermore, all diets were supplemented with 5 ml. of a complete vitamin solution (*14*) per 100 grams of diet. The animals were offered 15 grams daily of all diets, and records were kept of the amounts consumed. Each level of protein was fed for six days obtaining two three-day balance periods per level. Water was available at all times. The urine and feces were collected twice daily and placed under refrigeration. Every three days the individual collections were combined and analyzed for nitrogen by the micro-Kjeldahl method. Nitrogen retention was calculated from the nitrogen intake and nitrogen excretion data.

Studies in Children. The two cottonseed protein concentrates were used to prepared INCAP Vegetable Mixture 9 (*4, 6*), which contains 38% cottonseed flour. These mixtures were then fed to six children that had recovered from protein malnutrition, at a level of intake of 2 and 1 grams of protein per kg. of body weight per day, for nine days each, obtaining three three-day balance periods. Each child was first fed milk protein at the same two levels of dietary protein. The intake of calories was kept constant at 100 per kg. of body weight per day. Table I shows the age and weight of each child fed the two cottonseed flour samples and the sequence in which they were fed. The children were placed in metabolism beds, and feces and urine were collected continuously. Urine samples were collected in dark bottles containing 1 cc. of concentrated acetic acid and kept cold. Each three-day collection was brought to the laboratory and homogenized, and the urine volume and feces weight were measured. Aliquots of the food, urine, and feces were analyzed for nitrogen content using the Kjeldahl method.

Results

The chemical composition of the two samples studied is shown in Table II. Also shown in the table are the quality standards for edible cottonseed protein. Both flours meet with all specifications, with the CF-21 sample better than the B sample. The latter, however, contains slightly less available lysine.

The average essential amino acid content is also shown in Table II. Both samples contain similar concentrations of the different amino acids. The concentration of microbiologically determined lysine in both samples is higher than that for available lysine.

The results of the growth experiments in rats are presented in Table III. Maximum weight gain for the CF-21 flour and casein was found when they provided about 16% of protein. Similar level of protein from the B flour gave a slightly lower weight gain. Maximum PER values were obtained when casein provided 9.7%, CF-21 flour 12.4%, and B flour 11.3%.

The nitrogen balance of the two flours as determined in adult rats are shown in Table IV. The data show that cotton-

Table I. Description of Children and of Dietary Treatment

Child No.	Age, Months	Weight, Kg.	Sequence in Which Proteins Were Fed at Both Levels of Protein Intake
PC-120	48	17.69	Milk-VM9 (B)–VM9 (CF-21)
PC-125	40	13.28	Milk-VM9 (CF-21)–VM9 (B)
PC-126	47	11.59	Milk-VM9 (B)–VM9 (CF-21)
PC-135	33	12.46	Milk-VM9 (CF-21)–VM9 (B)
PC-136	27	10.60	Milk-VM9 (B)–VM9 (CF-21)
VV-25	60	19.16	Milk-VM9 (CF-21)–VM9 (B)

Table II. Chemical and Amino Acid Composition of B and CF-21 Cottonseed Flour

Components	Quality Standards	Cottonseed Flour CF-21	Cottonseed Flour B
Moisture, %	10.00	7.90	5.65
Fat, %	6.00	1.16	3.75
Crude fiber, %	5.00	2.60	4.31
Nitrogen, %	8.00	8.77	8.04
Ash, %	5.00	7.60	8.00
Free gossypol, %	0.060	0.025	0.057
Total gossypol, %	1.200	0.209	0.881
Tryptophan, mg./gram N		79	81
Lysine, mg./gram N		290	284
Methionine, mg./gram N		53	55
Cystine, mg./gram N		73	80
Phenylalanine, mg./gram N		222	240
Leucine, mg./gram N		313	330
Isoleucine, mg./gram N		231	262
Threonine, mg./gram N		241	243
Arginine, mg./gram N		720	763
Histidine, mg./gram N		207	209
Valine, mg./gram N		348	382
Tyrosine, mg./gram N		93	100
ϵ-NH_2 lysine, mg./gram N	225	234	216
ϵ-NH_2 lysine, gram/16 gram N	3.60	3.75	3.45

Table III. The PER of Casein, CF-21, and B Cottonseed Flours at Increased Levels of Protein Intake

Test Protein	Amount in Diet, %	Protein in Diet, %	Weight Gain,[a,b] Grams	PER
Casein	6.7	8.6	54	1.92
	11.2	9.7	126	3.19
	16.8	16.5	166	2.33
	28.0	25.2	166	1.52
	39.0	31.9	152	1.21
	50.4	43.1	150	0.97
CF-21	12.0	8.4	62	2.13
	20.0	12.4	142	2.44
	30.0	16.3	164	2.18
	50.0	27.4	163	1.28
	70.0	37.3	151	0.92
	90.0	48.3	110	0.64
B	12.0	7.5	46	1.81
	20.0	11.3	100	2.08
	30.0	16.7	141	1.85
	50.0	27.8	149	1.20
	70.0	36.2	152	0.94
	90.0	45.1	99	0.67

[a] Average initial weight, 45 grams.
[b] Experimental period, 28 days. All diets were supplemented with 4% mineral mixture (*11*), 5% refined cottonseed oil, 1% cod liver oil, and cornstarch to adjust to 100%. Furthermore, all diets were supplemented with 5 ml. of a complete vitamin solution (*14*) per 100 grams.

Table IV. Nitrogen Balance of Rats Fed Decreasing Levels of Nitrogen from Cottonseed Flours B and CF-21 (Average of 8 Rats)

Cottonseed Flour	Nitrogen, Mg./Three-Day Period				
	Intake	Fecal	Urine	Absorbed	Retained
B[a]	314	68	179	246	67
CF-21[a]	316	50	201	266	65
B[b]	261	56	135	205	70
CF-21[b]	269	42	158	227	69
B[c]	222	49	106	173	67
CF-21[c]	227	43	134	184	50
B[d]	176	38	82	138	56
CF-21[d]	183	35	96	148	52
B[e]	105	30	62	75	13
CF-21[e]	111	29	69	82	13
None	28	23	50	5	−45
None	29	24	45	5	−40

Cottonseed flour in diet: [a] 25%, [b] 20%, [c] 15%, [d] 10%, and [e] 5%.

Table V. Nitrogen Balance of Children Fed Skim Milk and Cottonseed Flours CF-21 and B at Two Levels of Protein Intake

Test Protein	Nitrogen, Mg./Kg./Day		
	Intake	Absorbed	Retained
Skim milk	305 (259–328)	255 (205–288)	82 (49–107)
Cottonseed flour B	302 (271–321)	218 (191–234)	72 (45–90)
Cottonseed flour CF-21	303 (272–314)	237 (219–261)	77 (54–101)
Skim milk	164 (151–176)	128 (115–145)	51 (25–66)
Cottonseed flour B	161 (131–182)	109 (86–127)	24 (4–42)
Cottonseed flour CF-21	163 (152–179)	117 (80–134)	18 (15–46)

seed flour B is equal to CF-21 flour. Nitrogen absorption data indicated that the digestibility of the CF-21 flour was slightly higher than the digestibility of the B flour; however, nitrogen retention was about the same for both.

Table V shows the results of the nitrogen balance data in the children fed the two flours and milk at 2 and 1 gram of protein per kilogram per day.

Statistical analysis of the values presented indicated that the differences in nitrogen intake between the two flours and between each flour and milk at the two levels of dietary protein were not significant. Nitrogen absorption of the B flour at both levels of intake was poorer than milk ($P < 0.01\%$). It was also poorer than CF-21 ($P < 0.05\%$) at the higher level of intake but was not significantly different at the lower level of intake. Nitrogen absorption of CF-21 was not significantly different from milk at either intake level. At the intake level of 1 gram of protein per kg. of body weight per day, only the nitrogen absorbed between milk and the B flour was significantly different at the 1% level.

Nitrogen retention values between the three proteins were not statistically significant at the 2-grams-of-protein-per-kg.-of-body-weight-per-day level of intake, but nitrogen balance for the cottonseed flours was significantly lower than that from milk at the 1-gram protein level of intake. The nitrogen retention figures for the two cottonseed flours at the lower level of protein intake were essentially the same.

Discussion

Because of the need to produce high quality cottonseed protein concentrates, efforts have been made either to modify technologies for oil extraction from oil-containing seeds or to develop newer technologies which could yield products of high quality and free of toxic compounds. The older technologies were designed to extract as much oil as possible from the seed, without paying attention to the protein quality of the residue. On the other hand, the modifications to the existing technologies and of newer methods are designed to manufacture products with little damage to the protein and still giving the same or similar yields of oil.

The results of the present study indicate that the existing prepress solvent method of processing, properly modified, is capable of producing cottonseed protein concentrates as good as the newer technologies for oil extraction. The most consistent difference between the two flours tested was the amount of absorbed nitrogen, which in both the rat and the child was highest for the CF-21 produced by the new technology of using hexane–acetone–water solvent (*9, 10, 12*). The B flour gave consistently lower nitrogen absorption values in both the rats and the children. This difference could be the result of the use of higher oil extraction temperatures in the production of the B flour as compared with the temperatures used for the preparation of the CF-21 concentrate. The effect of temperature can also explain the differences in the concentration of lysine. Acid hydrolysis of the two samples for the microbiological assay of lysine gave higher values than the method for available lysine. The amount of available lysine was, however, higher for the concentrate produced by the mixed solvent technique.

The PER assay indicated that the CF-21 concentrate has a higher protein value as compared to that of the B flour. This difference was to be expected since the former material showed higher concentrations of lysine, the amino acid most limiting for growth in cottonseed protein (*3*). However, the nitrogen balance studies carried out in the rat as well as the nitrogen balance in children at the two levels of protein intake, indicated equal utilization for the two samples tested. A possibility which could explain these findings is that since PER is based on weight gained and protein intake, highest weight gain will give higher PER. However, the highest weight gain may not necessarily be protein deposition as measured by nitrogen balance but could very well be fat, particularly if the protein has some kind of amino acid imbalance.

A second aspect of interest is that PER values above 2 are indications that the nitrogen retention in children fed adequate levels of protein is similar to that obtained from milk.

Finally, even though the absorption of nitrogen was less with the two flours than with milk, retention of nitrogen at intakes of 2 grams of protein per kg. of body weight per day was not statistically different from that of milk. This finding, referred to previously (*15*), may mean that the amino acids absorbed were in good balance for their effective utilization.

The results show that the modified technologies for oil extraction are capable of producing high quality material. While newer practical methods are developed, much could be done to alleviate protein-calorie malnutrition by using materials produced by the modified technologies in the elaboration of protein-rich vegetable food mixtures.

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METHODOLOGY

Determination of Cystine as Cysteic Acid after Low Voltage Paper Electrophoresis

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A procedure for the chemical determination of cystine includes as basic steps: oxidation of the samples with performic acid, hydrolysis with HCl, paper electrophoresis, ninhydrin reaction, elution, and colorimetric determination. This procedure when applied to a variety of food samples yielded accurate and reproducible results. With two electrophoresis cells as many as 12 samples can be assayed simultaneously.

Because carbohydrates destroy cystine during acid hydrolysis (*1*) the direct chemical determination of cystine is not reliable. Microbiological assays have been found to yield erroneous values because a short period of hydrolysis gave results that were too high; prolonged hydrolysis, results that were too low (*5*). Since cysteic acid is stable during acid hydrolysis, Schram, Moore, and Bigwood (*11*) recommended the oxidation of cystine to cysteic acid before hydrolysis and chemical assay. Methods for oxidation of cystine by performic acid have been improved to yield recoveries of 94% or more (*3, 8, 9*), but the subsequent separation from other amino acids by ion exchange is not easily adaptable to the simultaneous assay of several samples.

Hartel and Pleumeekers (*6*) used ion exchange paper chromatography for separating cysteic acid in wool hydrolyzates; in this laboratory a similar technique has been applied to foods after oxidation but with little success.

Mabry and Karam (*7*) reported that high voltage paper electrophoresis with a low ionic strength buffer separated cysteic acid from other amino acids. Low voltage paper electrophoresis was used by Diehl (*4*) for separating cysteic acid in wool hydrolyzates. In this laboratory, the latter method gave incomplete separation of cysteic acid from homocysteic acid and was not sensitive enough for the assay of food samples. The method described uses low voltage electrophoresis under conditions which permit the separation of homocysteic acid from cysteic acid and is sufficiently sensitive to be applied to the assay of food samples.

Experimental Procedure

Apparatus. Electrophoresis Cell. Durrum type paper electrophoresis cell (Spinco Model R) operated by a Spinco Duostat power supply.

Paper Strips. Whatman 3 MM paper strips 3.0 × 30.6 cm.

Paper Wicks. Grade EP 14, $2^2/_{32} \times 12^1/_2$ inches.

Spectrophotometer. Spectronic 20 with matched cuvettes of about 16-mm. inside diameter.

Evaporator. Craig rotary evaporator or similar.

Reagents. Performic Acid Reagent. Add 10 ml. of 30% H_2O_2 to 90 ml. of 88% formic acid. Allow the mixture to stand for 1 hour at room temperature. Cool to 0° C.

Hydrobromic Acid, 48% reagent grade HBr.

Sodium Hydroxide, 2*N* NaOH.

Hydrochloric Acid, 6*N* HCl.

Buffer Solution. To 700 ml. of H_2O add 30 ml. of formic acid and 120 ml. of glacial acetic acid. Dilute to 1 liter with H_2O.

Cysteic Acid Stock Standard Solution. Weigh 28 mg. of cysteic acid (equivalent to 20 mg. of cystine) and dilute to 100 ml. with H_2O.

Cysteic Acid Working Standard Solutions. Dilute 2, 3, 4, and 5 ml. of stock standard solution to 10 ml. with H_2O.

Ninhydrin Solution. 0.4% ninhydrin in ethyl alcohol containing 0.4% pyridine.

Copper Nitrate Solution. Mix 10 ml. of saturated copper nitrate solution with 0.2 ml. of concentrated nitric acid and make up to 1 liter with acetone.

Oxidation. This is essentially the method of Moore (*9*), in which some technical modifications have been incorporated. To a 125-ml. flat-bottomed flask with ground-glass joint add a sample containing about 1 mg. of cystine and 20 ml. of cold performic acid reagent and allow to stand at 0° C. for 4 hours with soluble proteins, or overnight with proteins that do not dissolve in the performic acid reagent. While swirling add 3.0 ml. of 48% HBr to the flask in the ice bath. Distill the free bromine at 40° C. under reduced pressure in a rotary evaporator and absorb the distillate into 25 ml. of 2*N* NaOH in the condenser. After the free bromine distillation is complete the last few milliliters of solution may be evaporated to near dryness at 60° C. without affecting the results. To minimize the tendency of HBr to condense in the stem of the rotary evaporator the level of water in the bath should reach the top of the reaction flask.

When the distillation is complete, raise the flask above the level of the condenser before the rotary evaporator is stopped, so that the condensation in the stem will run into the condenser