have been artifacts. The spectral absorption curve of the upper had much less fine structure, and had a cis-peak at a somewhat higher wave length.

Diols (IIIA). The major pigment of this fraction, and the most abundant by far, was identified as lutein and not capsolutein. On treating with hydrochloric acid in methanol and subjecting the product to a countercurrent distribution run with system I, nearly all of the material occurred as a fraction with N_{100} value of 40, in good agreement with the prior result of 38 for lutein monomethyl ether. The N_{100} values of lutein and capsolutein in this system were both 10, but the N_{100} value of capsolutein was unchanged after treatment with hydrochloric acid in methanol (*5*).

A very minor band on the column (IIIA-1) below lutein had spectral absorption maxima at 481 and 446 mμ. This may possibly have been the same as a nonepoxide diol found in red bell peppers (*5*), and tentatively identified as 6,6′-dideoxocapsorubin.

Above the lutein band was a much smaller, redder band (III A-4) which had similar spectral absorption maxima except the cis-peak was at 346 mμ instead of 338; the spectral absorption curve had much less fine structure. This was identified as zeaxanthin, and apparently consisted, at least in part, of cis-isomers which may have been artifacts.

Diepoxide Diols (IIIC). The major band of this fraction was violaxanthin (zeaxanthin-5,6,5′,6′-diepoxide); it was accompanied by smaller amounts of the corresponding 5,6,5′,8′-diepoxide isomers, the luteoxanthins, and by a much smaller amount of the 5,8,5′,8′-diepoxide isomers, the auroxanthins. Below the yellow auroxanthin band was a minor red band (IIIC-5) which had spectral absorption maxima near those of lutein. It was eluted much less readily than zeaxanthin and may have been a polyol such as hydroxycapsolutein (*5*).

Polyols (IV). The first band eluted from the column had pronounced spectral absorption maxima at 431, 405, and 384 mμ; these were only slightly changed on hydrochloric acid treatment, indicating that this substance was not a 5,6-epoxide such as sinensiaxanthin found in oranges (*7*). This band was eluted from a column much more rapidly than the auroxanthin band in fraction IIIC. The main constituent was neoxanthin, a 5,6-epoxide polyol of incompletely known structure, with much smaller amounts of the corresponding 5,8-epoxide isomers, the neochromes. When the major band (IV-2) was subjected to a countercurrent distribution run with system III (hexane-acetone-13% methanol), the N_{100} value was 64, in good agreement with previous results of 61-62 for neoxanthin from leaves.

As shown in Table III, lutein was the most abundant carotenoid, with somewhat smaller amounts of beta-carotene, violaxanthin, and neoxanthin. These are also the principal constituents of leaf carotenoids (*10*). Cholnoky *et al.* (*2*) recently investigated the carotenoids of green paprikas. Lutein was the most abundant constituent, with lesser amounts of beta-carotene, violaxanthin, "foliaxanthin," and "foliachrome." Small amounts or traces of beta-carotene-5,6-epoxide, mutatoxanthin, antheraxanthin, and apparently cryptoxanthin were also found. Three of the last four were not found in green peppers in the present work, two of which would be in the monoepoxide diol fraction (IIIB) which was not chromatographically investigated. Cholnoky *et al.* did not report a number of the minor constituents found in the present work, including phytoene, phytofluene, alpha-carotene, zeta-carotene, hydroxy-alpha-carotene, and zeaxanthin. "Foliaxanthin," a 5,6-epoxide, is apparently the same as neoxanthin; "foliachrome," reported by Cholnoky *et al.* as being the corresponding 5,8-epoxide, may be the same as neochromes, or, since it was found on the column below "foliaxanthin," possibly the same as luteoxanthins.

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HEAT EFFECTS ON SOYMILK

Indices of Protein Quality in Dried Soymilks

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The maintenance and evaluation of the nutritional quality of soybean products are of importance since such products often make up a significant part of an individual's food intake. This is the case when they are used as substitutes for animal milks in infant feeding and when they are used to elevate the protein content of otherwise inadequate diets. The use of animal feeding tests to obtain indices of protein quality (*13*) provides the most direct and reliable experimental evaluations of the products, but such tests are expensive and time consuming. Simpler and quicker methods for the control of the manufacture and procurement of soy products are widely recognized as being of value in ensuring high quality in the finished products (*14*).

For soybeans to provide a maximal contribution to nutrition, deleterious substances must be removed from the raw beans. Fortunately, through the application of heat, it is possible to inactivate such harmful materials as the trypsin inhibitors (*11*). To evaluate the adequacy of heat treatments a number of tests have been devised. Examples are the measurement of the extent of inactivation of urease (*3*) or trypsin inhibitors (*11*), the lowering of soluble nitrogen (*16*) or alkali-soluble solids (*14*).

Heating of soybeans introduces the possibility that some of the nutrients may

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Soymilk powders were prepared by various heating and drying procedures. Adequacy of heat treatment was evaluated by means of an assay for trypsin inhibitor. Soluble nitrogen and urease activity levels could not substitute for the extent of trypsin inhibitor destruction as an indicator of adequate heating. Overheating was accompanied by a decrease in available lysine and an increase in darkness of the product. Variations in free amino group levels depended on the conditions of heat treatment. Soluble nitrogen had no correlation to heat damage. Regression equations relating indices of protein damage to biological value are presented.

be damaged. Since the value of soybeans as a food is due in large part to their high content of protein, it is important to be able to measure the extent to which the nutritional quality of the protein may have been harmed during processing.

Destruction or modification of lysine in proteins can be measured by a procedure developed by Carpenter (*2*). This method yields a value for protein lysine available for use in the body for protein synthesis (or that lysine having a free epsilon amino group). Tests on animal proteins have shown a high correlation between available lysine content and biological value of the proteins (*1*). The estimation of available lysine has been applied to plant proteins (*1*, *15*) and mixtures of plant and animal proteins (*4*) with results that indicate that it may be useful in evaluating damage to proteins resulting from heating.

A type of protein or amino acid alteration that can occur during the processing of protein-containing foods results from the participation of amino groups in condensation reactions with other components of the food. By use of the formol titration, changes in the free amino group content can be estimated. Since proteins can become involved in browning reactions (*5*), the color of a product may also indicate protein damage. Therefore, the level of free amino groups and the color of the product were investigated as possible indices of protein quality in processed soymilks.

The purpose of this work was to compare various procedures used for evaluating adequacy of heating and possible protein quality damage in processed soybean milks. Nitrogen solubility and survival of urease and trypsin inhibitors were compared as indices of adequate heating. The nitrogen solubility as well as available lysine levels, free amino group content, and degree of browning were used to estimate protein damage. The correlation between these results and the biological value of the products was determined.

Procedure

Dehulled Clark variety soybeans were used in these studies. They were dehulled, soaked, ground with water, and then filtered. The details of the procedure have been described by Hand *et al.* (*7*). The experimental variables that were introduced into the process are presented in a later section, together with the data obtained.

Soluble nitrogen was determined by a method developed by Smith, Belter, and Johnsen (*16*). The soymilks were stirred in water, the mixture was filtered, and the nitrogen in the filtrate was measured. With the products studied it was not necessary to remove the fat before the water extraction.

The measurement of available lysine was carried out by the procedure of Carpenter (*2*), utilizing the reaction between fluorodinitrobenzene and the epsilon amino group of protein lysine. Recovery experiments in which epsilon dinitrophenyl lysine was added just prior to the acid hydrolysis step indicated that with these soy products a recovery of 78 to 82% was obtained. Data presented in this paper have therefore been corrected for a 20% loss during analysis.

Browning was measured as Hunter "L" values obtained using the photoelectric color difference meter described by Hunter (*9*). Plate No. 4 with an "L" value of 75 was used as a standard. With this instrument lower "L" values are associated with darker products.

The formol titration procedure followed was that described by Hawk, Oser, and Summerson (*8*). The results are expressed as equivalents of free amino groups per mole of nitrogen in the product.

The determination of residual trypsin inhibitors was carried out by using a modification of the assay developed by Learmonth (*10*), which compares the retardation of gelatin liquefaction by trypsin in the presence of various dilutions of heated and unheated soy products. The trypsin-gelatin mixture was no longer able to form a gel at 32° F. after 15 minutes' incubation at 98° F. The soybeans initially contained sufficient trypsin inhibitor such that 1 mg. of fresh soybean meal in 10 ml. of the trypsin-gelatin mixture would approximately double the incubation time needed for liquefaction. The results are expressed as per cent of the original trypsin inhibitors destroyed by processing. Urease was detected as described by Sumner and Somers (*18*).

Results

A comparison of methods for evaluating the adequacy of heat treatment of soymilk is given in Table I. The results obtained with representative products indicated that neither low soluble nitrogen nor the absence of urease were

Table I. Indices of Adequacy of Heat Treatments

Sample Description	Soluble Nitrogen, %	Urease	Residual Trypsin Inhibitor, %
Milk heated 2 minutes at 250° F.	29	−	36
5 minutes at 250° F.	35	−	10
8 minutes at 250° F.	39	−	16
32 minutes at 250° F.	35	−	5
30 minutes at 200° F.	18	−	10
60 minutes at 200° F.	24	−	14
120 minutes at 200° F.	24	−	4
Milk spray dried at 295° F.[a]	88	+	100
at 620° F.[a]	30	−	83
at 710° F.[a]	6	−	22
at 800° F.[a]	4	−	4
Soybeans steamed 5 minutes at 250° F.	21	−	8
15 minutes at 212° F.	46	+	20
30 minutes at 212° F.	40	+	10

[a] Inlet air temperature.

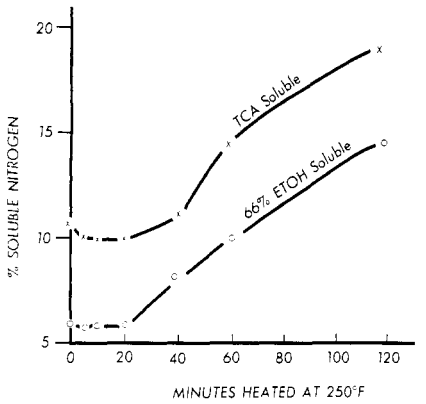

Figure 1. Effect of heating on the solubility of soymilk protein in 5% trichloroacetic acid (TCA) and in 66% ethanol

reliable indications of trypsin inhibitor destruction. During the heating of liquid milks, urease was inactivated more quickly than trypsin inhibitor but when dry beans were steamed the urease was inactivated more slowly than trypsin inhibitor. In milks, the soluble nitrogen changed very little after an initial decline, while trypsin inhibitor gradually decreased. The extent of trypsin inhibitor inactivation varied between lots of milk. At 200° F., the heating time needed to inactivate 90% of the trypsin inhibitor varied from 30 to 75 minutes, while at 250° F. the time needed varied from 5 to 10 minutes. Under spray-drying conditions, where the only heating was that taking place in the drier, the soluble nitrogen decreased more rapidly with rising temperatures than did the trypsin inhibitor.

Variations in the properties of dried soymilk caused by differences in heating time at 250° F. are shown in Table II. Per cent soluble nitrogen decreased during the early stages of heating but at longer times this quantity increased. During the first 10 minutes of heating, little change took place in the available lysine, free amino groups or Hunter "L" values, but later the free amino groups increased and the available lysine and Hunter "L" values decreased.

The increase in nitrogen solubility after prolonged heating, together with the increase in amino groups, suggested a possible splitting of soybean protein during heating. Further evidence of breakdown was obtained when the solubilities of the proteins in 5% trichloroacetic acid and in 66% ethanol were examined (Figure 1). The solubility of soybean nitrogen in these solutions showed a steady increase as a result of continued heating of the soymilk. After 121 minutes about 7% of the original protein had been broken down to fragments small enough to be soluble in these solvents.

Soymilk was much less sensitive to prolonged heating at 200° F. The data in Table III show little change in most measurements during a 6-hour heating period. There was a rapid initial drop in soluble nitrogen and a gradual decrease in amino groups and Hunter "L" values.

A further series of soymilks was prepared utilizing heat exchangers to obtain rapid heating and cooling of the milk and consequent close control of heating times. This series confirmed the earlier results showing that little change took place as heating was prolonged at 200° F. At 250°, changes were less than seen previously, presumably because of the shorter heating up and cooling down periods involved.

The inlet air temperature used in connection with spray drying had a marked effect on the protein indices. As can be seen in Table IV, higher temperatures were associated with lower nitrogen solubility, available lysine, free amino groups, and Hunter "L" values. The color differences were quite marked, and a charring effect was obtained at the high temperatures.

Most of this work has dealt with soymilks that were, in essence, water extracts of soybeans. Another type of product can be prepared by finely grinding dehulled soybeans to yield a material that can form a milk-like liquid when suspended in water. With such a process, the heat needed to destroy deleterious constituents such as trypsin inhibitors can be advantageously applied to the soybeans before grinding. The effect of various heat treatments on the protein quality indices is given in Table V. With this type of heating, there was no tendency for the soluble nitrogen or the free amino groups to increase as the heating became more severe. This contrasts with what takes place when liquid soymilks are heated.

The correlation between nonbiological indices of overheating and protein efficiency ratios (PER) (*6*) were calculated by the method described by Snedecor (*17*). Since maximum PER's were obtained when about 90% of the trypsin inhibitor had been destroyed those samples containing more than 10% of their original content of trypsin inhibitor were excluded from the computations. There was no significant correlation between soluble nitrogen and PER, but the free amino groups, Hunter "L" values, and available lysine were correlated with PER at the 99% confidence level. The standard error for regression equations relating laboratory tests and PER was quite high, ranging from 0.37 to 0.35 PER unit for the various indices. However, when the data on the milks made from steamed dry beans were considered separately from that on water extracted milks, the standard error terms were lower, especially in the case of available lysine. These regression formulas for dry soymilks are presented, together with the standard error of the estimated PER, in Table VI.

A multiple regression equation covering both types of milk is: $PER = -0.453 + 2.25L - 0.23L^2 + 207A - 2300A^2 - 0.239H + 0.0019H^2$, in which L = available lysine in grams per 16 grams N, A = equivalents of amino groups per mole of N, and H = Hunter "L" value. The standard error of the calculated PER value using this equation is 0.25. The use of such an equation re-

Table II. Protein Quality Indices Changes in Soymilk[a] Heated at 250° F. Prior to Drying

Time Heated at 250° F., Min.	Soluble Nitrogen, %	Available Lysine, Grams per 16 Grams N	Free Amino Groups, Eq. per Mole N	Hunter "L"
0	65	6.0	0.035	70
5	35	6.0	0.038	70
10	30	6.0	0.037	70
20	31	5.9	0.040	62
41	36	5.7	0.041	62
64	39	5.5	0.046	57
121	50	5.0	0.054	52

[a] Soymilk dried in freeze drier.

Table III. Protein Quality Indices Changes in Soymilk[a] Heated at 200° F. Prior to Drying

Time Heated at 200° F., Min.	Soluble Nitrogen, %	Available Lysine, Grams per 16 Grams N	Free Amino Groups, Eq. per Mole N	Hunter "L"
0	75	5.3	0.039	83
30	18	5.2	0.037	78
60	21	5.4	0.036	78
120	21	5.2	0.033	77
240	22	5.2	0.035	73
360	24	5.2	0.034	74

[a] Soymilk dried on vacuum roll.

Table IV. Effect of Spray Drying Temperature on Protein Quality Indices of Soymilk[a]

Spray Drier Temperature, °F.	Soluble Nitrogen, %	Available Lysine, Grams per 16 Grams N	Free Amino Groups, Eq. per Mole N	Hunter "L"
290	38	5.5	0.042	75
330	44	5.6	0.043	78
360	34	5.5	0.041	73
440	15	5.1	0.038	72
530	8	4.1	0.026	61
600	6	2.0	0.016	33

[a] Heated for 10 minutes at 250° F. before spray drying.

Table V. Protein Quality Indices of Milks[a] Prepared from Steamed Soybeans

Steaming Conditions		Soluble Nitrogen, %	Available Lysine, Grams per 16 Grams N	Free Amino Groups, Eq. per Mole N	Hunter "L"
Time, Min.	Temp., °F.				
5	250	21	5.2	0.049	73
10	250	11	4.9	0.046	72
20	250	8	4.9	0.043	67
40	250	8	4.4	0.041	59
15	212	46	5.0	0.050	81
30	212	40	5.1	0.050	80
60	212	19	5.0	0.047	75
120	212	12	4.7	0.046	69

[a] All products spray dried at 410° F. inlet air temperature.

quires that several indices be determined, but a reasonably accurate estimate of PER on soymilk with an unknown processing history can be obtained.

However, these statistical relationships do not apply to milks containing more than 10% of their original trypsin inhibitor.

Some samples were prepared to obtain information on drying methods (Table VII). Drum drying resulted in the greatest loss of nitrogen solubility and free amino groups, while the available lysine and Hunter "L" values compared favorably with the other drying procedures. Since all the products proved satisfactory in feeding tests, the variations seen in the measured values may be indicative of ranges that do not indicate damage to the nutritional quality. This table also contains a comparison of actual PER values and those calculated from the regression equation relating PER to available lysine in water-extracted milks.

The use of dye binding as an index of soybean meal quality by Moran *et al.* (*12*) has led the authors to attempt to relate heat damage to the ability of soymilk protein to bind the dye, orange G (*19*). The extent to which the dye was bound decreased as heat damage increased, but the differences were rather small. Under heating conditions where 20 to 30% of the available lysine was lost, the dye-binding ability of the soymilk decreased by 5 to 7%. This lack of sensitivity leads us to the conclusion that measurement of the binding of orange G, while it may be useful with soybean meal, is not a suitable method for use in the quality control of soymilk.

Table VI. Regression Equations Relating PER to Laboratory Tests

Equations[a]	Standard Error of Estimate of PER
WATER-EXTRACTED MILKS	
$PER = 0.49L - 0.72$	0.16
$PER = 29A + 0.71$	0.32
$PER = 0.027H - 0.04$	0.24
DRY-STEAMED BEAN MILKS	
$PER = 0.595L - 0.40$	0.12
$PER = 35A + 0.91$	0.16
$PER = 0.018H + 1.22$	0.15

[a] L = Grams available lysine per 16 grams N; A = eq. free amino groups per mole N; H = Hunter "L" value.

Discussion

Soybean products require heating at some stage to have high nutritional value. The nutritional value of soybeans is inversely proportional to the trypsin inhibitor content, and heating results in a destruction of the inhibitor (*11*). The level of trypsin inhibitor remaining in the soy product can therefore serve as an index of the adequacy of heat treatment. The other procedures tested in this work, soluble nitrogen and urease, gave only approximate estimates of destruction of trypsin inhibitor. Urease in soymilk was inactivated more readily than trypsin inhibitor; therefore its presence indicates undertreatment, but its absence does not ensure adequate heating. Soluble nitrogen varied widely with processing conditions, and many samples were found with low soluble nitrogen and still possessing significant amounts of trypsin inhibitors. Because the heating time needed to inactivate 90% of the trypsin inhibitor varied between lots of soybeans, in practice, each batch of product should be checked for trypsin inhibitor content.

Protein damage resulting from overheating was measured with the greatest accuracy through determination of the available lysine. Such a method detects any alterations in the amino acid, but under most conditions the major cause for the loss of available lysine is probably due to changes at the epsilon amino group. Although lysine is present in fairly high amounts in soybean protein and may not be the growth-limiting amino acid in soybean diets, the extent of its loss may provide an index of general heat damage to the nutritional quality of the soy protein. The principal disadvantages of the available lysine method are that it requires considerable technical proficiency and several days to obtain results. On the other hand, it has given excellent indications of soybean protein quality when used on soymilks heated and dried under a variety of conditions.

The measurement of browning resulting from processing also shows considerable promise in evaluating dried soymilks. This usefulness is due to the participation of proteins in browning reactions. This method is the easiest to apply, and useful information can be obtained with the eye alone. Therefore, it would be particularly well suited for the control of quality at the manufacturing plant.

Free amino groups have been shown to be highly correlated with the biological value of the soy protein. How-

Table VII. Comparison of Protein Quality Indices of Soymilks[a] Dried by Different Methods

Drying Method	Soluble Nitrogen, %	Available Lysine, Grams per 16 Grams N	Free Amino Groups, Eq. per Mole N	Hunter "L"	Observed PER	Calcd. PER
Spray dried, 330° F.[b]	44	5.6	0.054	78	2.22	2.02
Freeze dried	41	5.9	0.052	71	2.14	2.18
Atmospheric roll	20	5.7	0.047	77	2.16	2.08
Vacuum roll	43	5.5	0.051	80	2.23	1.98

[a] All milks heated at 250° F. for 10 minutes before drying.
[a] Inlet air temperature.

ever it is not safe to rely on this index since under some conditions, such as prolonged heating of milk at high temperatures, the measured amount of free amino groups increased while the quality of the protein was reduced.

While the measurement of soluble nitrogen has served as an excellent index of heat treatment for soybean oil meal, it was not satisfactory when applied to soymilks, possibly because the protein changes occurring in milk follow different patterns than those seen when dry beans are heated. For example, after an initial sharp drop in nitrogen solubility, further heating does not decrease and may even increase the per cent of soluble nitrogen in soymilk. As a result, soluble nitrogen levels of between 20 and 40% can be found in milks given a wide range of heating conditions and a variety of biological values.

A knowledge of the history of a dried soymilk facilitates the use of protein indices in evaluating possible protein damage. The relation between indices and PER was dependent to some extent on whether the heat was applied to dry beans or to liquid milks. Free amino groups were particularly sensitive to the method of heating. The most reliable indices were available lysine and Hunter "L" values.

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NUTRIENTS IN NUT KERNELS

Free Amino Acids in English Walnut (*Juglans regia*) Kernels

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THE SOLUBLE nitrogenous constituents in walnut kernels have not been characterized previously. It has been proposed (*16*) that nitrogen compounds may react with carbonyl compounds evolved from the degradation of sugars (*1, 5, 11*) or lipids (*14, 24, 28*) to form rancid flavors and odors. Recent studies on the mechanisms of rancidity in various protein foods (*20*) support this proposition. Two-directional filter paper chromatographic studies by the present authors of extracts of fresh and rancid walnut kernels suggested that the development of rancidity involved changes among the ninhydrin-positive constituents (unpublished data). The presence in walnuts of reducing and nonreducing sugars, as well as large amounts of unsaturated lipids, has been established. Characterization of the free amino acids and related nitrogenous constituents is a prerequisite to investigations on reactions between these compounds and the sugars, lipids, and their degradation products, and the influence of their products on walnut quality.

Attempts to identify the major nitrogenous constituents in kernels by filter paper chromatographic techniques only partially succeeded. Unequivocal identification of many spots was impossible because of insufficient material for chemical studies, the unavailability or nonspecificity of distinctive color reactions applicable to paper chromatograms, or diffuse spots which suggested concurrence of a number of compounds having similar chromatographic properties. Therefore, gradient elution ion exchange fractionation was employed for preliminary separation and isolation of the major constituents in sufficiently pure form to permit a study of their chemical, physical, and chromatographic properties.

Experimental

Paper Chromatography. Paper chromatography was employed for the analyses of ion exchange column eluates, purified fractions, and crystalline products. The techniques were procedures described previously (*18, 19*) modified (*17*) to improve sensitivity and reproducibility.

Solvent I. A stable mixture of *tert*-butyl alcohol : water : 85% formic acid in a ratio of 69.5:4.0:26.5 by volume at 24.0° C. (*14*).

Solvent II. Phenol : water : ammonia (*19*).