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Influence of Storage on Composition, Amino Acid Content, and Solubility of Soybean Leaf Protein Concentrate

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Soybean Leaf Protein Concentrate (LPC) was prepared by either acid (LPC pI) or heat (LPC Δ) precipitation. Freshly prepared, freeze-dried LPC pI contained 1.55% moisture, 10.92% nitrogen, and 9.60% lipid, whereas LPC Δ contained 2.67% moisture, 11.80% nitrogen, and 8.19% lipid. With the exception of the limiting amino acid methionine, the amino acid profile compared favorably with the FAO reference protein. LPC Δ was virtually insoluble in water adjusted from pH 1.50 to 11. LPC pI was >60% soluble at pH 2.0 and 10.0 and above but <10% soluble from pH 3.5 to 6.8. Samples of both LPC preparations were stored for up to 24 weeks at 27° in the presence of oxygen. There was significantly more isoleucine, leucine, and lysine in LPC Δ, whereas LPC pI contained more glutamic acid, glycine, and histidine, irrespective of storage time. Methionine, glutamic acid, and tyrosine varied significantly during storage; only tyrosine, however, exhibited a linear trend. Solubility profiles were not influenced by storage.

Leaf Protein Concentrate (LPC) is one of several novel sources of protein which could be effectively used to combat protein malnutrition. Leaves are an especially appropriate source of protein in the tropics, where malnutrition is acute and vegetation flourishes.

The merits of LPC as a protein supplement for humans have been described previously (Kinsella, 1970; Pirie, 1970). Briefly, the favorable aspects of LPC include high yields, a wide variety of potential sources, simplicity of extraction and preparation, and good nutritive value (Buchanan, 1969b; Protein Advisory Group, 1970; Stahmann, 1968).

The yields of LPC have been investigated in Europe, India, and the United States (Akeson and Stahmann, 1966; Joshi, 1971; Lexander *et al.*, 1970). Yields of 1500 and 1670 kg of LPC/ha/yr have been reported for alfalfa (*Medicago sativa* L.) grown in India, and cocksfoot (*Dactylis glomerata* L.) grown in England, respectively (Arkcoll and Festenstein, 1971; Singh, 1969). More protein may be obtained from leaves per unit of land area than from any other agricultural commodity (Swaminathan, 1967).

The nutritive value of LPC has been evaluated by amino acid analyses, enzymatic and dietary studies. The amino acid profile of LPC indicates that it is nutritionally superior to most cereal and legume seed proteins including cottonseed and soybeans; it also compares favorably with most animal proteins except milk and eggs (Byers, 1971; Gerloff *et al.*, 1965; Hartman *et al.*, 1967). *In vitro* studies using pepsin-pancreatin showed that LPC was superior to beef and casein, equivalent to milk and lactalbumin, and inferior to egg protein (Akeson and Stahmann, 1965). *In vivo* studies with animals and humans were recently summarized (Singh, 1971; Woodham, 1971). LPC was shown to be an effective protein supplement for diets consisting mainly of either rice, raggi, wheat, or groundnuts (Doraiswamy *et al.*, 1969; Garcha *et al.*, 1971; Subba Rau and Singh, 1971; Sur, 1967). LPC has also been used as a milk extender for infants and young children (Waterlow, 1962).

As with most sources of novel protein, there are some problems relating to the acceptance of LPC. The major objections have been related to the green color of the concentrate and the development of undesirable odors and flavors during storage (Protein Advisory Group, 1970). The lipid fraction of LPC is held responsible for most of the deteriorative changes which occur when LPC is stored (Buchanan, 1969a). The lipid content of LPC ranges from 3.9 to 12.0% for the ether extractives and from 20 to 28% for the hot chloroform–methanol extracts (Byers, 1971; Oelschlegel *et al.*, 1969; Spencer *et al.*, 1971). It is noteworthy that from 53 to 79% of the fatty acids present are polyunsaturated and, thus, susceptible to oxidation under the appropriate conditions (Betschart, 1971; Lima *et al.*, 1965). The oxidation of the lipid fraction of LPC has, in fact, been described under a variety of conditions (Buchanan, 1969a,c; Lea and Parr, 1961; Shah *et al.*, 1967). Thus, the products and/or intermediates of lipid oxidation have been implicated in the formation of undesirable odors and flavors and the concomitant decrease in the nutritive value of stored LPC (Buchanan, 1969a; Kohler and Bickoff, 1971; Subba Rau and Singh, 1971).

Few studies have been conducted on the stability of stored LPC. Subba Rau *et al.* (1967) developed methods of increasing the shelf life of wet LPC. Buchanan (1969c) investigated the effects of storage upon LPC prepared according to the Pirie process (Morrison and Pirie, 1961).

The objective of the present study was to observe the

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influence of storage at ambient temperature (27°) upon soybean LPC prepared by either acid precipitation at the isoelectric point (LPC pI) or precipitation by heat (LPC Δ). This paper reports the general composition of LPC, amino acid content, and the pH solubility profiles as a function of storage time.

EXPERIMENTAL SECTION

Soybean leaves were used because of their favorable protein content and the simplicity of harvesting them.

Sample Preparation. Soybean (*Glycine max.* L., "Hark") leaves were harvested 60 to 61 days after planting from plants 58 to 74 cm in height. The leaves were cleaned and prepared as previously described (Betschart and Kinsella, 1973); 100-g random samples were frozen at −30°, freeze dried within 6 hr after harvest at shelf temperatures ≤−7°, and subsequently stored at −30°.

The freeze-dried leaf samples were randomly removed from storage and the protein was extracted according to the method previously described (Betschart and Kinsella, 1973). The protein was precipitated from supernatant I with either concentrated HCl or heat. The isoelectric LPC was precipitated at pH 3.5; the heat coagulated LPC was prepared by adding the protein extract to water in which the temperature was maintained at 80–81°. Magnetic stirrers were used to ensure the uniform distribution of acid or heat. Both LPC pI and LPC Δ were collected on Whatman no. 1 filter paper and washed at least four times, or until the washings were clear, with a dilute solution of HCl (pH 4.0). The LPC was immediately frozen and freeze dried at a shelf temperature ≤−7° within 6 hr after preparation. The freeze-dried samples were ground with a mortar and pestle and passed through a 40-mesh sieve. Small portions of each sample (150 mg) were removed and stored in smaller vials for eventual lipid extraction. All of the samples, including the smaller portions, were stored in loosely capped glass vials at ambient temperature (27°) and in the absence of light. Three LPC Δ and two LPC pI samples were prepared for each storage period. Freshly prepared LPC which was not stored served as the control. Samples were removed from storage after 4, 8, 12, 16, 20, and 24 weeks and analyzed.

Analyses. *Moisture.* Immediately upon removal from storage duplicate 50-mg quantities of each sample were dried at 105° for 24 hr in a Stabil-Therm Gravity Oven (Blue Electric Co., Blue Island, Ill.).

Nitrogen. Triplicate 10-mg portions of each stored sample were analyzed by the micro-Kjeldahl method (McKenzie and Wallace, 1954).

Lipid. Chloroform–methanol (2:1 v/v) was used to extract the lipids from the 150-mg portions of each sample according to the method of Folch *et al.* (1957). The extract was evaporated under nitrogen and weighed.

Amino Acid Composition. Ten milligrams of each sample was defatted with chloroform–methanol (2:1 v/v) and held in a desiccator over Drierite for at least 12 hr or until constant weight was maintained. Duplicate 2-mg portions of each stored sample were hydrolyzed with 1 ml of 6 *N* HCl at 110° for 22 hr. The hydrolyzates were passed through a Millipore filter and, together with two additional washings, were evaporated under vacuum. Evaporated samples were dissolved in 2.5 ml of pH 2.2 citrate buffer. Analyses were conducted on a Beckman model 120C amino acid analyzer; 0.5 ml of the hydrolyzate was applied to each column.

LPC Solubility. The solubility of LPC as a function of pH at 25° was investigated. Solubility profiles were determined for the LPC pI and LPC Δ control samples and for those LPC pI samples stored 12 and 24 weeks. For each of the samples analyzed (*i.e.*, two LPC pI and three LPC Δ per storage period) 12 20-mg portions were suspended in pH-adjusted, distilled, deionized water. The pH values ranged from 1.5 to 12.0 and were adjusted with 0.1 *N* or 1.0 *N* NaOH or HCl. Samples were shaken for 1 hr at 25° on an Evapo Mix with the motor setting at 4 (Buchler Instruments, Fort Lee, N. J.). The pH of the solutions was checked every 15 min and readjusted when necessary. The final volume was made up to 10 ml and the solutions were centrifuged at 10,000 × *g*, 0°, for 15 min in a Sorvall RC2-B (Ivan Sorvall Inc., Norwalk, Conn.).

Duplicate 2-ml aliquots were removed from the supernatant and the soluble nitrogen was determined by micro-Kjeldahl analysis. The percent of LPC nitrogen solubilized was calculated as: % nitrogen solubilized = (nitrogen in supernatant/nitrogen in LPC sample) × 100.

Statistical Methods. An analysis of variance was conducted on each of the previously described sets of compositional data (Harvey, 1966). The sources of variation were method of preparation (acid or heat precipitation), storage time, and the interaction of preparation × storage time. The interaction mean square was used as the error term for methods of preparation whenever the interaction *F* ratio was greater than 1. Storage time and interaction were tested by the residual error, as was method of preparation when the interaction mean square was less than that of the residual.

Whenever there was a significant storage effect, the data were examined for possible trends and, where plausible, regression analyses were conducted. Linear regression equations were applied to the data on nitrogen content of LPC pI and LPC Δ, as well as to the tyrosine content of both types of LPC.

Table I. Composition of Freshly Prepared Soybean Leaf Protein Concentrate

	%	
	LPC, acid precipitate	LPC, heat precipitate
Moisture	1.55 ± 0.25[a]	2.67 ± 0.70
Nitrogen[b]	10.92 ± 0.03	11.80 ± 0.21
Lipid[b]	9.60 ± 0.05	8.19 ± 0.55

[a] Mean ± standard error. [b] Dry weight basis.

RESULTS AND DISCUSSION

All freshly prepared, freeze-dried LPC samples were prepared and evaluated in the same manner as the stored samples. The unstored samples served as controls for the storage study and also provided some general information about soybean LPC. The data relating to the unstored samples will be discussed initially. This will be followed by the results of the storage study.

Freshly Prepared LPC. *Moisture.* The moisture content of freeze-dried soybean LPC was, in general, similar to values reported for wheat LPC (Buchanan, 1969a). Soybean LPC Δ contained more moisture than soybean LPC pI (Table I). Since all of these samples were freeze dried at the same time under identical conditions, the observed differences would appear to be related to the method of preparing LPC.

Nitrogen. The nitrogen content of soybean LPC (Table I) compares favorably with LPC prepared from other sources (Byers, 1971; Gerloff *et al.*, 1965). Although most LPC contains from 8 to 10% nitrogen, the source of the protein can influence the nitrogen content. Oelschlegel *et al.* (1969) found that LPC prepared from several types of waste plant material contained from 2.8 to 6.9% nitrogen.

Soybean LPC Δ was slightly higher in nitrogen than LPC pI (Table I). This implies that the acid precipitation process coprecipitates more nonprotein contaminants than does the process of heat precipitation. The nature and safety of the nonprotein material should be more thoroughly understood.

Table II. Essential Amino Acid Composition

Sample	Ileu	Leu	Lys	Met	Phe	Thr	Try	Val	Reference
FAO reference protein[a]	4.2	4.8	4.2	2.2	2.8	2.8	1.4	4.2	FAO (1965)
LPC Δ avg of nine species[a]	5.3	9.8	6.3	2.1	6.0	5.2	1.6	6.3	Gerloff *et al.* (1965)
LPC Δ barley[a]	5.0	9.3	6.6	2.2	6.2	5.1		6.4	Byers (1971)
LPC Δ lupin[a]	4.9	9.8	6.7	1.7	6.2	5.0		6.3	Byers (1971)
LPC Δ avg soybean[a]	5.0	10.0	7.2	1.1	5.8	5.3		6.7	Present study
LPC pI avg soybean[a]	4.8	9.8	7.0	1.3	5.9	5.3		6.6	Present study
Glandless cottonseed flour[b]	3.3	5.6	4.3	1.2	5.3	3.0		4.5	Martinez *et al.* (1970)
Peanut meal[b,c]	4.0	6.7	3.1	1.1	5.4	3.4	1.3	4.7	Block and Weiss (1956)
Soy protein concentrate[b]	4.9	8.0	6.6	1.3	5.3	4.3	1.4	5.0	Meyer (1966)
Opaque 2 corn endosperm[c]	10.5	3.8	3.6	2.1	4.5	3.7		5.7	Bates (1966)
Rice, milled[b]	4.2	8.2	3.6	2.1	4.9	3.3		5.8	FAO (1970)
Wheat grain[b]	4.4	6.9	2.5	1.2	4.4	3.9	1.2	4.5	Buchanan (1969a)
Avg potatoes, vegetables, and fruits[b]	3.6	6.6	5.7	2.3	4.5	4.1	1.9	4.4	Block and Weiss (1956)
Avg meat, poultry, and fish[b]	6.3	7.7	8.1	3.3	4.9	4.6	1.3	5.8	Block and Weiss (1956)
Milk[b]	8.5	11.3	8.2	3.4	5.7	4.5	1.6	8.5	Block and Weiss (1956)
Eggs[b]	8.0	4.2	7.2	4.1	6.3	4.3	1.5	7.3	Block and Weiss (1956)

[a] Grams of amino acid/100 g of recovered amino acid. [b] Grams of amino acid/16 g of nitrogen. [c] Grams of amino acid/100 g of protein.

Lipid. The total extractable lipids of soybean LPC were approximately 8.2 and 9.6% of dry weight for LPC Δ and LPC pI, respectively (Table I). Unfractionated alfalfa LPC was reported to contain from 7.0 to 12.5% ether extractives (Doraiswamy *et al.*, 1969; Spencer *et al.*, 1971; Subba Rau *et al.*, 1969). Others, using ambient or hot chloroform–methanol (2:1 v/v), have found dry LPC contains 20 to 29% lipid (Buchanan, 1969a; Byers, 1971; Lea and Parr, 1961).

There are two possible explanations for the apparent discrepancy in lipid content and/or extractability. First, the soybean LPC may have contained less total lipids because of species differences or the method of preparation. In the present study the leaf extracts were sequentially centrifuged at 1000, 10,000, and 20,000 × *g* prior to heat or acid precipitation (Betschart and Kinsella, 1973). The highest *g* force may have removed a portion of the less soluble or "chloroplastic" protein which contains more lipid than the more soluble or "cytoplasmic" protein (Subba Rau *et al.*, 1969). Byers (1971) found 25 and 5.5% lipid present in the unfractionated and cytoplasmic LPC of barley. Although Byers used a force of 50,000 × *g* to prepare the cytoplasmic protein, this does not preclude the possibility that the preparation used in the present study was freed of a portion of the less soluble, lipid-rich protein fraction. The second explanation is that the lipid fraction of soybean LPC was less extractable. Since the preparations under discussion were freshly freeze dried at shelf temperatures $\leq -7°$, the latter explanation seems improbable.

Amino Acid Composition. The amino acid profiles of unstored soybean LPC were examined in terms of the influence of the method of preparation (acid or heat) and compared with other LPC preparations, cereal, and oilseed proteins. Only minor differences existed between the amino acid composition of soybean LPC pI and LPC Δ (Table II), *e.g.*, LPC Δ contained slightly more lysine than LPC pI. In contrast, others have reported 10 to 15% less lysine in the heat coagulated LPC preparation of three species (barley, lupin, chinese cabbage) as compared to the TCA precipitate (Byers, 1971).

The amino acid composition of soybean LPC was in agreement with the values reported for LPC of various origins (Table II) (Byers, 1971; Gerloff *et al.*, 1965). These observations reflect the findings that the amino acid profile of LPC is not significantly influenced by leaf species, variety, degree of maturity, or application of fertilizer (Gerloff *et al.*, 1965; Oelschlegel *et al.*, 1969). Byers (1971), however, did suggest that the methionine content might be species dependent in some instances.

Some minor differences were observed, however, in the amino acid composition of LPC prepared from various leaf sources. The lysine content was slightly higher in soybean LPC, whereas methionine was lower. The higher lysine content may be due to the method of preparing soybean LPC which allows for the removal of some of the less soluble chloroplastic protein as previously described. The less soluble fraction has been reported to contain less lysine, whereas the more soluble cytoplasmic fraction contains larger quantities of lysine than unfractionated LPC (Byers, 1971; Yemm and Folkes, 1953). It is of interest that the reported lysine content of cytoplasmic barley and lupin LPC, *i.e.*, 7.1 and 7.3 g/100 g of recovered amino acids, respectively, is similar to the soybean LPC prepared in the present study.

Not all investigators have reported differences in the amino acid profiles of the chloroplastic and cytoplasmic protein fractions (Gerloff *et al.*, 1965; Wilson and Tilley, 1965). One explanation for this discrepancy is that similar amino acid profiles were observed when the fractions were prepared by differential heating, *i.e.*, chloroplastic protein was collected after heating at 54 to 55°; cytoplasmic protein was precipitated when the remaining supernatant was heated to 80°. Differences in amino acid composition were observed, however, when these fractions were prepared by differential centrifugation (Byers, 1971). The possibility exists that these two methods of fractionation do not separate the same protein fractions although the respective fractions are given analogous terminology.

During acid hydrolysis, some methionine is converted to methionine sulfoxide. Since the quantity of methionine sulfoxide was negligible, the methionine values in the present study reflect the methionine content only and do not include the oxidation product. The low methionine values reported for soybean LPC may, therefore, reflect a small amount of acid destruction in addition to an initially low content of methionine. In general, if unfractionated LPC is supplemented with 1.5% (w/w) methionine, it is a well-balanced protein in terms of amino acid composition (Gerloff *et al.*, 1965; Smith, 1966). Cytoplasmic LPC contains an adequate amount of methionine irrespective of the method of preparation, *i.e.*, differential heating or centrifugation (Byers, 1971; Gerloff *et al.*, 1965). This characteristic of cytoplasmic protein in addition to its lysine content and the off-white color of the concentrate should provide impetus for further research on this fraction.

LPC contains more than adequate amounts of lysine compared to the FAO reference protein. Comparing soybean LPC with some other proteins, it frequently contains

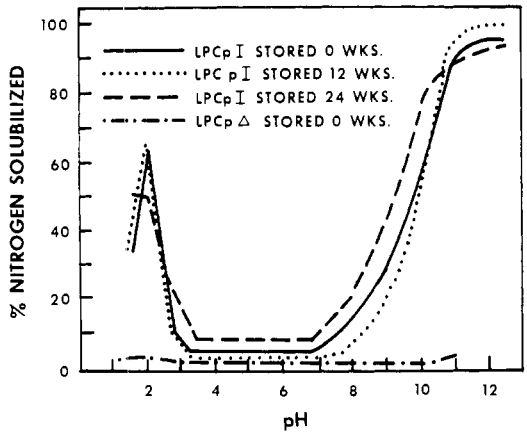

Figure 1. Solubility profiles of stored soybean leaf protein concentrate.

more lysine than soy protein concentrate and considerably more lysine than Opaque 2 corn (Table II). This intimates the potential use of LPC as a supplement for cereal-based diets such as rice, maize, and wheat, which are invariably low in lysine. LPC has, in fact, been used to supplement the cereal diets of humans as well as rats (Doraiswamy *et al.*, 1969; Shurpalekar *et al.*, 1966; Subba Rau and Singh, 1971).

The results of *in vivo* studies emphasize the limitations of amino acid analyses. The amino acid profiles of LPC are sometimes poorly correlated with biological indices (Buchanan 1969a; Henry and Ford, 1965; Subba Rau *et al.*, 1972). Discrepancies of this nature are to be expected since amino acid analyses do not reflect biological availability, digestibility, or the presence of antagonists and other toxic substances. Therefore, amino acid data should always be viewed as an indication of potential protein quality.

LPC Solubility. The solubility profile of a protein provides some insight into the extent of denaturation or irreversible aggregation and precipitation which may have occurred. It also gives an indication of the types of foods or beverages into which the protein might be incorporated.

Factors such as concentration, pH, ionic strength, and the presence of other substances influence the solubility of a protein. The influence of the concentration of soybean LPC pI was investigated. Solutions of 0.1, 1.0, and 5.0% (w/v) were 95-99% soluble when adjusted to pH 11.5. However, when solutions of 10% LPC pI were treated similarly, only 86% of the nitrogen was soluble. Since the quantities of LPC were limited, 0.1% solutions were used throughout the present study. The results should be applicable to solutions of up to 5.0%.

Freshly prepared LPC Δ was virtually insoluble at ambient temperature (25°) when exposed to pH values ranging from 1.5 to 11.0 (Figure 1). The process of heating to 80° apparently irreversibly denatures the protein. LPC Δ has also been reported to be sparingly soluble in solvents other than a pH adjusted aqueous medium. Arkcoll (1969) found that LPC, prepared by the Pirie process of heat precipitation, was less than 15% soluble in a solution of 50% urea in 0.1 *M* thioglycolic acid. In the presence of 5% sodium dodecyl sulfate, however, LPC was 22 to 25% soluble. It appears that neither hydrogen nor disulfide bonds are responsible for the limited solubility of LPC Δ.

The freshly prepared soybean LPC pI was more soluble at some pH values than was LPC Δ (Figure 1). At pH 2.0, 65% of the LPC pI nitrogen was soluble, and at pH 11.0 and above 90% was soluble. Solubility increased sharply at pH 9.0. The solubility profile of soybean LPC pI was similar to that of alfalfa LPC pI, except that the alfalfa LPC was more soluble at pH 2.0 (90% soluble) as well as at pH 5 to 9 (Lu and Kinsella, 1972). The enhanced solubility of the alfalfa LPC may be partially due to the conditions of extraction, *i.e.*, pH adjusted to 12 with NaOH and 70°. Under these conditions some depolymerization and disaggregation of the protein would be expected. The smaller subunits are, thus, more soluble. Also, the sodium ions present may be bound by the alfalfa leaf protein and increase the affinity of the protein for water.

Upon comparing the solubility profile of soybean LPC pI with that of the soybean leaf protein extract prior to precipitation (Betschart and Kinsella, 1973), it is apparent that the solubility of the protein is reduced as a result of the isoelectric precipitation. At pH 2.0 as well as pH 6.0 and above, the soybean leaf protein is completely soluble, as opposed to the diminished solubility observed in the soybean LPC pI (Figure 1). Conceivably, some irreversible changes occurred in the tertiary and possibly the secondary structure of the protein during the preparation of the isoelectric concentrate. This is especially critical since the pH of many of the foods and beverages into which LPC might be incorporated ranges from 4 or 5 to 7. It is imperative that the factors responsible for the decrease in solubility be investigated and, hopefully, minimized before LPC can be included in a wide variety of food formulations.

If some degree of protein denaturation and subsequent decrease in solubility is inherent in even the most favorable method of isolation, solubility may be enhanced by one of several methods. Sodium proteinate derivatives are usually more soluble than the unmodified protein (Lu and Kinsella, 1972; Meyer, 1966). Preferably, the proteinate should be prepared at pH 7 to 8 since racemization or destruction of the amino acid side chains may occur under more alkaline conditions (Hill, 1965; Tannenbaum *et al.*, 1970). Partial chemical or enzymatic hydrolysis also increases the solubility of protein nitrogen. With these methods it is important to be cognizant of the potential problems of undesirable odors and flavors which may accompany the degradation products of proteins. As the potential solubility of leaf protein is studied more thoroughly, the influence of salts and/or ionic strength, sugars, and other constituents which commonly occur in foods needs to be considered as well.

Stored LPC. The influence of storing LPC pI and LPC Δ at ambient temperature was evaluated using several criteria. The present paper reports the effects of storage upon the moisture, nitrogen, and lipid content, amino acid composition, and solubility as a function of pH.

Moisture. There were no observable trends in the moisture content of either LPC pI or LPC Δ. An analysis of variance revealed that neither method of preparation nor storage time accounted for a significant proportion of the variation among samples.

Nitrogen. The overall nitrogen content of stored LPC Δ (11.5 to 14%) was greater than that of LPC pI. The values for LPC Δ were slightly higher than most reported values (Gerloff *et al.*, 1965; Spencer *et al.*, 1971); the lower value of 11.5% was similar to the nitrogen content reported by Byers (1971). The method of centrifuging the supernatant and removing some of the less soluble protein, as previously discussed in this paper, may account for the slightly higher nitrogen content of the soybean LPC Δ as compared to LPC Δ prepared by others.

An analysis of variance revealed that a significant proportion of the variation in nitrogen content among the samples was due to the method of preparation as well as to the interaction of method of preparation × storage (Table III). There was also a significant linear effect as a function of storage time as well as within the interaction mean square. LPC pI exhibited a negative linear trend as storage time increased, whereas LPC Δ resulted in a positive slope. The respective linear regression equations are cited in Figure 2. These trends are difficult to

Table III. Analysis of Variance; Nitrogen and Lipid Content of Stored Soybean Leaf Protein Concentrate

Source	*df*	Nitrogen		Lipid	
		Mean square	*F*	Mean square	*F*
Method of LPC preparation, pI, Δ	1	48.798	17.73[b]	0.624	0.39
Storage time, weeks	6	1.116	1.68	3.584	1.84
Linear	1	5.241	7.90[a]	0.357	0.18
Remainder	5	0.291	0.44	4.229	2.17
Interaction, method × weeks	6	2.753	4.15[b]	1.610	0.83
Linear	1	15.485	23.36[b]	0.421	0.22
Remainder	5	0.207	0.31	1.848	0.95
Residual (rep, method × weeks)	21	0.663		1.946	

[a] Significant at the 5% level. [b] Significant at the 1% level.

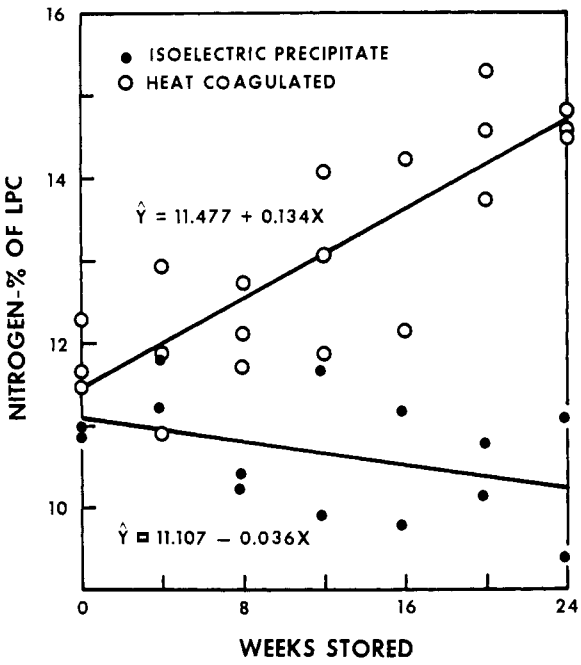

Figure 2. Data plot of nitrogen content of stored soybean leaf protein concentrate.

explain. All of the soybean leaves were harvested simultaneously and random samples were subsequently prepared for extraction. For each of the methods of preparation (*i.e.*, LPC pI and LPC Δ), all samples were treated in the same manner; from these preparations samples were then randomly selected for storage. Therefore, neither the original leaf material nor inconsistencies in the method of preparation would account for the observed linear trends in nitrogen content.

A possible explanation for the increase in nitrogen of stored LPC Δ may be microbial contamination. There were no observable changes, however, in the color, flavor, or odor of stored LPC Δ. Also, if microorganisms were responsible for the additional nitrogen, it would be interesting to investigate the reasons for their absence in the LPC pI. The hypothesis of microbial contamination warrants investigation since there are implications in practical situations when LPC might be stored at ambient temperatures in areas such as the tropics. The authors offer no explanation for the slight decrease in LPC pI nitrogen as a function of storage time.

Lipid. There was no observable trend in the lipid content and/or extractability of the lipid fraction of soybean LPC as a function of storage time (Table III). Others have reported a decrease in lipid extractability of stored LPC under a variety of conditions (Buchanan, 1969c; Lea and Parr, 1961; Shah, 1968). The decrease in extractability was accompanied by oxidation of the lipids, as indicated by oxygen absorption studies (Lea and Parr, 1961). Diminished extractability is postulated to be either the result of the formation of lipid complexes which are not extractable in lipid solvents or due to the formation of degradation products which are soluble in the aqueous phase of the lipid wash (Buchanan, 1969c). The likelihood that oxidation occurs is enhanced by the presence of unsaturated fatty acids in LPC lipids. From 53 to 79% of the fatty acids in LPC of eight different species was reported to be unsaturated (Betschart, 1971; Lima *et al.*, 1965). Also, chlorophyll, which is present in LPC, has been observed to function as a prooxidant (Hall and MacKintosh, 1964).

In contrast to the factors which favor oxidation there are many constituents in leaves which may act as antioxidants. Some of these are the phenolic compounds, α-tocopherol, some amino acids, and/or aromatic amines (Adamic *et al.*, 1970; Pratt, 1965; Shah, 1968). As LPC is isolated by either heat or acid, there is the possibility that the phenolic compounds which may have complexed with the protein, or α-tocopherol which may be part of the lipid fraction of LPC, have coprecipitated with the protein and, thus, would be expected to minimize oxidation.

The lack of an observed change in the extractability of LPC lipids in the present study may be due to several factors. Since lesser quantities of lipid were coprecipitated with the soybean leaf proteins, the likelihood of observable oxidation would be diminished. Also, natural antioxidants may have sufficiently curtailed the oxidation of lipids under the conditions of this study (27°). Finally, if oxidation did occur during storage, it was not sufficient to impair the extractability of the lipid fraction.

Amino Acid Composition. The amino acid content of stored soybean LPC pI and LPC Δ was examined to gain some insight into the influence of storage upon the potential protein quality. A cursory examination of the data involved plotting the means for each amino acid stored for the designated periods of time, *i.e.*, 0 to 24 weeks. Some variation among individual amino acids was apparent but there were few obvious general trends (Figures 3 and 4).

Analysis of variance of the data for the entire storage study showed that LPC pI contained significantly more glycine, glutamic acid, and histidine, whereas LPC Δ contained larger amounts of isoleucine, leucine, and lysine (Table IV). The quantitative differences of these amino acids resulting from method of preparation are listed in Table V.

The difference in the lysine content of the soybean LPC preparations is of interest since cereal-based diets are commonly limiting in this amino acid. It is evident, however, that the lysine content of both LPC preparations is greater than that of the FAO reference protein and is similar to that of egg (Table II). This reinforces the value of LPC as a supplemental protein to cereals and some oilseeds other than soybean. Although the two essential amino acids isoleucine and leucine are present in larger quantities in LPC Δ than in LPC pI, the isoelectric point concentrate contains more than adequate quantities of both. Thus, the differences in amino acid content associ-

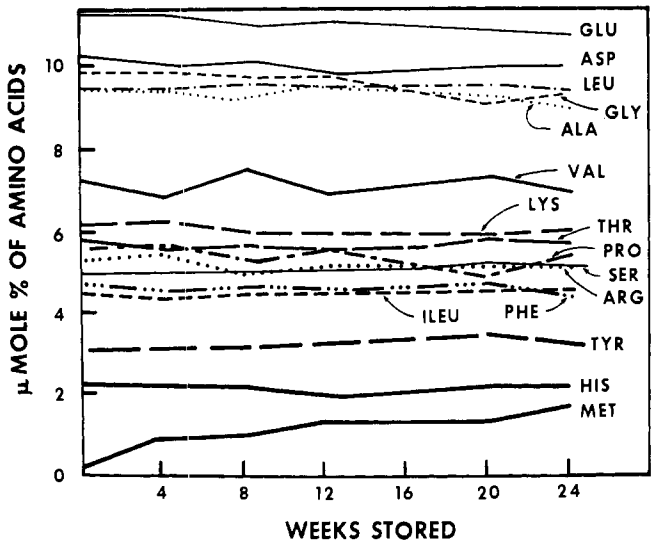

Figure 3. Mean amino acid composition of stored soybean leaf protein concentrate, acid precipitate (LPC pI).

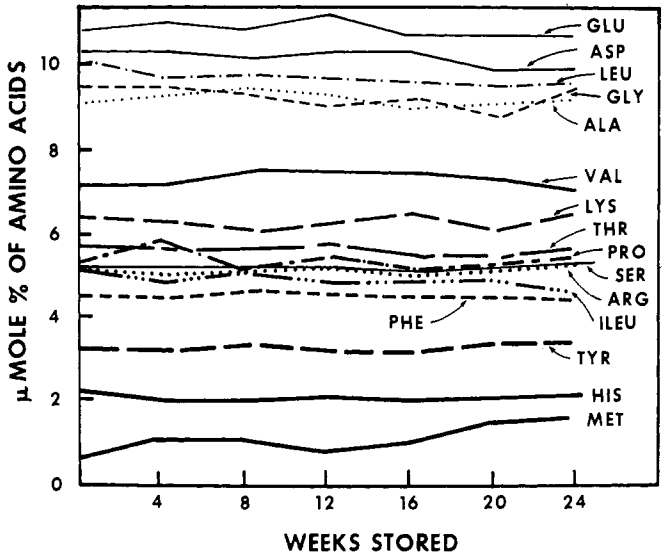

Figure 4. Mean amino acid composition of stored soybean leaf protein concentrate, heat precipitate (LPC Δ).

Table IV. Analysis of Variance; Amino Acid Composition of Stored Soybean Leaf Protein Concentrate

Source	*df*	Isoleucine Mean square	Isoleucine *F*	Leucine Mean square	Leucine *F*	Lysine Mean square	Lysine *F*	Methionine Mean square	Methionine *F*	Threonine Mean square	Threonine *F*
Method of LPC preparation, pI, Δ	1	0.403	12.82[b]	0.279	4.53[a]	0.293	7.66[a]	0.131	1.21	0.0617	1.73
Storage time, weeks	6	0.059	1.88	0.067	1.08	0.058	1.51	0.739	24.43[b]	0.0120	1.70
Interaction, method × weeks	6	0.011	0.37	0.042	0.68	0.030	0.78	0.108	3.57[a]	0.0357	5.05[b]
Residual (rep, method × weeks)	21	0.031		0.062		0.038		0.030		0.0071	

Source	*df*	Valine Mean square	Valine *F*	Glutamic acid Mean square	Glutamic acid *F*	Glycine Mean square	Glycine *F*	Histidine Mean square	Histidine *F*	Tyrosine Mean square	Tyrosine *F*
Method of LPC preparation, pI, Δ	1	0.071	0.89	0.399	6.62[a]	1.144	5.57[a]	0.107	6.47[a]	0.0003	0.02
Storage time, weeks	6	0.160	3.27[a]	0.131	4.61[b]	0.260	1.27	0.027	1.62	0.0400	5.08[b]
Interaction, method × weeks	6	0.079	1.62	0.060	2.12	0.086	0.42	0.014	0.86	0.0169	2.14
Residual (rep, method × weeks)	21	0.049		0.028		0.205		0.017		0.0079	

[a] Significant at the 5% level. [b] Significant at the 1% level.

ated with the method of preparation are not likely to be of major nutritional significance since the limiting amino acid, methionine, is not significantly different in the two LPC preparations.

Variations in the amino acid content of both LPC preparations were examined as a function of storage time. The analysis of variance revealed that glutamic acid, methionine, valine, and tyrosine varied significantly during storage. Trends of these amino acids were also observed. Although the content of tyrosine varied widely there was a significant linear component which correlated with increasing storage time (Figure 5). Since the amino acid data were expressed as μmol %, this implies that the tyrosine content of LPC was relatively more stable during storage than some of the other amino acids. A less likely interpretation would be that the tyrosine content increased during storage. It is conceded that the values obtained in the amino acid profiles are not independent variables. However, a general trend of stability or instability of the individual amino acids is gleaned from an application of the analysis of variance.

In general, it appears as though methionine was neither destroyed nor complexed during storage. Since others have reported methionine losses in the presence of oxidizing lipids (Roubal, 1971; Tannenbaum *et al.*, 1969) it appears that there was either a minimum of lipid oxidation in stored soybeam LPC or that those oxidation products which may have been present did not destroy methionine.

Although both glutamic acid and valine varied during storage, neither amino acid exhibited any predictable trends. They were merely present in less consistent quantities than other amino acids.

Table V. Amino Acid Composition of Stored Soybean Leaf Protein Concentrate (Means Which Are Significantly Different in Acid and Heat Precipitated LPC)

Amino acid	μmol %[a] LPC acid precipitate	μmol %[a] LPC heat precipitate
Isoleucine	4.64 ± 0.049	4.862 ± 0.050
Leucine	9.504 ± 0.043	9.686 ± 0.068
Lysine	6.097 ± 0.050	6.283 ± 0.049
Glutamic acid	11.075 ± 0.061	10.857 ± 0.059
Glycine	9.600 ± 0.142	9.230 ± 0.102
Histidine	2.175 ± 0.042	2.061 ± 0.029

[a] Mean ± standard error.

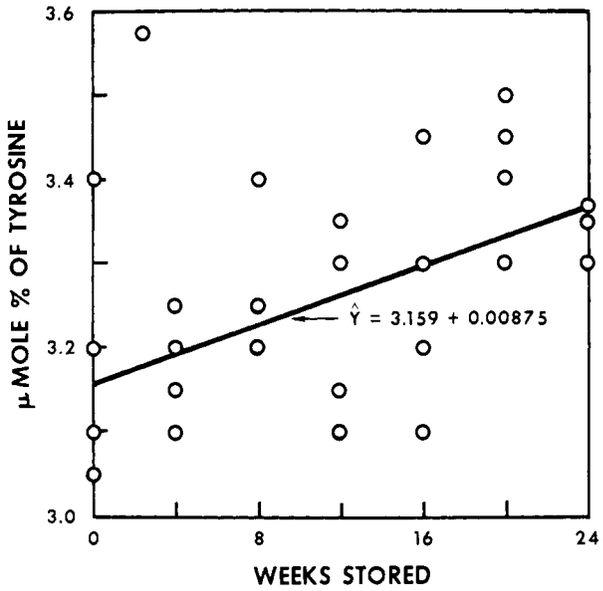

Figure 5. Data plot of tyrosine content of stored soybean leaf protein concentrate.

In two instances, *i.e.*, methionine and threonine, there was a significant interaction of the method of preparation × weeks of storage. Since there were no trends in the threonine content, this implies a different but nonpredictable trend between the methods of preparation as a function of storage.

On the basis of amino acid composition the stability of both LPC preparations stored at ambient temperature is encouraging. As with all amino acid analyses, however, one must recognize the limitations of the data generated by this technique. The results are dependent upon not only the initial amino acid composition of the material but also upon the method of sample preparation and acid hydrolysis

LPC Solubility. Since the freshly prepared soybean LPC Δ was virtually insoluble, the influence of storage upon its solubility was not investigated.

The solubility profile of soybean LPC pI was not markedly affected by storage for periods of up to 6 months (Figure 1). The solubilities of freshly prepared LPC pI and that stored 12 and 24 weeks were quite similar. The samples stored 12 weeks were slightly less soluble than the control at pH 3.2 to 9.5 and slightly more soluble at pH 10 to 12 (Figure 1). The LPC pI stored for 24 weeks was ~15% less soluble than the control at an acidic pH (1.2 to 2.0) and ~5% more soluble in an alkaline medium (pH 10 to 12).

A rapid decrease in the solubility of the proteins of fish protein concentrate and meat was reported in the presence of oxidizing lipids (Anderson and Rauesi, 1970; Olley and Duncan, 1965; Roubal, 1971). It was initially hypothesized that some of the unsaturated fatty acids of LPC might oxidize during storage at ambient temperatures and interact with the protein fraction, causing a decrease in solubility. The solubility profiles of stored LPC pI along with other evidence cited in this paper do not support this hypothesis.

Insofar as loss of solubility is a general indication of denaturation, very little denaturation occurred during the storage of LPC. The irreversible aggregation and insolubilization which did occur seem to have been the result of the methods of preparing LPC.

In addition to the influence of storage upon the general composition, amino acid content, and solubility of LPC, other factors such as *in vitro* digestibility and fatty acid content have been studied. The latter will be reported in subsequent papers.

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Isolation and Identification of the Components of the Tar of Hickory Wood Smoke

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The volatile components of hickory wood tar were fractionated by preparative gas chromatography. Individual fractions were analyzed on a Carbowax 20M 50 ft × 0.02 in. support coated open tubular column (SCOT) coupled to a mass spectrometer. The following compounds are among those being reported for the first time from hickory wood tar: 2-methyl-2-butenoic acid; benzaldehyde; 2-acetylfuran; 2-cyclohexenone; 4-propylguaiacol; 4-methylveratrol; 3-methyl-2-cyclopenten-1-one; 2,3-pentanedione; acetophenone; resorcinol; vinylphenol; 2,6-dimethylphenol; and 2-ethylphenol. A total of six aldehydes, eight ketones, four esters, six furans, 12 aromatic hydrocarbons, 32 phenols, and 13 acids were identified.

The smoking of foods as a method of preservation is one of the oldest methods known. Smoking not only partially dehydrates food during the process but also deposits compounds possessing antimicrobial antioxidant activities in the meat. With today's modern technology of food preservation, the smoking of foods is done for color and added flavor.

Most of the previous work in the literature on smoke flavor has been carried out on wood smoke itself. The identification of acids, carbonyls, alcohols, and other neutral components has been published (Fiddler *et al.*, 1967; Hamid and Saffle, 1965; Hoff and Kapsalopoulou, 1964; Jahnsen, 1961; Love and Bratzler, 1966; Porter *et al.*, 1964). Recently, work was carried out on the constituents of a liquid smoke solution (Fiddler *et al.*, 1970a,b). Much work has been done on the phenolic compounds and their role as contributors to the flavor of smoke foods (Fiddler *et al.*, 1967; Kornreich and Issenberg, 1972; Lustre and Issenberg, 1969; Tilgner *et al.*, 1962). The technological aspects of the smoking process have revealed the presence of phenols in smoked foods. Since phenolic compounds identified in foods are not normal components, they were attributed to the smoking process (Fiddler *et al.*, 1966; Foster and Simpson, 1961; Foster *et al.*, 1961; Porter *et al.*, 1964; Tilgner *et al.*, 1962; Ziemba, 1963).

We decided to look at a different aspect of smoke flavor by examining the tar of hickory wood smoke with a possibility of preparing a synthetic substitute.

ISOLATION

The material examined was a commerical sample obtained from Old Hickory Products Co., Atlanta, Ga. The material is a natural flavor product made from 100% hickory wood. As the hickory wood tar is a black viscous material, it was first dissolved in acetone (Matheson, Coleman and Bell, Spectroquality) and filtered to remove any carbon particles, and most of the solvent was removed by distillation using a Kuderna–Danish concentrator (Kontes Glass Co., Vineland, N. J.). The clarified sample (*ca.* 8 ml) was then used for our analytical work.

The acids present in the material were isolated and identified in the following manner. One kilogram of the hickory smoke concentrate was steam distilled and 2 l. of distillate were collected. A liter portion was made basic to pH 7.8 with sodium bicarbonate and the nonacidic components were removed by extracting with 3 × 150 ml of diethyl ether (Matheson, Coleman and Bell, ACS reagent grade). The aqueous mixture was then made acidic with concentrated hydrochloric acid to pH 1, saturated with sodium chloride, and extracted with 3 × 150 ml of diethyl ether. The ethereal extracts were combined, dried over anhydrous sodium sulfate, and filtered, and the solvent was removed using a Kuderna–Danish concentrator. The residue was then methylated using a diazomethane generator utilizing *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Co.). The methylated residue was then analyzed in the same manner as the clarified sample described below.

ANALYTICAL METHODS

Preparative gas chromatography was carried out on an F&M 770 preparative gas chromatograph equipped with a thermal conductivity detector utilizing an 8 ft × ¾ in. stainless steel column packed with 25% SE-30 on 60–80 mesh Chromosorb W A/W. The column temperature was programmed from 75° to 225° at 2°/min, with a helium carrier gas at a flow rate of 300 ml/min. The injection sizes for this separation were 2 ml. Two milliliters of the clarified sample was injected and the effluent collected as 12 distinct peaks which were trapped in capillary glass tubes cooled with crushed Dry Ice and sealed (Figure 1). The fractions were further resolved using an F&M 700 glc equipped with a thermal conductivity detector utilizing an 8 ft × ¼ in. stainless steel column packed with 20% Carbowax 20M on 60–80 mesh Chromosorb W A/W. The column temperature was programmed from 75° to 225° at 2°/min, with helium carrier gas at a flow rate of 80 ml/min. This column was used to trap out distinct peaks for nmr and ir analyses.

Nmr spectra of the samples in deuteriochloroform solution were recorded using a Varian HA-100 spectrometer. Tetramethylsilane was the internal reference compound. Sweep width was 1000 Hz, with a 50-Hz sweep offset.

Glc–ms analyses were carried out using an Aerograph 1520 gas chromatograph coupled to a Hitachi RMU-6E mass spectrometer. A 50 ft × 0.02 in. support coated open tubular (SCOT) stainless steel column coated with Carbowax 20M was used. The column temperature was programmed from 30° to 175° at 2°/min, with a helium carrier gas at a flow rate of 6 ml/min. The column was used with the column effluent split so that 5 ml was directed

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