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LOSSES OF AVAILABLE LYSINE DURING THERMAL TREATMENT OF SOYBEAN FLOUR, CONCENTRATE AND ISOLATE

By

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ABSTRACT

LOSSES OF AVAILABLE LYSINE DURING THERMAL TREATMENT OF SOYBEAN FLOUR, CONCENTRATE AND ISOLATE

By

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Soybean flour ground in the laboratory from whole soybeans, and concentrate and isolate supplied by Central Soya, Indiana, were analyzed for available lysine by HPLC and by spectrophotometric analysis based on the Sanger reaction. Values determined by the two methods in general agreed well.

The temperature effect on the availability of lysine was studied. From this study it can be said that thermal losses of available lysine on soybean flour, concentrate and isolate are an important factor that should be considered when processing is planned.

From this study it is concluded that among the reactions responsible for the losses of available lysine when reducing sugars and water are present the Maillard nonenzymatic browning reaction is an important one.

A kinetic study done for the soybean isolate demonstrates that thermal loss of available lysine can be described by a monomolecular reaction, and that temperature has a positive effect on the reaction rate.

Finally, the importance of moisture and carbonyl groups in accelerating the nonenzymatic loss of available lysine

was observed when glucose and water were added to the system.

Dedicated with all my love to my wife,

responsible for this achievement.

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INTRODUCTION

The interest in nutrient stability during food processing has traditionally concentrated on vitamins on the not unreasonable assumption that many of the vitamins can be seriously depleted by leaching, heat degradation, light and oxidation.

The content of amino acids in a protein need not quantitatively reflect its nutritive value since a limiting step in protein utilization is protein digestibility. Processing can both increase and decrease digestibility of proteins. Heat-induced protein denaturation can increase the ease with which the protein is hydrolyzed by intestinal track proteases, but heat can also degrade protein quality by degradation and by blocking the epsilon-amino group of lysine in intact protein.

The popularity of cereal type snack foods in this country and the fact that almost everywhere in the world people subsist on cereal based diets have created interest concerning the nutritive losses that these products suffer during thermal processing or storage.

A major source of nutritive loss during processing or storage is the deterioration in protein quality caused by

nonenzymatic browning frequently referred to as Maillard browning.

Practical nutrition is concerned with the nutritive values of diet, not of individual foods. The classification of individual food protein as good, bad or indifferent in meeting the body's requirements when fed alone, is of little significance in assessing the protein value of diets because of the supplementary relationships existing among food proteins, whereby amino acid deficiencies in one protein mixture are corrected by amino acid excesses in another, and vice versa.

The detection of such relationships by biological methods alone is a method that will give no clue to the way in which it may be improved by proper amino acid supplementation. When the results of amino acid analysis of several foods are shown in tables, one can predict with considerable accuracy the order in importance of the limiting essential amino acid.

Studies have shown lysine availability to be a reliable indicator of losses in protein quality due to Maillard browning. Another reason for choosing lysine loss is the fact that it is the amino acid most often limiting in cereal diets.

LITERATURE REVIEW

Available Lysine

Block and Mitchell in 1946 demonstrated the usefulness of amino acid analysis in predicting the nutritive value of the protein in a particular food and in the selection of balanced mixtures of proteins. They found that the biological value of a given protein is not only dependent on its content in essential amino acids but also on the relative properties of these amino acids.

At this time there was already some evidence that the difference between the observed nutritive value of the proteins in the diet and the one predicted from amino acid analysis were associated with the amino acid lysine and were not always accompanied by changes in the digestibility or in the amino acid composition of the protein. This was especially true in products that had been processed or stored under adverse conditions (Harris and Mattil, 1940; Block and Mitchell, 1946).

The bases for the current concepts of essential and non-essential amino acids, the former being those which cannot be synthesized by the body at a rate sufficient for optimum growth out of the material ordinarily available in

the diet, were formed during the first two decades of the century. Mendel (1923) summarized the results of these pioneer investigations as follows: "The proportion of protein in the diet may determine whether larger or smaller absolute amounts of the nitrogenous foodstuffs are consumed; but the actual intake of these is also modified by the character of the non-protein ingredients. When the absolute protein intake is small, the "law of minimum" may come into play to limit the efficiency of the whole because of the relative shortage of some essential amino acid. Conversely, when an animal ingests a very high quantity of some protein poor in an essential unit, the absolute amount of the latter thereby available from the great abundance of its precursor may suffice to promote nutritive effects that fail to appear on a lower plane of protein intake".

The nutritive value of a food protein depends also on the physiological availability of its essential amino acids. Amino acids are unavailable if they are in regions of a protein protected (chemically or physically) from action of proteolytic enzymes or if they are linked to other chemical moieties through bonds not readily broken by digestion (Finley and Friedman, 1973).

The fact that lysine (α,ε-diaminocaproic acid) may be apparently present but "unavailable" has drawn a lot of interest. Lysine is the only essential amino acid that still has a free amino group when in condensed form within

a peptide chain (Carpenter and Booth, 1973).

This is the reason why the nutritive value of vegetable proteins and of animal protein of inferior quality has been directly related to the lysine in these proteins (Boyne, Carpenter and Woodham, 1961; Mann, Carter, Frampton, Watts and Johnson, 1962).

Lysine is often the amino acid limiting the protein quality of mixed diets for man or animals. Cereals which are usually the staple energy source also supply a large proportion of protein but this cereal protein is seriously deficient in lysine. It is then of great importance to minimize the damage of lysine by controlling the processing of high protein foods (Carpenter, 1960).

Bjarnason and Carpenter (1969), in their work on the growing rat, concluded that the formation of amide linkages between the ε-NH_2 groups of the lysine units and some other molecule containing carboxyl groups may well be a cause of nutritional damage in heated proteins and that the essential changes that occurred in heating the pure protein may still be condensations between carboxyl and ε-NH_2 lysine groups but, if so, the products are largely indigestible.

Eldred and Rodney (1946) and Henry, Kon, Lea and White (1948) working with milk powders and casein-glucose models demonstrated that, even in relatively dry materials, the carbonyl groups of reducing sugars could react with the free amino groups of lysine and form compounds that had no

nutritional value.

Baldwin et al. (1951) and Jokinen and Reineccius (1976) studied the losses in available lysine due to thermal processing in protein model systems when reducing sugars are present. The reaction that takes place is apparently that of non-enzymatic browning (Maillard) between the free amino groups of the protein and the reducing sugar.

Several authors have investigated the fact that the properties of proteins are greatly modified when they have reacted with oxidized fats.

Tappel (1955) tried to define more precisely the interaction of oxidized unsaturated fats and proteins and observed that unsaturated fatty acids and esters could react with proteins forming rather stable complexes which could not be ruptured by solvent treatments. He attributed the stability of these complexes as being caused by a chemical union between the aldehydes produced during autoxidation of the lipid and the reactive amino groups of the proteins.

Narayan and Kummerow (1958) studied complexes formed between egg albumin and oxidized linoleic acid. They observed little or no complex formation when un-oxidized fatty acids were used. They found that covalent linkages did not seem to exist between the reactive groups of the protein and the lipide in complexes of this type. A possible structure on the basis of a large number of

hydrogen bonds was proposed.

Narayan, Sugai and Kummerow (1964) studied the nature of the reactions leading to the formation of complexes between oxidized lipids and proteins. They confirmed that the reactive groups in the protein were not covalently tied up to the lipid and that only oxidized or polymerized fatty acids were capable of complexing with egg albumin.

Nishida and Kummerow (1960) reported that oxidized methyl linoleate interacts in vitro with serum low density lipoproteins while unoxidized methyl linoleate does not.

Roubal and Tappel (1 and 2, 1966) reported that free-radical intermediates generated in peroxidizing unsaturated lipid-protein mixtures participate in the chain of reactions leading to considerable damage to proteins, enzymes and amino acids greatly modifying their properties. Among the most labile amino acids are methionine, histidine, cystine and lysine. The pattern of damage to proteins was similar to that observed in the case of radiation damage, being about one-tenth as effective as radiation damage.

Andrews et al. (1965) presented more evidence indicating that an interaction occurs between proteins and an autoxidizing unsaturated lipid. They used a model system approach with two purified proteins (gelatin and insulin) in the presence of methyl linoleate. They observed that lipid intermediates react with the ε-amino group of lysine and also with phenylalanine and glycine.

Baliga and Lyman (1959) studied the reaction between gossypol and purified cottonseed protein and found that gossypol reacts with the protein by combination with the free ε-amino group of lysine. With increased blocking of the free amino groups, tryptic and peptic digestion "in vitro", and the overall nutritional value of the protein progressively decreased. The reaction between protein and carbohydrate also decreased proteolytic digestion.

Lea, Parr and Carpenter (1960) observed a small but definite fall in the available lysine content of herring meal containing oil and stored in air. They explained this result on the basis of a reaction occurring between oxidation products of the oil and the ε-NH_2 groups of the lysine side chains in the protein molecules.

Badenhop and Hackler (1971) indicated that the effect of roasting on the amino acid composition of soybean protein is confined mainly to the destruction of the total and available lysine, cystine and histidine. The roasting of peanuts, tree nuts, coffee and other products includes a rapid dehydration followed by a partial pyrolysis. The process is carried out mainly for the desirable flavor changes produced in the product and other beneficial changes such as improved texture and color. Also the roasting of soybeans may improve nutritional quality by destroying substances such as trypsin inhibitors.

Due to the increasing importance of preserved milk as a source of high quality protein, Mauron et al. (1955) determined the nature and extent of amino acid alteration in milk manufactured according to four widely used processes. They found that lysine was the most seriously affected amino acid depending on the type of process used. Thus, they found no apparent destruction of lysine in boiled milk whereas a slight destruction took place in spray dried milk (3.6%) and sweetened condensed milk (4.8%). Destructions were appreciable in evaporated milk (8.4%) and average roller dried milk (13%), while in roller powder it was considerable (36.6%).

Ben-Gera and Zimmerman (1972) evaluated the loss of available lysine during the storage of several intact foodstuffs. Temperature (20, 30 or 40^{o}C), water activity (0.4 or 0.6) and environment were controlled during 24 months of storage. They reported available lysine losses of 87.6% in non-fat dry milk, 35.7% in cottonseed meal 33.3% in peanut meal, 30% in chick peas, 43.3% in wheat, 18.5% in rice, 41.9% in soybeans and 7.5% in soybean meal for the same storage conditions. Differences in loss of available lysine during storage of the various foodstuffs were attributed to quantity of reducing sugars present and occurrence of lipid oxidation.

According to Finley and Friedman (1973) cross-linking is probably the most important chemical mechanism restricting

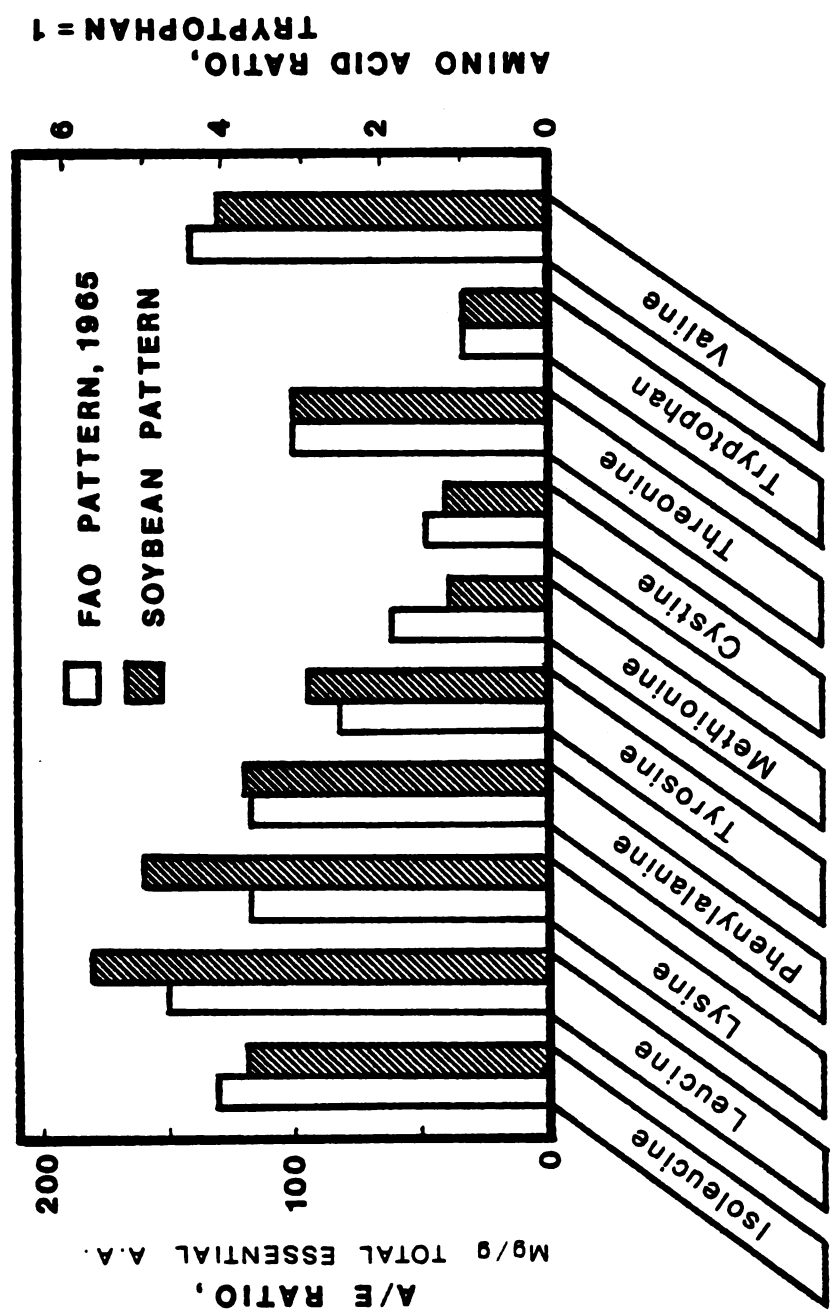

Figure 1. Essential amino acid pattern of soybean protein compared with the whole egg protein (Smith, A.K. and Circle, S.J., 1978.)

Table 1. Distribution of amino acids in protein from seeds

	g amino acid/16 g N				
	Sunflower seed flour	Wheat flour	Peanut flour	Sesame flour	Soybean flour
Amino acids essential for human nutrition					
Isoleucine	3.30	3.3	2.82	1.28	4.4
Leucine	4.77	6.2	6.46	3.94	7.5
Lysine	5.23	2.0	2.55	1.79	6.4
Methionine	1.91	1.6	1.93	1.26	1.4
Phenylalanine	2.62	4.8	3.67	2.19	5.2
Threonine	3.53	2.4	2.82	1.47	3.8
Valine	3.93	3.6	3.96	2.35	4.7
Nonessential for human nutrition					
Alanine	4.10	2.7	3.95	2.60	4.3
Arginine	8.43	3.5	13.12	14.89	7.3
Aspartic acid	7.70	3.6	7.84	7.29	11.9

Compiled from: Lawhon, Cater and Mattil (1972), FAO (1965) and Holt and Sosulski (1974).

biological utilization of lysine because of its ε-amino group. They also state that lysine becomes secondarily bound (nutritionally unavailable) as a result of chemical reaction of the ε-amino groups of lysine including the following: first, lysine may be buried in a protein matrix in a particular sequence of conformation which is slow to hydrolyze or is not hydrolyzed at all by animal proteases. Such lysine they say, may or may not appear to be chemically available by hydrolysis methods and yet be totally unavailable nutritionally. Secondly, lysine can be cross linked to an aspartyl or glutamyl residue on another protein or in the same protein molecule. Finally, they say that a more common reason for losses of available lysine may be reactions with reducing sugars which react with lysine in proteins to render it unavailable.

All these investigations have shown lysine availability to be a reliable indicator of losses in protein quality which can occur by different mechanisms depending on the conditions of processing of the protein containing product.

Lysine a Limiting Amino Acid

A lot of attention is being given to plant materials such as oilseed meals as sources of protein of high quality for both animal and human feeding.

The fact that cereals are the main source of protein intake for two thirds of the world population is well

recognized and, when compared with the reference amino acid pattern, all cereal proteins are deficient in the essential amino acid lysine. Therefore, lysine fortification of cereals could become a good approach to improve the protein quality of cereals and help solve the protein malnutrition problem (Tsao, Frey and Harper, 1978).

The fact that some proteins have several amino acids in a very small proportion when compared to others brings the term "limiting" which is no more than the one in least amount in the food (Lehninger, 1977).

In Table 1, the high value for lysine present in soybean can be observed and, as it is the limiting amino acid in almost every cereal and in many plant proteins, its importance when used as a supplementary cereal can be inferred.

The essential amino acid pattern of soybean protein is compared with that of whole egg protein (Fig. 1). This comparison reveals once more one of the most valuable attributes of soybean protein, namely the fact that it has a much higher content of lysine (Smith and Circle, 1978).

Available Lysine Determinations

The chemical estimation of lysine in foods can involve the measurement of total lysine or available lysine. Total lysine is generally determined after an acid hydrolysis treatment and does not necessarily reflect the amount of

lysine that is in a nutritionally available form, while available lysine is usually regarded as those lysine residues possessing free ε-amino groups (Peterson and Warthesen, 1979).

Sanger (1945) used 1-fluoro-2,4-dinitrobenzene (FDNB) for detection and quantitation of the different N-terminal amino groups in proteins with peptides. The reaction of FDNB with amino acids produced yellow derivatives with distinct chromatographic and spectrophotometric properties; so each free amino group became labelled with a dinitrophenyl (DNP) group that resisted subsequent digestion with acid. N-terminal lysine gave a ε-di-DNP-lysine and, where lysine was present in other positions, Sanger obtained ε-N-DNP-lysine which could also be separated by chromatography.

In 1955, Carpenter and Ellinger used a simple procedure to determine available lysine which proved useful for a range of animal materials to provide a measure of their nutritional value as a source of lysine. This procedure was based on the measurement of "FDNB-reactive" lysine.

Carpenter et al. (1957) directly measured the absorbance of the yellow, ether extracted acid hydrolysates of dinitrophenylated proteins in their estimation of available lysine.

In 1960, Carpenter (1960) studied the available lysine of a wide variety of animal and plant products and he

reacted the ε-dinitrophenylhydrazone-lysine with methyl chloroformate to produce an ether soluble lysine derivative as a way to reduce some of the error in the analysis. He took the difference in color intensity of the hydrolyzate before reaction with methyl chloroformate and after reaction and extraction with ether, as a measure of available lysine.

Baliga et al. (1959) reported a method for the accurate determination of free lysine ε-amino groups in samples of cottonseed meal, based on an adaptation of Sanger's procedure.

In 1963, Rao et al. used what they called a simple and rapid method for the estimation of available lysine. In their method, the ε-DNP-lysine produced is eluted from an ion exchange column with a solvent composed of three parts by volume of 3 N aqueous HCl and one part of methyl ethyl ketone; the product is then determined spectrophotometrically.

Selim (1965) proposed a micro determination of lysine in protein hydrolyzates based on the quantitative conversion of the amino acids into their copper salts, followed by treatment of the latter with FDNB; lysine being the only amino acid which yields a colored dinitrophenyl derivative that can be determined spectrophotometrically.

Kakade and Liener (1969), Ousterhout and Wood (1970) and Posati et al. (1972) used 2,4,6-Trinitrobenzene

sulfonic acid instead of 1-fluoro, 2,4-dinitrobenzene. Reasons offered for this are the possibility to use a smaller sample size and that, with this new reagent that could reduce the amount of reagent, diminishing thus the causes of error due to the absorbance imported by the excess. Another reason for the change of reagent is that the DNP-lysine obtained when using FDNB was destroyed to some extent due to the nitro groups during the hydrolysis.

Booth (1971) modified Carpenter's method (1960). He stated that methods that use FDNB or trinitrobenzene sulphonic acid for the determination of available lysine need a correction for loss. In the Carpenter method the dinitrophenylated material is digested under reflux in 5.8 M-HCl, and the yellow DNP-Lysine set free is measured colorimetrically. Part of this DNP-Lysine is lost during the acid digestion. Booth proposed to assess this loss by adding DNP-Lysine to duplicate samples, after the addition of the acid, and taking it through the rest of the procedure. The "recovery" of this added DNP-Lysine was used to provide a factor by which results are multiplied to correct for loss during the digestion. Booth (1971) suggested that the true loss of DNP-lysine from the test material can be roughly estimated as one half the percentage loss of added DNP-lysine.

Finley et al. (1972) used a colorimetric procedure based on Selim's work (1965) for determination of added lysine in fortified wheat and bulgur.

Details of a chemical method to determine available lysine using acetic acid and sodium nitrite are given by Allison et al. (1973). This method reports good correlation with other methods when used on leaf-protein concentrates for which other methods have not been very successful, in part due to the interference of plant pigments.

In 1967, White and Gauger reported an automated method for the simultaneous determination of lysine and total amino acids in seed hydrolysates. The lysine determination employs the enzyme L-lysine decarboxylase which specifically catalyzes the decarboxylation of lysine, producing an amine cadaverine, with subsequent measurement of the carbon dioxide liberated.

This later method was modified by Wall and Gehrke (1974), incorporating a dialysis block with a carbon dioxide gas, dialysis membrane in the system. The carbon dioxide is absorbed in an alkaline solution and the resulting decrease in pH is reflected in a reduction in indicator color. This modification they say will prevent the reagent from absorbing carbon dioxide from the air.

Datta (1976) estimated available lysine of compounded starter rations of poultry by treating the rations with FDNB followed by acid hydrolysis; the hydrolysate was

purified through a sephadex 0-50 column and the color was read spectrophotometrically.

A method of determination of available lysine by thin layer chromatography was reported by Datta and Datta (1977). This method is time consuming and highly costly.

Another method of analysis for available lysine using enzyme, in this case pronase, has been reported by Rayner and Fox (1978). Pronase does not release all amino acids from protein but this method still remains as a relative test.

The latest method found to determine total and available lysine is the one that uses high-pressure liquid chromatography reported by Peterson and Warthesen (1979). They avoid, by using high-pressure liquid chromatography, the interference of some compounds formed during the hydrolysis. Here the total lysine is determined by reacting hydrolysates with dansyl chloride and then separation and quantification of didansyl lysine.

MATERIALS AND METHODS

I. Soybean products

Three different types of soybean products were used for this study, viz, soybean seeds, soyafluff and promosoy. These products were supplied by Central Soya, Fort Wayne, Indiana. The three products were kept at approximately $4^{o}C$ in the dark to avoid as much as possible any decomposition during storage.

The soybean seeds were ground with the help of an industrial blender until they passed through a 100 mesh sieve.

Soyafluff 200 W 3040 Lot 865 and promosoy 100 4810 Lot 165 were freeze-dried products, so no preparations were necessary prior to the use of these products in the work. A micro-Kjeldahl analysis was done to determine the protein content. This method is described by the AOAC.

II. Available lysine determination by the spectrophotometric method

The method used to determine the available lysine was that described by Carpenter as modified by Booth (1971).

The samples were fine enough to pass through a .100 mm sieve. Ten ml of $NaHCO_3$ solution (8% w/v) were added to

approximately 1.0 g of sample placed in a round bottom flask. The flask was shaken gently to allow the sample to be wetted. The mixture was left to stand for 8-10 minutes.

A 0.5 ml of 1-fluoro,2,4-dinitrobenzene already dissolved in fifteen ml of ethanol was then added to the flask containing the sample. This mixture was shaken in a Burrell wrist action shaker at room temperature for four hours. After the shaking period, the ethanol was evaporated in a rotary evaporator. To assure the evaporation of the ethanol a weight loss of 12.5 g was checked.

The mixture was cooled and then mixed with 30 ml of 8.1 N HCl and refluxed for 16 hours.

After the 16 hours and while still hot, the condenser was washed with a little warm water and the flask then disconnected. The contents were filtered while still hot through a Whatman #2 filter paper and rinsed repeatedly with hot water until a 250 ml volume was reached. The hydrolyzates were stored at $4^{o}C$, in the dark, until used for further analysis.

Two ml of filtrate were pipetted into each of two stoppered test tubes, A and B. To prepare a blank the content of tube B was extracted with 5 ml of peroxide-free diethyl ether for three times. The ether was removed with a water aspirator and the remaining trace amounts were removed with the help of a steam bath. A drop of phenolphthalein solution was added and then a NaOH solution

(120 g/liter) was added until the first pink color appeared. Two ml of carbonate buffer pH 8.5 (19.5 g $NaHCO_3$ and 1 g Na_2CO_3 dissolved in 250 ml of water and pH adjusted to 8.5) were added. Under the fume hood 5-6 drops (approximately 0.06 ml) of methyl chloroformate was added. The tube was shaken and the pressure cautiously released and it was left to stand for 8 minutes. Then 0.75 ml of concentrated HCL was added with care and then with agitation to prevent frothing. The solution was then extracted with five ml of ether for four times as was described before. The tube was cooled and the content made up to 10 ml with water. Tube A was extracted three times with peroxide free diethyl ether in the same manner as before but the cold solution was made up to 10 ml with 1 N HCl.

The absorbance of both A and B were read at 435 nm against deionized water in a Bausch and Lomb Spectronic 20. Reading A minus B is the net absorbance attributable to DNP-Lysine.

With each set of samples analyzed, the absorbance of the standard solution prepared by dissolving 254 mg of mono-ε-dinitrophenyl lysine hydrochloride in 200 ml of 8.1 N HCl was determined by the same procedured followed by others for the samples. The only difference was that only two tenths of 1 ml of the standard solution was used in each run for tubes A and B.

To determine the effect of temperature on the availability of lysine, samples were heated at the different fixed temperatures (50°, 75°, 100°, 125° and 150°C) in a Precision model-29 oven.

III. Available lysine determination by high pressure liquid chromatography

The following procedure was followed for the determinations of available lysine using high pressure liquid chromatography.

Approximately 1.0 g of sample was treated in the same manner as before. After the acid hydrolysis, the sample was filtered while still hot and brought to a volume of 250 ml with water. Approximately 1 ml was then filtered through a 0.2 µm membrane filter. Separation and quantitation using a standard DNP-lysine was then accomplished by HPLC.

The liquid chromatograph used in this study consisted of a Waters Associates model-6000A pump, U6K injector and 400 absorbance detector fitted for determination of wavelengths of 436 or 254 nm. The detector output was recorded on a Hewlett Packard 3380A.

The wavelength used to detect DNP-lysine was 436 nm. The separation was accomplished on a µ Bondapack C_{18} column (3.9 mm idx30 cm. Waters Associates) with a mobile phase of 20% nanograde acetonitrile (Mallinckrodt) and 80%

0.01 M acetate buffer pH 4.0.

With a flow rate of 2.0 ml/min, DNP-lysine eluted in 12-13 minutes. Usually 40 µl was injected. For peak identification and quantitation, DNP-lysine HCL (Sigma Chemical Co.) was used as an external standard and at least four injections of different volumes were made each day. A 2,4-Dinitrophenol standard was also used to identify it together with the DNP-Lysine. The two peaks were recorded close to each other. The 2,4-dinitrophenol was eluted in 10-11 minutes.

IV. Fluorometric Analysis

The fluorescence developed from the different treatments given to Promosoy was measured. This was done using a Varian SF-330 spectrofluorometer equipped with a xenon lamp. Two grams of sample were treated at 100°C for 8 hours. The samples were heated alone, mixed with glucose, dissolved in water and mixed with glucose in water. After the samples were treated, they were dissolved in 30 ml of methanol and stirred overnight at 4°C. They were then filtered through a Whatman #4 filter paper and the filtrate was evaporated to dryness in a rotary evaporator. The dried material was resolubilized with 5 ml of methanol and its fluorescence measured. Excitation was 360 nm and emission 440. Methanol was used as a blank.

RESULTS AND DISCUSSION

I. Protein content of the products

Table 2 shows the results from the micro-kjeldahl analysis.

Table 2. Micro-kjeldahl analysis of soy flour, soyafluff and promosoy

	% Protein w/w	Average % Protein w/w
Soy flour	43	43
	43	
Soyafluff	49	47
	45	
Promosoy	65	64
	62	

% N x 6.25 = % protein

wet basis

II. Spectrophotometric studies

The method used for the analysis was that described by Carpenter (1960) as modified by Booth (1971) and was based on the Sanger reaction which involves a spectrophotometric determination.

Table 3. Available lysine of soy flour under different treatments (temperature and time)

(g of available lysine/100 g of protein)

Sample at a fixed temperature	Time (hours)						
	0	2	4	8	12	24	48
Soy flour at 50°C	5.9[a] ±0.19[b]				5.4[a] ±0.17[b]	5.9[a] ±0.00[b]	5.3[a] ±0.16[b]
Soy flour at 75°C	5.9[a] ±0.19[b]				5.5[a] ±0.01[b]	5.8[a] ±0.09	5.6[a] ±0.23[b]
Soy flour at 100°C	5.9[a] ±0.19[b]	5.5[a] ±0.04[b]	5.2[a] ±0.07[b]	4.9[a] ±0.25[b]	4.7[a] ±0.81[b]	4.5[a] ±0.19[b]	3.0[a] ±0.00
Soy flour at 100°C under N_2	5.9[a] ±0.19[b]				4.5[a] ±0.88	4.1[a] ±0.38	3.4[a] ±0.06
Soy flour at 125°C	5.9[a] ±0.19[b]				1.8[a] ±0.08[b]	1.7[a] ±0.08[b]	1.5[a] ±0.13[b]
Soy flour at 150°C	5.9[a] ±0.19[b]				1.8[a] ±0.01	1.8[a] ±0.01	1.6[a] ±0.21[b]
Defatted soy flour at 100°C	6.4[a] ±0.00[b]				6.4[a] ±0.11[b]	5.8[a] ±0.09[b]	4.7[a] ±0.07[b]

[a]Mean from two determinations.

[b]One standard deviation.

Table 4. Available lysine of soybean concentrate and isolate in different systems at different times under a fixed temperature

(g of available lysine/100 g of protein)

	Time (hours)						
	0	2	4	8	12	24	48
Soyafluff at 100°C	7.0[a] ±0.00[b]	6.3[a] ±0.42[b]	6.6[a] ±0.13[b]	6.3[a] ±0.06[b]			
Promosoy at 100°C	6.3[a] ±0.00[b]	6.0[a] ±0.08[b]	6.1[a] ±0.00	4.8[a] ±0.81[b]			
Promosoy + H_2O at 100°C	6.3[a] ±0.00[b]	6.4[a] ±	4.8[a] ±	4.1[a] ±			
Promosoy + glucose (dry system) at 100°C	6.3[a] ±0.00[b]	6.1[a] ±0.17	5.8[a] ±0.07	5.5[a] ±0.40[b]			
Promosoy + glucose + H_2O at 100°C	6.3[a] ±0.00[b]	5.5[a] ±	1.9[a] ±	1.6[a] ±			

[a]Mean from two determinations.

[b]One standard deviation.

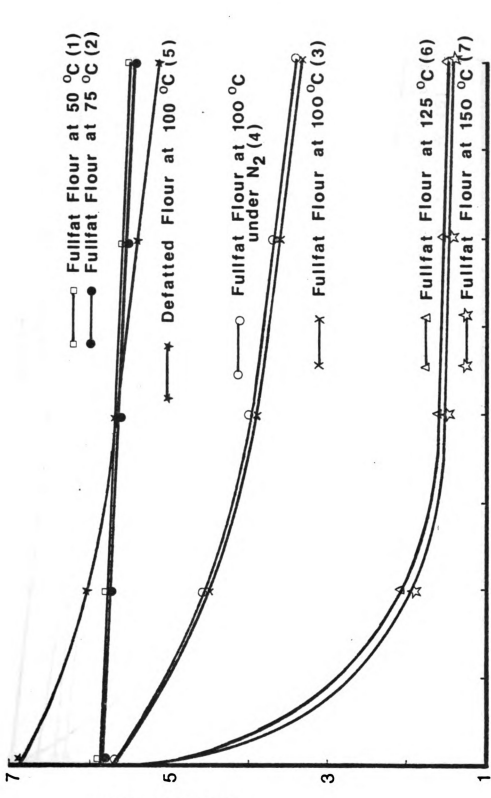

Figure 2. Available lysine vs time in soy flours.

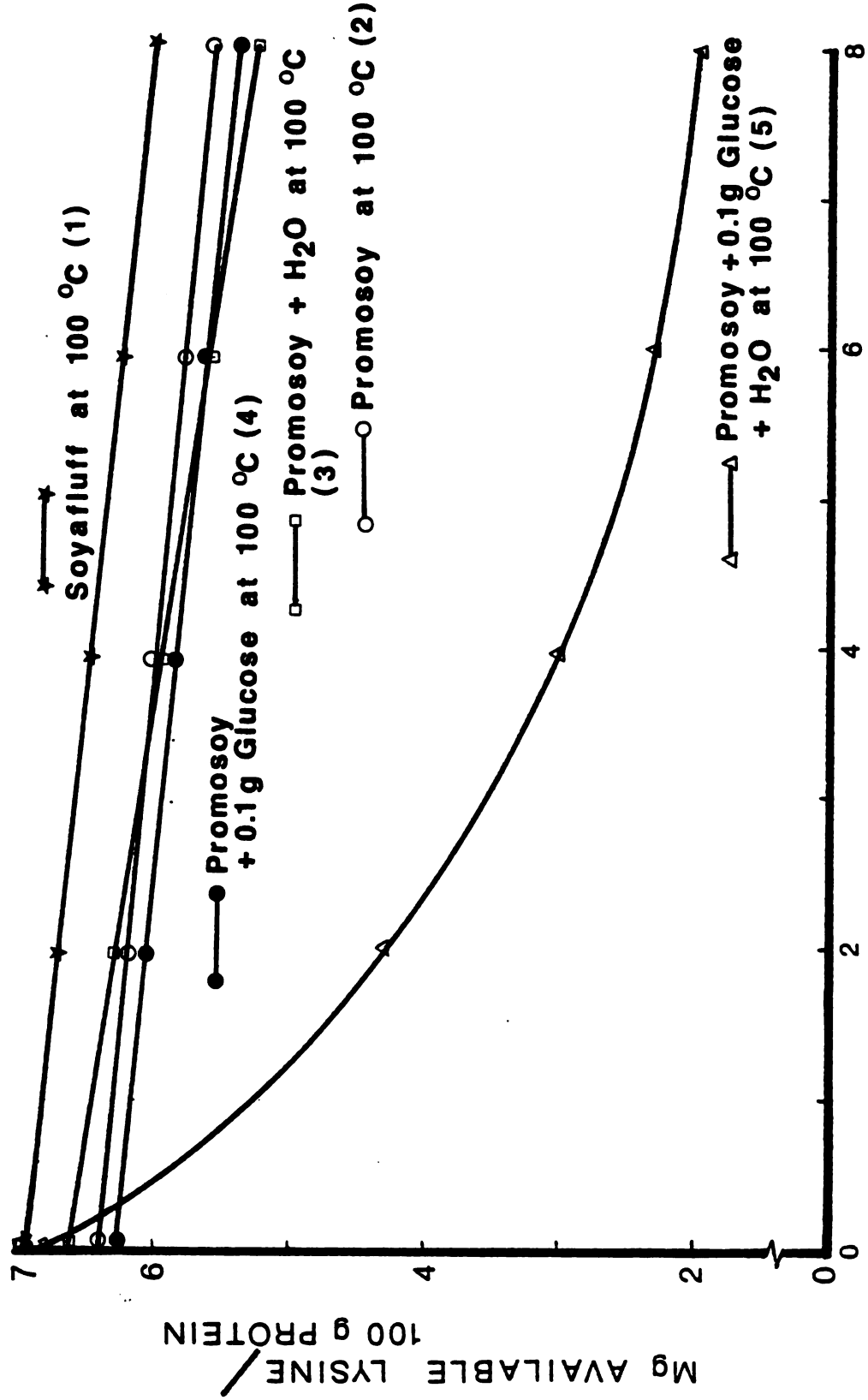

Figure 3. Available lysine vs time in a soy protein isolate.

The available lysine content was calculated from the following formula:

$$C = \frac{Ws}{As} \times \frac{Au}{Ah} \times \frac{Vh}{Wu\ P} \times 100$$

where:

C = g of available lysine/100 g of protein.

Ws = g of lysine in standard solution used in each run.

As = net absorption of the standard solution.

Au = net absorption of the unknown.

Ah = aliquot of filtrate.

Vh = total volume of filtrate.

Wu = weight of the sample in g.

P = protein content of the sample.

The results are shown in tables 3 and 4. From the results shown in tables 3 and 4 we were able to draw Figures 2 and 3 separating the soy flour analysis from those of Soyafluff and Promosoy.

From Figure 2 we can observe the definite effect of temperature on the availability of lysine. This is shown by curves 1, 2, 3, 6 and 7 representing the system full fat soybean flour at different temperatures. Here one can see that at higher temperatures the loss in available lysine is faster. Another fact that should be observed is that at low temperatures 50^{o}C and 75^{o}C there is no apparent difference in the effect on the loss of available lysine. These results agree with those reported by Tsao, Frey and Harper

(1978). In their work, they concluded that the thermal loss of available lysine in a rice-meal system can be described by a monomolecular reaction and that temperature has a definite positive effect on the reaction rate.

From the same figure, curve 4, which represents the soybean flour at 100°C under nitrogen, provides a basis for inference that the loss of available lysine is not due to an oxidation process because it shows the same pattern as the one that represents the soybean flour in an open system.

Another striking feature from this figure is shown by curve 5 which represents defatted soybean flour at 100°C. From this curve, it can be seen that it is mainly a thermal effect that affects the losses in available lysine. The curve has the same shape as the one that represents the fullfat soybean system. The displaced position of the curve #5 represents the changed lysine content due to removal of lipids from the system because in a defatted system the protein content will be increased accordingly.

These results agree with those presented by Baldwin, Lowry and Thiessen (1951). Their work was centered around the effects of heat on protein nutritional quality and more specifically on the effects of heat on proteins when reducing sugars are present during the processing. They concluded that the reaction that takes place is that of the nonenzymatic browning (Maillard) reaction between the free

amino groups of the protein and the reducing sugars. Figure 2 refers to soybean flour with a protein content of 43%. We know that reducing sugars are also present in flour, and that they can react with lysine in the protein to render it unavailable. They also concluded that increasing the temperature of processing from 100°C to 121°C affects adversely the protein of a casein-dextrose system. Increasing the temperature of a short time process (20 minutes) has more adverse effect than increasing the temperature on a long time process (120 minutes).

Figure 3 demonstrates that Soyafluff has a higher initial amount of available lysine when compared with that of Promosoy, although Soyafluff has, as it has been shown in Table 2, a 47% content of protein while Promosoy has 64% protein.

The work here was done at 100°C. This temperature was used because, from the prior work, shown in Figure 2, this is the temperature that shows its effects more effectively and in a shorter time. Consequently a study was made to determine suitable media in which nonenzymatic browning could take place.

From the curves 1 and 2 it can be observed that the losses of available lysine are similar in Soyafluff and Promosoy at 100°C.

Comparing Promosoy by itself and with glucose in a dry and wet system, it can be observed that there is little difference when the system is studied in a dry solid state, or when the study is made in a water solution. But, when Promosoy is mixed with glucose in the presence of water the loss of available lysine is greatly increased due to the combined effect of high temperature and probably non-enzymatic browning.

The need for water in the reaction is pointed out by Labuza, Tannenbaum and Karel (1970) who state that water has a dominant influence on the rate of browning in all carbonyl containing systems, whether the carbonyls are present in the initial system, or are formed during storage.

According to their investigations, browning increases with water content up to a maximum which depends on specific conditions. The reaction is complex and water not only accelerates the reaction but also shortens the induction period. The acceleration of the rate, they say, may be due to increased availability and mobility of reactants while the effect on induction time may indicate that formation of pigment has a different pathway at the lower humidity, which requires more time for color development or that sufficient intermediates must be built up before they can dissolve and react to form pigments.

Results show that in this system the losses of available lysine are probably due mainly to Maillard non-enzymatic browning and that sufficient water is necessary for it to proceed.

In 1979, Wolf, Thompson, Ahn and Hegarty reported that losses of available lysine in soy protein isolates during thermal processing go through three phases: a first order loss or rapid loss phase, a transition phase, and a no-loss phase. This change in reaction kinetics was not observed in this study. It was found that the losses of available lysine are represented by a first order reaction. The activation energy of available lysine degradation was calculated from the reaction rate constants obtained from Figure 4 and using Figure 5 and the Arrhenius equation.

$$K = Ae^{-\frac{E}{RT}}$$

where K is the slope from Figure 5 and A the intercept which may be referred to as the frequency factor. The value thus obtained was E = 38.12 Kcal/mole.

The activation energy found indicates that higher temperature processing of soy protein would cause significant increases in lysine losses. The data points for Figure 5 are the values of K from Figure 4. This figure shows that the first order rate reaction model postulated for the degradation of available lysine fit the experimental data well. These results agree with the work

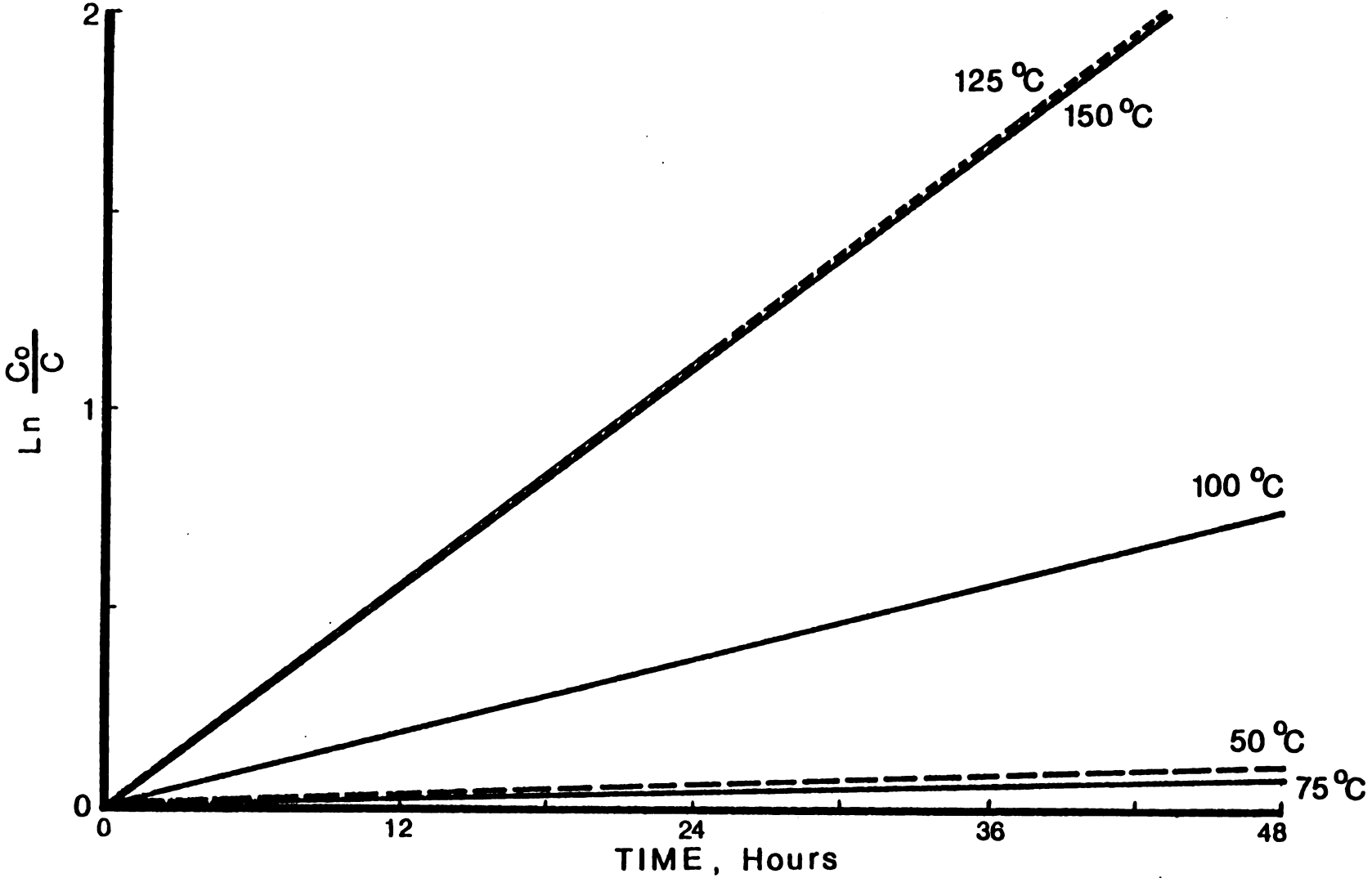

Figure 4. Losses of available lysine at different temperatures.

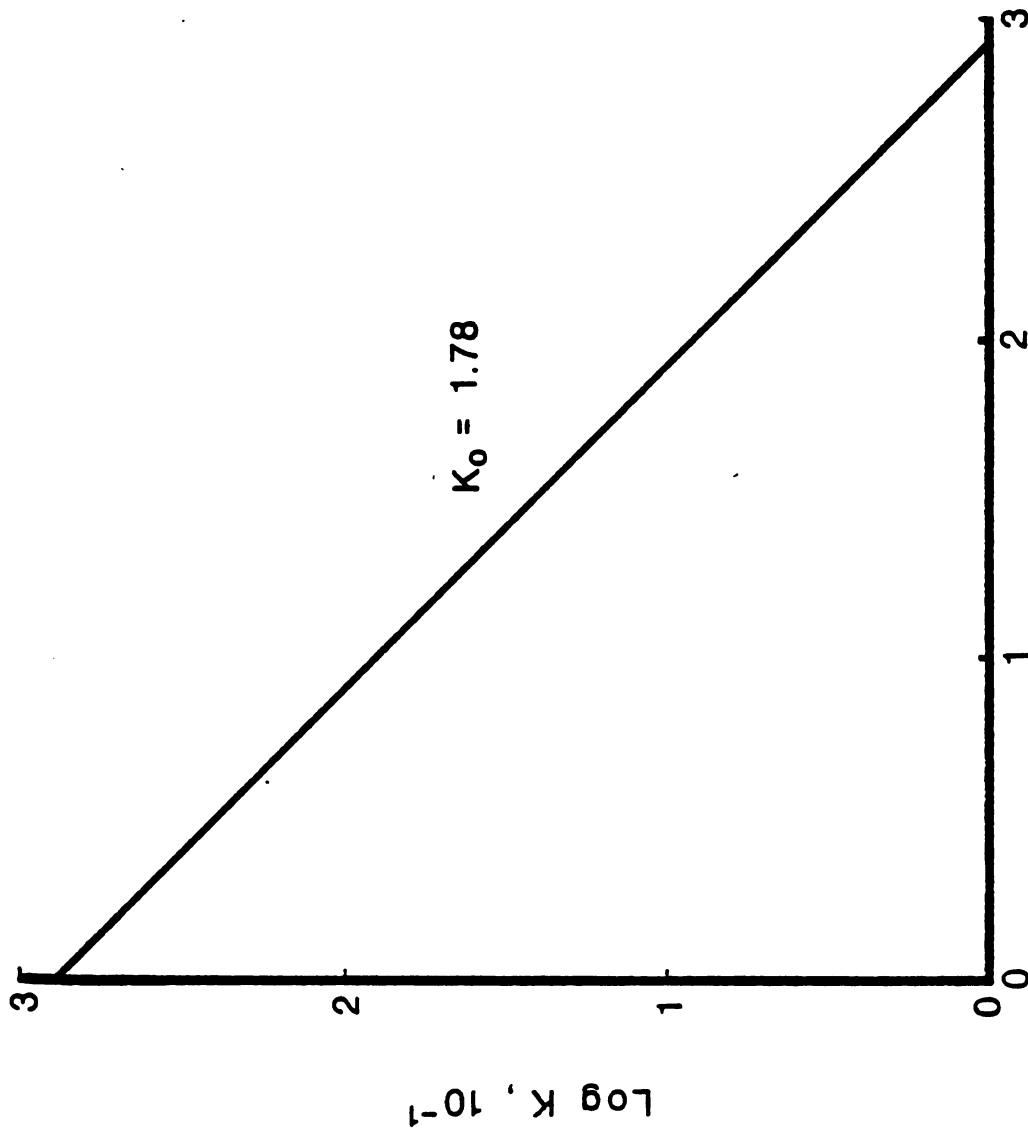

Figure 5. Rate of the losses of available lysine.

reported for casein by Lea and Hannan (1949) and single cell protein by Warmbier et al. (1976) and Wolf et al. (1978).

Of course, several problems exist in predicting nutrient losses. Each nutrient is destroyed at a different rate K, and K is a function of temperature as controlled by the activation energy E. The higher the activation energy, the more effect an increase in temperature has on the reaction.

This increase in rate with temperature is useful in that one can use accelerated storage tests to predict what would happen at lower temperatures.

III. High Pressure Liquid Chromatography studies

The HPLC method was compared to the direct FDNB spectrophotometric method described above. The same products used before were used to compare the methods.

The available lysine content using HPLC was calculated from the following formula:

$$C = \frac{H \times \frac{1000}{Iv} \times V_T}{S \times R \times Ws}$$

where

C = µg of available lysine/g of sample.

H = Height of the lysine peak on the chromatogram

Iv = Injection volume, usually 40 µl.

Vt = Reconstitution volume, 250 ml.

S = Slope from the standard curve.

R = Reproducibility factor from the curve.

Ws = Weight of the sample in g.

A standard curve was prepared at the beginning of the study. It is shown in Figure 6 with a slope of 7.4, an intercept of -13.2 and a correlation of 99.9.

The results obtained by this method are compared with those obtained with the spectrophotometric method in Tables 5 and 6.

As shown in the Tables, the results obtained from both methods are generally comparable. It should be noticed though that there are some differences which might be explained by the fact that in some instances we observed problems with the equipment used for HPLC.

The analysis of DNP-lysine by HPLC follows the same procedure as the spectrophotometric method of Carpenter (1960) and Booth (1971) through the hydrolysis step. The spectrophotometric procedure then requires ether extraction to remove α-DNP-amino acids and dinitrophenol formed from excess FDNB. In addition, a blank is prepared by reaction of the hydrolysate with methoxycarbonyl chloride followed by ether extraction to remove absorbance due to DNP-lysine. With the HPLC procedure, the filtered hydrolysate containing DNP-lysine was injected directly on to the liquid chromatograph without extensive sample clean up. It is important that the DNP-lysine is chromatographically separated from

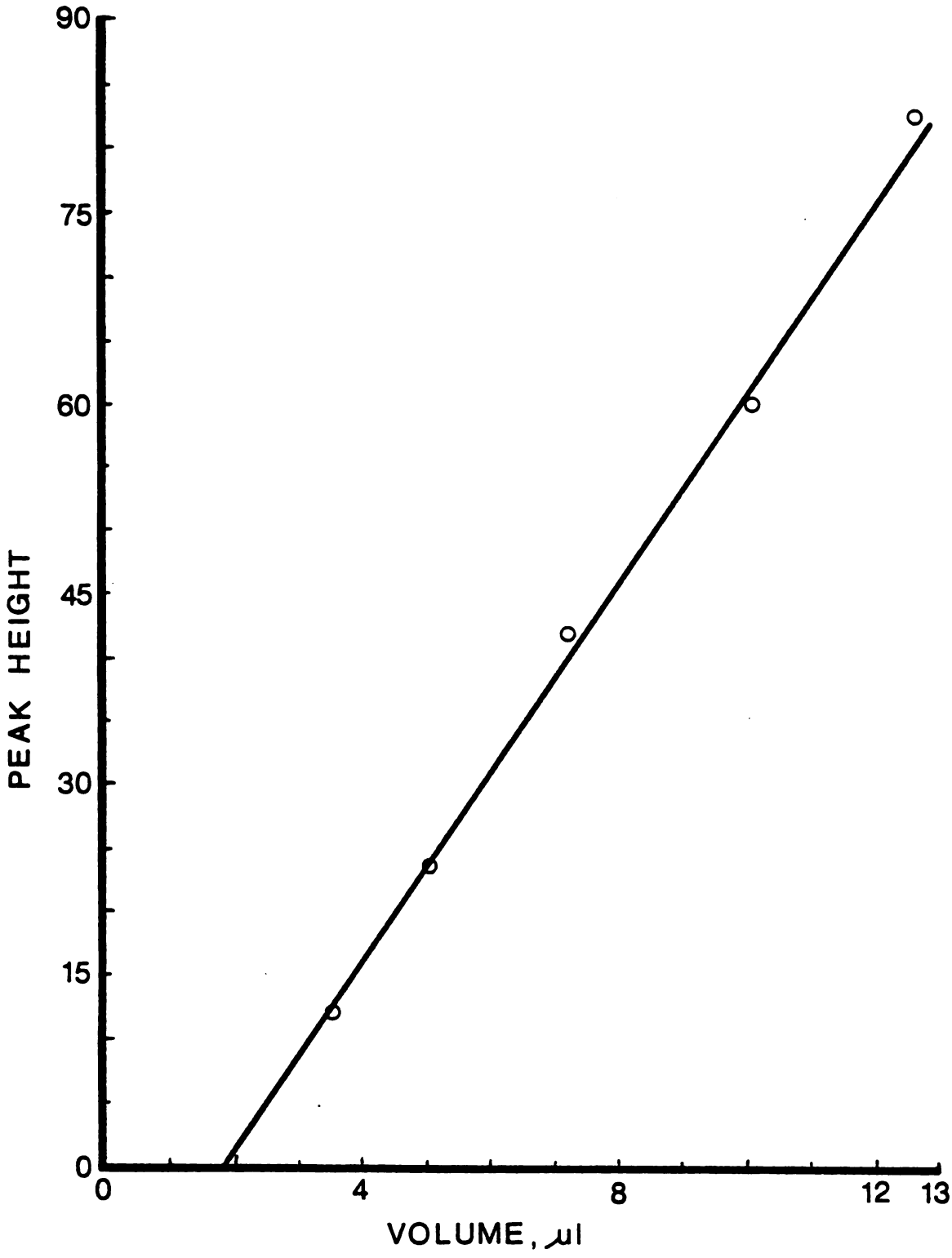

Figure 6. Standard curve for the DNP-lysine determination using HPLC.

Table 5. Comparison by spectrophotometric and HPLC methods of available lysine in soy flour and Promosoy treated under different conditions of temperature and time.

Sample at a fixed temperature		(g of available lysine/100 g of protein) Time (hours)						
		0	2	4	8	12	24	48
Soy flour at 50°C	1	5.5				5.4	5.9	5.3
	2	4.9				5.8	6.1	6.1
Soy flour at 75°C	1	5.9				5.5	5.8	5.6
	2	4.9				5.7	5.5	5.4
Soy flour at 100°C	1	5.9	5.5	5.2	4.9	4.7	4.5	3.0
	2	4.9	5.3	5.0	4.9	4.5	5.5	4.7
Soy flour at 100°C under Nz	1	5.9				4.5	4.1	3.4
	2	4.9				5.1	4.8	4.0
Soy flour at 125°C	1	5.9				1.8	1.7	1.5
	2	4.9				1.2	0.98	
Soy flour at 150°C	1	5.9				1.8	1.8	1.6
	2							
Defatted soy flour at 100°C	1	6.4				6.4	5.8	4.7
	2	6.5				6.1	5.4	4.8
Soyafluff at 100°C	1	7.0	6.3	6.6	6.3			
	2	6.7	6.6	6.8	6.2			

[1]Spectrophotometric analysis.

[2]High pressure liquid chromatography.

Table 6. Comparison by spectrophotometric and HPLC methods of available lysine in Promosoy, heated in presence and absence of glucose and water

(g of available lysine/100 g of protein)

Sample at 100°C		Time (hours)						
		0	2	4	8	12	24	48
Promosoy at 100°C	1	6.3	6.0	6.1	4.8			
	2	6.4	6.6	6.4	5.9			
Promosoy + H_2O at 100°C	1	6.3	6.4	4.8	4.1			
	2	6.4	6.2	4.9	4.0			
Promosoy + glucose system at 100°C	1	6.3	6.1	5.8	5.5			
	2	6.4	6.2	6.3	6.0			
Promosoy + glucose + H_2O at 100°C	1	6.3	5.5	1.9	1.6			
	2	6.4	5.3	2.0				

[1] Spectrophotometric analysis.

[2] High pressure liquid chromatography.

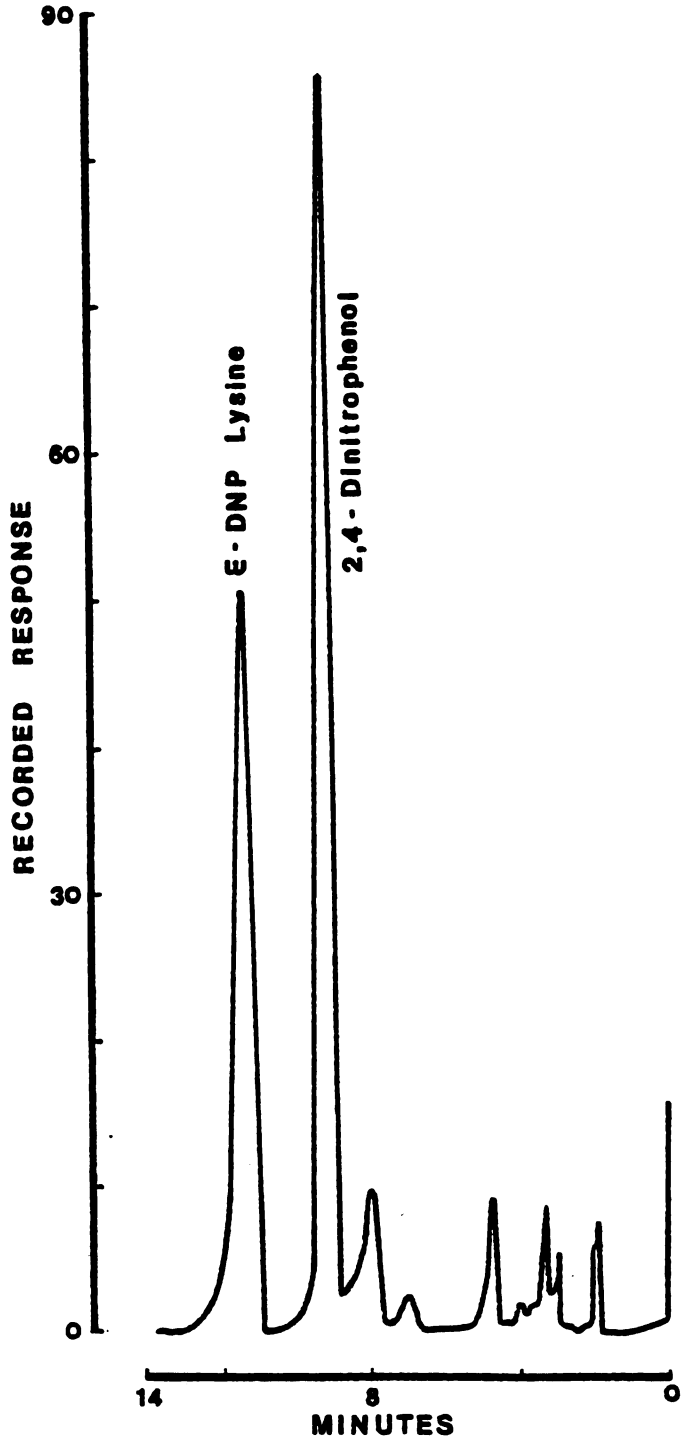

Figure 7. Chromatogram of the products from the reaction with FDNB and Promosoy heated at 100 °C for 2 hours.

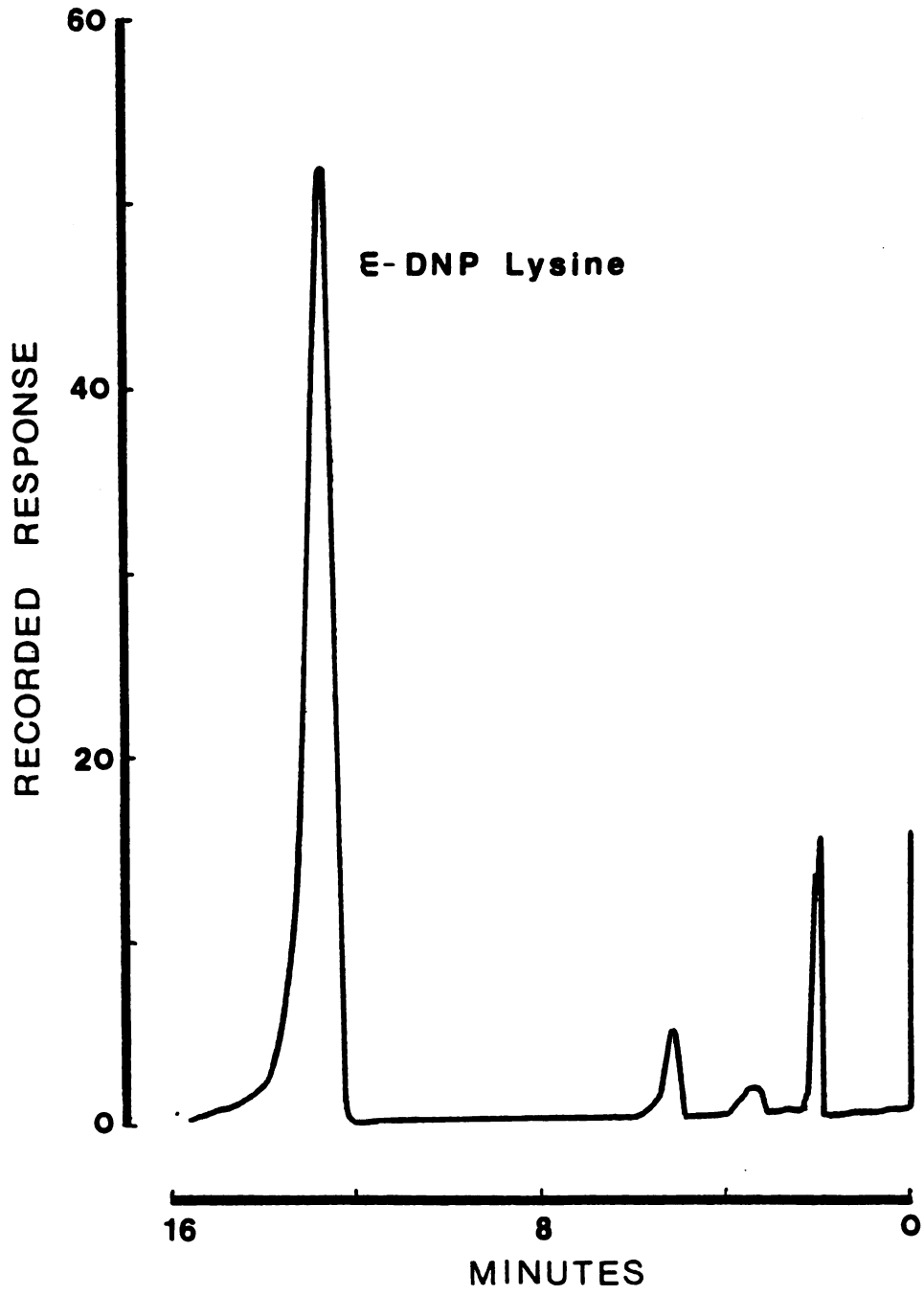

Figure 8. Chromatogram of the DNP-lysine standard.

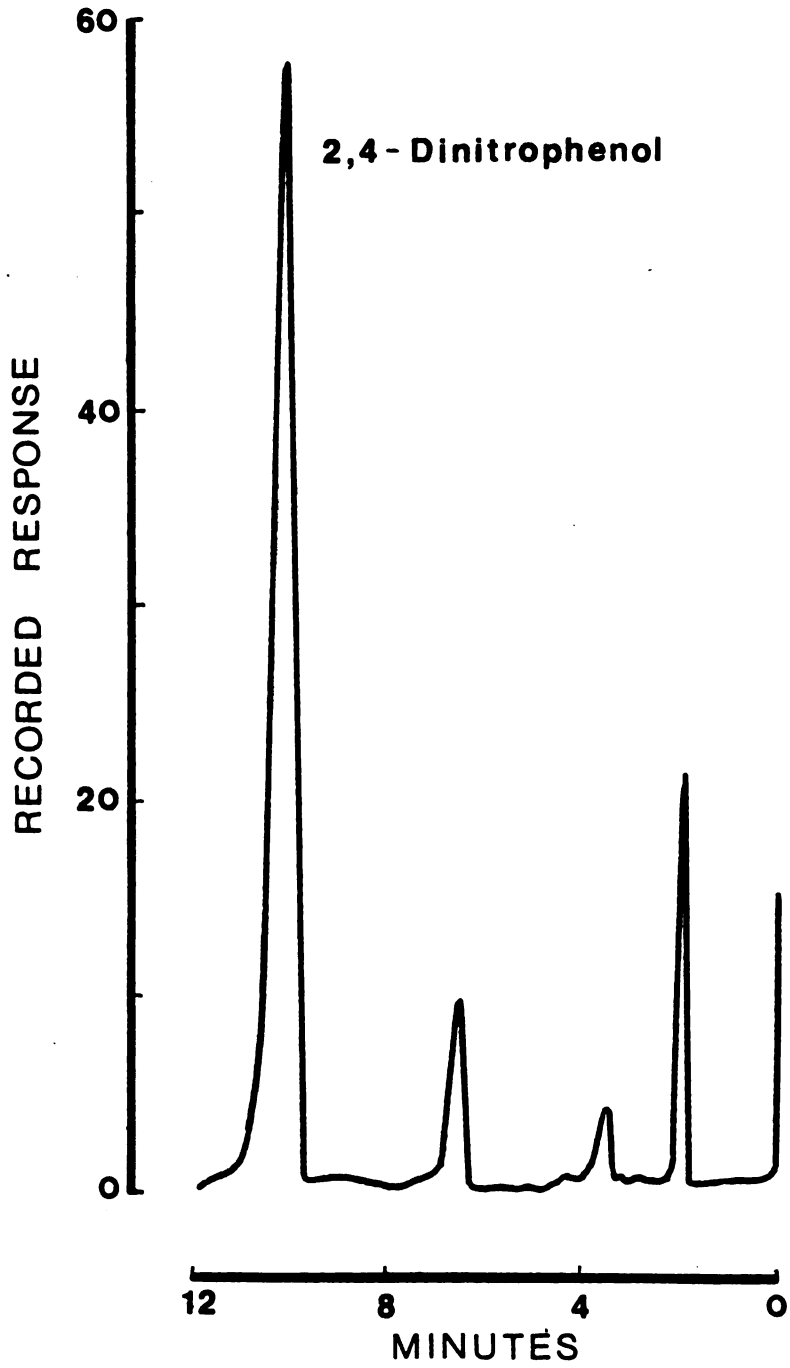

Figure 9. Chromatogram of the 2,4-dinitrophenol standard.

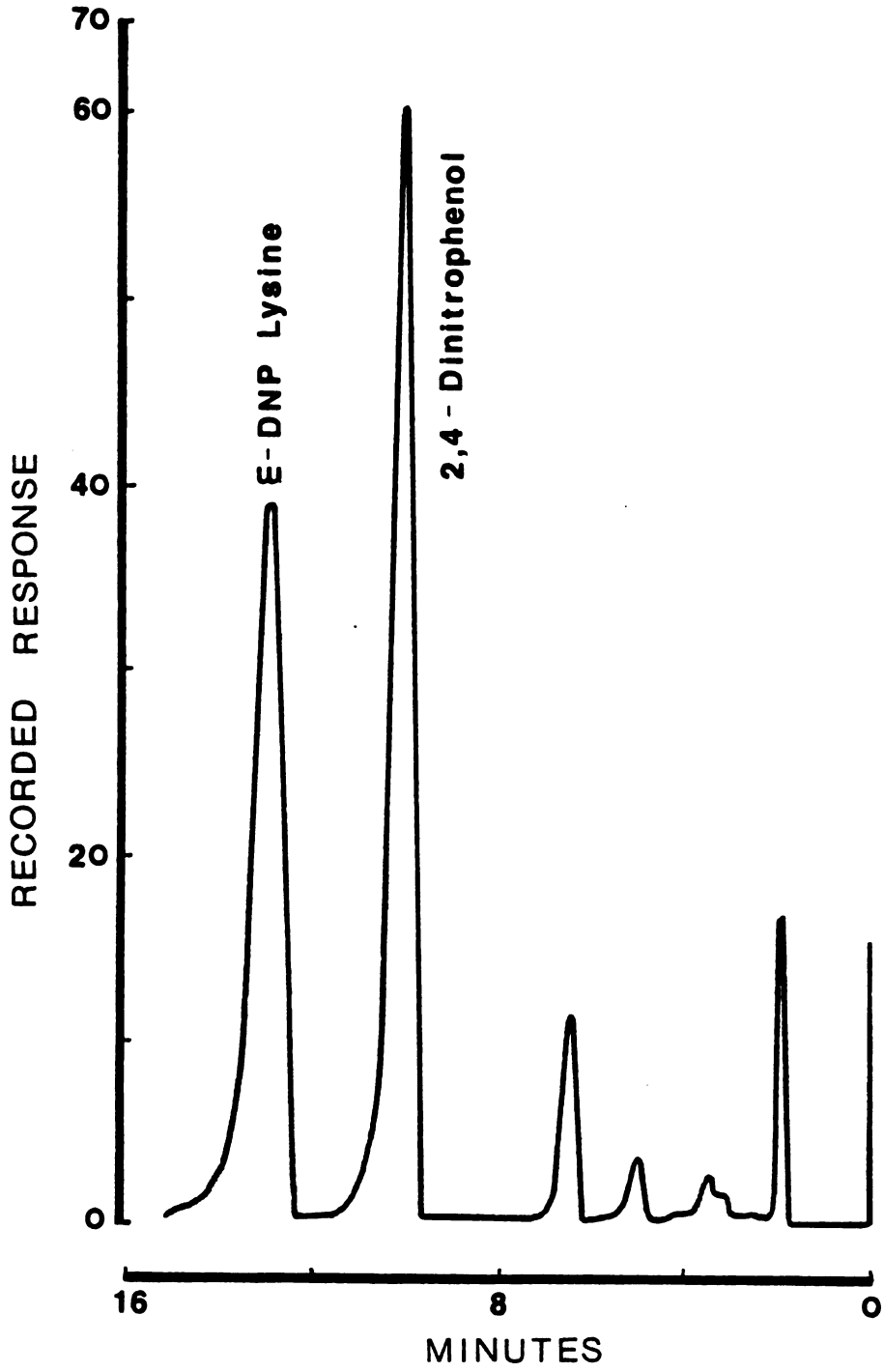

Figure 10. Chromatogram of a standard mixture [1:1] of DNP-lysine and 2,4-dinitrophenol.

dinitrophenol. Figure 7 is a chromatogram of products from one of the reactions and shows how the peaks are separated. Figure 8 shows a chromatogram of DNP-lysine standard used to identify and quantify the DNP-lysine present in the samples and Figure 9 shows a chromatogram of a 2,4-dinitrophenol standard solution. Figure 10 shows a chromatogram of a 1:1 mixture of DNP-lysine standard and 2,4-dinitrophenol standard that emphasizes the resolution capability of the equipment.

From the comparison given in Table 4 it can be observed that there was no finding of the difference reported by Peterson and Warthesen (1979) and attributed to carbohydrates causing overestimation of DNP-lysine in the spectrophotometric method. This could be explained by the fact that lesser amounts of glucose were used here. They worked between 0.5 and 80% cellulose and soy protein while only up to 10% glucose was used in this study. In an attempt to demonstrate even further that the reaction by which the losses of available lysine during a thermal process is mainly due to a Maillard nonenzymatic browning, the results of the fluorescence of the different Promosoy mixtures exposed for 8 hours at 100°C are given in Table 7.

Table 7. Fluorescence of different Promosoy systems

System	Fluorescence
Promosoy unheated	0.055
Promosoy heated at 100°C for 8 hours	4.74
*Promosoy with water heated at 100°C for 8 hours	4.44
**Promosoy with glucose in a dry system heated at 100°C for 8 hours	1.87
***Promosoy with glucose in water heated at 100°C for 8 hours	10.25
Methanol used as blank	0.0002

*System was a slurry of Promosoy (2 g) in 10 g of water.

**System was a mixture of Promosoy (2 g) and 0.1 g of glucose.

***System was a slurry of Promosoy (2 g) and glucose (0.1 g) in 10 g water.

Chio and Tappel (1969) showed that the crosslinking reaction of malonaldehyde with amino acids produces fluorescent chromophores with emission maxima at 460-470 nm when excited at 360-400 nm. The fluorescence of these compounds is due to a simple fluoro-chromo structure, the 1-amino-3-imino propene unsaturated system, R-N=C-C=C-N-R.

Malshet and Tappel (1973) defined the structural requirement for fluorescence in Schiff bases. These requirements are that the Schiff base be in conjugation with a fairly strong electron donating group such as -OH and $-NH_2$.

Our studies on the fluorescence of different Promosoy systems, shown on Table 7, demonstrate the formation of fluorescent compound which is probably a Schiff base. These further confirm that the losses in available lysine that we had been studying may be a product of a non-enzymatic browning reaction.

Although research on the Maillard reaction is in relatively good standing today, continuing efforts to emphasize its basic chemistry and implications with regard to nutritional and physiological effects, as well as food safety and technology, are eminently needed.

From the nutritional point of view, there is a substantial reduction in protein quality due to the non-enzymatic browning reaction during thermal processing and storage. If this can be eliminated or controlled, it would of course result in a substantial nutritional benefit to the consumer of these products.

As has been shown through this work, water is important for the browning reaction to proceed. This agrees with Labuza's generalized statement (1970) on the dependence of various deteriorative effects (lipid oxidation, non-enzymatic browning and growth of microorganisms) on water activity. Non-enzymatic browning increases as humidity increases up to a maximum in the intermediate moisture-range and then decreases again when water activity

approaches 1.

The fluorescence study was made to further prove the role of the non-enzymatic browning reaction in lysine losses in heated soy protein. It is recommended to continue this study by determining the minimal amount of water required for the reaction to proceed and to set the time-temperature-moisture distribution in food systems. As well as the effect of carbonyl groups in the system and how its relationship with the other three parameters affect the reaction.

To conclude, it can be said that the vast majority of the protein we eat has been treated in some way before consumption. This is also true for the protein concentrates and isolates that are used to supplement livestock rations and human foods.

Most processing of food is carried out without a significant degree of damage to the nutritional quality of its protein and in general, cooking increased digestibility. However, excessive heat can reduce the nutritive value of food proteins.

Any reactions occurring during heat treatment which lead to an impaired digestion, absorption or utilization of any essential amino acid like lysine can therefore reduce the nutritive value of the protein which depends on on the physiological availability of its amino acids.

Several types of damage can occur. One can be produced under mild conditions of heating and during prolonged storage. In this case Maillard reactions can occur between ε-amino groups of the lysine in the protein and sugar aldehyde groups. Lysine then becomes unavailable and there is little effect on other amino acids or on the digestibility of the protein (Henry et al., 1948). With increasing severity of heat treatments, damage can occur either in the presence or absence of carbohydrates, there is a fall in the digestibility of the protein as well as in the availability of lysine and of all the amino acids as a whole. However, lysine appears to be the amino acid primarily involved through the formation of cross-linkages within the protein (Carpenter and Booth, 1973).

In the absence of reducing sugars or other carbonyl compounds protein heat damage as measured by loss of ε-amino lysine and loss of nutritional value occurs at a much slower rate (Carpenter and Booth, 1973). However, after really severe or prolonged heating conditions both reactive lysine and nutritional value can be considerably reduced.

In the present work, we tried to study the effects of heat damage and of some treatments on the availability of lysine.

Food manufactures need to know how damage can occur, what reactions can take place during heat processing and storage, what measures can be taken to monitor the effects

of processing and what their nutritional consequences are.

SUMMARY AND CONCLUSIONS

This study was made to study lysine availability in soybean flour, concentrate and isolate after thermal treatment. Also to show the combined effects of carbonyl groups, moisture content and temperature on the availability of this essential amino acid.

During the development of the work, a comparison was made between the results given by a relatively new method of analysis such as High Pressure Liquid Chromatography with those obtained with the well known spectrophotometric analysis. It is concluded that HPLC is useful for the FDNB analysis of available lysine in that it eliminates several clean up steps in the Carpenter method (1960) as modified by Booth (1971). In addition, DNP-lysine is separated from compounds, presumably 2-amino-4-nitrophenol and 4-amino-2-nitrophenol, that could provide interference in measurements of absorbance in the spectrophotometric method.

The HPLC results in general appear to be in good agreement with those obtained by the Carpenter method and can be a useful method for a rapid determination of the lysine content in food.

The kinetic study allows us to establish that the thermal loss of available lysine in a soybean flour system can

be described by a monomolecular reaction. Temperature has a positive effect on reaction rate.

From the study it appears that the losses of available lysine during thermal treatments may be due to a Maillard nonenzymatic browning reaction. The Maillard reaction which occurs in food proteins leads principally to the blockage of lysine, which becomes biologically unavailable.

Finally, the importance of moisture and carbonyl groups in accelerating the nonenzymatic browning responsible in our study for the thermal losses of available lysine was observed.

To conclude, there is just not enough data to propose the right time-temperature-moisture relationship needed to establish a proper program. This program could then be used to control the temperature and moisture content to be used during storage and processing of food systems such as soybean flour, concentrate and isolate in order to minimize the losses of available lysine.

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