DETERMINATION OF L- AND D-METHIONINE AND L- AND D-VALINE IN SOY ISOLATE AND SOY FLOUR SAMPLES

by

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

As world population increases so will the demand for protein. Many sources of vegetable protein are being examined in an attempt to meet this need since they are less costly than proteins of animal origin. Soybean products may provide one of these alternate sources of protein.

Over the past decade use of soy protein has increased in the forms of flours, concentrates and isolates (Wolf and Cowan, 1975). The latter product is obtained from defatted soy flakes or flour and contains a greater amount of protein than the other two products (Smith and Circle, 1972).

During processing of some isolates, defatted meal is extracted in a dilute alkaline medium at near neutral or higher pHs and temperatures which vary among manufacturers (Smith and Circle, 1972). Among other effects, treatment with alkali may cause isomerization of the L-amino acids present in the protein (Lehninger, 1975), converting some of them to D-amino acids. Such effects have been observed in other proteins; for example, β-melanocyte-stimulating hormone (Geschwind and Li, 1964), casein (Levene and Bass, 1928;1929), and fish protein concentrate (Tannenbaum et al., 1970).

Evidence suggests that humans do not utilize some D-amino acids as well as the L-enantiomers (Kies et al., 1975; Zezulka and Calloway, 1976). If soy protein undergoes isomerization during processing of

isolates then the numerous products containing isolate may have impaired nutritional value.

The following experiment was designed to demonstrate the presence or absence of acidic and neutral essential D-amino acids in soy isolate and soy flour. The experimental technique was automated analysis of the L-leucine-N-carboxyanhydride derivatives of amino acids obtained from hydrolyzed soy isolate and soy flour samples.

CHAPTER II

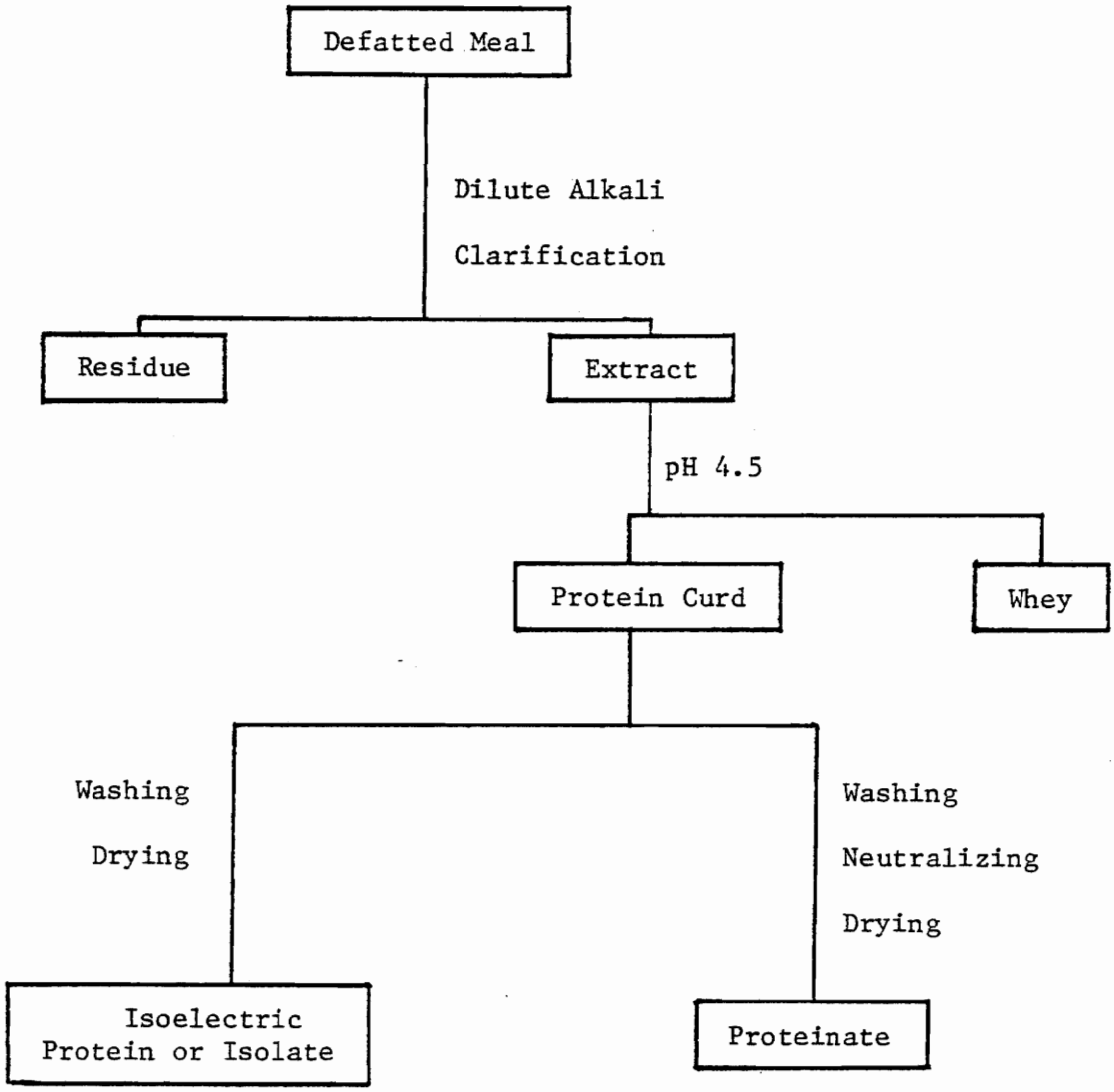
REVIEW OF LITERATURE

A. Production and Use of Soy Isolate

Defatted soybean meal or flour, the starting material for soy isolate, is prepared from cracked, dehulled and flaked soy beans (Wolf and Cowan, 1975). The oil is usually removed from the flakes by solvent extraction techniques (Kellor, 1974). The defatted flakes are subjected to moist heat, cooled (Kellor, 1974) and ground to the particle size appropriate for flours or meals (Horan, 1967).

A general overview of the processes involved in preparing soy protein isolates and proteinates from defatted soybean meal is contained in Figure 1. Soy protein isolates must contain not less than 90% protein on a moisture-free basis (Soybean Digest, 1966). In order to obtain such a concentrated protein product the water-insoluble polysaccharides, water-soluble sugars and residual low molecular weight proteins must be removed (Wolf and Cowan, 1975). This is accomplished by extracting the defatted soy meal with dilute alkali (Wolf and Cowan, 1975). Smith (1958) has observed that 92% of the protein in the defatted meal can be extracted with distilled water at a pH of about 6.6. However, low concentrations of NaCl, $CaCl_2$, or $MgCl_2$ reduce the dispersibility of the protein by almost half. This cationic effect can be overcome by raising the pH of the system thereby facilitating the extraction of soy protein.

The protein containing extract is separated from the residue or fibrous matter by screening, filtering or centrifuging; the pH



(Wolf, 1967)

Figure 1. Commercial isolation of soybean proteins.

of the extract is then adjusted to the isoelectric region (Meyer, 1967). The major globulins precipitate out and the resulting protein curd is separated from the whey by means of a filtering or centrifuging process (Meyer, 1967). The curd is washed and dried to yield the protein isolate (Wolf, 1967). The protein curd may be neutralized before drying thus forming the proteinate, a water dispersible isolated soybean protein (Wolf and Cowan, 1975).

Isolated soy protein (ISP) is incorporated into a number of commercial products for its nutritional and/or functional properties. For example, ISP is used in processed meat products, textured meat analogs, dairy-type foods, fruit-flavored beverages, infant formulas and special dietary foods and beverages (Meyer, 1971). Soy protein isolate may be used to supplement other plant protein foods, such as wheat, in macaroni and corn in corn-soy milk products, to improve their nutritional value (Sipos et al., 1974). Processed meat products such as sausage and coffee whiteners, whipped toppings and frozen desserts contain soy isolate for its ability to function as an emulsifier and an emulsion stabilizing agent (Meyer, 1967).

B. Effects of Processing on the Nutritive Properties of Soy Isolate and Other Proteins

Destruction of Amino Acids

Several authors have observed destruction of amino acids such as cystine (Badenhop and Hackler, 1970), cystine and serine (DeGroot and Slump, 1969; Bohak, 1964) and cystine, arginine, serine, threonine, isoleucine and lysine (Provansal et al., 1975) during alkali treatment

of vegetable proteins. Alteration of amino acid content may affect the nutritional properties of a protein. More specifically, an amino acid deficiency may result due to the inadequate supply of one or more essential amino acids (Harper and Benevenga, 1970). Since methionine is the limiting amino acid in soy protein, and cystine has a sparing effect on methionine (Harper, 1974) then destruction of cystine may exaggerate an amino acid deficiency. Formation of new compounds such as lysinoalanine, which will be discussed later, have also been observed and may be related to a decrease in the amount of cystine, serine and lysine present in vegetable proteins treated with alkali (DeGroot and Slump, 1969; Provansal et al., 1975).

Zinc Availability

Soy isolates have been found to cause deficiency symptoms associated with a decrease in the availability of certain minerals such as calcium, magnesium, manganese, copper, iron and especially zinc (Rackis, 1974). Formation of protein-phytic acid-mineral complexes during processing of isolates may be responsible for the increased requirement for zinc (Rackis, 1974). Lease (1970) speculated that, in soy meal, a carrier complex, composed of amino acids and a sugar moiety, forms a more stable complex with zinc than with phytic acid. During processing of isolates these carriers are either lost or decreased and zinc combines with phytic acid to form a complex. The nutritional quality of soy isolates may be improved by supplementation with zinc, or addition of chelating agents (Rackis, 1974) such as

ethylenediaminetetraacetate (EDTA), which increase the availability of zinc (Kratzer, 1959).

Trypsin Inhibitors

Trypsin inhibitors have also been implicated as affecting the nutritive value of soybeans. The inhibitor forms a complex with trypsin *in vitro* (Kunitz, 1945) and crystalline preparations of the inhibitor have been found to inhibit growth and cause pancreatic hypertrophy in rats (Rackis, 1965). The majority of human trypsin, however, is not inhibited by soybean trypsin inhibitor (Liener, 1977). Liener (1977) found that trypsin inhibitor alone was not sufficient to cause the observed growth inhibition in rats. He suggested that growth inhibition is due to the combined presence of trypsin inhibitor and soy protein in an undenatured and less digestible state. The presence of undigested protein and trypsin inhibitor in the intestinal tract causes the trypsin level to drop, a situation which causes pancreatic hypertrophy by stimulating the pancreas to produce more enzyme (Liener, 1977). Loss of endogenous protein as a result of pancreatic hypersecretion may be responsible for growth depression in rats (Liener, 1977).

Treatment with moist heat is usually sufficient to destroy trypsin inhibitor activity (TIA) (Rackis, 1965) and increase digestibility (Liener, 1977). Cogan et al. (1968) found that isoelectric protein made from unheated soybean meal had greater antitryptic activity than that made from toasted meal. In addition, Badenhop and Hackler (1973) observed decreased TIA in unheated soy milk obtained from

beans soaked in alkaline solutions of increasing pH. In this respect, exposing soybeans to alkaline conditions is a positive processing effect (Liener, 1977).

Lysinoalanine

New amino acid derivatives such as lysinoalanine (LAL) may be formed during alkaline extraction of soy protein (DeGroot and Slump, 1969). DeGroot and Slump (1969) observed formation of LAL accompanied by a decrease in lysine content and lowered net protein utilization (NPU) (Appendix 1) values in casein, soybean oil meal and animal protein concentrate treated at pH 12.0. These effects were accentuated by increasing time of exposure to alkali and heat. Bohak (1964) observed formation of LAL in papain, ribonuclease A, lysozyme and chymotrypsinogen treated at pH 13.1 and 40^{o}C for two hours. He postulated that LAL is probably formed by condensation of the α-amino group of lysine with a dehydroalanyl residue derived from cystine or serine through β-elimination. Woodward and Short (1973) detected cytomegalic alterations in the proximal tubule of the kidney in rats fed Alpha-Protein, an industrial grade isolated soy protein modified by alkaline hydrolysis, and soy protein subjected to alkali treatment in 0.1 N NaOH at 60^{o}C for eight hours. They related the condition to the appearance of lysinoalanine in amino acid analyses.

Lysinoalanine has been found in food ingredients, commercial food products and home-cooked foods some of which were alkali-treated (Sternberg et al., 1975). Sternberg et al. (1975) also found lysinoalanine in heated proteins not subjected to alkali treatment.

They suggested that heating may be responsible for lysinoalanine formation and reduced nutritional value of these foods as well as soy isolate.

L to D Isomerization of Amino Acids in Isolated Soy Protein

Observed changes in the nutritive value of isolated soy protein could be due to other factors. For example, isomerization has been implicated as contributing to decreased availability of amino acids in isolated soy protein (DeGroot and Slump, 1969).

Wesson (1975) extracted soy flour in aqueous and alkaline media of pHs 6.7, 9.0 and 12.0 at 60°C for increasing periods of time. Optical rotation measurements indicated that racemization of soy protein may occur at alkaline pHs of 9.0 and 12.0. Increased time of exposure enhanced the effect of racemization during alkali-treatment.

Isomerization of amino acids has been observed in other proteins. Geschwind and Li (1965) exposed β-melanocyte-stimulating hormone to 0.1 N NaOH for 15 minutes in a boiling water bath. Using a microbiological assay with organisms utilizing only the L-forms of amino acids, they observed that the amount of arginine, histidine, phenylalanine and methionine was significantly lowered after treatment. They attributed these findings to the isomerization of these amino acids in the hormone.

In early studies concerning the composition of protein molecules, Levene and Bass (1928) treated casein with dilute (0.1 N) and concentrated (5.0 N) NaOH at 25°C for 0-15 days. Based on optical rotation measurements they observed that the extent of isomerization increased

with lengthened exposure time and increased concentration of alkali. In a later study (1929), albumin, edestin and fibrin were tested in addition to casein. The proteins were exposed to 0.2 N, 1.0 N and 5.0 N NaOH for 1-5 days. Again, they observed that the percent isomerization of each protein increased with increasing exposure time and alkali concentration.

Pollock and Frommhagen (1968) exposed egg albumin, gelatin and soil humic and fulvic acids (polyphenolic polymers containing amino acids) to 0.5 N NaOH for 24, 48 and 96 hours at 25°C. In both the protein and the soil samples, alanine, aspartic acid, phenylalanine, glutamic acid and lysine exhibited the greatest percentages of D-enantiomer present as determined by gas chromatographic analyses. This was observed after 24 hours for the humic and fulvic acids and gelatin, and after 48 hours for the egg albumin. Complete racemization was not observed in any of the samples.

Heating is sometimes used in the preparation of soy isolates and may decompose a number of amino acids. Fujimaki et al. (1972) observed destruction of tryptophan, sulfur-containing amino acids and basic amino acids in roasted casein and lysozyme. Later studies by Hayase et al. (1973 and 1975) showed evidence of isomerization of glutamic acid, alanine, lysine and aspartic acid in the proteins during roasting.

C. Utilization of D- and DL-amino Acids

Berg (1959) states that since no proteins from animal tissue have been found to contain D-amino acids, dietary D-amino acids must be converted, by a process called inversion, to the L-form for use in

tissue synthesis and maintenance. The D-amino acid undergoes oxidative deamination to form the corresponding α-keto acid in the presence of D-amino acid oxidase (Winitz et al., 1960). This enzyme oxidizes a large variety of D-α-amino acids except D-glutamic acid (Burton, 1955). The α-keto acid formed in the oxidative deamination reaction then acts as an amino group acceptor in the presence of an amino group donor (another amino acid) and the appropriate amino-transferase (Lehninger, 1975). Kamath and Berg (1964) suggested that oxidative deamination is the major limiting step in D-amino acid utilization. Berg (1959) classified the D-isomers of the essential amino acids based on invertibility or the ability of the α-keto acid to successfully replace its D-analog in diets fed to rats; D-methionine and D-tryptophan were classified as readily invertible, D-histidine, D-phenylalanine, D-arginine, D-valine and D-leucine as moderately invertible and D-threonine, D-isoleucine and D-lysine as poorly invertible. In addition, decreased gastrointestinal absorption (Matthews and Smith, 1954) and cellular uptake (Christensen et al., 1952) may also affect the degree of D-amino acid utilization.

DeGroot and Slump (1969) recorded lowered net protein utilization values in rats fed isolated soy protein (ISP) extracted at pH's increasing from 7 to 12.2. The effect was accentuated by increasing exposure time and temperature. They also observed that the isoleucine, leucine, lysine, tyrosine, phenylalanine, methionine, threonine and histidine in the alkali-treated ISP were less susceptible to in vitro pepsin-pancreatin enzymatic release than the untreated ISP. This may be due to the stereospecificity of the pepsin, i.e. its inability to

recognize a D-amino acid substrate (Cuthbertson and Tilstone, 1972). However, several pepsins exist and each has a different activity in the presence of various substrates (Cuthbertson and Tilstone, 1972). The type of pepsin used in the study by DeGroot and Slump (1969) was not specified. Therefore, the pepsin may have been able to discriminate between D- and L-amino acids present in the ISP (Lehninger, 1975). The reduced enzymatic release would then have to be attributed to some other cause. When the alkali-treated isolate was supplemented with methionine, and a combination of lysine and methionine, NPU improved considerably. Addition of threonine caused further improvement. According to amino acid analyses before and after treatment, threonine content remained the same. The authors suggest that decreased utilization of threonine may have been caused by racemization during treatment with alkali.

In a series of studies reported in 1954 and 1955, Rose and his co-workers dealt with the quantitative determination of the requirements for eight essential amino acids in adult men. The nitrogen balance technique was used to determine the extent to which D, L or racemic mixtures of amino acids were utilized. The composition of the amino acid mixtures consumed in the studies included nine of the amino acids plus two additional sources of nitrogen. At least four of the nine amino acids were provided in the DL-form in amounts at least double the requirement of the L-amino acid. Each diet contained approximately 10.0 g of nitrogen per day.

Two subjects were involved in the tryptophan requirement study involving L- and DL-tryptophan (Rose et al., 1954). Both subjects

maintained positive nitrogen balance with 0.15 g L-tryptophan and a negative balance with 0.15 g DL-tryptophan. The experimenters concluded that DL-tryptophan is not as effective as L-tryptophan and that humans utilize little if any of the D-isomer. In contrast, rat studies demonstrated that D-tryptophan is almost equal to L-tryptophan in promoting growth (Oesterling and Rose, 1952).

In determination of the phenylalanine requirement, Rose et al. (1955d) found that 2.40 g of DL-phenylalanine or 1.75 g of L-phenylalanine maintained 28 subjects in positive nitrogen balance. Four individuals consuming D-phenylalanine in amounts equal to or greater than their minimum requirement for L-phenylalanine exhibited negative nitrogen balances.

The requirement for threonine was established after feeding both the D- and L-amino acid as well as a racemic mixture (Rose et al., 1955b). Positive nitrogen balances were obtained from the two subjects consuming 0.6 g L-threonine and 1.2 g DL-threonine. Negative balances comparable to those obtained for diets without threonine were observed in the subjects consuming D-threonine at the same level as L-threonine. Rose et al. (1955b) concluded that if any D-threonine is utilized by man it is too small to be detected by the nitrogen balance technique.

In studies dealing with the methionine requirement, Rose et al. (1955b) found that approximately as much D-methionine (1.0 g) as DL-methionine (0.9 g) gave positive nitrogen balance in the two subjects involved in the experiment. Therefore, it was proposed that D-methionine is as effective as DL-methionine in maintaining nitrogen

balance. Although L-methionine was not tested, the experimenters concluded that the requirement would be identical to DL-methionine.

Other studies report evidence to the contrary about methionine. Kies et al. (1975) supplemented "instant" oatmeal-based diets low in methionine with D, L, and DL-methionine at two levels (0.58 g/day and 1.16 g/day). The control diet contained no supplement. Subjects received 4.0 g of nitrogen per day from the oat diet. The mean nitrogen balance in subjects consuming the higher level of L-methionine was slightly positive while the subjects consuming the higher level of D-methionine had a mean negative balance comparable to the unsupplemented diet. DL-methionine supplementation at the higher level produced a negative balance approximately the same as that obtained with the lower level of L-methionine. Zezulka and Calloway (1976) studied the ability of D-methionine, N-acetyl-L-methionine and Na_2SO_4 as sulfur containing compounds to replace L-methionine in soy protein diets. They found only one man in positive nitrogen balance and five in negative or borderline balance in a D-methionine supplemented isolated soy protein diet. Subjects consuming the isolate plus L-methionine and N-acetyl-L-methionine containing diets exhibited positive nitrogen balances. The diet containing inorganic sulfate also produced negative or borderline nitrogen balances.

Two subjects were involved in the determination of the leucine requirement by Rose et al. (1955c). The D-isomer of leucine was fed at twice the level of minimal L-leucine requirements. A negative nitrogen balance was observed in both subjects and the experimenters concluded that the D-isomer was little used by man.

In feeding studies with isoleucine (Rose et al., 1955c), the D, L, and DL-forms of the amino acid were administered to determine the human requirement. DL-isoleucine, fed at twice the level of L-isoleucine known to be a minimum requirement for positive nitrogen balance, also induced positive balance in the two subjects consuming the diet. D-isoleucine administered at four times the level of L-isoleucine produced negative balance.

Two individuals ingesting D-lysine in amounts greater than or equal to their L-lysine requirements developed a negative nitrogen balance similar to that observed when the diet was devoid of lysine (Rose et al., 1955a). On the basis of these observations it was proposed that little, if any, if the D-isomer can be utilized by human men.

The results of the valine requirement study were quite similar to those involving both isoleucine and threonine. DL-valine administered at twice the level of L-valine, gave a positive nitrogen balance while D-valine given in the same amount as DL-valine induced a negative nitrogen balance (Rose et al., 1955e).

In rat studies, the D-isomers of essential amino acids were found to vary widely in their ability to promote growth when fed in place of the L-isomers (Berg, 1959). This may be due to the ability of D-amino acid oxidase to attack some D-amino acids more readily than others (Kamath and Berg, 1964). Kamath and Berg (1964) noticed a slight decrease in growth response in rats fed a diet containing one poorly invertible DL-amino acid and one readily invertible amino acid. The effect on growth was more pronounced after inclusion of all the

poorly invertible DL-amino acids in the diet. It appeared that the poorly invertible DL-amino acids collectively retard growth, possibly due to competition for the enzyme D-amino acid oxidase (Kamath and Berg, 1964).

D. Methods of Determining D- and L-Amino Acids

Three methods for determination of D-amino acids were taken into consideration: a gas chromatographic procedure, an ion-exchange procedure and an enzyme technique. The first, a gas chromatographic procedure, involved separation of N-trifluoroacetic (TFA) D and L amino acid isopropyl esters on N-TFA-L-valyl-L-valine cyclohexyl ester, an optically active stationary phase (Nakaparksin et al., 1970). This method was used by Hayase et al. (1973) in detecting racemization in roasted lysozyme and casein. One of the limitations of this and other derivatization procedures is that not all of the D and L amino acid present is converted to the derivative and extensive work is needed to determine the total amount of D and L amino acid present in a sample.

A second method was based on the separation of diastereomeric dipeptides by ion-exchange chromatography (Manning, 1972). Each DL-amino acid is reacted with an L-amino acid-N-carboxyanhydride in the cold at pH 10.4 for 2 minutes to form an L-L and an L-D dipeptide (Figure 2). The dipeptides are resolved on an amino acid analyzer by using a sodium citrate buffer of specific pH. In automated amino acid analysis, amino acids or dipeptides, are combined with ninhydrin and heated to give colored reaction products which are automatically recorded as a series of peaks corresponding to each dipeptide , the

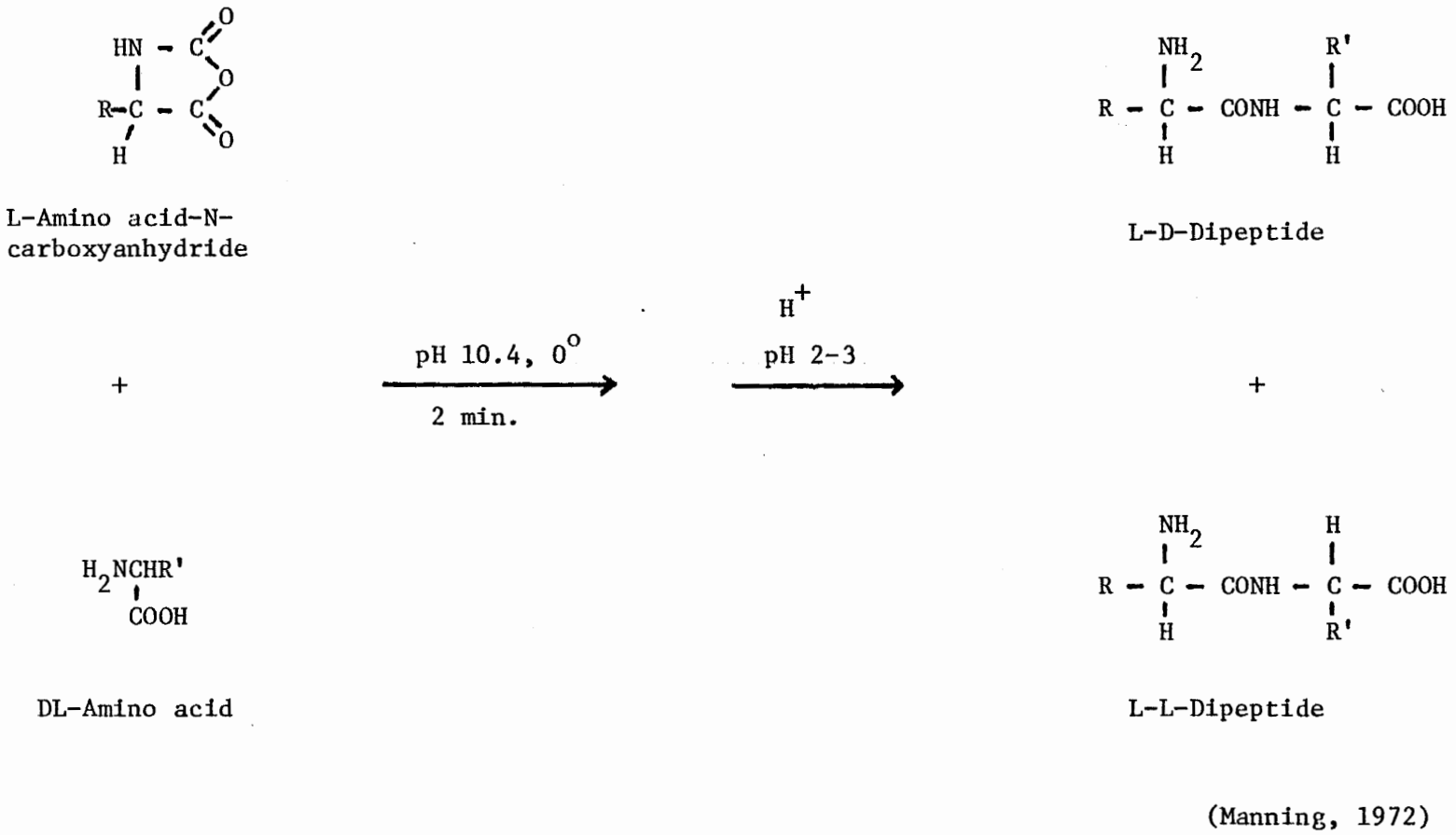


Figure 2. Formation of diastereomeric dipeptides.

unreacted derivatizing agent and the unreacted DL-amino acid. The same limitation as that encountered in derivatizing samples for the GC method applies.

A third method, specified by Hare (1969) in the determination of optical configuration of amino acids in fossils, is an enzyme technique involving the use of L-amino acid oxidase. This method would also involve use of the amino acid analyzer. L-amino acid oxidase from snake venom catalyzes the oxidation of a large number of L-amino acids, some more slowly than others (Wellner, 1971). The same enzyme obtained from rat kidney does not oxidize glycine, aspartic acid, glutamic acid, lysine, threonine or serine (Nakano and Danowski, 1971). The group which is not oxidized by the rat kidney enzyme is the same group which is slowly oxidized by the snake venom enzyme. Using this method (Hare, 1969), a sample containing a mixture of DL-amino acids would be run on the amino acid analyzer. Another sample having the same DL-amino acid content would have the enzyme plus catalase (to remove excess H_2O_2) added to it in a buffer of optimum pH. The amount of amino acid present in the sample-enzyme preparation would be substracted from the amount in the initial sample. The difference should demonstrate how much of each L-amino acid was present before oxidation. This could mean that the calculated L-amino acid content may not be representative of that contained in the sample if some of the amino acids are not completely oxidized.

Based on the equipment available, both the enzyme and ion-exchange chromatography methods were found to be acceptable. Primary

consideration was given to the ion exchange method while the enzyme technique was viewed as an alternative method.

CHAPTER III

OBJECTIVE

D-amino acids may be poorly utilized by humans (Kies et al., 1975; Zezulka and Calloway, 1976). Evidence of L to D isomerization in alkali-treated proteins has been observed (Geschwind and Li, 1965; Pollock and Frommhagen, 1968). The effects of alkali on commercially available food-grade soy protein products have not been reported. Wesson (1975) obtained indirect evidence of isomerization of amino acids in soy flour subjected to simulated commercial processing conditions by measuring optical rotation changes. This study was an attempt to extend Wesson's work by examining commercially available soy products and by using a more direct method of measurement.

The objective of this study was to determine qualitatively, and if possible quantitatively, L to D isomerization of amino acids in alkali-treated soy isolate by direct measurement of the proportions of dipeptide derivatives of the D- and L-amino acids obtained from hydrolyzed soy isolate samples. The dipeptide derivatives obtained from soy flour hydrolyzates were compared to those obtained from the soy isolate in an attempt to correct for L to D isomerization of amino acids which may have occurred during hydrolysis.

CHAPTER IV

MATERIALS AND METHODS

A. Equipment

A Technicon TSM Amino Acid Analyzer was used in separation of the diastereomeric dipeptides, amino acid fraction collection, and identification of the amino acids in the fractions. A Technicon NC-1P Amino Acid Analyzer was used to detect the presence of alloisoleucine in hydrolyzed soy isolate samples. Buffer pH and the pH of amino acid solutions used in the derivatizing procedure were measured on a Corning Digital 112 Research pH meter. A Mettler H6T analytical balance was used for weighing soy flour and isolate samples, amino acids and derivatizing agent while Mettler P163 and P1000 top loading balances were used for weighing buffer, 1% ninhydrin, 2mM hydrazine sulfate and 0.20 N NaOH solution components. The automatic fraction collector employed was Model 1205 made by Research Specialities Co. Eppendorf automatic pipets (20 ul, 50 ul, 100 ul and 300 ul) and an adjustable (0.01-9.99 ml) Gilson automatic pipet were used in the experiment.

B. Materials

L-leucine-N-carboxyanhydride was obtained from Vega Fox Biochemicals, Tucson, Arizona. Defatted soy flour and isolated soybean protein (tradenames Soyafluff 200 W and Promine D, respectively) were provided by Central Soya, Chicago, Illinois. DL-mixtures of the acidic and neutral essential amino acids (DL-methionine, DL-valine, DL-threonine and DL-leucine, assumed to be racemic, had previously

been obtained from the Sigma Chemical Co., St. Louis, Missouri. Other amino acids used in the experiment were L-isoleucine with D-alloisoleucine, D-threonine, D-valine, D-methionine, D-leucine, D-isoleucine, L-valine and L-leucine from Sigma Chemical Co., L-methionine, L-isoleucine and L-valine from ICN Pharmaceuticals Inc., and L-threonine from Nutritional Biochemicals Corp. Other chemicals used in amino acid analysis were 'Baker Analyzed' Reagents except for Piersolve, ninhydrin, thiodiglycol and Brij-35, which were specially purified by the Pierce Chemical Company, hydrazine sulfate, also made by Pierce, and (ethylenedinitrilo) tetraacetic acid disodium salt prepared by Eastman Kodak Company.

The pH 3.25, 4.25 and 5.25 buffers, cartridge equilibration buffer, 1% ninhydrin, 2mM hydrazine sulfate, 50% methyl cellosolve and 0.20 N NaOH solutions used in the amino acid analyzer for fraction collection and identification of amino acids contained in the fractions were prepared according to the directions in the TSM Operating Manual (Technicon Instruments Corp., Tarrytown, New York). The pH 6.5 buffer was prepared according to the instructions in the NC-1P Operating Manual (Technicon Instruments Corp., Tarrytown, New York). Borate buffer (0.45 M), used in the derivatization procedure, was prepared by dissolving 13.9 g of boric acid in 497 ml of freshly boiled distilled water and adding approximately 3.0 ml of 50% NaOH to bring the pH to 10.2 at 25^{o}C. The pH 4.0 buffer used to separate alloisoleucine and isoleucine was obtained by lowering the pH of the 4.25 buffer with 6 N HCl.

The buffers used in attempts to separate the diasteromeric dipeptides were pH 4.25 or 5.25 buffers, prepared as described above, or were derived from the pH 5.25 or pH 6.5 buffer. Sodium citrate buffers of pH's 4.5, 4.75 and 5.0 were obtained from the pH 5.25 buffer by lowering to the appropriate pH with 6 N HCl. The pH 5.45, 5.75, 6.0 and 6.25 buffers were obtained by adding 6 N HCl to the pH 6.5 buffer until the desired pH was reached.

C. Experimental Procedure

The experiment was carried out in several stages: determination of alloisoleucine in soy isolate; determination of dipeptide separation conditions; identification of peaks corresponding to the dipeptides, underivatized amino acid and unreacted derivatizing agent; preparation of soy flour and soy isolate hydrolyzates; and standard and sample collection and analysis.

Determination of Alloisoleucine in Soy Isolate

Formation of D-alloisoleucine from L-isoleucine is accelerated in alkaline solutions (Hare, 1969). Isoleucine contains two assymetric carbon atoms and has four stereoisomers: D-alloisoleucine, L-alloisoleucine, D-isoleucine and L-isoleucine. L-isoleucine isomerizes more easily to D-alloisoleucine than to D-isoleucine because the second assymetric carbon atom does not easily isomerize (Hare, 1969). Therefore, investigation into the presence of alloisoleucine in alkali-treated soy isolate was employed to yield evidence of possible isomerization of other amino acids.

D-alloisoleucine was eluted on the NC-1P Amino Acid Analyzer between methionine and isoleucine by using a pH 4.00 buffer (Marable, 1977). In order to determine the separation conditions of the methionine, isoleucine, and alloisoleucine, standard solutions of DL-methionine and L-isoleucine with D-alloisoleucine were prepared. To obtain a concentration of approximately 250 nmoles per ml of methionine and 125 nmoles per ml of L-isoleucine and D-alloisoleucine, 0.025 g of methionine and 0.025 g of a 1:1 mixture of L-isoleucine: D-alloisoleucine were dissolved in 100 ml of cartridge equilibration buffer in two separate volumetric flasks. Aliquots of 5 ml were taken from each flask and transferred to two 50 ml volumetric flasks. Each flask was brought to volume. The final concentrations were 0.025 mg/ml or 0.0125 mg/ml, respectively. Aliquots (20 ml) of these solutions were reserved in 125 x 15 mm screw cap test tubes and stored in the refrigerator. Aliquots of 300 ul were taken from each of the DL-methionine and L-isoleucine with D-alloisoleucine mixtures and placed in a 70 x 10 mm test tube. The solution was agitated and a 200 ul aliquot taken for analysis.

Next, a 200 ul aliquot of hydrolyzed soy isolate (500 ug protein per ml) was run on the NC-1P Amino Acid Analyzer using a normal buffer program (Appendix 2) with the pH 4.00 buffer replacing the pH 4.25 buffer. The alloisoleucine was identified by comparing the retention time of the peak found in the sample to the standard. Retention time was determined by marking the point of injection on the recorder tracing from the analyzer and measuring the length of

time required for the amino acid to reach the colorimeter and the maximum peak height to be recorded.

Basic Method for Derivatizing D and L Amino Acids

The method of Manning (1972) was followed for determination of essential D-and L-amino acids by ion-exchange chromatography. The method described the conversion of amino acid enantiomers into diastereomeric dipeptides. The derivatizing agent, L-leucine-N-carboxyanhydride, was kept stored in the freezer in a jar containing Drierite. The jar was removed from the freezer and allowed to come to room temperature before opening. For standard solutions, 20 umoles of each L-or D-amino acid or 20 umoles of a DL-amino acid mixture were weighed out into a 150 x 25 mm screw cap test tube, dissolved in 2 ml of borate buffer and placed in an ice bath. While the solution cooled, L-leucine-N-carboxyanhydride, the derivatizing agent used with neutral and acidic amino acids, was weighed into a 40 x 10 mm test tube and stoppered with a cork. L-leucine-N-carboxyanhydride must be maintained under dry conditions to avoid degradation. For this reason, the test tubes were stoppered immediately after weighing. A 20% molar excess of the derivatizing agent was used based on the number of moles of amino acid to be derivatized. A Vortex-Genie mixer, stop watch, test tube plus ice bath, weighed amount of derivatizing agent, bottle of 1 M HCl and an adjustable automatic pipet set at 0.8 ml were brought into a walk-in freezer at $0^{o}C$. Each tube was mixed for a few seconds and the derivatizing agent dumped into the swirling solution. The solution was mixed for two minutes with two second stopping intervals

every thirty seconds. The derivatization reaction was stopped by acidifying the solution to pH 1-3 with 1 M HCl. The pH of the reaction mixture was tested with pH paper. Final concentration of the solutions used as standards was 20 umoles of amino acid in the derivatized and underivatized state per 2.8 ml. The solution was kept frozen until needed for analysis. Each derivatized amino acid was chromatographed on a Technicon TSM Amino Acid Analyzer using a buffer of specific pH to determine elution characteristics. Manning's method recommended diluting the derivatized mixture by half or more using a pH 2.2 citrate buffer. By eliminating the dilution step, the concentration of the derivatized mixture could be increased and a smaller aliquot taken for analysis.

The method of Manning (1972) specified use of an automatic amino acid analyzer equipped with a 0.9 x 62 cm column packed with Beckmann-Spinco AA-15 resin. In addition, the recommended column temperature and flow rate are 52^{o}C and 50 ml per hour, respectively. Separation of the dipeptides under the above mentioned conditions required 0.20 N sodium citrate buffers of pHs 3.25, 4.25 and 5.42.

The amino acid analyzer available was a Technicon TSM model equipped with a column less than half the specified length (0.7 x 27.5 cm) and packed with Chromobeads, Type C-3 resin. Normal operating conditions of the TSM are 0.5 ml per minute flow rate and 60^{o}C column temperature. Since these conditions vary from those specified in the Manning (1972) method, it was necessary to experiment with the column temperature, flow rate and buffer pH to obtain good separation of the dipeptides.

Determination of Conditions for Separation of D-L and L-L Dipeptides

Samples of DL-methionine, DL-leucine, DL-threonine, DL-valine containing 20 umoles each, and L-isoleucine with D-alloisoleucine containing 10 umoles of each stereoisomer, were derivatized according to the method of Manning (1972). A 20 ul aliquot of the derivatized mixture provided adequate sample for good separation and peak measurement of the dipeptides on the TSM Amino Acid Analyzer.

Attempts were made to separate the dipeptides using the buffers specified in the Manning (1972) method with poor results. Buffers of higher pH were experimented with until good separation was obtained. The pHs of the recommended buffers, experimental buffers and the actual buffers used for separation of the various dipeptides are contained in Table 1. Both column temperature and flow rate were altered in the separation of the valine dipeptides at several pHs. The column temperature variations were 50^{o}C and 55^{o}C while the flow rate was changed to 0.4 ml/minute and 0.33 ml/minute. Column temperature and flow rate played such a minor role both separately and together in changing retention times and improving resolution, that buffer pH was chosen as the principal means of improving separation. Retention time was determined for each observable peak.

Peak Identification

When adequate separation of the dipeptides was obtained the next step was identification of the observable peaks. More specifically, it

Table 1

Recommended, Experimental and Actual Buffer pH's Used in Separation of Amino Acid Dipeptides

Amino Acid Dipeptides	Buffer pH		
	Recommended (Manning, 1972)	Experimental	Actual[a]
L-leucine-L-methionine	4.25	4.25	5.00
L-leucine-D-methionine		4.50	
		4.75	
		5.00	
		5.25	
L-leucine-L-valine	4.25	4.25	5.00
L-leucine-D-valine		4.50	
		4.75	
		5.00	
		5.25	
L-leucine-L-leucine	5.42	6.25	5.45
L-leucine-D-leucine		6.00	
		5.75	
		5.45	
L-leucine-L-isoleucine	5.42	6.25	5.45
L-leucine-D-alloisoleucine		6.00	
		5.75	
		5.45	
L-leucine-L-threonine	3.25	4.00	5.00
L-leucine-D-threonine		4.23	
		3.28	
		5.00	
		5.25	

[a] Column temperature and flow rate were 60°C and 0.5 ml/minute, respectively.

was necessary to determine which peaks represented the L-D and L-L dipeptides, the unreacted derivatizing agent and the underivatized amino acid.

The D- and L-forms of methionine, valine, isoleucine, leucine, and threonine were derivatized separately (Manning, 1972), using a 20 umole sample of each, and analyzed at the appropriate pH (Table 1) in order to determine the identity of each dipeptide peak based on retention time. Solutions of DL-methionine, L-isoleucine with D-alloisoleucine, DL-leucine, DL-threonine and DL-valine were prepared in concentrations of 25 nmoles/50 ul and analyzed to determine the retention times for the underivatized amino acid and the unreacted derivatizing agent.

Retention times are expressed in Table 2. Although the retention times for the amino acid, its derivatives, and the unreacted derivatizing agent varied slightly with the daily operating conditions of the amino acid analyzer, the derivatives and the unreacted reagents were eluted in the same order.

Derivatization of Methionine Dried in Buffer

One umole of DL-methionine was dissolved in 20 ml of pH 3.25 buffer for drying in a vacuum dessicator. It was anticipated that this concentration of methionine would result from the collection of twenty 100 ul aliquots of soy flour or soy isolate hydrolyzate. Calculations were based on the following information. The flow rate of the amino acid analyzer was 0.5 ml/minute. The timing device on the fraction collector was set for 1 tube/30 seconds, i.e. it

Table 2

Relative Retention Times of the Dipeptide Derivatives, the Unreacted Derivatizing Agent and Unreacted Amino Acids[a]

Amino Acid or Dipeptide	Retention Time (minutes)
DL-leucine	17.7
L-leucine-L-valine	19.7
L-leucine-D-valine	23.0
DL-valine	16.3
DL-leucine	17.7
L-leucine-L-threonine	16.5
L-leucine-D-threonine	19.1
DL-threonine	15.2
DL-leucine	17.3[b]
L-leucine-D-alloisoleucine	24.8
L-leucine-L-isoleucine	21.6
L-isoleucine with D-alloisoleucine	17.3
DL-leucine	17.7[b]
L-leucine-L-leucine	22.0
L-leucine-D-leucine	25.2
DL-leucine	18.1[b]
L-leucine-L-methionine	22.6
L-leucine-D-methionine	26.6
DL-methionine	18.1

[a] Retention time is determined by marking the point of injection on the recorder tracing from the analyzer and measuring the length of time required for an amino acid or dipeptide to reach the colorimeter (570 nm) and the maximum peak height be recorded.

[b] Retention times vary with the daily operating conditions of the amino acid analyzer.

[c] See Table 1 for operating conditions for the amino acid analyzer.

collected 0.25 ml/tube. Based on calculations from a sample tracing methionine was eluted from the bottom of the column over a period of two minutes. This corresponded to four fraction collection tubes. Drying this sample of methionine in buffer was designed to simulate the actual dried sample which would result from collection of fractions from the soy flour and soy isolate hydrolyzates. The amount of methionine dried in buffer and derivatizing agent used are expressed in Table 3. A 100 ul aliquot of the derivatized mixture, containing approximately 17 nmoles of methionine in the derivatized and underivatized form, was applied to the column and produced two rather small but not immeasurable dipeptide peaks. It appeared that calculation of peak area could be made if the tracing were electronically amplified. Although amplification was not used for this particular sample it was employed for the methionine in the hydrolyzate samples.

Preparation of Soy Flour and Soy Isolate Hydrolyzates

Promine D (approximately 0.05 g) and Soyafluff 200 W (approximately 0.10 g) samples were weighed into 20 ml break-seal ampules. Soy Flour samples 6, 9 and 10 and Soy Isolate samples 4 and 13 were the only hydrolyzates from which amino acids were collected. Break-seal ampules have a colored blue band about the neck which is easily broken following hydrolysis. Ten ml of 6 N HCl were added to each sample. The ampules were flushed with nitrogen gas and sealed under a slight vacuum using a propane torch (Webb, 1977). The samples were hydrolyzed for 24 hours at 110^{o}C, cooled to room temperature and filtered through Whatman #4 filter paper into 40 ml glass beakers.

Table 3

Approximate Sample Amino Acid Content and L-leucine-N-carboxyanhydride Used in Derivatization

Sample and Amino Acid[a]	Estimated Maximum Amount of Amino Acid Present in Sample (umoles)[b,c]	L-leucine-N-carboxyanhydride (mg)
Soy Isolate 13		
Met	1.25	0.2
Val	4.25	0.8
Soy Isolate 4		
Met	0.31	0.2
Val	1.85	0.4
Soy Flour 9		
Met	0.80	0.2
Val	2.80	0.5
Soy Flour 6		
Met	0.40	0.2
Soy Flour 10		
Val	5.10	1.0
Methionine Dried in pH 3.25 buffer	1.00	0.2

[a]Soy Isolates 4 and 13 are separate hydrolyzates of Promine D; Soy Flours 6, 9 and 10 are separate hydrolyzates of Soyafluff 200W.

[b]Method of calculation is given in Appendix 4.

[c]Table 4 expresses the total volume (ml) in which each amino acid is contained.

Filter paper was more effective than glass wool as a filtering aid. The entire filtrate was dried in a vacuum dessicator over NaOH pellets and redissolved in 10 ml cartridge equilibration buffer. This solution was transferred to a 125 x 15 screw cap test tube and kept frozen until needed.

Calculation of Elution Time

Before fractions could be collected, it was necessary to calculate the elution times for each neutral and acidic essential amino acid. Elution time is defined as the time it takes for an amino acid to travel from the point of injection to the fraction collector. Determination of elution time is based on the retention time of the neutral and acidic essential amino acids. Elution time was calculated as follows. Methylene blue was injected into a reagent line (hydrazine sulfate) of the analyzer and the dye tracked and timed from the base of the column to the first flow cell (570 nm) of the colorimeter. Retention time minus the latter gave column elution time (or the time it takes for an amino acid or dipeptide to be eluted from the base of the column) for each amino acid. To this time was added the time it took for the effluent to travel the length of the tubing attaching the column to the fraction collector.

Fraction Collection

The specific amino acids to be derivatized from both a standard amino acid mixture and the soy isolate and flour samples were obtained by collecting, in fractions, the amino acids as eluted from the column

of the amino acid analyzer. Fractions were collected from a standard amino acid mixture to simulate conditions for collecting fractions from the soy isolate and soy flour samples. Culture tubes (150 x 18 mm) were used for fraction collection.

Twenty 100 ul aliquots from a standard amino acid mixture were applied to the column with each 100 ul sample containing approximately 50 nmoles of each L-amino acid. These samples were collected over a period of several days. The same set of culture tubes was used repeatedly for the collection of all samples. On each day of sample collection a recorder tracing was made of the amino acids in the standard mixture and the elution time for the five neutral and acidic essential amino acids determined. The elution time was multiplied by a factor (tubes/minute) obtained from the timing device on the fraction collector to determine the location (tube number) of a specific amino acid.

Fractions were collected from soy isolate and soy flour hydrolyzates in a manner similar to the collections from the standard amino acid mixture. Both a standard and a sample tracing were made before collecting fractions from each hydrolyzate. This was done to determine the number of nmoles of amino acid present in the sample based on a known amount in the standard. An example is given in Appendix 3.

A sufficient number of fractions were collected from each of two hydrolyzed soy isolates (Promine D) and two hydrolyzed soy flour (Soyafluff 200 W) samples (Table 3) to provide approximately 1 or more umoles of amino acid. Forty 150 ul samples were applied

to the column for fraction collection from one isolate while forty 100 ul samples were used from a second isolate. Thirty-seven 100 ul samples were applied from each of two soy flour hydrolyzates.

Following each day's collection, the tubes were transferred to test tube racks and covered with plastic caps. Each test tube rack was labelled and the tubes refrigerated until they could be composited. Details of compositing are contained in the next section.

Initially, plans called for the collection of threonine, valine, methionine, isoleucine, and leucine. Later, this was modified to threonine, valine and methionine. Methionine and threonine are, respectively, the first and second limiting amino acids in ISP (DeGroot and Slump, 1969) and are, therefore, of special interest. Isomerization of valine was observed in alkali-treated egg albumin and gelatin (Pollock and Frommhagen, 1968).

Standard and Sample Analysis

Following collection of fractions from soy isolate and soy flour samples, the tubes believed to contain methionine, valine and threonine were tested by measuring retention time against a standard. In this way the identity of the tube contents was established. Aliquots taken for analysis contained approximately 200 ul. After a test tube was found to contain methionine, valine or threonine, tubes on either side were tested to determine the beginning and ending points of elution for each amino acid.

For each hydrolyzate sample and standard, the fractions containing threonine, methionine and valine were dried in a vacuum dessicator.

This was accomplished by pouring the contents of one tube (or fraction), known to contain the amino acid, into a 150 x 25 mm screw cap test tube. Each fraction was dried before another one was added. This process was repeated until all the fractions containing a given amino acid had been dried.

The contents of the test tubes appeared to bubble over when greater than 2 ml were added. This was attributed to the presence of Brij-35, a detergent, in the elution buffer. The problem was eliminated by adding less than 2 ml to the test tubes. Later, several fractions were added to the test tube at a time and immersed in a dry ice-acetone bath for 2-3 minutes before drying. This eliminated the foaming problem.

Approximate amino acid content of the dried fractions as well as the amount of derivatizing agent necessary for the reaction were calculated. An example of these calculations is given in Appendix 4. The approximate amino acid content and amount of derivatizing agent used are contained in Table 3. The dried amino acid contained in each test tube was redissolved in borate buffer and titrated to approximately pH 10.2 with 1.0 N NaOH (Manning and Moore, 1968). A Corning combination electrode was used to check the pH. The samples were derivatized as specified earlier.

Each derivatized amino acid from the soy flour and soy isolate samples was run on the amino acid analyzer and the diastereomeric dipeptides were separated at the appropriate pH (Table 1). The L-L and L-D dipeptides, prepared from the crystalline DL-amino acids, were also run in order to determine peak identity and calculate

height times width at one-half height (H x W @ 1/2 H). This measurement is used as a basis of comparing peak areas of the dipeptides. The H x W @ 1/2 H of the standard dipeptide derivatives, the ratio of nmoles of L to D amino acid from derivatized standard applied to the column, and the H x W @ 1/2 H measurements of the soy flour or soy isolate dipeptide derivatives were incorporated into a formula to obtain the ratio of nmoles of derivatized L to D amino acid from the sample applied to the column. This formula is as follows:

$$\frac{\text{Ratio of nmoles of L to D amino acid from derivatized standard applied to column}}{\text{Ratio of H x W @ 1/2 H of peaks for L-L to L-D dipeptides in standard}} = \frac{\text{Ratio of nmoles of L to D amino acid from derivatized sample applied to column (unknown)}}{\text{Ratio of H x W @ 1/2 H of peaks for L-L to L-D dipeptides in sample}} .$$

CHAPTER V

RESULTS AND DISCUSSION

A. Presence of Alloisoleucine in Soy Isolate

Alloisoleucine in hydrolyzed soy isolate was identified by comparing the retention time of alloisoleucine in the standard to that of the sample. Although the alloisoleucine peak was observable, it was too small to be measured. Based on this observation, it appeared that a small fraction of the amino acid underwent isomerization. This provided evidence that a similar condition might exist for other amino acids.

B. Sources of Error Incurred During Sample Collection

Two possible sources of error were encountered during collection of the sample amino acids. The timing device on the fraction collector was set at 22 seconds/tube or 2.7 tubes/minute. At the end of an 81 minute buffer program, the fraction collector should have been positioned over tube 220. In actuality, the program ended at tube 220±5.

Retention and elution times for the amino acids varied on a daily basis. Although one sample tracing was made per day in order to calculate both retention and elution times, the operating conditions of the TSM may have varied during the course of the day thereby making the initial calculations inaccurate. These errors were sufficient to contaminate the threonine, valine and methionine with other amino acids during fraction collection.

C. Analysis of Dipeptides Obtained from Standard Amino Acid Mixture Collections

Isoleucine, valine, threonine and methionine, collected from the standard amino acid mixture, were dried and derivatized. It was assumed, mistakenly, that the calculated tube numbers (Appendix 5) of each amino acid were the same as the actual tube numbers. Consequently, the dried and derivatized fractions did not contain enough of the amino acid or were contaminated by additional amino acids. No results are included for this section.

D. Hydrolyzate Sample Size Used for Fraction Collection

Aliquots of hydrolyzates used in fraction collection ranged from 150 ul for one soy isolate sample to 100 ul for another isolate and two soy flour samples. In the future it would be advisable to double the concentration of the hydrolyzate solution so that a smaller aliquot (50 ul) could be taken for analysis. An aliquot of this size is more adequately held by the cartridge resin and improved resolution would be obtained.

E. Handling of Soy Flour and Soy Isolate Amino Acids and Their Derivatives

Some of the fractions containing methionine, valine and threonine obtained from the first hydrolyzate, Soy Isolate 4, were mixed together before checking the identity of the tube contents. For this reason only one-half to one-third of the collection mixture containing each amino acid was available for derivatization (Table 3). Tube contents

were identified by amino acid analysis of a 200 ul aliquot taken from each tube and comparing against a standard amino acid mixture tracing. An example of this is found in Figures 3 and 4. The methionine from Soy Flour 10 was dropped during the derivatization process and was not used. This sample was replaced by methionine from Soy Flour 6 which had previously been collected.

The methionine, valine and threonine, collected from soy flour and soy isolate samples, were each handled differently (Table 4). This was related to the amount of buffer in the fractions containing each amino acid and the amount of amino acid collected. For example, the valine from Soy Isolate 13 was eluted in approximately 72 ml of the 3.25 buffer while the threonine from the same isolate was eluted in 16 ml. As the sample was dried, the buffer salts and amino acids were left behind. Large amounts of buffer salt required more 1 M NaOH to reach pH 10.2 than did smaller amounts. Additional 1 M HCl was required to overcome the buffering capacity of the solution at pH 10.2 and to stop the derivatization reaction by bringing the pH of the mixture to 3.

In all cases, the derivatization mixture was more dilute than anticipated. The methionine and valine dipeptides from all samples were too dilute to be measured from recorder tracings. This necessitated concentration of the samples. Aliquots of four or five ml, depending on the amount of derivatization mixture available, were taken from each of the dipeptide mixtures and placed in 25 x 50 mm flat bottom vials. These were put in the vacuum dessicator to reduce the volume. Each sample was removed from the dessicator when buffer

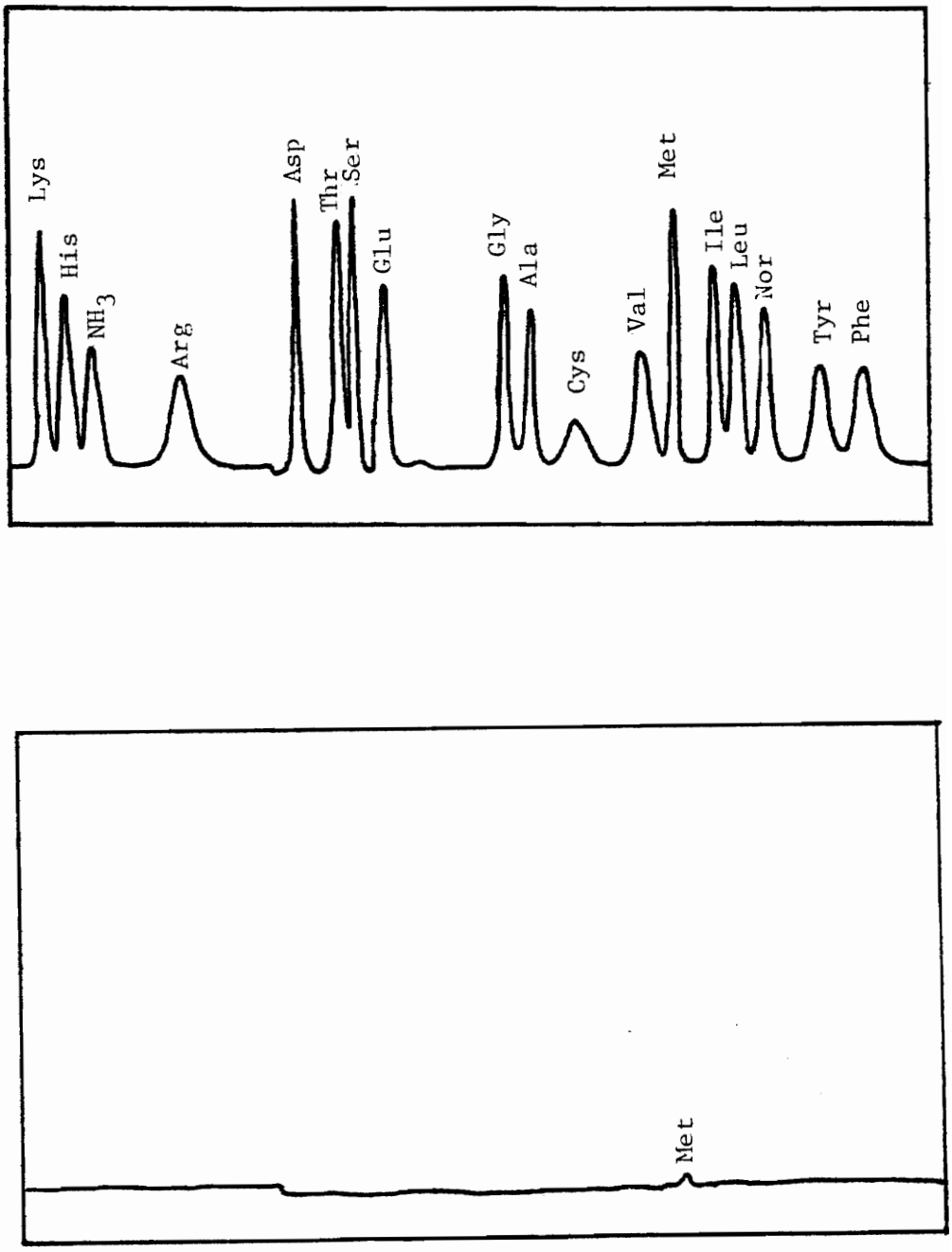


Figure 3. Standard amino acid mixture tracing (top) and tracing of tube number 155 contents (bottom) obtained from Soy Flour 9.

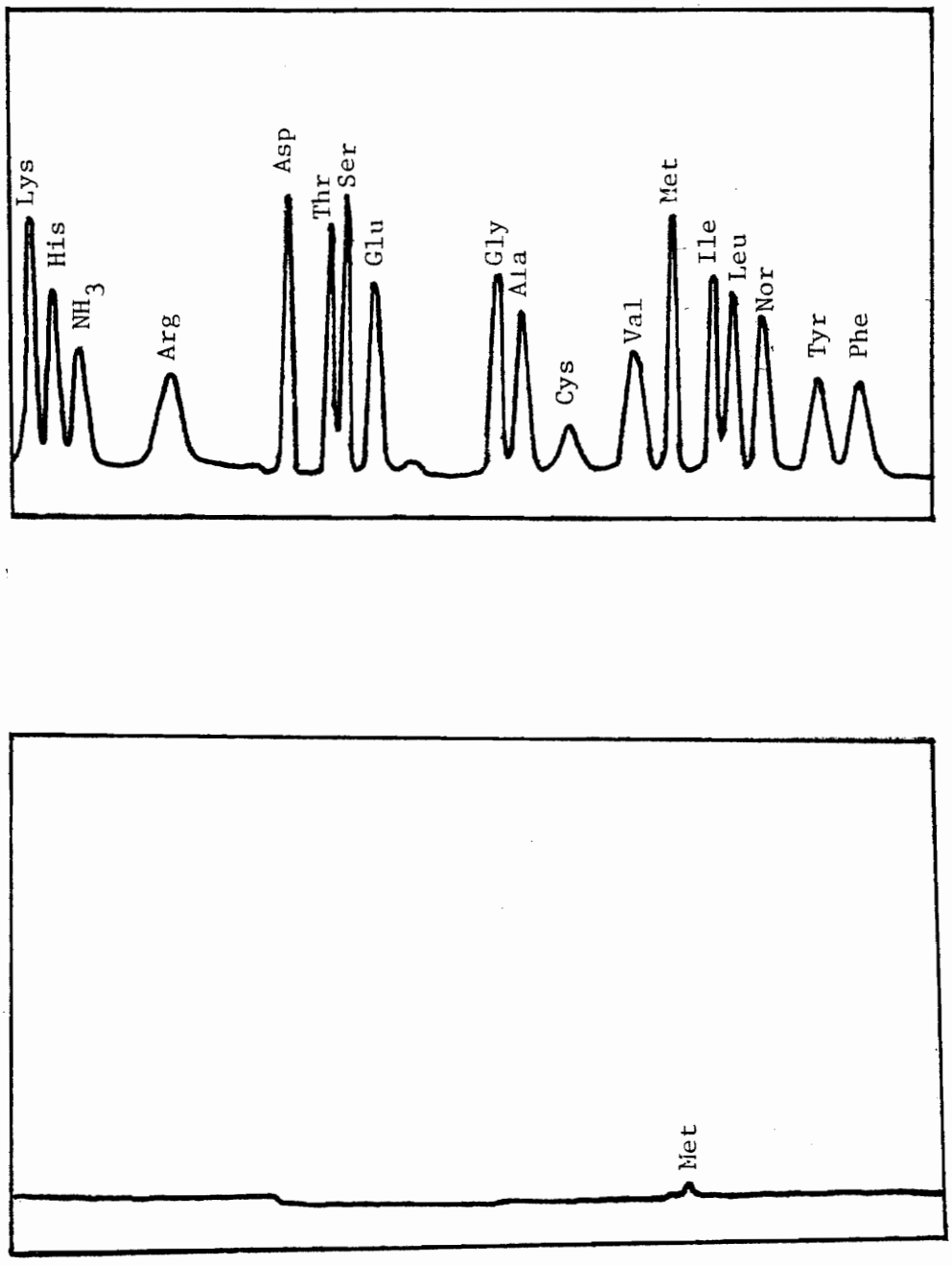


Figure 4. Standard amino acid mixture tracing (top) and tracing of tube number 162 contents (bottom) obtained from Soy Flour 9.

Table 4

Handling of Samples

Sample and Amino Acid[a]	Volume of Reagents Added to Dried Samples, Total Volume and pH of Total Volume			
	1 M NaOH (ml)	1 M HCl (ml)	Total Volume (ml)	Measured pH of Total Volume
Soy Isolate 13				
Met	7.5	4.0	13.5	10.22
Val	18.4	4.8	25.2	10.27
Soy Isolate 4				
Met	2.2	0.8	5.0	10.20
Val	4.2	3.2	9.4	10.25
Soy Flour 9				
Met	6.9	3.7	12.6	10.27
Val	16.9	3.2	22.1	10.29
Soy Flour 6				
Met	1.2	1.6	4.8	10.24
Soy Flour 10				
Val	17.6	4.8	24.4	10.27
Met dried in Buffer 1	3.0	0.8	5.8	10.18

[a] 2 ml of 0.45 M Borate Buffer were added to each sample.

crystals began to appear in the solution. At this point, the valine dipeptides could be separated and easily measured. The methionine dipeptides, however, had to be electronically amplified 2-3 times the normal amount before measurable peaks could be obtained.

Further studies employing this method of D- and L-amino acid determination should be modified to minimize the difficulties attributed to dilute samples. This may be accomplished by using a more concentrated form of NaOH for titrating the dried amino acids to pH 10.2, or by removing the buffer salts from the collected amino acids prior to drying using a desalting ion-exchange resin.

The threonine collected from all samples was contaminated by aspartic acid. This was due to the inaccuracy of the timing device on the fraction collector and/or the variation in elution time as mentioned previously. The threonine samples were derivatized but the results not included.

F. Dipeptides Present in Soy Isolate and Soy Flour Samples

Examples of recorder tracings expressing the valine and methionine dipeptides present as well as the unreacted derivatizing agent and underivatized amino acid, in the soy flour and soy isolate samples are contained in Figures 5-14. The soy flour and isolate dipeptides were analyzed on different days. On each day of analysis, the dipeptides previously prepared from the DL-amino acids were separated prior to the sample dipeptides. This provided a basis for comparing the height times width at half-height (H x W @ 1/2 H) of the sample dipeptides to the standard dipeptides.

The tracing in Figure 5 represents the methionine standard run on the same day as the methionine dipeptides contained in Soy Isolate 13 (Figure 6) and Soy Flour (Figure 7). The identities of each peak, established by derivatizing D-methionine and L-methionine are represented in Figure 8 and 9, respectively. The tracings from Soy Isolate 13 and Soy Flour 6 were electronically amplified three-fold over the standard. Amplification, of course, amplifies noise as well as the signal itself. This noise is recorded as a series of short spikes which make accurate measurement of dipeptide peaks (H x W @ 1/2 H) difficult.

None of the valine dipeptides from the soy flour and isolate samples required amplification. The valine dipeptide standard in Figure 10 was run on the same day as the valine dipeptides in Soy Flour 9 (Figure 11) and Soy Isolate 13 (Figure 12). The valine dipeptide standard peaks were identified by comparison to the derivatives obtained from L-valine (Figure 13) and D-valine (Figure 14).

G. Peak Measurement

The ratios of the H x W @ 1/2 H measurements of the soy flour and soy isolate valine and methionine dipeptide peaks, as well as their respective standards, are contained in Table 5. The measurement of H x W @ 1/2 H was made in cm^2 and is proportional to peak area.

The standard L-L dipeptide peaks (H x W @ 1/2 H) appeared to be slightly smaller than those of the standard L-D dipeptides for both valine and methionine standards (Table 5). The methionine dipeptide standard run with the Soy Isolate 4 sample was the exception. This

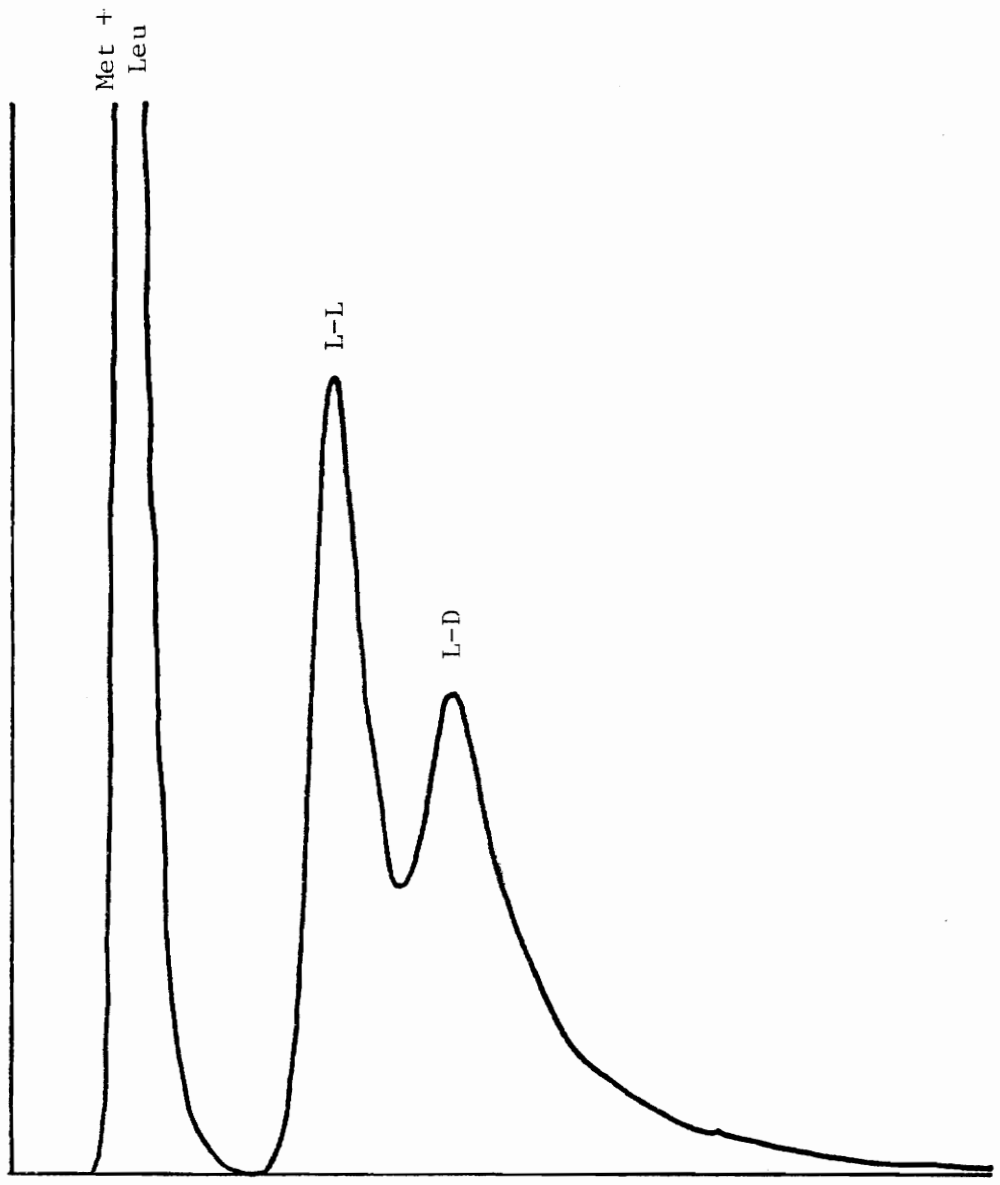


Figure 5. L-D and L-L methionine dipeptides used as standards for Soy Isolate 13 and Soy Flour 6.

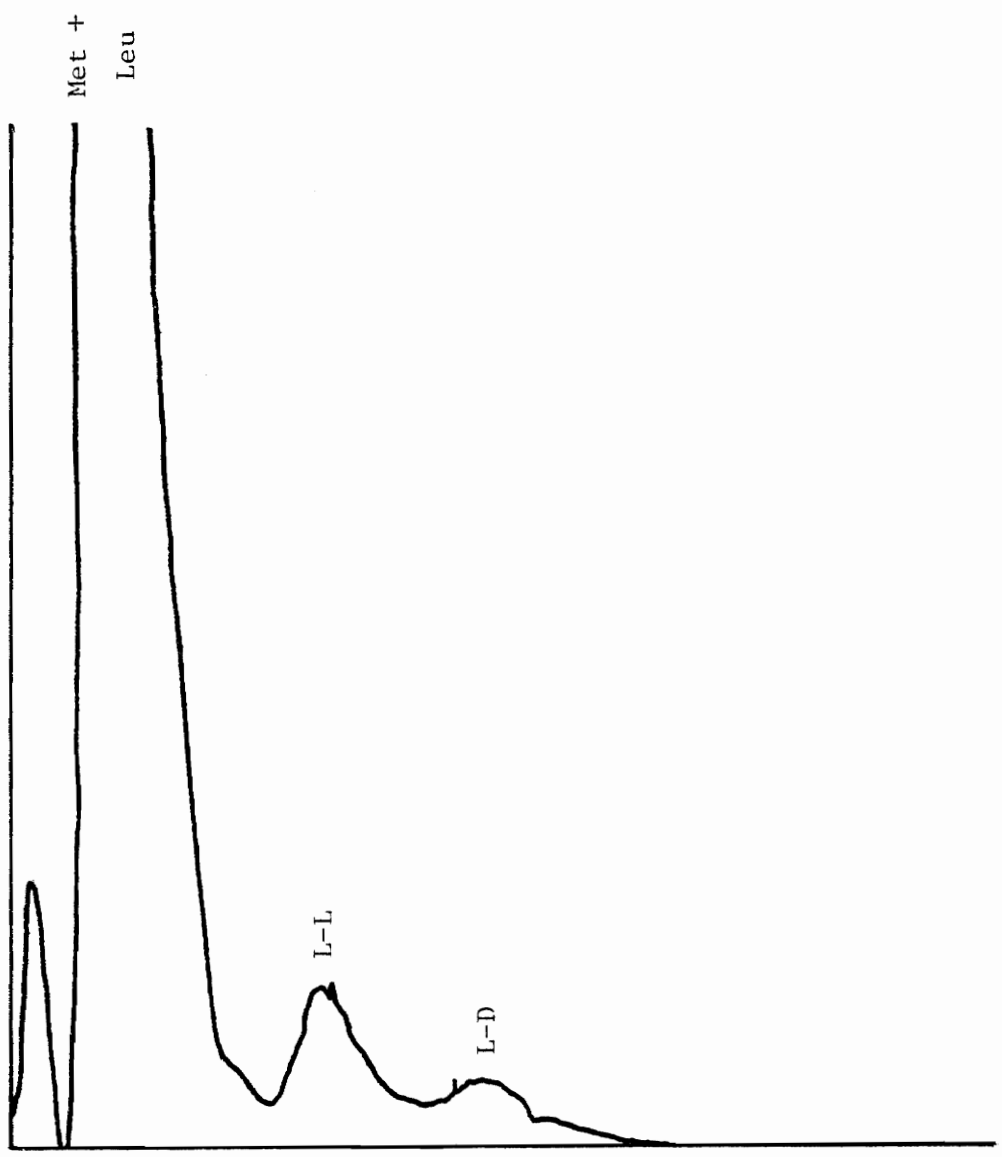


Figure 6. Three-fold amplification of L-D and L-L methionine dipeptides obtained from Soy Isolate 13.

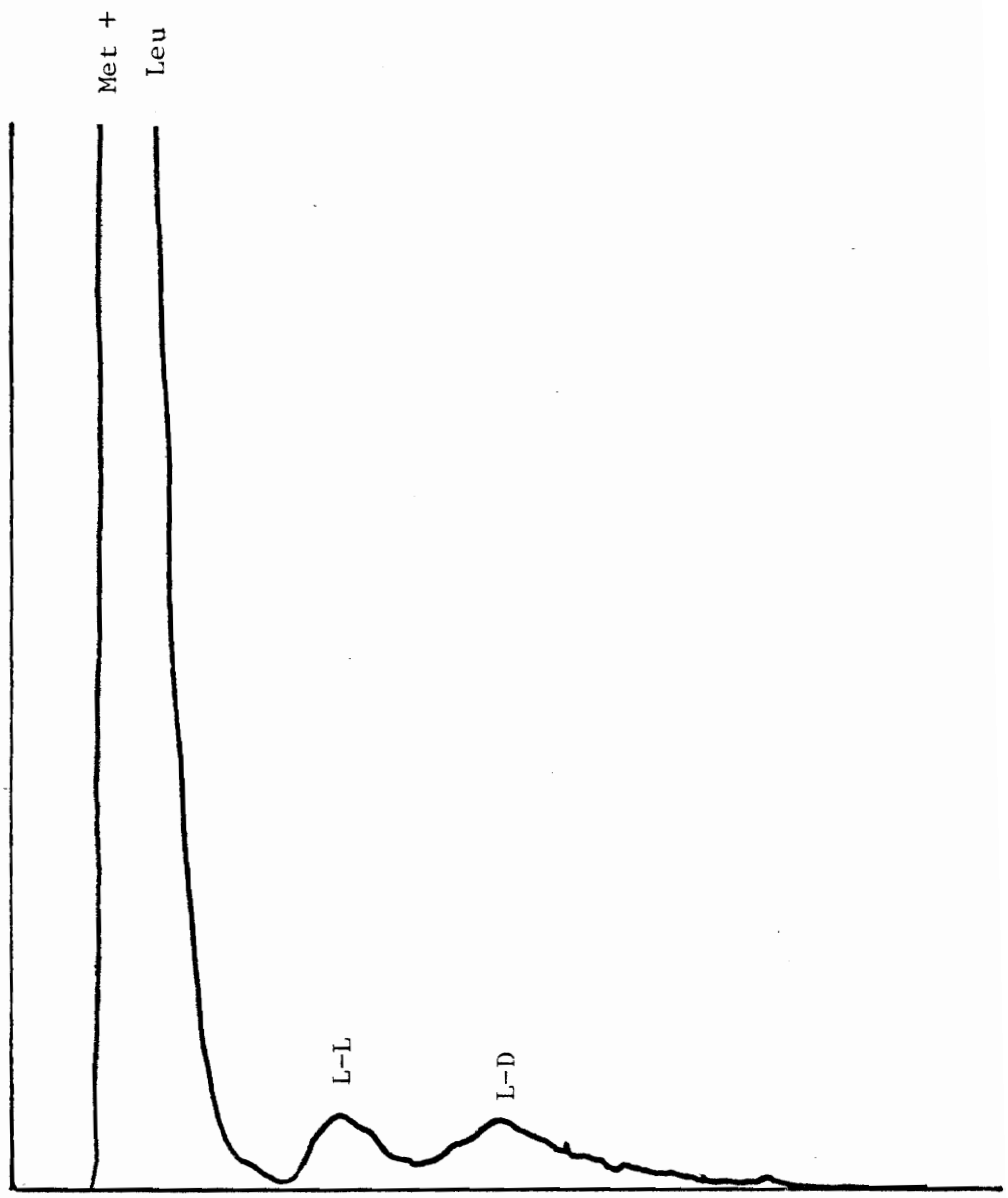


Figure 7. Three-fold amplification of L-D and L-L methionine dipeptides obtained from Soy Flour 6.

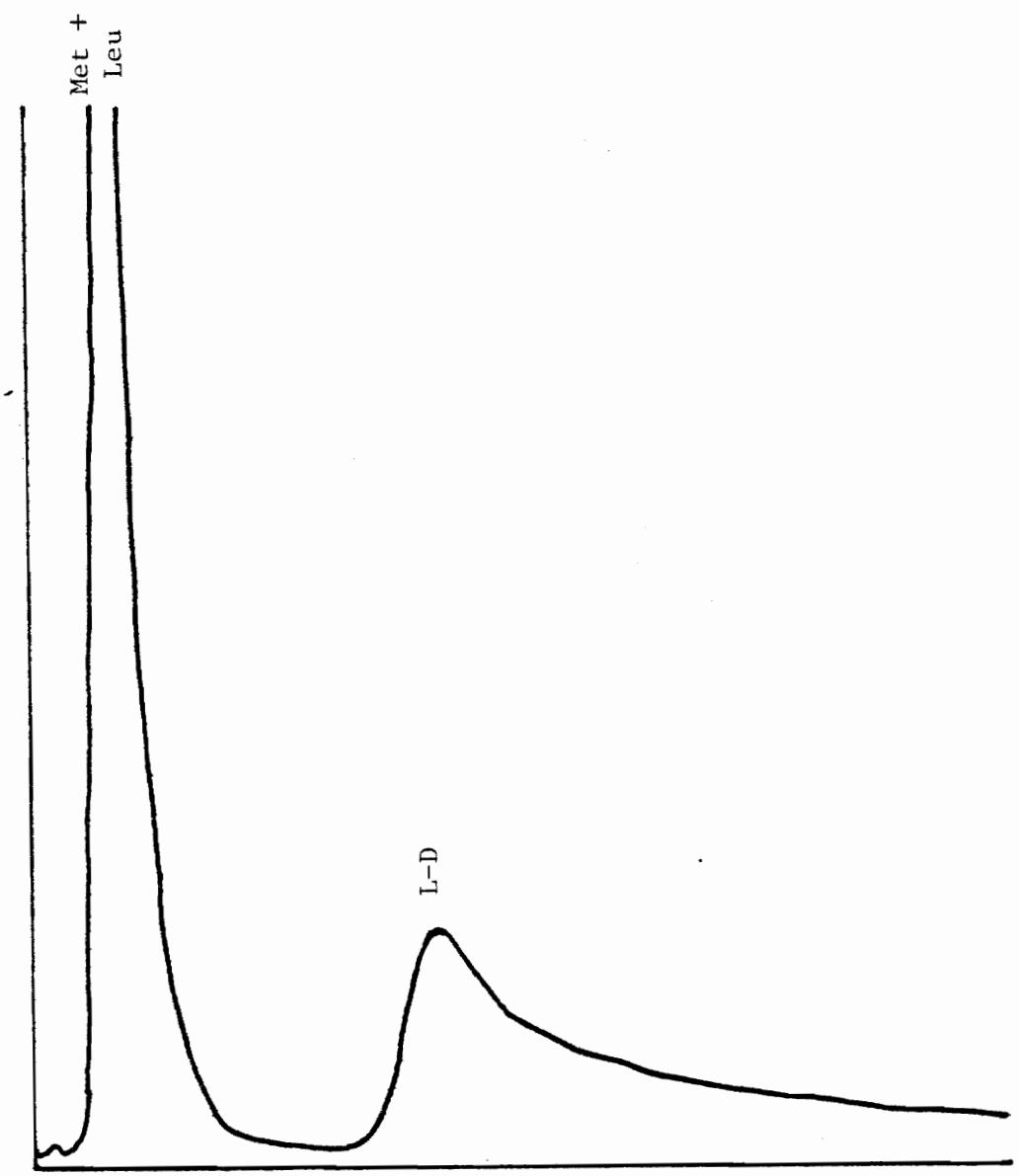


Figure 8. L-D dipeptide derivative prepared from D-methionine.

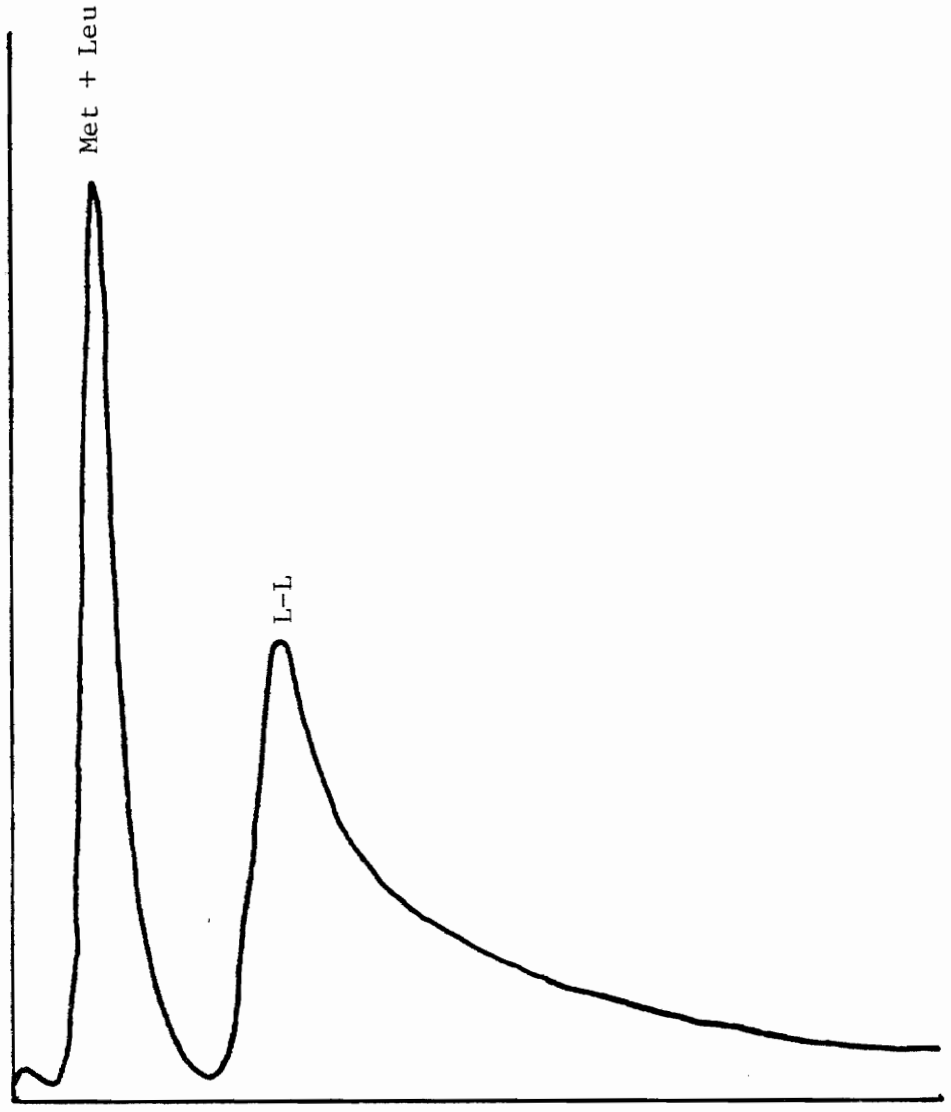


Figure 9. L-L dipeptide derivative prepared from L-methionine.

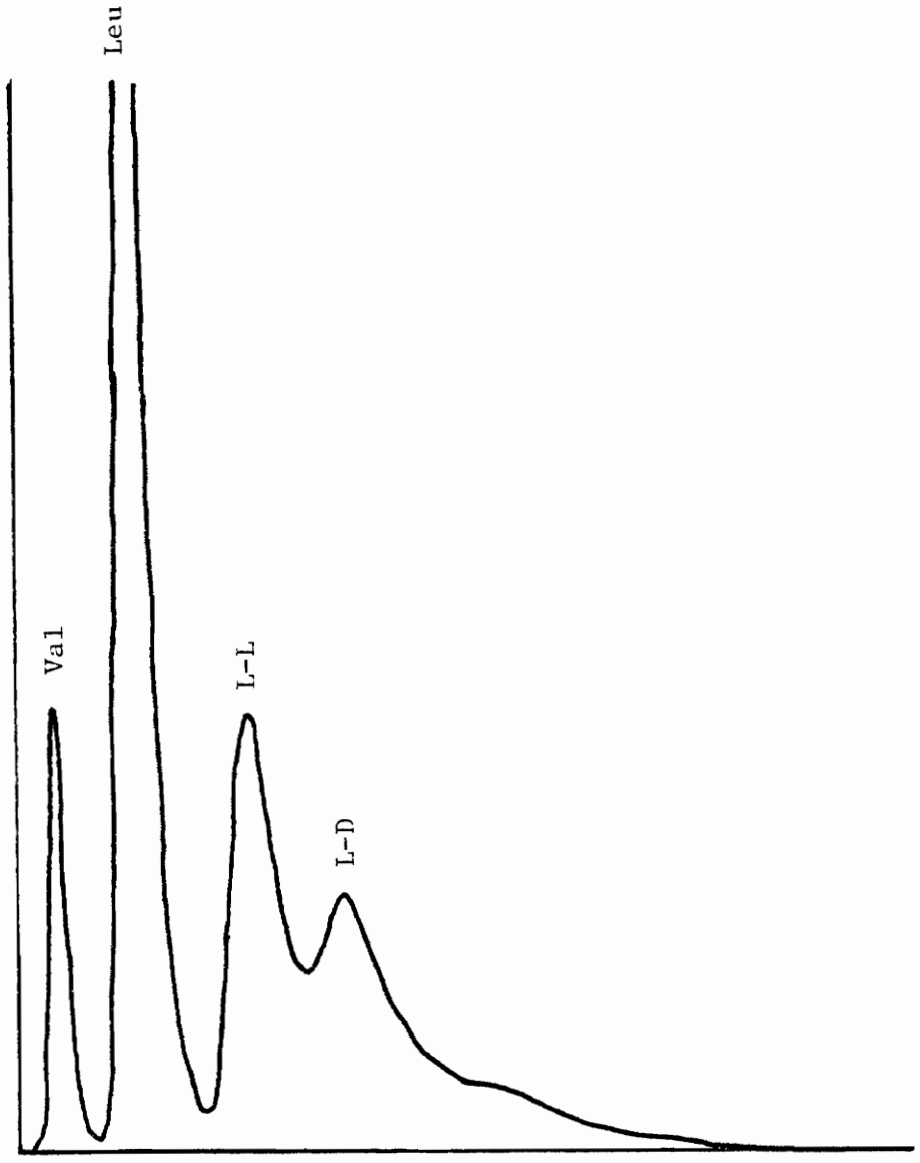


Figure 10. L-D and L-L valine dipeptides used as standards for Soy Flour 9 and Soy Isolate 13.

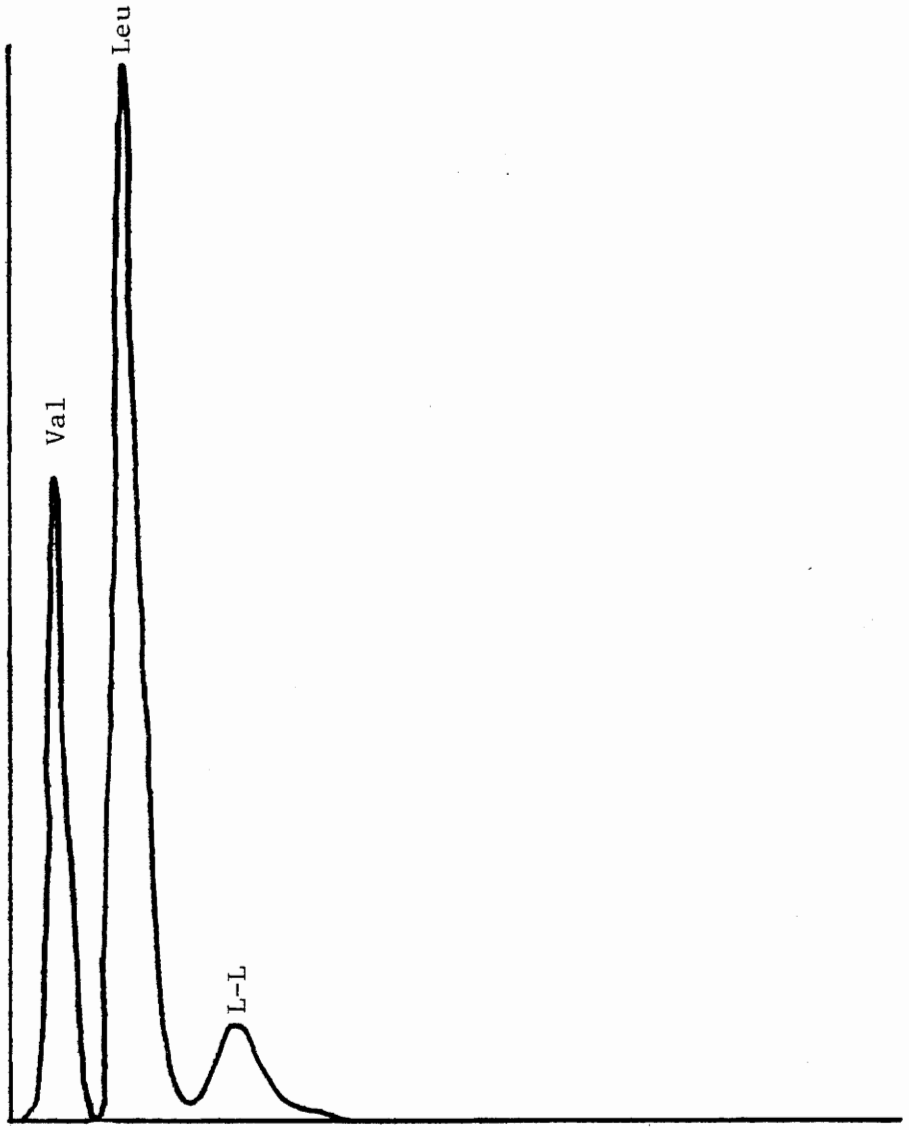


Figure 11. L-L valine dipeptide obtained from Soy Flour 9.

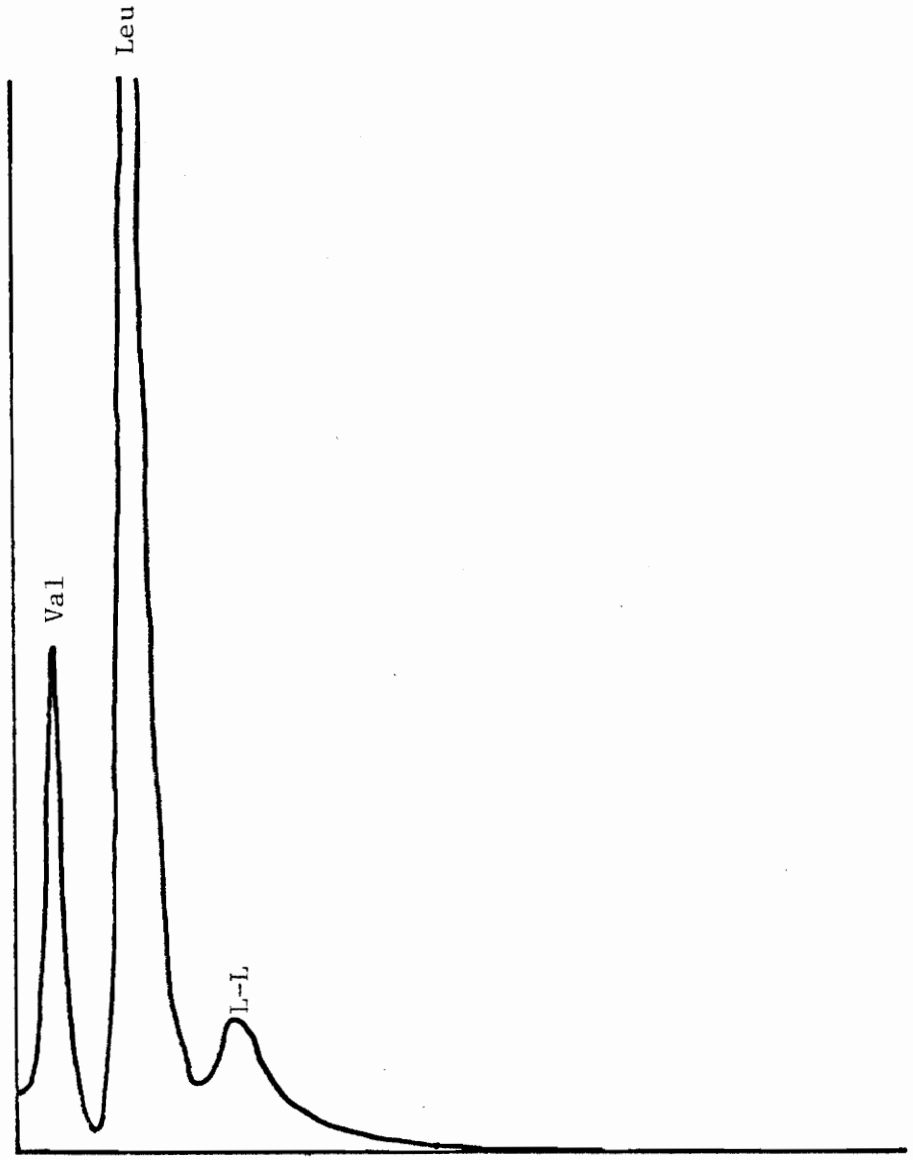


Figure 12. L-L valine dipeptide obtained from Soy Isolate 13.

Figure 13. L-L dipeptide derivative prepared from L-valine.

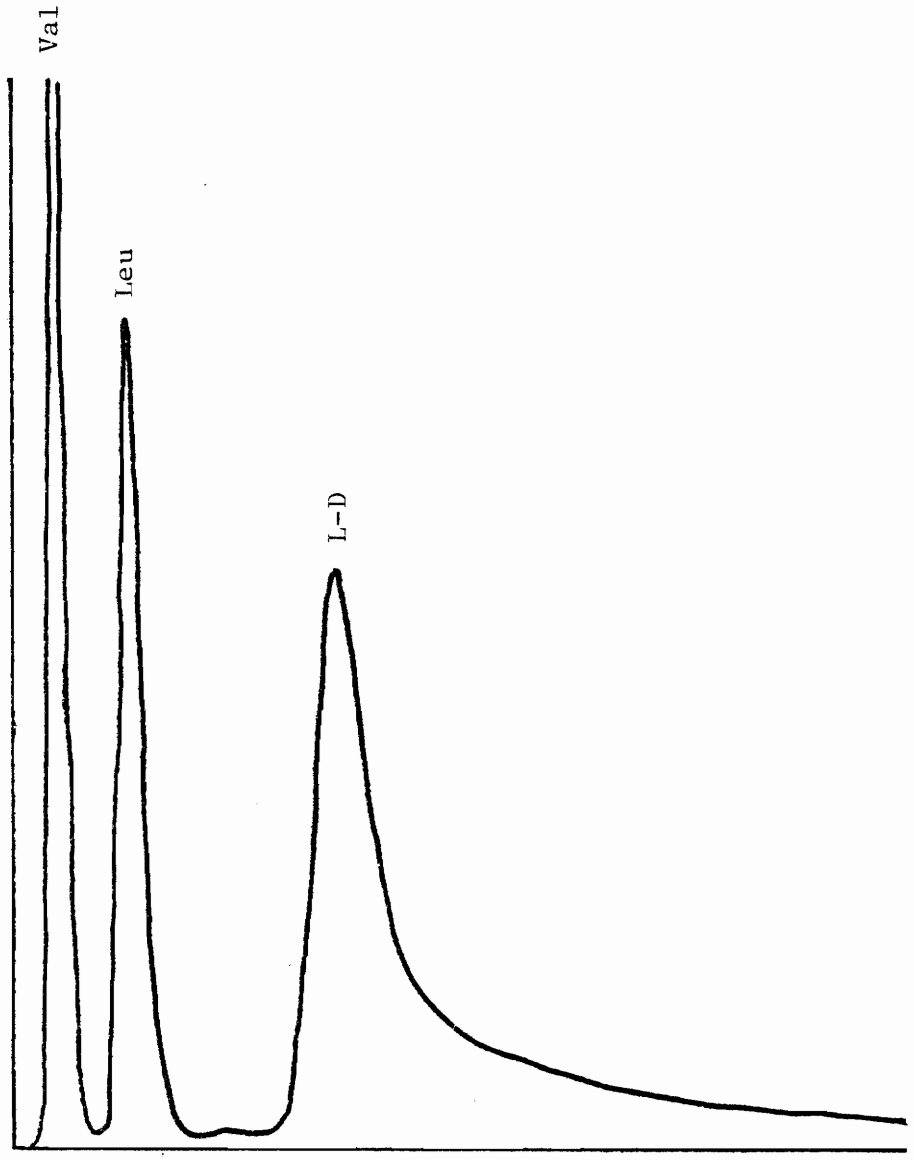


Figure 14. L-D dipeptide derivative prepared from D-valine.

Table 5

Ratios of Height Times Width at One-Half Height[a] of
L-L to L-D Dipeptides in Samples and Standard

Amino Acids and Samples	L-L/L-D Dipeptide Peak Measurements[b]: Standard	L-L/L-D Dipeptide Peak Measurements[b]: Sample
Valine		
Soy Isolate 13	4.2/4.9 = 0.86	1.1/0 = 0
Soy Isolate 4	4.2/4.9 = 0.86	0.4/0 = 0
Soy Flour 9	4.2/4.9 = 0.86	0.8/0 = 0
Soy Flour 10	4.5/4.8 = 0.94	1.7/0 = 0
Methionine		
Soy Isolate 13	9.2/9.3 = 0.99	2.1/1.2 = 1.75
Soy Isolate 4	14.9/18.6 = 0.80	0.8/1.0 = 0.80
Soy Flour 9	11.7/11.8 = 0.99	1.6/1.9 = 0.84
Soy Flour 6	9.2/9.3 = 0.99	1.1/1.4 = 0.78
	Average Ratio = 0.99[c]	

[a] H x W @ 1/2 H in cm^2.

[b] H x W @ 1/2 H for L-L/H x W @ 1/2 H for L-D.

[c] Results from Soy Isolate 4 omitted.

was the only methionine standard which was electronically amplified beyond the normal amount. The L-L to L-D ratio of the standard run with Soy Isolate 4 was discarded and an average value computed from the remaining ratios for use in calculating the ratio of L- to D-amino acid in the samples. The L-L/L-D methionine dipeptide peak measurements obtained from Soy Isolates 13 and 4 are inconsistent. Further work is required in order to make a concrete statement about the reproducibility of the L-L and L-D valine and methionine dipeptide peaks used as standards and the methionine dipeptides obtained from soy isolate.

H. D- and L-methionine and D- and L-valine Content of Soy Flour and Soy Isolate Hydrolyzates

It appears from the results in Table 6 that valine did not undergo isomerization in either the soy flour or soy isolate samples. Only the L-enantiomer is present in detectable amounts. The opposite is true of methionine. Both the D and L forms are present in the soy flour and isolate samples. Within the limits of experimental error, the hydrolyzates of Soy Isolate 4 and Soy Flour 6 and 9 contain a 1:1 mixture of D- and L-methionine (Table 6). Isomerization from 100% L to greater than 50% D amino acid is not possible under the conditions of this experiment. The measurement of greater than 50% D-methionine may be attributable to the difficulties encountered in measuring the peak H x W @ 1/2 H of each dipeptide or other experimental errors.

Although the extent of isomerization of methionine can not be stated in quantitative terms, it appears from the results (Table 5 and 6) that substantial isomerization (racemization in three cases, in

Table 6

Ratio of nmoles of L- to D-Amino Acids Applied to Column from Standards and Samples

Amino Acids and Samples[a]	Ratio of L- to D-Amino Acids Applied to Column from Samples (nmoles)[b]	% L-enantiomer Present	% L-enantiometer Present
Valine			
Soy Isolate 13	0	100	0
Soy Isolate 4	0	100	0
Soy Flour 9	0	100	0
Soy Flour 10	0	100	0
Methionine			
Soy Isolate 13	1.8	64	36
Soy Isolate 4	0.8	44	56
Soy Flour 9	0.8	44	56
Soy Flour 6	0.8	44	56

[a] The ratio of L- to D-amino acids in the standard was 1.

[b] Computed from data in Table 5 using formula on page 37. An average peak ratio for the methionine standards was used.

fact) has indeed occurred. The opposite is true for valine. Since the D and L forms of methionine are found in both soy isolate and soy flour samples, it is difficult to know when the isomerization occurred. Manning (1970) measured the amount of racemization during acid hydrolysis by hydrolyzing free amino acids in tritiated HCl and determining the amount of ^{3}H incorporation into the amino acid. He compared these results with those from the derivatization reaction using the free amino acids. The results for valine and methionine are found in Table 7. Methionine was observed to isomerize at a slightly faster rate than valine. Neither 2.2% nor 2.7% D-isomer content compares with the results obtained in this study. However, Manning (1970) states that the amino acids in some peptide sequences may undergo increased racemization during hydrolysis. Therefore, the isomerization may have occurred during acid hydrolysis.

Some other factor, in addition to acid hydrolysis, may be responsible for the observed isomerization. Defatted soy flakes are subjected to moist heat during preparation of soy flour. Since soy isolates are prepared from soy flour, the effects of heat, if any, on the protein could be seen in both products, but the effects in soy isolate would be expected to be larger due to the greater extent of processing. Hayase et al. (1975) observed 8.8% and 5.0% isomerization of valine in casein and lysozyme, respectively, roasted at 230°C under air. Isomerization of methionine was not measured. If heat is responsible for isomerization, it appears to have had a greater effect on methionine than valine.

Table 7

Isomerization of Free L-Valine and L-Methionine During Acid Hydrolysis[a]

L-Amino Acid	% D-isomer as Determined by ^{3}H-incorporation	L-D dipeptide
Valine	0.2	0.7
Methionine	2.7	2.2

(Manning, 1970)

[a] Amino acids hydrolyzed at 110°C for 22 hours.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The presence of D-and L-methionine and valine in soy flour (Soyafluff 200 W) and soy isolate (Promine D) was determined by ion-exchange chromatography of the derivatized amino acids. Each D-or L-amino acid was chromatographed as a L-D or L-L dipeptide. Conditions for separating the L-L and L-D dipeptides of isoleucine, leucine and threonine were also examined.

Both the soy isolate and soy flour samples were observed to contain L-valine, L-methionine and D-methionine. The presence of what appears to be almost a racemic mixture of methionine in soy isolate and soy flour may further reduce the nutritive value of these products which are already low in methionine. In addition, food products containing soy isolate or soy flour may be similarly affected.

Further work is required to clarify the exact proportions of D-and L-methionine present. This may be accomplished by comparison of results obtained in this study with results obtained from another method. It may be interesting to continue the course of this study by investigating the presence of D-and L-forms of the remaining essential amino acids in these and other soy flour and isolate samples.

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APPENDIX 1

Net Protein Utilization

$$\text{Net Protein Utilization (NPU)} = \frac{\text{Food Nitrogen Retained}}{\text{Food Nitrogen Intake}}$$

(Pike and Brown, 1975)

APPENDIX 2

Normal Buffer Program - NC-1P

Buffer pH	Pumping Time (min)
3.25	52
4.25	21
5.25	35

APPENDIX 3

Determination of Standard and Sample

Amino Acid Content (nmoles)

Example:

Sample: Standard (50 nmoles each amino acid/100 ul)

Factor = nmoles ÷ H x W

Amino Acid	Height	Half Height	Width (@ half height)	H x W	nmoles	Factor
Valine	1.7	0.85	1.2	2.04	50	24.51
Methionine	3.6	1.80	0.62	2.23	50	22.42

Sample: Soy Isolate 13 (100 ul aliquot)

nmoles = H x W x Factor

Amino Acid	Height	Half Height	Width (@ half height)	H x W	Factor	nmoles
Valine	4.3	2.15	0.96	4.13	24.51	101.18
Methionine	2.1	1.05	0.63	1.32	22.42	29.66

APPENDIX 4

Calculations of Approximate Content of Amino Acids from Dried Fractions and Derivatizing Agent

1. Amino Acid Content (nmoles) in 100 ul aliquot of hydrolyzate sample[a] x Number of aliquots taken from hydrolyzate sample =

Estimated Maximum Possible Amount of Amino Acid from Dried Fractions (nmoles)

2. Weight of 1 umole L-leucine-N-carboxyanhydride (g/umole) x Approximate Content of Amino Acid from Dried Fractions (umoles) +

Weight of 20% Molar Excess of L-leucine-N-carboxyanhydride (g) = Total Weight of Derivatizing Agent (g)

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[a] Based on calculations in Appendix 3.

APPENDIX 5

Calculation of Tube Numbers of Collected Amino Acids

1. Beginning Tube Number

   a. Retention Time at Beginning of Amino Acid Peak − Time Effluent Takes to Travel from Base of Column to Colorimeter = Column Elution Time

   b. Column Elution Time + Time Effluent Takes to Travel from Base of Column to Fraction Collector = Elution Time

   c. Elution Time x Fraction Collection Rate (tubes/minute) = Beginning Tube Number

2. Ending Tube Number

   a. Retention Time at Ending of Amino Acid Peak − Time Effluent Takes to Travel from Base of Column to Colorimeter = Column Elution Time

   b. Column Elution Time + Time Effluent Takes to Travel from Base of Column to Fraction Collector = Elution Time

   c. Elution Time x Fraction Collection Rate (tubes/minute) = Ending Tube Number

VITA

Laura Marcella Gilbert was born in Detroit, Michigan on October 10, 1952. She attended parochial schools in Detroit and Louisville, Kentucky. In December 1974 she received a Bachelor of Science degree in Nutrition and Food Science from the University of Kentucky, Lexington, Kentucky. She started graduate studies at Virginia Polytechnic Institute and State University in September 1974. As a graduate student in the Department of Human Nutrition and Foods she was assigned graduate teaching responsibilities in the Food Selection and Preparation and Food Service Management classes. Presently, she is employed as an Instructor/Extension Specialist in the Department of Human Nutrition and Foods at Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Laura Marcella Gilbert

DETERMINATION OF L- AND D-METHIONINE AND L- AND D-VALINE IN SOY ISOLATE AND SOY FLOUR SAMPLES

by

Laura Marcella Gilbert

(ABSTRACT)

L to D isomerization of amino acids in a commercially prepared alkali-treated food-grade soy isolate was investigated. A soy flour product was also investigated as a control which had not been alkali-treated. Methionine and valine collected from two soy flour and two soy isolate samples were coupled with L-leucine-N-carboxyanhydride. If both D- and L-forms were present this would produce diastereomeric dipeptide derivatives. The derivatives were separated by ion-exchange chromatography using a TSM Amino Acid Analyzer. Comparison of the sample methionine and valine dipeptide peaks to standard dipeptide peaks revealed the presence of L-valine, L-methionine and D-methionine in both soy flour and soy isolate samples. Methionine was substantially isomerized in both products. D-valine was not observed in either soy flour or soy isolate samples. Thus, isomerization of methionine appeared to occur at a faster rate than valine. Acid hydrolysis, toasting of soy meal, and alkaline treatment are all conditions which may be responsible for the isomerization of methionine.