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Kinetics of protein quality change in extruded cowpea:corn flour under varied steady-state storage conditions

by

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INTRODUCTION

Even with the advent of increased individual and governmental support and awareness, the food crisis in less developed countries remains a significant problem (Berg, 1974). Central to this issue are concerns for both the quality and availability of food. While a critical need exists for both calories and protein, questions concerning the amount and quality of dietary protein may be more important than energy (Scrimshaw, 1981). Solutions to food problems in less developed countries have generally been in the form of outside intervention and provision of foodstuffs, or outside assistance in economic and agricultural development. However, implementation of traditional processing technology is often not economically feasible for less developed nations (Jansen et al. 1978). Recently, low-cost extruder cookers (LEC) have been used for the development of cereal:legume blends intended for use in less developed countries (Jansen et al. 1978). The development and evaluation of a high-protein, nutritionally sound legume:cereal based weaning food supplement processed with LEC technology has been a recent accomplishment of this department. Despite the increased interest in the incorporation of LEC technology in less developed countries, there are relatively little data available to adequately

describe changes in protein quality either during processing or with subsequent storage.

Heat treatment applied to foods generally increases food protein quality through destruction of heat-labile nutritional factors, and protein denaturations that improve in vivo digestibility (Liener, 1981). Application of severe heat, or long-term exposure to moderate heat can produce significant decreases in protein quality through Maillard non-enzymatic browning reactions (Hurrell and Carpenter, 1981). Maillard reactions produce decreases in food protein quality through decreases in protein digestibility, and destruction of essential amino acids (Mauron, 1981). Because lysine is a limiting amino acid in most cereal-based products, and because lysine is the principal amino acid destroyed during Maillard reaction, the impact of these reactions in high-quality protein supplement foods may be significant.

Accurate knowledge of rates of deteriorative processes in food systems, and factors which affect these rates are of great importance to the food scientist. However, comparatively little data are available concerning kinetics of nutrient quality changes in foodstuff with varied processing or storage conditions. Application of kinetic models to changes in protein quality and non-enzymatic

browning during foodstuff processing or storage have provided much useful information, but are available for a very limited range of products (Labuza et al. 1977; Chen et al. 1983). The purpose of the present research was to examine kinetic changes in protein quality of a legume:cereal based product exposed to varied storage conditions. The following objectives were formulated for the present research:

- Examine kinetic behavior of protein quality of a cowpea:corn flour blend exposed to varied temperature and moisture storage conditions.
- Determine kinetic parameters and evaluate the temperature dependence of reactive lysine losses and non-enzymatic browning reactions in the product.
- Determine the experimental error for estimation of the kinetic parameters.
- Evaluate the effect of the extent of non-enzymatic browning on changes in <u>in vitro</u> protein digestibility of the product.
- Describe potential processing and storage implications for products of this composition and optimum storage conditions to minimize protein nutritional quality loss due to non-enzymatic browning.

- Develop an adequate data base of kinetic information with this food system that will enable future work to be conducted, and that will allow future development of preliminary evaluations and predictions of shelf-life for this product.

REVIEW OF THE LITERATURE

The Maillard Reaction

Discovery and significance

One of the most common chemical reactions occurring in a food system exposed to heat is the development of brown pigments with subsequent characteristic flavor development. Since the discovery in 1912 by the French chemist Louis Maillard (1912) that the formation of brown or melanoid pigments occurred when a solution of lysine and sucrose was heated, the reaction has been the area of extensive research interest (Fenney et al. 1975; Hurrell, 1980; Kawamura, 1983).

The Maillard reaction is a term used to characterize a great number of related reactions taking place during heating of food. Melanoidin pigments and flavor compounds resulting from the browning of food are usually desirable for satisfactory sensory properties of heated foods (Hurrell, 1980). Occasionally, however, processing methods or subsequent abusive storage treatment of finished products may result in excessive browning. Maillard browning remains a major problem during both processing and storage of dehydrated or semi-moist foods (Labuza et al. 1977). Production of dark pigments, losses in protein solubility,

and subsequent decreases in nutritional value are the possible deteriorative effects of browning. It is the purpose of this review to describe the proposed chemistry of the Maillard reaction, to describe crucial physical factors that effect the extent of the reaction, to examine the methods currently available for quantitation of Maillard reactions, and finally, to examine the effects of browning on the protein quality of foodstuffs.

Chemistry of the Maillard reaction in model systems and foods

The browning reactions occurring in foods may be broadly generalized into three basic types. The first of these, the browning due to the classical Maillard reaction, is characterized by carbonyl-amine interactions and will be the only type considered in detail in this review.

This kind of reaction is called non-enzymatic browning because no enzyme catalysis is involved at any step in the reaction sequence. A second type of browning occurs when polyhydroxy carbonyl compounds, ranging from simple monosaccharides and complex carbohydrates to polylhydroxy carboxylic acids, are heated to high temperatures in the presence of catalytic concentrations of acid or base (Shallenberger and Birch, 1975). These processes, termed caramelization reactions, also play an important role in

color and flavor development of foods. These roles are reviewed elsewhere (Hodge, 1967). A final type of browning reaction occurs via enzyme catalyzed oxidations of polyphenolic compounds such as chlorogenic acid, catechols, catechin, and the poly-carbonyl ascorbic acid (Mason, 1956). Browning of this type is termed enzymatic browning and is of major importance in all facets of fresh produce processing (Matthew and Parpia, 1971).

The first detailed scheme of non-enzymatic browning, and one widely used today, was presented by J. E. Hodge (1953). The reaction may be conveniently divided into three stages, the early stage which is characterized by aldose-amine condensations with little color development, the advanced stage where intermediate browning reaction products produce yellow to light brown pigmentation, and the final stage characterized by intense brown color and flavor development accompanied by strong absorbance in the ultraviolet spectrum by the end products.

The early stages It is generally agreed that the first step in the Maillard reaction sequence is a simple condensation (Figure 1) between a carbohydrate compound possessing a free reducing hemi-acetal or hemi-ketal carbonyl group and the amine group of a free or protein bound amino acid (Hodge, 1953).

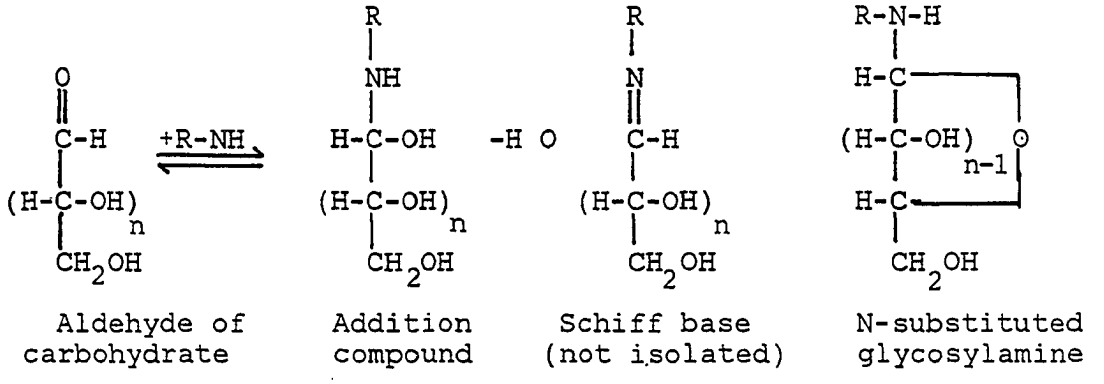

FIGURE 1. Initial stages in the Maillard reaction

The carbonyl group, however, may also be provided by oxidation products of lipids, other proteins, certain vitamins, polyphenols, or certain food additives, and are not solely limited to carbohydrates (Liardon and Hurrell, 1983). On subsequent dehydration, a N-substituted glycosamine is formed in equilibrium with a Schiff base intermediate (Fenney and Whitaker, 1982). Early kinetic studies using simple sugar:amino acid model systems demonstrated that sugar-amine condensation occurs at rates proportional to the alkaline strength of the system (Hodge, 1952). Therefore, the extent of browning reactions would be expected to be greater with higher reaction medium pH. The reaction is also subject to acid or base catalysis (Hodge, 1953). The early stages of non-enzymatic browning occur without any overt changes in product color (Mauron, 1981).

Pentose sugars are generally more reactive than hexose sugars, and monosaccharides more reactive than disaccharides or poly-saccharides in terms of the overall Maillard reaction (Ellis, 1959). The reactivity of a carbohydrate compound in the early stages of browning is not always as clear cut, however. Glucose in solution exists almost entirely in one of several α- or β-pyranose isomers with trace amounts of the free carbonyl form, but is a very reactive sugar in N-substituted glycosamine formation (Fenney and Whitaker, 1982). Fructose, on the other hand, is less reactive than the aldoses in glycosamine formation but it is considered one of the most reactive reducing sugars in the overall Maillard sequence. The amino groups participating in non-enzymatic browning may be contributed by free amino acids or proteins, as well as additive compounds possessing amine groups (Mauron, 1981). The most reactive of the constituent amino acids in a foodstuff is lysine with its free ε-NH_2 group (Bjarnson and Carpenter, 1970). Major involvement of lysine in browning of casein has been well documented (Finot et al. 1968; Henry et al. 1948). Participation of other basic amino acids such as histidine and arginine has been found as well (Evans and Butts, 1951; Patton et al. 1954), but are of lesser importance compared to lysine. The major form of lysine

present in foodstuffs following Maillard reactions is the deoxyketosyl-lysine derivative. The reactivity of other amino acids in browning reactions remains a largely uninvestigated area. Cysteine has been shown to be less stable in the presence of reducing sugars than in model systems without sugar (Miller et al. 1965). Methionine contents in autoclaved casein:glucose mixtures decreased as heat induced browning increased (Rao and McLaughlan, 1967). Rates of methionine loss, however, were slower compared to rates of lysine loss. Tryptophan destruction may also occur in browned proteins (Dworschak and Hegedus, 1974). These latter amino acids, however, especially cysteine and tryptophan, are also very heat-labile making discrimination between losses due to thermal treatment and losses due to participation in non-enzymatic browning difficult.

The N-substituted glycosamine formed via reactions between reducing sugars and simple amine compounds, such as primary aromatic amines and alkylamines, is relatively stable. However, the same reaction using simple amino acids produces products which are unstable and difficult to isolate (Mauron, 1981). This is presumably due to a rapid conversion of the glycosamine to the 1-amino-1-deoxy-2-ketose (Figure 2) by the Amadori rearrangement (Hodge and Rust, 1953; Gottschalk, 1952).

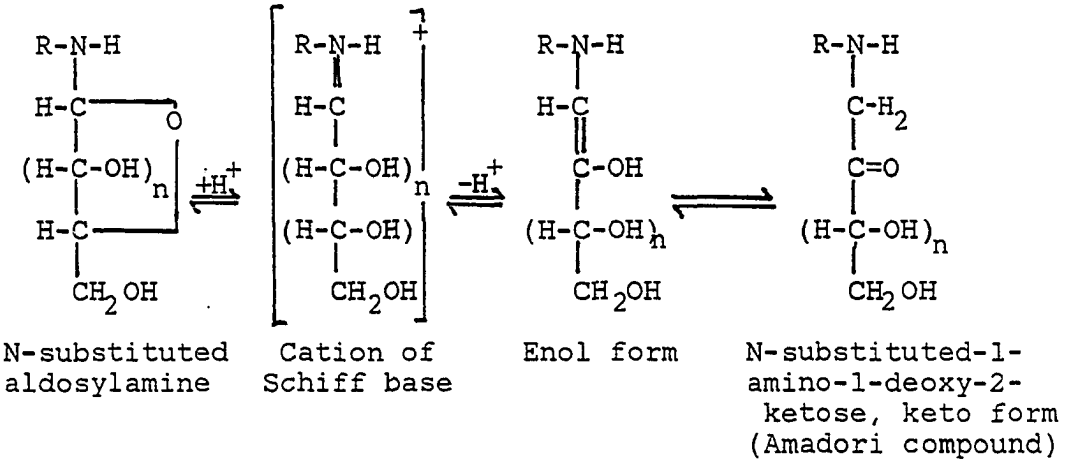

FIGURE 2. The Amadori rearrangement

The critical step in the Amadori rearrangement is the formation of a protonated cation of the Schiff base. Subsequent prototrophic shifts through an enol intermediate result in the Amadori compound (Fenney and Whitaker, 1982). For aldoses such as α-D-glucopyranose the Amadori compound is the 1-amino-1-deoxy-2-ketosyl derivative. Ketoses, such as α-D-fructopyranose, proceed through a ketosylamine addition compound to form the 2-amino-2-deoxy-glucopyranose (Figure 3) by the so-called Heyn's rearrangement (Reynolds, 1965; Kort, 1970).

The advanced stages Upon formation of the Amadori or Heyn's compound further degradation is thought to proceed through three possible pathways (Hodge, 1953). Advanced stage reactions during non-enzymatic browning produce yellow

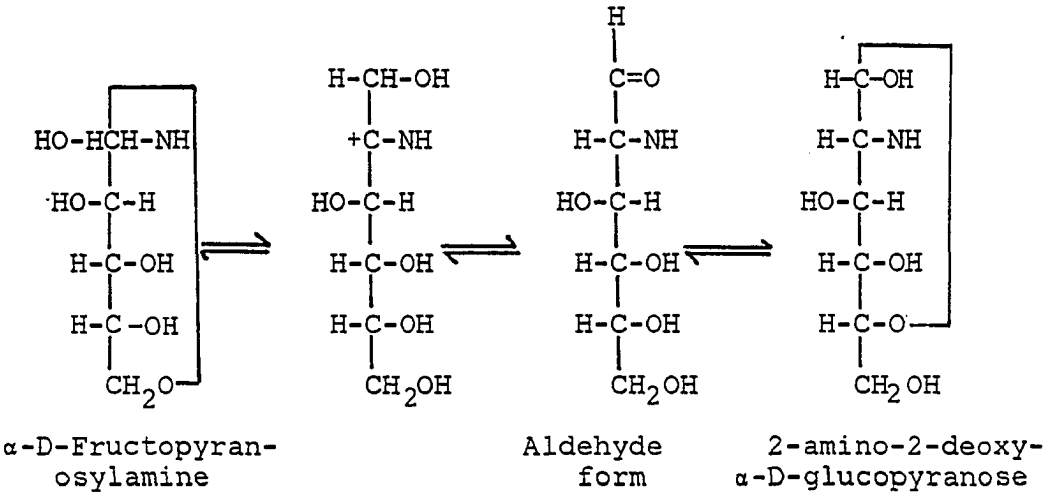

FIGURE 3. The Heyn's rearrangement

to light brown color formation as well as some flavor compounds. The first of these pathways involves 1-2-carbon enolization, and deamination at C_1 to produce a 3-deoxyhexosone (Figure 4). Subsequent dehydration and cyclization result in 5-hydroxymethyl-2-furfuraldehyde (Gottschalk, 1952; Feather and Russell, 1969). Furfuraldehydes are a very commonly isolated flavor compound characteristic of browning reaction (Hodge et al. 1972).

A second pathway is thought to involve C_2 and C_3 enolization of the rearranged compound, followed by C_1 deamination (Figure 5) to form 2,3-dicarbonyl compounds (Feather and Russell, 1969).

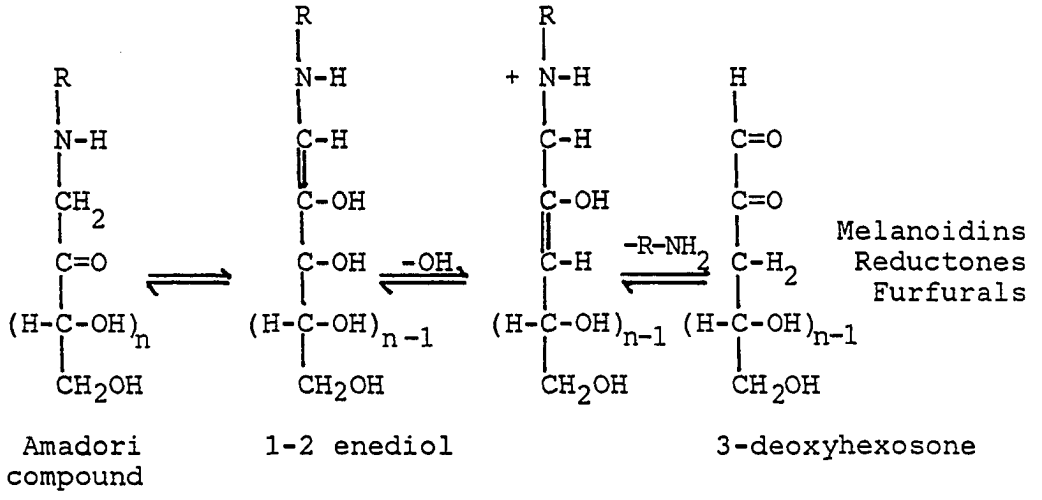

FIGURE 4. 1-2 Enediol degradation pathway

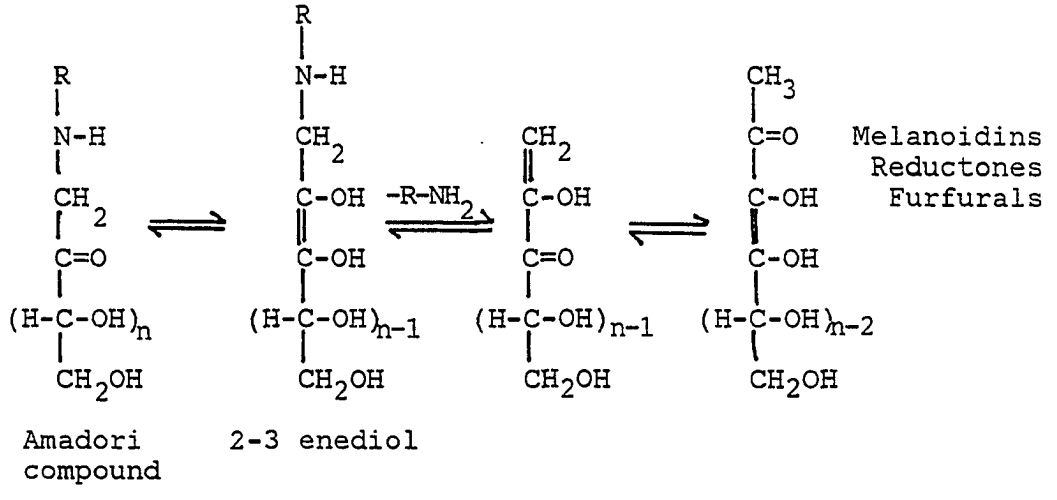

FIGURE 5. 2-3 Enediol degradation pathway

Fission products such as reductones, dicarbonyls, and keto-aldehydes are also commonly isolated from browned

systems. Some end product flavor compounds produced from this pathway are acetaldehyde, diacetal, and pyruvaldehyde.

A third pathway is the Strecker degradation (Figure 6) involving the reaction between α-amino acids and dicarbonyl compounds. Some of the dicarbonyls are formed as Maillard intermediates in the second pathway (Gottschalk, 1972). Volatile aldehyde derivatives of the amino acids (Strecker aldehydes) are important flavor compounds in breads (Folkes and Gramshaw, 1981), and are very commonly isolated in heated potato products (Sullivan, 1981).

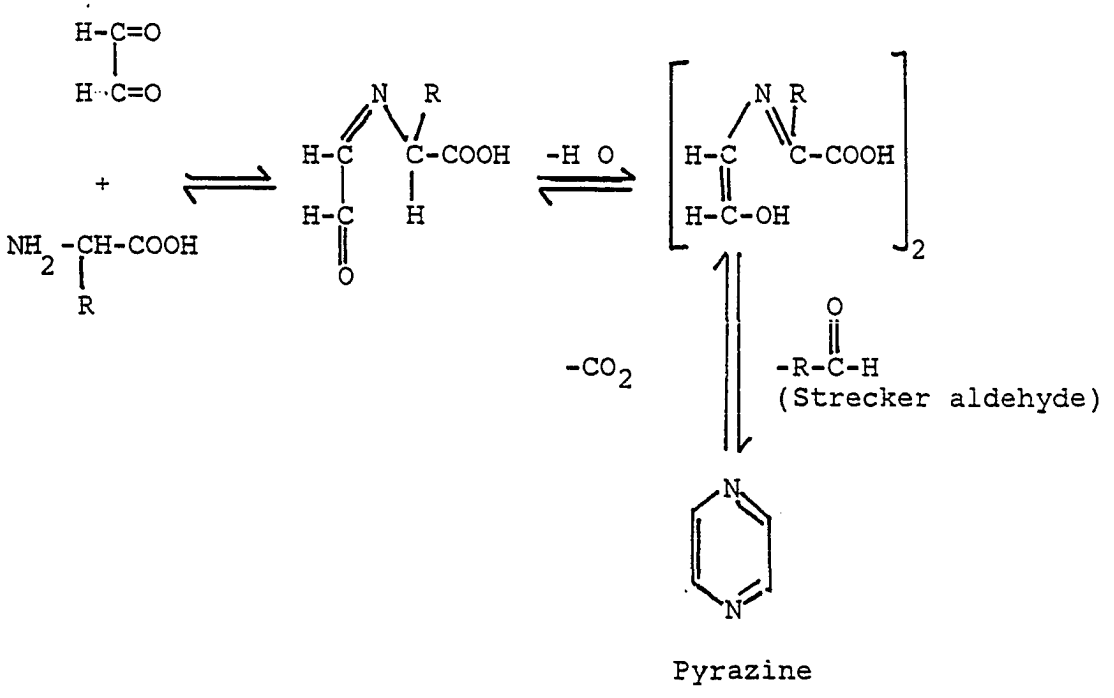

FIGURE 6. The Strecker degradation

Strecker degradation may result in formation of a large family of pyrazine compounds which are an important component of nutty-roasted food flavors (Maga and Sizer, 1973). Strecker aldehydes may also condense with themselves, with other furfural compounds, or with other products of the advanced stage reactions to initiate brown pigment formation.

The final stages In the final stages of the Maillard reaction, the intermediates formed during previous stages polymerize to form unsaturated highly colored polymers (Hodge, 1953). Despite the extensive research devoted to non-enzymatic browning, the final stage reactions that produce the melanoid color compounds are poorly understood. Aldol condensation (Rice et al. 1948), aldehyde-amine polymerizations (Hodge, 1953), and formation of heterocyclic amines such as pyrroles, pyridines, and pyrrolydines are all involved in melanoid formation. Unsaturated carbonyl compounds and furfurals may also polymerize with proteins to form water insoluble brown pigments (Reynolds, 1965). Much of the difficulty in understanding the final stages of Maillard browning resides in the fact that such a large number of highly reactive compounds are generated that study of specific pathways is difficult. Further, melanoids themselves are difficult to

isolate and characterize (Mauron, 1981) although they are relatively unreactive. In addition to the possible deleterious effect of off-flavor and pigment development, final stage Maillard reactions have also been reported to increase the toughening of foods (Labuza, 1973).

Physical and chemical factors effecting the rates of non-enzymatic browning

Several physical or chemical factors present in a system may work separately or cooperatively to effect the extent of a chemical reaction. Three factors classically affect rates of non-enzymatic browning: pH, temperature, and water activity (a_w).

Much of our understanding of the mechanisms of non-enzymatic browning, and the factors which influence browning have been derived from work with simple model systems containing protein or amino acids, and carbohydrate. Simple solutions of a sugar and an amine brown at rates that are proportional to the alkaline strength of the reaction medium, and the reacting amine group. Lea and Hannah (1949), using a casein:glucose model system, demonstrated that alkaline pH values greatly accelerated browning reactions while acidic values exhibited an inhibitory effect.

Wolfe et al. (1977a) used a soy protein and glucose model system to evaluate initial stages of browning. Reaction rates were increased as pH increased from 4.0 to 8.0. An interaction between pH and glucose concentration was noted as glucose levels were more critical in influencing reaction rates at high pH. This interaction is probably due to increased glucose mutarotation and enolization at higher pH values that produce more carbonyl groups to react with amines (Speck, 1958). Lee et al. (1984) used a glucose-lysine model system and studied Maillard reaction kinetic parameters as a function of reaction time, temperature, and pH. The formation of brown pigment, monofructosyl- and di-fructosyl-L-lysine were, followed to evaluate browning.. Reaction rates increased parabolically with increasing pH from 4 to 8. The authors noted a pH 6 break point in rate constants for all temperatures studied. Reaction rate constants increased to a maximum at this pH, and decreased at higher pH values. The effect of pH on initial rates of browning has been attributed to the increase in mutarotation of the carbohydrate moiety, which would increase the amount of the free carbonyl formation, and to the pK_a dependent deprotonation of the amine group in alkaline solution (Fenney and Whitaker, 1982). Therefore, at low pH, the

protonated amine would not be expected to react with reducing sugars.

Increases in temperature have an obvious effect on the reaction rate. This relation is true for most chemical reactions. Maillard (1912) first determined that increasing the temperature increased the amount of pigment formation. Using a casein-glucose model system, Lea and Hamnah (1949) employed the Van Slyke reaction to follow the temperature dependence of amino nitrogen losses due to browning. A 40,000 fold increase in the rate of amino-nitrogen loss was found with a temperature increase of 0 to 80° C. Hurrell and Carpenter (1974) used an albumin and glucose model system to follow losses of the ε-NH_2 group of lysine as a function of both time and temperature. Interestingly, reasonably similar losses were found in samples stored for 30 days at 37°C compared to samples autoclaved for 15 minutes at 121° C. The importance of this finding to the browning of food systems exposed to short or moderate term increases in temperature, as would occur during abusive storage conditions, will be examined further later.

The effect of changes in water content is a crucial component in the study of browning reactions in food systems. Water both contributes to the textural and structural properties of a food and may interact with many

components in the food that will affect nutritive quality and storage stability (Rockland, 1969). An effective indication of moisture relations in a foodstuff is the expression water activity (a_w). a_w is defined as the chemical reactivity of water in a given system and is more commonly defined in the following equation (1):

$$a_w = \frac{V}{V_0} = \frac{ERH\%}{100} \qquad (1)$$

Where V = vapor pressure of water in the foodstuff, and V = vapor pressure of pure water, ERH = equilibrium relative humidity at which no moisture loss or gain to the atmosphere is found for the food. The range of a_w values is from 0.0 for a completely dry state to 1.0 for pure water. The most convenient method to evaluate the effect of changes in a_w on moisture content in a food is with a sorption isotherm (Labuza, 1968). The sorption isotherm of a food is best described as a plot of water content as a function of a_w at a specific temperature. One method for isotherm measurement is to place the food material over appropriate saturate salt solutions which give a specific equilibrium relative humidity, and then measure changes in moisture content of the food material. The importance of a_w in a food system is illustrated by the fact that a hygroscopic food stuff and a non-hygroscopic food stuff may have vastly different

moisture contents at an identical a_w value. This difference may greatly affect the rates of deteriorative processes in the product. The isotherm may be subdivided into several general regions depending upon the state of the water molecules present (Labuza, 1968). Sorption of water at low a_w levels (0.00 - 0.20) is thought to occur in a single layer or monolayer throughout the food material (Rockland, 1969). Successive water molecule layers are then formed as the a_w increases. This region of adsorption of additional water molecule layers occurs roughly from a_w values 0.20 - 0.60. The remaining region (0.6 - 1.0) corresponds to capillary condensation of water in the structure of the food (Labuza et al. 1970). Water in this state is readily available as both solvent and reactant in deteriorative processes. Using ^{14}C-labeled glucose, Duckworth and Smith (1963) determined that water freely diffuses in a foodstuff at a_w values approximating the monolayer.

The effect of variations in a_w on the extent of non-enzymatic browning reactions has been reviewed by several authors (Labuza, 1968; Eichner, 1975; Troller and Christian, 1978). Very little browning normally occurs at a_w values corresponding to the mono-layer moisture level and below. Rates of browning will increase to a maximum in the a_w region of 0.5 - 0.7 and will decrease at values greater than

0.8 (Troller and Christian, 1978). Browning maxima have been found to vary greatly depending upon product composition. The decreased rate observed at high a_w is presumably due to simple reactant dilution. Hannah and Lea (1952), found negligible browning pigment development in ε-N-acetyl-L-lysine and D-glucose model systems at 20% relative humidity (RH). Browning maxima in this system occurred at 40% RH. The same workers (Lea and Hannah, 1949), found maximum browning rates at 70% RH in a casein and glucose dry model system. The data suggested that both decreases in amino nitrogen and color development were a_w dependent. This relationship held true at all temperatures used, 37, 70, and 90°C. This general pattern of minimum browning at very low and very high a_w's with a maximum rate in the intermediate moisture range has been found true in most foods (Labuza et al. 1970).

Analytical Methods for Quantitation of Maillard Browning

Because of the importance of non-enzymatic browning reactions to the processing, physical characteristics, storage stability, sensory acceptability, and nutritive value of many foods, much attention has been given to methodology for qualitative and quantitative estimation of browning in foodstuffs. These methods have focused on two

crucial aspects of the reaction. First, the characteristic color development during browning has given rise to a number of colorimetric assays to measure color development in a browned food system. Second, since amino acids or proteins are a required reactant, much research has been devoted to methods that evaluate chemical changes in critical amino acids during browning. Most interest in chemical changes of proteins and amino acids due to Maillard browning reactions has focused on lysine.

Quantitation of browning pigment

The formation of colored compounds during the Maillard reaction provided the first means of quantitation available. Visual, or spectrophotometric, evaluation of color change is the basis for these procedures. Color evaluation instruments, such as Hunterlab Difference colorimeters or spectroreflectometers, have also been employed as a non-destructive means to evaluate color changes in foodstuffs (Bookwalter and Kwolek, 1981).

Choi et al. (1949) developed a method involving tryptic digestion and aqueous extraction of the melanoid pigments from dried milk powders that is the basis for most current methods. Subsequent modifications of the initial procedure including the use of a multi-enzyme system, a variety of buffers, centrifugation steps, and several filtration

techniques have all been reported in the literature (Labuza, 1973; Warmbeir et al. 1976a). Warmbeir et al. (1976a) reported that the enzymatic digestion technique of Choi et al. (1949) was both accurate and reproducible for non-enzymatic browning evaluation in a wide variety of foodstuffs.

Available lysine after Maillard reactions in foodstuffs

As previously mentioned, the ε-NH_2 group of lysine is the major amine reactant in Maillard reactions. Lysine is also an essential amino acid for which losses due to processing treatment or storage could potentially impact nutritional quality. Several analytical methods for measurement of lysine losses in non-enzymatically browned foodstuffs have been developed. These may be classified into chemical, enzymatic, microbiological, and biological methods based upon animal growth or plasma response to lysine in the diet.

Chemical methods for lysine Chemical methods developed to measure lysine loss attempt to differentiate between those lysine units that possess free unreacted ε-NH_2 groups, and those lysine units whose ε-NH_2 group has reacted with reducing sugars, or other carbonyls. Since the latter would not be biologically available for protein synthesis, the generic name given assays of this type is 'available'

lysine procedures. However, the theory that blocked lysine groups are totally biologically unavailable has been questioned (Carpenter and Booth, 1973; Hurrell and Carpenter, 1981).

Lysine products arising from early Maillard reactions are biologically unavailable, but glutamyl-lysine formed during heat treatment of proteins in the absence of carbohydrates has been shown, upon digestion, to be utilized as a source of lysine by the rat (Hurrell et al. 1976; Waibel and Carpenter, 1972). Further, severe heat treatment of proteins may produce large decreases in protein solubility and digestibility even when some ε-NH_2 groups of lysine remain unblocked (Hurrell et al. 1977). For these reasons, many workers have replaced the 'available' lysine with 'reactive' lysine.

Accurate and reproducible procedures for reactive lysine estimation would ideally interact only with free ε-NH_2 groups of lysine, and not other compounds generated during Maillard reactions. Reaction of the derivatizing reagent with non-lysine amine groups, and of reaction with later Maillard products has presented a problem for many procedures (Booth, 1971).

Because lysine, as well as any amino acid, is ultimately destroyed during advanced Maillard reactions, the

analysis of changes in total lysine by conventional ion-exchange chromatography would seem a simple method for measurement of reactive lysine. However, biologically unavailable lysine forms, such as the N-substituted glycosamines (deoxyketosyl-L-lysine), will yield free lysine upon standard acid hydrolysis in 6N HCl leading to serious overestimation of reactive lysine (Hurrell and Carpenter, 1981).

1-Fluoro-2,4-dinitrobenzene reactive lysine

The first chemical method for quantitation of lysine losses during Maillard-type reactions, and the method largely used today, involves the reaction of the free ε-NH_2 group with Sanger's reagent, 1-fluoro,-2,4-dinitrobenzene (FDNB). FDNB reacts with amino groups under alkaline conditions, and after acid hydrolysis, the resulting yellow N-ε-dinitrophenyl-lysine (DNP-lysine) is measured colorimetrically. This method is also referred to as the 'direct' FDNB method since chemically reactive lysine is measured directly as DNP-lysine. Carpenter and Ellinger (1955) first employed FDNB to measure reactive lysine but significant interference from ε-DNP-arginine, and interference from excess levels of dinitrophenol limited its accuracy. Subsequent modifications to the original procedure (Carpenter, 1960; Booth, 1971) using

methoxycarbonyl-chloride to correct for N-ε-DNP-arginine and histidine interference, and the yellow colored humin compounds generated during acid hydrolyis of carbohydrate containing compounds have greatly increased the utility of the procedure.

The FDNB method of Carpenter has several limitations. FDNB is not water soluble, and the ethanol used as a carrier solvent for the reaction may not be compatible with the protein of some foods (Carpenter and Booth, 1973). One major problem with the FDNB method is that in samples containing high levels of starch or other polysaccharide, variable losses of the ε-DNP-lysine (10-30 %) generated during acid hydrolysis may occur (Hurrell and Carpenter, 1981).

Since DNP-lysine is among the most stable amino acid derivatives to acid hydrolysis under normal conditions (Sanger, 1945), it is thought that reductive, and thus decolorizing, breakdown products of carbohydrate during acid hydrolysis are responsible for the ε-DNP-lysine losses. This limitation is usually overcome through the direct quantitation of DNP-lysine destruction in the sample and the application of an appropriate correction factor, or the use of previously published correction factors (Booth, 1971). Small coefficients of variation (1-5%) are reported in the

literature for the FDNB reactive lysine procedure. A further drawback to this method is that a 16 hr hydrolysis is used which results in a 2 day period required to complete the assay. In addition, FDNB has been shown to react with some later Maillard compounds which yield DNP-lysine upon acid hydrolysis (Finot and Mauron, 1972; Carpenter and Booth, 1973).

In response to the above limitations of direct reaction of FDNB with lysine, Rao et al. (1963) developed an alternative method using FDNB. The theory behind this procedure is that lysine may combine with reducing sugars as well as other compounds that make it unable to react with FDNB, but some of these products will still yield small amounts DNP-lysine during acid hydrolysis. Therefore, a measure of chemically reactive lysine can be obtained if both total lysine content of the sample and the amount of ε-DNP lysine released from other compounds are measured. This provides the basis for the indirect, or 'by difference' FDNB methods. Roach et al. (1967) further improved the procedure which uses ion-exchange chromatography for measurement of total lysine content and treatment with FDNB in the standard method. Reactive lysine is then calculated indirectly by the 'difference' between total lysine and the lysine remaining in solution after separate hydrolysis of

the FDNB treated protein. This procedure, though more tedious, has the advantage of eliminating interference by carbohydrate and other colored compounds such as humin. The 'difference' value represents the lysine groups in the protein that are both reactive and have free ε-NH_2 groups.

2,4,6-trinitro-benzenesulfonic acid reactive lysine Because of the carrier solvent incompatibility, and length of assay time problems with the FDNB method, an alternative method using 2,4,6-trinitro-benzenesulfonic acid (TNBS) and colorimetric determination of reactive lysine as N-ε-trinitrophenyl-lysine derivative (TNP-Lysine) was introduced by Kakade and Liener (1969). The principal advantages of this method are the use of a water soluble derivatization reagent and shorter hydrolysis time (90 min).

Several serious disadvantages also exist. TNP-lysine is even more susceptible to reduction in the presence of carbohydrate than is DNP-lysine (Posati et al. 1972). The TNBS reagent also reacts with the Schiff base compound of early Maillard reactions which may lead to serious over estimation of reactive lysine (Hurrell and Carpenter, 1981). Poor reproducibility of the TNBS method has also been reported (Hall et al. 1973) when it was applied to a wide variety of samples. The TNBS method probably has greatest utility in samples low in total carbohydrate or free of carbohydrate.

Measurement of reactive lysine by guanidination O-methyl-isourea (MIU) reacts with free-NH_2 groups to yield homoarginine (Bujard and Mauron, 1964), which upon acid hydrolysis, is quantitated by conventional ion-exchange chromatography, or gas chromatography (Nair et al. 1978). Accurate estimations of reactive lysine by the MIU reaction have been reported for both early and advanced stages of non-enzymatic browning. Routine application of the MIU method to reactive analysis remains difficult, however, because of the 48 hr reaction time required. The fact that different reaction pH values are needed depending upon sample composition, and the obligatory use of an amino acid analyzer or gas chromatograph (Maga, 1981) also make routine application of this method difficult.

Reduction with sodium borohydride Another compound that has been used to directly measure reactive lysine is sodium borohydride (Couch and Thomas, 1976), which reduces the deoxyketosyl-lysine (Amadori compound) generated during early Maillard reactions to a derivative stable to acid hydrolysis. Lysine released after acid hydrolysis following borohydride treatment represents the unreacted lysine units.

The furosine method The basic amino acid ε-N-(2-furosylmethyl)-L-lysine occurs as one of the many

degradation products of fructosyl-lysine (Hurrell and Carpenter, 1981). Finot and Mauron (1972) generated a model compound, N-formyl-(ε-N-deoxyfructosyl)-L-lysine, which upon acid hydrolysis yielded 32% furosine. Ion-exchange chromatography can then be used to quantitate the amount of furosine in the browned sample. Increasing furosine concentration is inversely proportional to reactive lysine and can, therefore, be used to evaluate the extent of maillard reactions.

Dye-binding methods The use of dye-binding capacity to quantitate protein content has been used for some time (Udy, 1971). Very good agreement has been reported between dye-binding capacity and the contents of FDNB-reactive lysine, histidine, and arginine in foodstuffs (Hurrell and Carpenter, 1975). Further modifications of the dye-binding procedure (Hurrell and Carpenter, 1979) use propionic anhydride as a specific propionylation reagent for lysine. The basis for the procedure is that the difference between the total basic amino acid dye-binding capacity of a foodstuff and the dye binding capacity of a propionylated sample yields a measure of the propionylated lysine. This is called the dye-binding lysine (DBL) method. The method has been demonstrated to be reasonably satisfactory with severely heated samples, but lacks sensitivity for

application to samples undergoing only early Maillard reactions (Hurrell and Carpenter, 1981).

Biological methods

Microbiological and enzymatic methods for reactive lysine The use of micro-organisms with absolute requirements for particular nutrients has long been employed for qualitative nutrient analytical work. Nearly all early total lysine values for foodstuffs were obtained with microbial assays (Ford, 1981). Several workers have applied this technique to the measurement of reactive lysine. Bell et al. (1977) used a mutant Escherichia coli strain and developed an accurate, rapid method for lysine determination by following induction of a lysine-dependent enzyme unique to this organism. Apart from this procedure, few organisms have been found that have an absolute lysine requirement.

Several proteolytic organisms have been used, however. The protozoan Tetrahymena pyriformis W has amino acid requirements similar to man and is often used as a microbial assay for lysine (Shorrock, 1976). Warren and Labuza (1972) used Tetrahymena to evaluate early non-enzymatic browning reactions in a casein:glucose model system. The microbial assay was found to provide comparable reactive lysine values, when compared to the FDNB procedure, for advanced stages of browning. The Tetrahymena assay was found to overestimate reactive lysine in early browning stages.

In an attempt to mimic proteolytic conditions in mammalian digestion, a number of *in vitro* procedures utilizing proteolytic enzymes have been developed for measurement of reactive lysine. Trypsin, with an active site specific for lysine, has been used (Carpenter and Booth, 1973), as well as several other enzymes. These methods however, because of very large variability, and poor correlations to traditional methods, have found limited utility for reactive lysine measurement.

In vivo methods for reactive lysine The ability of an organism to digest a protein, and to absorb and utilize the constituent amino acids, is the basis for protein quality determination. Digestibility and bioavailability of individual amino acids can be measured through several methods (Carpenter, 1981). Measurement of the digestibility of lysine in heat treated or browned proteins is obtained by difference between food and fecal lysine content (Waring, 1969; Soares and Kifer, 1971; Varnish and Carpenter, 1971). These methods, however, are complicated by methodological problems, such as synthesis and utilization of lysine by intestinal flora, that render interpretation of lysine digestibility values difficult.

The same basic principles are involved in the measurement of lysine bioavailability by following plasma

response to dietary protein. Studies of this type have provided valuable information regarding the in vivo utilization of Maillard and heat-damaged proteins (Rao and McLaughlan, 1967).

Nutritional bioassays utilizing unit weight gain per unit of a particular nutrient have also been used to evaluate lysine bioavailability (Carpenter and Booth, 1973). Their method has been the basis of many protein quality bioassays. Nevertheless, it also is complicated by the same procedural and interpretational difficulties associated with protein quality procedures such as the protein efficiency ratio.

Comparative studies between reactive lysine methods

In an effort to examine the accuracy and reproducibility of the various reactive lysine procedures, several comparative studies have been reported in the literature. Hurrell and Carpenter (1974) evaluated the TNBS, FDNB-Lysine (by difference), MIU, furosine, and borohydride methods, using the direct FDNB-lysine procedure as a reference. Using model systems consisting of purified albumin and glucose taken to early and advanced stages of Maillard reactions, the direct FDNB-lysine and MIU procedures most accurately quantitated lysine losses with minimal error. The latter methods were determined to be the most applicable for the

widest variety of samples. Both the TNBS and FDNB-lysine 'by difference' methods severely overestimated reactive lysine during early Maillard damage.

Rayner and Fox (1978) compared the FDNB, Silcock 'difference', and a procedure using enzymatic digestion with a pronase, for determination of reactive lysine in beef muscles in the presence of added glucose. The workers concluded that the pronase hydrolysis method was perhaps, more sensitive than either the direct or difference FDNB method. While the pronase method may indeed be more accurate in muscle tissue, the applicability of the method to a wide variety of sample types is probably limited.

Creamer et al. (1976) evaluated the FDNB 'by difference' and MIU procedures, and a rat growth assay, for available lysine in heat treated milk powders. In comparisons between the chemical methods, the MIU procedure produced significantly smaller standard deviations and was more reproducible when compared to the indirect FDNB method. The rat bioassay produced large (15-20 %) coefficients of variation, is a common problem in reactive lysine bioassays (Hurrell and Carpenter, 1981). The authors concluded the MIU procedure produced the most accurate and reproducible values. These results are in agreement with those of Hurrell and Carpenter (1974).

Jokinen et al. (1976) compared the direct FDNB and TNBS procedures for reactive lysine losses in a soy:glucose model system. Mean reactive lysine values and standard errors for the TNBS method were found to be 7-12% compared to soy reactive lysine literature values of 5-6%. The authors concluded the TNBS method, though less time consuming, did not provide sufficient accuracy or reproducibility. Opstvedt and Carpenter (1976) compared the FDNB, FDNB by difference, and dye-binding chemical methods, and a chick growth method, for an evaluation of reactive lysine in fish meal. The direct FDNB method gave the best statistical correlation to the animal assay ($r = +0.91$) compared to the other chemical methods.

The ideal method for estimation of reactive lysine would be rapid, simple, and would provide accurate estimates through the early, advanced, and late Maillard reaction stages. The FDNB method, even with the documented disadvantages, still remains the most accurate procedure for all stages of the Maillard reaction, and for the greatest variety of foodstuffs (Hurrell and Carpenter, 1981). Biological methods, though perhaps more relevant to extrapolation of reactive lysine to protein quality, remain difficult and impractical for routine analytical work.

The Effect of Maillard Browning Reactions on Nutritional Quality of Proteins

When protein-containing foods are severely heated during processing or subsequent storage there is often a decrease in nutritive value. McCollum and Davis (1915) first demonstrated that significant losses of the nutritive value of milk occurred upon exposure to heat. These decreases were attributed to deteriorative changes in casein. Mitchell and Block (1946) concluded that decreases of biological parameters of protein quality seen in explosion puffed and roller dried cereals were associated with losses of essential amino acids.

Much of the early work concerning effects of Maillard reactions on protein quality has been done with dehydrated milk products, these being the only protein food with a large reducing sugar content (Finot and Mauron, 1972). Henry et al. (1939) studied the changes in protein quality in dried skim milk powders processed by spray drying or roller drying. They reported no change in protein value due to processing. However, many other workers (Schroeder et al. 1953; Kraft and Morgan, 1951) have documented 50-75% loss of protein nutritive value in milk powders heat treated under similar conditions. The conflicting nature of those early reports can probably be attributed to wide differences

in techniques used to process the powder, species dependent variation, and methodological errors in in the protein quality bioassays used by these workers. Mauron and Mottu (1958) compared the differences in protein quality in milk powders under carefully defined spray drying or roller drying processing conditions. Protein efficiency ratio (PER) decreases in roller dried powders ranged from 3 to 82% and reactive lysine losses ranged from 18 to 72%. The decreases in protein quality were nearly completely reversed with lysine supplementation of the animal ration. Bjorck et al. (1983) evaluated the effect of extrusion processing on protein nutritional value of a protein enriched cereal-based biscuit. Maillard reactions during extrusion decreased biological value (BV) from 73 to 60% and net protein utilization (NPU) value from 72 to 57%. Linear correlations ($r = +0.99$) were obtained for the relationship between FDNB-Lysine and biological measures of protein quality.

Changes in protein quality under varied storage conditions as a result of non-enzymatic browning have also been extensively studied. Henry et al. (1939) examined changes in PER values in spray dried skim milk powders stored at 37°C constant temperature and at variable moisture content. The powders underwent marked moisture dependent decreases (from 88 to 65%) in BV that were entirely restored

upon lysine supplementation of the ration. Decreases in protein quality were greater at a_w 0.55 than at 0.30. Browning in corn-soy-milk powder blends (Bookwalter et al. 1980; Bookwalter and Kwolek, 1981) stored for 28 days at 54°C or 14 days at 60°C resulted in a 28% decrease in reactive lysine, and 45% decrease in PER. Groundnut products (Anantharaman and Carpenter, 1969) stored at 75°C and 6% moisture for 13 weeks underwent significant decreases in both FDNB reactive lysine, and protein biological value. Total reactive lysine losses were greater when 16% sucrose was added compared to addition of 22% starch.

Maillard browning reactions in a variety of staple foods are thought to cause decreases in protein quality in one or more of the following ways. First, the decreases in bioavailability and actual destruction of essential amino acids, resulting from Maillard reactions, severely affect the protein quality of a non-enzymatically browned foodstuff. Lysine is a first limiting essential amino acid in many cereal and cereal:legume blended products and losses during processing or storage may significantly reduce protein quality. Amadori compounds of lysine are not biologically available (Hurrell and Carpenter, 1981). This conclusion does not appear to be wholly true for Schiff base compounds. Finot et al. (1977) found bioavailability, as

per cent utilization, of fructose and lysine model Amadori compounds to range from 5-9 % in the rat model, and Schiff base compound utilization to be nearly 100%. These findings may be of importance in view of the fact that 2-15% of the lysine in heated milk products may be in the Schiff base form (Finot and Mauron, 1972). Second, decreases in protein digestibility arise due to several events in the Maillard reaction. Erbersdobler (1976) found that during in vitro enzymatic hydrolysis, only 10% of the lysine residues with fructose blocked ε-NH_2 groups were released. Steric hindrance of trypsin action was postulated. Significant decreases in apparent protein digestibility were found in heat treatment (80 to 121°C) of casein and glucose or fructose model systems (Knipfel, 1981). Reactions between the ε-NH_2 groups of lysine and the carboxyl group of either aspartic or glutamic acid residues (Hurrell and Carpenter, 1976; Valle-Riestra and Barnes, 1970) may also form crosslinkages through Maillard reactions which are resistant to the action of digestive enzymes.

Third, possible toxic or mutagenic compounds generated by advanced and final stage Maillard products may further interact with proteins to produce decreases in nutritive value. Knipfel (1981) found significant decreases in food intake in rats fed diets containing autoclaved casein and

reducing sugars. Tanaka et al. (1977) found significantly increased liver weights, and increases in biochemical parameters indicative of hepatic damage in rats fed browned albumin as a sole protein source. Barnes et al. (1983) isolated several mutagenic N-heterocyclic primary amines from a variety of proteinaceous foods. While it seems certain that products of severely browned protein foods exhibit mutagenic activity in short term assays, such as the Ames mutagenicity test, the long term potential implications of these findings have not been extensively investigated and will require much further work (Lee et al. 1981).

Application of Steady and Non-Steady State Kinetics to Changes in Reactive Lysine and Non-Enzymatic Browning

Accurate knowledge of deteriorative changes that occur in a food system, and the magnitude of parameters that affect those changes, is valuable knowledge to the food scientist. An examination of the literature reveals, however, that comparatively little data are available concerning kinetics of nutrient quality change with processing or storage for many foodstuffs. Carpenter and Booth (1973) noted a lack of kinetic information concerning decreases of reactive lysine in foodstuffs during non-enzymatic browning. Rates of nutrient deterioration in a

food can usually be represented by a simplified zero- or first-order reaction rate expression (Labuza, 1979).

As with the Maillard reaction, much of the available data regarding reactive lysine loss have been derived from model systems. Lea and Hannah (1949), in studies on Maillard reactions in casein:glucose model systems, determined that reactive lysine losses were best described by a mono-molecular first-order rate equation. First-order rates for lysine loss have also been documented for other model and real food systems (Tsao et al. 1978; Lee et al. 1984). Labuza et al. (1977), reported that the rate of development of brown melanoid pigment from Maillard reactions is best described by zero-order kinetics. However, FDNB-reactive lysine in soy protein isolate systems has been shown to proceed through three phases instead of the simple first-order process (Wolfe et al. 1977b). The first phase was found to be best described by first-order processes. Phase two showed a statistically significant increase in FDNB-reactive lysine, and the third phase was characterized by a zero-loss period where the model system was stable to further lysine loss. The authors suggested that these data, though in contradiction to most other work, indicate that different protein sources may behave differently with respect to changes in reactive lysine.

Kinetic studies have also been useful in examining parameters affecting browning during varied storage conditions. Fabriani and Frantoni (1972) examined the effect of varied storage temperature on FDNB reactive lysine losses in pasta. Significant losses (90%) were found with high temperature holding for 15 to 20 months. Warmbier et al. (1976a), in storage studies with an intermediate moisture model foodsystem of casein and glucose, adjusted to a_w values of samples from 0.14 to 0.86. Browning maxima occurred at a_w 0.45 to 0.55. The rate of non-enzymatic browning was 33 fold faster at 45°C compared to 25°C. Steady-state as well as non-steady-state (sine-wave temperature fluctuation) temperature regimes were used to examine the kinetics of browning and protein quality change in whey powder (Labuza and Saltmarch, 1981a). Both FDNB-reactive lysine, and Tetrahymena thermophilis procedures were used to evaluate protein quality. Storage temperatures of 25, 35, 45, and 55°C , and a_w values of 0.33, 0.44, and 0.65 at each temperature were used. Significant browning and protein quality losses, as measured by either the chemical or microbial method, occurred at a_w 0.44. The authors concluded that significant protein quality loss could occur during normal food processing or warehouse storage if moisture content was not controlled.

Labuza et al. (1982) used the same protocol to evaluate browning and protein quality changes in egg noodles upon storage. As expected, both browning and protein quality losses increased with increasing temperature and a_w. Twenty-five percent losses of reactive lysine occurred in 40-50 weeks at 25°C, whereas losses of the same magnitude at 45°C were seen in 6-12 weeks. The significant losses at 'consumer' storage conditions may indicate that short term temperature abuse could be detrimental to protein nutritional quality of stored products. In further work with pasta (Chen et al. 1983), significant temperature dependent protein quality losses, as measured by FDNB-reactive lysine, or the Tetrahymena Thermophila growth assay, occurred after 1 year at any temperature above 30°C.

Despite the fact that studies examining the effects of varied processing and storage parameters on kinetics nutrient quality change have increased in the last 5 years, many authors have strongly suggested more work be done in this area and that particular emphasis be given to increasing the variety of food products investigated (Chen et al. 1983; Labuza and Saltmarch, 1981a; Labuza et al. 1977).

METHODS AND MATERIALS

Cowpea:Corn Powder Production

California black-eyed peas (Vigna sinensis) and stone milled corn flour were obtained fresh from a local food cooperative. One to two hr prior to extrusion processing the whole cowpeas were preground in a Fitzpatrick hammer mill equipped with a 0.093" screen. This pregrinding was found to both facilitate extrusion and to produce good product uniformity. When performed just prior to processing, the grinding did not contribute significantly to the development of lipoxygenase catalyzed off-flavor compound production. Since the ultimate intended use of the product is as a weaning food supplement in underdeveloped countries, a formulation of cowpea:corn flour was devised to meet minimum FAO/WHO standards of crude protein and energy content. A 70:30 (w/w %) cowpea:corn flour mixture was found to best meet these requirements and was used for the present research. Fifty to 100 lb batches of the mixture were weighed and uniformly blended in a commercial animal feed mixer. Because of the low total lipid content of the blend, corn oil was blended in at a level of 2% (w/w) to facilitate extrusion.

The raw mixture was then extruded with an Insta-Pro 2000R low-cost extruder cooker (Triple F Feeds, Inc., Des Moines, IA) at a barrel temperature of 170°C. The extruder barrel was brought to the correct temperature by extrusion of raw whole soy beans prior to extrusion of cowpea:corn mixture. The first portion of the later mixture was then discarded to avoid product cross contamination by the extruded whole soy beans. The resultant pellets were spread on drying sheets, allowed to cool at room temperature and were packaged in Kraft paper bags. Upon return to campus, the product was then ground to pass a 100-mesh sieve in a Fitzpatrick hammer mill. A random 10 kg portion of the ground product was taken with a riffle sampler and was stored at -80°C until it was used subsequently.

Crude Protein/Total Nitrogen

Total nitrogen was determined on replicate samples of the cowpea:corn flour by the standard macro-Kjeldahl procedure (Am. Assoc. Cereal Chem. Off. Methods 46-10). Digestions and distillations were performed with a Tecator block digestor model DS-6 (Tecator, Inc., Boulder, Colo.) and a Tecator KJELTEC I Distillation system. Total crude protein contents were calculated using a conversion factor of 6.25. Total nitrogen values are expressed on a dry sample basis.

Trypsin Inhibitor Activity

As an indicator of the adequacy of processing, trypsin inhibitor activities were determined in triplicate on samples of the raw ingredients, the raw formulation, and the extruded product using the standard method of Kakade et al. (1974). Values were calculated as Trypsin Inhibitor Units (TIU)/ mg dry solids.

Sorption Isotherm

A sorption isotherm for the flour was obtained using the Proximity Equilibration Cell (PEC) technique described by Lang et al. (1981). The cells were fashioned from capped Mason jelly jars containing the following appropriate saturated salt solutions giving the indicated a_w (Greenspan, 1977) [LiCl - 0.11; $KC_2H_3O_2$ - 0.22; $MgCL_3$ - 0.33; K_2CO_3 - 0.44; $Ca(NO_3)_2$ - 0.55; $NaNO_2$ - 0.65; NaCl - 0.75; $(NH_4)_2SO_4$ - 0.82; KNO_3 - 0.95]. Aluminum weighing boats with pre-cut holes of approximately 4 cm in diameter were fit with 5.5 cm circles of Whatman No. 1 filter paper. Following a 24 hr equilibration period, the weight of the aluminum dish and paper support was recorded to the nearest 0.1 mg. Approximately 1.7 g of sample was weighed into each cell, sealed, and the weight changes of the sample cells were used for each a_w. Following equilibration, sample moisture

contents of quadruplicate samples were determined by drying in a vacuum oven for 18 hr at 70°C and 29.2 ins Hg.

Equilibration/Sample Preparation

Approximately 400 g of cowpea:corn flour were humidified over specific saturated salt solutions in vacuum desiccators which had been covered to exclude light. A temperature controlled environment of 25°C was used for the equilibration procedure. The saturated salts chosen and their respective a_w's were: K_2CO_3 - 0.44; $Ca(NO_3)_2$ - 0.54; and $NaNO_2$ - 0.65. a_w's chosen for the present study were selected to mimic conditions of moisture changes that could occur during processing or storage in a humid tropic/sub-tropic environment. An a_w of 0.75 was originally included, but was later omitted because of obvious mold growth in the flour. No mold growth was found in any of the remaining samples. Vacuums were broken approximately every 2-3 days, the product was stirred, and the vacuum re-applied. The flour was allowed to equilibrate for approximately 4 weeks. Final equilibrated a_w of the flour was measured with a Beckman JEL- 20 relative humidity monitor (Beckman Instruments, Englewood, NJ). When triplicate sample measurements taken from random locations in the equilibrating flour yielded the target a_w, the sample was considered equilibrated.

Triplicate 5 g samples of the equilibrated flour from each a_w were sealed in Wheaton screw-cap glass sample vials and stored at 25, 35, 45, and 55°C for 15-21 weeks. The temperatures were selected to mimic exposure of the product to abusive temperature storage in tropical/sub-tropical environments. Sample bottles stored at 25 and 35°C were double dipped in paraffin to prevent moisture loss. Bottles at 45 and 55°C were double sealed with Parafilm laboratory film both screwed directly to the bottle and surrounding the cap. Samples were withdrawn from the incubators at three week intervals. Samples were then transferred to aluminum weighing dishes and moisture was removed by drying in a vacuum oven at ambient temperature and 28.5 ins Hg for 48 hr. Previous experimentation with samples equilibrated at the highest a_w used showed that the 48 hr period was sufficient to dry the sample as adequately as conventional heated vacuum oven methods.

Non-Enzymatic Browning

Determination of the extent of non-enzymatic browning in the samples was made with a modification of the enzymatic digestion method of Choi et al. (1949). The assay is based on the principle that the Maillard reaction brown pigments can be extracted in an aqueous solution after enzymatic

digestion of the pigments from the peptide chains. Approximately 0.35 g of sample was accurately weighed into a 50 ml Erlenmeyer flask. Fifteen mls of distilled water was added, the solution was mixed well, and was allowed to equilibrate in a 37°C shaking water bath for 30 minutes. An enzyme solution (9.0 mg/ml) of bovine pancreatic protease (#-4630, Sigma Chemical Co., St. Louis, Mo.) was prepared in distilled water and adjusted to pH 7.5 with 0.1N NaOH or 0.1N HCL. Samples were then adjusted to pH 7.5, and one ml of enzyme added to each. Following digestion for 30 minutes in the water bath, 1.0 ml of 50% (w/w) acetic acid was added to stop the reaction. The flasks were allowed to stand in the bath for an additional 15-20 minutes. The hydrolyzate was centrifuged at 10,000 g for 15 minutes and the supernatant decanted into a 10 ml glass leur-lock syringe. The sample was filtered through a Millipore Swinnex 13 filler unit fitted with a 13 mm Gelman Gn-6 membrane filter (0.45 uM retention size). If the filtrate was cloudy it was refiltered. The optical density of the filtrate was measured at 420 nm against a distilled water blank carried through the same procedure. Sample browning was expressed as 0.1 absorbance unit/gm dry solid. Duplicate analyses were performed for each sample.

Reactive Lysine Determination

FDNB-reactive lysine was determined by method of Carpenter (1960) modified by Booth (1971). Several additional modifications also were made. Sample sizes to give approximately 12 mg of lysine (1.2-1.5 g) were weighed accurately into 100 ml round bottom flasks. Ten mls of 0.94 M $NaHCO_3$ was added to each of the flasks, and they were shaken at 37°C for 1 hr in a shaking water bath. Fifteen mls of 1-fluoro-2,4-dinitrobenzene (FDNB) (Nutritional Biochemicals, Chicago, Ill.) solution containing 0.4 ml FDNB/15ml 95% ethanol was added to each flask, and the flasks were shaken at room temperature for 3 hr. Thereafter, the procedure followed was that described by Booth (1971).

The following additions to the procedure were made. Triplicate aliquots of the filtered hydrolyzate were carried through the procedure. Blanks were prepared in duplicate. Aliquots containing known contents of dinitrophenyl-L-lysine-hydrochloride (Sigma Co., St. Louis, Mo.) were carried through each analysis as standards. Ethyl ether was prepared fresh every 2 days by glass re-distillation from a dilute sulfuric acid/ferrous sulfate solution and storage over clean iron wire. Ether held more than 48 hr was again re-distilled and stored as described above prior to

analysis. FDNB-reactive lysine values reported were calculated as mg FDND-reactive lysine/100 g dry solids. FDNB-reactive lysine values reported here are uncorrected.

Given the fact that all samples withdrawn from storage could not be analyzed simultaneously, the order of analysis was randomized by temperature and a_w to remove any experimental error introduced by the order of sample analysis.

In vitro Protein Digestibility

The in vitro protein digestibility of the samples was determined with the multi-enzyme procedure of Satterlee et al. (1982). Triplicate analyses were performed using ANRC casein as a reference standard. Hydrolyzate pH (+- 0.001) was recorded with a Fisher Accumet pH meter (Fisher Scientific Co., Pittsburgh, PA).

Experimental Design

The overall purpose of this research was to investigate the effects of differences in a_w, temperature, and length of storage on in vitro measures of protein quality, and the kinetics of lysine loss due to non-enzymatic browning. Replication at temperature, a_w, as well as sample replication, were considered important in examining the

kinetics of protein quality changes as accurately as possible. Given this goal, and logistical constraints upon incubator availability, a 4x3 factorial randomized block design was devised. Individual samples were processed and stored at 25, 35, 45, or 55°C with a_w's of 0.44, 0.55, or 0.65 within each temperature. The variable 15-21 week storage period was defined as the block. Grouping and assignments of sample temperatures and a_w were all performed randomly as described by Cochran and Cox (1950). The experiment was then repeated for three storage periods to provide the desired replication at temperature and a_w effects.

The rationale for selection of this protocol was to provide greater statistical accuracy in the prediction of the kinetic parameters over the single study design commonly employed in similar storage stability studies. The experiment was designed to examine the affect of varied steady-state storage conditions on protein quality change, and was not designed to examine protein quality changes resulting from extrusion processing. Because operation of the extruder used to process the cowpea:corn flour cannot be strictly controlled, the same 10 kg random sample was used for the entire study. Therefore, batch to batch variation in protein quality of the extruded product was not considered in this design.

Statistical Analysis

Statistical analyses were performed using the Statistical Analysis System (SAS). Differences due to main effects of a_w, temperature, and length of storage time were evaluated using linear regression analysis (Cochran and Cox, 1950). Comparisons between individual factors in the experimental model were made using sub-division of sums of squares. Probability levels of less than or equal to 0.05 were considered significant for all statistical procedures.

Kinetic parameters for Maillard browning reactions in the cowpea:corn flour were calculated as follows. Reaction rate constants (k) were determined after application of linear regression to determine the best-fit regression lines based upon a first-order rate equation (2):

$$A_L = A_o e^{-kt}$$

Where A_o = FDNB reactive lysine concentration at time zero, and A_L = FDNB-reactive lysine concentration after storage for t weeks, k = the first-order reaction rate constant, and t = time. Log (e) transformation was performed for lysine loss data to determine the first-order rate constant. Slopes derived from the three replications of the study were combined to allow calculation of mean values, and standard errors for prediction of k.

Temperature dependence of the reaction was evaluated by several methods. Application of the Arrhenius model (Moore, 1972) allows calculation of the Arrhenius activation energy (E_a) following a plot of ln k vs. 1/T (equation 3).

$$k = k_o e^{-Ea/RT}$$

Where k = first-order reaction rate constant, k_o = frequency factor, Ea = energy of activation, R = gas constant, and T = reaction temperature (degrees Kelvin). Linear regression analysis was applied to this plot to determine the best-fit regression line and residual errors. The Q_{10} value is a measure of the effect of 10 degree incremental increases in temperature on the k of a given reaction. Q_{10} values were calculated using equation (4):

$$Q_{10} = \frac{\text{rate K at temp A + 10'°C}}{\text{rate K at temp A}} \qquad (4)$$

Statistical accuracy for determination of the above kinetic parameters was based upon replication of kinetic parameters for the purpose of calculation of standard errors, and followed the recommendations of Lenz and Lund (1980).

RESULTS AND DISCUSSION

Crude Protein and Trypsin Inhibitor Inactivation

Crude protein contents based on Kjeldahl N analysis are given in Table 1. The cowpea:corn flour blend was formulated to comply with the weaning food supplement for crude protein level suggested by the FAO/WHO. The crude protein content of the blend was 18.7% and was formulated to provide 5 g of protein/100 g when reconstituted with a 1:5 ratio of product to water.

TABLE 1. Crude protein content of the LEC processed cowpea:corn flour blend

Sample	%N	% Crude protein
Raw cowpea	3.6 ± 0.0[a]	22.6[b]
Raw corn flour	1.4 ± 0.1	9.2
Cowpea:corn flour (raw)	3.0 ± 0.2	18.5
Cowpea:corn flour (extruded)	3.0 ± 0.3	18.7

[a] Values given are x ± S.D. quadruplicate determinations.

[b] % N x 6.25.

The distribution of trypsin inhibitor (TI) in the raw materials, TI level in the raw cowpea:corn flour blend, and

the effect of low-cost extruder cooker (LEC) processing on TI activity are given in Table 2.

TABLE 2. Change in trypsin inhibitor activity with LEC processing in the cowpea:corn flour food system

Sample	TIU		
	Raw	Extruded	% Change
100% Cowpea	10.6 ± 0.5 [a]	1.7 ± 0.2	-84
Cowpea:corn flour blend	10.6 ± 0.3	1.7 ± 0.1	-85

[a] Values given are Trypsin Inhibitor Units (TIU)/mg dry solids for triplicate determinations.

Trypsin inhibitors are a class of heat-labile protease inhibitors present in many legumes (Liener and Kakade, 1980). These proteins are thought to bind and sterically inhibit digestive proteases and, therefore, can decrease the protein nutritional quality of the food product. Several workers (Liener, 1981; Rackis, 1981) have shown that in animal rations with either purified TI or significant endogenous TI, reductions in total TI activity through heat treatment of greater than or equal to 80% result in product protein quality equivalent to the protein quality of TI free rations. LEC processing at 170° C in the product used here

resulted in a reduction in TI activity from 11.6 to 1.7 TUI/mg, which is 84% destruction.

Sorption Isotherm Determination

Mean percent moisture values achieved with the proximity equilibration cell (PEC) for a spectrum of a_w values are given in Table 3.

TABLE 3. Sorption isotherm moisture contents for cowpea:corn flour

Saturated salt solution	a_w	H_2O (g/100gm solids)
LiCl	0.11	4.4 ± 0.01 [a]
$KC_2H_3O_2$	0.22	5.0 ± 0.1
$MgCl_3$	0.33	5.3 ± 0.2
K_2CO_3	0.44	6.4 ± 0.3
$Ca(NO_3)_2$	0.55	7.0 ± 0.1
$NaNO_2$	0.65	9.6 ± 0.1
NaCl	0.75	11.9 ± 0.4
$(NH_4)_2SO_4$	0.84	14.1 ± 0.2
KNO_3	0.94	21.7 ± 0.3

[a] Values given are x ± S.E. for quadruplicate determinations.

At the a_w values used in the present experiment, 0.44, 0.55, and 0.65, the moisture contents were 6.4, 7.0, and 9.6%, respectively. Moisture contents at the a_w values corresponding to mono-layer levels ($\sim$ 0.11) were 4.4%. The sorption isotherm determined with the PEC technique is given in Figure 7.

A plateau region can be seen from a_w values of 0.11 to approximately 0.55, after which moisture content increases more rapidly with increased a_w. Five basic types of isotherms are recognized for water adsorption (Brunauer, 1945). The sigmoidal shape of the cowpea:corn flour isotherm classifies it as a Type II isotherm. Although specific isotherms for cowpea:corn flour blends are not available in the literature, the isotherms available for both dried pea flour and for corn flour are very similar (Iglesias and Chirife, 1982).

Kinetic Analysis of FDNB-Reactive Lysine Losses

Individual variation in first-order rate constant estimation

First order reaction rate constants calculated from the slopes of the best-fit linear regression lines for FDNB-reactive lysine loss under varied temperature and a_w storage are given in Table 4.

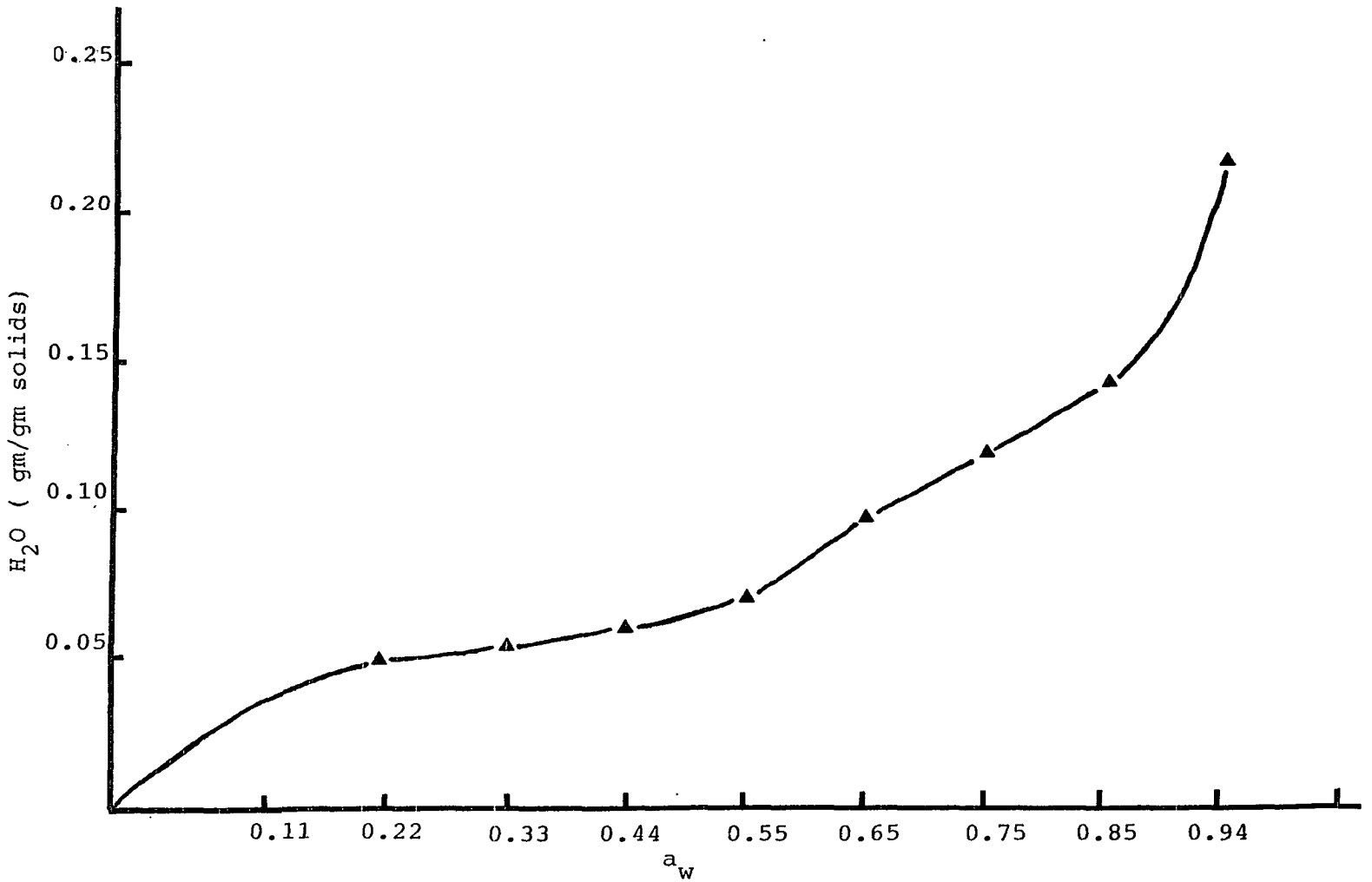

Figure 7. Sorption isotherm at 25°C for the LEC processed cowpea:corn flour

TABLE 4. First order rate constants for FDNB-reactive lysine loss in LEC processed cowpea:corn flour under varied storage conditions

Temperature(°C)	Rep	a_w 0.44	0.55	0.65
25	1	9.2 [a]	11.3	12.6
		(0.784) [b]	(0.765)	(0.950)
	2	12.7	9.2	11.4
		(0.810)	(0.920)	(0.930)
	3	6.2	6.3	6.0
		(0.600)	(0.260)	(0.250)
35	1	12.0	9.2	13.9
		(0.930)	(0.730)	(0.600)
	2	12.3	14.7	9.2
		(0.730)	(0.770)	(0.850)
	3	9.3	12.4	15.3
		(0.624)	(0.852)	(0.850)
45	1	----	------	------
	2	8.7	16.2	11.8
		(0.672)	(0.860)	(0.870)
	3	17.8	18.7	20.8
		(0.850)	(0.890)	(0.980)
55	1	20.7	15.2	19.6
		(0.880)	(0.910)	(0.780)
	2	18.0	16.2	20.1
		(0.500)	(0.890)	(0.863)
	3	19.5	24.5	33.6
		(0.854)	(0.920)	(0.992)

[a] Values given are first-order rate constants (k) x 10^3 in $weeks^{-1}$.

[b] Coefficient of determination (r^2) for best-fit linear regression line.

An important aspect of this research was to provide a

statistically appropriate measure of the variability with which the rate constants can be estimated in this system. Because single replications of a design of this type is the common approach to FDNB-reactive lysine loss kinetic analysis, the variability in the rate constant is usually given over a 95% confidence interval based upon one value for a given rate constant. While this does provide useful information, a more appropriate variance estimate, such as the standard deviation or standard error, is more desirable. Table 4 provides calculated rate constants for each replication, and at each temperature and a_w. It is hoped that this approach will better illustrate rate constant variation. As seen in Table 4, there is considerable variability among replicates of rate constants across the a_w values used. R^2 values for some replicates were low ($r^2 < 0.50$) suggesting some non-linearity. Figure 8 provides a typical first-order plot FDNB-reactive lysine loss at 25°C and varied a_w.

Since plots of every replicate would be redundant, examples at each temperature are provided. Absolute losses of FDNB-reactive lysine over 15 wks at 25°C when averaged over all replicates were -12.7, -14.3, and -16.5% for a_w's of 0.44, 0.55, and 0.65, respectively. The poorest r and r^2 values were found at 25°C where the least change occurred.

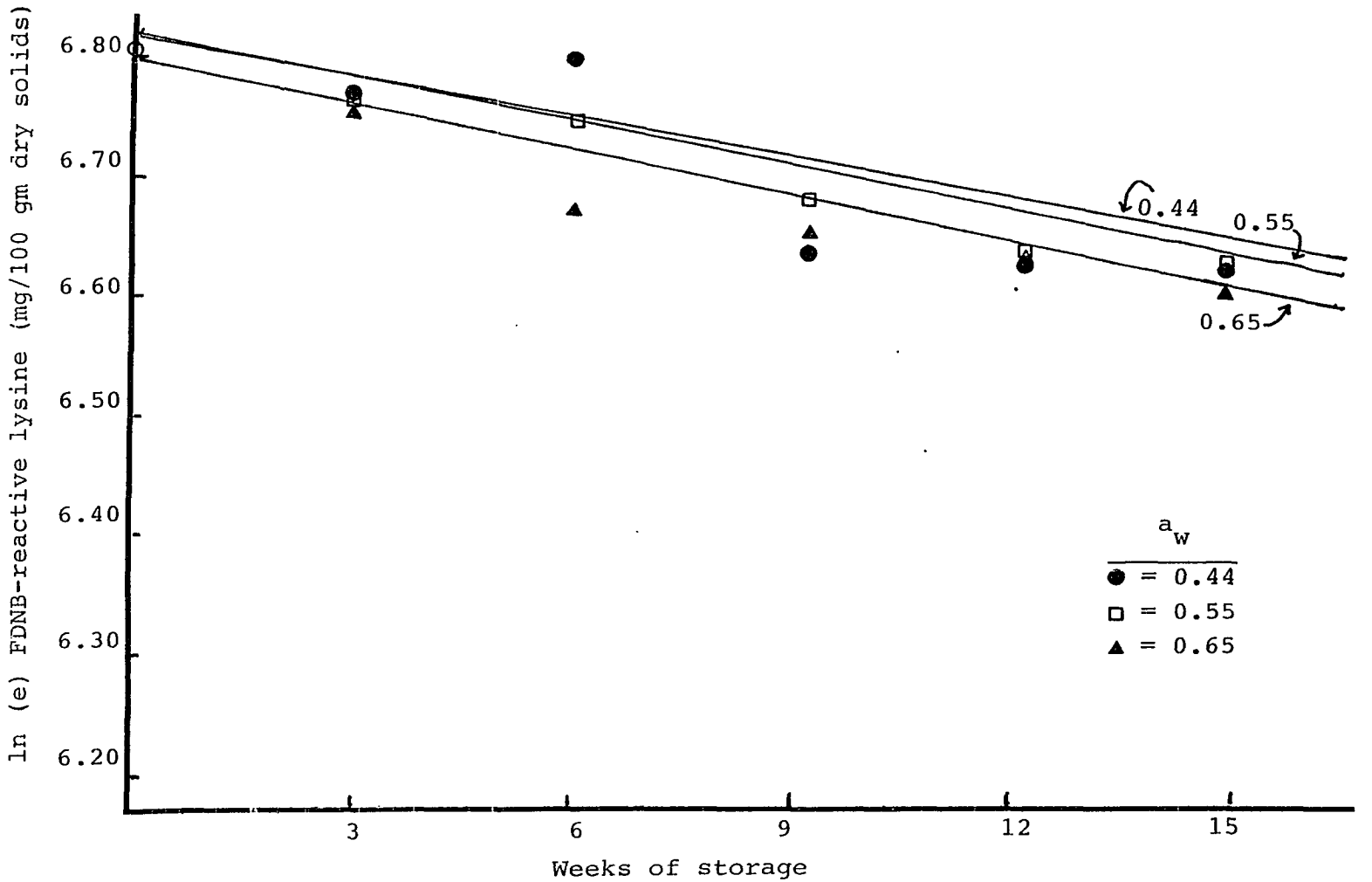

Figure 8. FDNB-reactive lysine loss in LEC processed cowpea:corn flour at 25°C and varied a_w

The problem of poor correlations, non-linearity, and increased variability at ambient storage temperature is a problem that also has been found in whey protein systems (Labuza and Saltmarch, 1981a) and in pasta (Labuza et al. 1982; Chen et al. 1983) over the same temperature and a_w storage conditions. The problem may be due to the fact that the length of time of most storage studies (< 48 wks) may not be sufficient to obtain a true estimate of the rate constant at these lower temperature storage conditions.

Individual rate constants and r^2 values for FDNB-reactive lysine losses at 35°C are improved over those from 25°C (Table 4). Based on zero-time FDNB-reactive lysine values, end-point reactive lysine losses after 15 wks of storage at 35°C were -18.1, -15.6, and -17.0% for a_w's of 0.44, 0.55, and 0.65, respectively. A typical first-order plot of lysine losses at 35°C is given in Figure 9.

The rate constants and r^2 values for FDNB-reactive lysine stored at 45°C are given in Table 4. The first 15 wk period at 45°C produced rate constants that were lower than these for 25°C and r^2 values of < 0.30. The reason for these results remains unclear. Because this pattern was not repeated in subsequent replication of the 45°C storage period, these data were not included in Table 4. The data used to derive the rate constants, however, were included in

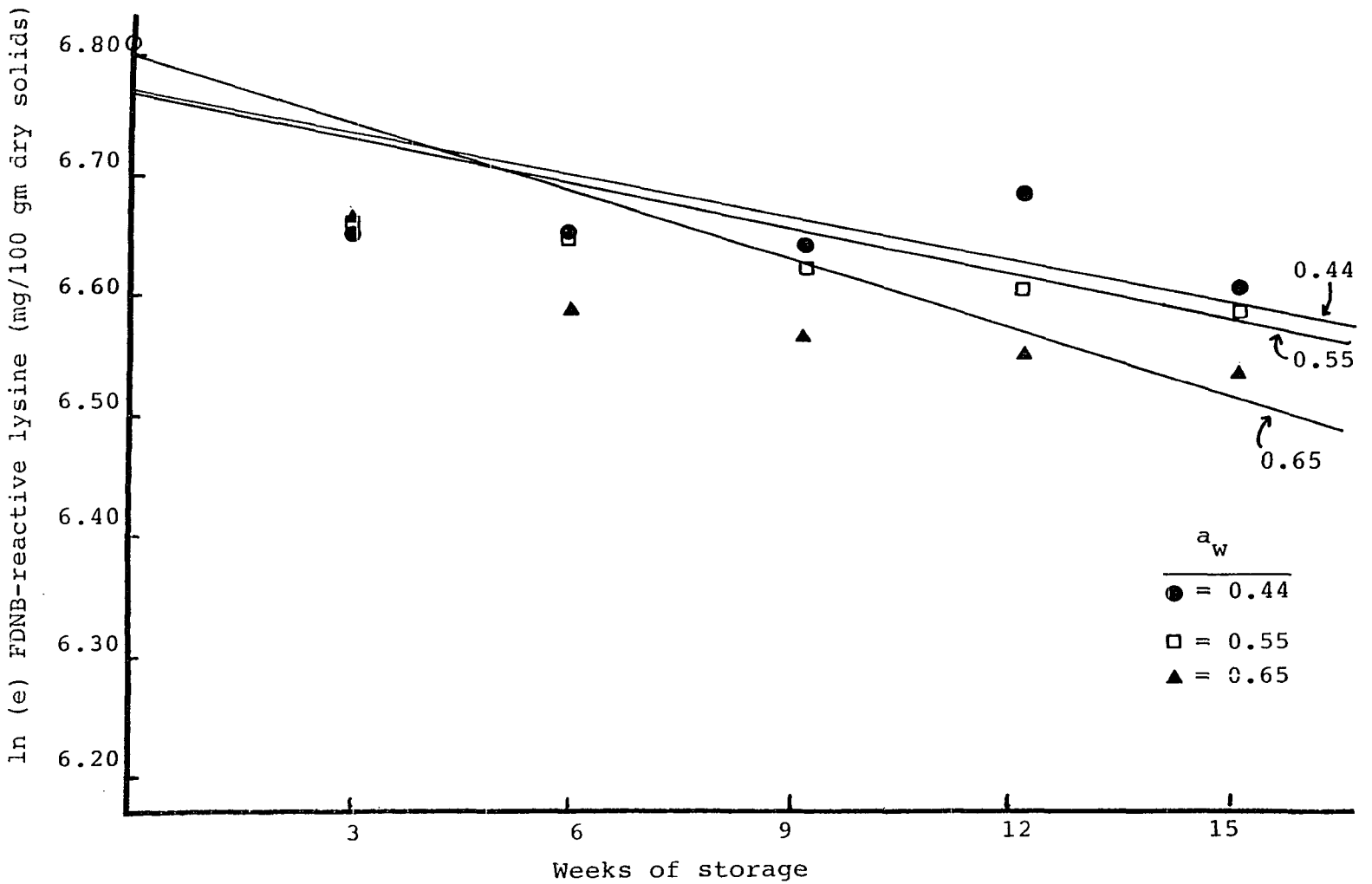

Figure 9. FDNB-reactive lysine loss in LEC processed cowpea:corn flour stored at 35°C and varied a_w

the statistical analysis of the experiment. Compared to zero-time FDNB-reactive lysine values, end point reactive lysine losses during storage at 45°C were -19.6, -23.5, and -21.4% for a_w's of 0.44, 0.55, and 0.65, respectively. Mean FDNB-reactive lysine loss values for some points during the 45°C storage periods were still more scattered than is desirable (Figure 10).

First order reaction rate constants for FDNB-reactive lysine loss for replications of the 15wk storage periods at 55°C are found in Table 4. Some low r^2 values are found during high temperature storage as well, but this trend was not consistent across all replications. Final FDNB-reactive lysine losses were -22.0, -24.1, and -31.3% for a_w's of 0.44, 0.55, and 0.65%, respectively. One trial at 55°C and a_w = 0.65 storage was extended to 18 to 21 wk storage. After 18 wks, FDNB-lysine losses were -37.2% and after 21 wks, the losses were -46.8% when compared to initial values (Figure 11).

The problem of data-point scatter resulting in some low r^2 values is apparent at low a_w at this temperature. One possible solution to this problem in this system would be to decrease the sampling intervals to 2 wks which would increase the number of data points and could improve the r^2 value.

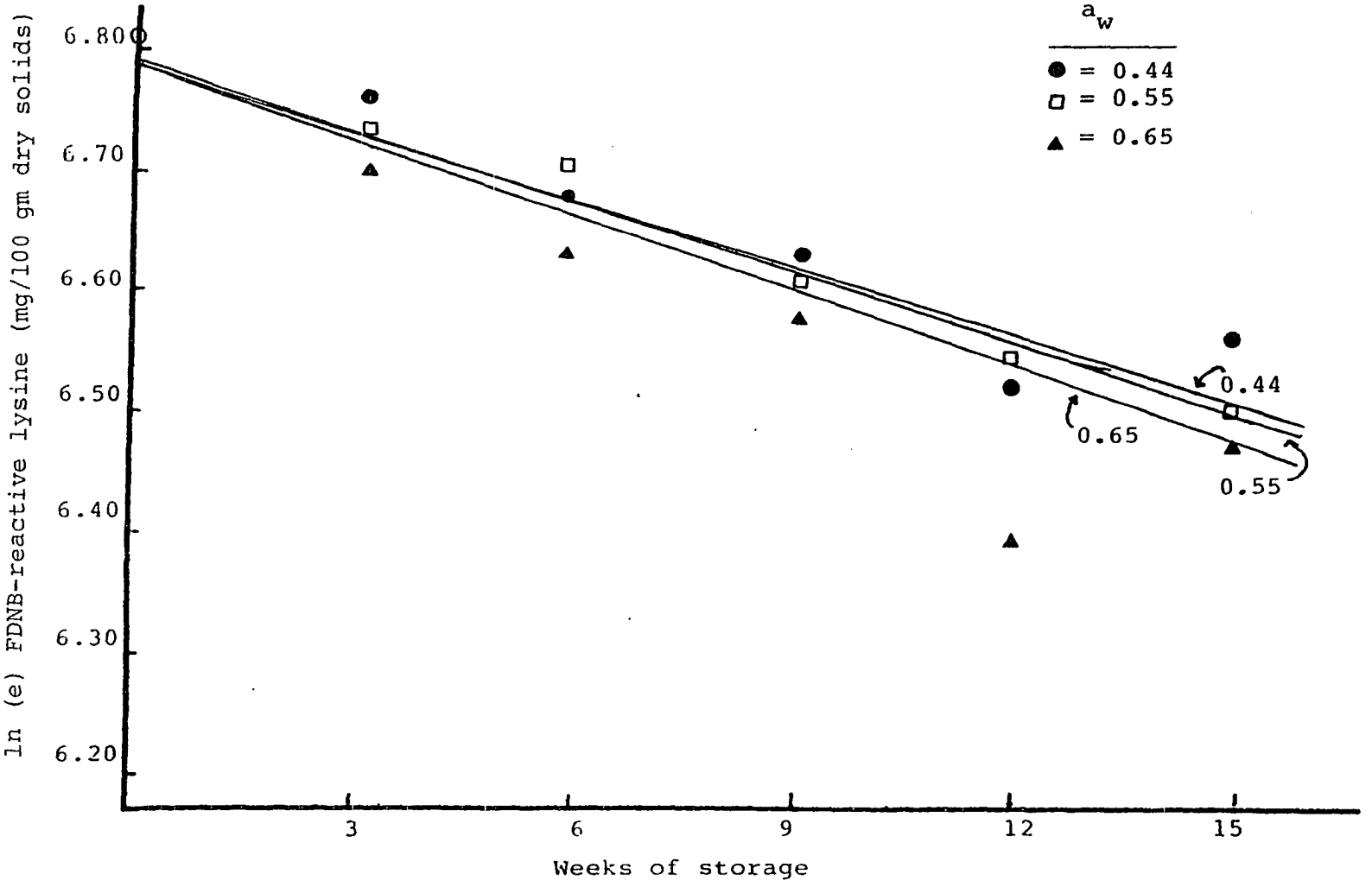

Figure 10. FDNB-reactive lysine loss in LEC processed cowpea:corn flour stored at 45°C and varied a_w

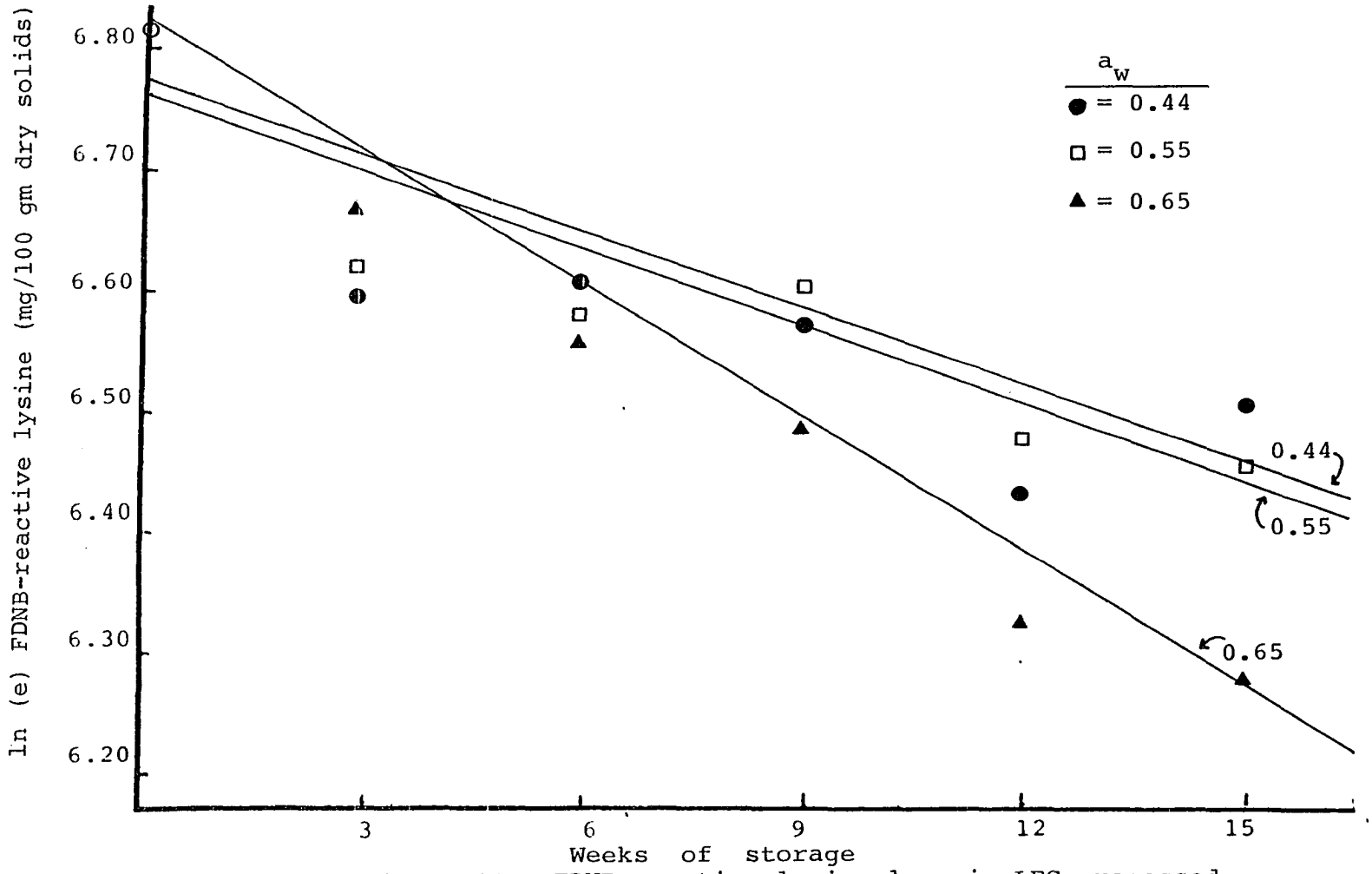

Figure 11. FDNB-reactive lysine loss in LEC processed cowpea:corn flour stored at 55°C and varied a_w

An important aspect of any kinetic work is the precision with which experimental data are obtained. A measure of this precision was obtained by determining both the average standard deviation across all sample means, and the relative percent variation across all sample means. The mean standard deviation across all FDNB-reactive lysine determinations was ± 16.4 mg FDNB-reactive lysine/100 g dry solids. The relative percent variation across all samples was ± 2.1 %. The average coefficient of variation for the procedure was 2.0 %. Labuza et al. (1982) report a 2-4 % relative variation for FDNB-reactive lysine analysis in pasta. Booth (1971) found an overall coefficient of variation of 3.8% when the FDNB-lysine procedure was applied to a wide variety of samples. Analytical precision of the FDNB-reactive lysine analysis in this study is well within these literature values.

Main effects of temperature and a_w on FDNB-reactive lysine loss

A summary of rate constants ± standard error of the mean (S.E.M.) for FDNB-reactive lysine losses in LEC processed cowpea:corn flour under varied storage conditions is given in Table 5.

Values range from a low of 8.92×10^{-3} $week^{-1}$ for 25°C and a_w = 0.55 to 25.9×10^{-3} $week^{-1}$ at 55°C and a_w = 0.65.

TABLE 5. First order rate constants (k) + S.E.M. for FDNB-reactive lysine loss in cowpea:corn flour under varied storage conditions

Temperature (°C)	a_w 0.44	0.55	0.65
25	9.4 ± 1.5 [a]	8.9 ± 1.2	10.6 ± 1.6
	16 [b]	13	17
35	11.2 ± 0.8	12.1 ± 1.3	12.8 ± 1.4
	7	10	11
45	13.3 ± 3.0	17.4 ± 0.8	16.3 ± 3.2
	24	5	19
55	18.5 ± 1.3	18.1 ± 0.9	25.9 ± 3.4
	7	5	13

[a] Values given are x ± S.E.M. for k x 10^3 in $weeks^{-1}$.

[b] Values given are relative percent error (S.E.M./rate constant).

The variability (given %S.E.M. of rate constant) ranged from a low of 5 to a high of 24% (Table 5). Percent error is largest at lower temperatures where the least change occurs, and it is lowest at higher temperatures where the most change occurs. The advantage of this design is evident when compared with 16-67% variation in rate constants over the

same temperature and a_w ranges when $\pm$ 95% confidence intervals are used (Labuza et al. 1982).

Water content and a_w both influence reaction kinetics of deteriorative processes in foods (Labuza, 1980). At any temperature, many deteriorative reactions (including non-enzymatic browning) increase exponentially with increases in a_w above the mono-layer (Labuza et al. 1977). The effect of a_w on FDNB-reactive lysine loss is seen clearly in Table 5. Rate constants increase with temperature and increase at a_w's within each temperature. Maximum values are at the highest a_w with the exception of 45°C where the rate was maximum at a_w of 0.55. Statistical analysis of the temperature, a_w and time effects on FDNB-reactive lysine losses are given in Table 6.

As expected, temperature, a_w, and storage time had significant effects ($p < 0.01$) on FDNB-reactive lysine losses. Of greater interest, however, are the differences among the main treatment effects on lysine loss, and the nature of the response of this food system to increases in temperature and a_w. Length of storage time would be expected to have a significant impact on lysine losses.

Comparison of F-values for temperature and a_w effects (Table 6) suggest that in this food system increasing temperature has a more significant effect on the rate

TABLE 6. Statistical analysis of FDNB-reactive lysine loss kinetics

Source	df	M.S.	F-value	Level
Block (Blk)	2	0.0298 [a]	13.88	p<0.01 [b]
Temperature (Temp)	3	0.00750	35.71	p<0.001
Linear	(1)	0.2175	103.57	p<0.001
Lack of fit (LOF)	(2)	0.004	1.78	NS
Blk*Temp (error a)	6	0.0021		
A_w	2	0.0230	11.5	p<0.01
Linear	(1)	0.0447	22.35	p<0.01
LOF	(1)	0.0013	0.65	NS
Blk*Temp+ Blk*Temp*A_w (error b)	14	0.0020		
Time	5	0.2016	100.0	p<0.001
Linear	(1)	0.9817	490.0	p<0.001
LOF	(4)	0.0046	2.28	NS
Time*Temp	15	0.0070	3.4	p<0.001
Lin*Temp	(3)	0.0274	13.7	p<0.01
LOF	(12)	0.0017	0.85	NS
Time*A_w	10	0.002	0.92	NS
Temp*Time*A_w	30	0.0038	0.10	NS
Residual	108	0.002		
Total	203			

[a] Mean square error.

[b] Not significant.

constants for lysine loss than does increase in a_w. The influence of increasing temperature and a_w are linear ($p <$

0.01) and no significant evidence of non-linearity was found. Jokinen et al. (1976), in a study of the effects of glucose, temperature and a_w on FDNB-reactive lysine losses in a soy protein model system, found linear temperature but non-linear a_w effects. However, it cannot be absolutely concluded that increases in a_w have a linear effect on lysine loss kinetics in the present study because only a narrow a_w range was used for storage. Further study with a wider range of a_w's along the LEC cowpea:corn flour isotherm must be done to confirm the a_w response of this system. Kinetics of FDNB-reactive lysine loss in soy isolate model systems appear to follow first-order kinetics up to a 50% loss, and then appear to follow a zero-order model (Wolfe et al. 1977a). Although a first-order model was found to best describe the FDNB-reactive lysine losses in the cowpea:corn flour system, the maximum absolute losses were only 48% when compared to zero time values. Further work with this system is suggested to determine whether a first-order kinetic model best describes data for FDNB-reactive lysine losses of greater than 50%. Comparisons of rate constants for FDNB-reactive lysine losses in the present study to previous work shows that in both pasta (Labuza et al. 1982) and whey powder (Labuza and Saltmarch, 1981a) their reaction rates are faster, and that the magnitude of temperature and a_w

effects are larger. For example, a 15-fold increase in lysine loss rate constant at a_w = 0.65 was found between storage at 25°C and 55°C in pasta. A 3-fold increase under these same conditions was found in the present study. Several explanations for the differences are possible. First, because of the differences in sorption isotherms of foodstuffs, large differences in moisture contents can occur at identical a_w. The sorption isotherm for pasta showed that at a_w of 0.44 moisture content is approximately 11.5%, and at a_w of 0.65, moisture is nearly 15%. Percent moisutre at the same a_w's for the cowpea:corn flour was 6.4 and 9.5%, respectively. In a theoretical comparison of reactive lysine rate loss constants in two foodstuffs whose total moisture contents at an a_w of 0.50 are 5 and 15%, respectively, temperature effects may be expected to have a greater impact on lysine losses in the lower moisture product when compared to the higher moisture product. More chemically reactive water available to participate as a solvent produces faster rates of browning.

Second, some differences could be attributed to variation in total protein and reducting sugar content. FDNB-reactive lysine losses would be expected to be more rapid in systems containing high levels of reducing sugar, such as dried whey. Although no reducing sugar analyses were performed in the present experiment, or the study with

pasta, it is possible that the difference in total carbohydrate could result in more total reducing sugar and, therefore, result in increased rates of FDNB-reactive lysine loss. Dried milk products contain significant amounts of reducing sugar and would be expected to brown rapidly when heated.

An important facet in kinetic analysis of nutrient changes in foods is confirmation of the kinetic model best describing any change. As previously mentioned, losses of FDNB-reactive lysine due to temperature and a_w changes are best described by a first-order equation. The linear component of time in this study was significant ($p < 0.001$) whereas the non-linear component, as might be important if zero- or second-order kinetic models best described the data, was not significant (Table 6). The linear effect of time was significant ($p < 0.01$) across temperatures indicating that slopes of the best-fit regression lines (and therefore the rate constants) are significantly different (Table 6). The Time x a_w interaction, however, was not significant, which further suggests that the main effects of a_w may not be linear over the entire a_w spectrum. The nature of the response of protein quality change in this system when exposed to varied environmental conditions should provide valuable information to the food processor handling products of similar composition.

The magnitude of % FDNB-reactive lysine losses was such that only after 21 wks of storage at the highest temperature and a_w (55°C and 0.65) did losses approach the 50% decrease required for a half-life (-46.8%). Absolute substantiation of first-order rate constants theoretically requires the reaction to be carried out through 2 half-lives (Labuza, 1979). Where applicable, it is desirable to carry a reaction through 5-6 half-lives (Lenz and Lund, 1980). Over the 15 wk periods of this experiment only 12-18% losses of FDNB-reactive lysine occurred with 25°C and 35°C storage which may reduce reliability of the rate constants even with high r^2 values (Labuza, 1979).

Very rarely, however, can practical experiments examining FDNB-reactive lysine losses under varied storage conditions be designed to carry the reactions through 2 half-lives at every temperature. Because of the fact that rates of lysine loss will be dependent upon the particular products sorption isotherm, storage periods required to achieve losses equal to 1-2 half lives will vary greatly. In the cowpea:corn flour system used for this experiment, storage periods of 110-160 wks (>2 yrs) would be required to achieve 2 half-lives at 'ambient' storage temperatures of 25 or 35°C. Food processors in this country, or any country, are not likely to have inventory stockpiled for 2 yrs. In

the case of surplus commodities stored in government commodity programs, there is opportunity for storage periods in excess of 24 months.

Options available to scientists to increase reliability of kinetic data for FDNB-reactive lysine loss are to increase storage time, or to design experiments to more accurately evaluate experimental error and variability. Since the cowpea:corn system used here was stored for 15-21 wks, the latter option was chosen to improve reliability of the kinetic data.

Temperature dependence of FDNB-reactive lysine losses in cowpea:corn flour under varied storage conditions

Kinetic parameters that describe temperature dependence of reactive lysine losses in the cowpea:corn flour are given in Table 7.

The energy of activation (E_a) was derived from the slope of the best-fit regression line relating ln k vs. 1/T(°K) (Moore, 1972). Because the mean rate constants from Table 5 were used, no measure of standard error is provided. The Arrhenius plots used to determine the E_a values at a_w's of 0.44, 0.55, and 0.65, and 95% confidence intervals for the regression lines, are given in Figure 12, Figure 13, and Figure 14, respectively.

TABLE 7. Temperature dependence of FDNB-reactive lysine loss in cowpea:corn flour under varied storage conditions

	a_w 0.44	a_w 0.55	a_w 0.65
E_a	4.4 [a]	4.9	6.0
r^2 (ln k vs. 1/T)	0.966 [b]	0.931	0.971
Q_{10}			
average	1.3	1.3	1.4
range	1.2-1.4	1.1-1.4	1.3-1.6

[a] Energy of activation (kcal/mol).

[b] Coefficient of determination (r^2) for best-fit regression line.

The E_a is defined as the minimum energy state of molecules participating in a reaction, and it also provides a measure of the temperature dependency of the reaction (Labuza, 1980; Chen et al. 1983). E_a values for FDNB-reactive lysine loss in the present work were 4.4, 4.9, and 6.0 kcal/mol, for a_w values of 0.44, 0.55, and 0.65, respectively. E_a values reported in the literature for a limited number of foodstuffs range from 10-38 kcal/mole indicating a wide variation in temperature sensitivity for FDNB-reactive lysine loss. Increases in a_w may increase or

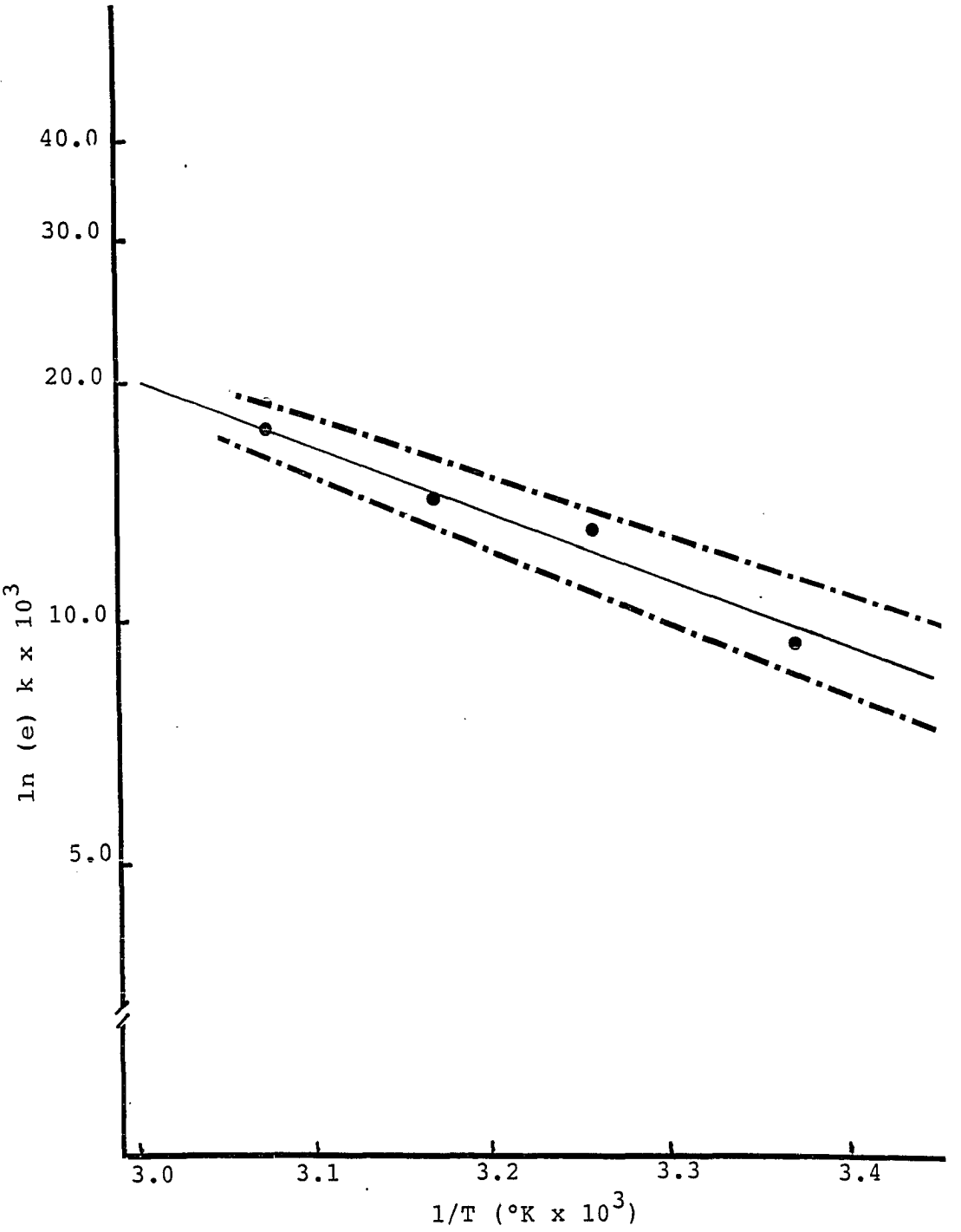

FIGURE 12. Arrhenius plot of steady state rate constants versus reciprocal of absolute temperature for a_w = 0.44

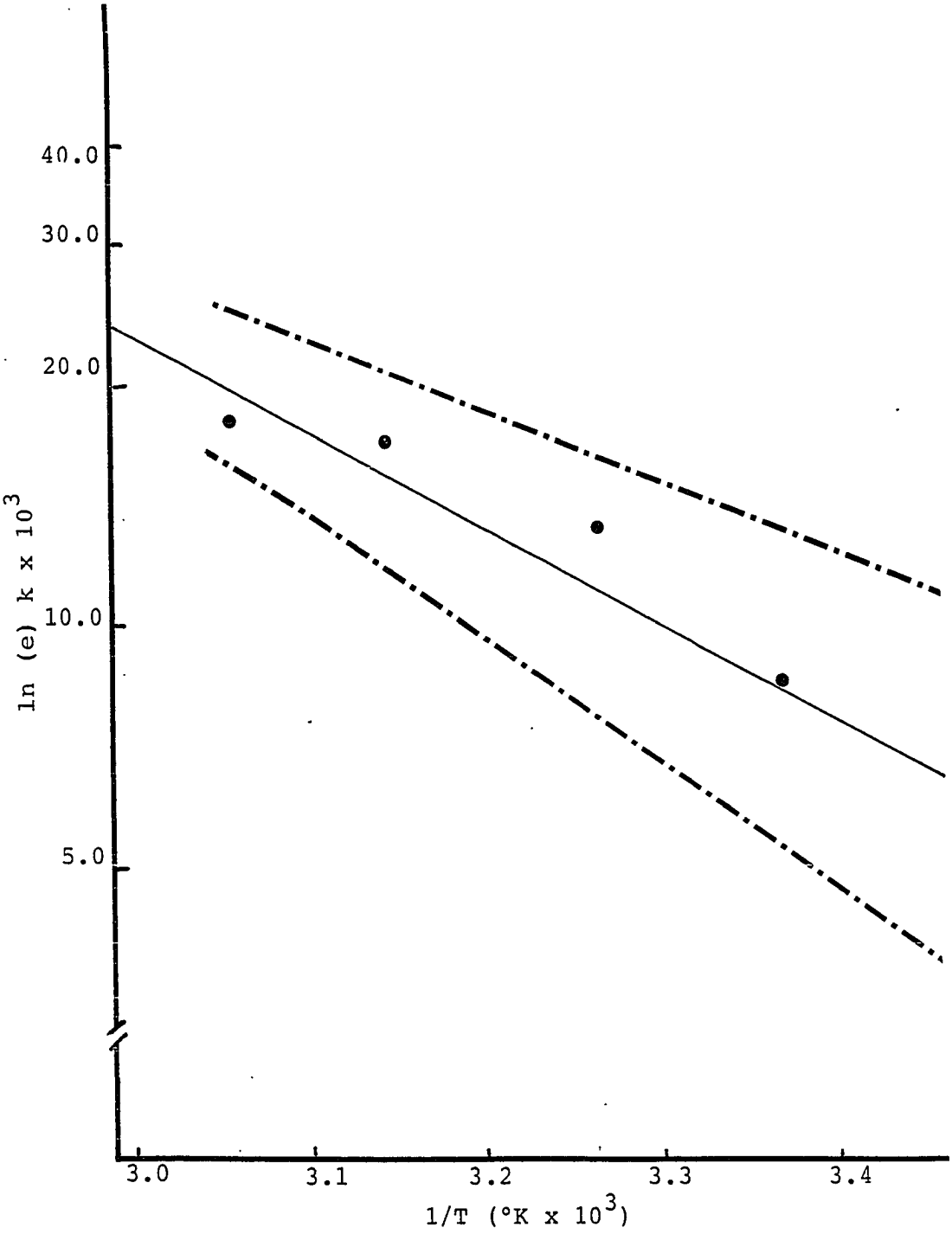

FIGURE 13. Arrhenius plot of steady state rate constants versus reciprocal of absolute temperature for a_w =0.55

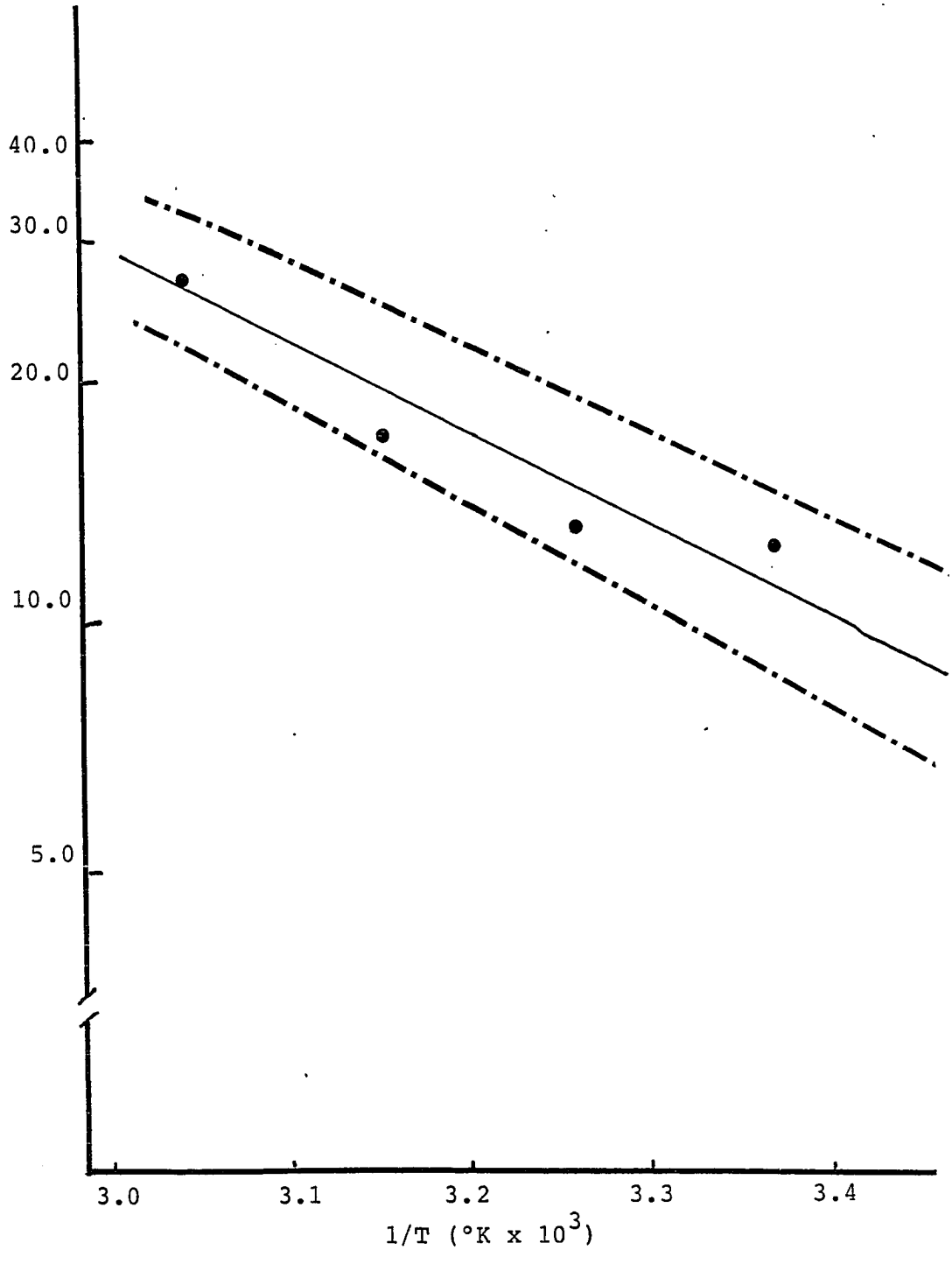

FIGURE 14. Arrhenius plot of steady state rate constants versus reciprocal of absolute temperature for a_w =0.65

decrease the E_a of a deteriorative process, depending upon the food system (Labuza, 1980). E_a values for FDNB-lysine losses in pasta have been shown to be independent of sample a_w (Labuza et al. 1982). E_a values given in Table 7 do show dependence upon a_w, but it is not known whether this dependence is significant. Q_{10} values (the reaction rate increase with a 10°C increase in temperature) are fairly constant which indicates that there are probably insignificant changes in E_a with a_w changes (Saltmarch et al. 1981). Q_{10} values reported in the literature for FDNB-reactive lysine losses for a limited number of foodstuffs range from 1.4-4.7 (Labuza and Saltmarch, 1981b).

Kinetic Analysis of Non-enzymatic Browning Reactions

Results of non-enzymatic browning analyses on the LEC processed cowpea:corn flour yielded only limited information. Quantitation of extractable brown pigment by a modification of the method of Choi et al. (1949) showed no differences in browning for any temperature below 55°C. Only product stored at 55°C and a_w = 0.65 developed sufficient browning to allow calculation of kinetic rate constants (Table 8).

Browning rate constants were calculated based on a zero-order equation (Labuza, 1980; Saltmarch et al. 1981).

TABLE 8. Kinetics of non-enzymatic browning in LEC processed cowpea:corn flour stored at 55°C and a_w = 0.65

Temperature(°C)	1	2	3	X + S.E.
55	11.7 [a] (0.850) [b]	10.5 (0.850)	11.0 (0.950)	11.1 ± 0.3

[a] Values given are rate constant (k) x 10 increase in absorbance per g solid/week^{-1}.

[b] Coefficient of determination (r^2) for best-fit regression line.

Several explanations for this outcome are possible. Because the appearance of brown pigments in the Maillard reaction occurs during the final stages of the process, rates of browning would be expected to be slower and show some lag time compared to FDNB-lysine loss. Browning reactions are influenced by temperature and a_w change in the same manner as reactive-lysine loss. Because formation of the characteristic melanoid pigments during non-enzymatic browning occurs during latter stages of the reaction, a lag time often occurs from beginning of storage to development of measurable browning. Warren and Labuza (1977) found the induction period for browning reactions to be inversely proportional to a_w. At lower a_w's the rates of reactant diffusion and reactant mobility are lower which contributes

to decreased rates of browning (Eichner and Karel, 1972). However, the formation of colorless Amadori compounds during early stages of the Maillard reaction continues over longer periods of time without noticeable browning. This relation is clearly seen in the 12-24 % losses of FDNB-reactive lysine found in this experiment without visible browning. The principal reason for lack of browning in the LEC processed cowpea:corn system is probably the low moisture contents over the a_w range which was used. It is doubtful that increasing length of storage would produce measurable browning because no noticeable change occurred within 15 wks. It seems unlikely that significant browning would occur in a system showing no visible changes in color even if the induction period were > 15 weeks. A further possible explanation for the lack of observable browning may lie with the assay itself. Warmbeir et al. (1976a) report an accuracy of $\pm$0.001 in the browning assay. These results were obtained in model systems, however. Figure 15 provides the data points and best-fit regression lines for browning at 55°C and a_w = 0.65.

There is considerable scatter of the data points at all a_w's. Excessive scatter in browning data points around both temperature and a_w was noted by Labuza et al. (1982) for pasta under storage conditions similar to those used in the

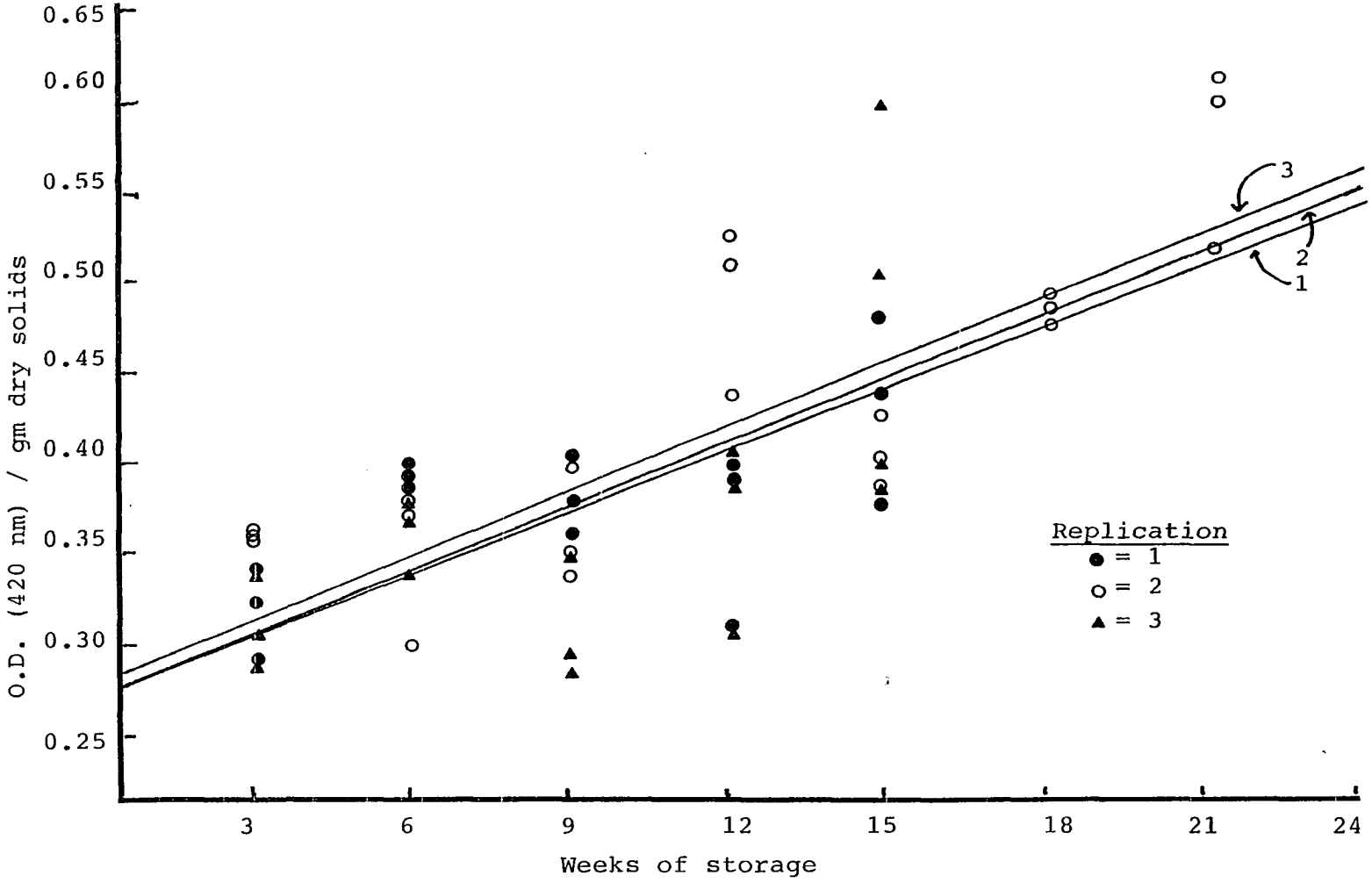

Figure 15. Non-enzymatic browning in LEC processed cowpea:corn flour under storage at 55°C and a_w = 0.65

present study. This variation might suggest that the browning assay, while seemingly accurate and reproducible in simple model systems, may need improvements for suitable application to food systems, or it may need to be adapted to each individual food system. In experiments examining rates of non-enzymatic browning, the observed data point scatter may suggest that collection of accurate kinetic data for browning and, therefore, prediction of shelf-life of foodstuffs is difficult.

Lack of observed browning in the LEC processed cowpea:corn system over the range of temperatures and a_w used in this study suggest that under normal, or even abusive storage, reductions in quality due to browning reactions will very likely not be significant.

Nutritional Significance and Storage Implications

The ultimate utility of kinetic data describing nutrient changes in foods is in their prediction of nutrient quality changes. The times required to achieve a 25% ($T_{¼}$) reduction in FDNB-reactive lysine under specified storage conditions are given in Table 9.

Losses of this magnitude would be biologically significant from a nutritional labeling standpoint (Labuza et al. 1982). The choice of the 25% reduction ($T_{¼}$) as a

TABLE 9. $T_{\frac{1}{4}}$ for FDNB-reactive lysine loss in LEC processed cowpea:corn flour under varied storage conditions

Temperature (°C)	a_w 0.44	0.55	0.65
25	37.0[a] (27.3-55.9)[b]	39.0 (30.6-55.3)	34.8 (27.5-58.5)
35	30.9 (28.2-37.3)	28.6 (23.5-37.7)	27.1 (22.6-37.5)
45	26.1 (19.5-39.8)	19.9 (18.5-21.4)	21.2 (16.6-29.4)
55	18.7 (16.7-22.7)	19.1 (17.3-21.3)	13.6 (10.3-17.7)

[a] Values given are mean time in weeks.

[b] Range of times obtained.

possible critical end point value for shelf-life of the cowpea:corn flour was based upon several factors. Firstly, because the product is intended for use as a high-quality protein supplement, any significant decrease in FDNB-reactive lysine might be expected to decrease the protein nutritional quality of the product. Secondly, reductions of FDNB-reactive lysine of this magnitude are currently being used to evaluate shelf-life and, therefore, could provide a basis for comparison to literature values (Labuza et al. 1982). Finally, based upon the essential amino acid pattern

of the cowpea:corn flour, and comparison to the FAO/WHO reference pattern, any losses of FDNB-reactive lysine result in its becoming the first limiting essential amino acid. If supplementation of the product is intended, the $T_{\frac{1}{4}}$ values will be helpful in evaluating supplementation requirements. The $T_{\frac{1}{4}}$ values show the same effects of variation in temperature and a_w seen in the rate constants. These data indicate that significant losses may occur in stored product that is thermally abused, especially under high humidity storage conditions. In tropical or sub-tropical countries, this may be of practical importance to storage of products of this composition. It is also possible that significant losses could occur under 'consumer' storage conditions, i.e. 25-30°C., over storage periods of < 40 wks. The wide range of $T_{\frac{1}{4}}$ values obtained for lower temperature storage compared to 45 or 55°C storage suggests that accurate estimation of T is difficult. However, these data should be useful for approximation of nutritional quality changes in the cowpea:corn flour system. Although decreases are much more rapid at higher temperatures, it seems unlikely that a product would be exposed to these conditions for any significant time.

The statistical relationship of changes in FDNB-reactive lysine and browning reactions to changes in protein

quality has been previously discussed. Because biological measures of protein quality were not determined in this experiment, the exact biological effects of changes in reactive lysine is uncertain. However, comparisons can be made to previous work with similar losses. Lysine losses of 37% in a freeze-dried cod + glucose system, which are similar to high temperature/high a_w losses in this experiment, produced a net protein utilization (NPU) decrease from 91 to 18 (Hurrell and Carpenter, 1977). Tanaka et al. (1977) found significant decreases in protein efficiency ratios of egg albumin stored 10 days at 37°C and 68% relative humidity. Bjorck et al. (1983) found NPU decreased from 71.1 to 57.7 with a 33% decrease in FDNB-reactive lysine in a protein fortified biscuit.

The in vitro protein digestibility of the most browned cowpea:corn flour samples decreased only 4% (Table 10) compared to digestibilities of zero time samples.

Although decreases in protein digestibility might be expected in browned samples, animal feeding studies with a casein:glucose model system showed only a 9% decrease in protein digestibility after 30 days storage at 37°C and 68% RH (Henry et al. 1948). Protein efficiency ratios dropped from 2.42 for the untreated control to 0.20 for the 30-day samples. This may suggest that protein digestibility data

TABLE 10. Change in *in vitro* protein digestibility in LEC processed cowpea:corn flour after 15-21 wks storage under varied storage conditions

Temperature(°C)	Weeks of storage	a_w 0.44	0.55	0.65
Baseline = 85.4				
25	15	80.4[a]	80.8	81.2
35	15	82.1	84.0	83.5
45	15	82.6	81.4	81.5
55	15	82.3	82.4	81.7
	18	--	--	80.8
	21	--	--	82.7

[a] Values given are means of duplicate determinations.

alone do not adequately reflect protein quality changes in browned foodstuffs.

The question of bioavailability of early Maillard products has been discussed previously. Because observable browning in the cowpea:corn flour system occurred only in samples stored at 55°C and a_w of 0.65, the FDNB-reactive lysine losses observed with storage are probably due to early Maillard reactions. N-substituted glycosamine derivatives (Amadori compounds) are not biologically available (Hurrell and Carpenter, 1981). However, several synthetic Schiff base compounds have been shown to be nearly

100% utilized by the rat (Finot et al. 1977). The distribution of Schiff base and Amadori-lysine derivatives in the cowpea:corn flour is not known, but the only known food system containing significant amounts of the Schiff base form of lysine is dried milk powder (Hurrell and Carpenter, 1981). Therefore, it can probably be concluded that the FDNB-reactive lysine lost during early Maillard reactions in the cowpea:corn flour system is biologically unavailable.

Under storage conditions used in this experiment, decreases of reactive lysine of 25% or more are certainly possible in tropical or sub-tropical environments, or with thermal abuse. Because of the strong statistical relationship of FDNB-reactive lysine loss to decreases in biological measures of protein quality such as the PER, NPU, and BV, losses of this magnitude may result in significant decreases in protein nutritional quality.

SUMMARY AND CONCLUSIONS

Maillard non-enzymatic browning reactions are a primary problem during storage of dry and semi-moist foodstuffs. The reactions between carbonyl groups, contributed primarily from reducing sugars, and the amine groups of proteins can significantly affect the protein nutritional quality of the foodstuff. A series of experiments was designed to examine the effects of varied steady-state storage conditions on the kinetics of FDNB-reactive lysine loss, and non-enzymatic browning reactions in low-cost extruder cooker (LEC) processed cowpea:corn flour.

Rate constants for FDNB-reactive lysine loss ranged from 8.9×10^{-3} $weeks^{-1}$ for storage at 25°C and a_w of 0.55 to 25.9×10^{-3} $weeks^{-1}$ for storage at 55°C and a_w of 0.65. Maximum rates of lysine loss were found at the highest a_w (0.65) at all temperatures with the exception of storage at 45°C where maximum rates of lysine loss were found at a_w of 0.55. A simplified first-order kinetic equation was found to best describe FDNB-reactive lysine loss in this system. Statistical analysis of the main treatment effects of temperature and a_w showed linear effects on lysine loss with increases in both temperature and a_w. Temperature effects were more significant, however, compared to a_w effects. Because a narrow range of a_w's was used for this experiment,

it is possible that the linear effect of a_w may not be significant over a wider range of a_w's. The lower range of a_w's used for this research (0.44, 0.55) are in a plateau region of the product sorption isotherm that changes only slightly in percent moisture with large changes in a_w. The effects of a_w change were less significant than effects of temperature change on lysine losses in the system because the lower a_w values may not be significantly greater than mono-layer levels of moisture. Further, the a_w's used for storage of the cowpea:corn flour product are slightly higher that the normal a_w of the product (¬ 0.33) when extruded. This suggests that at a_w values likely to be encountered during processing and normal storage conditions, the a_w effect may still be of less importance compared to temperature. However, because the lower a_w values values used in this experiment fall within a plateau region in the isotherm, further work should be done with lower a_w values to determine if FDNB-reactive lysine loss is decreased as moisture contents approach the mono-layer. Rates of deteriorative processes in foods are generally at a minimum at mono-layer moisture levels (Labuza, 1980). Temperature dependence of FDNB-reactive lysine loss in the cowpea:corn flour food system was low. Over the 15-21 wk course of the experiments, only samples stored at the highest temperature and a_w developed quantitatable browning.

Differences in the effect of temperature and a_w on FDNB-reactive lysine loss, and the lack of observable browning in most stored samples can best be explained by examination of the cowpea:corn flour sorption isotherm. Little browning occurs at a_w's corresponding to the mono-layer moisture levels in a foodstuff, and reaction rates increase as water is added in successive layers above the mono-layer (Labuza et al. 1977). At lower moisture levels, reactant diffusion is slow, which increases diffusion resistance, and decreases reactant solubility (Eichner and Karel, 1972). The lower a_w's used in this experiment (0.44, 0.55) probably did not provide sufficient moisture to allow the Maillard reactions to proceed to the extent required for development of browning pigment even under the higher temperature storage conditions.

These conclusions have several potential implications for processing and storage of products of this composition. Results suggest that significant changes in product sensory quality due to the development of excessive non-enzymatic browning are probable only under abusive temperature and a_w storage. Even with such abusive storage, 6-9 wks would have to elapse before the development of noticeable pigmentation would be seen in the product.

From a practical standpoint, however, significant losses of FDNB-reactive lysine (> 25%) are very possible under ambient storage conditions of 25 to 30° These changes could affect the protein nutritional quality of the product especially where lysine is the first-limiting essential amino acid. These data should provide an adequate foundation for future development of predictive models for changes in protein quality in products of similar composition. Because the experiment was not designed to evaluate changes in protein quality in the product as a result of extrusion, and because operation of the extruder used here is not strictly standardized, extrapolation of these results to different lots of the product may not be appropriate. Further work must be done to determine if the results obtained in the present study can be applied to different lots of the product produced under different processing conditions.

Further, these data suggest that control of process and storage temperature, and length of storage time may have a greater impact on minimizing reductions of protein nutritional quality due to losses of FDNB-reactive lysine than control of a_w. Future work with additional a_w values, and perhaps with longer storage times, is needed to substantiate the nature of the response of this food system to a_w change.

BIBLIOGRAPHY

American Assoc. of Cereal Chemists. Method 46-10, Approved Methods Vol. 2. Am. Assoc. of Cereal Chem., St. Paul, Minn.

Anantharaman, K., and Carpenter, K. J. 1969. Effects of heat processing on nutritional value of ground nut products. I. Protein quality of groundnut cotyledon for rats. J. Sci. Food Agric. 20:703-708.

Barnes, W., Spingarn, N. E., Gould, C., Vuolo, L. E., Wang, and Weisenburger, J. H. 1983. Mutagens in cooked foods: Possible consequences of the maillard reaction. Pages 485-506 in G. R. Waller, and M. S. Feather, eds. The Maillard reaction in foods and nutrition. ACS Symposium Series No. 215. American Chemical Society, Washington, D.C.

Bell, G., Higgins, C. F., Payne, G. M., and Payne, J. W. 1977. Use of E-coli to determine available lysine in plant proteins. Pages 107-123 in Nutritional evaluation of cereal mutants. International Atomic Energy Agency, Vienna.

Berg, A. 1974. The nutrition factor. The Brookings Institution, Washington, D. C.

Bjarnson, J., and Carpenter, K. J. 1970. Mechanisms of heat damage in proteins. Br. J. Nutr. 24:313-329.

Bjorck, I., Noguchi, A., Asp, N., Cheftel, J. C., and Dahlqvist, A. 1983. Protein nutritional value of a biscuit processed by extrusion. J. Agric. Food Chem. 31:488-492.

Bookwalter, G. N., and Kwolek, W. F. 1981. Predicting protein quality of corn-soy-milk blends after non-enzymatic browning. J. Food Sci. 41:771-715.

Bookwalter, G. N., Bothast, R. J., Kwolek, W. F., and Gumbman, M. R. 1980. Nutritional stability of corn-soy-milk blend after dry heating to destroy salmonellae. J. Food Sci. 45:975-980.

Booth, V. H. 1971. Problems in the determination of FDNB available lysine in proteins. J. Sci. Food Agric. 22:658-668.

Brunauer, S. 1945. The adsorption of gases and vapors. Vol. 1. Princeton University Press, Princeton, N. J.

Bujard, E., and Mauron, J. 1964. Guanidination, an alternative approach to determination of available lysine in foods. Proc. 6th Int'l. Congress (Edinburgh).

Carpenter, K. J. 1960. The estimation of available lysine in animal protein foods. Biochem. J. 77:604-610.

Carpenter, K. J. 1981. Individual amino acid levels and bioavailability. Pages 239-254 in C. E. Bodwell, L. Adkins, and D.T. Hopkins, eds. Protein quality in humans. Assessment and in vitro estimation. Avi Publ. Co., Westport, Conn.

Carpenter, K. J., and Booth, V. H. 1973. Damage to lysine in food processing: Its measurement and its significance. Nutr. Abstr. Rev. 43:424-451.

Carpenter, K. J., and Ellinger, G. M. 1955. Estimation of available lysine in protein concentrates. Biochem. J. 61:xi-xx.

Chen, J. Y., Bohnsack, K., and Labuza, T. P. 1983. Kinetics of protein quality loss in enriched pasta in a sine wave temperature condition. J. Food Sci. 48:460-464.

Choi, R. P., Koncus, A., O'Mally, C., and Fairbanks, B. 1949. A proposed method for determination of color in dry products of milk. J. Dairy Sci. 32:580-585.

Cochran, W. G., and Cox, G. M. 1950. Experimental design. 2nd ed. John Wiley & Sons, Inc., New York, N. Y.

Couch, J. R., and Thomas, M. C. 1976. A comparison of the chemical and biological methods for the determination of available lysine in fish meals. J. Agric. Food Chem. 24:943-948.

Creamer, L. K., Matheson, A. R., and Lohrey, E. E. 1976. Assessment of two chemical assays for available lysine. N. Z. J. Dairy Sci. Technol. 11:96-99.

Duckworth, R. B., and Smith, G. M. 1963. Diffusion of solutes at low moisture levels. Pages 252-267 in J. M. Leitch, D. N. Rhodes, eds. Recent Advances in Food Science. Butterworths, London.

Dworschak, E., and Hegedus, M. 1974. Effect of heat treatment of the nutritive value of proteins in milk powder. Acta Aliment. 3:337-347.

Eichner, K. 1975. The influence of water content on non-enzymatic browning in dehydrated foods and model systems and the inhibition of fat oxidation by browning intermediates. Pages 417-429 in R. R. Duckworth, ed. Water relations of foods. Academic Press, New York, N. Y.

Eichner, K, and Karel, M. 1972. The influence of water content and water activity on the sugar amino browning reactions in model systems under varied conditions. J. Agric. Food Chem. 20:218-222.

Ellis, G. P. 1959. The maillard reaction. Adv. Carbohydr. Chem. 14:63-128.

Erbersdobler, H. 1976. Amino acid availability. Pages 103-139 in D. J. Cole, K. N. Boorman, P. J. Buttery, D. Lewis, and H. Swan, eds. Protein metabolism and nutrition. Butterworths, London.

Evans, R. J,. and Butts, H. A. 1951. Heat inactivation of the basic amino acids and tryptophan. Food Res. 16:415-421.

Fabriani, G., and Frantoni, A. 1972. Changes by processing on the nutritive value of some cereal products. Bibliotneca Nutritio Et Dieta 17:196-202.

Feather, M. S., and Russell, K. R. 1969. Deuterium incorporation during conversion of 1-amino-2-deoxy-D-fructose derivatives to 5-(hydroxymethyl) -2-furfuraldehyde. J. Organ. Chem. 34:2650-2652.

Fenney, R. E., and Whitaker, J. R. 1982. The maillard reaction and its prevention in food protein. Pages 201-229 in J. Cherry, ed. Protein deterioration: Mechanism and functionality. ACS Symposium Series no. 206. American Chemical Society, Washington D. C.

Fenney, R. E., Blankenhorn, G., and Dixon, N. 1975. Carbonly-amine reactions in protein chemistry. Adv. Protein Chem. 29:135-203.

Finot, P. A., and Mauron, J. 1972. Blockage of lysine by the maillard reaction. II. Properties and chemistry of N-(ε-deoxyfructosyl-and N-(deoxy-1-D-lactosyl)-L-lysine. Helv. Chem. Acta 55:1153-164.

Finot, P. A., Bricout, J., Viani, R., and Mauron, J. 1968. Identification of a new lysine derivative obtained upon acid hydrolysis of heated milk. Experimentia 24:1097-1099.

Finot, P. A., Bujard, E., Mottu, F., and Mauron, J. 1977. Availability of the true Schiff's bases of lysine. Chemical evaluation of schiff's base between lysine and lactose in milk. Pages 343-365 in M. Friedman, ed. Protein Crosslinking. Plenum Press, New York, N. Y.

Folkes, D. J., and Gramshaw, J. W. 1981. The flavor and volatiles of bread crust. Prog. Food Nutr. Sci. 5:369-376.

Ford, J. E. 1981. Microbiological methods for protein quality assessment. Pages 278-300 in C. E. Bodwell, J. S. Adkins, and D. T. Hopkins, eds. Protein quality in humans. Assessment and in vitro estimation. Avi Publ. Co., Westport, Conn.

Gottschalk, A. 1952. Some biochemically relevant properties of N-substituted fructosamines derived from amino acids and N-arylglucosylamines. Biochem. J. 52:455-460.

Gottschalk, A. 1972. Interaction between reducing sugars and amino acids under neutral and acidic conditions. Pages 141-156 in A. Gottschalk, ed. Glycoproteins, Vol. 5a. Elsevier, Amsterdam.

Greenspan, L. 1977. Humidity fixed points of binary saturated aqueous solutions. J. Res. of NSBA-Physics and Chem. 81A:89-94.

Hall, R. J., Trinder, N., and Givens, D. I. 1973. Use of 2,4,6, trinitro-benzenesulfonic acid for determination of available lysine in protein concentrates. Analyst 98:673-686.

Hannah, R. S., and Lea, C. H. 1952. Studies of the reaction between proteins and reducing sugars in the dry state. 5. The reactivity of the terminal amino groups of lysine in model systems. Biochim. Biophys. Acta 9:293-305.

Henry, K. M., Houston, J., Kon, S. K., and Osborne, L. W. 1939. The effect of commercial drying and evaporation on the nutritive properties of milk. J. Dairy Res. 10:272-293.

Henry, K. M., Kon, S. K., Lea, C. H., and White, J. C. 1948. Deterioration on storage of dried skim milk. J. Dairy Res. 15:292-363.

Hodge, J. E., 1953. Chemistry of browning reactions in model systems. J. Agric. Food Chem. 1:928-943.

Hodge, J. E., and Rust, C. E. 1953. The amadori rearrangement under new conditions and its significance for non-enzymatic browning reactions. J. Am. Chem. Soc. 75:316-322.

Hodge, J. E. 1967. Non-enzymatic browning reactions. Pages 70-98 in H. W. Schultz, E. A. Day, and L. M. Libbey, eds. Chemistry and physiology of flavors. AVI Publ. Co., Westport, Conn.

Hodge, J. E., Mills, F. D. and Fischer, B. C. 1972. Compounds of browned flavor derived from sugar-amine reactions. Cereal Sci. Today 17:34-40.

Hurrell, R. F. 1980. The maillard reaction in flavor. Pages 95-134 in I. C. Morton, and A. J. Mcleod, eds. Food flavors. Elsevier, Amsterdam.

Hurrell, R. F., and Carpenter, K. J. 1974. Mechanisms of heat damage in proteins. 4. The reactive lysine content of heat damaged materials as measured in different ways. Br. J. Nutr. 32:589-604.

Hurrell, R. F., and Carpenter, K. J. 1975. The use of three dye binding procedures for the assessment of heat damage to food proteins. Br. J. Nutr. 33:101-115.

Hurrell, R. F., and Carpenter K. J. 1977. Maillard reactions in food. Pages 168-184 in T. Hoyem and O. Kuale, eds. Physical, chemical and biological changes in food caused by thermal processing. Applied Science, London.

Hurrell, R. F., and Carpenter, K. J. 1979. Reactive lysine in food stuffs as measured by a rapid dye binding procedure. J. Food Sci. 44:1221-1227.

Hurrell, R. F,. and Carpenter, K. J. 1981. The estimation of available lysine in foodstuffs after maillard reactions. Prog. Food Nutr. Sci. 5:159-176.

Hurrell, R. F., Carpenter, K. J., Sinclair, W. J., Otterburn, M. S., and Asquith, R. S. 1976. Mechanisms of heat damage in proteins. 7. The significance of lysine containing isopeptides and lanthionine in heated proteins. Br. J. Nutr. 35:383-395.

Iglesias, H. A., and Chirife, J. 1982. Handbook of food isotherms: Water sorption parameters for food and food components. Academic Press, New York, N. Y.

Jansen, G. R., Harper, J. M., and Odeen, L. 1978. Nutritional evaluation of blended foods made with a low-cost extruder cooker. J. Food Sci. 58:406-415.

Jokinen, J. E., Reineccius, G. A., and Thompson, D. R. 1976. Losses in available lysine during thermal processing of soy model systems. J. Food Sci. 41:816-818.

Kakade, M. L., and Liener, I. E. 1969. Determination of available lysine in proteins. Anal. Biochem. 27:273-280.

Kakade, M. L., Rackis, J. J., McGhee, J. E., and Puski, G. 1974. Determination of trypsin inhibitor in soy products: A collaborative analysis of an improved procedure. Cereal Chem. 51:376-382.

Kawamura, S. 1983. Seventy years of the maillard reaction. Pages 3-20 in G. R. Waller, and M. S. Feather, eds. The maillard reactionin foods and nutrition. ACS Symposium No. 215. American Chemical Society, Washington, D. C.

Knipfel, J. E. 1981. Nitrogen and energy availabilities in foods and feeds subjected to heating. Prog. Food Nutr. Sci. 5:177-192.

Kort, M. J. 1970. Reactions of free sugars with aqueous ammonia. Adv. Carbohydr. Chem. Biochem. 25:311-349.

Kraft, R. A. and Morgan, A. F. 1951. The effect of heat treatment on the nutritive value of milk proteins. J. Nutr. 45:567-575.

Labuza, T. P. 1968. Sorption phenomena in foods. Food Technol. 22:263-272.

Labuza, T. P. 1973. Optimization and storage stability of intermediate moisture foods. NASA Contract Report. L. B. Johnson Space Center, Houston, Texas.

Labuza, T. P. 1979. A theoretical comparison of losses in foods under fluctuating temperature sequences. J. Food Sci. 44:1162-1166.

Labuza, T. P. 1980. The effect of water activity on reaction kinetics in food deterioration. Food Technol. 34:36-41.

Labuza, T. P. and Saltmarch, M. 1981a. Kinetics of browning and protein quality loss in whey powders during steady and nonsteady state storage conditions. J. Food Sci. 47:92-96.

Labuza, T. P., and Saltmarch, M. 1981b. The non-enzymatic browning reaction as affected by water in foods. Pages 605-650 in L. B. Rockland, and G. F. Stewart, eds. Water activity: Influences on food quality. Academic Press, New York, N. Y.

Labuza, T. P., Tannenbaum, S. R., and Karel, M. 1970. Intermediate moisture foods. Food Technol. 24:543-550.

Labuza, T. P., Warren, R. M., and Warmbier, N. C. 1977. The physical aspects with respect to water and non-enzymatic browning. Pages 379-418 in M. Friedman, ed. Protein Crosslinking. Plenum Press, New York, N. Y.

Labuza, T. P., Bohnsack, K., and Kim, M. N. 1982. Kinetics of protein quality change in egg noodles stored under constant and fluctuating temperature. Cereal Chem. 59:142-148.

Lang, K. W., McCune, T. D., and Steinberg, M. P. 1981. A proximity equilibration cell for determination of sorption isotherms. J. Food Sci. 46:936-938.

Lea, C. H. and Hannah, R. S. 1949. The effect of water, of pH and of temperature on the primary reaction between casein and glucose. Biochim. Biophys. Acta 3:313-335.

Lee, C. M., Sherr, B. and Koh, Y. N. 1984. Evaluation of kinetic parameters for a glucose-lysine maillard reaction. J. Agric. Food Chem. 32:379-382.

Lee, T. C., Kimiagar, M., Pintauro, S. J. and Chichester, C. O. 1981. Physiological and safety aspects of maillard browning of foods. Prog. Food Nutr. Sci. 5:243-256.

Lenz, M. K., and Lund, D. B. 1980. Experimental procedures for determining kinetics of food components. Food Technol. 34:50-55.

Liardon, R., and Hurrell, R. F. 1983. Amino acid racemization in heated and alkali treated proteins. J. Agric. Food Chem. 31:432-437.

Liener, I. E. 1981. Factors affecting the nutritional quality of soy products. J. Am. Oil Chem. Soc. 58:406-415.

Liener, I. E., and Kakade, M. L. 1980. Protease inhibitors. Pages 8-68 in I. E. Liener, ed. Toxic constituents of plant foodstuffs. 2nd ed. Academic Press, New York, N. Y.

Maillard, L. 1912. Action des acides amines sur les sucres: Formation des melanoidines par voie methodologique. C. R. Acad. Sci. 154:66-68.

Maga, J. 1981. Measurement of available lysine using the quanidination reaction. J. Eood Sci. 46:132-134.

Maga, J. A. and Sizer, C. E. 1973. Pyrizines in foods: A review. J. Agric. Food Chem. 21:22-30.

Mason, H. S. 1956. Structures and functions of the phenolase complex. Nature 177:79.

Matthew, A. G., and Parpia, H. A. 1971. Food browning as a polyphenol reaction. Adv. Food Res. 19:75-145.

Mauron, J. 1981. The maillard reaction. A critical review from the nutritional standpoint. Prog. Food Nutr. Sci. 5:5-35.

Mauron, J., and Mottu, F. 1958. Relationship between in vitro lysine availability and in-vivo protein evaluation in milk powders. Arch. Biochem. Biophys. 77:312-327.

McCollum, E. V., and Davis, M. 1915. The cause of loss of nutritive efficiency of heated milk. J. Biol. Chem. 23:247-254.

Miller, E. L., Carpenter, K. J., Morgan, C. B., and Boyne, A. W. 1965. Availability of sulfer amino acids in protein foods. Assessment of available methionine by chick and microbiological assays. Br. J. Nutr. 19:249-267.

Mitchell, H. H., and Block, R. J. 1946. Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem. 163:599-620.

Moore, W. J. 1972. Physical chemistry. 6th ed. Prentice Hall, Englewood Cliffs, N. J., 324 pp.

Nair, B. M., Laser, A., Burvall, A., and Asp, N. 1978. Gas chromatographic determination of available lysine. Food Chem. 3:283-291.

Opstvedt, J., and Carpenter, K. J. 1976. Application of chemical and biological assay procedures for lysine to fish meals. J. Agric. Food Chem. 24:389-393.

Overend, W. G., Peacock, A. R., and Smith, J. B. 1951. Reactions at position of carbohydrates. I. The polorographic reduction of carbohydrates. J. Chem. Soc. London. 3487-3496.

Patton, A. R., Solander, R. C., and Piano. M. 1954. Lysine destruction in casein glucose interaction measured by quantitative paper chromatography. Food Res. 19:444-450.

Posati, L. P., Hebinger, V. H., DeVillbliss, E. D., and Pallansh, M. J. 1972. Factors affecting the determination of available lysine in whey with 2,4,6-trinitro-benzenesulfonic acid. J. Dairy Sci. 55:1660-1665.

Rackis, J. J. 1981. Significance of soya trypsin inhibiitors in nutrition. J. Am. Oil Chem. Soc. 58:495-501.

Rao, M. N., and McLaughlan, J. M. 1967. Lysine destruction in casein. J. Assoc. Off. Anal. Chem. 50:704-707.

Rao, S. R., Carter, F. L., and Frampton, V. L. 1963. Determination of available lysine in oilseed meal proteins. Anal. Chem. 35:1927-1930.

Rayner, C. J., and Fox, M. 1978. Measurement of available lysine in processed beef muscle by various laboratory procedures. J. Agric. Food Chem. 26:494-497.

Reynolds, T. M. 1965. Chemistry of non-enzymatic browning. I. Adv. Food Res. 12:1-51.

Rice, R. G., Kertesz, Z. I., and Stolz, E. H. 1948. Color formation in furfural systems. J. Am. Chem. Soc. 69:1798-1800.

Roach, A. G., Sanderson, P., and Williams, D. R. 1967. Comparison of methods for the determination of available lysine value in animal and vegetable protein sources. J. Sci. Pol. Agric. 18:274-278.

Rockland, L. B. 1969. Water activity and storage stability. Food Technol. 23:1241-1252.

Saltmarch, M., Vagnini-Ferrari, M., and Labuza, T. P. 1981. Theoretical basis and application of kinetics to browning in spray dried whey food systems. Prog. Food Nutr. Sci. 5:379-400.

Sanger, F. 1945. The free amino groups of insulin. Biochem. J. 39:507.

Satterlee, L. D., Kendrick, J. G., Marshall, H. F., Jewell, D. K., Ali, R. A., Heckman, M. M., Steinke, H. F., Larson, P., Phillips, R. D., Sawar, G., and Slump, P. 1982. In vitro assay for predicting protein efficiency ratio as measured by rat bioassay: Collaborative study. J. Assoc. Off. Anal. Chem. 65:798-809.

Schroeder, L. J., Iacobellis, M., Lees, H., and Smith, A. H. 1953. The effect of heat on the nutritive value of milk proteins as influenced by water and fat. J. Nutr. 50:351-360.

Scrimshaw, N. S. 1981. Nutritional significance of protein. A global view. Pages 3-18 in C. E. Bodwell, J. S. Adkins, and D. T. Hopkins, eds. Protein quality in humans: Assessment and in vitro estimation. Avi Publ. Co., Westport, Conn.

Shorrock, C. 1976. An improved method for the assay of available lysine and methionine in foodstuffs using Tetrahymena pyroformins W. Br. J. Nutr. 35:333-341.

Shallenberger, R. S., and Birch, A. G. 1975. Sugar Chemistry. Avi Publ. Co., Westport, Conn.

Soares, J. H., and Kifer, R. R. 1971. Evaluation of protein quality based on residual amino acids of the illeal contents of chicks. Poultry Sci. 50:41-45.

Speck, J. C., Jr. 1958. The Lobry de-Bruyn-alderda Van Ekenstein Transformation. Adv. Carbohydr. Chem. 13:63-79.

Stott, J. A., and Smith A. 1966. Microbiological assay of protein quality with Tetrahymena pyroformis W. 4. Measurement of available lysine, methionine, arginine and histidine. Br. J. Nutr. 20:663-673.

Sullivan, J. F. 1981. Control of non-enzymatic browning in the dehydration of fruits and vegetables. Prog. Food Nutr. Sci. 5:377-393.

Tanaka, M., Kimiagar, M., and Lee, T. C. 1977. Effect of maillard browning on nutritional quality of protein. Pages 321-341 in M. Freidman,. ed. Protein crosslinking. Nutritional and medical consequences. Plenum Press, New York, N. Y.

Troller, J. A. and Christian, J. H. B. 1978. Water activity and food. Academic Press, New York, N. Y. 63 pp.

Tsao, T. F., Frey, A. L., and Harper, J. M. 1978. Available lysine in heated fortified rice meal. J. Food Sci. 43:1106-1108.

Udy, D. C. 1971. Improved dye-method for estimating proteins. J. Am. Oil Chem. Soc. 48:29A-33A.

Valle-Riestra, J., and Barnes, R. H. 1970. Digestion of heated damaged egg albumin by the rat. J. Nutr. 100:873-896.

Varnish, S. A., and Carpenter, K. J. 1971. A comparison ileal and fecal analysis to determine the availability of dietary amino acids. Proc. Nutr. Soc. 30:70A. (Abstract)

Waibel, P. E., and Carpenter, K. J. 1972. Mechanism of heat damage in proteins. 3. Studies with ε-(ψ-L-glutamyl)-L-lysine. Br. J. Nutr. 27:509-515.

Waring, J. J. 1969. The nutritive value of fishmeal, meat and bone meal, and field bean meal as measured by digestibility experiments on a diet colostomized foul. Br. Poultry Sci. 10:155.

Warmbeir, H. C., Schnickles, R. A., and Labuza, T. P. 1976a. Effect of glycerol on non-enzymatic browning in a solid intermediate moisture model food system. J. Food Sci. 41:528-531.

Warmbeir, H. C., Schnickles, R. A., and Labuza, T. P. 1976b. Non-enzymatic browning kinetics in an intermediate moisture model system: Effect of glucose to lysine ratio. J. Food. Sci. 41:981-983.

Warren, R. M., and Labuza, T. P. 1977. Comparison of chemically measured available lysine with relative nutritive value measured by a Tetrahymena bioassay during early stages of non-enzymatic browning. J. Food Sci. 42:429-431.

Wolfe, J. E., Thompson, D. R., and Reineccius, G. A. 1977a. Initial losses of available lysine in model systems. J. Food Sci. 42:1540-1544.

Wolfe, J. E., Thompson, D. R., and Reineccius, G. A. 1977b. Kinetics of available lysine loss during thermal processing of a soy protein isolate. J. Food Proc. Preserv. 1:271.

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