IMPROVING *IN VITRO* PROTEIN DIGESTIBLITY AND DETERMINING PROTEIN NUTRITIONAL QUALITY BY MODELLING

by

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(Under the direction of Robert Dixon Phillips)

ABSTRACT

Casein, sorghum, cowpea, extruded sorghum, and extruded cowpea samples were treated with bile salts and solublized and non-solublized fractions were separated by TCA precipitation. Digested samples were then analyzed for protein content in TCA experiments over a 6-hour time period. Bile salts were not effective in improving *in vitro* protein digestion. Simultaneous proteolysis and dialysis were also used as a method of *in vitro* protein digestion. Digestibility was measured at 2, 4, 6, 8, and 12-hour time periods by analyzing dialysates for protein content and amino acid profile. Amino acid profiles were obtained from High Performance Liquid Chromatography (HPLC). Protein quality of dialyzed samples was determined from protein and amino acid analysis. PDCAAS values were calculated and compared to that of in vivo findings. *In vitro* enzymatic digestion can be a reliable tool in determining overall protein nutritional quality.

INDEX WORDS: *In vitro*, TCA precipitation, Dialysis, Protein Digestibility, Amino Acids, HPLC, PDCAAS, AAACAAS, *In vivo*, Protein quality

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B.S., Southern University and A&M College, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

Athens, Georgia

2003

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December 2003

DEDICATION

I dedicate this thesis to my family and friends, who have supported me and shown me unconditional love throughout the way

ACKNOWLEDGEMENTS

I would like first to thank God, from which all of my blessings and achievements have been made possible. I would also like to thank Dr. Robert Dixon Phillips for his guidance and education throughout my masters' studies and Dr. Louise Wicker and Dr. Ronald Eitenmiller for serving on my committee. I would also like to thank Dr. F.K. Saalia for his ultimate guidance, support, knowledge, friendship, and assistance in helping to plan and make this research a success.

My appreciation goes to: Lary Hitchcock for his support in completing this research project. I extend my thanks and best wishes to my fellow colleagues and classmates, many of whom I embarked upon my educational journey with in the fall of 2000. I extend special thanks to Dwayne Wright, Tony Edmunds and Dr. Arthur Hinton whose guidance and wisdom on many life decisions helped to form the career path that I now follow.

I would like to extend very special thanks to Adrianne M. Johnson for being an undergraduate and graduate classmate of 7 years whose loving support, motivation, and friendship have been a cornerstone for all of my achievements past, present, and future.
I would like to also extend my eternal thanks to my family: Mom, Dad, Brian, Katie, Damond and Dakala. Without your presence in my life, no accomplishment would be complete.

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CHAPTER 1

INTRODUCTION

Protein, along with carbohydrates and lipids are considered to be the basic building blocks of all matter. Gerard Johann Mulder identified protein, the Greek word meaning "primary" in the Nineteenth Century (Nakai and Modler 1996). Proteins contribute to key body functions, including blood clotting, fluid balance, production of hormones and enzymes, vision, and cell growth and repair (Wardlaw and Insel 1996). Proteins are composed of twenty different amino acids, which have a basic structure with varying side chains. The differences in these side chains define each protein and give it its specificity and functional abilities. Proteins are joined by amide links otherwise known as peptide bonds and the resulting chains are termed polypeptides. Although, nitrogen (N) is the most distinguishing element found in proteins, they also contain carbon, oxygen, hydrogen, and sulfur. In most foods, amino-nitrogen accounts for approximately 16% of the protein weight, however the amino-N content may range from 13.4% to 19.1% due to varying amino acid compositions (Nielson 1998). Of the amino acid structures 20 are well recognized and considered to be "classical constituents of proteins". These amino acids are classified as "indispensable" or "essential", meaning that they must be obtained through the diet to maintain life while the others are termed "dispensable" or "nonessential" implying that they do not have to be obtained through dietary consumption (RDA 1989).

Protein quality is defined, as the ability of a food to meet the protein nutritional needs of an organism or individual species. In any given organism this is indicated by how well protein is digested, absorbed, and utilized for other life sustaining processes. The essential amino acid that is found in the least quantity is defined as the limiting essential amino acid. The limiting essential amino acid ultimately determines the nutritional value of the protein for humans (Wardlaw and Insel).

Several methods of determining protein nutritional quality have been employed throughout the 20th century. Many of these employ data from animals such as rats and swine and then extended to humans. While initial results in most of these studies looked promising in the area of determining protein nutritional quality for humans, most were modified by subsequent research. Many of these methods were based upon the premise that a linear relationship exists between a protein's quality and the amount of protein is consumed. This was proven to be an incorrect assumption, as protein quality was shown to vary with intake (Phillips 1981).

In vitro enzymatic assays of protein digestibility were later developed to provide conditions that could simulate many of the digestive processes found in the human stomach. One method, TCA precipitation, can be used to obtain digestibility measurements by precipitating and separating digested proteins in solution from those that are undigested. Another characteristic of several methods for measuring digestibility features the use of crude enzymes, which resemble many of the digestive enzymes that were used in these experiments. Dialysis was also determined to be an effective means of reproducing protein digestibility, *in vitro*, due to its ability to prevent the accumulation of digested products and subsequent reaction inhibition when coupled with the use of digestive enzymes. By determining the digestibility of proteins and amino acid availability in this manner, data gatherered from these experiments can be used to predict values that can determine protein nutritional quality. PDCAAS (Protein Corrected Digestibility Amino Acid Scores) and AAACAAS (Amino Acid Availability Corrected Amino Acid Scores) can both be obtained from the use of *in vitro* assays.

The current research, about which this thesis is written, involves the development of an improved method of protein digestibility. This is in addition to the long-term goal of this laboratory in developing an improved method of predicting protein nutritional quality from

reliable *in vitro* data. This method should also be able to exhibit an equilateral relationship with that of in vivo assay findings; ultimately resulting in the end of the need for cumbersome and costly human and animal studies used to determine protein quality.

CHAPTER 2

REVIEW OF LITERATURE

PROTEIN

Early in the Nineteenth Century, the Dutch chemist, Gerard Johann Mulder coined the word “protein” from the Late Greek word *proteos* meaning “primary” (Nakai and Modler 1996). It was at this time that the true importance of protein was identified. It was also discovered that proteins play a functional as well as a genetic role in human development. Proteins can be found abundantly in all cells and tissues and are the primary constituents of the molecular machines found within the human body. Proteins are unbranched polymers of amino acids linked head to tail, from carboxyl group to amino group, through formation of covalent peptide bonds, a type of amide linkage (Garrett and Grisham 1999). Proteins are constituted from twenty different amino acids, which are joined together by amide links, known as peptide bonds, thereby, forming long chains of amino acids called polypeptides or proteins. Nitrogen is the most distinguishing element found within proteins (Nielson 239). Nitrogen content is a reflection of the amino acid bioavailability. In most foods, amino-nitrogen accounts for approximately 16% of the protein weight, however the N content may range from 13.4% to 19.1% due to varying amino acid compositions (Nielson 239). There are 20 amino acids that are well known and considered “classical” constituents of proteins. These amino acids are divided into groups according to their necessity in the human diet. Those that may be present in the diet but can be omitted without threatening life are called dispensable or nonessential amino acids (NEAA) (RDA 1989). Those that are required to maintain life are called indispensable or essential amino acids (EAA) (RDA 1989). The essential amino acids include leucine, isoleucine, valine, phenylalanine, tryptophan, histidine, threonine, methionine, and lysine. While the nonessential amino acids consist of proline, arginine, and tyrosine. Cysteine and tyrosine are conditionally essential amino acids meaning that they can be synthesized from the metabolism of

methionine and phenylalanine, respectively. Therefore, methionine and phenylalanine can be used for the synthesis of cysteine and tyrosine. This phenomenon is called the "sparing" effect of cysteine and tyrosine. Although nonessential amino acids are mutually interchangeable as sources of nonspecific nitrogen, there is experimental evidence suggesting that some amino acids commonly classified as nonessential should be present in the diet of rapidly growing animals to promote maximal growth and nitrogen retention and, therefore, maximal utilization of amino acids (Tuan et al. 1999a).

The factors affecting protein and amino acid nutrition include the organism's requirements for essential amino acids as well as non-specific nitrogen and protein quality. Protein quality is dependent on the levels of essential amino acids present in the protein and the digestibility of the protein and of each amino acid (Tuan et al. 1999b). Protein requirements are defined as the amount of good quality (high and balanced content of EAA) protein needed to maintain a desirable state of health and nutrition: "… the lowest level of dietary protein intake that will balance the losses of nitrogen from the body in persons maintaining energy balance at modest levels of physical activity" (FAO/WHO/UNU, 1985). The definition of protein requirement extends to include the elderly, children, and pregnant or lactating women. This includes the amount of protein required for deposition of tissues or secretion of milk.

Dietary Reference Intake values (DRI) were established in 1997 to present a set of values for nutrients, and their specific uses (DRI 2002). DRI's were not used as replacements for the previously used Recommended Dietary Allowances (RDA 1989), but rather as intermediate extensions to data already presented within the text of the RDA's. They are used in such a way that the assessment of nutrients must be applied to a specific population of individuals. DRI values are comprised of 5 individual reference values (DRI 2002): (1) Recommended Dietary

Allowances (RDA); (2) Adequate Intake (AI); (3) Tolerable Upper Intake Level (UL); (4) Estimated Average Requirement (EAR); (5) Estimated Energy Requirement (EER). By determining the lowest acceptable intake value that will meet an individual's need, the adequacy of these values can be assessed.

- *Recommended Dietary Allowance (RDA):* the average daily dietary nutrient intake level sufficient to meet the nutrient requirement of nearly all (97 to 98 percent) healthy individuals in a particular life stage and gender group.
- *Adequate Intake (AI):* the recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group (or groups) or apparently healthy people that are assumed to be adequate – used when an RDA cannot be determined.
- *Tolerable Upper Intake Level (UL):* the highest average daily nutrient intake level that is likely to pose no risk of adverse health effects to almost all individuals in the general population. As intake increases above the UL, the potential risk of adverse effects may increase. *
- *Estimated Average Requirement (EAR):* the average daily nutrient intake level estimated to meet the requirement of half the healthy individuals in a particular life stage and gender group. *
- In the case of energy, an *Estimated Energy Requirement (EER)* is provided: it is the average dietary energy intake that is predicted to maintain energy balance in a healthy adult of a defined age, gender, weight, height, and level of physical activity, consistent with good health. In children and pregnant and lactating women, the EER

is taken to include the needs associated with the deposition of tissues or the secretion of milk rates consistent with good health.

Table 2.1 shows values of current U.S. recommended allowances of reference (ideal) proteins (RDA 1989). Table 2.2 shows amino acid patterns of the ideal dietary protein for various age groups.

PROTEIN QUALITY EVALUATION

Protein Nutritional Quality refers to the ability of a food to meet the protein nutritional needs of an organism or individual species. Protein quality can serve as an index to how well a protein is digested, absorbed, and utilized for cell growth and maintenance. Therefore, the quality of a protein is determined by the quantity and balance of each amino acid provided the availability of these amino acids after digestion, and the individual's requirements (Tuan et al 1999a). Although poor digestibility or impaired amino acid bioavailability may influence nutritional value, the content of the single most limiting essential amino acid in a protein in the primary factor that determines the nutritional value of the protein for humans (Bodwell 1977a). Although the amino acid composition of a protein is fundamentally related to its nutritional quality, protein quality cannot be reliably predicted from amino acid composition alone. Bioavailability includes a protein's digestibility, absorbability, and metabolic utilizability. Several approaches have been made toward the evaluation of the nutritional quality of protein. This includes both short-term and long-term nitrogen balance studies in humans, intake-response assays in animals, biochemical indexes in animals and humans, *in vivo* and *in vitro* assays, enzymatic digestion procedures, and methods based on amino acid composition data

Table 2.1: U.S. Recommended Allowances of Reference Protein

Category	Age	Weight	Recommended Dietary Allowance	
	(years)	(kg)	(g/kg)	(g/day)
Both Sexes	0-0.5	6	2.2	13
	0.5-1.0	9	1.6	14
	1.0-3.0	13	1.2	16
	4.0-6.0	20	1.1	24
	7.0-10.0	28	1	28
Males	11.0-14.0	45	1	45
	15.0-18.0	66	0.9	59
	19.0-24.0	72	0.8	58
	25.0-50.0	79	0.8	63
	50+	77	0.8	63
Females	11.0-14.0	46	1	46
	15.0-18.0	55	0.8	44
	19.0-24.0	58	0.8	46
	25.0-50.0	63	0.8	50
	50+	65	0.8	50

Modified from Table 6-4 in RDA 1989.

Table 2.2: Suggested Patterns of Amino Acid Requirements

Essential Amino Acids	Infant mean	Suggested pattern of Requirement (mg/g crude protein)			
		Infant Range	2-5 years	10-12 years	Adult
Histidine	26	18-36	19	19	16
Isoleucine	46	41-53	28	28	13
Leucine	93	83-107	66	44	19
Lysine	66	53-76	58	44	16
Methionine + Cysteine	42	29-60	25	22	17
Phenylalanine + Tyrosine	72	63-118	63	22	19
Threonine	43	40-45	34	28	9
Valine	55	44-77	35	25	13

(Bodwell 1985). *In vitro* assays can provide a fast, cheap, and effective solution to many of the problems often associated with *in vivo* assays. However, *in vitro* assays can only be considered fully proven and effective when based upon data from a reliable *in vivo* assay. Biological as well as *in vitro* methods are discussed and reviewed here. Biological nitrogen assays for protein quality evaluation show that it is difficult to separate the effects of each protein where more than one is consumed (Hackler 1977). Biological assays yield data that reflects maintenance and growth as functions of protein quality. Biological Value (BV), Nitrogen Balance (NB), and Nitrogen Balance Index (NBI) are methods of assessing protein quality through the measurement of biological Nitrogen.

Biological Value and digestibility measurements can give a complete evaluation of dietary proteins by measuring the fecal and urinary nitrogen of a subject a test protein diet and then correcting for the amounts excreted when a nitrogen-free diet is fed (Thomas 1909). Studies that determine biological value (BV) and digestibility measure the fecal and urinary nitrogen of a subject fed a test protein diet and correct for the amounts excreted when a nitrogen-free diet is fed (Tuan 1995). Biological Value is calculated as:

$$BV = (\text{retained N} \times 100)/\text{absorbed N}$$

$$= \frac{I - (F - F_o) - (U - U_o)}{I - (F - F_o)} \times 100$$

I = N intake

F = fecal N

F_o = fecal nitrogen from a N-free diet

U = urinary N

U_o = urinary nitrogen from a N-free diet

Bressani et al (1973) showed that BV determined in human and animal assays produced data that were well correlated to each other. This was refuted by Bodwell (1977b), after it was proved that the rat and human assays correlated well only because proteins were fed at variable intake levels unlike that of the rat. This resulted in exaggerated estimates of the nutritive value of the proteins values recovered from the rat assays.

Nitrogen Balance Studies have been used to measure the amount of nitrogen in the diet, feces, and urine to estimate the adequacy of diets or to determine nutritional requirements (Hegsted 1976). Nitrogen balance is calculated as:

$$NB = I - F - U$$

The NB method is most sensitive to nitrogen intake and this sensitivity often leads to misleading results through an overestimation of endogenous nitrogen use for growth and maintenance and an underestimation of exogenous nitrogen losses through sweat, skin, and hair (Hegsted 1976).

Nitrogen Balance Index (NBI) establishes a linear relationship between absorbed nitrogen and nitrogen balance in the region of negative nitrogen balance. This linearity extends into positive balance but becomes curvilinear as nitrogen balance increases. Nitrogen Balance Index is calculated as:

$$NBI = -a \pm bN_A$$

Although considered an effective method of establishing protein requirements by Bressani et al. (1973), use of the NBI is not advantageous when considering the time needed to obtain N retention values at three or four levels of N intake (Tuan 1995).

BV and NBI are very similar within a range of intake levels which results in nitrogen balance from slightly above to slightly below the nitrogen equilibrium level for a specific protein (Bodwell 1977b).

PER

The protein efficiency ratio (PER) was first introduced by (Osborne et al) in 1919. The protein efficiency ratio served as a way of numerically expressing the growth-promoting value of protein (Jansen 1978). The PER was calculated by dividing the gain in weight by the weight of the protein consumed.

$$\text{PER} = \frac{\text{weight gain, g}}{\text{protein consumed, g}}$$

Due to several limitations within the PER, the PER is used mainly in feeding experiments with small animals and has been used in studies with infants (Sheffner 1967). It was widely recognized by nutritionists that the PER reflected the amino acid requirements for the rat rather than the actual human amino acid requirements (Madl 1993). Another criticism of the PER is that it makes no allowances for the maintenance requirement in rats (Phillips 1981). Data variability is also seen in proteins of varying nutritional quality. With growing recognition of the limitations of the PER method, a change to other assays was indicated.

NPR

The Net Protein Ratio (NPR) was first assessed by Bender and Doell (1957) as a way to correct for the severe limitations found within the PER assay. The NPR corrects for the PER's inability to consider protein requirements of protein maintenance requirements of animals. The NPR is calculated as:

$$\text{NPR} = \frac{\text{wt gain of test group + wt loss of protein-free group}}{\text{protein intake}}$$

McLaughlan (1972) reported that the NPR is almost equivalent to the PER plus 1.5 and requires a shorter time to perform the experiment. Similarities also exist between the NPR and NPU

however the NPR method is less demanding since body weight rather than body nitrogen or body moisture is used as a measure of response (Phillips 1981).

McLaughlan and Keith (1975) developed a method which was a modified version of the PER and NPR. This hybrid method takes into account the "presumed" maintenance requirement that was not accounted for in the earlier assays.

NPU

Net protein utilization (NPU) was an assay designed in 1953 by (Bender and Miller). The NPU is almost identical to the NPR assay; however, it assesses measurements of body nitrogen as compared to measurements of body weight used in the NPR. NPU is calculated as:

$$\text{NPU} = \frac{\text{(body N – body N of protein free group)}}{\text{N intake}}$$

Protein quality determinations from the NPU depend upon the level of protein fed. The NPU can be derived from the carcass gain from fecal and urinary excretion or directly from carcass analysis (Pellett 1973). Body water was often measured instead of carcass nitrogen due to inconsistencies formed from the latter and correlated well with body nitrogen (Phillips 1980). NPU is similar to BV in that BV is measured as the amount of nitrogen absorbed rather than the amount of nitrogen consumed as seen in the NPU.

In studies with humans:

$$\text{Biological Value (BV)} \times \text{digestibility} = \text{NPU}$$

Thus, BV values will be generally higher than those of the NPU (Hegsted 1974). McLaughlan (1972) noted that depending upon the level of the protein; the NPU tends to exaggerate lysine deficiencies. This is often seen from data that attempts to predict protein nutritional quality from

assays that use rats as test subjects. One of the major discrepancies between rat and human data is that the estimated requirements for total essential amino acids, expressed as mg/g protein, are much higher for rats than for humans at comparable degrees of maturity (Bodwell 1977b).

SLOPE RATIO ASSAYS

Slope ratio assays are multiple-dose (protein level) methods where three or four levels of the test protein are fed (Tuan 1995). The Relative Nutritive Value (RNV) assay and the Relative Protein Value (RPV) assays are measured in such a way that a response curve is generated and slopes yield protein quality values (Hegsted and Chang 1965). McLaughlan and Keith (1975) concluded that RPV is the best when compared to the NPR and RPV.

PROBLEMS IN THE DEVELOPMENT AND APPLICATION OF METHODS FOR ASSESSING PROTEIN QUALITY

Over time, a standardized method for determining protein quality has eluded the health and nutrition organizations of the world. In November of 1991, (Madl 1993) the FDA mandated the use of Protein Digestibility-Corrected Amino Acid Score (PDCAAS) for determining protein quality in foods intended for children over 1 year old and for adults. The PER method was retained for determining protein quality for foods used by infants. Casein was decided upon as an appropriate reference standard due to its high protein content. The PDCAAS includes calculating the protein content, amino acid composition, and the digestibility of the food based upon a standardized rat balance method. With serious questions as to the reliability of estimates made by the PER, the FDA was prompted to move toward a more reliable method of estimating overall protein nutritional quality. This move was based on the fact that since PDCAAS is based

on human amino acid requirement it is more appropriate for evaluating the protein quality of foods intended for human consumption as well as the fact that PDCAAS has been recognized by established international organizations such as the FAO, WHO, and UNU. These changing views suggest that questions still remain in the search for an "ideal" method of protein quality evaluation (Henley and Kuster 1994).

C.E. Bodwell (1977b.) discussed the advantages and disadvantages in having an "ideal assay" for evaluating protein nutritional quality. Bodwell (1977b.) stated that, "For estimation of protein nutritive value for humans, an ideal assay would provide quantitative information about the nutritive value of a protein as a single source or protein for humans and its potential value when consumed with other proteins. Furthermore, the ideal assay would accurately reflect differences in protein quality for nutrition labeling purposes. Hegsted proposed an ideal assay of protein quality that compensated for low quality proteins by increasing the total quantity of the protein itself. By feeding animals more of a low quality protein, the performance of the animal would be elevated by the increased feeding of the same protein. In human applications, the requirements for a specific protein could be calculated simply by knowing the requirement of a given individual for a given protein and the nutritive value of that protein. Egg protein has been used as a reference standard in determining protein nutritional quality. In 1977, (Hackler) commented that humans needed an *ideal protein* for protein nutritional quality evaluation that could cover all of the protein requirements. One suggestion was that by supplementing lower nutritionally valued proteins with other proteins would combine to form a protein without deficiencies normally just seen in single protein-containing foods. It was later suggested by Mauron (1973) that amino acid composition and availability could possibly be the best measure of defining a single or group of proteins nutritional value. In under-developed and third world

countries, the task of improving protein nutritional quality is an important challenge due to diets heavy in cereal and legumes, some of which may not supply the quality and quantity of required proteins. Mensa-Wilmot et al (2001) investigated the possibilities of developing infant formulas with adequate protein quality for weaning infants in tropical Africa. After extensive analysis of the formulas for nutrition and physical properties it was determined that the nutritional status of the children could be enhanced by the formulas.

NONLINEAR METHODS

The previously mentioned methods of protein nutritional quality evaluation are all based upon the premise that a linear relationship exists between a protein's quality and the amount of protein that is consumed (Satterlee et al 1979, Phillips 1981, 1982, Tuan et al 1999a/b,). The saturation kinetics model, a model based upon non-linear intake-response relationships was developed by Mercer and his colleagues (Morgan et al, 1975; Flodin et al., 1977; Mercer and Gustafson, 1984). Protein intake levels were measured along with the intake-response relationships of various animals and monitored to describe the organism's response to graded levels of nutrient intake. By describing the organism's response in a curvilinear model, the models proved that the animal response leveled off at higher nutrient intake levels. While linear methods often estimate quality at a single point (PER, NPR, NPU) or assume that quality is independent of intake over a chosen range (linear regression), quality in the saturation kinetics model changes continuously and can be estimated at any value of intake (Phillips 1981). This is proven true at lower levels of protein intake; and response and quality cease to change only at infinitely high intakes where the intake-response curve reaches an asymptote. This model is based on the same considerations as the principle of enzyme kinetics. The concept of saturation

kinetics is based on the principle of diminishing returns, where the efficiency of nutrient utilization decreases as the protein requirement is approached based on some rate-limiting step (Phillips 1982). Therefore, proteins must be fed at varying levels to validate the models. The model was proven to be effective by its ability to fit the entire range of intake-response data. Phillips (1981) showed that protein quality varies with intake. The model includes several variables from the originators of the model and others proposed by varying researchers of the saturation kinetics model (Morgan et al 1975, Phillips 1981):

$$r = \frac{bK_I + RmaxI^n}{K_I \pm I^n}$$

Where:

r = response of the organism

R_{max} = asymptotic response at high levels of intake

I = Nutrient intake

n = apparent kinetic order or slope factor

b = calculated ordinate intercept

K_I = nutrition constant

additionally:

K.5 = intake at half-maximal response (Rmax $\pm$ b)/2

r/I = efficiency of intake utilization

$dr/dI = [(Rmax - b) (nK_I I^{n-1})] / [(K_I \pm I^n)^2]$

$I_{zero\ response}$ = intake at 'maintenance' = $(-bK_I/Rmax)^{1/n}$

$I_{max\ eff}$ = intake at maximum efficiency

$= [(-B \pm B^2 - 4AC)^{1/2} / 2A]^{1/n}$, where

$A = Rmax/K_I$

$B = [Rmax(1-n) \pm b(1 \pm n)]$

$C = bK_I$

If a wide range of intake-response data is considered, good quality proteins tend to fit a sigmoidal saturation kinetics curve ($n \rightarrow 2$); while poor quality proteins fit a more nearly hyperbolic curve ($n \rightarrow 1$) (Phillips 1981). This reaffirms the ability of the saturation kinetics model to describe the response-intake relationship of organisms that have received diets of varying protein content and quality. Computer iteration is necessary to solve the equations generated by varying levels of protein found within the diet. This could prove to be costly and tedious work, although, the model was shown to fit a wide range of response-intake data while proving that protein quality varies with intake. With this basic conclusion, several drawbacks to the saturation kinetics method also became apparent. Sufficient curvilinearity is needed by the data in order for the model to converge to a useful solution. Secondly, proteins with low quality must be fed in higher amounts than those of higher quality to reach a certain plateau. This amount of diet material used within the assays may be exceedingly large with a large number of experimental animals; therefore it is possible to assume that animals may not be able to eat enough of a poor quality protein to show curvilinearity. Phillips (1982) modified the saturation kinetics model to fit those proteins with low quality and those that exhibited slight curvilinearity. Constant values were assigned to b and R_{max}, based on the fact that responses at zero, and very high protein intakes are characteristics of the test subjects rather than the protein being fed. The revised model also made it easier to determine protein quality through the reduction of standard errors when compared to the original model.

Other non-linear models that were developed include the monomolecular, logistic, and

Gompertz models (Richards 1959; Nelder 1961). Although these models used different biological assumptions, similar results were obtained from each. Phillips (1981) compared many of the previously mentioned linear models to the saturation kinetics model and arrived at conclusions that would suggest that the linear methods are flawed in their linear assumptions.

Although non-linear models provide a better, more accurate fit to intake-response data compared to linear methods of protein quality evaluation, they do require a great deal more effort. These assays remain costly and can become extremely complex in their evaluation. Non-linear quality parameters provide the best chances at developing a completely effective assay for the quality evaluation of proteins. By acquiring meaningful and reliable data from an *in vitro* study to calculate non-linear parameters, many of the hassles associated with obtaining data through in vivo studies, could be eliminated.

IN VITRO ASSAYS

In vitro studies on the digestion and absorption of proteins have long been sought after to deliver accurate and reliable data about the digestion of proteins. This has been an elusive task for several reasons: (1) Animal tests provide the standard of comparison for comparable *in vitro* data; however, the animal tests used do not all yield the same information. (2) There are differences in the nutritive values of different proteins of similar amino acid content, and wide variations in the nutritive quality of different samples of the same protein (Sheffner 1967). These variations come from differences in strain, raw material sources, and from resultant changes in processing. *In vitro* assays of protein quality take into account all the characteristics of proteins, which may affect bioavailability. Enzymatic digestion methods can be used to measure amino-acid availability or to estimate the biological value of a protein (Mauron 1970).

An appropriate *in vitro* measure of digestibility would reflect general changes in amino acid availability thereby granting a useful approach to evaluating protein nutritional quality for humans. Enzymatic hydrolysis was first proposed as a way to improve the *in vitro* evaluation of protein quality and to obtain a single corrective index related to either biological or nutritive value measured *in vivo* (Mauron 1970).

PDR

One method of *in vitro* enzymatic hydrolysis is the PDR (pepsin digest-residue). Sheffner et al (1956) devised an amino acid index that combined the pattern of essential amino acids released by *in vitro* pepsin digestion with the amino acid pattern of the remaining protein to produce an integrated index, the pepsin digest-residue amino acid index. Egg protein was used as a reference standard to compare against the digestion of flour (soy and white wheat), egg albumin, lactalbumin, defatted egg, and casein. The method involves the incubation of protein with pepsin for 24 hours at 37 C. Total amino acid content was determined by preparing acid hydrolysates of each sample and then autoclaving each protein for 16 hours at 120C. Alkaline hydrolysates used in the determination of tryptophan and tyrosine were autoclaved at 120 C for 8 hours. *Leuconostoc mesenteroides* P-60 was used for the assay of cysteine, histidine, lysine, methionine, phenylalanine, tyrosine, and valine. *S. faecalis* was used to determine isoleucine, leucine, threonine, and tryptophan (Sheffner et al 1956). The PDR was calculated by the use of formulas, which were derived by determining the pattern of essential amino acids in the pepsin and residue stages, and the amount of amino nitrogen as a group containing the amino acid pattern. The PDR represents the net protein utilization (NPU) of protein by the microorganisms and demonstrates excellent correlation with NPU values of a number of representative proteins

when tested in growing rats. The most influential factor associated with the use of peptic digestion may be seen in its influence on subsequent digestion of pancreatin in the PPD, PPDD methods. While the PDR method does not provide a complete view of enzymatic digestion, it does provide a view of the importance of peptic digestion in the patterns associated with amino acid absorption. Also, the calculations associated with the PDR may be misleading in that they were developed on a trial and error basis and may seem to pad the high level of correlation with NPU values (Mauron 1970).

PPD

Akeson and Stahmann (1964) proposed the PPD method (pepsin pancreatin digest) as a modification of the PDR. Protein was incubated with pepsin for 3 hours at 37 C and following the subsequent addition of pancreatin; the digestion mixture was incubated at 37 C for an additional 24 hours. After the pepsin and pancreatin digestions and an acid hydrolysis, amino acid analysis was done on soluble species by the use of ion-exchange chromatography. Calculations of the PPD were similar to the PDR method except that amino acid concentrations were reported as grams per 100g of total amino acid rather than as milligrams per gram of protein as seen in the former. Results showed that PPD values were higher than those of the PDR and a suitable correlation was found between that of PPD values and biological values. Casein, whose peptic digestibility is lower than that of most other proteins, displayed a higher digestibility with the addition of pancreatin to the digestion mixture (Mauron 1970). The improved correlations of the PPD can be directly attributed to the fact that the addition of pancreatin extends the digestion beyond just that of a peptic digestion. A disadvantage to the PPD method involves the use of picric acid, which is too volatile for some analyses. The

accumulation of products absorbed in digestion as seen *in vivo* suggested that the amino acid pattern *in vitro* could be distorted and the enzymatic reaction could possibly be inhibited.

PPDD

As a solution to the disadvantages presented by the PPD method, Mauron et al (1970) devised a method of simultaneously digesting and dialyzing first with pepsin at 30 C for 16 hours and then with pancreatin. The sample was then transferred to a dialysis bag and incubated for 24 hours at 50 C. Twenty-seven fractions of digestate were taken over the 24-hour time period for future amino acid analysis. The PPDD showed that the aromatic acids leucine and methionine were readily released followed by the basic amino acids. The other amino acids were poorly released, specifically the dicarboxylic amino acids and proline, which fell well below the sensitivity of automatic chromatography (Mauron 1970). The PPDD was calculated in much the same way as the previously mentioned PDR amino acid index. The PPDD provides information on amino acid availability as well as the time sequence of amino acid patterns released upon digestion.

Marable and Sanzone (1981) discussed the PDR, PPD, and PPDD methods and concluded that (1) the theoretical basis for attempting to improve on simple amino acid analysis is unsound, (2) the experimental approaches are incorrect, or at least incomplete, (3) the results of handling the data during the final calculations are misleading. The calculations use geometrical means, which has a leveling effect on the individual amino acid ratios. Thereby, making it an incorrect calculation due to the fact that a high digestibility rate for one amino acid will not compensate for the low rate of another amino acid in that protein's nutritional quality. Therefore, the basic failures of the PDR and PPDD methods lie not in the experimental

methodology but rather in the calculations and manipulation of data generated from these experiments. Marable and Sanzone (1981) proposed an equation that would eliminate some of the problems associated with the previously mentioned calculations of PDR and PPDD methods. The equation predicts biological value based upon an exponential formula.

$$BV = 100(1 - e^{-K(ai/ei)L})$$

The equation establishes a constant K (which is approximately equal to 2.9) and compares $(a_i/e_i)_L$ which is the limiting amino acid rate of release to that of the same amino acid in egg protein. This theory provides a firm basis that allows consideration of amino acids and rates of digestibility in ultimately determining protein nutritional quality by moving away from many of the linear concepts that had been developed previously but with poor correlations. The success of the Marable and Sanzone approach suggests that a more general solution to predicting overall protein quality might be to develop a way of estimating non-linear equation parameters, e.g., n and K.5 of the saturation kinetics model (Phillips 1981), from the available amino acid content of proteins.

Tuan et al (1999a) conducted a series of studies that sought to establish a mathematical relationship between overall protein quality and *in vivo* protein digestibility/amino acid availability. Later, this research's emphasis shifted to the development of a reliable *in vivo* assay for measuring overall nutritional quality (Tuan et al 1999b). Casein, extruded cowpea, sorghum, and extruded sorghum were fed to pigs, because of their physiological similarities to the human digestive system (Miller and Ullrey 1987). Casein, extruded cowpea, sorghum, and extruded sorghum also exhibit a wide range of nutritional qualities that can supply nitrogen intake-response data, which in turn can be used to calculate equation parameters by fitting experimental data to linear models (nitrogen balance versus nitrogen intake) and the saturation kinetics model

for the evaluation of protein quality. Two theoretical concepts were derived from this work (Tuan et al 1999b). First, improvements to the saturation kinetics model made by Phillips (1982) can positively correct the previous errors found in linear models that were associated with the minimum and maximum responses to protein intake of a population and fit a wide range of dietary proteins including those of low and high qualities. Second, the idea of an *ideal protein* arose from the requirements of essential and non-essential amino acid profile and the nitrogen associated with their availabilities. The research proved that integrated protein quality could be predicted from the most limiting EAA by a simple mathematical relationship, which involved the development of AAACASS (Amino Acid Availability Corrected Amino Acid Score). AAACASS gives the ultimate measure of any digestibility assay by predicting protein nutritional quality based upon its single most determining factor – amino acid availability of the most limiting essential amino acid.

Another goal of this lab was to look at the effects of processing, namely extrusion cooking, in the assessment of overall protein quality. Phillips and Baker (1987) and Tuan et al (1999a/b) used extrusion cooked sorghum and cowpea to demonstrate the effects of processing on some cereals and legumes. By comparing lower protein digestible food products to high quality proteins such as casein or egg protein that digest at almost 100% efficiency, one is often left with data that provides evidence of significant differences in protein digestibility as well as amino acid availability that may lead to a reliable determination of protein quality.

Casein serves as of one the two major categories of milk proteins. Casein exhibits a special property in that it becomes insoluble and forms curds at a pH of 4.6. Curds are the milk precipitate that contains casein and forms readily in an acidic medium while whey is the liquid that remains from curds of clotted milk. Curds are comprised of 4 representative forms of

casein proteins (caseinates): α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein (McWilliams 1997). Casein exhibits a rate of *in vitro* digestibility between 83 and 92%. This supports the use of casein as a reference standard for comparing its digestion to that of other proteins and the subsequent evaluation of protein nutritional quality in *in vivo* and *in vitro* experiments (FDA 1991).

Cowpea, also known as the black-eyed pea, is a major plant food found in many developing countries in Africa. Cowpea is often cooked as a seed and used in many other dishes. Cowpea is an attractive source of nutrition due to the fact that it is drought resistant and lower in antinutritional factors than can be seen in many others legumes. However, cowpea does have a relatively low digestibility. Cowpea proteins have an apparent protein digestibility of approximately 72% (Sing and Rachie 1985). A 100-gram sample of cowpea usually contains about 22.8 grams of protein (Watt and Merrill 1963). Food products such as akara, a deep-fat fried cowpea product, have shown to be improved nutritionally due to the extrusion-cooked forms of the seed used in its preparation. Cowpeas resemble other legumes in their potential contribution to protein nutrition.

Sorghum, an important cereal crop primarily grown in developing countries, serves as a staple food for these nations. Sorghum, a seeded grain, is mainly used within the food and beverage industries in developing countries and as a feed grain in developed countries such as the U.S. Sorghum proteins consist mainly of prolamins and are generally less digestible than those of other cereals. Their digestibility is negatively correlated with total protein content, total prolamin protein, cross-linked prolamins, and beta-prolamin digestibility (Dendy 1995). A 100-gram portion of sorghum usually contains about 11.0 g of protein (Watt and Merrill 1963). *In vitro* digestion of sorghum is enhanced by extrusion processing with proper control of

temperature and pH; however, a reduced measure of *in vitro* digestion can be seen when data is recorded from cooked sorghum (Dendy 1995).

At the dawn of the current millennium, current research has changed its focus to that of carbohydrate research. However, the question still exists as to the feasibility and importance of protein research. While current trends in the food and nutrition industries suggest that a reduced carbohydrate, increased protein diets may be an ideal diet of choice. This lab views that the importance of understanding protein quality and digestibility is brought even moreso the forefront of research thus wholly justifying the need for a reliable, accurate, and improved method of measuring protein nutritional quality and *in vitro* digestibility.

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CHAPTER 3

IN VITRO PROTEIN DIGESTIBILITY AND AMINO ACID AVAILABILITY

INTRODUCTION

Protein digestion is an accurate measure of nitrogen utilization by the human body and the essential and non-essential amino acids. These amino acids serve as the building blocks for many of the physiological processes found within the human body. Although poor digestibility of proteins and impaired availability of amino acids have a noticeable impact upon the nutritional value of a protein, the single most limiting essential amino acid is the primary factor that determines the nutritional value of protein in humans (Bodwell 1977). Most methods for assessing the quality of nutrients in higher organisms have been based upon the assumption of a linear relationship that exists between nutrient intake and growth response (Ware et al 1980). However, this has been proven to be incorrect, as many of the assays do not provide a good estimate of nutritional quality and the constituent amino acid profile (Bodwell 1977). This is not a reliable assumption due to the non-linear biological changes (i.e. growth and maintenance) that occur in humans and animals. Linear methods such as nitrogen balance have been used to evaluate protein quality by measuring the balance between protein synthesis and breakdown (Tuan et al 1999a). Linear methods that have tried to relate animal data to human needs are biological value, net protein utilization, nitrogen balance index, nitrogen growth index, and relative protein value. These methods have provided inconsistent data that cannot be used to derive conclusions about the estimated essential amino acid requirements of man.

Bile salts are lipid “detergents” that may serve as useful agents in the digestion of proteins. While bile salts are commonly known for their emulsifying properties in lipids, structural chemistry suggest that they might increase protein solubility, further enhancing protein digestibility. Hydrophobic interactions between protein and bile salts should leave the polar, end of the bile salts exposed and readily solvated by polar constituents of liquids (i.e. water),

thereby allowing for greater solvation hence digestibility. The importance of protein to overall diet and health has been recognized for many years. However, more focus is being geared toward the quality of protein sources (Tome and Bos 2000). The cost of testing food products and designing models in animal subjects for research data is expensive as well as time consuming. Costs involved in animal studies must cover all phases of research and experimentation as well as costs related to proper care of the animals. The fundamental problem found in most *in vivo* assays is the inability to develop a quantitative model by which true amino acid availability and overall quality within the same animal model can be correlated (Tuan et al 1999b). Problems often associated with *in vitro* studies include the inherent weaknesses found within the mathematical methods used for measuring protein quality. There is also a lack of suitable models correlating available essential amino acid profiles to overall quality and a lack of correlation between *in vivo* and *in vitro* data (Vachon et al 1987). Factors such as protein configuration, intramolecular bonding, modification of amino acid side chains, inert barriers such as cell walls, antinutritional factors and processing are known to affect amino acid availability (Tuan et al 1999a).

Starchy legumes such as cowpea are an important source of nutrition in developing countries within Africa, Latin America, and Asia. Cowpea is consumed in a variety of ways within these countries and is eaten with yam, maize, or rice, or as moin-moin or akara (Phillips and McWatters 1991). Cowpeas contribute to the diet with their relatively high protein content of 25%, and to the quality of dietary protein by forming complimentary mixtures with staple cereals (Phillips and Baker 1987). Although there is a large consumption amongst the industrialized countries of these nations, cowpeas are often underutilized because of their low sociocultural status. Most of these societies view cowpeas as a bland food, stressing the need for

novel products as well as questioning its digestibility and contribution to the nutritional pool. Careful processing, namely extrusion cooking, improves the protein nutritional quality of cowpea (Phillips and Baker 1987). This coupled with the lowered presence of heat stable and heat labile antinutritional factors such as trypsin inhibitors found within cowpea, add to its nutritional properties as well. However, storing legume seeds at elevated moisture contents and temperature reduces nutritional quality of cowpea protein when coupled with most processing methods, especially extrusion (Phillips and Finley 1989). Thus, it is widely known and accepted that the nutritional quality of cowpea is mostly affected by its amino acid profile and its protein digestibility.

Cereal grains, such as sorghum, are important food cereals found in many parts of Africa, Asia, and the semi-arid tropics worldwide (Dendy 1995). Sorghum is used in the production of porridges, flat breads, and alcoholic beverages. However, sorghum usage is impeded by its poor digestibility. The poor digestibility of sorghum is part of its poor protein nutritional quality. Sorghum's protein content falls between 8 and 10 percent, and that protein is low in lysine and other essential amino acids. Sorghum protein digestibility is negatively correlated with the presence of tannins. Moreover, protein cross-linking may be the greatest contributing factor to the lack of protein digestibility seen in sorghum (Dendy 1995).

Digestibility and availability are the two most important factors to be considered when determining the nutritional quality of food proteins. Enzymatic hydrolysis was used to evaluate the digestibility and availability of proteins. Several methods including one and two-step processes have been developed over time. The one-step processes included the use of pepsin, trypsin, papain, and a combination of trypsin, chymotrypsin, and peptidases (Hsu et al 1977). The one-step processes represent the initial phase of protein digestion, peptic digestion. Two-

step processes were introduced to combine the peptic digestion with that of a hydrolytic pancreatic digestion (Akeson and Stahmann, 1964; Stahmann and Woldegiorgis, 1975; Mauron et al 1970). Trypsin (Saunders et al 1973) was also used as a pancreatic digestion measure in other trials. Both peptic and pancreatic digestion steps were arranged according to the optimum requirements for enzyme activity in the digestive system. Peptic digestion was carried out in a 37 C hydrochloric acid solution of about pH 2 which is similar to that found in the human stomach. Pancreatic enzymes were used with a phosphate buffer adjusted to neutrality (7.5 pH) with an incubating temperature of 37 C. Some other methods used sulfuric acid in peptic digestions while even others determined protein digestion by evaluating the reduction in pH with a non-buffered solution over a 10-minute time period (Hsu et al 1977)

Enzyme-substrate (E:S) ratio was determined to be the most important factor in completing the digestions reactions successfully (Robbins 1978). Also, optimization of the E:S ration yields an accurate account of digestibility (Robbins 1978). The ratio of enzyme to substrate has an influence on the reaction rate and the size of peptides produced (Steinhart and Kirchgessner 1973b). Therefore, one may assume that the enzymatic release of amino acids will vary due to the E:S ratio. However, when comparing the release of individual amino acids to the protein digestibility (or nitrogen release in this case), a correlation should be made between the two. Comparisons between digestion methods are hard to make due to the fact that when using enzymatic preparations with different known activities, results will be specific to the enzyme and substrates used.

Proteolysis often leads to an accumulation of digestion products and their subsequent interactions, which may ultimately result in inhibition of the enzymatic reactions. Dialysis provides a means of avoiding the problems associated with proteolysis *in vitro*. However, it has

been noted that proteolysis should be stopped when differences in hydrolysis rates can still be seen (Gauthier et al 1986). Steinhart and Kirchgessner (1973a) carried out the digestion inside a dialysis bag. This procedure provided a separation of proteolytic products from the mixture during digestion and reduced the possibility of product-related inhibition of reaction. Gauthier, Vachon, and Savoie (1986) later expanded this method. Their aim was to measure the effect of alkaline treatment of proteins on protein digestibility and enzymatic release of amino acids. Savoie and Gauthier (1986) created a digestion "cell" that was capable of simulating a controlled gastro-intestinal cycle of digestion. The dialysis cell used a two-step proteolysis at 37 C, a 30-minute pepsin digestion at a pH of 1.9, followed by a digestion with pancreatin at pH 7.5 with a 1000 MW dialysis bag which was washed with a neutral phosphate buffer solution to collect all digested material over a 24-hour period. The digestion cell allows for the study of proteolytic activators or inhibitors, nutrient interactions as well as nutrient availability (Savoie and Gauthier 1986).

Crude enzymes that closely resemble those found in the gut should be used in enzymatic *in vitro* assays (Marable and Sanzone 1981). Crude enzymes interact with food proteins in a way that is more consistent with the way the human stomach works as compared to purified proteolytic enzymes that may not offer a representative picture of protein digestion. The choice of enzymes to be used in an assay is directly related to their specific action on the protein and it will ultimately influence the remaining digested products. This is especially important due to the fact that within proteins, some peptide bond are less susceptible to hydrolysis by digestive enzymes than others, so that some amino acids are released rapidly while others are slowly digested (Robbins 1978).

In this study, TCA precipitation with the added effect of bile salts preceded the dialysis experiments. TCA provides a method of separating soluble and insoluble fractions of digested materials. Dialysis was chosen due to its ability to prevent the accumulation of digestion products and provide selectivity of digested proteins and amino acid for further analysis (Robbins 1978). A modification of the *in vitro* digestion method of Gauthier et al (1986) was chosen because of its satisfactory extent of proteolysis for use in analysis. Also, freeze-drying of 25-35 ml of dialyzed sample proved to be important in the analysis and recovery of nitrogen and future amino acid work. The concentration of the buffer solution versus the amount of soluble nitrogen is one that requires a fairly large amount of the liquid to be analyzed to obtain reliable data. Freeze drying provided an effective means of concentrating the buffer solution for analysis while at the same time not altering its composition. With data recovered from the previously mentioned enzymatic assay procedures, all samples were plotted as time versus digestibility or availability.

Extrusion cooking has been shown to produce food products that have desirable sensory properties while at the same time are nutritionally adequate with varying textures (Phillips and Baker 1987). Extrusion cooking places plant proteins in areas of high temperature and high shear for a short time. This treatment may improve or cause considerable damage to the nutritional quality of the proteins in the extruded materials by various mechanisms, depending on residence time, temperature, moisture, pH, shear rate, and their interactions, the nature of the proteins themselves; and the presence of carbohydrates, lipids, or other reactive species (Phillips and Finley 1989).

MATERIALS AND METHODS

Pancreatin (SIGMA, 4 x U.S.P.) and pepsin (SIGMA, 2800 units/mg) were used to initiate and complete the enzymatic reaction. One normal, Sodium hydroxide (NaOH), 0.1N hydrochloric acid (HCl), 0.1N, and 7.5 pH sodium phosphate buffer solutions were used to aid in the *in vitro* digestion. 1000 MW Termamyl (Novo Nordisk Novozymes, Franklenton, NC), an amylase, was used to aid in the digestion of extruded products. Five protein sources were chosen to study *in vitro* protein digestibility: 1. Casein 2. Raw sorghum 3. Raw cowpea flour 4.Extruded cowpea flour 5. Extruded sorghum flour.

EXTRUSION COOKING

Cowpeas and sorghum flour were milled and extruded using the Wayne single screw extruder (Wayne Machine and Die Corporation, Totowa, NJ). The extruder was equipped with a 3mm id-die, a screw of 5:1 compression ratio, and run at 150 rpm's. The barrel temperatures were set at 150 C (zones 2 and 3 closest to the die). Both cowpea and sorghum meal were adjusted to 20% moisture and choke fed into the extruder port. Samples were collected at steady state conditions. Samples were then dried at 70 C in a forced air oven and milled using a Retsch mill (Retsch GmbH, Haan, West Germany) fitted with a 0.8mm discharge aperture screen.

TCA PRECIPITATION STUDY

Samples containing 250 mg of protein were digested. In the case of extrudates, substrates formed viscous suspensions were first treated with α-amylase (Termamyl, (pH optimum 6.5; temperature optimum, 90C) to hydrolyze gelatinized starch, liquefy, and allow for proper *in vitro* digestion. Samples were suspended in boiling water and treated with 0.1 ml, equaling 1100 units (mg DE/min/μl) at ~100C for 5 minutes. They were then adjusted to, or if not pretreated, suspended in 17 ml of 0.1 N HCl for 10 minutes. The suspension was stirred and

incubated at 37°C for 20 minutes. Afterwards, the pH was adjusted to 1.9 with 1 N NaOH. A pepsin solution of 1.3 mg/ml (2800 units/mg, E:S ratio of 1:250) in 0.1N HCl was added to initiate thehydrolysis. Hydrolysis was stopped after 30 minutes by raising the pH to 7.5. Ten milligrams of pancreatin (4x U.S.P., E:S ratio of 1:25) were added to 10 ml of 0.1N 7.5 pH sodium phosphate ($NaPO_4$) buffer, and the solution was added to the pepsin digest to initiate the next reaction. The samples were removed from the shaking water bath after 6 hours of digestion and made to a final volume of 50 milliliters. To stop the reaction, the digestate were added to 10% w/v (5 grams/50 ml) TCA to precipitate large protein molecules (Figure 3.1).

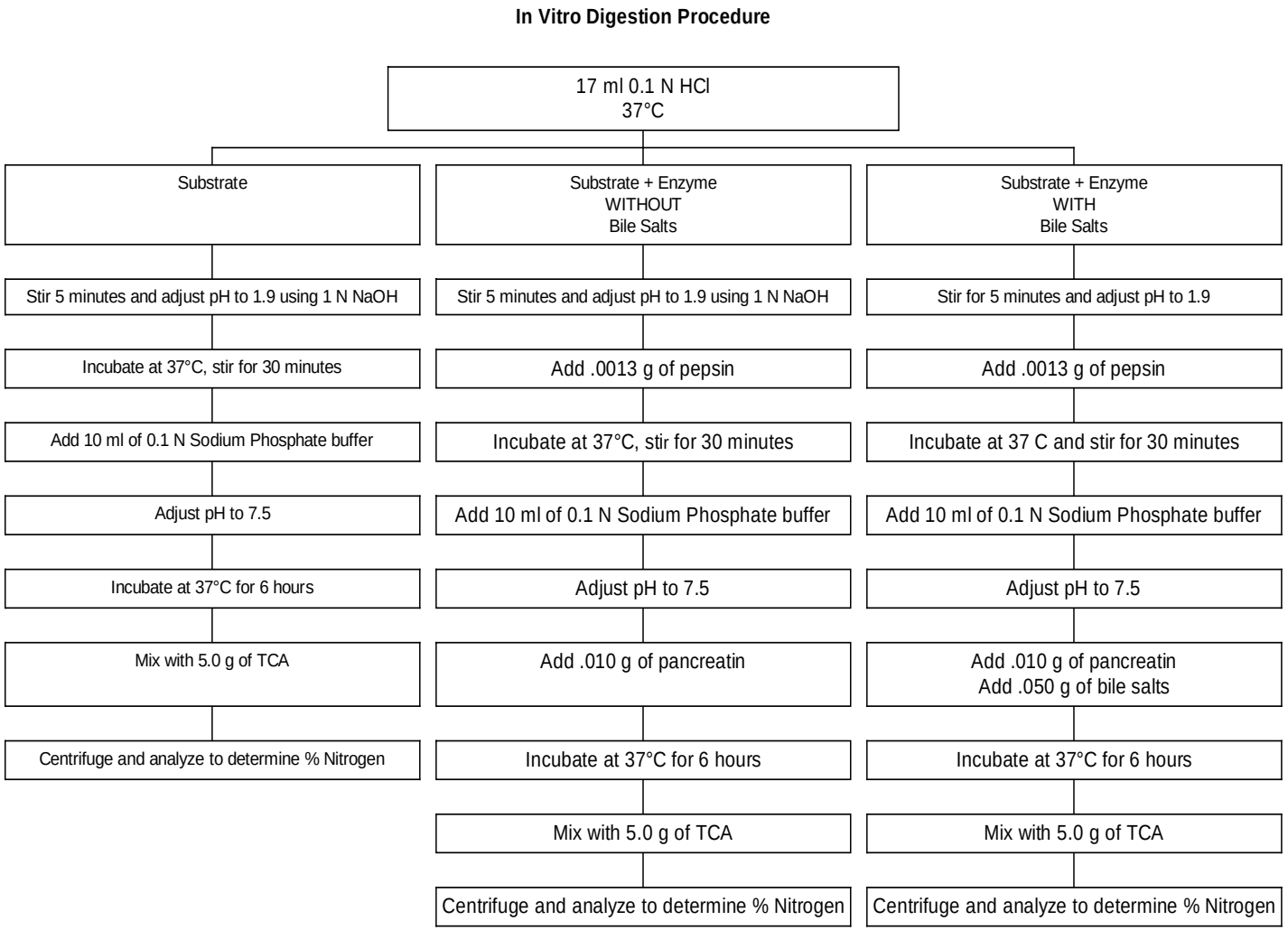
DESIGN OF EXPERIMENT

In each experiment, 3 samples: substrate alone, enzyme alone, and a combination of the substrate and enzyme were subjected to the pH adjustment/ incubation sequence. A 50/50 % mixture of sodium cholate (NaC) and sodium deoxycholate acid (NaDC) was used. In one series 0.05% bile salts were added along with the pancreatin. In the other, bile salts were omitted. The suspension was centrifuged for 20 minutes at 18900-x g (12500 RPM). The clear supernatant was then sampled for nitrogen content by the Dumas combustion method using a LECO FP-2000 analyzer.

DIALYSIS STUDY

A modification of the method of Gauthier et al (1986) was used to evaluate the hydrolysis of proteins using mammalian enzymes. The digestion was described using a two-step hydrolysis to simulate continuous gastric and pancreatic digestion. Two hundred fifty mg (protein - N X 6.25) of non-extruded samples were suspended in 17 mL of 0.1N HCl in a 50 ml Erlenmeyer flask. Two hundred fifty mg of extruded samples were pretreated with amylase as described in TCA precipitation. All digestion mixtures, extruded and non-extruded, were then shaken at

Figure 3.1: TCA precipitation and analysis procedure



37°C for 15 minutes. One ml of pepsin (2800 units/mg protein) solution (1.3 mg/ml prepared in 0.1 N HCl) was added and the pH was adjusted to 1.9 to initiate the enzymatic peptic digestion. The digestion was carried out in a shaking water bath at 37°C for 30 minutes and stopped by raising the pH to 7.5 with 1N NaOH. The pancreatic reaction was initiated by adding 10 mg of pancreatic enzymes prepared in a sodium phosphate buffer (0.1M, ph 7.5) solution (1 mg/ml). To prevent microbial growth, 50 ppm of thimerosal was added to all buffer solutions. The digestate was then transferred into 1000 molecular weight cut-off dialysis tubing and submerged into a 250 ml Erlenmeyer flask with 200 ml of 0.1M, pH 7.5 sodium phosphate buffer circulating around the dialysis tubing. The dialysate flasks were then labeled as 2,4,6,8, and 12-hour time intervals for each sample. After 2, 4, 6, 8 and 12 hours the corresponding flask of buffer was removed from the water bath, transferred to storage bottles, and placed in a minus18 C freezer for future analysis. Twenty-five milliliters of dialysate was then freeze-dried (model Genesis SQ 25, Gardiner, NY) for 96 hours to allow for complete dehydration. Freeze dried samples were then stored in desiccators prior to analysis to prevent rehydration from atmospheric moisture. Samples were then analyzed for nitrogen using the LECO®Nitrogen/Protein analyzer (model FP 2000, St. Josephs, MI) based on the Dumas method and the crude protein content was calculated (% nitrogen value x 6.25). Moisture content of food samples was determined by AOAC procedure 925.09. Starting materials casein, cowpea flour, sorghum flour, and extrudates were analyzed for moisture content as well and then analyzed using the LECO®Nitrogen/Protein analyzer (model FP 2000, St. Josephs, MI)

AMINO ACID ANALYSIS

Undigested starting materials, and dialysate buffer samples were analyzed for amino acid content.. Dialysate buffer samples were then freeze-dried (model Genesis SQ 25, Gardiner, NY)

for 96 hours to allow for complete dehydration. Freeze dried samples were then stored in desiccators prior to analysis to prevent rehydration from atmospheric moisture. Samples were then hydrolyzed for amino acid analysis using a method similar to that of Tuan and Phillips (1995). Twenty-five to fifty milligrams of sample proteins - freeze-dried dialysate buffer samples and undigested starting materials (as determined by LECO analysis) - was added to 5 ml of a 2.5% phenol solution containing 12N HCl and 3 ml of a 2% DTDPA (dithiodiproprionic acid) solution in 1N NaOH was then added. Next, 0.5 ml of an internal standard solution, norleucine was added to the hydrolysate. Finally, 1.5 ml of double deionized, filtered water was added to they hydrolysate to equal the 5 ml of 12 N HCl resulting in a 6 N HCL solution. Hydrolysis tubes were then sealed, placed under a vacuum, and flushed with argon. 3 cycles of purging the tubes with argon and then vacuuming were used to remove all oxygen from the tubes. Tubes were placed in a heating block at temperature 145°C and allowed to hydrolyze for 75 minutes. Afterwards, the tubes were removed and allowed to cool. The cooled hydrolysates were then subjected to the AccQ Tag dilution method for amino acid analysis (Waters Corporation 1996). Four milliliters of hydrolysate was added to a 100ml volumetric flask (10 in 250) of deionized filtered water. Five ml of the hydrolysate sample was filtered and stored for subsequent amino acid analysis. HPLC samples were prepared by mixing 10 ul of filtrate with 70 ul of AccQ Fluor (derivitization) Borate buffer, and then vortexing 20 ul of AccQ Fluor reagent in a low-volume vial insert. After which, samples were heated for 10 minutes at 55°C and allowed to cool. Samples were then analyzed using a Waters HPLC. The reverse-phase Waters AccQ Tag column (C-18) (3.9 x 150 mm) used a binary gradient consisting of solvents A (5.1 pH acetate buffer) and solvent B (60% acetonitrile, 40% water solution with a 50-minute flow at 1 ml/min.

PROTEIN DIGESTIBILITY AND AMINO ACID AVAILABILITY CALCULATIONS

Protein digestibility was calculated by first determining the amount of protein found in the freeze dried samples of either 25 or 50 ml. The dialyzable protein (nitrogen) content in 230 ml of the buffer solution was then calculated. The total weight of nitrogen subjected to digestion was calculated by correcting sample weight for moisture and then compared to the total weight of dialyzable nitrogen to give percentage of protein digestibility.

Amino acid content was calculated by first converting the HPLC reading of pico moles per 5 micro liters of hydrolysate injected to determine the grams of amino acid in 100 micro liters of the derivitization mixture. Next the value was multiplied to determine the grams of amino acids in 10 micro liters of diluted hydrolysate. This value was then corrected to 100 ml of diluted hydrolysate. The next factor of dilution was obtained by determining the grams of amino acid in 4 ml of hydrolysate by using a factor of 2.5. Pico moles were converted to moles by a factor of 10^{-12}. Next the percent protein value obtained from LECO analysis was used to determine the grams of available amino acids found in the freeze-dried sample. This value was then calculated in 25 ml (amount of dialysate freeze-dried) of buffer solution. Finally, the available amino acids in 230 ml of buffer solution were then determined. The amount of amino acids made available from the hydrolysis procedure was then divided by the total amount of amino acids present at the start of the digestion from the starting materials. A correction to the total amount of amino acid recovery from the starting material (casein, raw sorghum, raw cowpea, extruded cowpea, and extruded sorghum) was applied to determine the total amino acids available in the digesta.

STATISTICAL ANALYSIS

TCA samples were analyzed in triplicate. Mean values were then calculated and standard deviations of the triplicate analyses were used to determine error. Mean values for individual amino acids were then determined in dialysis experiments. Starting material samples, casein, cowpea, sorghum, extruded cowpea, and extruded sorghum, were subjected to hydrolysis for amino acid analysis in triplicate. Mean values were then determined for individual amino acids and used to calculate the percentage of amino acid available in each sample (grams of amino acid per 100 gram of sample). Dialysate samples were hydrolyzed and subjected to amino acid analysis in duplicate. Mean values were then used to determine availability.

RESULTS AND DISCUSSION

In vitro protein digestibility results acquired from TCA precipitation showed that digestibility ranged from (Figure 3.2) 100% $\pm$ 1.6 in casein, 62% $\pm$ 2.3 in cowpea, and 25% $\pm$ 0.82 in sorghum samples that were not treated with bile salts. In samples where bile salts were added, the digestibilities of casein, cowpea, and sorghum were generally unaffected. While showing a digestion higher than that of its raw counterparts, extruded cowpea (81% $\pm$ 1.1) and extruded sorghum (40% $\pm$ 0.85) showed no changes in digestibility when treated by bile salts (Figure 3.3). These results are in contrast to preliminary results (not presented here) in which it appeared that the addition of bile salts significantly increased the digestibility of some proteins. We suspect that this was due to difficulties with some analytical techniques being employed. This suggests that our hypothesis, that bile salts might have a role in protein digestibility, is incorrect.

The interaction between detergents and lipids involves the hydrophobic interaction between the hydrocarbon ‘tail’ of the detergent and the corresponding tail of the fatty acids in the

Figure 3.2: TCA digestibility of raw casein, cowpea, and sorghum with and without bile salts

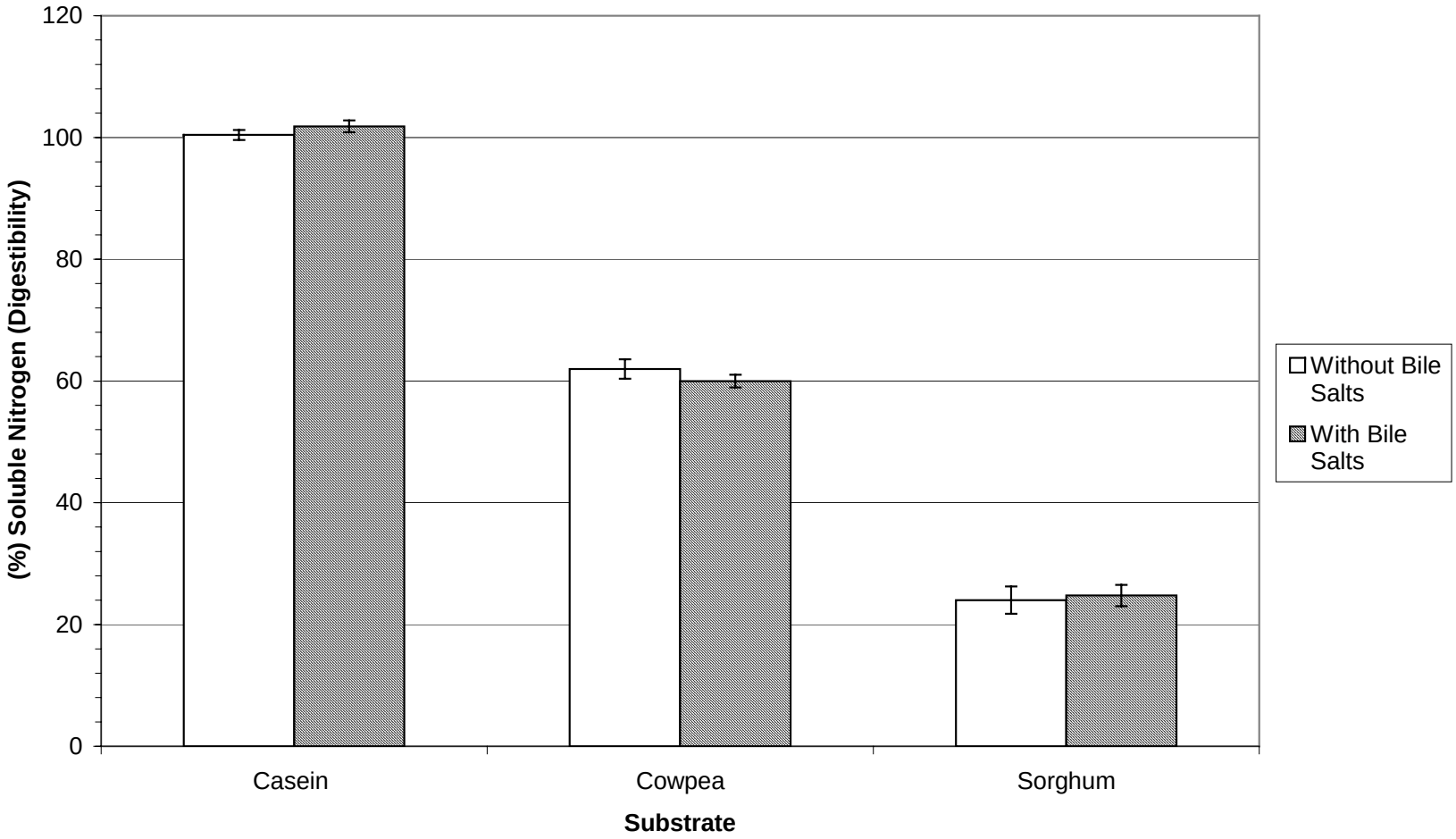
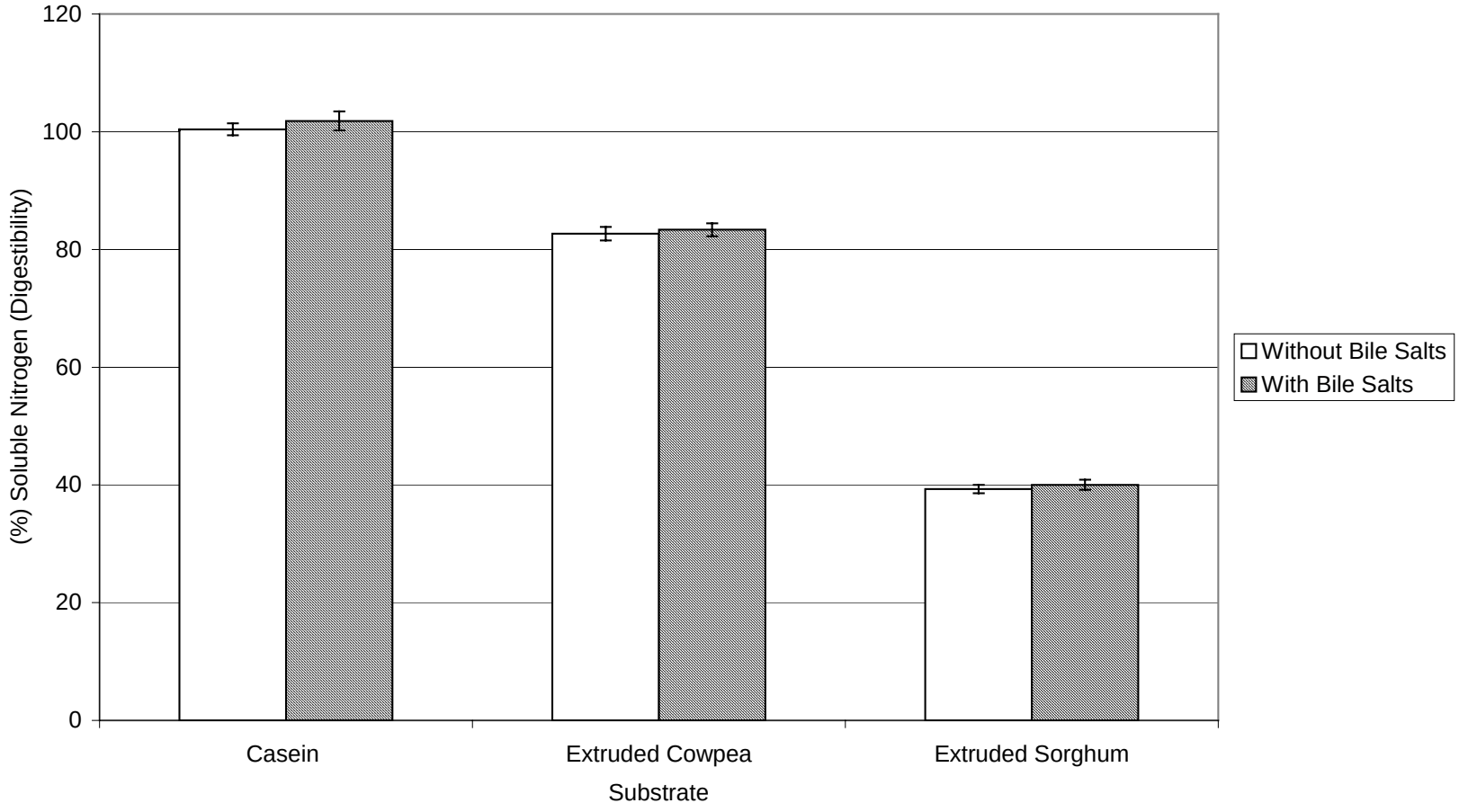


Figure 3.3: TCA digestibility of casein, extruded cowpea, and extruded sorghum with and without bile salts



triglycerides. The polar 'head' of the detergent is solvated by water, causing the lipid to dissolve, making them more accessible to lipases. However, the lack of effect of bile salts on protein digestion suggests that, even if hydrophobic cores are formed during proteolysis, the presence of bile salts doesn't enhance their digestion. The *in vitro* digestibilities determined for casein (101.6% $\pm$1.6) and extruded cowpea (88%$\pm$ 1.1) in this study were similar to those from the *in vivo* study of Tuan et al (1999a). Results for both raw and extruded sorghum yielded considerably lower digestibilities than that of the *in vivo* findings.

Simultaneous proteolysis and dialysis was used to test the *in vitro* protein digestibility and amino acid availability of casein cowpea, sorghum, extruded cowpea, and extruded sorghum. The protein contents of casein, cowpea, sorghum, extruded cowpea, and extruded sorghum were found to be 88%, 23%, 8%, 23%, and 8% respectively. This range of varying protein content was chosen to allow for comparison between samples of low and high protein content and proteins anticipated differing in digestibility. Protein digestibility values for casein (120%) was comparatively higher than that of Tuan et al (1999b) at ~102% and sorghum (59%) were similar to values obtained from the in vivo study of Tuan et al (1999b). However, the protein digestibility of the extrudates did not increase greatly over their raw counterparts, in contrast to the results of (Tuan et al 1999a) who reported that the digestibility of sorghum increased from 56% to 91% after extrusion. Extruded cowpeas protein digestibility of 63% did not differ much from the digestibility of raw cowpea (57%).

Amino acid profiles of the starting material samples were recovered and compared to that of those values for casein, cowpea and sorghum established by the USDA's Food Consumption Tables (Watt and Merrill). Table 3-1 illustrates the amino acid profiles of casein, cowpea, and sorghum literature values while Table 3-2 illustrates recoveries from the hydrolysis and amino

acid analysis of the casein, cowpea, sorghum, extruded cowpea, and extruded sorghum samples**.** Extruded cowpea and extruded sorghum sample values were based upon and compared to the literature values of their non-extruded counterparts. Tables 3-1 through 3-5 show the protein digestibilities and amino acid availabilities for dialyzed and hydrolyzed samples. The amino acid availabilities represented in these tables show recovery amounts based upon the total amount of amino acids present in the starting material. Thus, the low recovery rates shown in these tables are not reflective of the amount of amino acids that were actually recovered from our hydrolysate samples, i.e. freeze-dried samples. Rather, they are representative of the total amount of amino acids present in the dialysate. In order to correct this, amino acid recoveries were calculated to the total amount of amino acids present in the hydrolysate, which was considerably lower than those actually seen in the total dialysate. When comparing the amino acid recoveries to the total amount of amino acids in the hydrolysate sample to that of the analyzed sample, the correction in some cases resulted in unrealistically high values; thus, reducing the ability to obtain reasonable AAACAAS values. It is believed that errors within the hydrolysis method contrary to those steps usually implemented to prevent the deterioration of methionine and cysteine, the sulfur containing amino acids, aided in the formation of erroneous data. Amino acid profiles showed consistency through the first 6 hours of digestion; however, inconsistencies were recorded after the 6^{th} hour. The rate of digestibility/availability increased over the 12-hour time and in some cases began to plateau at 6-8 hours. Lysine values in sorghum samples showed a great deal of variability while in casein, cowpea and extruded cowpea samples lysine availability resulted in reasonable amounts. And while there was not a large increase in protein digestibility between cowpea and extruded cowpea samples, each of the individual essential amino acids increased in extruded cowpea compared to the raw sample.

Another source of variability in the sorghum and extruded sorghum results may have been due to the low recoveries seen in dialysate samples. Cysteine availability in cowpea and extruded cowpea samples were 50% and 54% availability. Availability of essential amino acids in casein exhibited a uniform trend of being high.

Table 3.1: Uncorrected Protein Digestibility/Amino Acid Availability values of Casein

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	cysteine	isoleucine	leucine	phenylalanine	methionine
2	33.60	5.85	6.00	5.23	4.20	6.87	4.49	3.99	12.42	43.93
4	53.29	12.64	12.50	12.48	9.30	13.26	9.73	9.26	12.42	43.93
6	80.98	15.91	15.49	14.01	11.30	15.67	12.30	10.51	15.10	53.35
8	122.04	16.95	16.60	16.69	13.99	21.39	15.36	12.84	17.24	58.76
12	120.29	18.11	18.68	19.65	15.23	23.52	16.53	14.74	18.96	60.96

Table 3.2: Uncorrected Protein Digestibility/Amino Acid Availability values of Cowpea

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	cysteine	isoleucine	leucine	phenylalanine	methionine
2	23.43	2.65	0.68	1.98	0.82	0.99	1.06	0.54	1.22	3.47
4	47.09	6.20	2.62	9.29	3.19	2.20	3.29	2.75	4.24	7.50
6	63.44	7.18	4.09	7.62	3.84	3.36	3.97	3.46	4.90	7.39
8	49.24	9.17	5.91	8.30	4.65	3.78	4.36	3.67	4.93	10.79
12	57.05	8.53	4.93	12.11	4.99	4.34	5.79	4.83	6.81	9.96

Table 3.3: Uncorrected Protein Digestibility/Amino Acid Availability values of Sorghum

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	cysteine	Isoleucine	leucine	phenylalanine	methionine
2	12.49	2.65	0.68	1.98	0.82	0.99	1.06	0.54	1.22	3.47
4	22.23	6.2	2.62	9.29	3.19	2.2	3.29	2.75	4.24	7.5
6	24.10	7.18	4.09	7.62	3.84	3.36	3.97	3.46	4.9	7.39
8	39.22	9.17	5.91	8.3	4.65	3.78	4.36	3.67	4.93	10.79
12	59.49	8.53	4.93	12.11	4.99	4.34	5.79	4.83	6.81	9.96

Table 3.4: Uncorrected Protein Digestibility/Amino Acid Availability values of Extruded Cowpea

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	cysteine	isoleucine	leucine	phenylalanine	methionine
2	18.12	2.64	2.88	3.14	2.75	2.18	2.71	2.75	3.33	8.50
4	29.99	4.12	4.60	4.34	4.01	3.01	3.84	4.20	4.73	10.61
6	45.62	5.26	5.90	5.75	5.73	4.16	6.10	5.71	6.63	11.70
8	56.60	5.94	6.30	6.55	6.03	4.25	6.61	6.25	7.06	13.42
12	63.93	7.00	7.96	6.23	6.39	4.66	6.08	6.57	7.09	13.50

Table 3.5: Uncorrected Protein Digestibility/Amino Acid Availability values of Extruded Sorghum

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	cysteine	isoleucine	leucine	phenylalanine	methionine
2	23.43	2.13	3.88	5.49	2.13	1.34	2.6	1.43	2.56	5.99
4	47.09	5.08	6.3	8	3.6	2.59	4.24	2.4	4.05	9.55
6	63.44	7.84	9.51	11.06	6.25	2.97	7.23	3.2	5.85	11
8	49.24	9.52	10.1	11.9	7.44	4.01	7.3	4.65	7.37	14.6
12	57.05	9.07	12.23	11.43	6.15	2.82	11.82	3.62	6.09	12.31

Table 3.6: Corrected Protein Digestibility/Amino Acid Availability values of Casein

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	cysteine	isoleucine	leucine	phenylalanine	methionine
2	33.60	47.35	48.60	42.35	34.01	55.66	36.41	32.36	31.45	182.53
4	53.29	68.97	68.25	68.11	50.76	72.40	53.11	50.55	67.78	239.75
6	80.98	102.50	99.78	90.25	72.79	100.94	79.19	67.67	97.22	343.52
8	122.04	129.97	127.33	128.01	107.29	164.02	117.78	98.50	132.21	450.48
12	120.29	139.30	143.69	151.18	117.19	180.94	127.17	113.43	142.00	469.90

Table 3.7: Corrected Protein Digestibility/Amino Acid Availability values of Cowpea

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	cysteine	isoleucine	leucine	phenylalanine	methionine
2	23.43	20.53	27.92	16.02	23.11	20.55	23.97	24.51	27.88	60.24
4	47.09	40.16	47.90	36.93	47.50	44.90	50.23	53.28	54.31	111.87
6	63.44	52.16	63.56	54.66	64.45	52.05	70.18	73.29	77.14	143.63
8	49.24	47.32	56.80	44.50	49.31	41.02	52.73	45.59	47.09	112.68
12	57.05	50.26	57.24	54.70	56.91	50.10	65.47	57.65	58.61	130.37

Table 3.8: Corrected Protein Digestibility/Amino Acid Availability values of Sorghum

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	Cysteine	Isoleucine	leucine	phenylalanine	methionine
2	12.49	71.22	18.48	53.21	22.09	26.73	28.62	14.74	32.73	92.23
4	22.23	75.18	31.75	112.58	38.66	26.68	39.90	33.39	51.36	90.94
6	24.10	81.82	46.55	86.77	43.79	38.35	45.29	39.41	55.84	84.22
8	39.22	138.43	89.30	125.32	70.25	57.11	65.89	55.46	74.54	162.99
12	59.49	192.69	111.49	273.37	112.85	98.06	130.91	109.06	153.88	224.81

Table 3.9: Corrected Protein Digestibility/Amino Acid Availability values of Extruded Cowpea

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	cysteine	isoleucine	leucine	phenylalanine	methionine
2	18.12	32.04	34.88	38.07	33.37	26.42	32.80	33.33	40.33	102.92
4	29.99	39.41	44.04	41.54	38.38	28.80	36.79	40.19	45.25	101.45
6	45.62	60.32	67.69	65.96	65.69	47.74	69.94	65.51	76.07	134.16
8	56.60	67.51	71.59	74.41	68.44	48.25	75.05	70.99	80.22	152.30
12	63.93	81.57	92.70	72.50	74.45	54.33	70.84	80.22	82.51	157.09

Table 3.10: Corrected Protein Digestibility/Amino Acid Availability values of Extruded Sorghum

Time (hours)	Protein Digestibility	Essential Amino Acids								
		histidine	threonine	lysine	valine	Cysteine	isoleucine	leucine	phenylalanine	methionine
2	23.43	47.50	58.87	83.35	32.41	20.45	39.49	21.81	38.86	90.97
4	47.09	74.10	91.85	116.70	52.59	37.82	61.83	35.11	59.14	139.31
6	63.44	122.46	148.63	172.84	97.73	46.49	113.03	50.03	91.37	171.91
8	49.24	160.28	170.09	200.33	125.32	67.54	122.91	78.38	124.14	245.75
12	57.05	169.51	228.61	213.60	115.01	52.87	129.83	67.67	113.87	230.14

Table 3.11: True Protein Digestibility and Ileal Availability (%) of Essential Amino Acids in Pigs fed 10% Protein Diets

Protein/ Amino Acid	Whole Sorghum	Extruded Sorghum	Extruded Cowpea	Extruded blend	Casein
Protein	55.7 ± 10.0 d	90.5 ± 9.3 abc	88.0 ± 4.1 abc	89.1 ± 6.9 abc	101.6 ± 2.9 a
Lysine	30.2 ± 24.5 c	83.9 ± 10.1 b	91.6 ± 4.5 ab	88.9 ± 5.9 ab	100.2 ± 1.4 a
Threonine	48.1±4.9 c	83.6 ± 8.6 b	85.5 ± 4.6 b	84.0 ± 5.4 b	96.3 ± 4.0 a
Leucine	71.8 ± 6.5 c	91.9 ± 3.2 b	88.9 ± 4.8 b	92.1 ± 3.7 b	100.1 ± 1.7 a
Isoleucine	40.7 ± 14.8 c	89.2 ± 11.3 ab	78.6 ± 6.4 b	84.4 ± 8.8 b	98.7 ± 3.9 a
Valine	62.8 ± 17.3 c	87.4 ± 7.0 b	87.3 ± 10.7 b	89.6 ± 4.5 ab	99.9 ± 1.8 a
Methionine	48.5 ± 27.8 b	91.7 ± 8.8 a	81.4 ± 14.1 a	82.8 ± 12.4 a	99.3 ± 5.2 a
Cystine	55.1 ± 21.5 c	78.9 ± 9.4 b	78.9 ± 11.3 b	70.3 ± 19.3 bc	102.0 ± 13.6 a
Phenylalanine	72.5 ± 7.6 c	92.2 ± 3.4 b	92.3 ± 4.0 b	92.6 ± 2.4 b	99.9 ± 1.6 a
Tyrosine	68.0 ± 8.4 c	90.1 ± 4.2 b	89.7 ± 4.6 b	90.8 ± 3.5 b	99.8 ± 1.5 a
Histidine	62.6 ± 7.1 c	84.9 ± 2.4 b	89.6 ± 5.8 b	89.5 ± 4.4 b	99.7 ± 2.1 a

* Within rows, means with the same letter are not significantly different ($p<0.1$).

Mean and standard deviation of values determined in 4 animals.

** Taken from Tuan et al (1999a)

CONCLUSION

TCA precipitation and dialysis experiments provided data that was inconsistent at times with the patterns normally associated with protein digestibility and amino acid availability in vivo (Tuan et al 1999). Bile salts did not suitably enhance protein digestibility through TCA precipitation, and the chemical interactions by which bile salts emulsify lipids do not provide any insight as to an improved method of protein digestibility. A methodology must be engineered that can ensure the proper release of amino acids while at the same time, maintain its integrity for analytical purposes. Shown in some cases throughout this experiment, protein digestibility is a function of the sum of the individual amino acid availabilities.

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CHAPTER 4

MODELLING *IN VITRO* PROTEIN DIGESTIBILITY AND AMINO ACID AVAILABILITY

INTRODUCTION

General saturation curves have been developed in the past to describe a wide variety of nutrient-response relationships in varying organisms (Morgan et al 1975). These curves accurately predict experimentally observed responses. Response parameters that are analogous to V_{max} and K_m in enzyme kinetics can be generated from intake/response data. Other linear, semi-logarithmic, and quadratic equations have been developed to describe the nutrient-response curves of higher organisms. Most nutrient-response curves tend to "plateau out", i.e. to approach an asymptotic or limiting response at high nutrient intake (Morgan et al 1975). Upon reaching this asymptote, most curves resemble either hyperbolic saturation curves or the Michaelis-Menten type or sigmoidal saturation curves.

The Hill Equation describes sigmoidal saturation curves. The Hill Equation allows for the treatment of both hyperbolic and sigmoidal occurrences. Secondly, curves generated from the general equation are constructed to intersect the ordinate axis at any point required from the experimental data (Morgan et al 1975). The equation reads:

$$y = (Y_{lim}X^n)/(K + X^n)$$

where:

y = velocity or saturation fraction

Y_{lim} = asymptotic velocity or saturation fraction

X = concentration of ligand or substrate

n = apparent kinetic order of the velocity or saturation fraction with respect to X as X approaches zero

K = characteristic constant of the system, having the property that for $X = K^{1/n}$, $y = Y_{lim}/2$

It should be possible to fit the release of ‘digestible’ protein or amino acids to the Hill equation, predicting equation coefficients including Dmax which would be analogous to Y_{lim}. The resulting equation should represent the best estimate of how protein digestion varies over time for a given substrate. Interpretation of nutritional responses, which demonstrate the “saturation phenomena”, has important implications for the estimation of nutritional requirements such as determining minimal or optimal intake levels (Morgan et al 1975). The purpose of this research is to establish a mathematical relationship between *in vitro* protein digestibility and amino acid availability and *in vivo* digestibility results of similar protein sources that vary in quality.

Proteins, in particular, specific “indispensable” or “essential” amino acids, are essential components of the human diet. Protein quality is determined by the quantity of each essential amino acid, the species requirement, and the availability of the individual amino acids after digestion (Madl 1993). For a long time, protein quality measurements were made using the PER (Protein Efficiency Ratio) method. The widely accepted method measured the weight gain in growing rats to determine protein quality. Seemingly effective, the method was later determined to be an inaccurate measure of protein quality, in part because it reflected the amino acid requirements for the rat rather than actual human amino acid requirements. The PER compared protein sources to a casein reference of 2.5 PER and classifying them as either “high quality” or “low quality”. The Food and Agricultural Organization (FAO) and World Health Organization (WHO) addressed the need for amino acid requirements for humans that would be based upon growth and other physiological needs. These organizations later established the requirements for using the PDCAAS (Protein Digestibility Corrected Amino Acid Score) for determining protein quality in foods intended for children over 1 year old and adults. The PER method was retained

for the evaluation of protein quality in infant foods. The newly implemented PDCAAS provides an accurate method of determining protein quality based upon its amino acid profile to be compared with that of predetermined amino acid requirements in humans. PDCAAS requires that protein digestibility/amino acid availability can be accurately measured *in vitro*. This would eliminate the use of costly *in vivo* assays to determine protein nutritional quality.The PDCAAS is determined as follows (Henley and Kuster 1994):

1. Analyze for proximate nitrogen (N) composition
2. Calculate protein content (N x 6.25 or specific AOAC conversion factor)
3. Analyze for essential amino acid (EAA) profile
4. Determine the amino acid score (uncorrected):

   $$\text{Uncorrected amino acid score} = \frac{\text{mg of EAA in 1g of test protein}}{\text{mg of EAA in 1g of reference protein}}$$

5. Analyze for digestibility
6. Calculate the PDCAAS:

   PDCAAS = Lowest uncorrected amino acid score x protein digestibility

(1999b)PDCAASAmino Acid Availability Corrected Amino Acid Scores (AAACAAS)*in vivo*protein digestibilitiesamino acid present *in vitro*estibilitiesamino acid availabilities.

METHODS

Data obtained from the enzymatic dialysis of casein, cowpea, sorghum, extruded cowpea, and extruded sorghum reported in the previous section was used to calculate protein digestibility and amino acid availability. Results of protein digestibility and amino acid

availability were analyzed using the nonlinear procedure (NLIN) for determining integratable data and the Marquardt method of the Statistical Analysis System (SAS Institute, Cary, North Carolina). This data was then fitted to the Hill equation to calculate Dmax, R-Squared, K, and n values. These values were then substituted into the Hill Equation and curve-fitted plots for points on the curves were calculated and later used to make plots of protein digestibility and amino acid availabilities for each sample. Dmax values were also used to calculate the PDCAAS by multiplying the Dmax of each protein by the amount (grams) of amino acid per 100 gram found in the starting material and then dividing it by the ideal protein value (Henley and Kuster 1994). The lowest value among the essential amino acids would then determine the limiting essential amino acid for that sample; however, Dmax values of the individual amino acids were generated and then multiplied by the weight of that particular amino acid in the starting material. This value was then divided by the ideal protein value the limiting essential amino acid was determined from the lowest amino acid availability.

RESULTS AND DISCUSSION

Table 4.1 shows Dmax values from the non-linear regression of protein digestibility data. These Dmax values resembled the protein digestibility values of Tuan et al (1999a). Curve-fitted Dmax values for protein digestibility give a better view of the digestibility that is often seen in tested swine experiments (Tuan et al 1999a). One aspect of protein digestibility that the Dmax values do not show is the curvilinear rate of digestion often seen with the “saturation phenomena”. Table 4.1 also displays the n, K, and R-squared values for individual proteins digested. protein digestibility Table 3.1

used to.This to data Tuan et al .for the digestibility of casein (101.6%). Dmax values (Table 4.1) when compared to protein digestibility data of Tuan et al (1999a) (Table 3.11), show

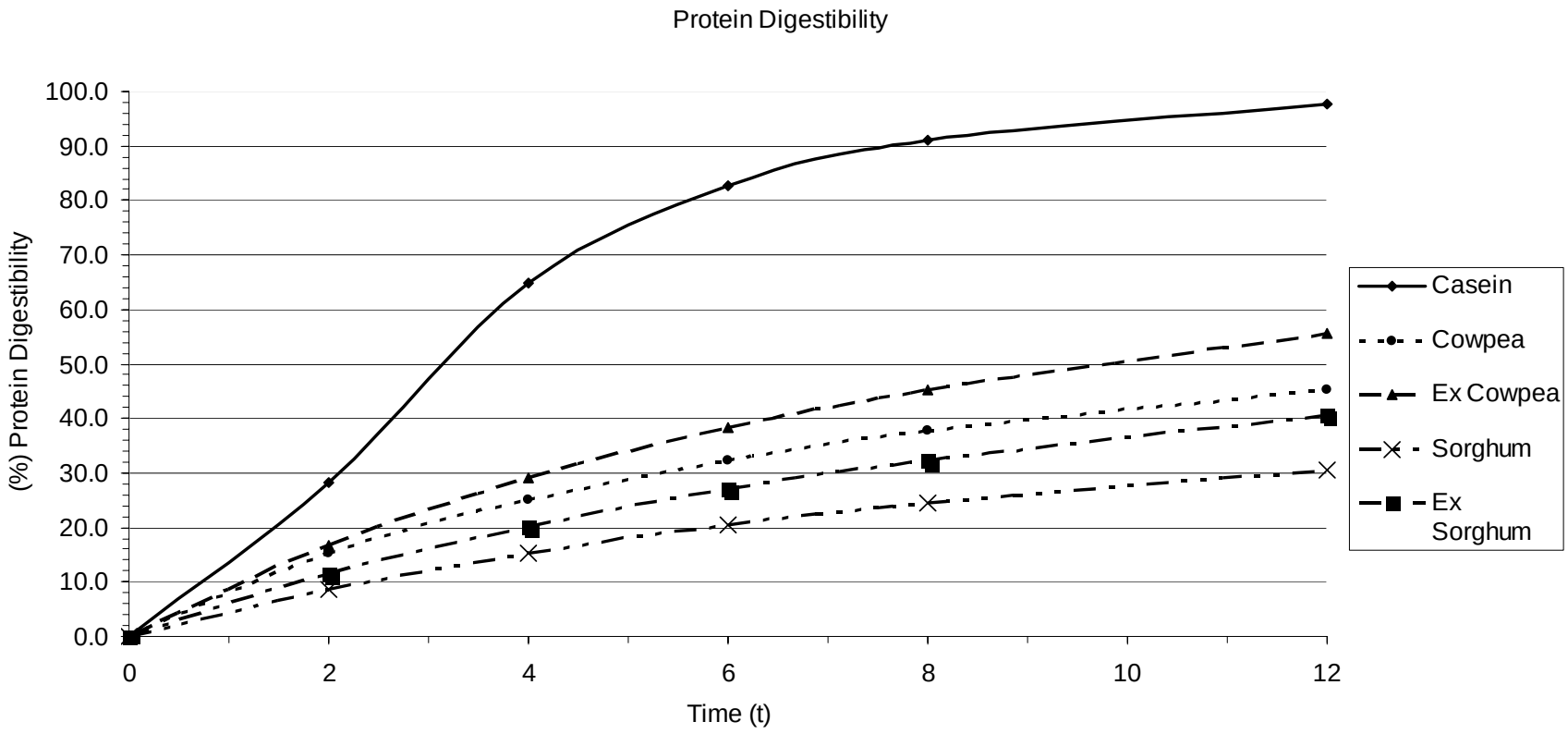
similarities between the *in vitro* and *in vivo* studies. Casein, extruded cowpea, sorghum, and extruded sorghum all show comparable Dmax values to the *in vivo* protein digestibilities of Tuan et al (1999a).

Amino acid availability plots revealed that outlying values were created by the overestimation and the failure to converge toward meaningful data was often seen in samples with low protein content. *in vivo*

Table 4.1: non-linear regression values for Hill Equation coefficients

	Dmax	R-Square		K
Casein	102.8	0.91	2.18	11.99
Cowpea	75.95	0.97	0.99	7.99
Extruded Cowpea	100.36	0.99	1.01	9.99
Sorghum	61	0.93	1	12
Extruded Sorghum	81	0.98	1	12

Figure 4.1: Curve-fitted Protein Digestibility



f*in vivo*1999a Table 3.11. as well. Amino acid availability were affected by low recoveries of amino acids in dialysate samples. Amino acid availability seen through these values (Figure 4.2 – Figure 4.6) does not correlate with protein The lack of correlation between overall protein and amino acid availability within the samples may be due to poor recoveries of amino acids throughout the dialysis and subsequent errors in the hydrolysis procedure for amino acid analysis

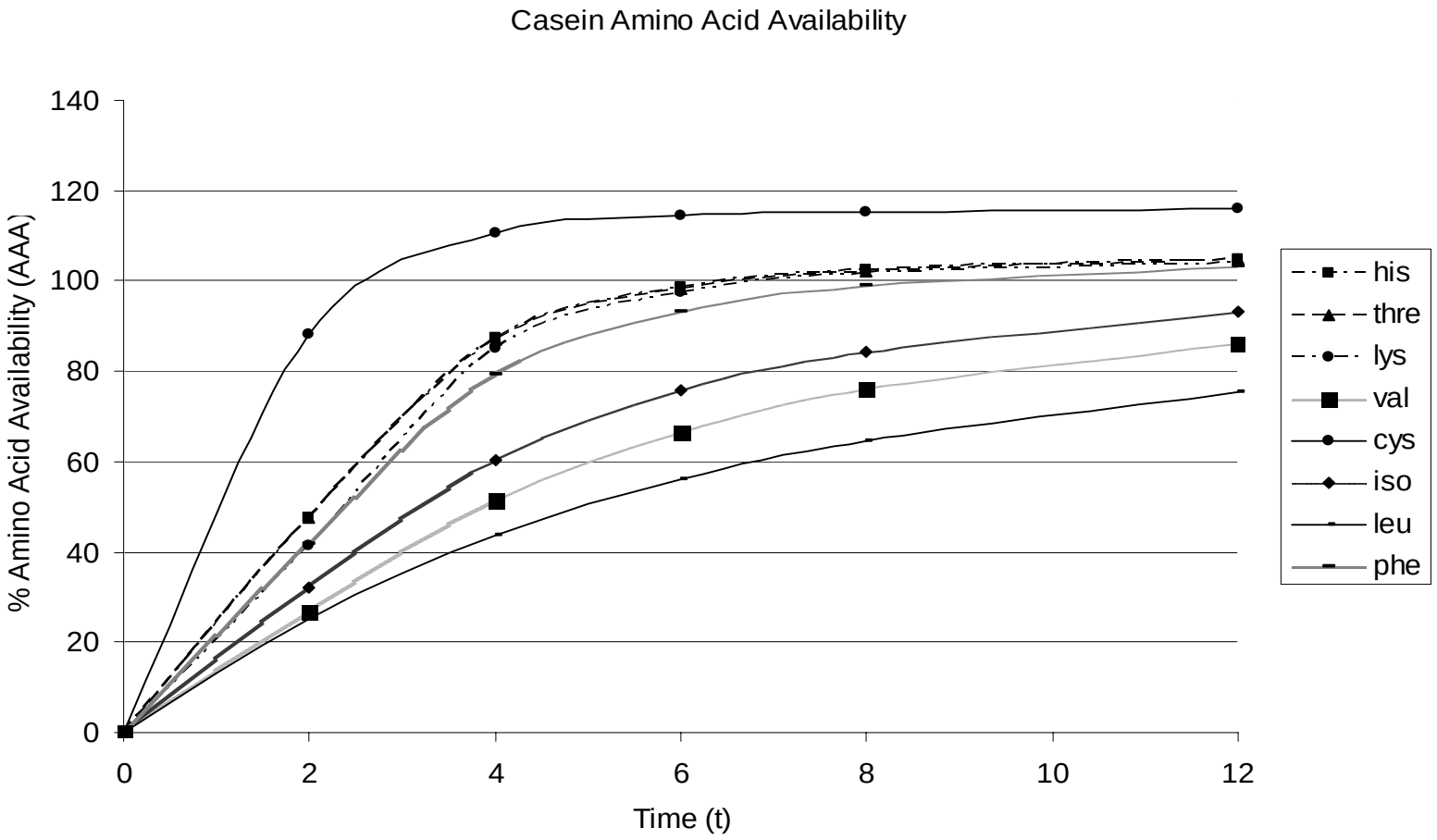


Figure 4.2: Curve-fitted Casein Amino Acid Availability

Figure 4.3: Curve-fitted Sorghum Amino Acid Availability

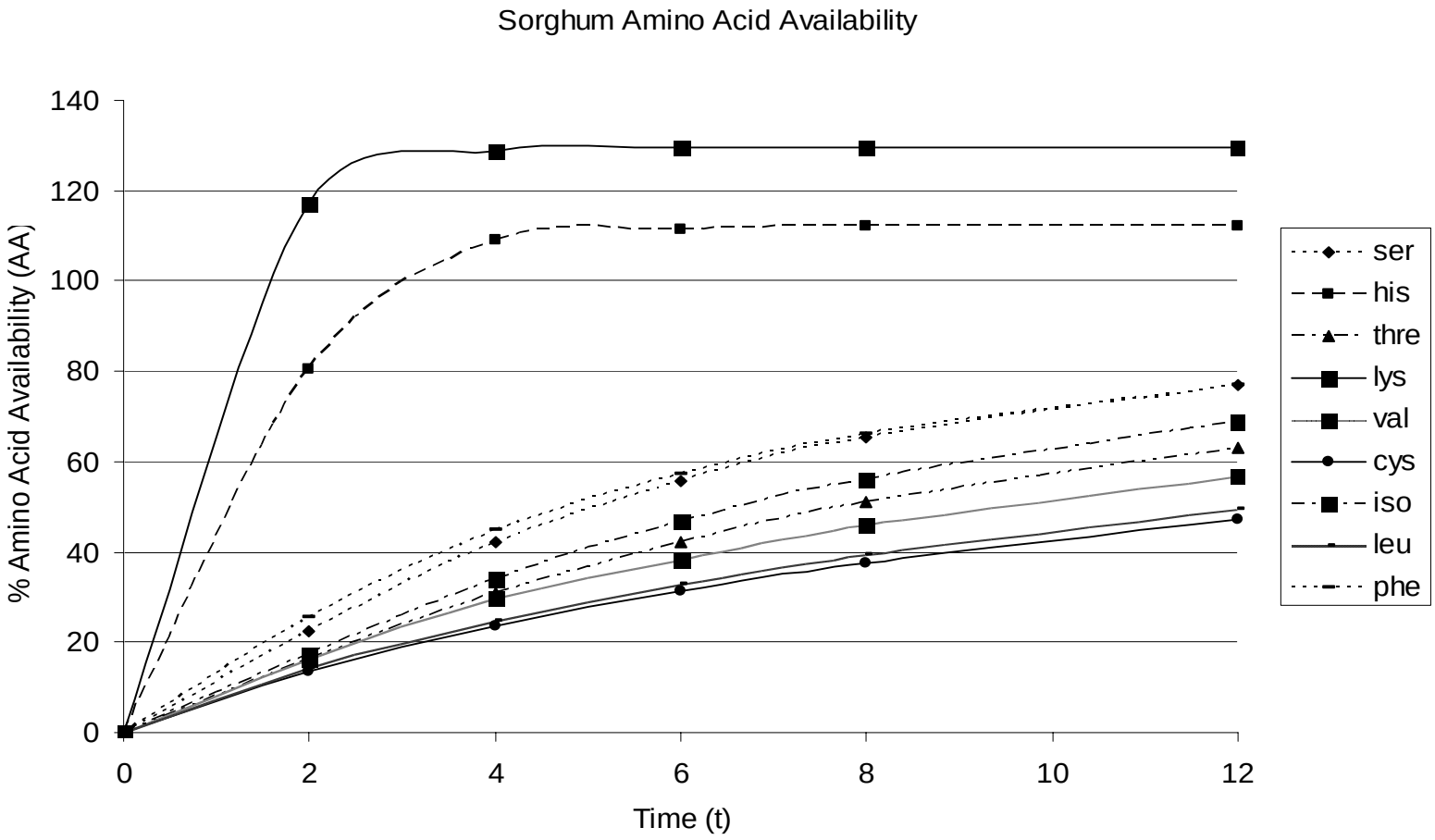


Figure 4.4: Curve-fitted Cowpea Amino Acid Availability

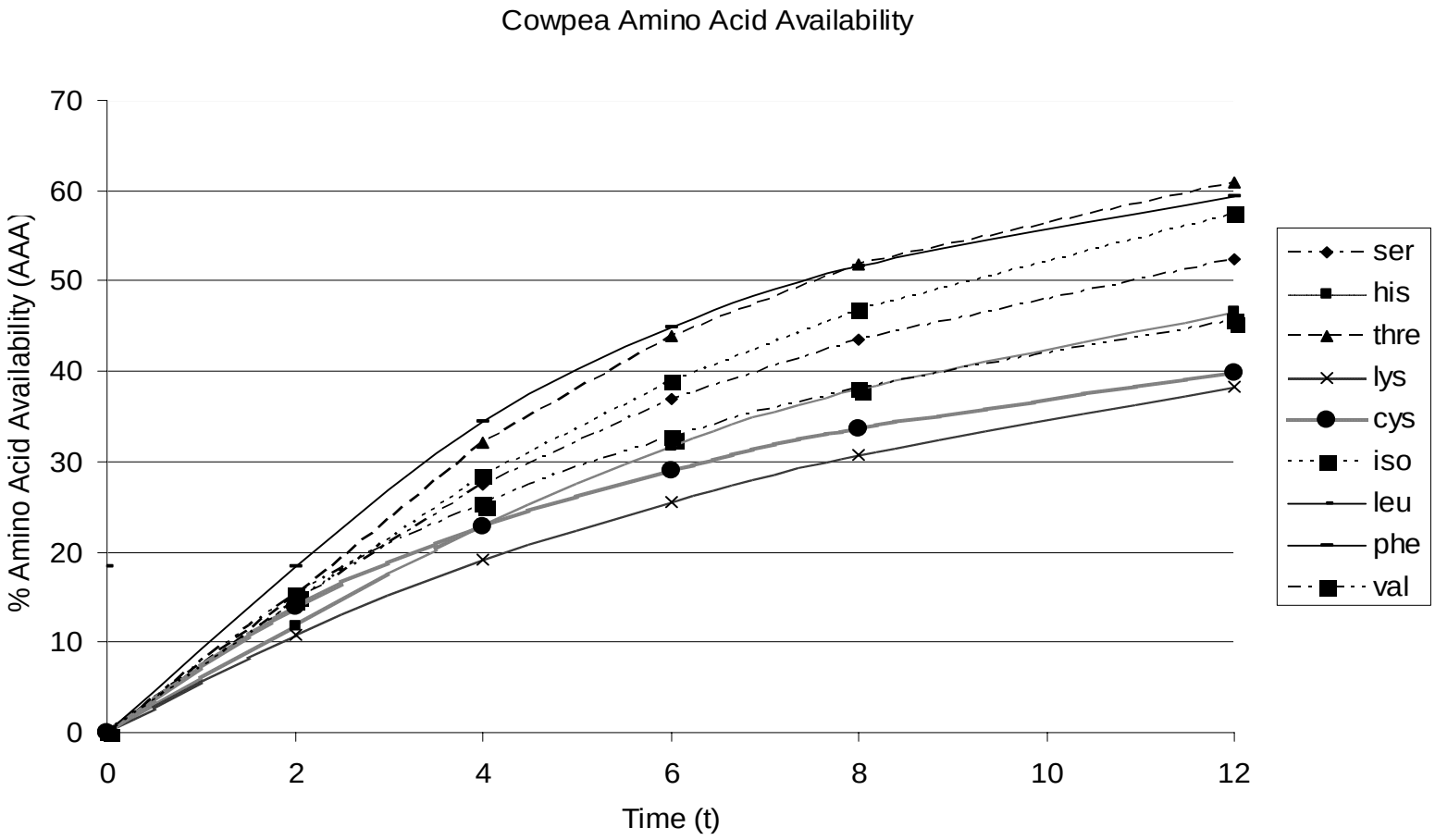


Figure 4.5: Curve-fitted Extruded cowpea amino acid availability

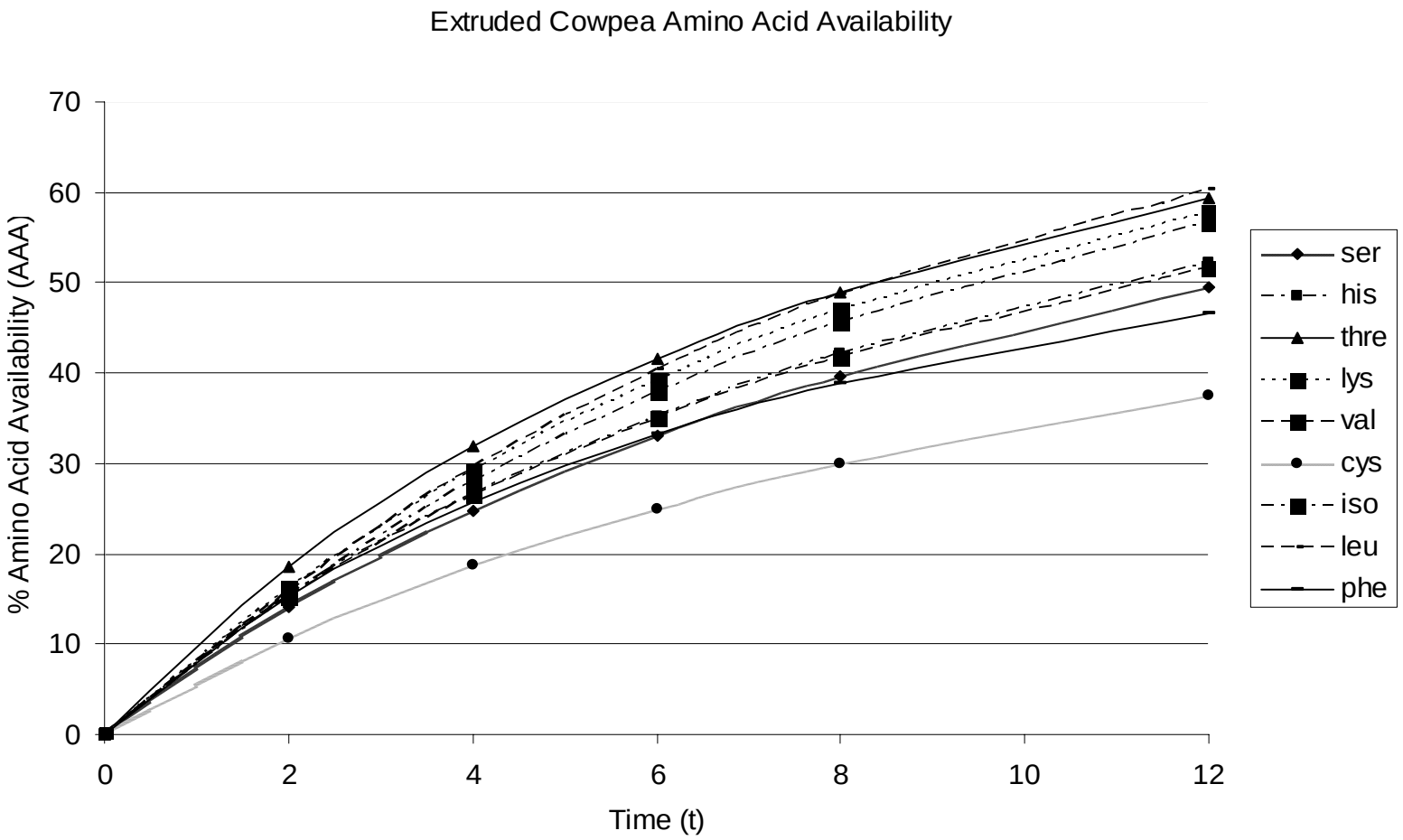


Figure 4.6: Curve-fitted Extruded sorghum amino acid availability

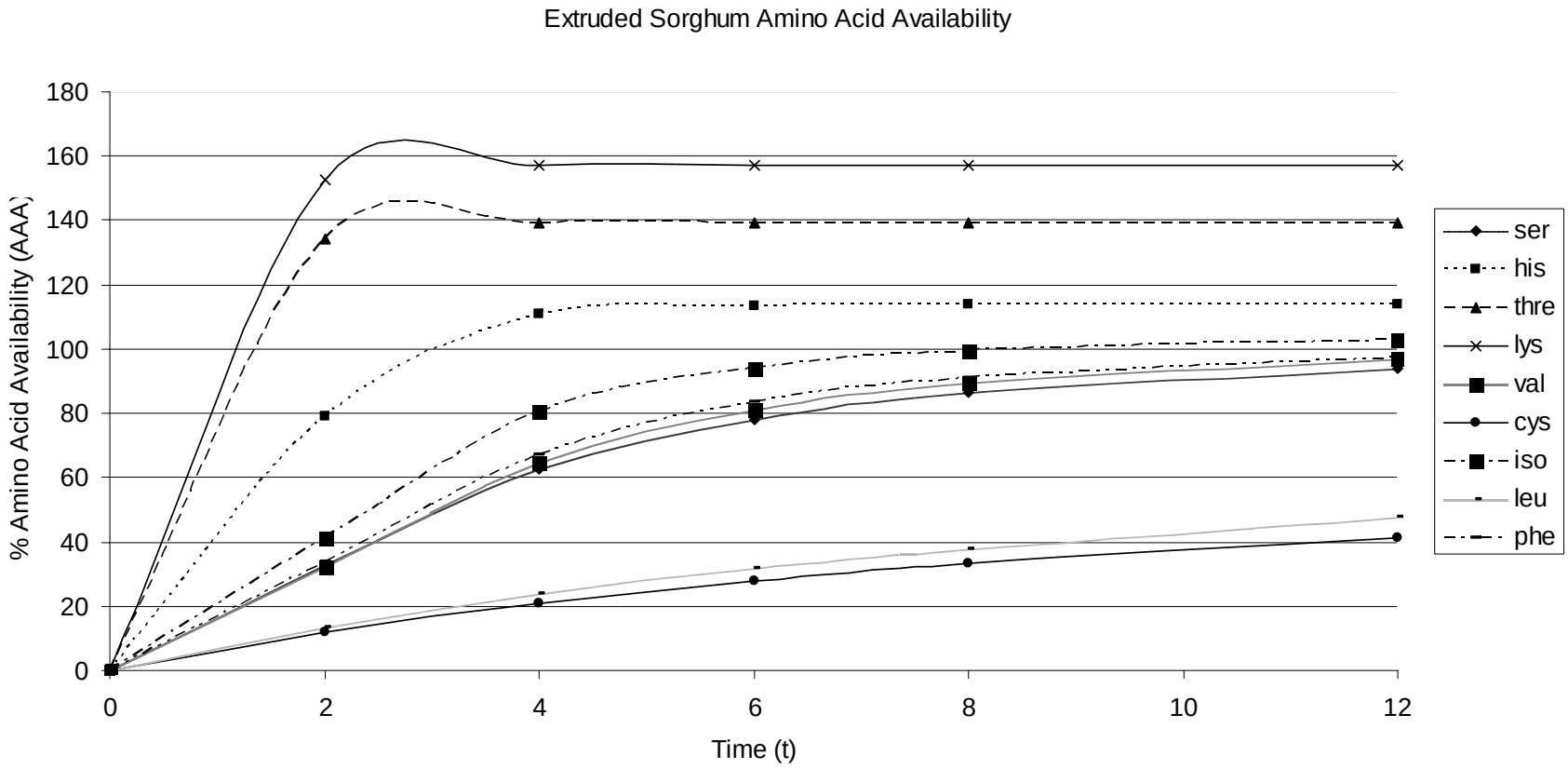


Table 4.2: *In vitro* protein digestibility PDCAAS

Essential Amino Acid	Casein	Sorghum	Cowpea	Extruded Sorghum	Extruded Cowpea
Histidine	1.94	1.06	1.51	1.25	2.15
Threonine	1.47	0.72	0.97	0.83	1.31
Lysine	1.73	0.42	0.99	0.45	1.37
Valine	1.91	1.12	1.02	1.30	1.43
Cysteine + Methionine	0.91	1.18	0.81	1.33	1.11
Isoleucine	1.90	1.00	1.08	1.19	1.56
Leucine	1.70	1.35	0.81	1.62	1.18
Phenylalanine + Tyrosine	1.93	1.07	1.10	1.28	1.61

CONCLUSION

PDCAAS values generally agreed with *in vivo* data of Tuan et al (1999a). Although the PDCAAS results showed promise in the areas of recovery, recovery for individual amino acids was poor. This in turn resulted in the inability to calculate any AAACAASvalues. Amino acid recovery was compromisedby sulfur containing recoveries results. There can only be consistency with the amino acid data when recoveries through the freeze-drying process can quantitatively be examined.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

In vitro protein digestibility can be used as a method of determining protein nutritional quality. Secondly, *in vitro* protein digestibility offers many benefits toward cost reduction in determining protein nutritional quality. *In vitro* protein digestibility can also offer a realistic approach and insight into many of the processes that humans go through when digesting.

The first thing that must be done in order to establish a "working" measure of *in vitro* protein digestibility is to gather data from an in vivo study and correlate it with that of an *in vitro* study of the same materials. When this correlation is made then and only then can *in vitro* digestibility be considered as an effective means of determining protein nutritional quality. The need for better analytical techniques in *in vitro* protein determination is the only way to quantitatively acquire information regarding amino acids and their individual profiles. Overall digestibility and sample recovery size is also important. Therefore, the need for an improved measure of *in vitro* protein digestibility exists. PDCAAS can be calculated from all *in vitro* sources of protein digestibility.