Variations of Amino Acid Standardized Ileal Digestibility in Soybean Meals

Elizabeth Maria Ramirez

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Jeffery Escobar, Chair

Mark D. Hanigan

Jason M. Scheffler

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ABSTRACT

Soybean meal (SBM) is a staple proteinaceous feedstuff in diets for monogastric animals like poultry and swine. It is known that soybeans contain several anti-nutritional factors that, if untreated, results in decreased quality and bioavailability of amino acids (AA). Thermal processing via heat treatment of soybeans and SBM is essential for inactivation of these anti-nutritional factors; however, over-processing may result in extensive AA damage, particularly lysine. Feeding heat damaged SBM has been proven to be an inefficient source of AA for monogastrics as they cannot be used for any metabolic function. In typical corn-soybean meal diets for pigs and poultry, lysine is the first- and second- limiting AA, respectively. Currently, laboratory procedures are unable to accurately determine digestible lysine in SBM. The objective of this thesis was to compare SBM AA digestibility obtained from 28-day old broilers to values obtained from an *in vitro* digestion procedure. The correlation between AA concentration in the SBM and its *in vivo* standardized ileal digestibility (SID) was also analyzed. Twenty-four SBM samples (21 from U.S.A., 2 from Canada, and 1 from Mexico) were analyzed. *In vivo* lysine SID ranged from 69-93%. Results indicated no correlation ($r = -0.16$ to 0.21; $P = 0.33$ to 0.98) between analyzed AA content in SBM and *in vivo* SID. An increase in lysine SID was associated with an increase in the SID of phenylalanine, leucine, isoleucine, valine, tyrosine, alanine, threonine, glutamate, aspartate, methionine, histidine, and glycine ($r^2 = 0.63$ to 0.93; $P < 0.001$). Poor association was determined between lysine proline, arginine, and serine ($r^2 = 0.14$ to 0.43; $P = 0.001$ to 0.003). Lastly, results indicated no association ($r^2 = 0.00$ to 0.08; $P = 0.17$ to 0.99) between *in vivo* and *in vitro* SID for any of the AA tested. In summary, it appears that

lysine may be a good indicator for SID estimations for most essential AA; however, SBM content of a particular AA is not a good indicator of its digestibility. Additionally, current *in vitro* digestibility techniques seemed inadequate in identifying *in vivo* SID differences and further analytical improvements are needed.

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Chapter 1

Introduction

Soybeans have been widely used in the agricultural industry as a source of protein, especially for monogastric animals. Production of soybeans has increased from 1.078 million metric tons in 1980 to 1.756 million metric tons in 2009; however, prices have also increased with soybeans being $7.57/bushel in 1980 to $11.95/bushel in 2011 (USDA, 2011). This increase in price is due the increased use of corn in ethanol production. Increasing the allotment of corn for ethanol production adds to the demand of corn used for livestock feed. Most of the corn produced in the U.S. is produced in the mid-western states (i.e. Iowa, Illinois, Nebraska, etc). Coincidently, these are the same states that also produce the majority of the soybean crop. The competition over land for crop production results in the increase in price. With this increase in price, it becomes more important for farmers to achieve precise feeding of amino acids (AA) to obtain consistently high quality products.

Soybeans are generally used for soy oil production, and in that process, soybean meal (SBM) is obtained. This byproduct is used as a feed ingredient for livestock. In the U.S., the poultry and swine industries consume about 12.7 and 6.9 million metric tons, yearly, respectively (USB, 2011).

Soybeans contain several anti-nutritional factors; compounds present in the feed that negatively effect or reduce the growth of the animal. The most notable ones in soybean are Kunitz-trypsin inhibitors (Kunitz, 1946; Baker 2000). Briefly, the trypsin inhibitors bind to trypsin found in the lumen of the small intestine, preventing the activation effect of trypsin on the zymogens released from the pancreas. The lack of active enzymes, such as chymotrypsin and trypsin, results in decreased digestion of dietary protein. Presence of dietary proteins in the

lumen of the small intestine results in inhibition of the cholecystokinin (CCK) negative feedback to the pancreas, continuing secretion of enzymes from the pancreas (Rackis et al., 1986). Feeding raw soybeans to monogastric animals result in decreased growth performance and hypertrophy of the pancreas due to the presence of trypsin inhibitors (Herkelman et al., 1991). Inactivation of these inhibitors can be accomplished through heating of the bean, which occurs during the processing of soy oil extraction and meal production (Synder and Kwon, 1987; Liener, 1994; Purushotham et al., 2007; Goebel and Stein, 2011).

Heating time greatly affects the quality of SBM. Insufficient heating results in inadequate inactivation of trypsin inhibitors, while overheating results in overproduction of melonin compounds via the non-enzymatic Maillard reaction (Hodge, 1953; Ford, 1973; Brien and Morrissey, 1989; Pahm et al., 2008). Lysine is particularly susceptible to the Maillard reaction due to its exposed ε-NH_2 group (Pahm et al., 2008). Its condensation product forms a deoxyketosyl compound after Amadori rearrangement, making the new lysine product unavailable for metabolic functions (Hurrell and Carpenter, 1981).

Storage of the beans and the SBM may also affect the quality of the SBM. Inappropriate handling may result in an increase in split beans resulting in increased oil oxidation and rancidity. Beans should be optimally stored at 5-8°C with a moisture content of about 10%. Beans containing more than 10% moisture should be dried to prevent bacterial or mold growth (Herrman, 2002).

Continuous SBM analysis has the potential to improve precision formulation of diets and subsequent reductions in nitrogen excretion from animals. The ideal analyses will quantify, quickly and accurately, digestible AA and lysine in particular since this is the most heat liable AA. The objective of this thesis was to compare the digestibility of AA in SBM obtained from

28-day old chicks to those obtained from an *in vitro* digestion procedure. The correlation between AA concentration in SBM and its *in vivo* standardized ileal digestibility (SID) was also analyzed. Finally, the association between lysine SID and SID of other AA was determined.

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Chapter 2

Review of Literature

2.1 Introduction

Soybeans are processed to extract oil and the co-product of this process is dried and becomes soybean meal (SBM), which is primarily used to feed animals. Soybean meal (SBM) makes up an integral part of monogastric diets mainly for their protein content and amino acid (AA) profile. Soybean meal is relatively low is sulfur AA (i.e. cysteine and methionine), which are abundant in cereal grains. Thus, combination of corn and soybean meal, for example, provides an ideal AA profile for the growth of poultry and swine. Yet unprocessed soybeans cannot be fed to monogastric animals because of their high content of anti-nutritional factors, primarily trypsin inhibitors.

The trypsin inhibitors block the negative feedback from CCK to the pancreas by binding to the trypsin found in the lumen of the small intestine. This prevents trypsin from activating zymogens released from the pancreas, which decreases dietary protein breakdown (Rackis et al., 1986). Thermal treatment reduces trypsin inhibitors but overheating results in formation of D-isomer AA, lysinoalanine, and other AA-derived compounds that animals cannot use for growth or metabolic processes (Friedman and Liardon, 1985).

The negative reaction associated with excess thermal treatment of SBM is the Maillard reaction. This reaction occurs between an amino group and a reducing sugar (Hodge, 1953). Due to the ε-amino group, lysine (Lys) is the most liable AA to heat damage.

In corn-soybean based diets, Lys is the first limiting AA for swine and second limiting for poultry. Therefore, development of appropriate laboratory techniques to quantify digestible or bioavailable Lys in SBM would have a profound impact on monogastric animal nutrition.

Furthermore, because of its sensitivity to heat damage, lysine digestibility could be used as a qualitative or quantitative evaluator of protein quality. Providing animal nutritionists with an adequate and efficient means of analyzing SBM will allow for consistency in formulation of diets containing SBM and animal performance.

2.2 Monogastrics

2.2.1 Typical Monogastric Diet in US

Feed constitutes the highest production cost in animal enterprises. For monogastric animals in the U.S., such as poultry and swine, feeds are mainly formulated with ground corn and SBM, and supplemented with fats, vitamins, and minerals. Soybean meal is an excellent source of AA, particularly lysine. It contains about 2,230 and 3,180 kcal/kg of metabolizable energy (ME) for poultry and swine, respectively (NRC, 1994, 1998). Corn is typically the major source of energy with 3,350 to 3,420 kcal/kg ME for poultry and swine, respectively. The high sulfur AA content of corn is an ideal complement to the lower content in SBM and hence the basis for their vast combined use in animal feeding.

Corn and SBM based diets are low in calcium and moderate in phosphorus (NRC, 1994). Over half of the phosphorus found in cereal grains is found in the organically bound form, also known as phytic acid. Neither poultry nor swine can digest phytic acid, resulting in phosphorus requirements being expressed as non-phytate phosphorus. Corn has 0.08% non-phytate phosphorus and SBM has 0.65%, which is not enough to fulfill the nutritional need of poultry and swine (NRC, 1994). Thus, corn-SBM based diets are commonly fortified with calcium and non-phytate phosphorus sources like steamed bone meal, mono- or di-calcium phosphate, sodium phosphate, and limestone (NRC, 1994, 1998).

2.2.2 Monogastric Gastrointestinal System

Pigs and poultry (i.e., chickens, turkeys, ducks, geese, and quail) have simple stomach digestives systems with subtle differences. The pig digestive tract is composed of a teethed mouth (i.e., mastication capacity), esophagus, stomach, small intestine, cecum, and large intestine (**Figure 2.1)**. Initial breakdown of the feed occurs at the mouth, where the teeth physically break down the feed to smaller particle sizes while it mixes with amylase-containing saliva. Once ingested, the digesta moves through the esophagus and enters the stomach. The stomach is the site of pepsin release, an enzyme that initiates the break down of proteins into peptides. At the level of the small intestine, most other nutrients, such as lipids, starch, protein and peptides, vitamins and minerals, are further digested by pancreatic and brush-border enzymes. Absorption of AA occurs exclusively in the small intestine. Fermentation occurs mainly in the cecum and large intestine, where there is also absorption of water and short-chain fatty acids, contributing energy to the animal (Stevens and Hume, 1995).

The chicken gastrointestinal tract has a toothless beak (i.e., no mastication), esophagus, crop, proventriculus, ventriculus (gizzard), small intestine, paired ceca, and large intestine (**Figure 2.1**). The crop is an extension of the esophagus and functions to hold feed for future digestion. Though the crop holds feed, little to no digestion occurs here (Stevens and Hume, 1995). The proventriculus is the site of acid and pepsinogen secretion. The gizzard is the muscular organ charged with particle size reduction. Like in pigs, the small intestine is the exclusive site of AA absorption in poultry. Chickens are also unique in that they contain paired ceca. Since digesta passage rate is faster in poultry compared to pigs, the ceca acts as a fermentation chamber while decreasing the passage rate of the digesta. In the chicken about a

third of its total ME can be obtained from post-small intestinal absorption (Stevens and Hume, 1995; Wenk, 2001).

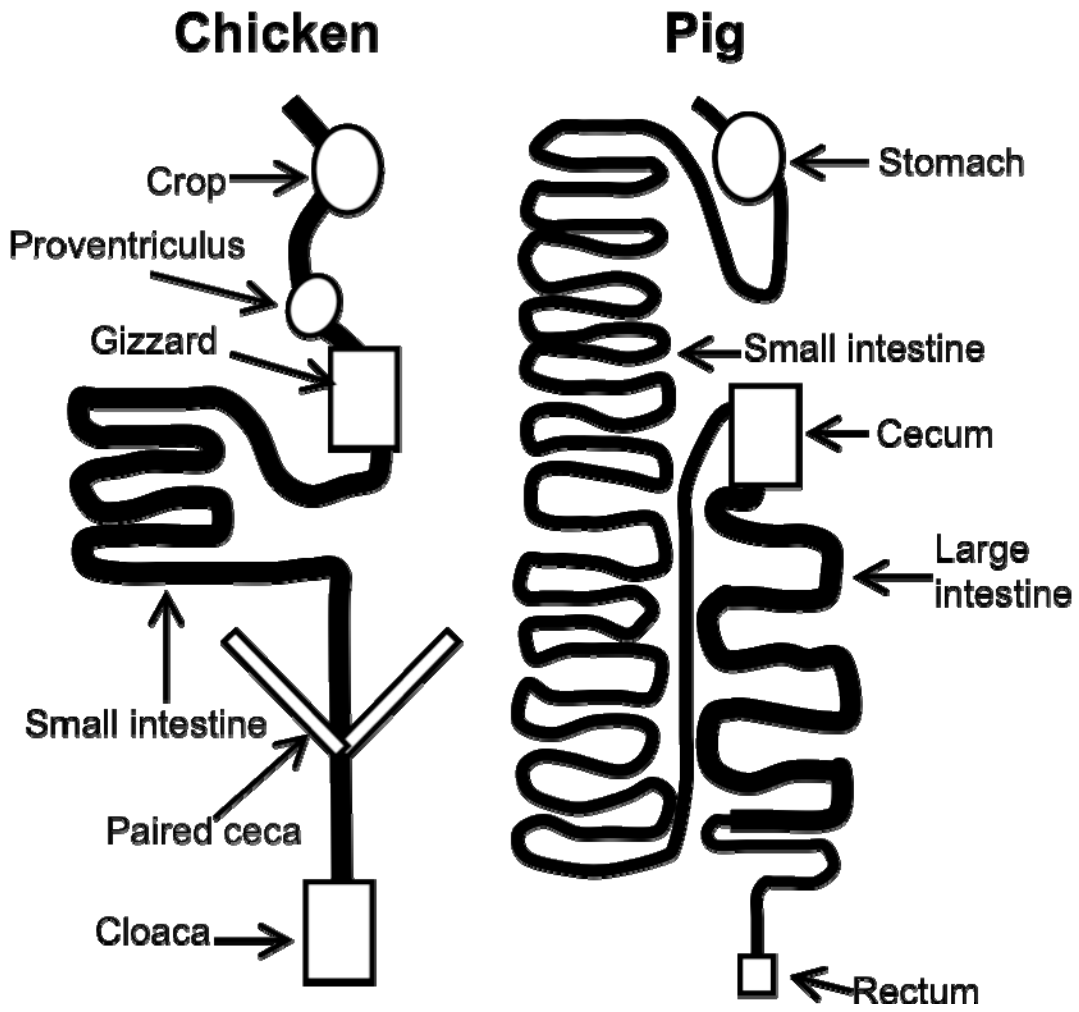

Figure 2.1. Schematic of avian and porcine gastrointestinal tracts (Stevens and Hume, 1995).

2.3 Soybean

2.3.1 Soybeans

The soybean is a crop of great significance to the agricultural industry, widely used as a source of AA for animals. The AA composition of SBM is complementary to that of other cereal grains. Its low content of sulfur-AA but high levels of lysine are an ideal complement to cereal

grains (e.g., corn, wheat, sorghum). Though variations may be found depending on variety, location, and agronomic practices, soybean contain on average 40% protein, 20% lipid, 35% starch, and 5% ash on a dry matter basis (Synder and Kwon, 1987).

2.3.2 Structure of Soybeans

The soybean has several structures that are important to note because they vary in nutrient composition, and processing will have an affect on the nutritional quality of the meal (**Table 2.1**). The cotyledons, which comprise 90% of the seed, contain the majority of protein in structures called protein bodies that range from 2-20 μm in diameter. Lipids, also found in the cotyledon, are located in structures called spherosomes and are 0.2-0.5 μm in diameter. The next largest component of the soybean seed is the hull. During processing, the hull is normally removed and has very little, if any, nutritional contribution in diets for monogastrics. The hypocotyl comprises the smallest part of the seed (2%) but contains a significant portion of protein and lipid (Wolf, 1977).

Table 2.1. Structural and proximate composition of a soybean seed (adapted from Wolf, 1977).

Fraction	Protein (N x 5.71), %	Fat, %	Carbohydrate, %	Ash, %
Whole bean (100%)	40	21	34	5
Seed coat (8%)	9	1	86	4
Cotyledon (90%)	43	23	29	5
Hypocotyl (2%)	41	11	44	4

The pair of cotyledon found in each soybean provides the majority of nutrients used for growth. Splitting the cotyledons causes extensive damage to the bean, as it results in deterioration of oil and impairs separation of gum and oil fractions during oil extraction.

Excessive moisture is detrimental to soybeans because it enhances spoilage during storage (Synder and Kwon, 1987).

2.3.3 Processing of Soybeans

Soybeans go through several steps during processing to produce two major substances: oil and meal. First, seeds are cleaned to ensure a final product free of debris or other contaminants as well as to minimize damage to the processing equipment. Then, hulls are removed due to low oil content and high fibrous contribution to the meal. To facilitate hull removal, the beans should contain about 10% moisture and thus beans are usually subjected to thermal drying processes. Soybeans are exposed to streams of hot and cool air and are then allowed to temper for 1-5 days. Soybeans are then roll cracked (i.e., counter-rotating, corrugated, or fluted) to remove the hulls using light jet-streams of air. Cracked soybeans are conditioned with steam in preparation for flaking via two smooth rolls; facilitating the oil extraction process (Synder and Kwon, 1987).

Hot hexane and other solvents are used to extract oil. Solvents are recovered from both oil and flakes, recycled, and used again while the soy oil is refined. This refining process entails degumming, alkali refining, bleaching, hydrogenation, winterization, and deodorization, preparing oil for distribution (Synder and Kwon, 1987). The desolventizer-toaster is a common method to remove solvents while inactivating trypsin inhibitors in a single step. Finally, desolventized flakes are subjected to hammer mill treatment to reduce particle size. The addition of hulls allows for adjustment of crude protein from 44-49%. (Synder and Kwon, 1987).

2.3.4 Handling and Storage of Soybeans and Soybean Meal

Handling and storage of soybeans and SBM are important when talking about the quality of end products. Increase in breakage, storage time, moisture, and other factors will decrease quality of both main products: oil and meal. Mechanical handling can increase the amount of splitting, resulting in bean deterioration particularly during extended storage (Herrman, 2002).

There are three major components that will affect bean quality during storage: temperature, moisture content, and duration. Temperatures between 5-8°C are optimal for soybean storage if their moisture content is at 10% to reduce spoilage (Herrman, 2002).

2.3.5 Kunitz-Trypsin Inhibitors

One of the major reasons soybeans are exposed to thermal treatment is to increase the nutritive value of the meal via reduction of Kunitz-trypsin inhibitors. Negative effects of feeding raw soybeans to animals have been known since the early 1900s, but it wasn't until the 1940's that the mechanisms were understood. Kunitz (1946) discovered what are now called trypsin inhibitors. Trypsin inhibitors bind to trypsin in the small intestine. Trypsin is responsible for protein digestion and activation of pancreatic protease zymogens for further protein digestion. Presence of dietary protein in the small intestine, due to bound trypsin, inhibits negative feedback from cholecystokinin to the pancreas. This results in increased secretion from the pancreas, leading to hypertrophy of the pancreas (Rackis et al., 1986;Herkelman et al., 1991).

Most trypsin inhibitors reside in the cotyledons, which are concentrated in the meal after oil extraction, resulting in the presence of trypsin inhibitors in SBM (Horisberger and Tacchini-Vonlanthen, 1983a,b; Horisberger et al., 1986; Anderson and Wolf, 1995). Thus, thermal treatment considerably reduces the presence of trypsin inhibitors in SBM compared to raw seeds (**Table 2.2**). Table 2.2 demonstrates differences in trypsin inhibitor activity between raw and

toasted SBM. Trypsin inhibitor activity was measured by trypsin inhibition (Anderson and Wolf, 1995).

Heat used to inactivate trypsin inhibitors in SBM is generally provided by the desolventizer-toaster, which effectively deactivates about 90% of trypsin inhibitors. When exposing SBM to heat treatment, it is essential that an accurate temperature and exposure time be utilized.

The effects of under, adequate, and over exposure to heat treatment on chick growth performance and pancreas weight has been previously documented (**Figure 2.2 and 2.3**) (Herkelman et al., 1991). Exposing meal to insufficient heat treatment will result in inadequate deactivation of trypsin inhibitors, whereas excessive treatment will cause degradation of essential AA, most notably lysine, via the Maillard Reaction (Synder and Kwon, 1987). It is clear that performance is greatly reduced when raw soybeans are fed due to high trypsin inhibitor presence. Increasing exposure of soybeans to heat improves growth performance, with optimization occurring at the 40 min time period (Herkelman et al., 1991).

Table 2.2. Trypsin inhibitor content of soybeans and its processed products (Anderson and Wolf, 1995).

Trypsin Inhibitor Activty		
Type	**mg/g sample**	**mg/g protein**
Whole soybeans	16.7-27.2	34.7-122.6
Raw flour	28-32	57.8
Toasted flour	7.9-9.4	15.9

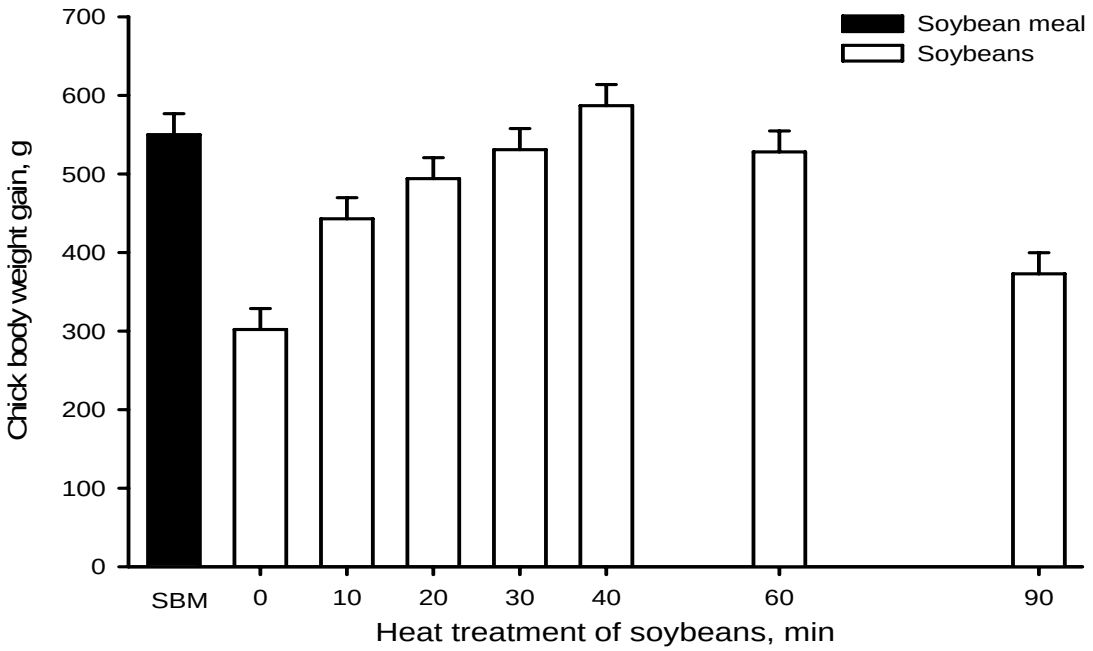

Figure 2.2. Weight gain of broilers fed soybean meal or soybeans heated for varying lengths of time (Herkelman et al., 1991).

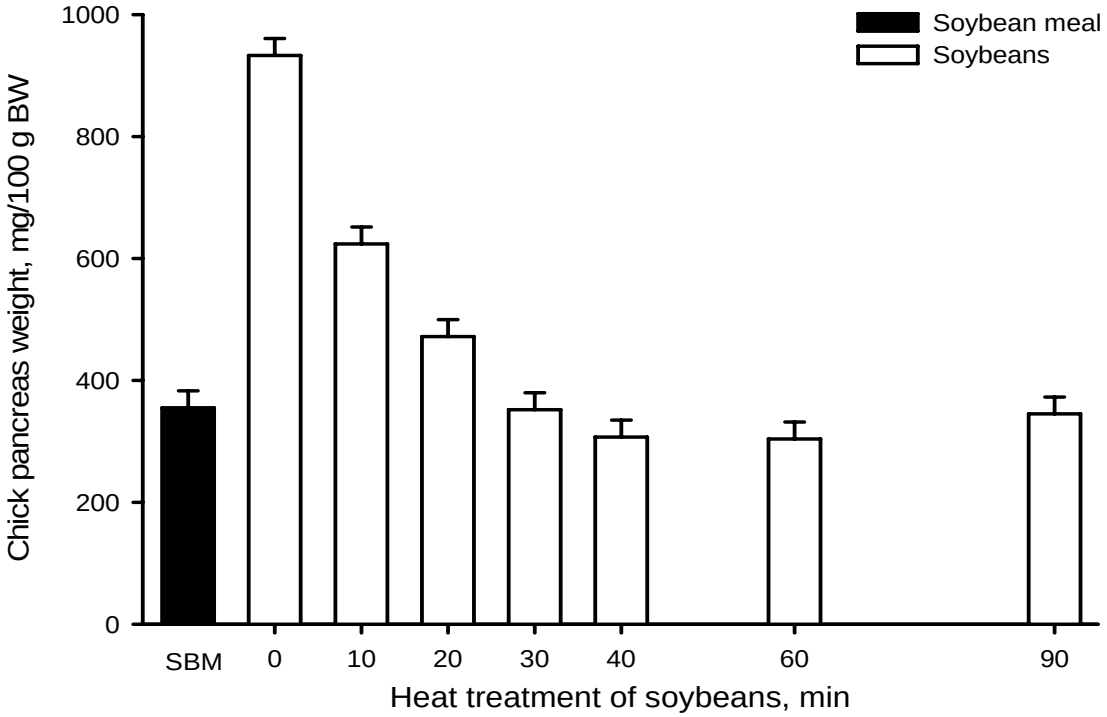

Figure 2.3. Pancreas weight of broilers fed soybean meal or soybeans heated for varying lengths of time (Herkelman et al., 1991).

2.3.6 Maillard Reaction

During the heating process, the combination of reducing sugars, AA, and heat accelerates the Maillard reaction in SBM. This non-enzymatic reaction is responsible for the browning associated with cooking and contributes unique colors and flavors to foods and feedstuffs at the expense of nutritional value.

Though the Maillard reaction is complex, it can generally be divided into 3 major stages (**Figure 2.4**). First, condensation of amino and carbonyl groups results in production of glycosylamine; forming a deoxyketose or deoxyaldose via Amadori rearrangement. Next, Amadori compounds breakdown via two possible pathways resulting in formation of 1,2-eneaminol at low pH or 2,3-enediol at high pH. The final stage is production of melanoidin pigments. Though not entirely understood, it is believed that melanoidin pigments are created through aldol condensation and aldehyde-amino polymerization. (O'Brien and Morrissey, 1989; Hodge, 1953)

The Maillard reaction produces desirable compounds in the human food industries, like baking and meat products. These compounds, however, are not appreciated in monogastric animal diets because of reduced AA bioavailability. Cross-linked products created between sugars and lysine during Maillard reaction, such as lysinoalanine, are the main reason for deterioration in protein quality of feedstuffs. In this form, lysine is no longer bioavailable to the animal for metabolic uses like growth (Gabert et al., 2001; Freidman, 1996; O'Brien and Morrissey, 1989). Lysine is the first and second limiting AA in diets fed to swine and poultry, respectively, and SBM is the primary source of AA in most commercial diets. Therefore, heat damage of SBM is highly detrimental to the growth performance of pigs and poultry and hence increases production costs for producers and purchase price for consumers.

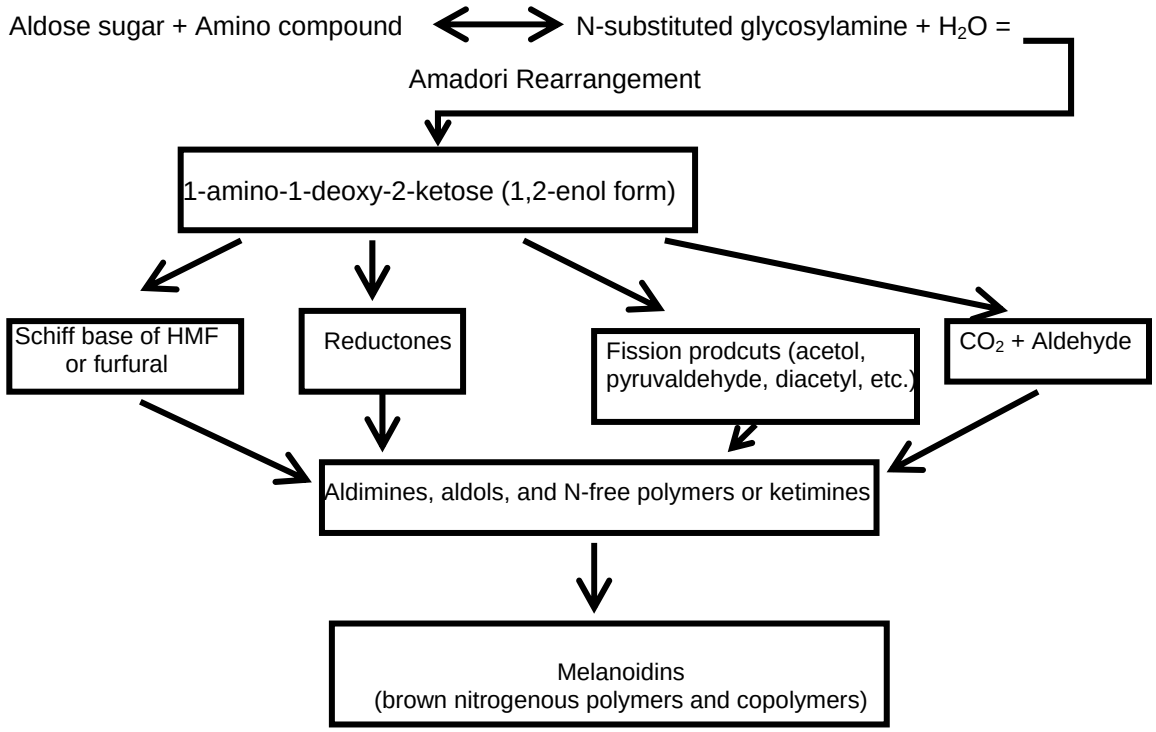

Figure 2.4. The Maillard Reaction (adapted from Hodge, 1953).

2.4 Conclusion

SBM constitutes the main source for AA in the diets for most animals. Because of their simple stomach anatomy, poultry and swine are most susceptible to the negative nutritional consequences of heat damaged SBM. Ensuring high AA quality in SBM is essential to formulate precise diets for pigs and poultry to achieve maximum performance and reduced nitrogen excretion. Proper processing of SBM is needed to reduce trypsin inhibitors while maintaining protein quality and AA integrity. Therefore, appropriate development of accurate and precise analytical methods to determine lysine digestibility in SBM are needed.

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Chapter 3

Variations of Amino Acid Standardized Ileal Digestibility in Soybean Meals

3.1 Abstract

Heat treatment of soybean meal causes amino acid (AA) damage, particularly to lysine. Birds and pigs cannot use heat-damaged AA for growth. Lysine is the first- and second-limiting AA in corn-soybean meal diets for pigs and poultry, respectively. Currently, there is no reliable laboratory analysis for the determination of digestible lysine in soybean meal. The objective of this thesis was to compare the digestibility of AA in SBM obtained from 28-day old chicks to those obtained from an *in vitro* digestion procedure. The correlation between AA concentration in SBM and its *in vivo* standardized ileal digestibility (SID) was also analyzed. Finally, the association between lysine SID and SID of other AA was determined.

A total of 24 soybean meal samples were obtained from different processing plants across the U.S (n=21), Canada (n=2) and Mexico (n=1). Our results indicate a poor correlation between analyzed AA content in soybean meal and standardized ileal digestibility (SID) coefficients obtained in growing broilers. There are significant positive correlations between the SID of lysine with several essential AA. These correlations indicate that heat damage also affects the digestibility of other AA and that lysine can be a good indicator of overall protein and AA quality. Thus, once lysine SID is quantified in soybean meal samples, prediction equations can be utilized to determine SID of other essential AA. This implies a similar approach to the ideal protein concept, to which poultry and swine nutritionists are well familiarized. Therefore, efforts to develop analytical methods to determine digestible AA should be concentrated on SID lysine. Finally, correlation between poultry SID for AA and values obtained for the same soybean meal samples were poorly correlated mainly due to overestimation of the in vitro procedure.

3.2 Introduction

Soybeans are processed to extract oil and the co-product of this process is dried and becomes soybean meal (SBM), which is primarily used to feed animals. Soybean meal makes up an integral part of monogastric diets mainly for their protein content and amino acid (AA) profile. Soybean meal is relatively low in sulfur AA, which are abundant in cereal grains. Thus, combination of corn and SBM, for example, provides an ideal AA profile for the growth of poultry and swine.

Unprocessed soybeans cannot be fed to monogastric animals because of their high content of anti-nutritional factors, primarily trypsin inhibitors (Kunitz, 1946). Inactivation of these inhibitors can be accomplished through heating, which occurs during the process of oil extraction and later drying (Synder and Kwon, 1987). Insufficient heating results in inadequate deactivation of trypsin inhibitors whereas overheating results in formation of D-AA, lysinoalanine, and other compounds that cannot be used for growth or metabolic processes (Friedman and Liardon, 1985).

The Maillard reaction or non-enzymatic browning occurs between a free amino group of an AA and reducing sugars in the presence of heat. Because of its ε-amino group, lysine (Lys) is the most liable AA to heat damage via Maillard reaction (O'Brien and Morrissey, 1989; Hodge, 1953).

The amount of Lys available for growth can be determined by digestibility studies and it is known as digestible Lys. Animal studies are expensive and time consuming and therefore are not suitable for routine evaluation of SBM batches. Conventional AA analysis cannot detect the amount of digestible Lys because the indigestible Lys fraction is converted back to Lys during the acid hydrolysis required to breakdown proteins into free AA for analysis. Thus, conventional

AA analysis provides "total lysine" content from which animal nutritionist must estimate the digestible lysine content. In the U.S., SBM usage in animal production is a driving force to set soybean value.

Development of laboratory procedures for rapid analysis of AA digestibility in SBM batches will allow nutritionist to control variation in SBM batches and hence formulate more precise diets for poultry and swine. Because Lys is the most sensitive AA to heat damage, its digestibility could be used as a qualitative and quantitative evaluator of whole protein quality. The objective of this study was to use an established in vitro digestion procedure to quantify AA digestibility. To accomplish this goal, we determine AA digestibility in 24 different SBM samples in growing broilers and in the laboratory. In addition, AA digestibility for all soybean meal samples was also assayed in growing pigs.

3.3 Materials and Methods

3.3.1 Poultry Trial

The Virginia Tech Institutional Animal Care and Use Committee approved all animal procedures.

A total of 480 straight-run Cobb 500 (Cobb Vantress Inc., Wadesboro, NC) birds were obtained from a commercial breeder (Broadway, VA) at day-of-hatch. Birds were transported to Virginia Tech and housed in a thermostatically controlled brooder battery divided into pens. Each pen was equipped with raised wire floors, height-adjustable nipple waterers, and feeder. The brooder battery was located in a discretely ventilated room with 100% clean air (i.e., no recirculation), under constant negative pressure, temperature controlled by an automated system,

and under continuous fluorescent lighting. Broilers had ad libitum access to water and feed unless otherwise indicated.

From day 1 to 21 of age, birds consumed a standard commercial starter diet that met or exceeded the nutritional recommendations from the breeder (Cobb-Vantress, **Table 3.1**). At 21 days of age, birds were individually weighed, stratified by body weight and randomly assigned to pens. Five birds were placed in each of 48 stainless-steel pens (Alternative Design, Siloam Springs, AR) and 4 pen replicates were run for each of 24 different SBM samples. The study was replicated once for a total of 96 pens of 5 birds each. From 21 to 26 d of age, birds consumed the semi-purified experimental diet, which was formulated to meet or exceed the breeder recommendations for nutrient with the exception of AA (**Table 3.1**). For all SBM samples, it was assumed that they contained 47.5% crude protein and it was the only source of protein and AA in the broiler test diets. Chromium oxide (Cr_2O_3) was used as an indigestible marker according to standard procedures to quantify AA digestibility (Lemme et al., 2004; Stein et al., 2007).

On d 28, birds were asphyxiated with CO_2, euthanized by cervical dislocation, and dissected. The ileal (from the vitelline diverticulum to 2 cm proximal to the ileo-cecal junction) contents from each of the 5 birds per pen were manually stripped into labeled bags to obtain a composite sample. Ileal contents were immediately frozen in liquid nitrogen and stored at -80°C until freeze-dried for Cr_2O_3 and AA content analysis.

Before analyses, SBM and diet samples were ground in a mill fitted with a 1 mm screen. Digesta samples were ground using a coffee grinder. A ground subsample was dried overnight at 105°C for determination of dry matter (DM) content.

3.3.2 Chromic Oxide Analysis

Chromic oxide was used as the indigestible marker. Briefly, ground subsamples of diets and digesta were subjected to dry-ashing at 450°C overnight, and then to sulfuric:perchloric acid hydrolysis for colorimetric quantification of Cr_2O_3 content at 440 nm as previously described (Fenton and Fenton, 1979).

3.3.3 Amino Acid Analysis

A ground subsample was hydrolyzed with 6 M HCl at 100°C for 24 hours for AA analysis as previously described (Albin et al., 2000). Acid hydrolyzed AA were derivatized with phenyl isothiocyanate (Acros Organics, Geel, Belgium), and separated using a 2695 Alliance HPLC equipped with a 30-cm Pico-Tag column, 2487 UV/Vis detector, and Empower software (all from Waters Corp., Milford, MA) using previously described conditions (Cohen et al., 1989). Amino acid concentrations are expressed in g/100 g of digesta, SBM, or diet.

3.3.4 <u>In-Vivo</u> A*mino Acid Digestibility*

Digestibility coefficients for apparent ileal digestibility of AA (AID) were calculated based on the following equation (Stein et al., 2007):

$$\text{AID (\%)} = \{ 1 - [(AA_{Digesta} / AA_{Feed}) \times (M_{Feed} / M_{Digesta})] \} \times 100$$

where AID is the apparent ileal digestibility of an AA, $AA_{Digesta}$ is the AA content in the ileal digesta, AA_{Feed} is the AA content in the feed, M_{Feed} is the Cr_2O_3 concentration in the feed, and $M_{Digesta}$ is the Cr_2O_3 concentration in the ileal digesta; all on a DM basis.

Standardized ileal digestibility coefficients were calculated using the following formula (Lemme et al., 2004):

$$SID\ (\%) = AID\ (\%) + [\ (AA_{End}\ ) / (\ AA_{Feed}\ ) \times 100\ ]$$

where SID is the standardized ileal digestibility of an AA, AID is the apparent ileal digestibility coefficient of an AA (%), AA_{End} is the basal endogenous AA losses (g/kg DM), and the AA_{Feed} is the AA content of the feed (g/kg DM). Values for basal endogenous AA losses were adopted by the regression method using enzymatically hydrolyzed casein (Golian et al., 2008).

3.3.5 In Vitro D*igestion and Amino Acid Digestibility*

In vitro digestion was performed on 24 SBM samples using previously published conditions (Boisen and Fernandez, 1997; Noblet and Jaguelin-Peyraud, 2007; Wilfart et al., 2007) with modifications. This procedure consists of a two-step digestion process mimicking gastric and small intestinal phases of porcine digestion. Briefly, 0.1 g of ground SBM sample was mixed with 5 mL of 0.1 phosphate buffer (pH 6.0), HCl to pH 2.5, and 200 µL of freshly made porcine pepsin in distilled water (Sigma-Aldrich, St. Louis, MO) in a screw-cap borosilicate tube for 2 hours at 39°C under gentle rocking. Porcine pepsin was added to achieve 23.45 U of enzyme per mg of SBM sample. Then, 2 mL of 0.2 M phosphate buffer (pH 6.8) and 1 mL of 0.6 M NaOH were added to each tube and pH was adjusted to 6.8 with NaOH. A 200-µL aliquot of freshly made porcine pancreatin (Sigma-Aldrich) in distilled water was added to achieve 20 USP units of pancreatine per mg of SBM sample. Tubes were re-capped and incubated under rocking at 39ºC for an additional 4 hours. Upon completion, 200 µL of 10

mg/mL of phenylmethanesulfonyl fluoride (Sigma-Aldrich) in methanol were added to stop enzymatic activity. Tubes were immediately vortexed and centrifuged at 4,000×g for 15 min at ambient temperature. Supernatants were discarded and samples were dried under vacuum at 45°C for 12 hours, or until completely dry. Samples were allowed to cool to ambient temperature, weighed, and subjected to acid hydrolysis with 6M HCl (i.e, 60 µL per mg of dry pellet). After acid hydrolysis AA were quantified as previously stated.

3.3.6 Statistical Analysis

Data were tested for normality using the PROC UNIVARIATE procedure of SAS (SAS Inst., Inc., Cary, NC). Regression equations between poultry Lys SID and poultry SID for all other individual AA were obtained with the PROC MIXED procedure (SAS). The PROC CORR procedure of SAS was used to determine correlation coefficients between poultry SID and in vitro SID for each individual AA.

3.4 Results and Discussion

3.4.1 Standardized Ileal Digestibility of Amino Acids

Digestibility results indicated no correlation between poultry SID and AA levels in SBM (r = -0.16 to 0.21; P = 0.33 to 0.98; **Figures 3.1 to 3.8**). Heat damage reduces quality of AA that animals can use for metabolic processes (Chang et al., 1987; Fontaine et al., 2007; Boucher et al., 2009). When samples are subjected to strong acid hydrolysis, many chemical changes occur (i.e. disaggregation of Maillard reaction compounds) thus transforming non-bioavailable AA back to

their unmodified structure. This may explain the low correlation found between poultry SID and AA levels in the SBM.

Lysine is the AA most affected by heat (i.e. Maillard browning reaction) (Aburto et al., 1996). In feed formulation, Lys is also the AA used in the concept of the ideal protein, in which diet requirements for other essential AA are based as a percentage of the Lys requirement (Mack et al., 1999). Previous studies have demonstrated that digestible Lys and bioavailable Lys do not differ in SBM samples (Batterham et al., 1990). For these reasons, we sought to determine if Lys SID in poultry could be used as an indicator of overall protein and AA quality and digestibility. Our results indicated that the AA phenylalanine, leucine, isoleucine, valine, tyrosine, alanine, threonine, glutamine, and aspartate were strongly associated with Lys digestibility (r^2 = 0.76 to 0.93; $P < 0.001$; **Figures 3.9 to 3.13**). Our results indicate that for these AA, Lys may be used as a qualitative marker to estimate their respective SID. Methionine, histidine, and glycine had a less positive association with Lys digestibility (r^2 = 0.63 to 0.64, $P < 0.001$; **Figures 3.14 to 3.15)**. Though the relationship was not as high as the previously mentioned AA, Lys may still be used to estimate their respective SID. Proline, arginine, and serine demonstrated the lowest association with Lys digestibility (r^2 = 0.14 to 0.43, P = 0.001 to 0.003; **Figures 3.16 to 3.17**).

Several linear prediction equations were developed to estimate AA SID based on Lys SID (**Table 3.2)**. This estimation method based on Lys SID is similar to the ideal protein concept readily used in the poultry industry (Emmert and Baker, 1997). Our results indicate that research efforts should be concentrated to determine Lys SID, which will facilitate the SID determination of the other essential AA. Furthermore, determination of Lys SID appears to be a good indicator of overall protein and AA quality in SBM samples.

3.4.2 In Vivo *vs.* In Vitro *Standardized Ileal Digestibility*

Distribution of *in vivo* Lys SID is shown in **Figure 3.26**. Standardized ileal digestibility of Lys determined in growing broilers ranged from about 65 to 95%. We hypothesized that a lower quality SBM would have lower *in vivo* AA SID; one that would be strongly associated to the *in vitro* AA SID. Interestingly, this association was not significant in the 16 AA tested (r^2 = 0.00 to 0.08; P = 0.17 to 0.99; **Figures 3.18 to 3.25**).

In all cases, the *in vitro* digestibility values were all higher then the *in vivo* digestibility values, in agreement with previous studies (Boisen and Fernandez, 1995). Our *in vitro* digestion failed to show any differences in AA SID, due to lack of sensitivity by the *in vitro* assay (i.e. *in vitro* digestibility was the same for all samples). There are several possible explanations for this lack in variation. Reduced particle size is directly correlated with enhanced digestibility *in* vivo (Wondra et al., 1995). Similarly, grinding samples to particle sizes of 1 mm or less likely contributed to high digestibility values. Incubation length is also associated with increased digestibility. Previous work has indicated Michaelis-Menten like behavior for in vitro digestion of organic matter and crude protein (Wilfart et al., 2007; Wilfart et al., 2008). We evaluated several combinations of incubation time for both the gastric and small intestinal phases (data not shown). Regardless of incubation time, high in vitro AA digestibility coefficients were obtained.

One assumption of the in vitro digestion process is that soluble proteins are considered digested. To test the contrary, we used trichloroacetic acid (TCA) to precipitate high molecular weight proteins in solution. Unfortunately, results were inconclusive due to the high presence of salts in the pellet resulting in interference with further acid hydrolysis and AA quantification.

Quantification of AA in the supernatant was also performed. Addition of TCA resulted in lower AA levels in the supernatant, but no differences were detected among samples (data not

shown). Our next steps are to evaluate different concentrations of pepsin and pancreatin in combination with varying incubation times and coarsely ground samples.

Other studies testing total tract *in vivo* energy digestibility with *in vitro* energy digestibility have indicated high correlations in SBM (Boisen and Fernandez, 1997). Post-ileal degradation of dietary fibers and the energy utilization associated with it is much higher in SBM samples then in other feedstuffs; which also changes with the variation in the SBM used (Boisen and Fernandez, 1997). This was not accounted for in the Boisen and Fernandez (1997) study and may have an impact in the prediction equations developed for SBM, as they used energy as their measurable variable when predicting *in vivo* and *in vitro* digestibility. Other studies that have demonstrated a positive relationship between *in vivo* and *in vitro* digestibility of crude protein and amino acids used mixed diets in the development of their prediction equations (Boisen and Fernandez, 1995). Using mixed diets, as opposed to a single feedstuff, contain crystalin AA, which are theoretically digested completely. Measuring digestibility as total nitrogen digestibility, as was the case, may result in incorrect AA digestibility calculations.

The source of endogenous losses was also different. Though we corrected for endogenous losses using previously determined values (Golian et al., 2008), our prediction equations did not provide the same results as previously stated. The prediction equations of others (Boisen and Fernandez, 1995) corrects for endogenous losses via another prediction equation developed to utilize the undigested dry matter from *in vitro* digestion. The prediction equations previously developed are thought to be better used in estimating amino acid digestibility in mixed feeds, as opposed to a single feedstuff, as was conducted in this thesis.

3.5 Conclusion and Future Work

Determining quantity and quality of AA in SBM prior to its use as a feed ingredient is important, particularly when feed prices continue to increase. On the one hand, AA levels in SBM appear to have little correlation with in vivo SID values. On the other hand, using a generalized SID coefficient for multiple SBM batches appears to be a reasonable practical approach for animal nutritionists. Evaluation of other more "gentler" *in vitro* digestion procedures will continue aiming to establish a quantitative relationship between AA digestibility in pigs and broilers. This entails further assessing the *in vitro* assay used by analyzing variations in SBM particle size, digestion incubation times, and enzyme concentrations.

Tables

Table 3.1. Starter and test diets fed to broilers, as fed-basis.

Ingredient	Broiler starter diet, %	Broiler test diet, %
Corn	58.54	- - -
Corn starch	- - -	51.19
Soybean meal, 47.5%	34.80	42.10
Soy Oil	2.03	2.00
Dicalcium phosphate	1.56	1.72
Limestone	1.73	1.61
DL-Methionine	0.50	- - -
L-Lysine-HCl	0.13	- - -
L-Threonine	0.02	- - -
Vitamin premix[1]	0.11	0.15
Mineral premix[2]	0.12	0.50
Salt	0.46	- - -
Chromic oxide	- - -	0.40

[1]Provided per kg diet: vitamin A, 7334.7 IU/kg; vitamin D_3, 1010.4 IU/kg; vitamin E, 42.1 IU/kg; vitamin K, 7.3 ppm; biotin, 0.3 ppm; choline, 549.7 ppm; folic acid, 1.4 ppm; niacin, 31.2 ppm; pantothenate, 21.1 ppm; riboflavin, 6.4 ppm; vitamin B_{12}, 27.6 µg/kg.

[2]Provided per kg diet (ppm): Cu, 5.9; Fe, 96.2; Mn, 29.4; Se, 0.2; Zn, 96.2; I, 0.3.

Table 3.2. Regression equations for the relationship between AA standardized ileal digestibility (SID) and lysine SID

Amino acid	Intercept	Std. error	Slope	Std. error	P-value
Asparagine	7.66	7.72	0.91	0.09	<0.001
Glutamine	-7.8	7.0	1.08	0.08	<0.001
Serine	21.74	20.89	0.74	0.24	0.001
Glycine	16.71	11.35	0.8	0.13	<0.001
Histidine	25.56	21.23	0.71	0.25	<0.001
Arginine	21.53	15.15	0.73	0.18	0.001
Threonine	12.09	9.13	0.88	0.11	<0.001
Alanine	8.88	7.73	0.9	0.09	<0.001
Proline	62.88	14.4	0.31	0.17	0.003
Tyrosine	2.82	4.82	0.94	0.06	<0.001
Valine	19.17	7.46	0.8	0.09	<0.001
Methionine	15.82	11.11	0.82	0.13	<0.001
Isoleucine	10.73	5.8	0.89	0.07	<0.001
Leucine	5.21	5.87	0.94	0.07	<0.001
Phenylalanine	6.51	7.55	0.92	0.09	<0.001

Figures

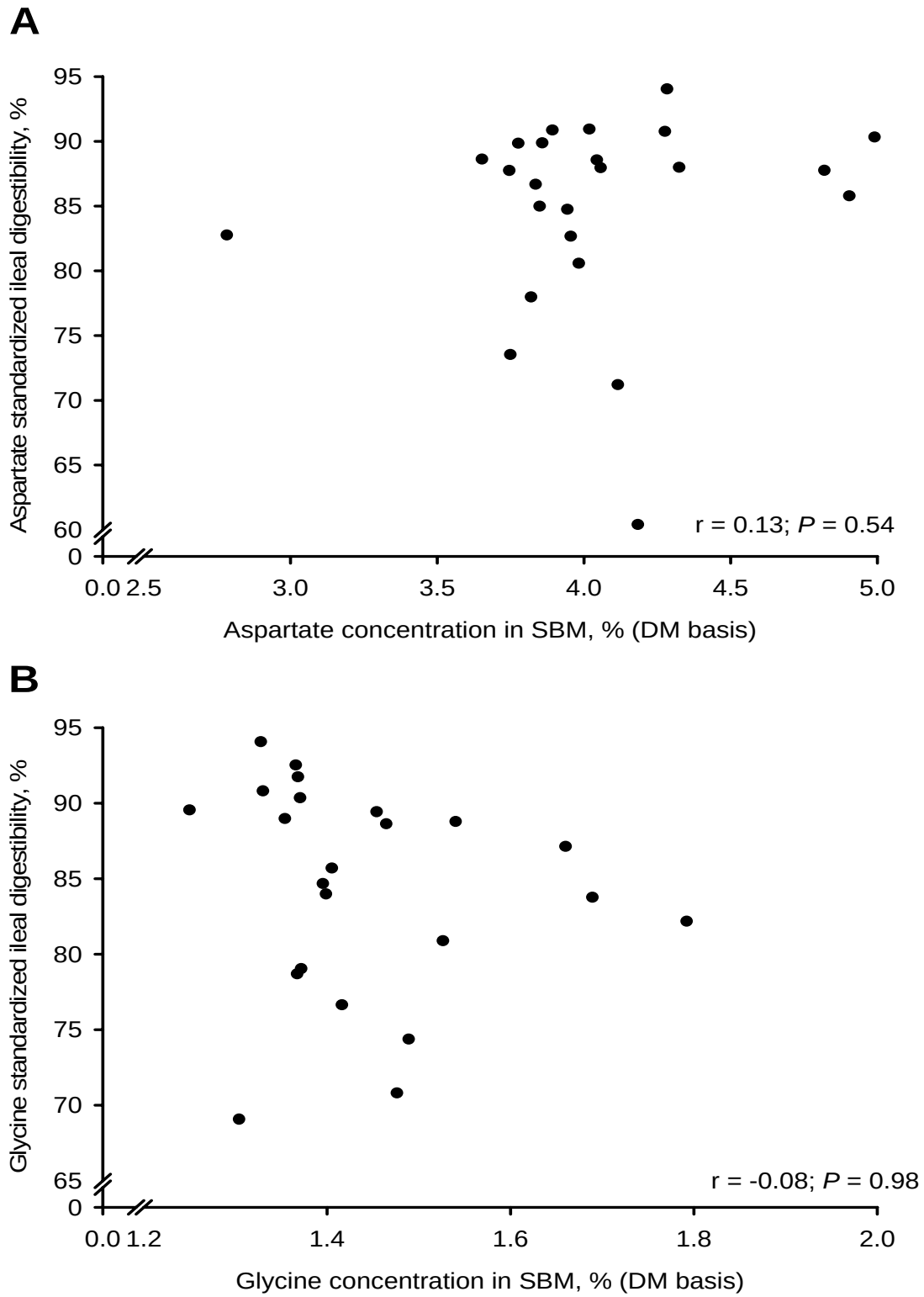


Figure 3.1. Correlation between standardized ileal digestible aspartate (A) and glycine (B) with their respective concentrations in 24 samples of soybean meal.

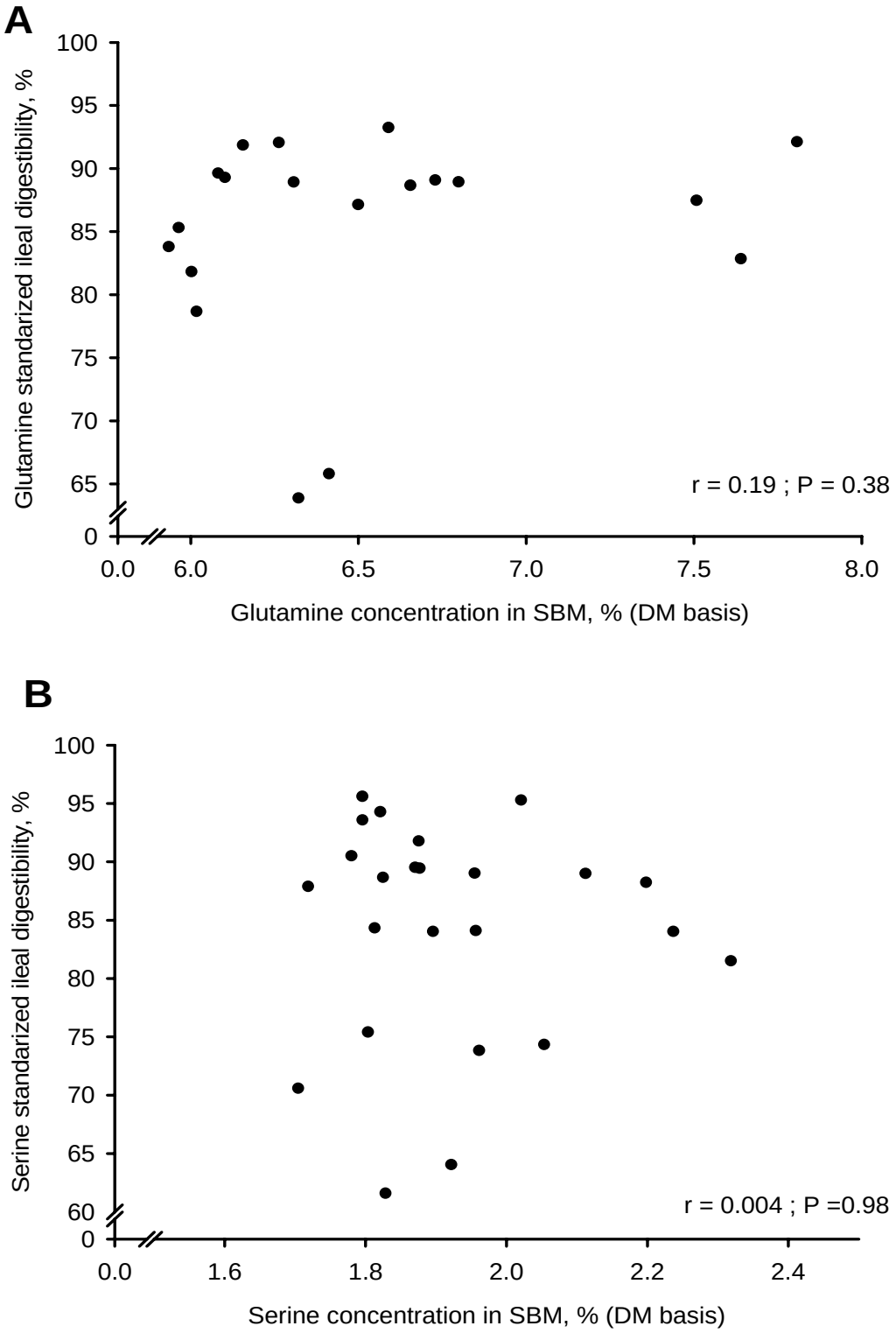


Figure 3.2. Correlation between standardized ileal digestible glutamine (A) and serine (B) with their respective concentrations in 24 samples of soybean meal.

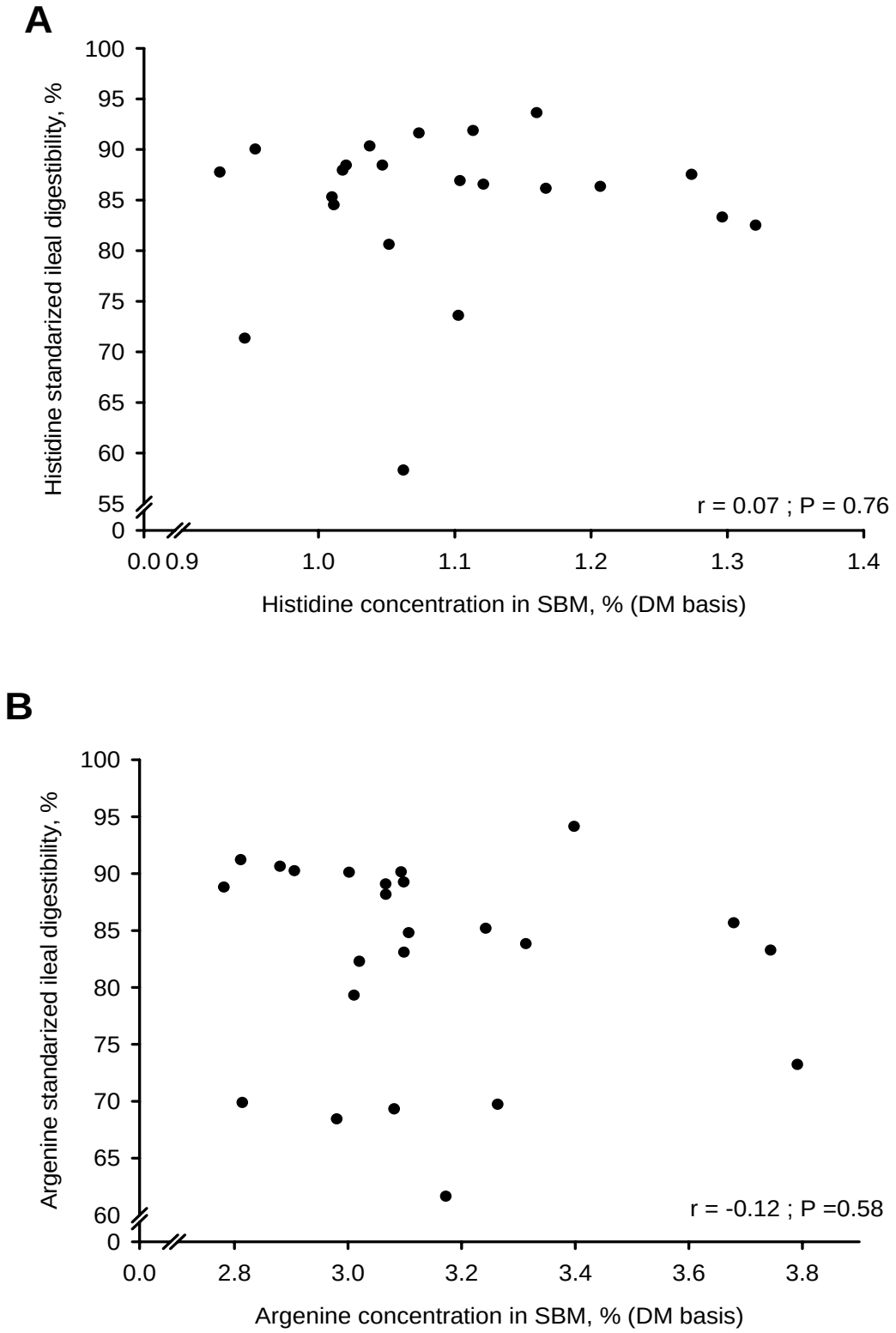


Figure 3.3. Correlation between standardized ileal digestible histidine (A) and argenine (B) with their respective concentrations in 24 samples of soybean meal.

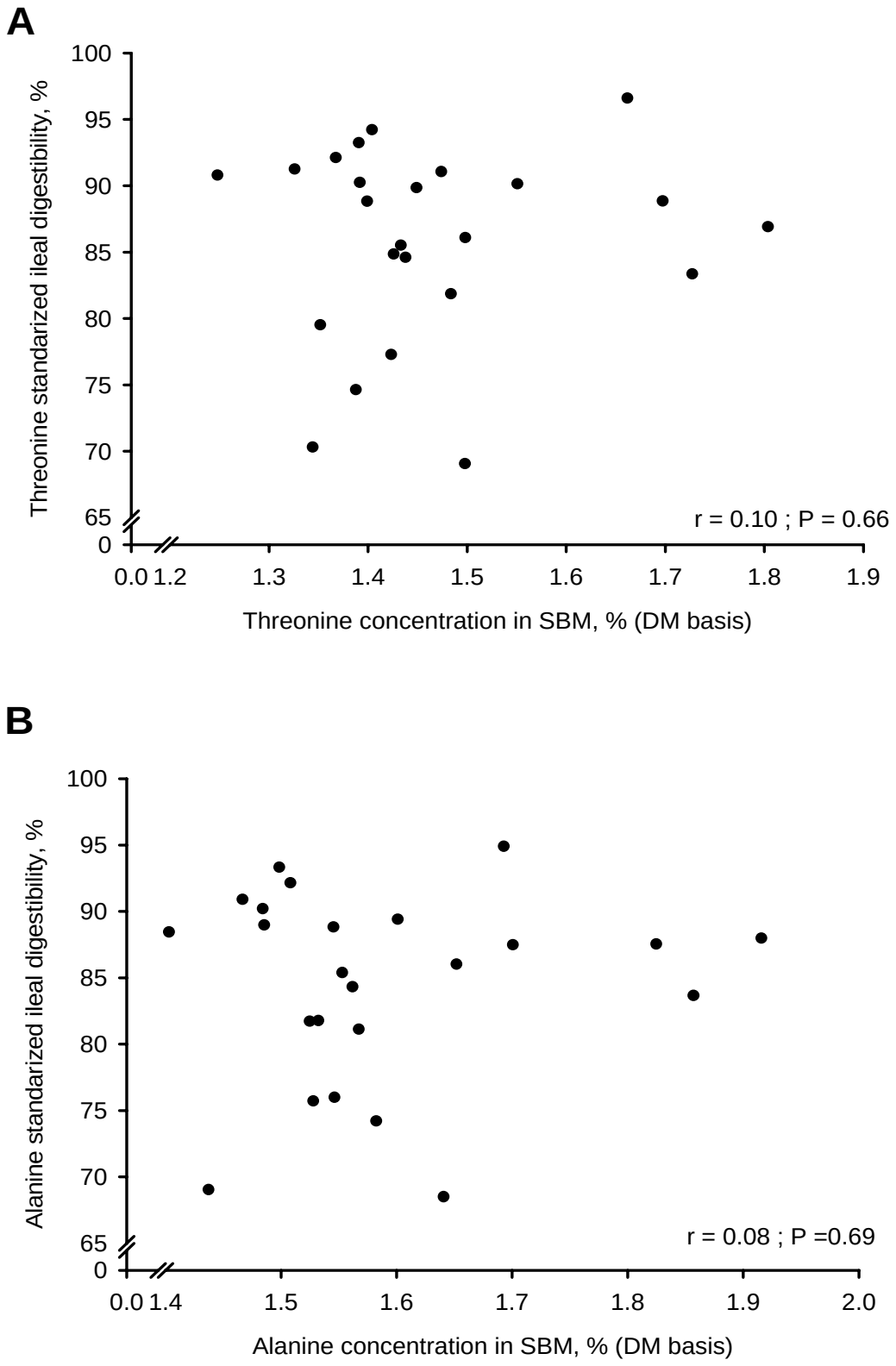
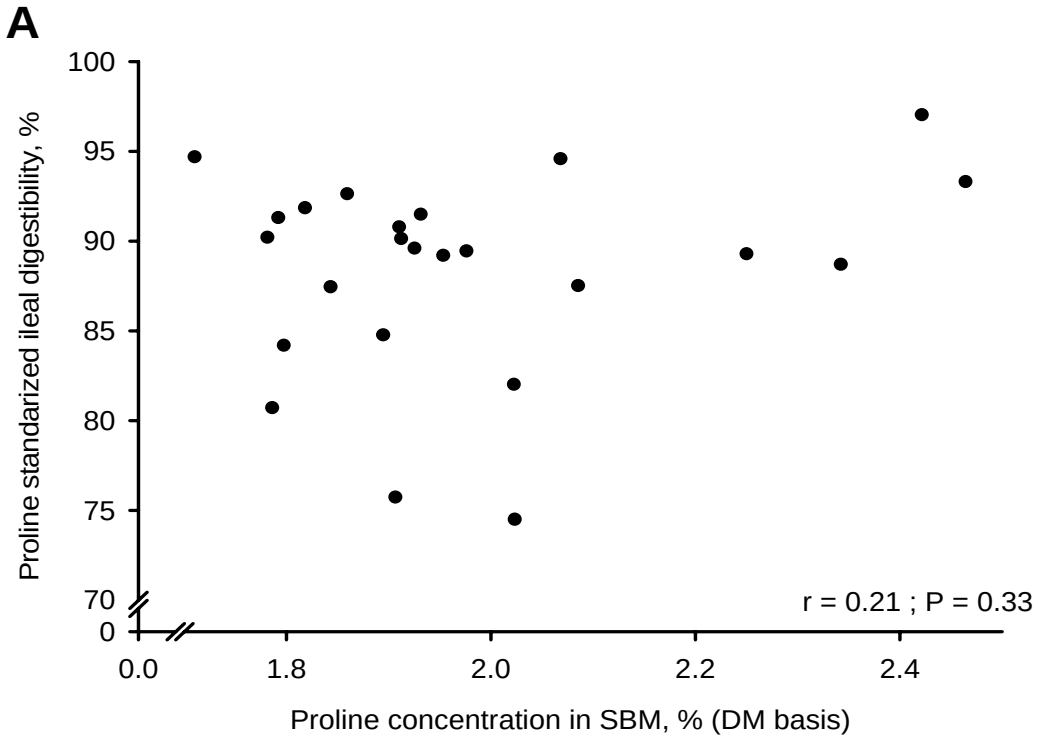


Figure 3.4. Correlation between standardized ileal digestible threonine (A) and alanine (B) with their respective concentrations in 24 samples of soybean meal.



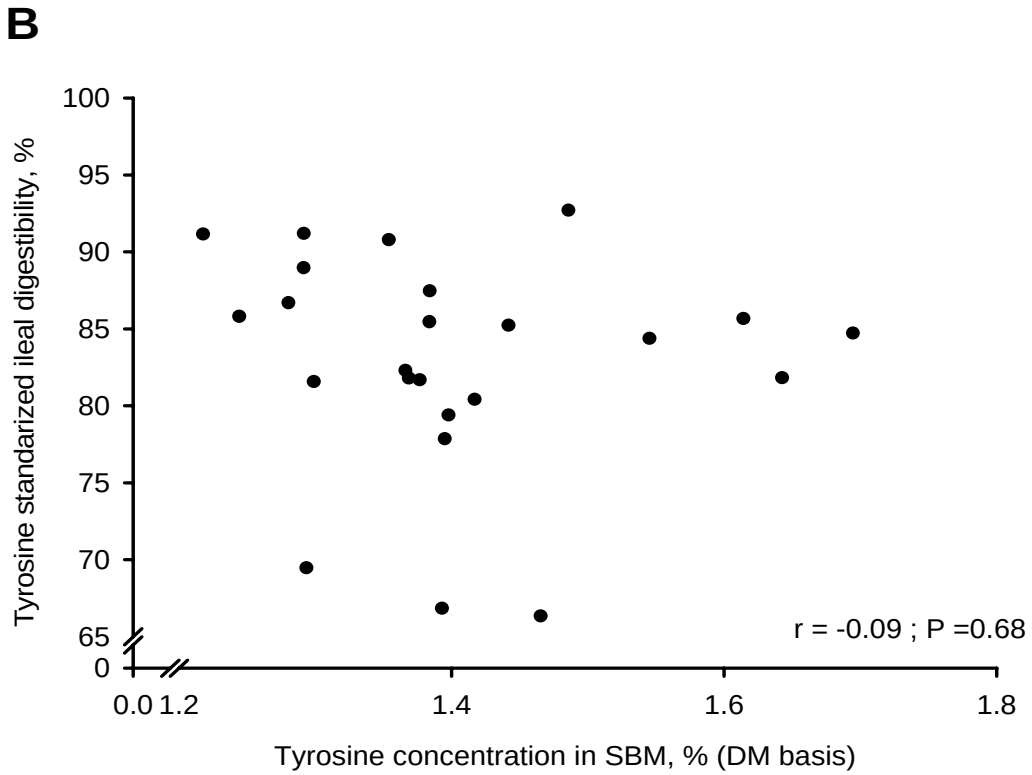


Figure 3.5. Correlation between standardized ileal digestible proline (A) and tyrosine (B) with their respective concentrations in 24 samples of soybean meal.

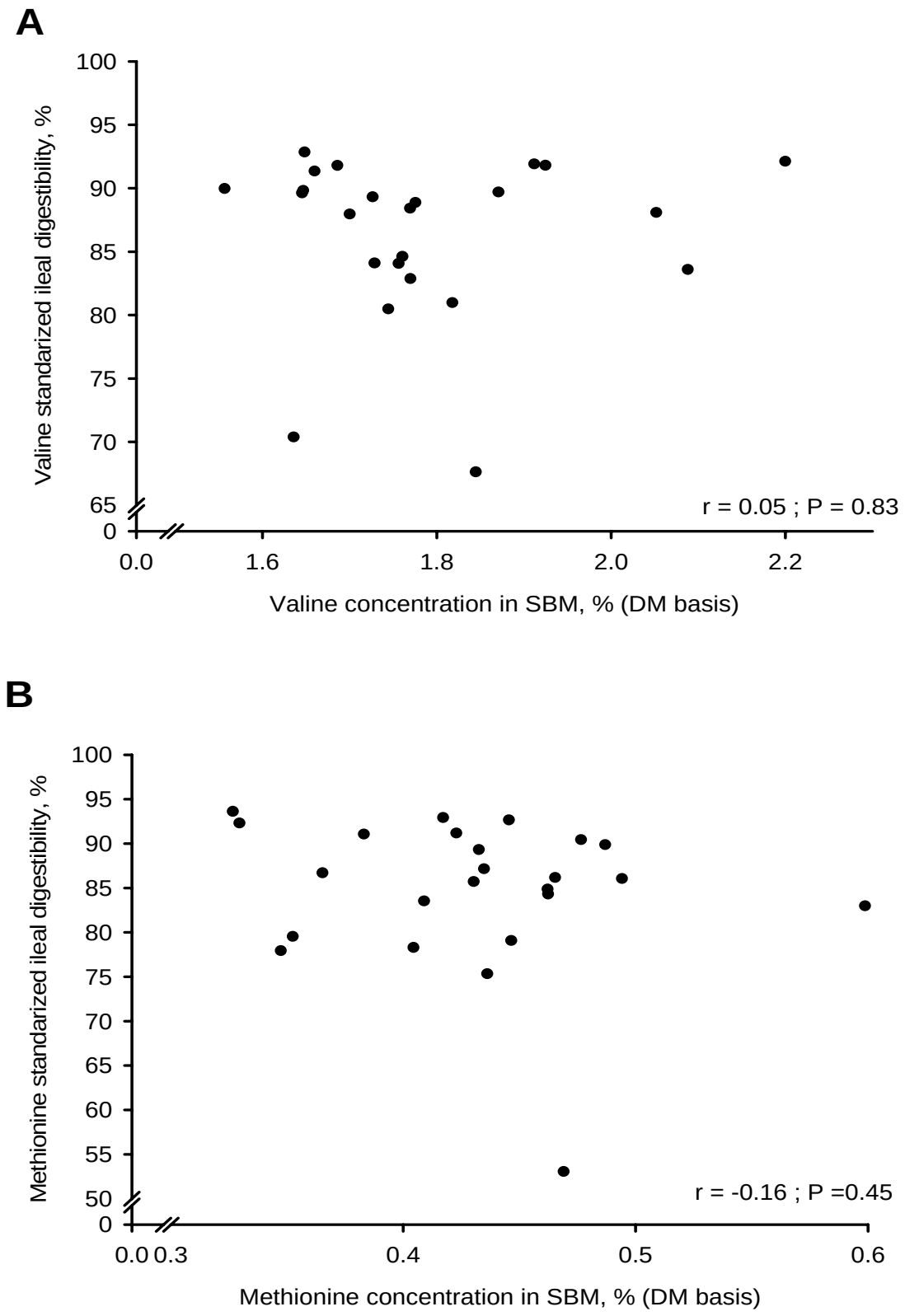


Figure 3.6. Correlation between standardized ileal digestible valine (A) and methionine (B) with their respective concentrations in 24 samples of soybean meal.

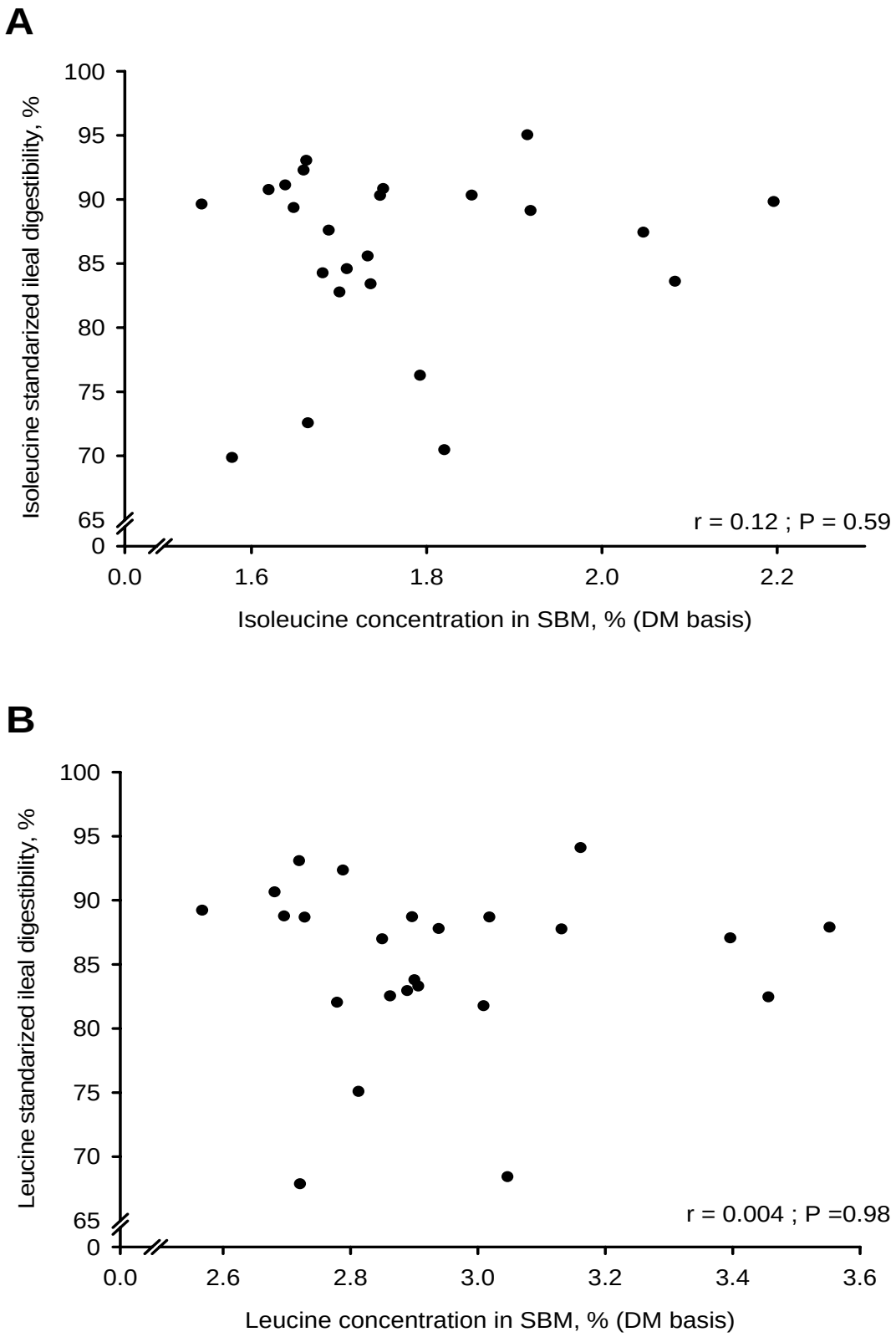


Figure 3.7. Correlation between standardized ileal digestible isoleucine (A) and leucine (B) with their respective concentrations in 24 samples of soybean meal.

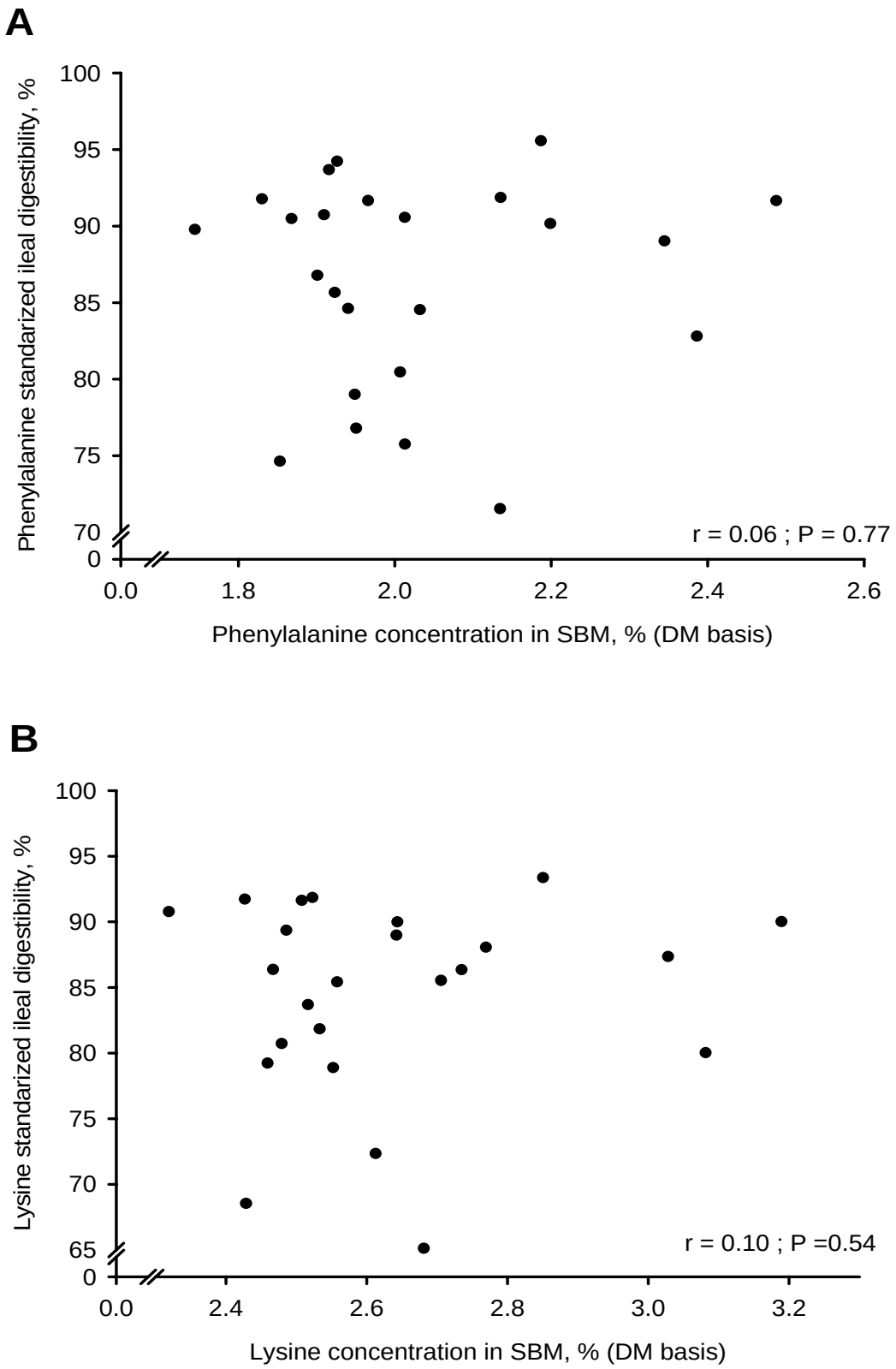
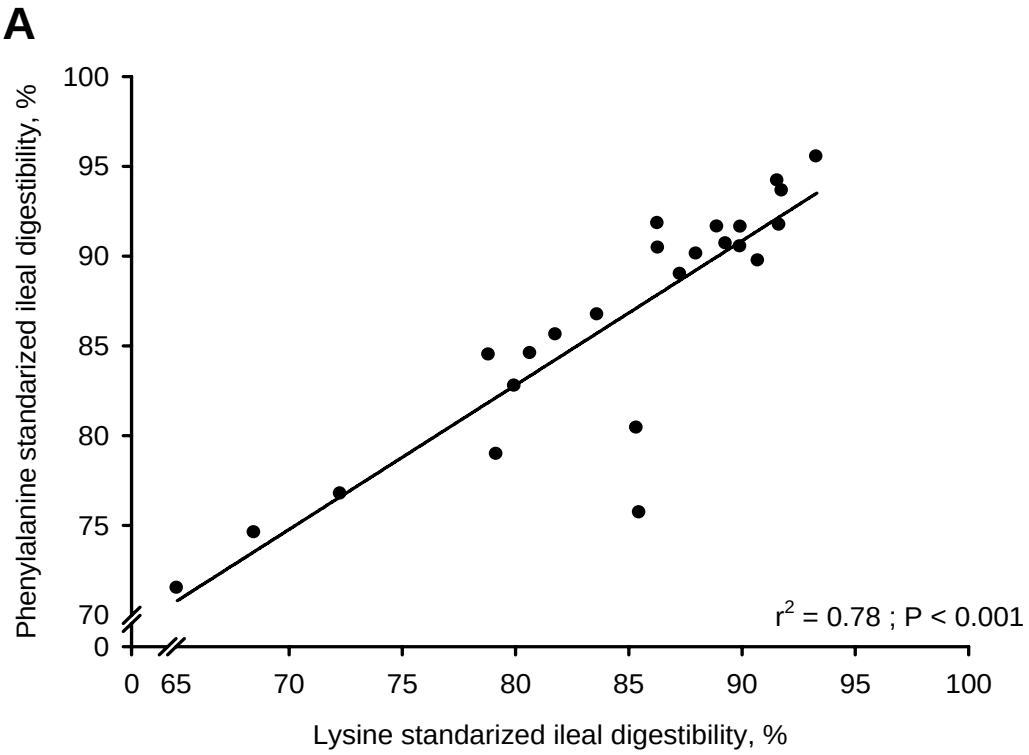


Figure 3.8. Correlation between standardized ileal digestible phenylalanine (A) and lysine (B) with their respective concentrations in 24 samples of soybean meal.



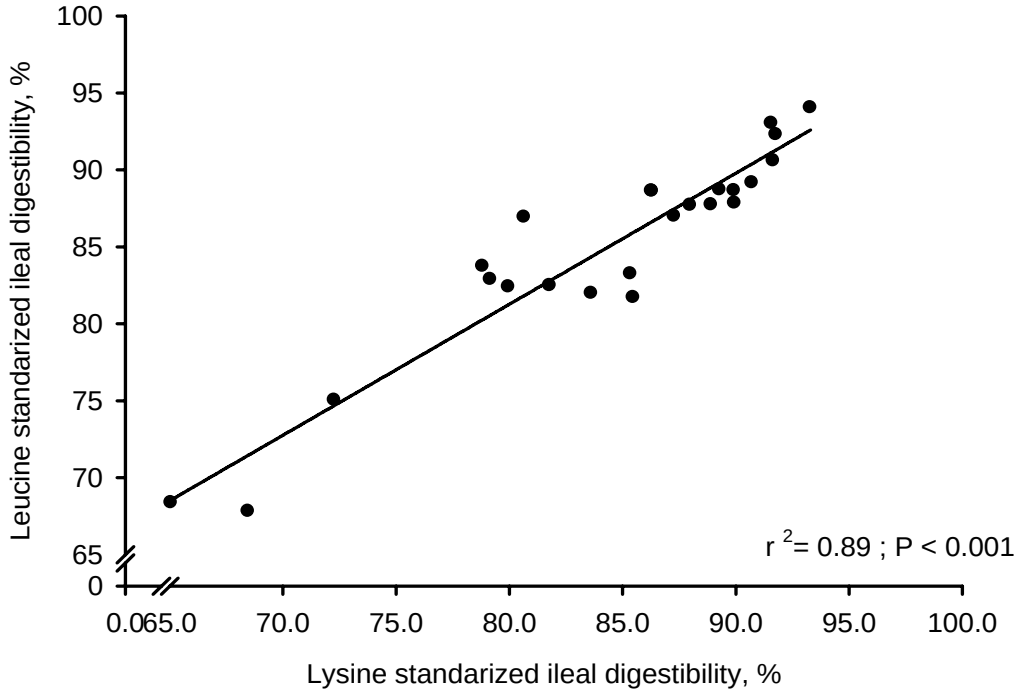


Figure 3.9. Relationship between standardized ileal digestible (SID) phenylalanine (A) and leucine (B) with lysine SID in 24 samples of soybean meal.

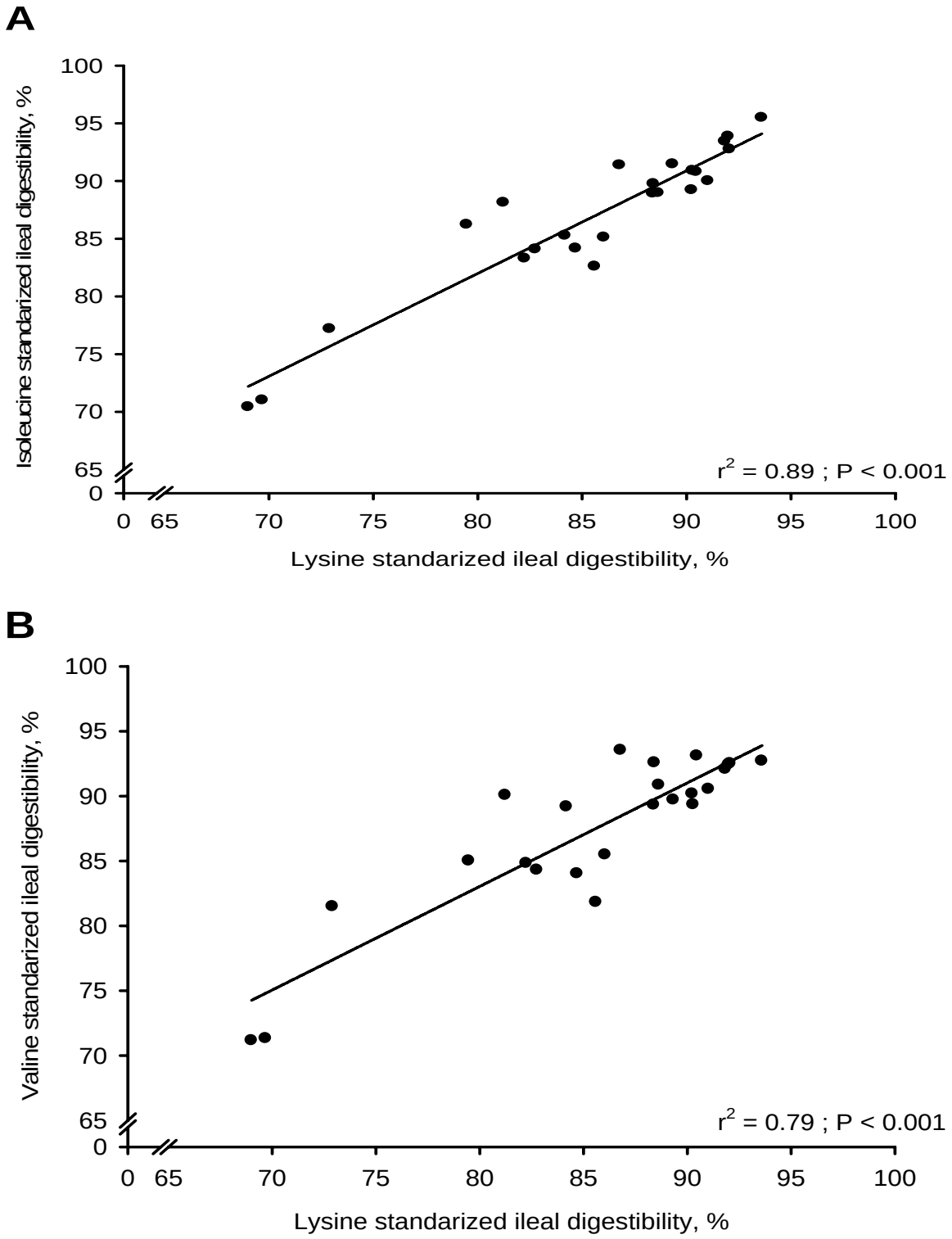


Figure 3.10. Relationship between standardized ileal digestible (SID) isoleucine (A) and valine (B) with lysine SID in 24 samples of soybean meal.

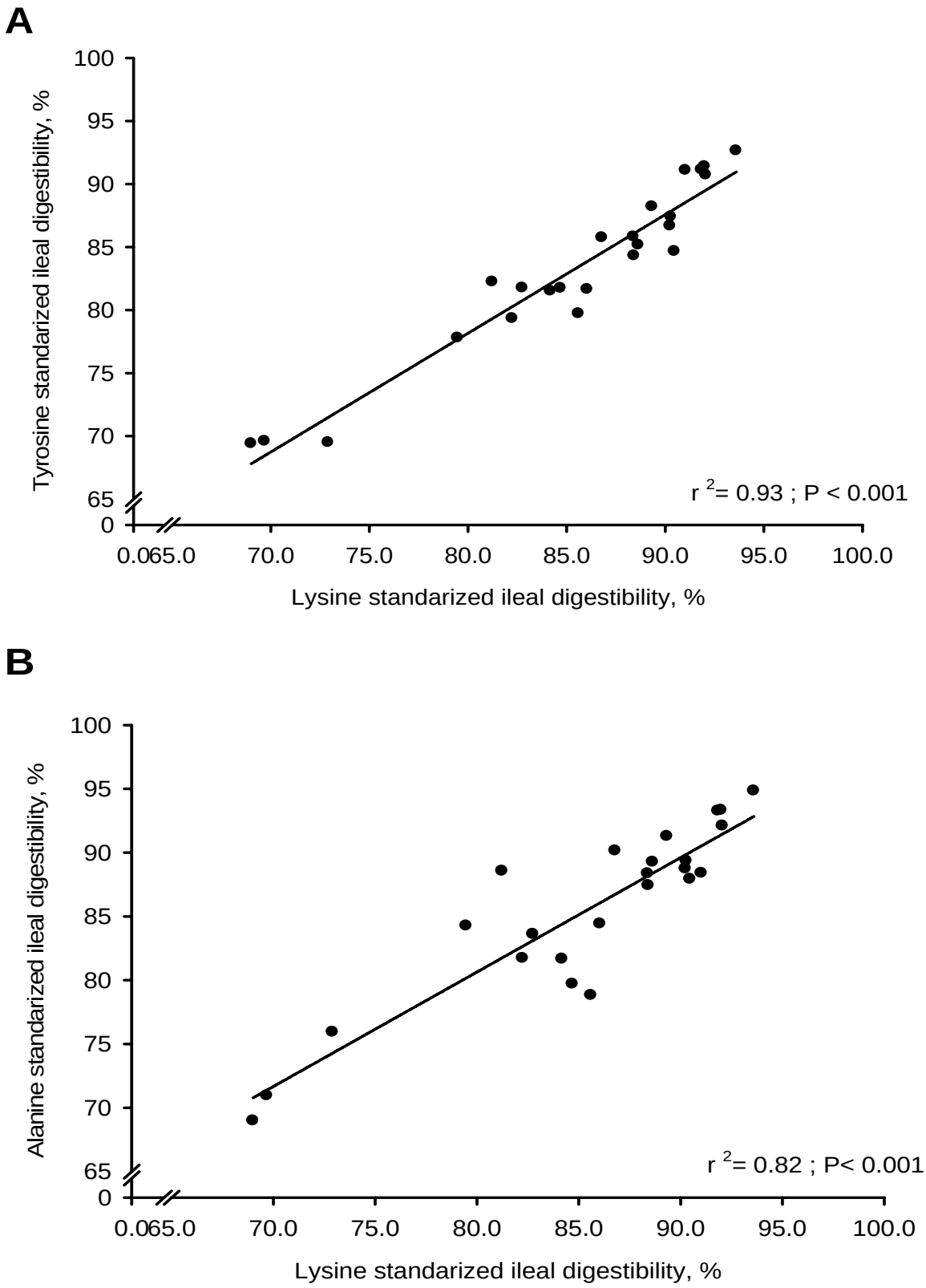


Figure 3.11. Relationship between standardized ileal digestible (SID) tyrosine (A) and alanine (B) with lysine SID in 24 samples of soybean meal.

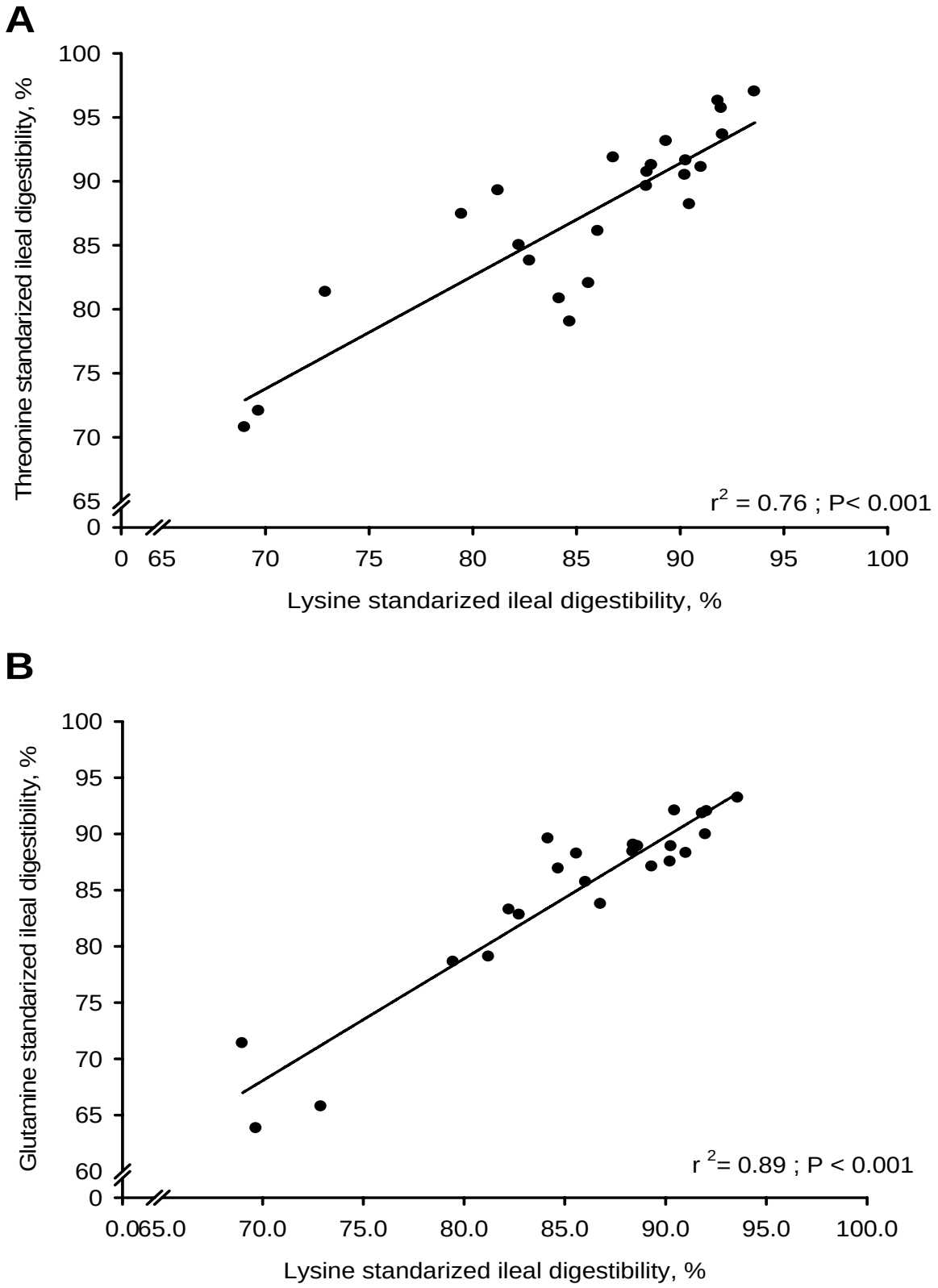


Figure 3.12. Relationship between standardized ileal digestible (SID) threonine (A) and glutamine (B) with lysine SID in 24 samples of soybean meal.

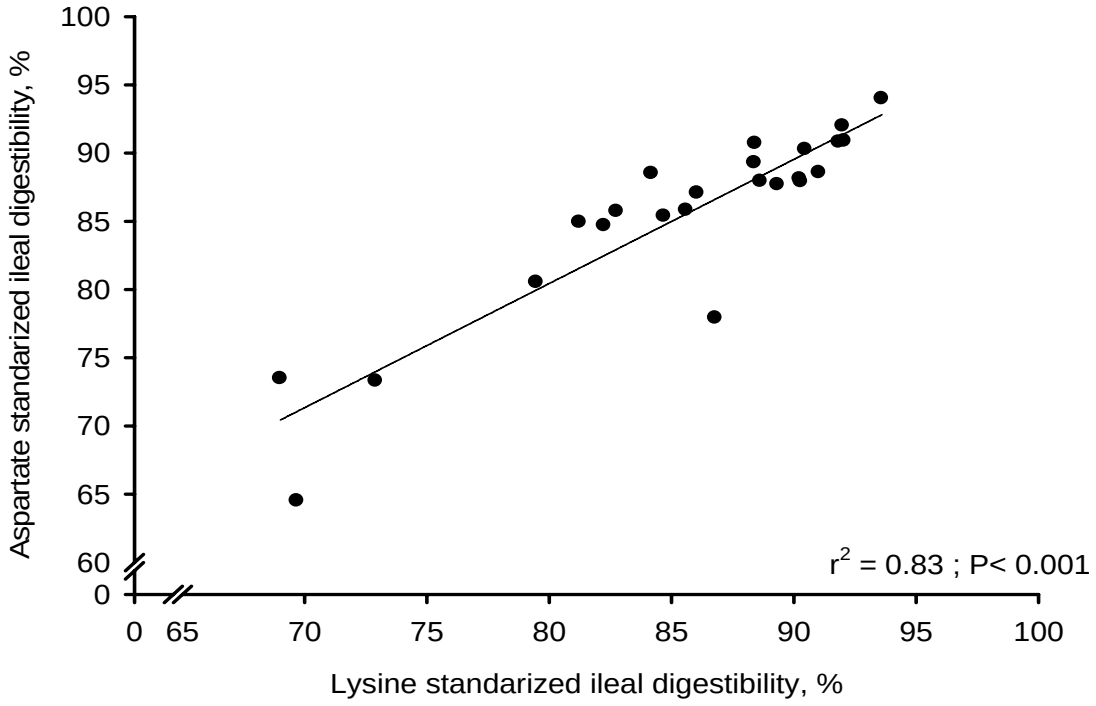


Figure 3.13. Relationship between standardized ileal digestible (SID) aspartate with lysine SID in 24 samples of soybean meal.

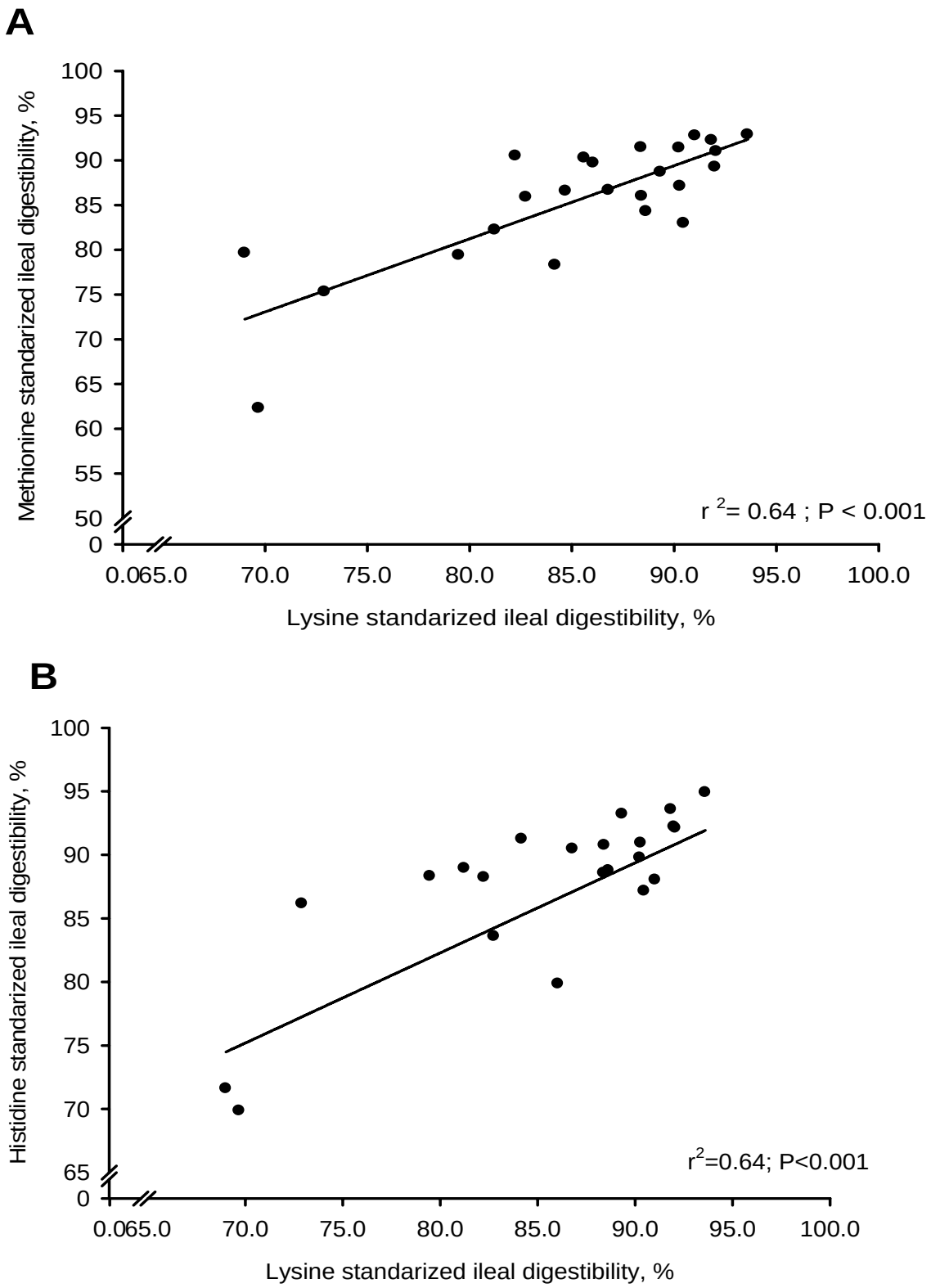


Figure 3.14. Relationship between standardized ileal digestible (SID) methionine (A) and histidine (B) with lysine SID in 24 samples of soybean meal.

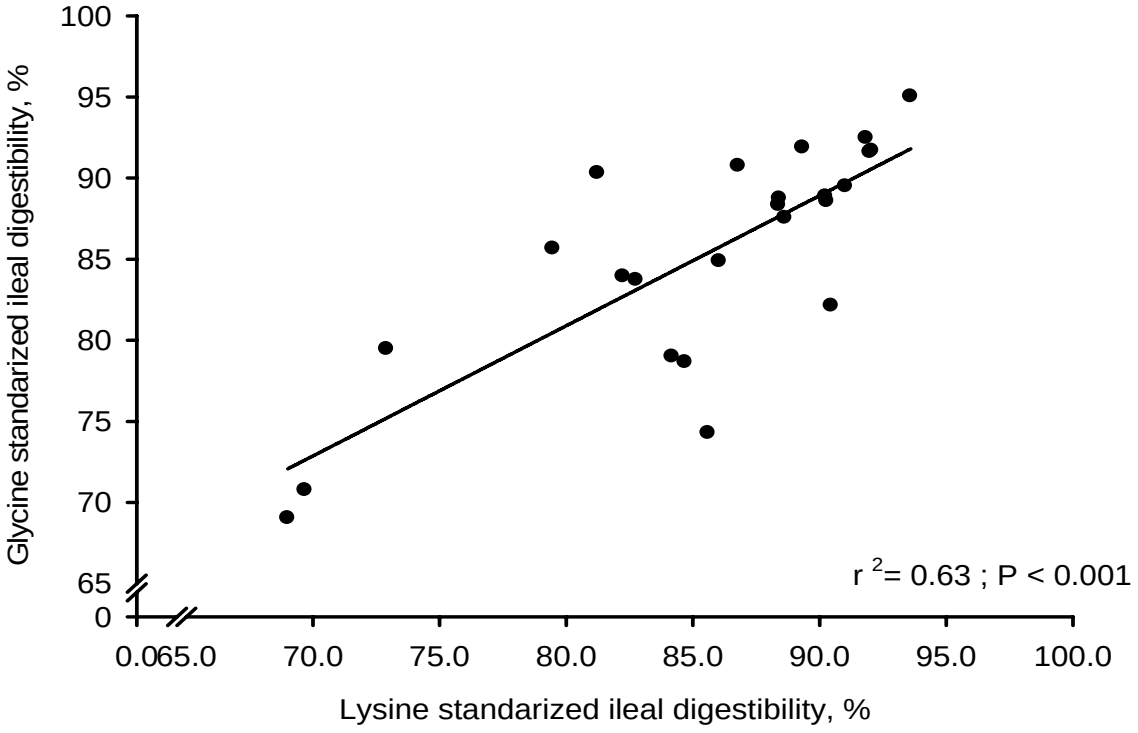


Figure 3.15. Relationship between standardized ileal digestible (SID) glycine with lysine SID in 24 samples of soybean meal.

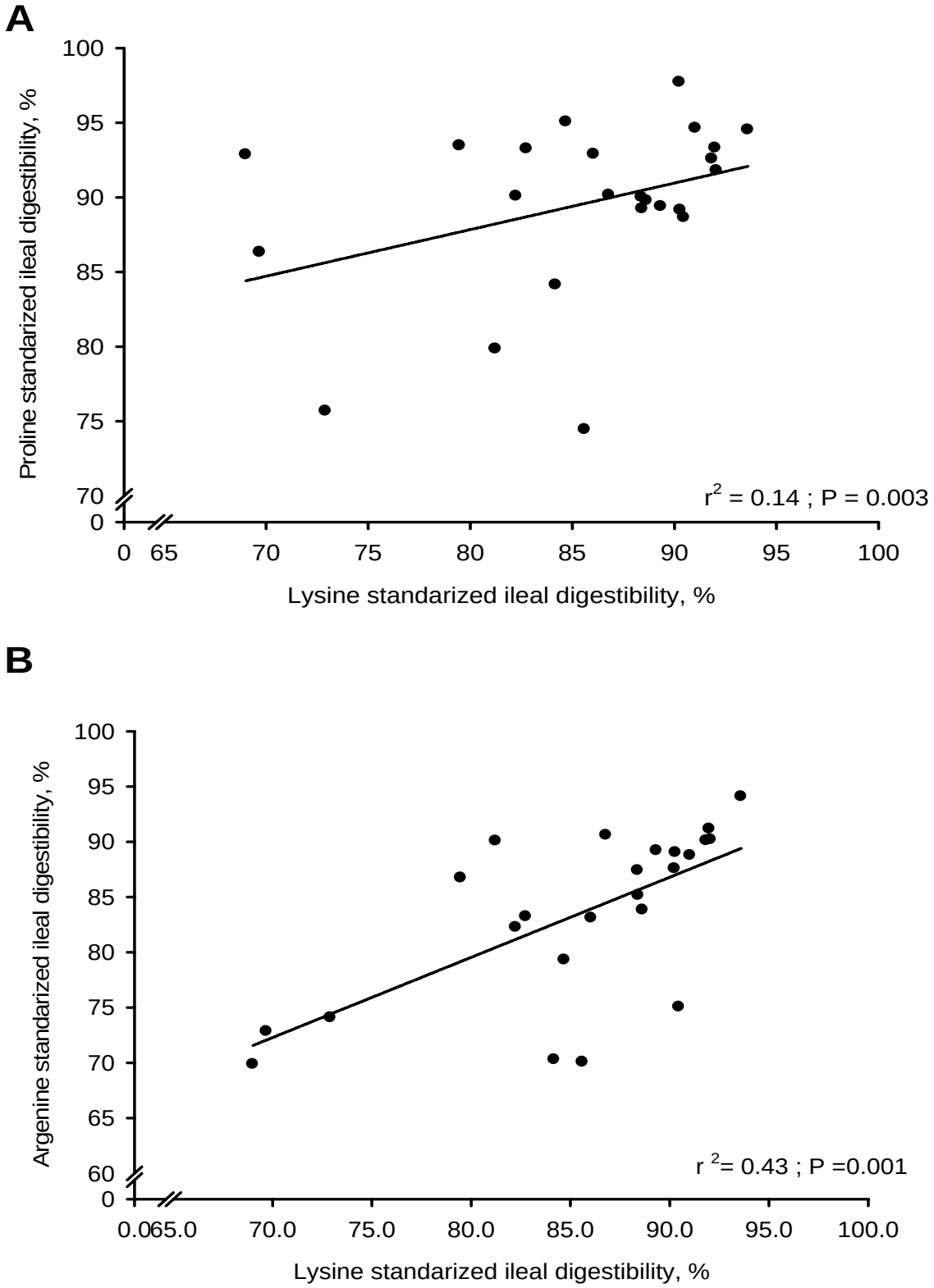


Figure 3.16. Relationship between standardized ileal digestible (SID) proline (A) and argenine (B) with lysine SID in 24 samples of soybean meal.

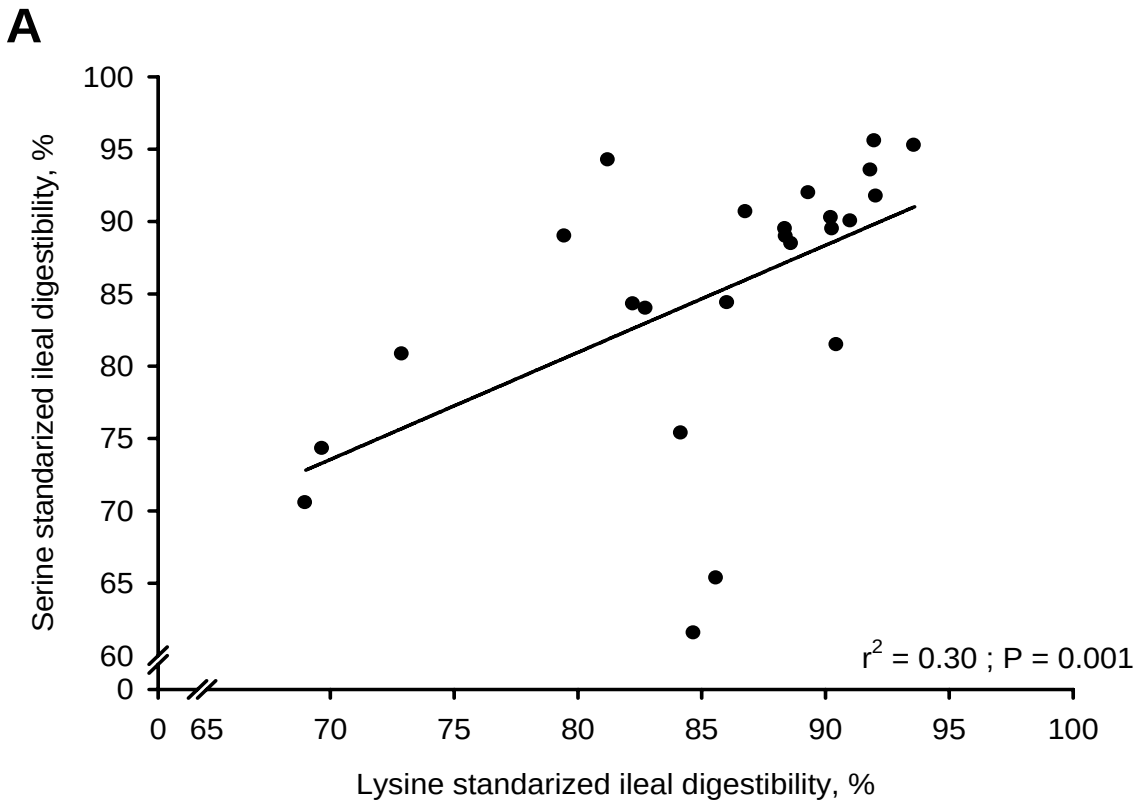


Figure 3.17. Relationship between standardized ileal digestible (SID) serine with lysine SID in 24 samples of soybean meal.

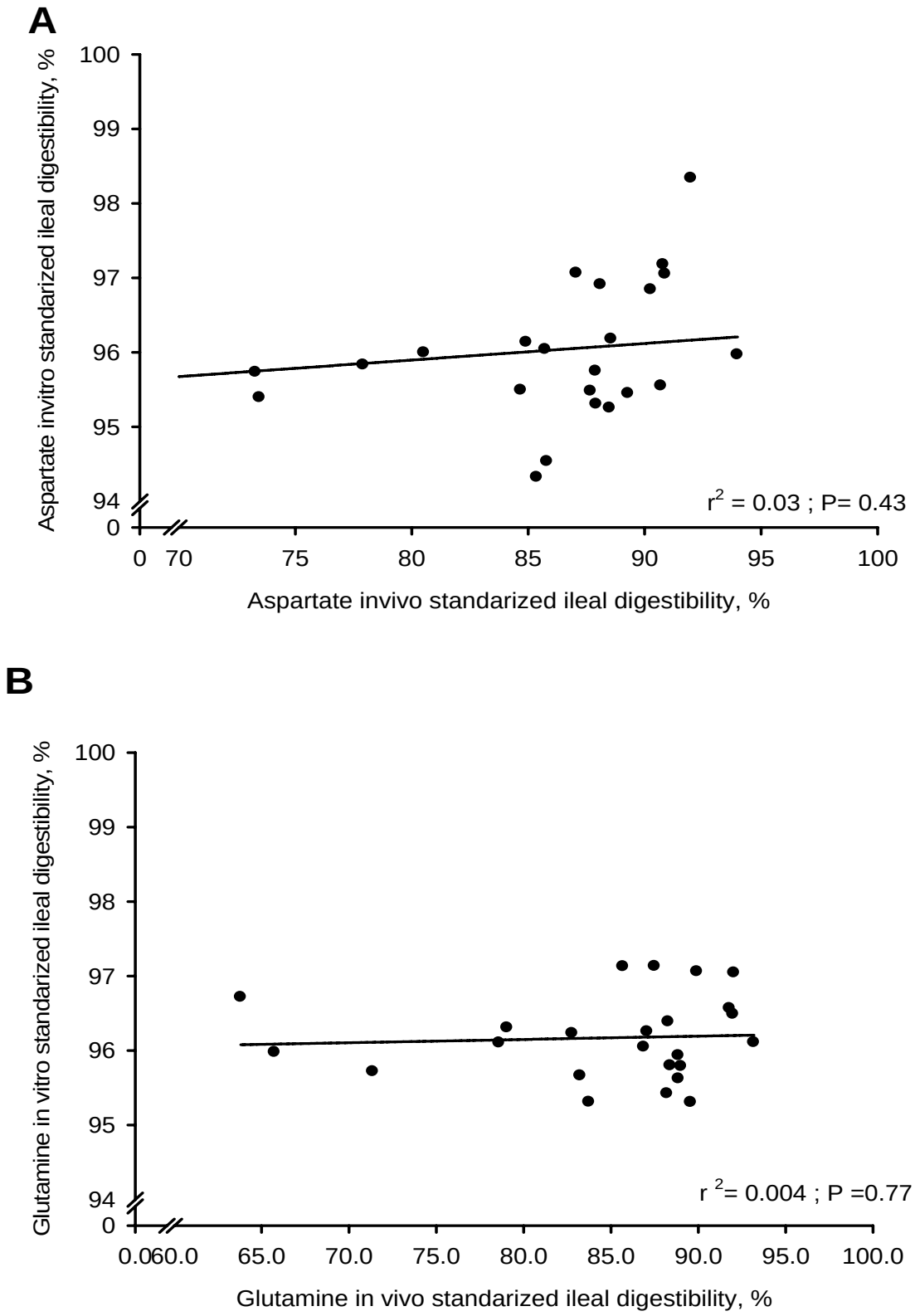
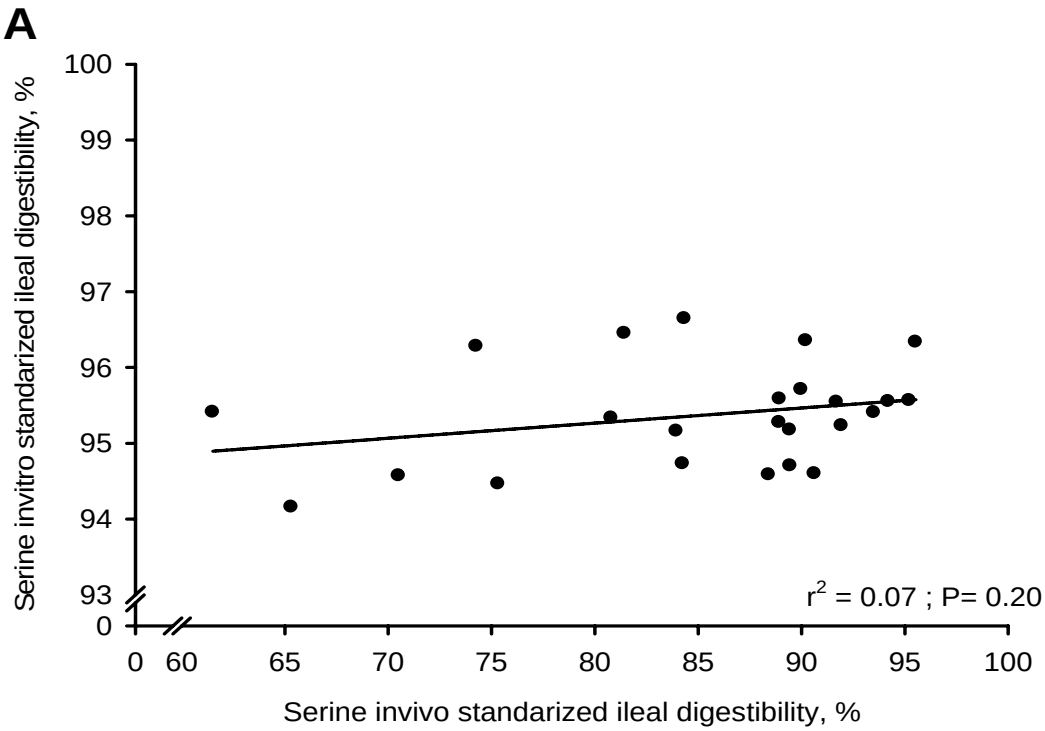


Figure 3.18. Relationship between *in vivo* and *in vitro* SID for aspartate (A) and glutamine (B).



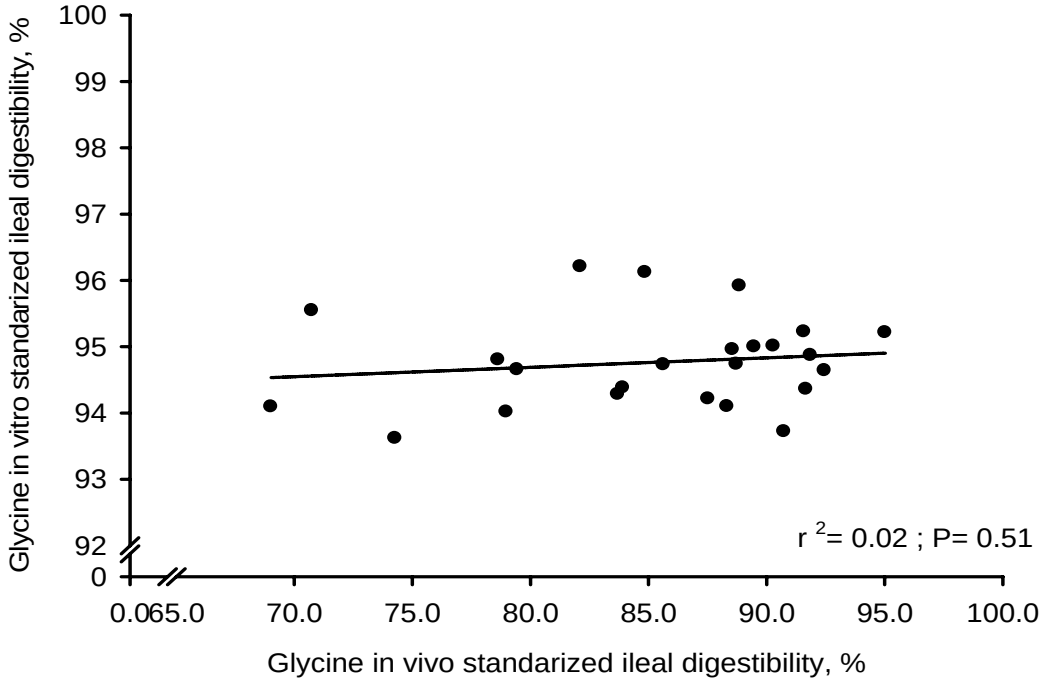
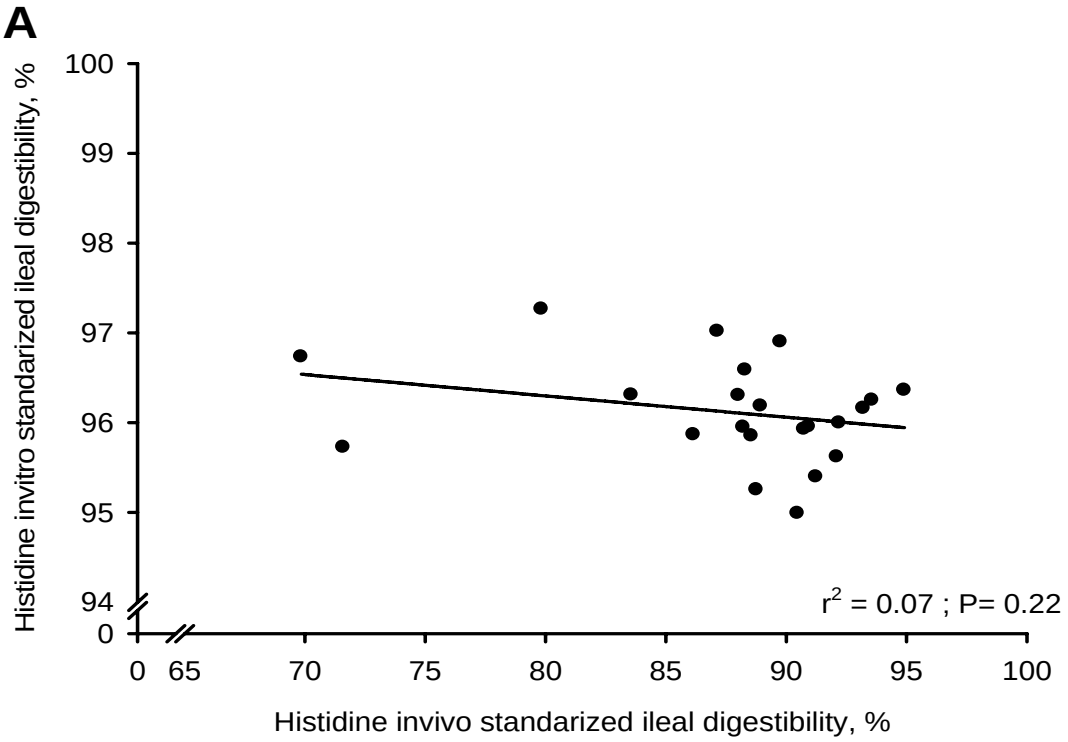


Figure 3.19. Relationship between *in vivo* and *in vitro* SID for serine (A) and glycine (B).



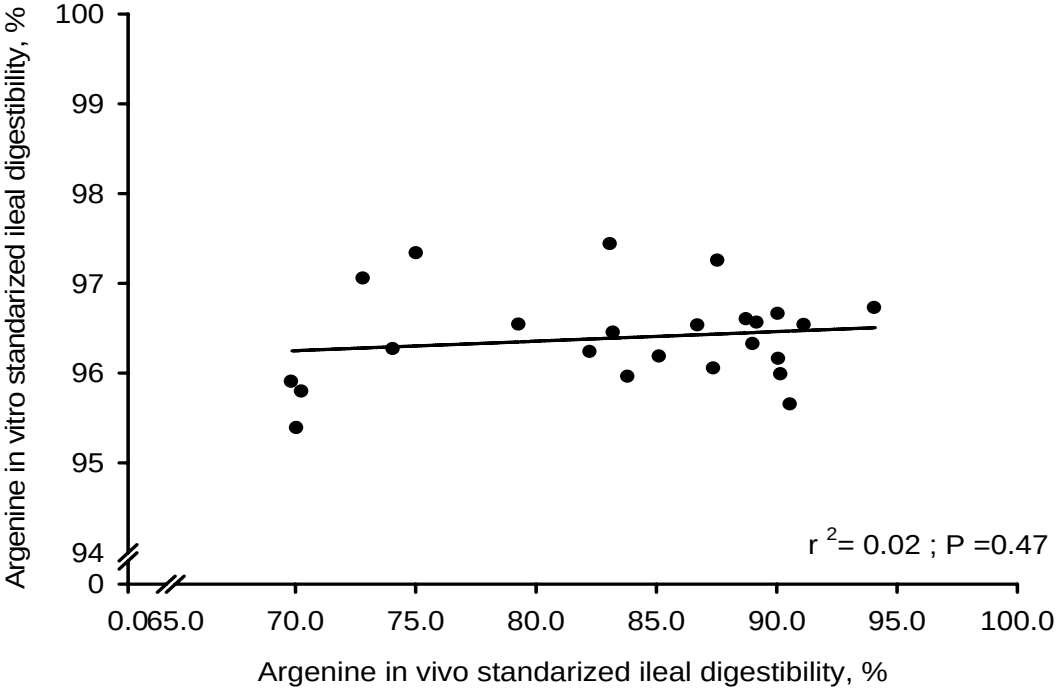


Figure 3.20. Relationship between *in vivo* and *in vitro* SID for histidine (A) and argenine (B).

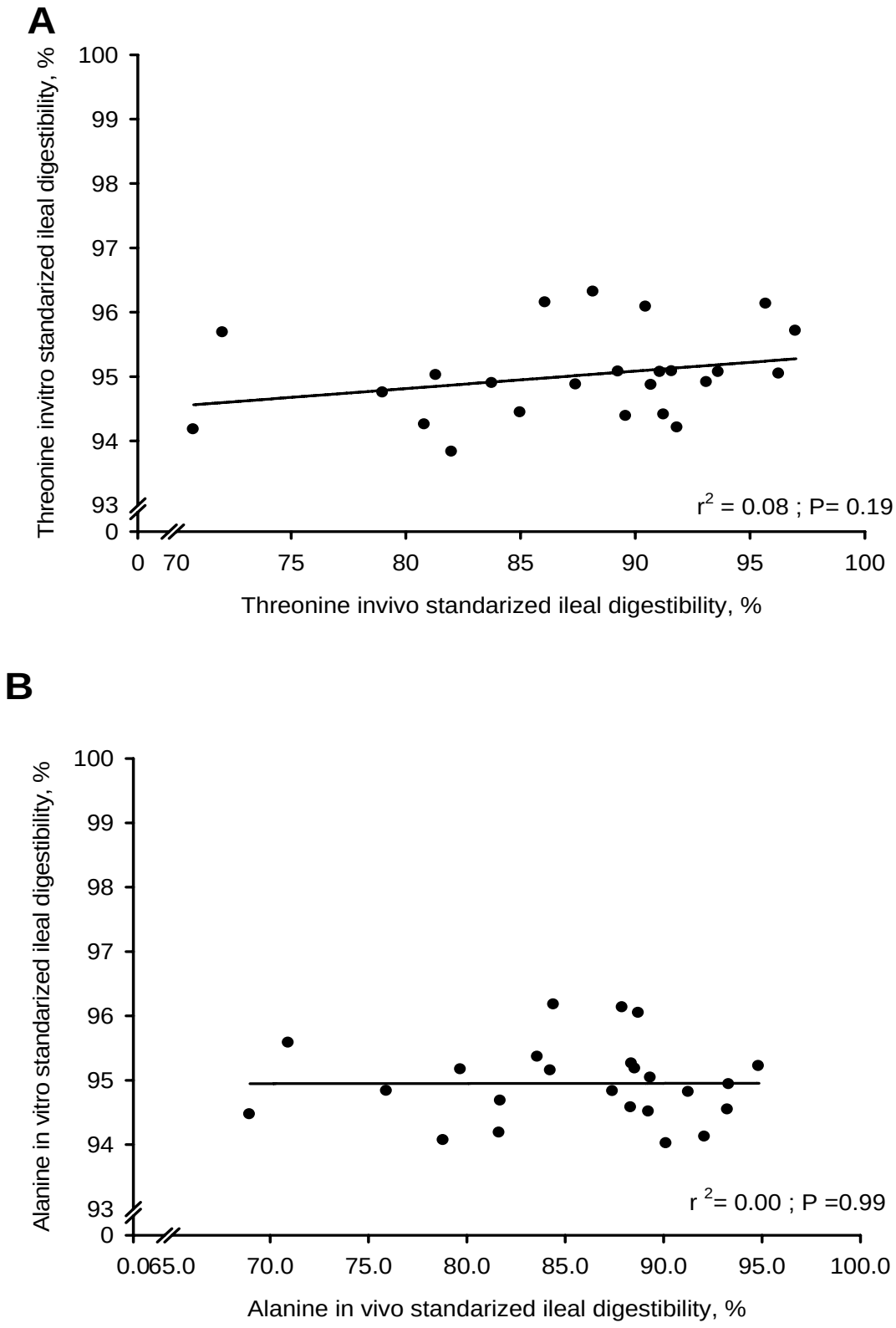


Figure 3.21. Relationship between *in vivo* and *in vitro* SID for threonine (A) and alanine (B).

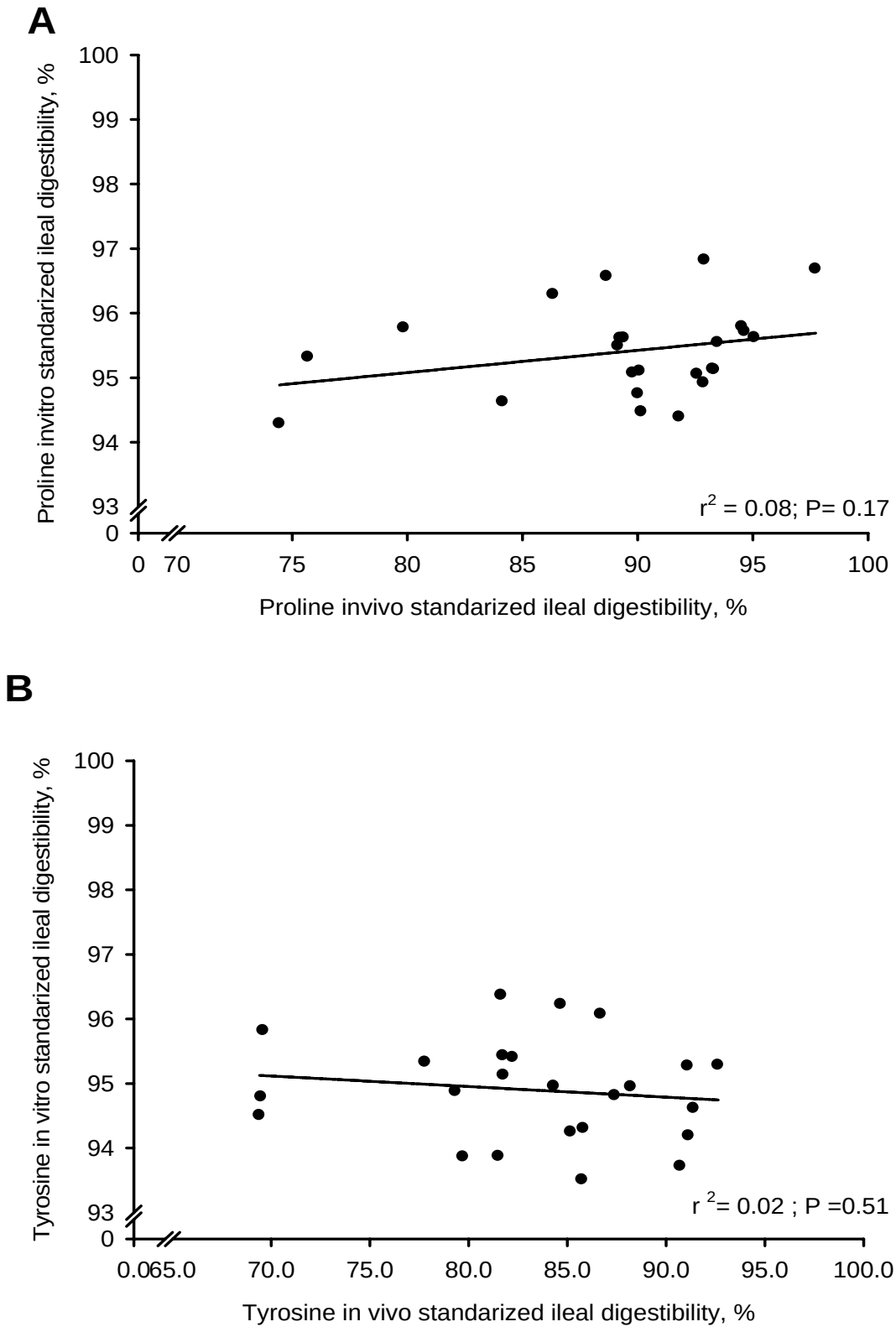


Figure 3.22. Relationship between *in vivo* and *in vitro* SID for proline (A) and tyrosine (B).

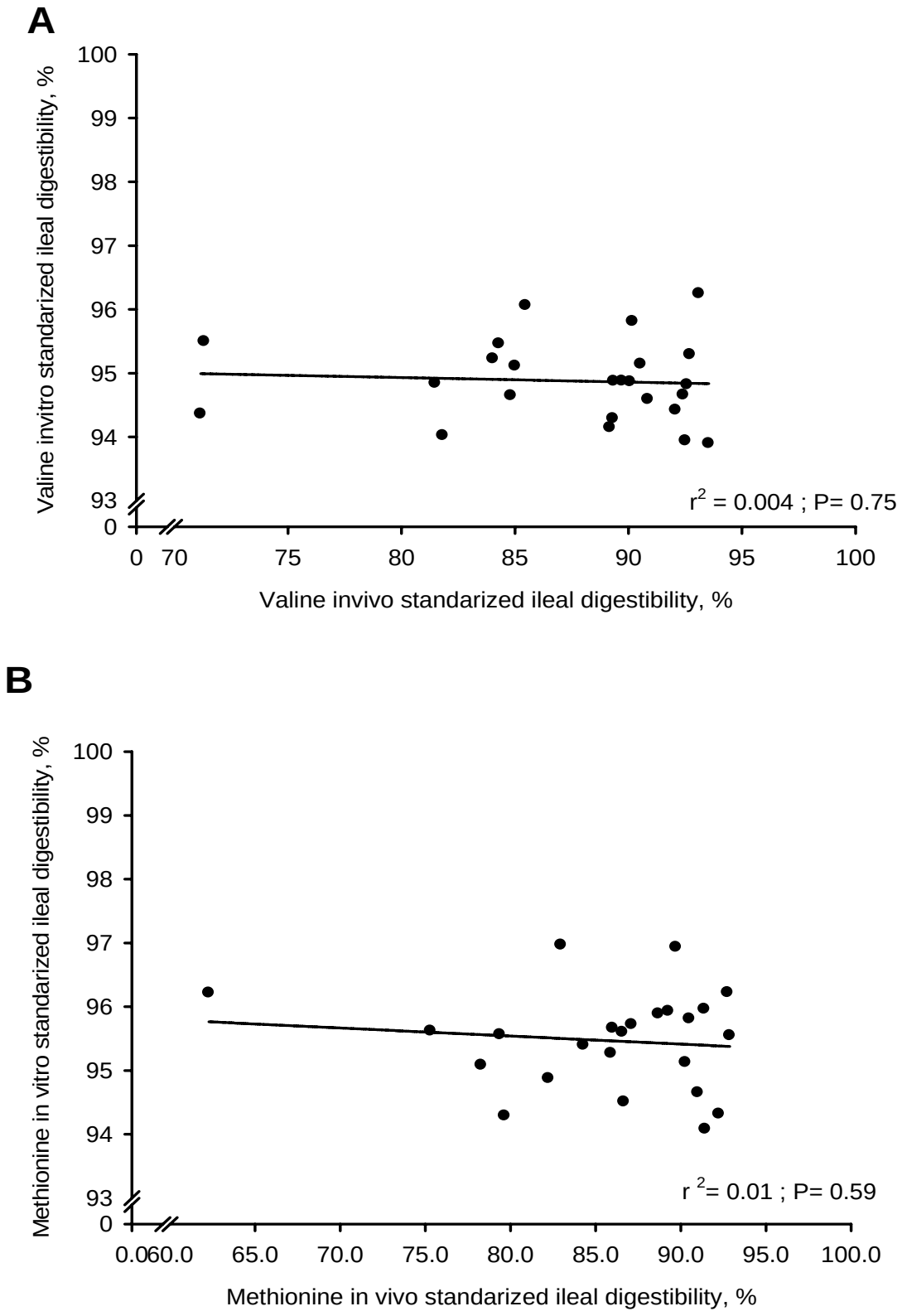
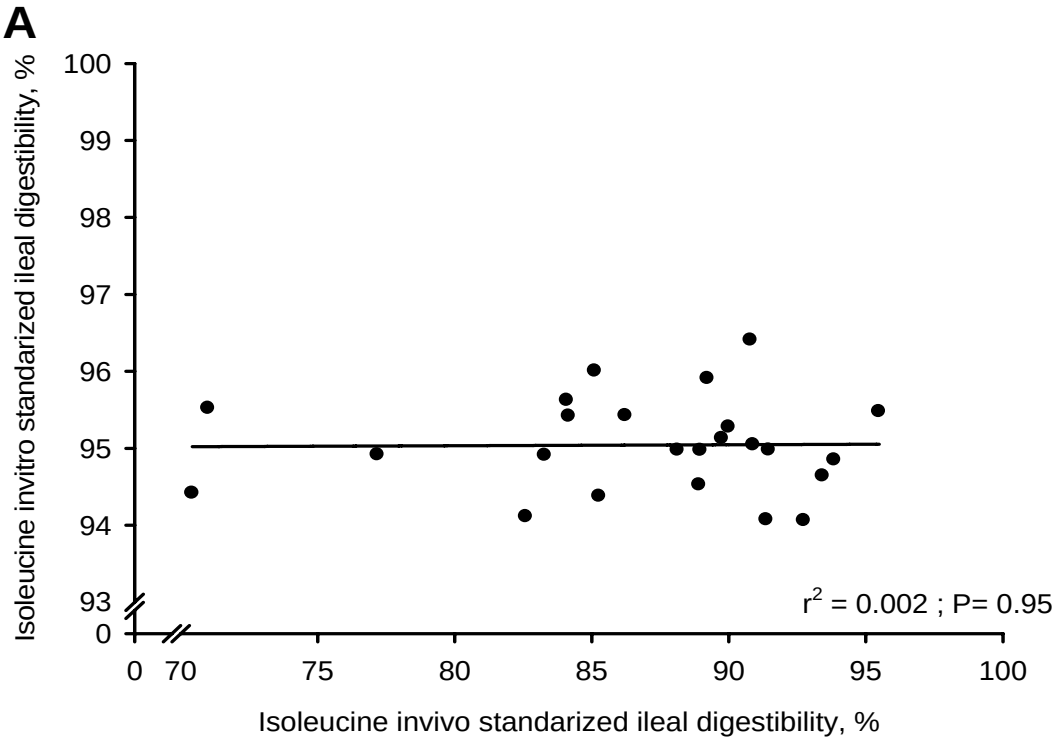


Figure 3.23. Relationship between *in vivo* and *in vitro* SID for valine (A) and methionine (B).



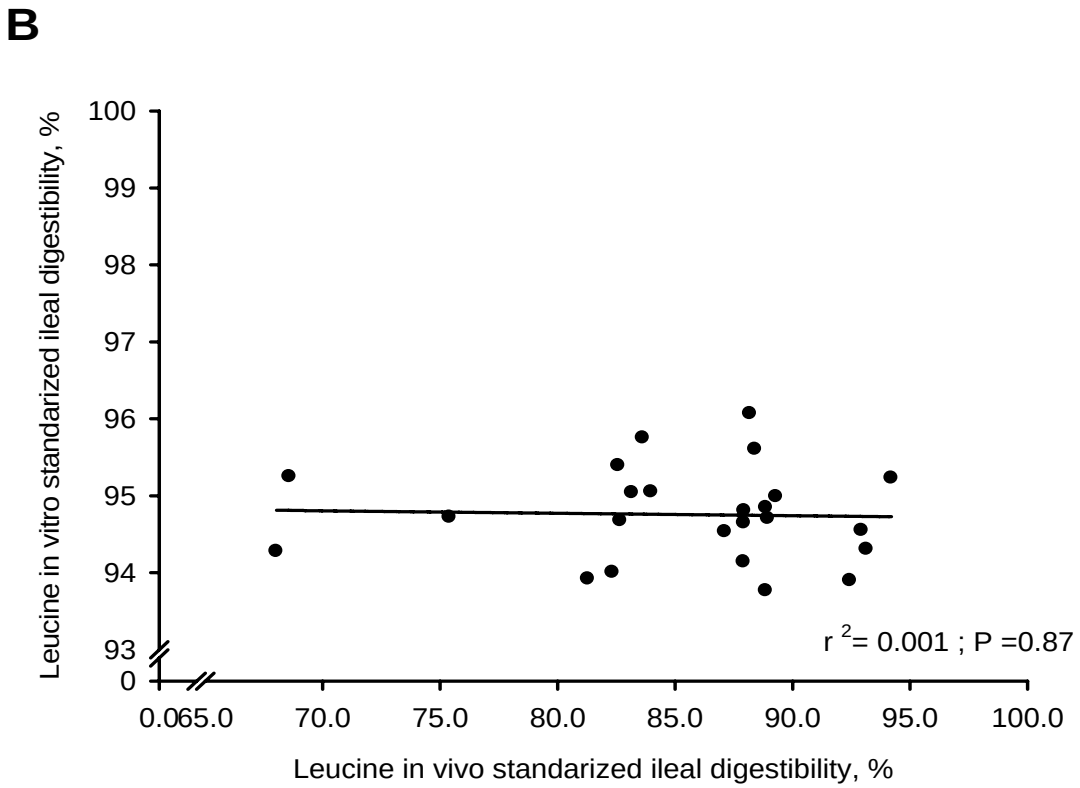


Figure 3.24. Relationship between *in vivo* and *in vitro* SID for isoleucine (A) and leucine (B).

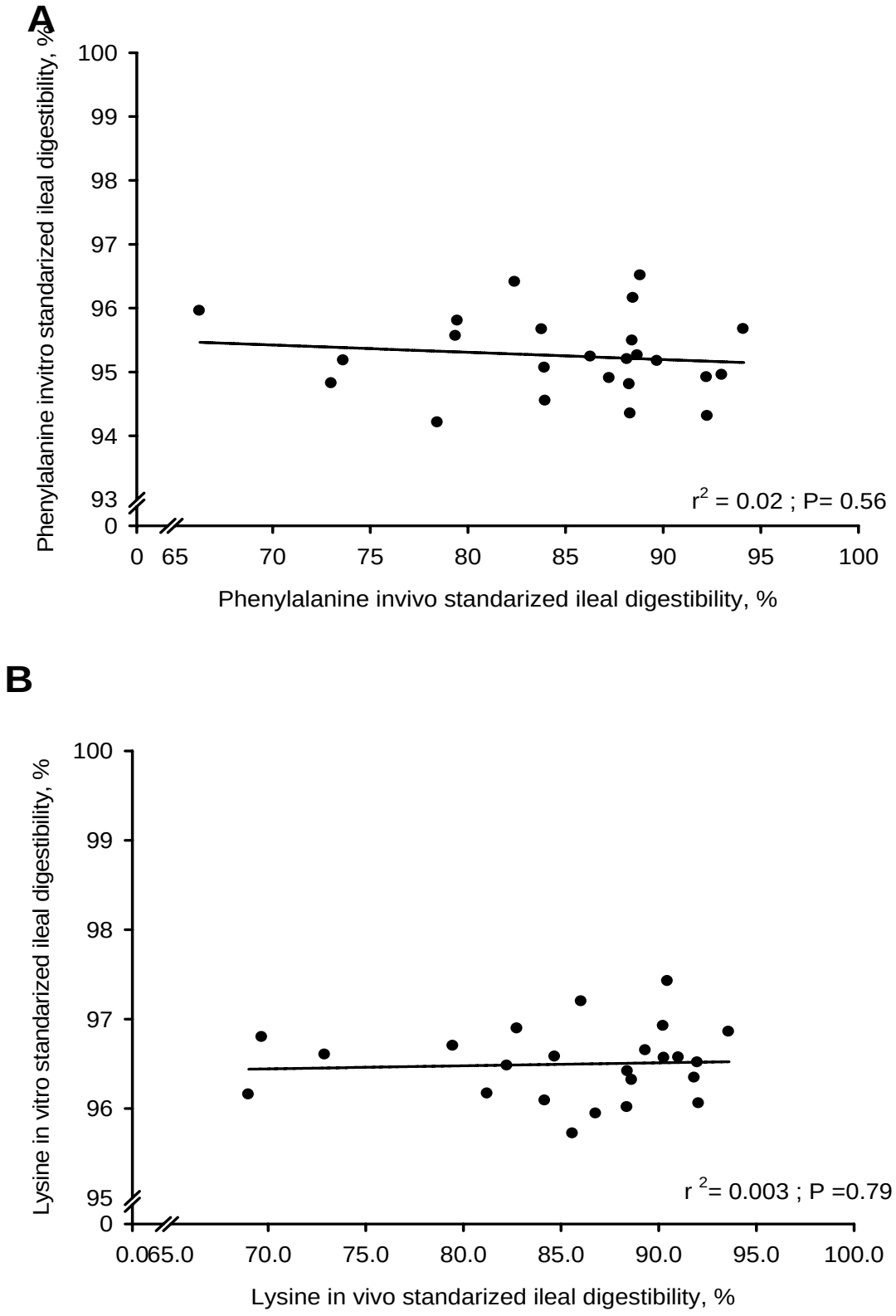


Figure 3.25. Correlation between *in vivo* and *in vitro* SID for phenylalanine (A) and lysine (B).

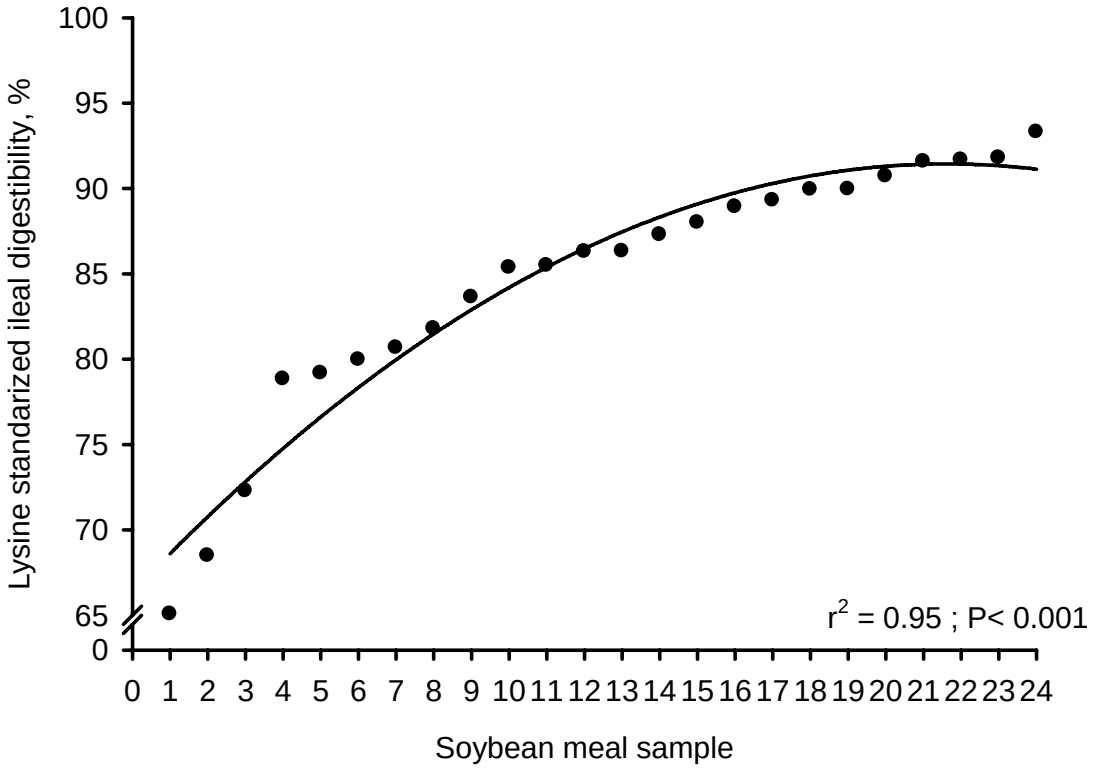


Figure 3.26. Distribution of standardized ileal digestible lysine in 24 samples of soybean meal.

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