Reliability of a microbial assay assessing lysine and methionine concentrations in maize (*Zea mays* L.) kernels

by

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GENERAL INTRODUCTION

Introduction

Since the domestication of maize (*Zea mays* L.) people have been altering either the physiological appearance of the plant or one of its chemical components. One of the problems with maize is that it does not have a balanced protein composition; meaning the grain is nutritionally deficient in some of the essential amino acids. Animal producers overcome this problem by supplementing the animals' ration with soybean meal, or other sources of amino acid additives. This route of action can be very costly and burdensome to the producer. One way to overcome this obstacle is for plant breeders to develop a maize variety that has higher levels of the limiting amino acids. There are several methods available to the breeder to improve the nutritional content of maize. These methods include using traditional plant breeding selection, mutants, or biotechnology. In order for plant breeders to successfully improve the quality of the maize kernel they need to have a breeding population with variation for the traits of interest and a reliable assay to detect plants that express the desirable traits. This literature review will focus on the feasibility of developing plants for increased amino acid levels, and the assays that are used to determine the amino acid content.

Essential Amino Acids and Animal Nutrition Requirements

Two types of digestive systems exist in animals used for production, ruminant and non-ruminant, with each system placing different demands on the organism. Non-ruminant animals, also known as monogastric animals, lack the microbial-digestive system of ruminant animals that provide the ruminant animal with the necessary amino acids for proper development. Monogastric animals are not able to synthesize all of their required amino acids so they must obtain them directly from their diet. These required amino acids that must be supplied in the diet are known as essential amino acid (Berg, 2002). This is in contrast to a nonessential amino acid that the organism can synthesize from metabolites produced in the body. The amount of essential amino acids required fluctuates throughout an organism's life as the nutritional requirements change. If an essential amino acid is not present in a

monogastric animal's diet, the animal's body will begin to degrade protein to accumulate the needed amino acid. The rate of protein breakdown will exceed the rate of protein synthesis resulting in a negative nitrogen balance. This excess breakdown of protein results in a high level of nitrogen excretion (Berg, 2002). To avoid this negative effect, the animals must ingest a properly balanced diet consisting of all necessary amino acids. Among the list of essential amino acids are lysine, methionine, and tryptophan. These three amino acids are limiting in maize-based rations.

Maize protein is comprised of four different fractions that are defined by their solubilities (Osborne, 1897). The fractions are albumins (soluble in water), globulins (soluble in saline solution), prolamines or zeins (soluble in relatively strong alcohol), and glutelins (soluble in alkali solutions), which are present in the kernel in varying amounts. On average, the albumins and globulins each comprise 3% of total seed protein content, the glutelins make-up 34% and the zeins encompass 60% of the protein content (Hallauer, 2001). Given that the zeins constitute most of the kernel protein they have the most influence over the quality of the protein. Osborne and Clapp (1908) analyzed the zein proteins and reported deficiencies in two of the essential amino acids, lysine and tryptophan.

Animal studies focusing on the nutritional nature of corn confirmed the finding of Osborne and Clapp. Willcock and Hopkins (1913) reported when mice were fed zein or zein plus tyrosine rations they did not live very long. However, when the mice were fed zein plus tryptophan rations the life expectancy of the mice increased. This finding was still being reinforced a century later by studies conducted by Peter et al. (2000) on the weight gain, feed intake, and feed efficiency in chickens fed a fortified corn meal diet verses a corn-soybean diet. By removing, in various combinations, the fortified amino acids from the corn meal, there was a significant decrease in the above listed traits. This study concluded the limiting order of amino acids in corn is lysine, tryptophan, threonine, valine, isoleucine, histidine, cystine, and methionine. By adding the limiting amino acids to the animal's corn-based ration the animal's performance and life span increased.

Studies on Methods to Improve Nutritional Value

Traditional Selection

Protein quantity

A number of maize breeders have implemented traditional breeding methods to increase the protein quantity present in the kernels. The Illinois long term selection experiment implemented in 1896 by C.G. Hopkins at the University of Illinois is the most well known. This experiment was initiated to determine if the chemical composition of the kernel could be altered by selection.

Hopkins knew that the environment could greatly influence the protein quantity. Before he would start a selection program for protein quantity he needed to know if there were some heritable factors for protein. To test this, he planted 50 selected ears from the Burr's white population in a field with uniform soil conditions. After harvest and analysis the distributions of the protein and oil concentrations were compared to data from ears grown in highly diverse environments. The uniform environment data, by comparison, had deviations much larger than one would expect if the ears had been grown in a varied environment. This was evidence enough for Hopkins that there were heritable factors governing the protein and oil content (East and Jones, 1920). Understanding his heritability assumption, Hopkins began an ear-to-row mass selection program for stains of maize characterized by high or low protein, or by high or low oil content (Hopkins, 1899).

Smith (1908) reported that this selection process was effective in increasing and decreasing the protein and oil concentrations. Winter (1929), Woodworth et al. (1952), Leng (1962), Dudley et al. (1974), and Dudley and Lambert (1992) have all reported on the progress this selection program has made throughout the years. After 90 generations of selection, this program has resulted in high and low protein levels of 32% and 4%, with high and low oil levels of 22% and 1.0%, respectively (Dudley and Lambert, 1992). These are significant changes from the initial population of 10.9% protein and 4.69% oil content (Hopkins, 1899). The low strain for oil has approached a physiological limit where the viability of the seeds are so low that after 87 generations selection this strain was no longer selected for. In addition, the low strain of protein has leveled off at 4%.

With the plateau of the low protein and oil strains, is there enough genetic variation preserved in the population by this selection program to continue to see progress? Shortly after the program was initiated, Davenport and Rietz (1907) studied the four strains being selected and concluded, "The variability was not sensibly reduced during the ten years of rigid selection." They also believed that even after significant advances have been accomplished, there would still be "abundant variability on which to base future selection, and that if the limits of improvement are ever reached it will be for some reason other then the failure of variability" (Davenport and Rietz, 1907). After 65 generations of selection, Dudley et al. (1974) reported that there was significant genetic variability present in all of the selection areas in the population. Later in 1992, after 90 generations of selection, Dudley and Lambert concluded, with the exception of the low protein and oil strains, the remaining strains have genetic variation present and continued progress with this program should be possible.

Another program with the objective of altering the protein quantity was the program started in 1912 at the Connecticut agricultural experiment station. This program, although not as long-term as the Illinois program, used two methods of selection in their program. The first method was to make selections out of self-pollinated lines and the other was to make selections in alternately crossed and selfed lines. The result from the self-pollinated lines was that they could produce lines high in protein in a relatively short amount of time. In the second method of selection, the station was able to produce ears in six years that were as high in protein as the Illinois station had produced in 25 years (East and Jones, 1920).

With the ability to increase the quantity of protein available what was changing in the kernel composition for this phenomenon to occur? Hopkins (1899) reported that there was a physical difference between the high and low protein kernels. Frey et al. (1949) reported that by selecting for an increase in the total protein level, there was an increase in the zein fraction of the endosperm proteins. As it has already been stated, the zein fraction is nutritionally deficient in lysine and tryptophan. By selecting for high protein levels, the result was a seed that had a poor protein quality. Was it possible to select for kernels that had high protein quality?

Protein quality

Zuber, and Helm (1972) undertook a project to determine if the lysine content in a non-mutant strain could be increased through recurrent selection. At this point in history many studies had been conducted to illustrate there was variation for amino acid composition in maize. Zuber and Helm chose three open-pollinated varieties that were higher then average for lysine content as their material to work with. One selection cycle consisted of making selections, two generations of intermating, followed by a selfing generation. After two cycles of selection, the range of lysine content was still the same between the two cycles; however, the mean lysine content was increased. The protein levels were monitored during this selection process, and they did not show any significant change. Since the cycles were conducted in different years the difference seen in the lysine content could have been caused by environmental effect. To test this theory, the original population and the two selected cycles were grown together in the same year (Zuber, 1975). The mean lysine values for the various cycles grown in the same year showed an increase in lysine content for two of the three populations studied. Protein content of the various cycles grown in the same environment showed a gradual increase with the exception of one population in which there was a decrease in protein content between the two selection cycles. This population that had a decrease in protein was the same population that did not exhibit an increase in lysine for those cycles. The correlations of protein content and lysine content for all three populations grown in the same environment were significant at the 1% level. This study concludes that lysine content can be increased by recurrent selection in some populations without the use of endosperm mutants.

Mutations and Mutation Breeding

Natural occurring mutations

There are several mutations that can alter the endosperm protein quality. Some of these mutants are *opaque-2*, *opaque-6, opaque-7, floury-2, floury-3, defective B30*, and *mucronate* just to list a few (Bjarnason, and Vasal, 1992). The most well characterized mutants are the *opaque-2*, and *floury-2* genes. Both of these mutants cause an increase in the level of lysine present in the kernel. The *opaque-2* mutant was first described by Jones and

Singleton in the early 1920's at the Connecticut Agricultural Experiment Station (Emerson et al, 1935). However, it wasn't until 1963 when Mertz et al. (1964) made the discovery that *opaque-2* increased the lysine content by 69% over the level of normal maize. The following year, Nelson (1965) determined that the *floury-2* mutant could alter the lysine and tryptophan levels in maize. The *floury-2* mutant was first isolated by Mumm (1935) at the Illinois Agricultural Experiment Station. The *floury-2* mutant, in many genetic backgrounds, would increase total kernel protein up to 50% without a decrease in the lysine levels as was seen in previous studies (Nelson, 1973).

The breeding community was in a frenzy to incorporate these mutations into their elite lines to produce lines that had high protein quality and nutritional value. After extensive breeding efforts in the early 1970's it soon became apparent that the pleiotropic effects of the mutations were a problem, to the extent that most of the research centers and institutions discontinued their *opaque-2*, and *floury-2* breeding programs. The major problems associated with the mutant alleles were reduced grain yields, soft chalky dull phenotype, poor acceptance of the kernel phenotype or appearance, increased moisture content resulting in slow dry down after physiological maturity, increased vulnerability to ear rot, increased kernel breakage during mechanical harvest, greater damage by stored grain insect pests, and a lower rate of germination. Other hindrances included a thicker pericarp, reduced cob weight, reduced kernel weight and density (Vasal, 2001; Bjarnason and Vasal, 1992).

Even though the *floury-2* mutant increased lysine to levels very similar to that of the *opaque-2* mutant, the *floury-2* mutant had significantly lower nutritional values (Nelson, 1973). In separate feeding trials conducted on weanling rats and weanling pigs, the animals fed *floury-2* maize did not grow as rapidly as those fed *opaque-2* maize (Veron, 1967; Gipp, 1968; Klein et al, 1970). Due to the lower nutritional value of *floury-2*, the mutant gene was not highly utilized in developing high-lysine maize. These results also indicate that the research efforts for high-lysine maize should be focused on overcoming the problems associated with the *opaque-2* gene and utilizing it.

Some of the known high lysine mutants alone would exhibit the characteristic soft, chalky phenotype; however, when in combination with another mutant they would often express reduced levels of the unacceptable traits. One such combination that was mildly

successful was the *sugary-2 opaque-2* strains. At Purdue University and at CIMMYT the *sugary-2 opaque-2* combination was shown to have an increase in kernel hardness, a reduction of ear rot over that of the single *opaque-2* mutant gene, and a higher tolerance to mechanical harvest. The protein content in the combination was as high as the single *opaque-2* strains and in some backgrounds was even higher (Mertz, 1992). However, the downfall of the double mutant combination was the yields were reduced by so much that it was not economically feasible for producers.

Another strategy employed by CIMMYT to improve the detrimental pleiotropic effects of the *opaque-2* gene was to capitalize on the recognition of *opaque-2* endosperm modifier genes discovered by Paez et al in 1969. These modifier genes altered the appearance of the *opaque-2* mutants, giving them a hard appearance instead of the soft, chalky one of the unmodified *opaque-2*. It appeared that a whole range of problems associated with the *opaque-2* gene could be remedied by using certain modifying gene complexes. In the 1980's, by successfully combining the *opaque-2* gene with the right set of modifier genes, CIMMYT was able to develop high-quality protein populations, referred to as QPM populations, that yielded comparably to their respective non-*opaque-2* populations (Vasal, 2001). *Opaque-2* strains were tested in feeding trials using growing swine when it was discovered that the lysine present in the *opaque-2* corn was nutritionally available as the lysine in the non-*opaque-2* corn (Kornegay, 1975).

Induced mutations

Another approach to increasing the concentration of essential amino acids in the maize storage proteins is to induce mutations in the biochemical pathway controlling amino acid biosynthesis. The production of amino acids is maintained by a control mechanism called feed-back inhibition. In this process, the final product of the pathway often inhibits the enzyme that catalyzes the first step in the reaction (Berg, 2002). Analogs of the amino acids can act like the natural amino acids, in the sense that they can induce feed-back inhibition and stop the production of the natural amino acids. However, in most cases, the analogs do not replace the natural amino acids in the body's chemical processes (Brock et al, 1973) and therefore these analogs are toxic to the plant. Mutants that develop resistance to these toxins either over-produce the enzyme needed in the first step of the biosynthetic pathway or the

enzyme at the first step of the pathway develops insensitivity to the feedback molecule. If the mutant is then grown in the absence of the analog, an over-production of the natural amino acid occurs (Widholm, 1972).

One success story of this method is the increase in tryptophan levels via resistance to the 5-methyltryptophan (5MT) analog in maize lines. Kang and Kameya (1993) used two different mutagenic treatments of EMS on tissue cells to derive their mutagenic material. The strains were screened for resistance to 5MT and selfed. The amino acid levels in the M_1 calli and seeds were analyzed and found to be approximately two to three times the levels of the control plants. Specifically in the calli the amount of tryptophan was 5.8 times higher then the control. In the seeds the tryptophan levels were 4.5 times higher then the control. These results indicate that there is another method for improving the quality of protein available to plant breeders.

Biotechnology Approaches

With the advancement of technology, it is now possible to genetically alter the nutritional quality of numerous crops using techniques such as chimeric genes and gene silencing. In the development of enhanced genes there are two sources of genes, heterologous and endogenous genes. There has been much research conducted using heterologous genes to improve the plants amino acid balance. Two main sources of heterologous genes for increased methionine that will be discussed are the Brazil nut, and the sunflower.

The Brazil nut was selected as a candidate for chimeric genes because of its high methionine levels, 18% (Sun et al, 1987). Altenbach et at. (1989) developed transgenic tobacco plants that expressed elevated levels of methionine in the seeds. The transgenic seeds expressed a 30% increase of methionine over the control lines. In another study by Altenbach et al. (1992) winter canola was modified with the Brazilian nut gene and contained 33% more methionine then the controls. Thus, it is possible to use this gene and successfully increase the levels of methionine in a various crops. A gene from the sunflower has also been identified as being high in methionine providing avenues for transgenic plants. Molvig et al (1997) obtained a 94% increase in methionine levels in lupins with the transgenic gene encountering no statistically significant change in the levels of the other amino acids, total nitrogen nor yield reduction. In an animal feeding study, the transgenic lupin gave significant

increases in the performance of the animals demonstrating the possibilities of using the sunflower gene to increase animal performance. Both of these studies indicate that it is possible to use heterologous sources of genes to increase the level of methionine.

One notable study using an endogenous protein was the work of Lai and Messing (2002) as they utilized the natural variation in the maize genome to increase the levels of methionine in the seed kernel. The Dzs10 protein is a seed storage protein that contains high levels of methionine. While this protein is present in maize, the low expression levels are attributed to the low levels of mRNA accumulation during endosperm development due to post-transcriptional regulation. To increase this expression, a gene was developed in which the 5`UTR and promoter were replaced with endosperm-specific storage protein genes that are not post-transcriptionally regulated. The 3` region was replaced with a transcript of cauliflower mosaic virus. The effect of this gene was an increase in the Dzs10 accumulation resulting in increased methionine levels. Not only was there an increase in the methionine levels but the enhanced methionine was nutritionally available to monogastric animals.

The second type of technology discussed here is the use of gene silencing to alter the nutritional quality of maize. The zein fraction is the most abundant protein in the endosperm, while the α-zeins comprise >70% of the total zein fraction. This specific zein fraction appears to be represented in only a few transcripts (Woo et al, 2001) that make it the ideal target for gene silencing. Huang et al. (2004) used gene silencing to reduce the amount of the 19-kDa α-zein fractions in the maize endosperm. The α-zein gene coding sequence was inserted in antisense orientation into an expression cassette containing the 3`UTR of the nopaline synthase gene and γ-zeinA promoter. The transgenic lines examined demonstrated reduced levels of the α-zein fraction with an increase in the other seed proteins. The percent of lysine, tryptophan, and methionine in the transformed lines were 4.23, 1.03, and 3.43 compared to the control line of 2.94, 0.79, and 2.38% respectively. The essential amino acids increased significantly over the control line as a result of the decreased α-zein fraction.

These studies demonstrated the feasibility of enhancing the nutritional quality of food crops produced via transgenic methods; however, these techniques have disadvantages that need to be acknowledged as well. One general problem with chimeric genes is the challenges faced with plant transformation, and expression of the proteins in the transgenic plant.

Heterologous proteins may be expressed and accumulated right after germination, but then sometimes the levels begin to decline during development (Altenbach et at, 1989). Molvig et at (1997) reported problems with regeneration of the grain legumes and the unstable expression of the protein in the target host. The Brazilian nut gene for increased methionine content has shown to contain allergenic properties (Nordlee et at, 1996). In endogenous chimeric genes, often genomic imprinting and negative dominance may become a hindrance when increasing the methionine levels (Lai and Messing, 2002).

Amino Acid Assays

An essential characteristic of a successful breeding program for increased amino acids is to have to ability to analyze large numbers of samples accurately and efficiently. Three main methods are used in quantifying the concentrations of amino acids: bioassays, chemical assays and chromatography.

Bioassays

Microorganisms first were implemented for quantifying growth stimulating substances in the early 1900s (Wildiers, 1901). The first organisms used in the detection and determination of biological availability of amino acids were the ciliate protozoan *Tetrahymena pyriformis*, and the lactic bacteria *Lactobacillus, Streptococcus, Leuconostoc* spp. among other strains. These strains were faster growing and cheaper then animals while being more physiologically meaningful then chemical assays. Shankman et al (1943) used auxotrophic strains of *Lactobacillus arabinosus* to determine the amino acid concentration of eight different amino acids. The concentrations of the amino acids were based on the amount of lactic acid that was produced. There were several drawbacks associated with these early microbial assays. First of all, the procedures were cumbersome, the bacterial medium was complex to compose, contamination was a problem, and the cultures needed to be incubated approximately four days. Further more, the density of the sample was determined by manually counting the number of cells ml^{-1} of culture under a microscope. This left the measurements produced by the assay unreliable and subject to human error (Payne et al, 1977). In 1995, Wright and Orman proposed a microbiological assay utilizing *Pediococcus cervisiae* to measure methionine in maize and soybean seeds. When the amino acid of

interest is limiting, the bacterial growth, measured as turbidity, reflects this and is proportional to the amino acid concentration. Although this method may not be as accurate as other methods available, it provides a means for screening large numbers of samples in a short time, which is desirable to plant breeders.

The use of *Escherichia coli* auxotrophic for specific amino acids was proposed to solve some the problems with the early microbial assay strains. *E. coli* was more desirable because they could be auxotrophic, and plasmids could be inserted into the *E. coli* genome to acquire different traits. The growth medium is simple and easily prepared. The whole assay can be done in a matter of a few days. A few traits that can be expressed by adding plasmids to the bacteria are antibiotic resistance and bioluminescence. Froelich et al (2002) determined that adding antibiotic genes to *E. coli* and growing them on selective media did not have a negative impact on the bacteria's growth and reduced the levels of contamination from foreign bacteria. To make microbial assays even faster, bioluminescent genes have been inserted which allow for detectable differentiation as soon as four hours (Erickson et al, 2000; Diaz and Ricke, 2003). The bioluminescence method has been compared to conventional optical density measurements with measurements 98% correlated.

Chemical Methods

Deciding that microbial assays and chromatographic techniques were expensive, tedious, and time consuming, other methods were developed. Hernandez and Bates (1969) developed a chemical method utilizing iron chloride to characterize Papain-hydrolyzed protein for the detection of tryptophan. This method was extensively used at CIMMYT in their maize breeding program. Another method proposed by Sastry and Tummuru (1985) used a colored product of the reaction between tryptophan, thioglycolic acid and sucrose to measure tryptophan levels. This method proved to be highly sensitive, simple, and rapid.

Chromatography

The American Organization of Analytical Chemists recognizes ion exchange chromatographic methods for determining the concentrations of several amino acids (AOAC, 1995). Chromatographic methods provide a higher level of sensitivity and accuracy to the

amino acid concentration measurements; however, these methods are expensive, laborious, and slow making them prohibitive for most plant breeding programs (Prasanna et al, 2001).

Thesis Organization

This thesis is comprised of a general introduction, the findings of a single study, and a general conclusion. The focus of the study was to determine if a microbial assay was reliable for the selection of material by plant breeders. This study shows not only is the assay able to detect genetic differences between samples, but also do so at a highly consistent rate. A simulation program was conducted to determine the optimum assay protocol.

This study was written by the primary author with the guidance and assistance of Dr. Lamkey and Dr. Scott. All of the chemical analyses for lysine and methionine content were conducted by the primary author in Dr. Scott's lab with technical support for grinding seeds. The primary author conducted all of the data analysis.

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RELIABILITY DETECTED IN MICROBIAL ASSAY

Introduction

It has been recognized that the genetic constitution of the maize kernel may have an impact on its chemical composition (Doty et al., 1946). Seed protein is made up of four fractions of proteins: albumins, prolamins, globulins, and glutelins. Of these proteins, the prolamin class zeins are present at the highest amounts, accumulating up to 60% of total protein. Osborn and Clapp (1908) analyzed the composition of the zein proteins and made the discovery that they were deficient in lysine and tryptophan. Both of these are essential amino acids for monogastric animals. To improve the nutritional quality of the protein in maize would require an increase in the content of these two essential amino acids.

In breeding programs, where thousands of samples are analyzed each week, there *is* a need for simple, inexpensive, high-throughput, precise, and accurate assays. Selection programs to increase the quality of the maize protein require the adaptation and utilization of amino acid assays. The accepted method of the AOAC International is expensive and time consuming. At the University of Missouri – Columbia where samples are analyzed using the AOAC methods, turnaround takes 8-10 days costing in excess of $44 per triplicate sample (Experimental Station Chemical Laboratories, 1996). In attempt to address these problems, a microbial assay with a turnaround of 3-4 days costing $0.50 per triplicate sample was critically reviewed. A plant breeder with the intention of increasing the quality of maize protein needs to know the reliability of assays used for selection. However, it is necessary to have some approximation of the extent to which accuracy is compromised.

The objective of this study was to answer the question, is the auxotrophic microbial assay described below sufficiently reliable to detect genotypic differences between samples? To answer this question, this objective was broken down into several components. First, we set out to determine the major sources of variation responsible for phenotypic variation between samples. The sources of variation considered were weighing the sample, repeated measurements on each sample, replication of measurements on samples from the field, and spatial variation between and within assay plates. Secondly, we determined the statistical

repeatability of this assay. Finally, we used estimates of the variance components for each source of variation to develop a simulation that allows optimization of the protocol.

Materials and Methods

Genetic Material

The maize samples analyzed in this study are from the BS11 synthetic maize population. Dr. W.L. Brown at Pioneer developed the BS11 population by inter-mating nine prolific cultivars and two semiprolific hybrids (Troyer, 2004). Two of the cultivars were tropical; the other seven were southern, temperate cultivars. The two hybrids used were adapted to central Iowa. Mass selection in the BS11 population began in 1967 and was continued in the manner described in Weyhrich et al. (1998). Seed from the fifth cycle of recurrent selection improvement, abbreviated BS11(S2)C5, was used in this study. One control was used in the amino acid assay, the inbred line B101, which is known to be high in methionine (Hallauer and Wright, 1995).

Field Procedure

Seeds from the BS11(S2)C5 population were sent to the Chile winter nursery in fall of 2002 and 150 individual plants were self pollinated to produce S_1 lines. In the summer of 2003, ninety randomly chosen S_1 lines from the population were mechanically planted in Carroll and Ames, IA. The experimental plots were planted in a 10 X 9 row-column lattice design with two replications at each location. Two row plots were used with 76 cm (30 in) between the 5.47 m (18 ft) long rows. The plots were over-planted and thinned to obtain an equal number of plants at both locations, with a final density of approximately 62,191 plants ha^{-1} (25,168 plants $acre^{-1}$). The plots were allowed to open pollinate. Plots were harvested mechanically using a plot combine and a sample was collected from each plot using a sampling device in the combine. Each sample weighed approximately 1.0 kg (2 pounds).

Assay Procedure

Grain samples from 14 entries were selected from both locations based on whole-kernel NIR analysis. The selection criterion was to capture the full range of protein variation present in evenly distributed samples. Five samples selected were relatively high in protein,

five samples were relatively low in protein and four had an average protein concentration. Each field sample was represented by three sub-samples each consisting of 30 flat kernels. Each of these sub-samples was separately ground to a fine powder in a Stein mill. The ground samples were stored in plastic snap cap containers at 4°C until they were analyzed. The 14 field samples, a control (B101), and the five chemical standards comprised the entries on a plate. The five chemical standards were known concentrations of methionine and lysine. For methionine the concentration of the standards were 0.1, 0.2, 0.5, 0.6, 0.8 mM. For lysine, the standards were 0.1, 0.25, 0.75, 1, 2 mM. All entries were arranged in a Randomized Complete Block Design with three replications on a single 96-well microplate (fig 1). Each plate contained one grind of each sample and each grind was replicated on three plates. Thus, a total of nine plates contained all three grinds, each in triplicate (Fig. 2).

Protein hydrolysis

The protein hydrolysis process is analogous to the protein enzymatic hydrolysis process that is described by Wright (1995). At the time of analysis, 10 ± 0.2 milligrams of ground sample powder was weighed into the appropriate wells on a 96 v-well plate. 200 μl of a 0.2M HCl-KCl pH 2 buffer containing 0.04 mg pepsin was added to each well. The plates were covered with an adhesive sealing film and placed in an incubating shaker for a minimum of seven hours at 37°C, 225 rpm. Then the plates were centrifuged for 20 minutes at 3,000 rpm to collect the ground sample in the bottom of the plate. Using sterile technique, the hydrolyzate was transferred to a new sterile 96 flat-well plate.

Preparation of innoculum

The innoculum was prepared using sterile technique, containers, and utensils. Using the Yale University *E. coli* genetic stock centers coding system, the P4x methionine strain used in this study was number 261 (Jacob and Wollman, 1957, 1961). The KL334 lysine strain was number 4345 (Brige 1974). The methionine innoculum was prepared in 5 mL M9 media (Sambrook and Russel, 2001) containing 120 μl of 1 mg/1 mL methionine, and an auxotrophic colony. The lysine innoculum was prepared in 5 mL M9 media containing 240 μl of 1 mg/1 mL lysine plus a lysine auxotrophic colony. The inoculums were incubated at 37° C, 225 rpm, 15 hours for the methionine innoculum and 18 hours for lysine innoculum.

Methionine and lysine assay

After protein hydrolysis, the hydrolysate was allocated to two sterile flat-well plates, one for the methionine assay and one for the lysine assay. Using sterile technique, 5 μl hydrolysate per well for methionine and 10 μl per well for lysine were distributed to the respective plate. 5 μl of the five chemical standards were dispensed into the appropriate wells on each amino acid plate. In addition to the standard solutions, 5 μl of pepsin buffer (no pepsin) was added to the standards on the lysine plates so that the final volume of analyte was the same in all wells. Into each well, 2 μl of the appropriate innoculum and 100 μl of M9 media were added. Once again the plates were covered with adhesive sealing film and incubated for 16 hours for the methionine assay and 18 hours for the lysine assay. At the end of the incubation time, the plates were shook for 30 seconds at 1,000 rpm before the optical density (OD) was determined at 595 nm using a microplate reader. Three OD measurements were taken of each plate.

Statistical Procedures

Description of models

The data was analyzed in two steps. The first analysis was carried out on single plates individually to check for outliers, determine if the weight of the sample was an important source of variation, and calculate the repeatability of the assay. After analyzing the data from the single plates, the analysis was run across all of the plates together to estimate variance components. The data was analyzed using either the proc glm or proc mixed procedures in the SAS system (SAS Institute, 2000).

The value for each sample was estimated using a generalized linear model, referred to as model I, consisting of the following terms:

$$y_{ij} = \mu + b_i + t_j + \epsilon_{ij}$$

where

y_{ij} = observed value in the ijth well;

μ = overall mean;

b_i = block effect due to the ith block (i = 1, 2, 3);

t_j = treatment effect of the j^{th} entry (j = 1,...20)

ϵ_{ij} = error associated with the ij^{th} well observation

All of the factors in this model are treated as random effects. To identify outliers, the raw data was analyzed using model I and the Anscombe Tukey test. If a data point fell outside the predicted range determined by a function of the error variance, it was considered to be an outlier and removed. In order to determine if the mass of the sample had a significant effect, the data was analyzed using model I with the mass fit as a covariate; the ANCOVA model used for this will be referred to as model II. The repeatability, r, of the OD values was calculated to determine the reproducibility of the OD measurements. The formula used to calculate the repeatability was:

$$r = \frac{\sigma_S^2}{\sigma_S^2 + \sigma_E^2}$$

where σ_S^2 is the variance among genotypes and σ_E^2 is the variance of the error. These variances were calculated using the REML estimates from model I.

To determine if the repeated measures increased the precision of the OD readings, the repeated measures model, referred to as model III, was used:

$$y_{ijklmn} = \mu + e_i + g_{j(i)} + p_k + pe_{ik} + b_{l(k)} + m_{m(k)} + \beta_n + \epsilon_{ijklmn}$$

where

y_{ijklmn} = observed value in the $ijklmn^{th}$ well;

μ = Overall mean;

e_i = entry effect of the i^{th} entry (i = 1, ... 20);

$g_{j(i)}$ = grind effect of the j^{th} grind nested in the i^{th} entry (j = 1, 2, 3);

p_k = plate effect of the k^{th} plate (k = 1, ... 9);

pe_{ik} = interaction effect between the i^{th} entry and the k^{th} plate;

$b_{l(k)}$ = block effect of the l^{th} block nested on the k^{th} plate (l = 1, 2, 3);

$m_{m(k)}$ = measurement number effect of the m^{th} measurement number nested on the k^{th} plate (m = 1,2,3);

β_n = time effect of the n^{th} time (n = 1, 2, 3);

ϵ_{ijklmn} = error associated with the ijklmnth well observation.

All of the terms in the repeated measures model are random except for the time effect, which is a fixed effect. This model can be clarified using figure 3. Each sample was composed of three ground subsamples. This repeated sampling gives rise to the grind term being nested in the sample term. Each grind was then replicated onto three plates creating the plate term. The interaction between the i^{th} entry and the k^{th} plate arises from the same sample being replicated on a single plate three times, one time in each block. The blocks are intuitively nested on the plates as is the measurement number nested on the plate. Figure 3 only represents two of the 14 samples; however, the structure was present among all of the samples tested.

Model IV was the model used to estimate the variance components across all of the plates in this experiment. Only the first set of measurements taken was used in model IV. This model is described as follows:

$$y_{ijkl} = \mu + e_i + g_{j(i)} + p_k + pe_{ik} + b_{l(k)} + \epsilon_{ijkl}$$

where

y_{ijkl} = observed value in the ijklth well;

μ = overall mean;

e_i = entry effect of the i^{th} entry (i = 1, ... 20);

$g_{j(i)}$ = grind effect of the j^{th} grind nested in the i^{th} entry (j = 1, 2, 3);

p_k = plate effect of the k^{th} plate (k = 1, ... 9);

pe_{ik} = interaction effect between the i^{th} entry and the k^{th} plate;

$b_{l(k)}$ = block effect of the l^{th} block nested on the k^{th} plate (l = 1, 2, 3);

ϵ_{ijkl} = error associated with the ijklth well observation.

All of the terms in this model are random.

Simulation program

Once estimates of the variance components were obtained from model IV, a simulation program was initiated to use the components and test possible assay procedures. The model used, referred to as model V, to generate the simulations was:

$$y_{ijkl} = \mu + e_i + g_{j(i)} + p_k + pg_{jk} + b_{l(k)} + \epsilon_{ijkl}$$

Where

y_{ijkl} = observed value in the ijklth well;

μ = overall mean;

e_i = entry effect of the ith entry (i = 1, ... 20);

$g_{j(i)}$ = grind effect of the jth grind nested in the ith entry (j = 1, 2, 3);

p_k = plate effect of the kth plate (k = 1, ... 9);

pg_{jk} = interaction effect between the kth plate and the jth grind;

$b_{l(k)}$ = block effect of the lth block nested on the kth plate (l = 1, 2, 3);

ϵ_{ijkl} = error associated with the ijklth well observation.

All of the terms in the model are random. A single iteration of the model generated 100 possible outcomes dependent on the terms in the model. Different assay protocols were analyzed using this model. For each step in the assay that was tested the Pearson and Spearman correlations were generated by using proc corr in the SAS system. The Pearson correlation is the correlation among samples BLUP values generated in the 100 simulations with the true value. The Spearman correlation is the correlation among sample rankings across the simulations. Two different sets of assay parameters were analyzed. The first parameter set, set 1, contained all of the variance components calculated from model IV. The second set of parameters, set 2, had the grind, block, and the interaction between the plate and grind effects set to zero. The two sets of parameters were compared against each other to determine if the effects set to zero had an impact on the outcome. A graphical representation of the correlations for the various assay protocols and parameter sets were developed using proc capability in the SAS system. All of the data were analyzed using either the proc mixed, proc correlation, or proc capabilities procedures in the SAS system (SAS Institute, 2000).

Results

Repeatability of the Assay

The repeatability was calculated to determine the precision of the measurements given by the assay. The variances used to calculate the repeatability were obtained from the BLUPS from model I. A list of the repeatabilites for the nine plates for both amino acids is given in Table 1. Nineteen of the twenty-seven methionine repeatability values were above 90% while the remaining eight were between 81% and 89%. In the lysine assay all of the repeatability values, with the exception of one were above 90%. These repeatability values give a clear indication that this assay is capable of detecting genetic differences among the entries analyzed.

Identification of Steps in the Assay Contributing to Error

Adjusting for variation in sample mass.

Since mass of each sample could vary from 9.8 to 10.2 mg, observed differences in amino acid content could be solely due to differences in sample mass. If this is the case, it may be necessary to adjust the OD measurements for the actual sample mass. To determine if this was necessary, model II was used to fit sample mass as a covariate. The F-values and their significance levels for the mass covariate term are listed in Table 2. Only one out of 27 covariates were significant in the lysine assay while none of the mass covariates were significant in the methionine assay. Thus we conclude that for the range of sample masses tested it is not necessary to adjust the measurements for the mass of the sample.

Repeated measurements on each plate.

In order to increase the precision of the measurements, the OD values on each plate were measured three times. The time effect from model III was highly significant ($P < 0.0001$) in both assays. This gives a clear indication that the time between measurements is important. Examination of the error values of each measurement revealed that they increase over time. Table 3 contains a list of the error values from model III for the three measurements. Repeated measures did not increase the precision of the measurements, so only the first reading of each plate was used in subsequent analysis of the data.

Ability to distinguish genotypic differences

One of the objectives of this study was to make sure that the assay protocol was able to detect genotypic differences between the entries despite other sources of variation in the assay. There are several steps in the assay that contribute to the error. The variance components for the sources of variation in the assay were estimated using model IV and are listed in table 4. Individual variance components are discussed in the following paragraphs. Despite the significance of some of the other sources of variance, the entry source of variance is still significant for both amino acids, and thus we are able to detect genotypic differences among the samples tested.

Number of times a field sample should be replicated.

For each field sample, three independent grinds were analyzed. This was done to determine if the measurement taken from a single grind would represent all of the variation present in the field sample. If the three grinds were significantly different from each other this would indicate that all of the variation present in one field sample was not being captured in a single thirty-kernel grind. Table 4 lists the variance components for different steps in the assay estimated using model IV across all nine plates. In both the methionine and lysine assays the grind effect was not statistically significant. These findings suggest that grinding a single 30-kernel sample from the field sample is sufficient for representing that sample.

Spatial variation within and between plates.

The spatial variation was examined within and between plates. It is important to understand the spatial variation so that it can be accounted for when designing and analyzing experiments. In this study, the plate and block terms represent the spatial variation between and within plates, respectively. The variance components, listed in table 4, were calculated for both methionine and lysine using model IV. In the methionine assay, the variation within plates was significant while the variation between plates was not significant. For the lysine assay, the spatial variation was not significant within a plate; however, it was significant between plates. Thus when comparing samples from the methionine assay it is important to

account for spatial variation in the context of within plate variation, while for the lysine assay the spatial variance is accounted for in the between plates variance. Both of these sources of spatial variation indicate that there is variation across the plate and between plates that must be controlled when comparing samples.

Simulation Program

One of our objectives was to determine an optimal assay design considering levels of replication at different steps in the assay, and if and how these replications should be blocked. Using the variance components calculated from model IV, a statistical simulation program was implemented to simulate different assay protocols. The simulations were conducted using model V. The first assay step examined was the number of plates to replicate an experiment over. In this simulation an experiment was replicated over one to six plates. The Pearson and Spearman correlations were calculated for the lysine and methionine assays and graphically represented in histograms (Fig 4, 5, 6, 7). The histograms clearly indicate a diminishing point of returns as the number of plates increase. Even though the standard deviations decrease and the measurements become more precise, there is an increase in the laboratory cost for replicating across more plates. The gain from going from three to four plates is smaller then the gain from two to three plates. For this assay under these conditions it is practical to use three plates for both methionine and lysine.

Another way to present this data is to compare the correlation means for each plate to the number of plates an experiment was replicated over (fig 8, 9, 10, 11). The slope of the curve for lysine begins to slowly decrease after replicating on three plates. The same trend is present in the methionine assay curves although it is a more pronounced decrease after the third plate. Once again, given the financial increase with replicating over a greater number of plates, and the diminishing returns indicated by these graphs, three plates may be the desired number of plates to replicate an experiment over.

The second parameter examined was the number blocks on each plate. The simulation program was run several times adjusting the number of blocks on a plate ranging from one to four blocks. In the block simulations, the experiment was replicated over three plates. Figures 12, 13, 14, and 15 are the histograms of the different blocks per plate for the Pearson and Spearman correlations for lysine and, methionine respectively. The LSD values (0.0039, and

0.0049) for the lysine Pearson and Spearman correlations, respectively, lead to the conclusion that the number of blocks per plate in the lysine assay does not make a statistically significant difference. However, when calculating the LSD values in the methionine assay (0.0096, 0.0116) for the Pearson and Spearman correlations, respectively, utilizing one block per plate is statistically different then using 2, 3, or 4 blocks per plate. Taking into consideration the number of samples to be analyzed and the results of this simulation there is no loss if only one block was used in the lysine assay; however, for the methionine assay one block is desirable.

To reinforce the significance of the variance components given by the variance components model, a second set of variance parameters were analyzed in the simulation program. In the second set of parameters the grind, plate by grind, and block effects were all set to zero. The histograms for the Pearson and Spearman correlations for the number of plates to replicate an experiment over, for both lysine and methionine, were very similar to the first set of simulations (fig 16, 17, 18, 19). The curve of the correlations means regressed over the number of plates, figures 20, 21, 22, and 23 indicated also that three plates were the ideal number of plates to replicate an experiment over. The conclusion from comparing the histograms from parameter set 1 and parameter set 2 was that the model terms that were set to zero did not have a large impact as a source of variation in the assay.

Discussion

Sources of Variation

The primary objective of this portion of the study was to estimate the main sources of variation in the study and their variance components. The partitioning of the variance components allows for the estimation of the roles that the individual steps of the assay and the environment play in the optical density measurements. In the words of Falconer and MacKay (1996), "The question of 'relative importance' can be answered only if it is expressed in terms of the variance attributable to the different sources of variation. The relative importance of a source of variation is the variance due to that source, as a proportion of the total…variance."

An important aspect of any assay is that it is precise enough to detect differences in the entries analyzed. In the case of this assay, the entry effect was relatively important for both the methionine and lysine assays. This means that at the basic level the assay is achieving what plant breeders desire, distinguishable amino acid measurements.

Another area of interest was whether the variation of the samples collected in the field could be represented in a single thirty-kernel grind. The results of this study state that the variation between the three thirty-kernel grinds represents a minimal portion of the variance in this assay. This is not concluding that thirty kernels is the optimum number of kernels to use for this assay, but that the variation from a single field sample can be adequately represented in one thirty kernel grind. By having to only grind one set of kernels for each entry instead of three, the manual labor needed for this assay is reduced, thus making it more time effective for plant breeders to use. Future work may include analyzing the optimum kernel number to use to reduce the amount of seed needing to be ground.

Grinding the kernels is one of the few labor-intensive steps in this assay, however, it doesn't compare to the time and patience needed to weigh the ground samples into the 96-well plates. This step requires the operator to meticulously handle minute portions of the samples and dispense it into the correct wells. The most challenging part of weighing the samples is obtaining the correct mass. This study concluded that within the range of 9.8-10.2 mg, the mass of the sample did not have an effect on the amino acid measurements. This makes the assay a little more flexible in that the operator does not have to accurately weigh out 10 mg of sample each time. If another study expanded the allowable mass range of the assay, it would greatly benefit the assay.

The individual plate effect was a significant source of variation in the lysine assay while the block-within-plate effect was significant for methionine. What both of these represent is essentially a microenvironment, just on different levels. Each one is influenced by a unique set of factors including small differences in allocating the solutions into the plate, hot spots from a specific location in the incubator, or simply by chance. Knowing where the spatial variation lies is important when comparing samples. Given the opportunity, replication on a plate and across several plates should be practiced, as it would be beneficial. Also, incorporating a row-column design on the plates might help address this issue.

Another factor critiqued in this assay procedure to increase reliability was the repeated measures readings. It is important to critically review in this assay how many times the plates need to have their OD measurements taken. The time effect between the when the measurements were taken plays a significant role in the OD measurements. When looking at the error values for the repeated measures model, it is apparent that there is a general trend of increasing values. This would indicate that as more measurements are taken, there is the introduction of more inaccuracy instead of precision. The error values may be increasing due to the bacteria continuing to grow. The best method of collecting the OD measurements would be to take only one measurement thus saving time and expense needlessly running the spectrometer.

Examining the repeatability of the assay revealed that the repeatability of this assay was excellent. Most of the repeatability values were above 90% indicating that the assay is working very well. By having high repeatability values, plant breeders can be assured that this assay will give consistent results, which is important when comparing hundreds of samples for selection.

One must note that this assay is highly dependent on the accuracy and consistency of the operator. From weighing out the samples to consistently pipetting the solutions into the wells, everything the operator does can contribute either in a positive or negative manner on the OD measurements produced by this assay. Therefore, to ensure consistent results, the same operator should collect a whole data set.

Optimum Assay Protocol Utilizing the Simulation

The purpose of a simulation is to test various situations that would otherwise be impossible or expensive to test. This section deals with a simulation using the variance components estimated using the current protocol to create the optimum assay protocol.

The reason for conducting this simulation program was to determine what assay protocol would give the most practical results. The first step examined was the number of plates to replicate an experiment over. There are diminishing returns as the number of plates increase. Outlier plates can be identified when more then two plates are analyzed, and the labor-intensive step of weighing the samples can be limited with fewer plates. Balancing the

rate of diminishing returns with the practically of conducting this assay, three plates was determined to be desirable.

The second step reviewed was the number of blocks on a single plate. The simulation projected that in the lysine assay no single replication number was statistically significant; however, in the methionine assay one block is statistically different then 2, 3 or 4 blocks. This difference in number of block per plate between the two assays may be a result of the operator, or simply by chance. Inaccuracies in allocating the hydrolyzate or innoculum across the plate in the methionine assay may cause one block to appear to be more precise instead of concluding the number of blocks is irrelevant. Another study could be conducted to confirm that the replication number on a single plate for either assay is unimportant.

A second set of parameter values were analyzed using the simulation in which the grind, block, and plate by grind interaction variance terms were set to zero. These terms are believed to have minimal influence on the OD measurement. Once again, given the diminishing returns with the increase in plate number, three plates are projected to be the practical number of plates to replicate an experiment over. This supports the conclusion that the terms that were set to zero are not vital in determining the number of plates to replicate over.

The implications of the simulation are that three plates, utilizing one block per plate is adequate. Contrasting the two parameter sets in the simulation confirms that the block term, grind term and plate-by-grind interaction are not large sources of variation in this assay. The simulation projected the optimum protocol to be what is currently being conducted. No major changes necessary and plant breeders should take comfort in using this assay to screen for amino acids.

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Tables and Figures

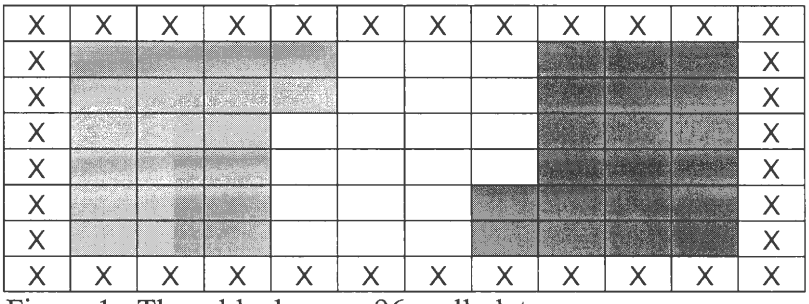

Figure 1. Three blocks on a 96-well plate.

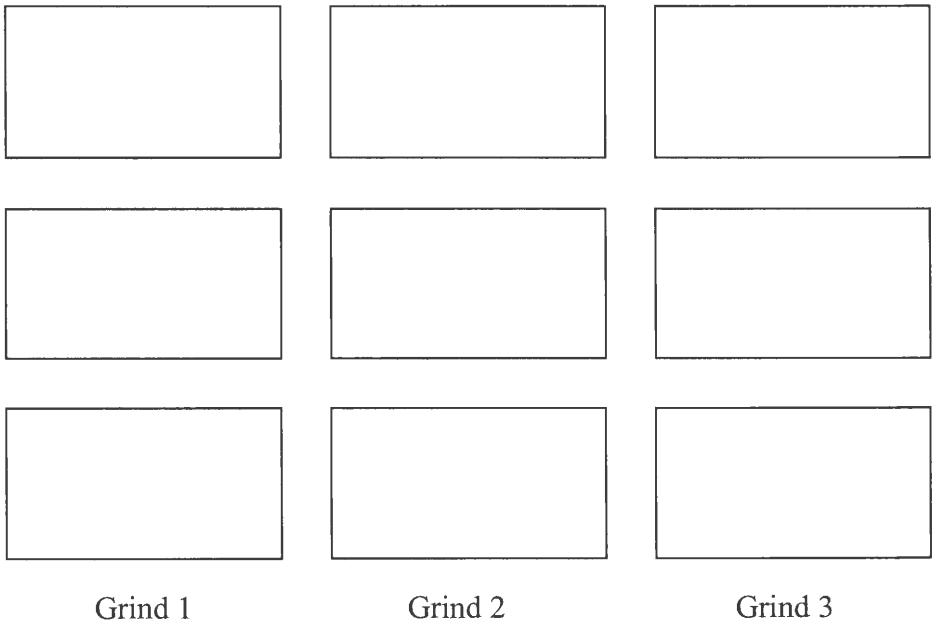

Figure 2. Replication of grinds across plates. Each rectangle represents a single plate.

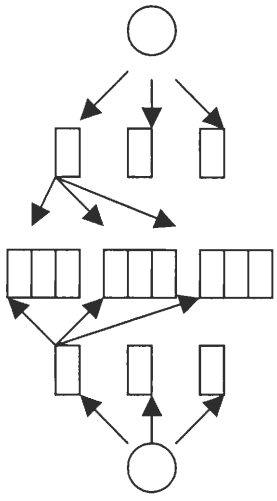

Figure 3. Layout of the experimental design.

Table 1. Repeatability (r) of OD readings with 90% confidence intervals.

	amino acid: methionine								
	measurement								
	1			2			3		
		confidence interval			confidence interval			confidence interval	
plate	r	lower	upper	r	lower	upper	r	lower	upper
1	0.85	0.68	0.92	0.94	0.87	0.97	0.95	0.90	0.97
2	0.80	0.54	0.88	0.88	0.75	0.93	0.90	0.79	0.94
3	0.90	0.80	0.95	0.88	0.74	0.93	0.93	0.86	0.96
4	0.98	0.96	0.99	0.96	0.93	0.98	0.94	0.89	0.97
5	0.93	0.86	0.96	0.95	0.90	0.97	0.96	0.93	0.98
6	0.87	0.71	0.92	0.92	0.83	0.96	0.95	0.91	0.98
7	0.94	0.87	0.97	0.93	0.85	0.96	0.87	0.71	0.92
8	0.98	0.96	0.99	0.96	0.92	0.98	0.81	0.58	0.89
9	0.97	0.94	0.98	0.98	0.95	0.99	0.89	0.78	0.94

	amino acid: lysine								
	measurement								
	1			2			3		
		confidence interval			confidence interval			confidence interval	
plate	r	lower	upper	r	lower	upper	r	lower	upper
1	0.98	0.96	0.99	0.98	0.95	0.99	0.97	0.95	0.99
2	0.96	0.92	0.98	0.94	0.89	0.97	0.95	0.91	0.98
3	0.98	0.96	0.99	0.97	0.95	0.99	0.97	0.94	0.98
4	0.99	0.98	0.99	0.99	0.97	0.99	0.98	0.97	0.99
5	0.99	0.98	0.99	0.99	0.98	0.99	0.99	0.98	0.99
6	0.93	0.87	0.96	0.92	0.84	0.96	0.94	0.87	0.97
7	0.96	0.91	0.98	0.96	0.91	0.98	0.97	0.93	0.98
8	0.94	0.88	0.97	0.93	0.87	0.94	0.94	0.88	0.97
9	0.90	0.80	0.95	0.90	0.80	0.95	0.87	0.72	0.93

Table 2. F-values from model II, ANCOVA mass covariate.

	amino acid					
	methionine			lysine		
	measurement			measurement		
plate	1	2	3	1	2	3
1	0.08	1.73	1.27	0.07	2.24	0.15
2	1.68	0.34	0.34	0.68	0.11	0.22
3	2.93	1.34	0.18	0.12	0.06	0.01
4	1.13	2.14	0.13	0.55	0.73	0.05
5	1.92	0.04	0.11	0.01	0.61	0
6	0.97	0.18	0.04	1.91	6.24*	2.39
7	0.34	0	0.38	1.16	0.81	0.04
8	0.48	0.54	0.5	0.36	0.21	0.6
9	0.37	0.56	0.31	0	0	0.01

* Significance at the 0.05 probability level.

Table 3. Errors from model III, repeatability model. Note how the error values usually increase with the increasing measurement number.

	amino acid					
	methionine			lysine		
	measurement†			measurement†		
plate	1	2	3	1	2	3
1	4.9940	4.2351	4.3153	0.4344	0.6961	0.9340
2	4.1168	5.1558	11.9218	0.6693	1.1930	1.1508
3	2.5798	10.3586	12.9748	0.3929	0.7192	1.0524
4	0.2338	0.7625	3.3927	0.2092	0.3943	0.4983
5	0.4439	0.6005	0.9664	0.1286	0.2227	0.1985
6	3.1994	4.2753	4.0686	1.1477	1.7659	1.6577
7	0.2002	0.6619	4.8949	0.7412	0.8014	0.8212
8	0.1799	0.8171	12.0652	1.0983	1.4896	1.6763
9	0.4564	0.6046	4.7081	0.6948	0.6948	1.0612

† The values have been multiplied by 1,000.

Table 4. Covariance parameter estimates with large sample confidence intervals for methionine and lysine.

	amino acid					
	methionine			lysine		
covariance		confidence interval			confidence interval	
parameter	estimate	lower	upper	estimate	lower	upper
entry	**2.1139****	1.0686	5.9904	0.2101*	0.0984	0.7197
grind(entry)	0.1906	0.0605	2.8122	4.80E-20	N/A	N/A
plate	0.9786	0.3844	5.7168	0.2597*	0.1073	1.2777
plate*entry	0.2753*	0.1104	1.4973	0.2141**	0.1200	0.4857
block(plate)	0.4223*	0.2057	1.3072	0.0259	0.0068	1.3370
residual	2.0268***	1.6998	2.4587	0.7862***	0.6590	0.9545

*,**,*** Significance at the 0.05, 0.01, 0.001 probability levels, respectively.

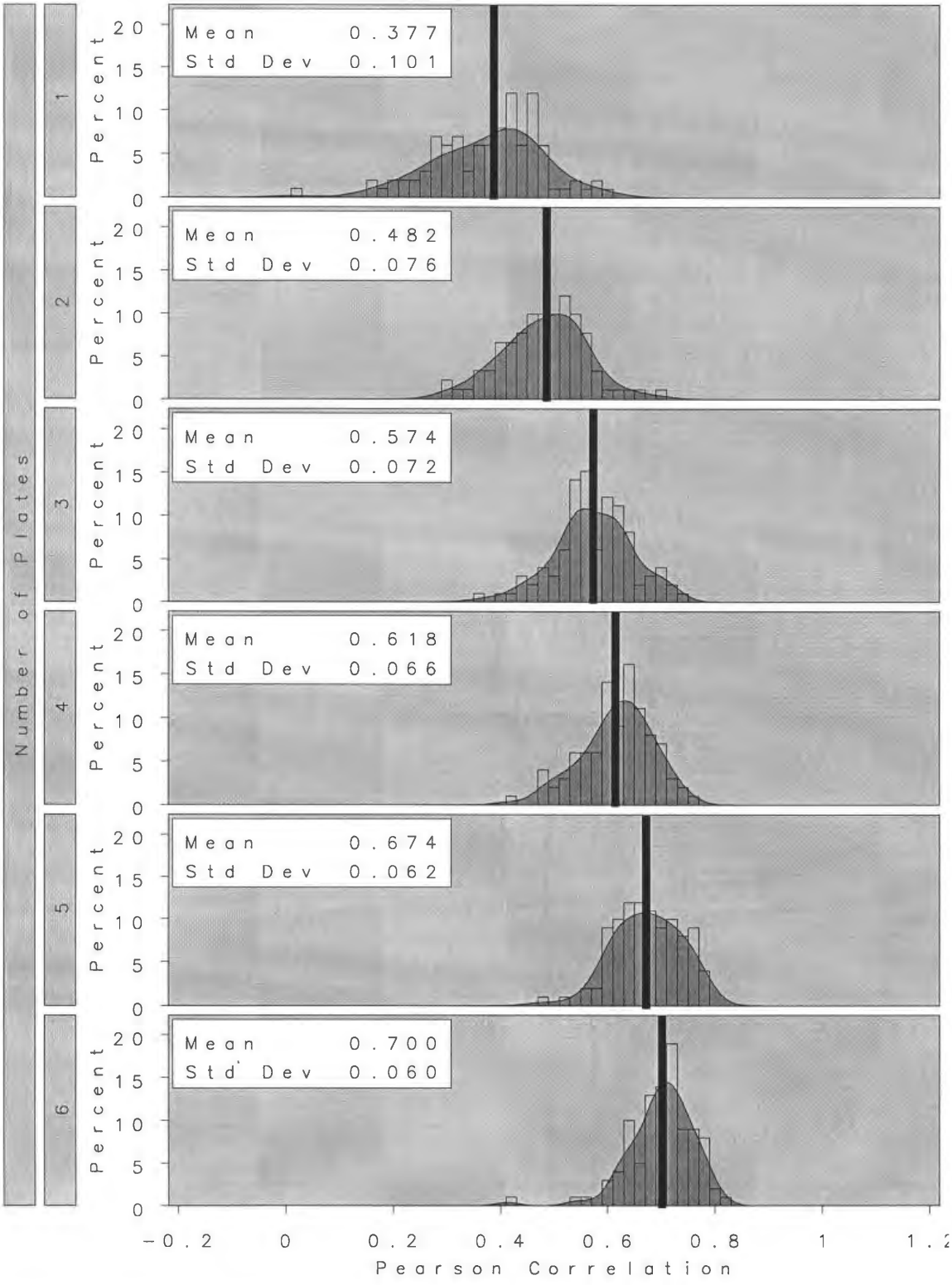

Figure 4. Histogram of Pearson correlations for varying number of plates in the lysine assay, using parameter set 1.

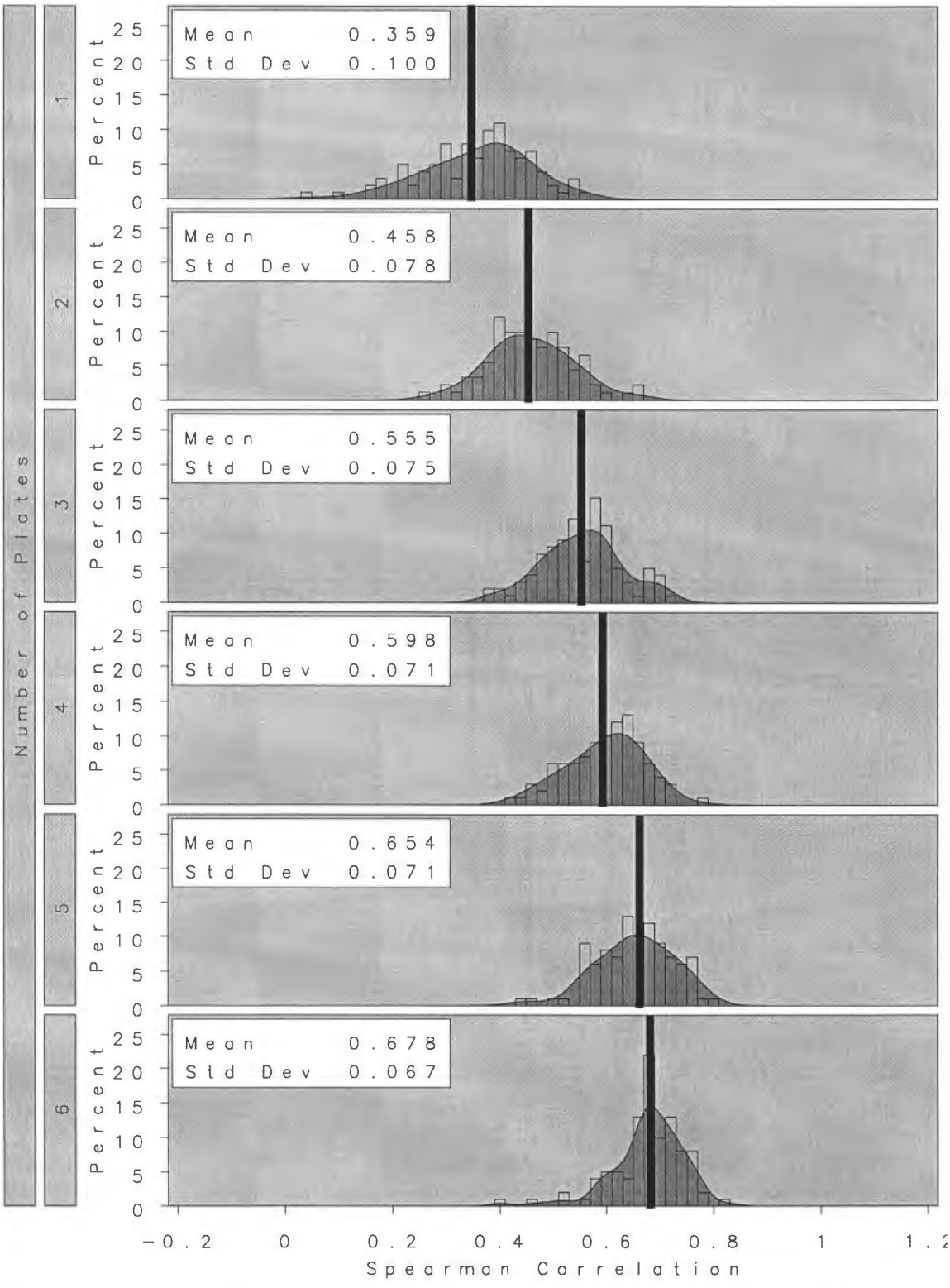

Figure 5. Histograms of Spearman correlations for varying number of plates in the lysine assay, using parameter set 1.

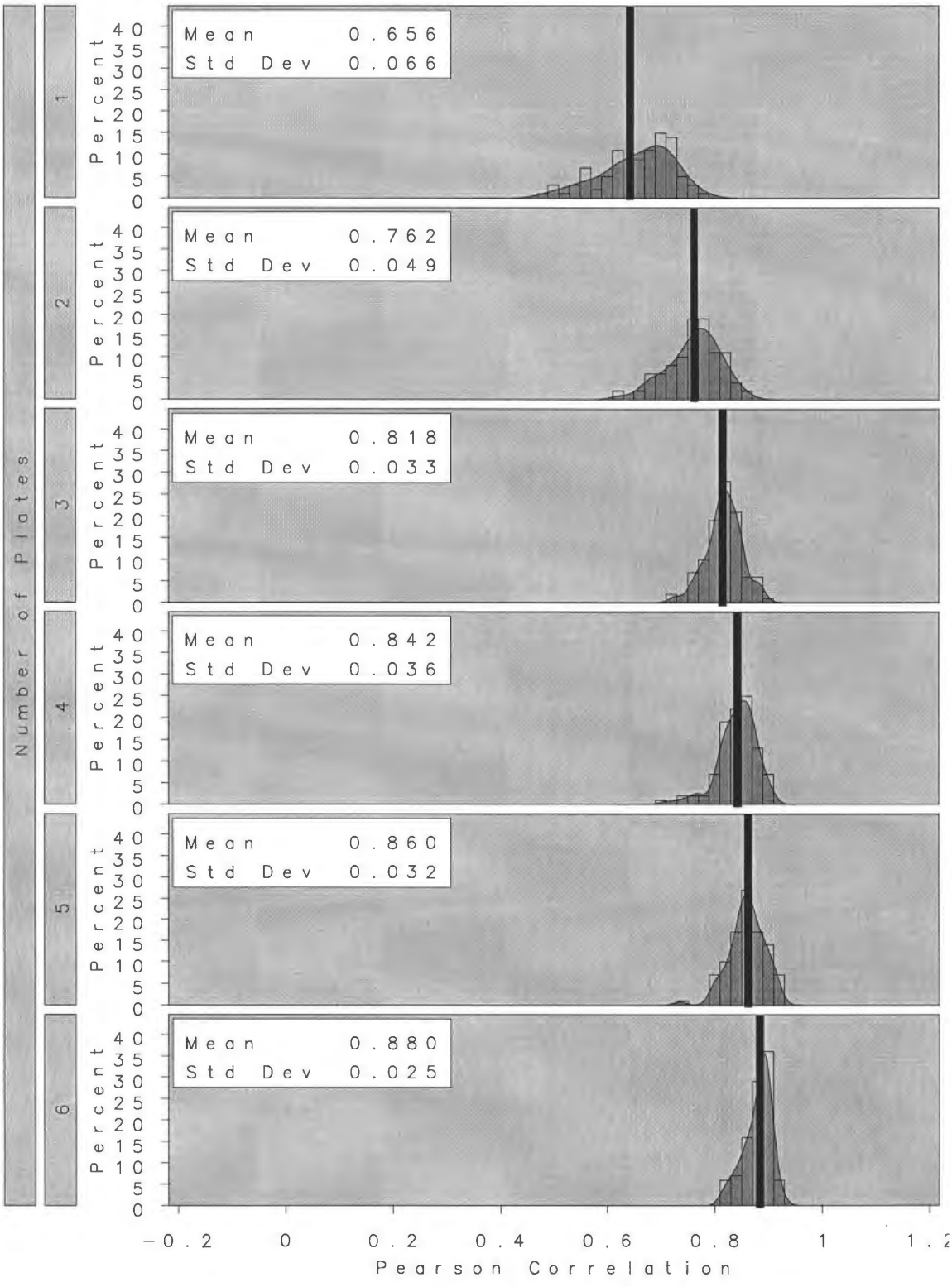

Figure 6. Histogram of Pearson correlations for varying number of plates in the methionine assay, using parameter set 1.

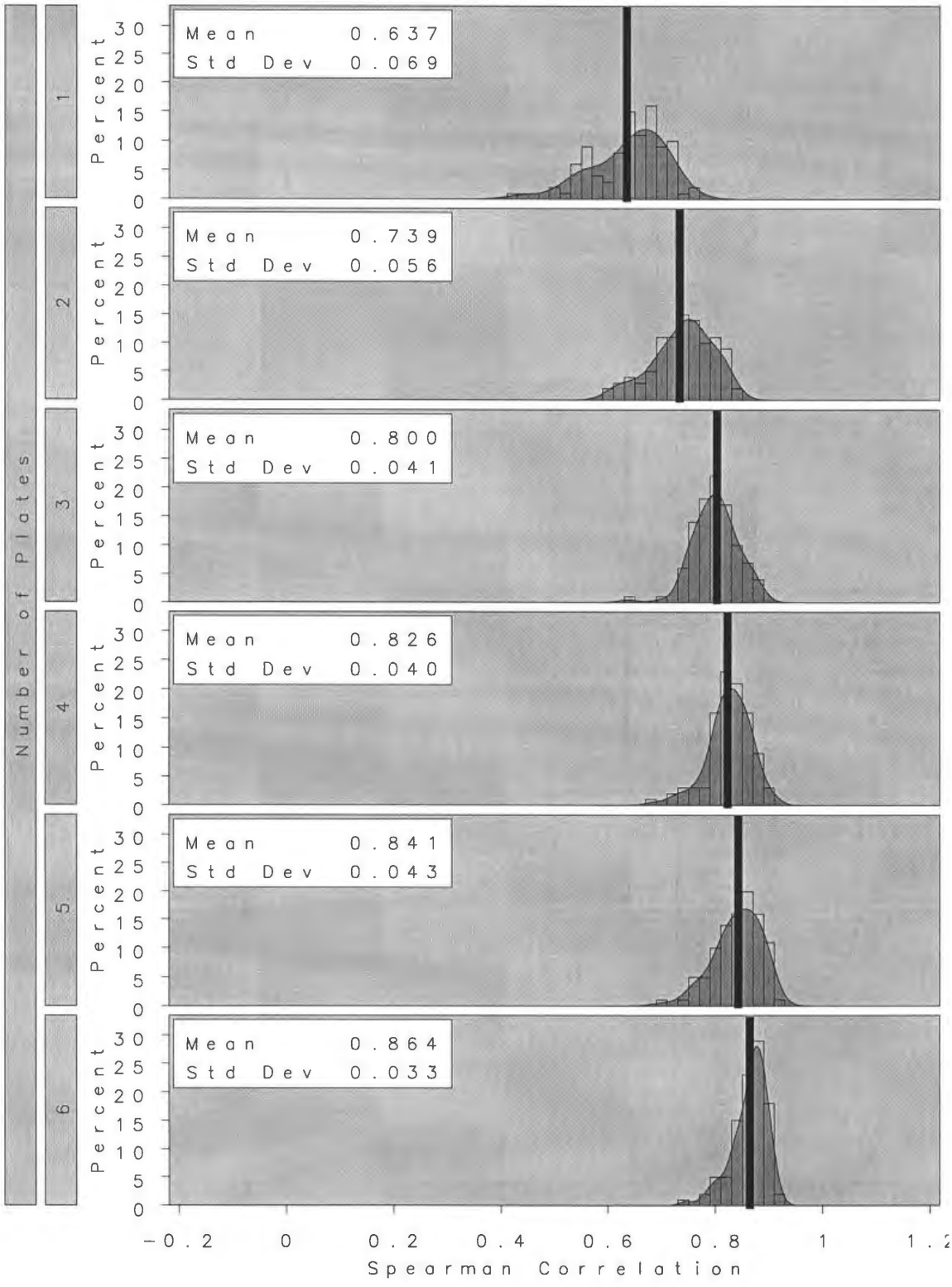

Figure 7. Histogram of Spearman correlations for varying number of plates in the methionine assay, using parameter set 1.

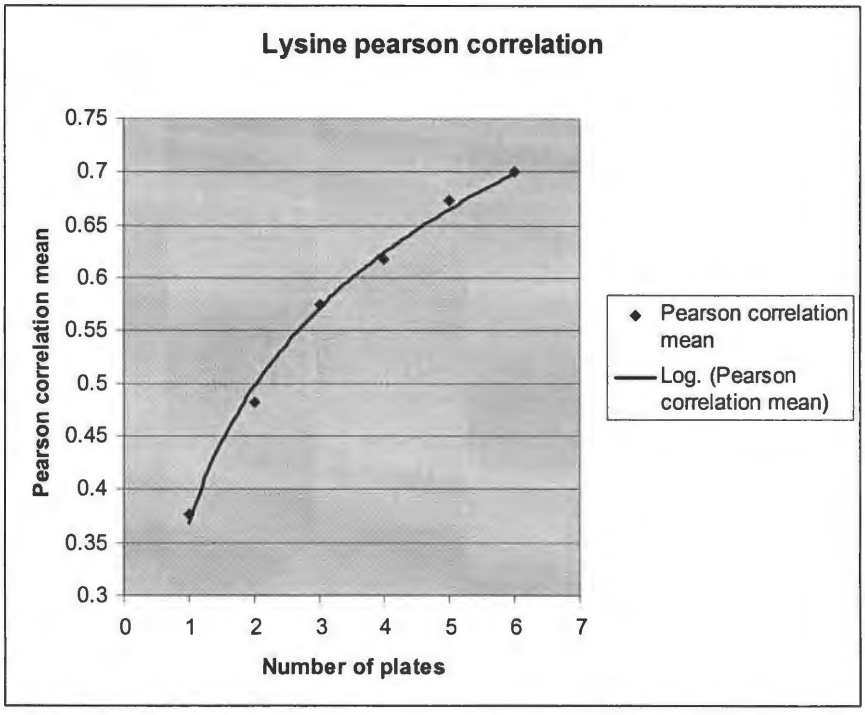

Figure 8. Regression of Pearson correlation means on number of plates for the lysine assay, using parameter set 1.

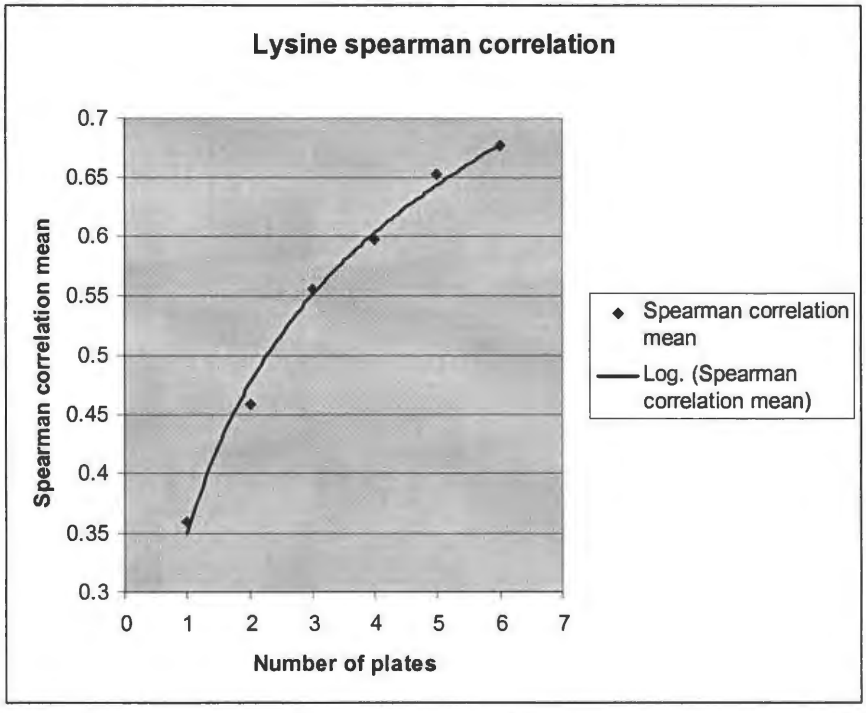

Figure 9. Regression of Spearman correlation means on number of plates for the lysine assay, using parameter set 1.

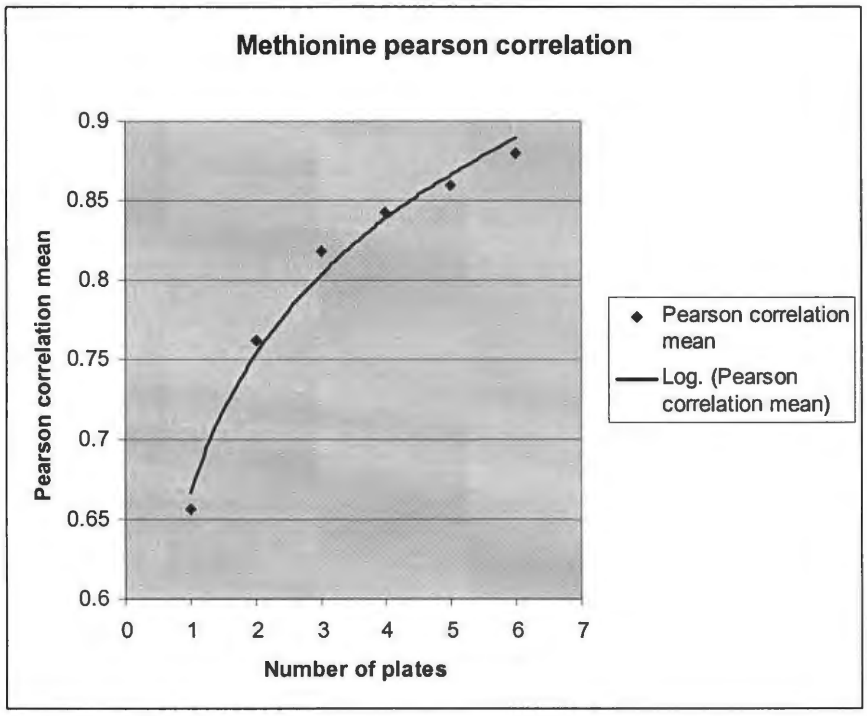

Figure 10. Regression of Pearson correlation means on number of plates for the methionine assay, using parameter set 1.

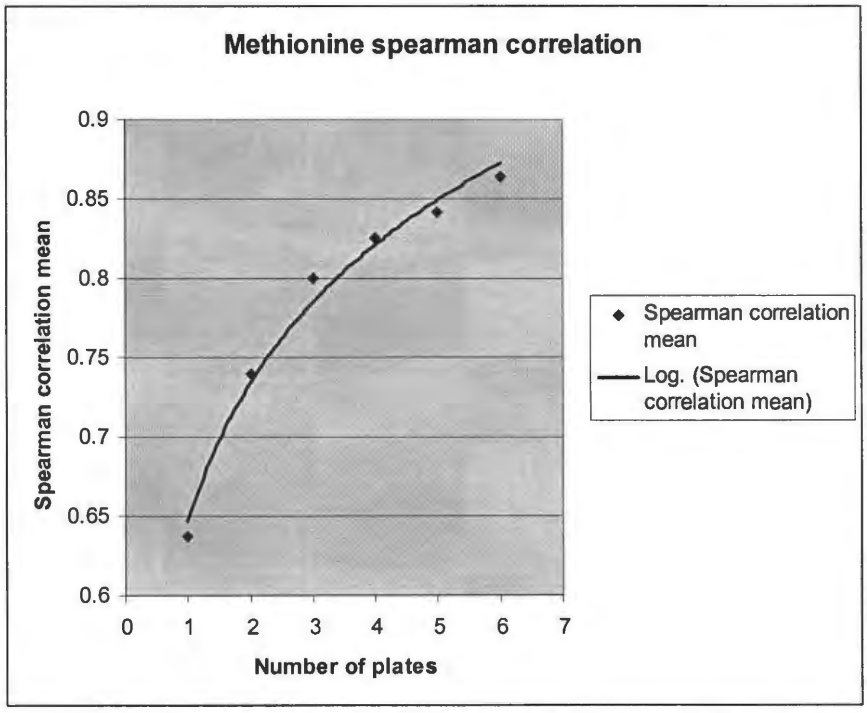

Figure 11. Regression of Spearman correlation means on number of plates for the methionine assay, using parameter set 1.

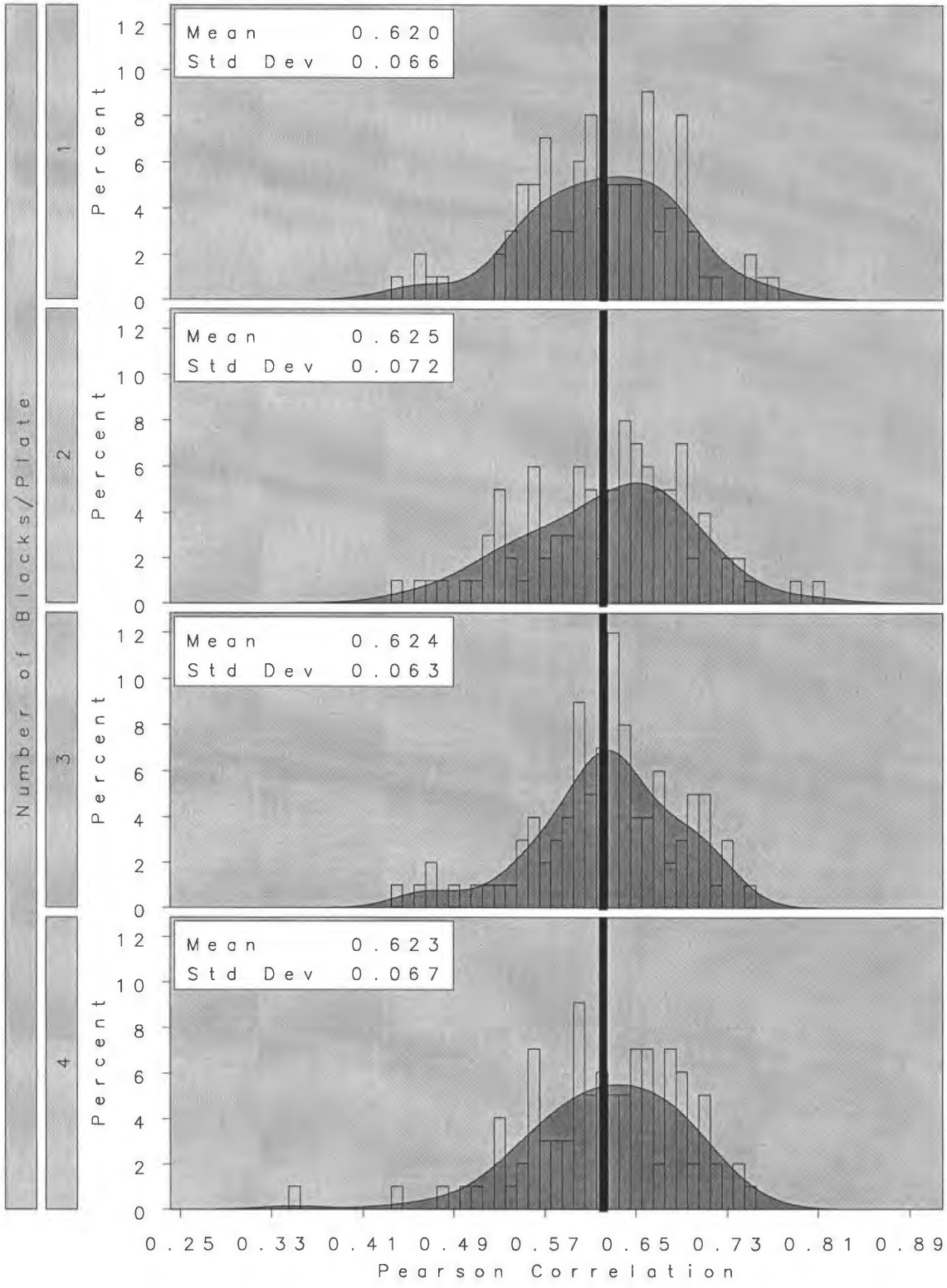

Figure 12. Histograms of Pearson correlations for varying number of blocks per plate in the lysine assay, using parameter set 1.

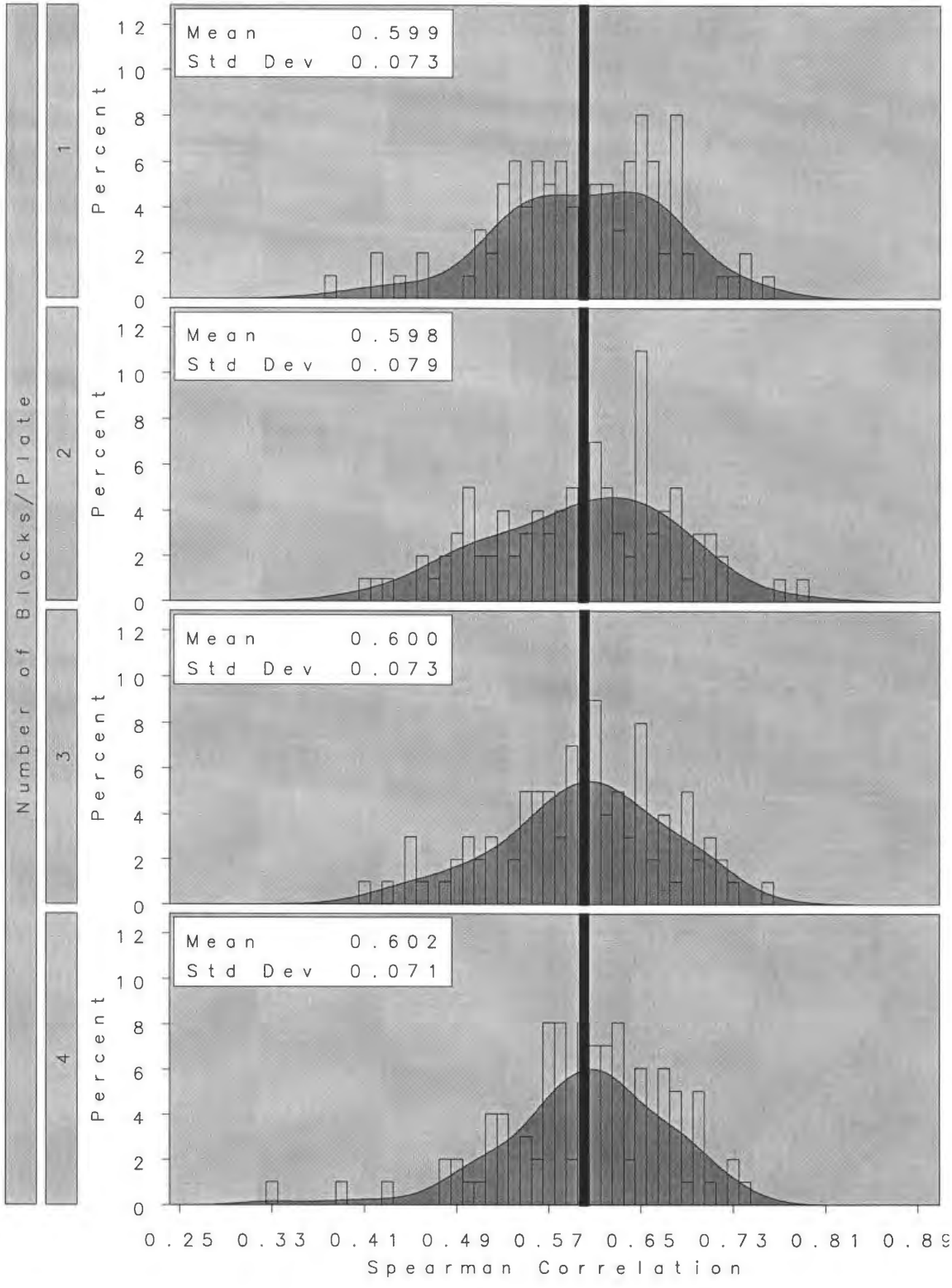

Figure 13. Histograms of Spearman correlations for varying number of blocks per plate in the lysine assay, using parameter set 1.

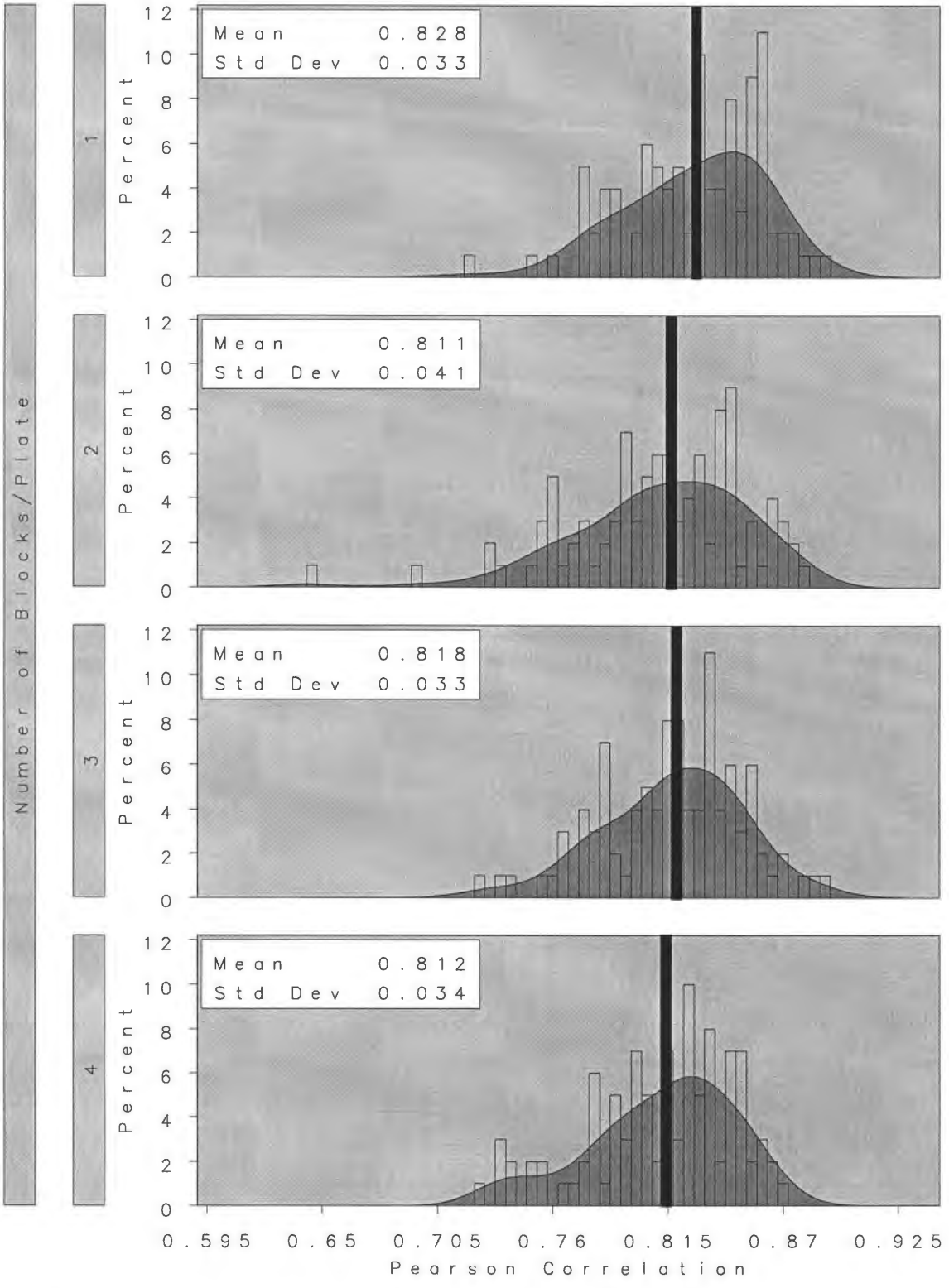

Figure 14. Histograms of Pearson correlations for varying number of blocks per plate in methionine assay, using parameter set 1.

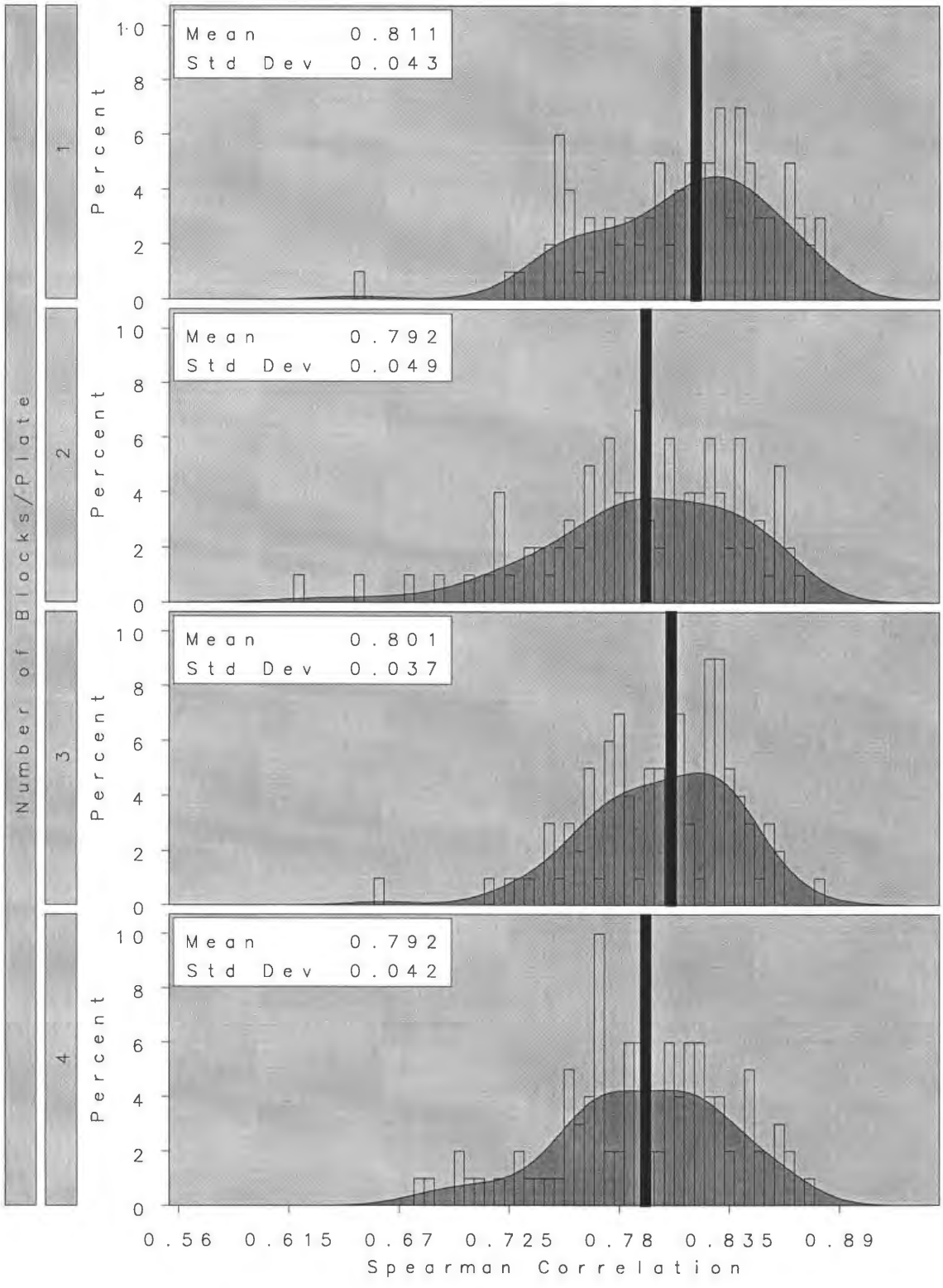

Figure 15. Histogram of Spearman correlations for varying number of blocks per plate in the methionine assay, using parameter set 1.

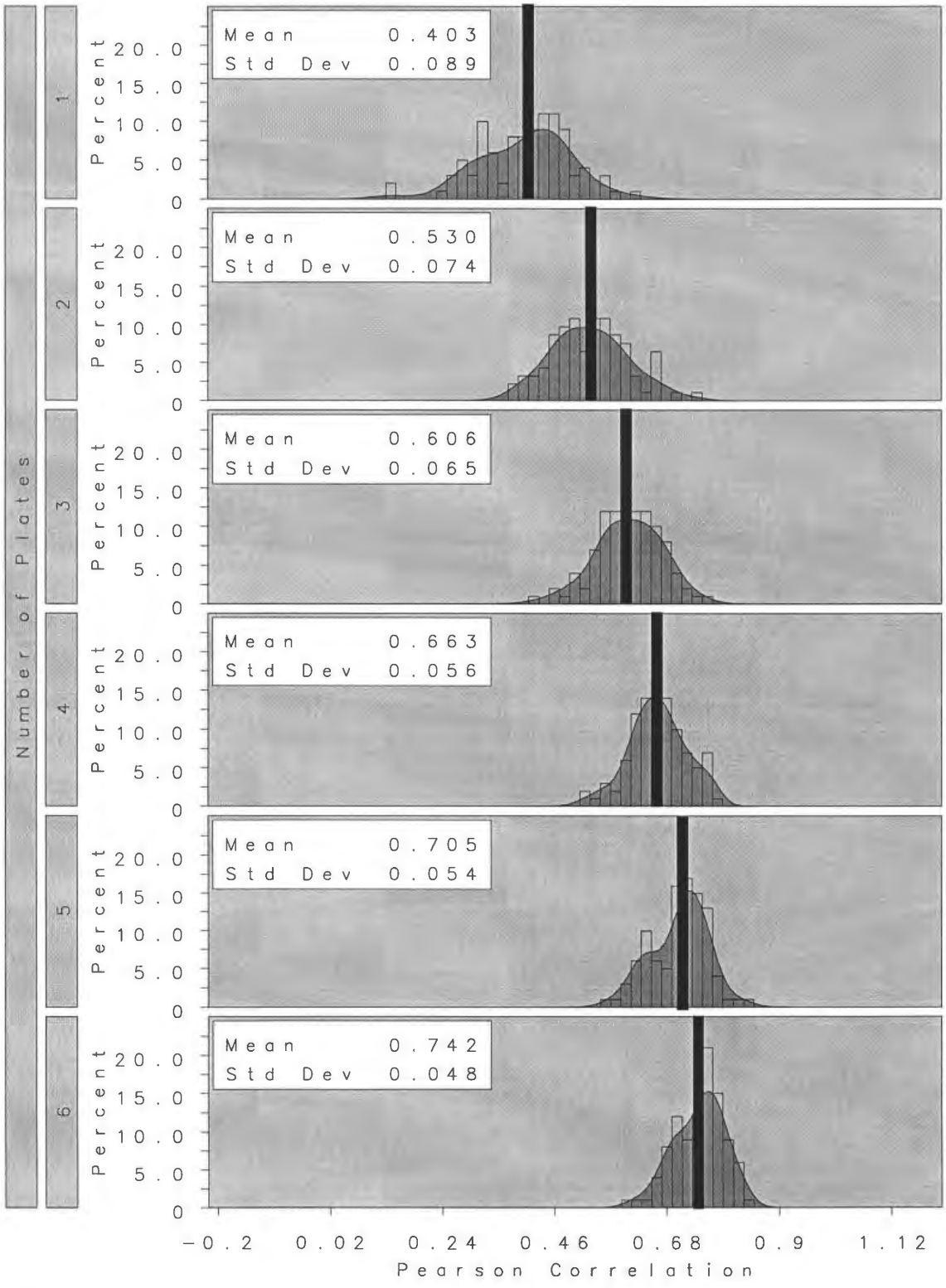

Figure 16. Histogram of Pearson correlations for varying number of plates in the lysine assay, using parameter set 2.

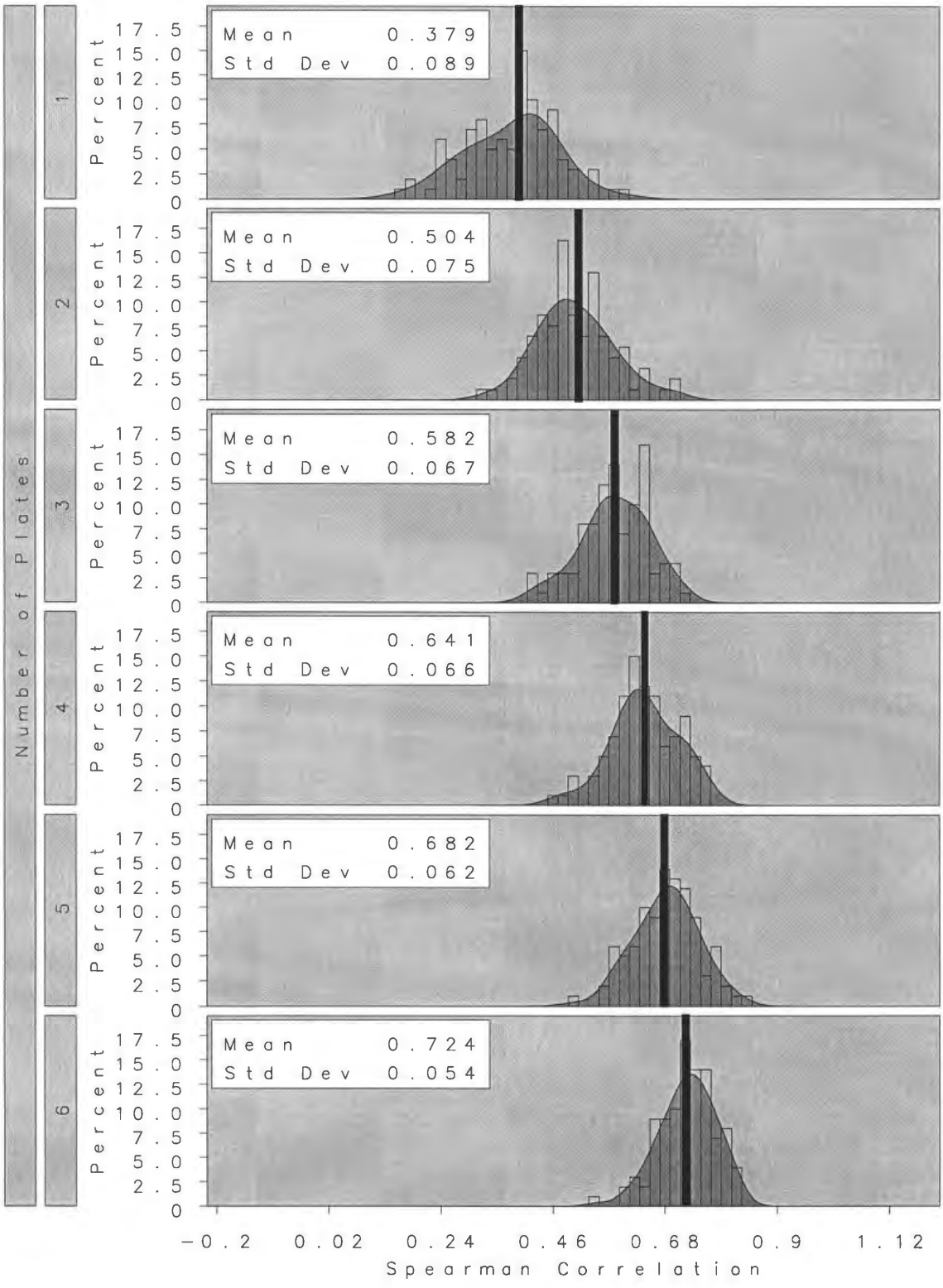

Figure 17. Histogram of Spearman correlations for varying number of plates in the lysine assay, using parameter set 2.

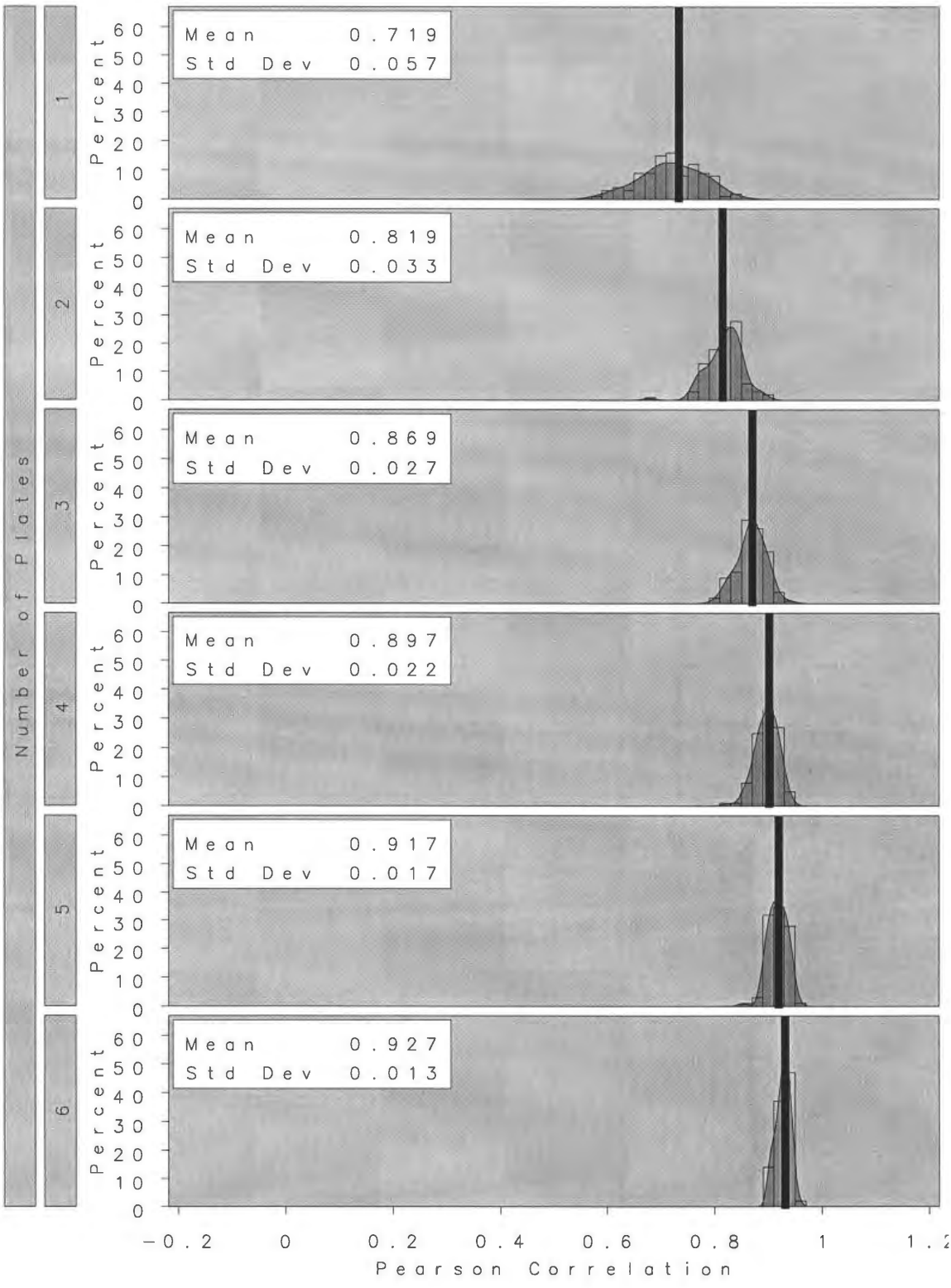

Figure 18. Histogram of Pearson correlations for varying number of plates in the methionine assay, using parameter set 2.

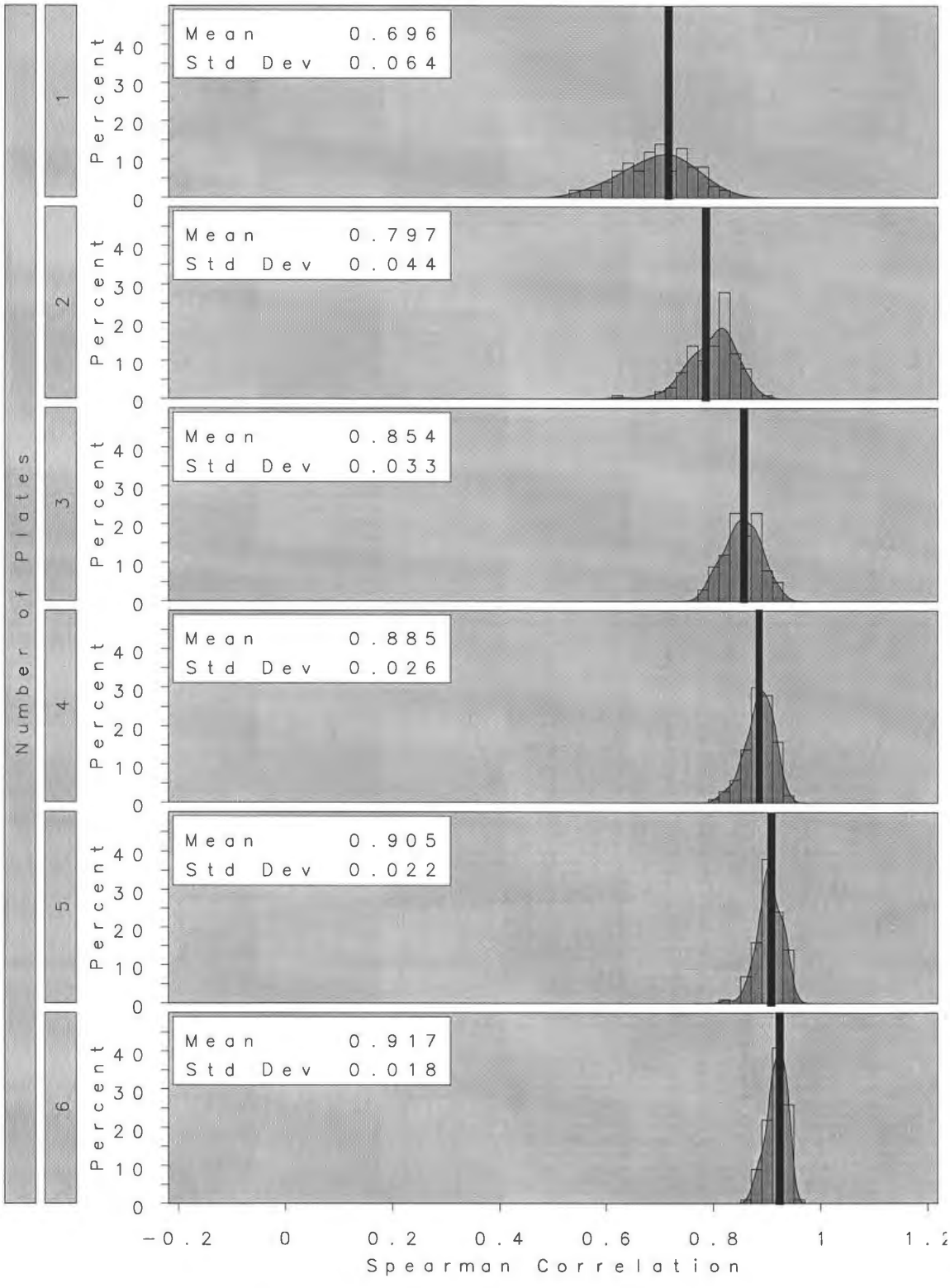

Figure 19. Histogram of Spearman correlations for varying number of plates in the methionine assay, using parameter set 2.

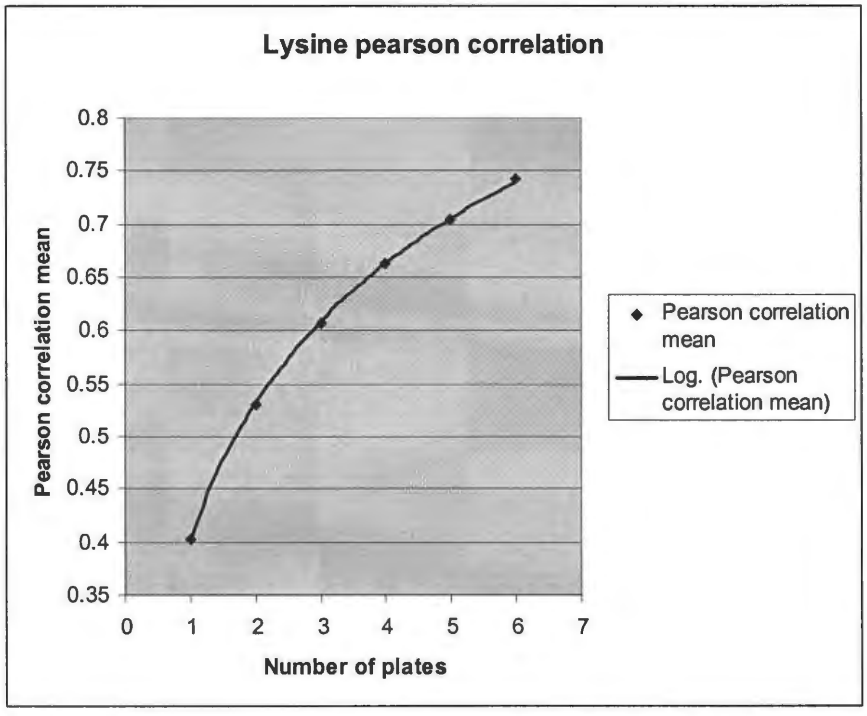

Figure 20. Regression of Pearson correlation means on number of plates for the lysine assay, using parameter set 2.

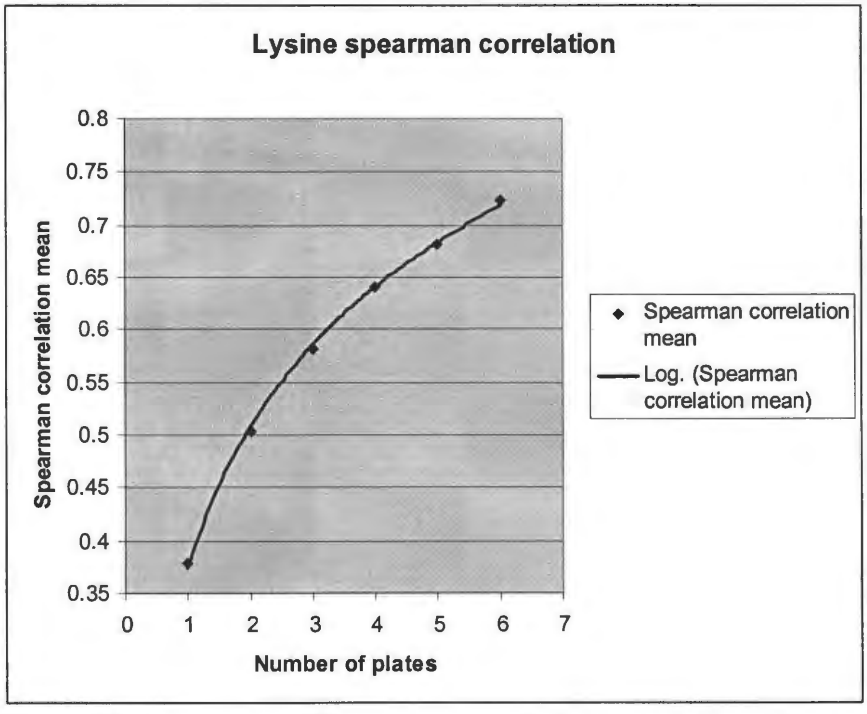

Figure 21. Regression of Spearman correlation means on number of plates for the lysine assay, using parameter set 2.

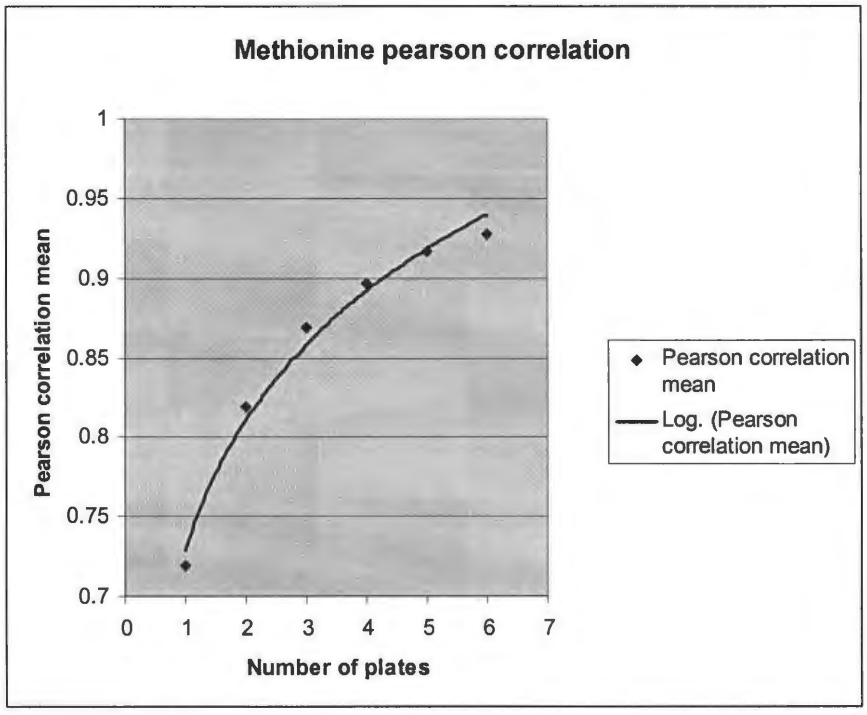

Figure 22. Regression of Pearson correlation means on number of plates for the methionine assay, using parameter set 2.

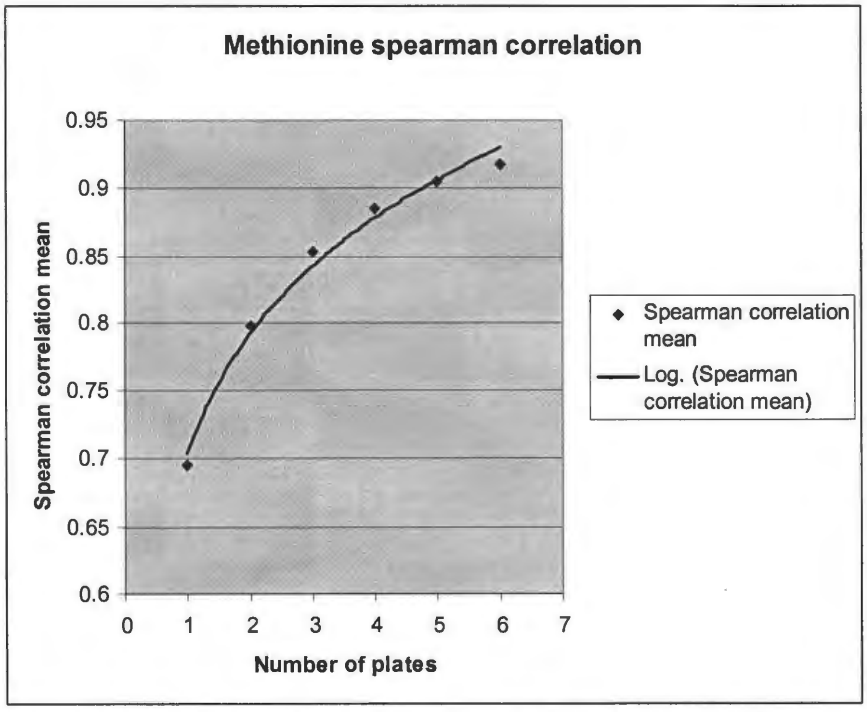

Figure 23. Regression of Spearman correlation means on number of plates for the methionine assay, using parameter set 2.

GENERAL DISCUSSION

General Discussion

Maize is extensively used as feed for animals; however, it is nutritionally limiting in lysine and methionine. Its nutritional qualities could be improved to reduce the dependence on amino acid supplements. Plant breeders have been able to improve the quality of maize by working with traditional recurrent selection programs, mutants, and biotechnological methods. All of these avenues have proven to be successful in altering the quality of maize protein. Regardless of the method employed to increase the limiting amino acids, an inexpensive, high throughput, reliable assay is required.

In this study, a microbial assay was reviewed and found to be reliable for amino acid measurement. The repeatability of this assay was extremely high, with most of the repeatability values being above 90%. The assay was sensitive enough to detect genotypic differences between the entries. Only one replication of each field sample was necessary. In a simulation program, three plates is the practical number to replicate entries over in this assay. The block, grind, and plate by grind interaction effects were all determined to not be a significant source of variation in this assay. Any plant breeder can take comfort in this assay that the entries submitted would be consistently analyzed.

Future Work

Future work should include a study to determine the optimum number of kernels to use per grind. This would prevent the needless grinding of seed in the case where seed is limiting. To ease the tedious labor of weighing samples into the individual wells, a study could test a larger range of sample weight values to find the critical limit where the mass does influence the OD readings. Also, to improve the creditability of this assay, one would need to send sample to be analyzed using the AOAC International methods at the University of Missouri-Columbia testing center and compare them to the results obtained using this microbial assay. Finally, correlations with NIR data could be made which, pending the level of correlation, could provide another means for screening amino acid content.

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I wish to express my gratitude and appreciation to the professors of my committee program. Dr. Lamkey, I would like to thank you for your patience and understanding throughout my duration here, and for your statistical explanations. Dr. Scott, thank you for your guidance and patience not only in the lab, but in writing this manuscript as well. Dr. Nettleton, thank you for your help in the design of this experiment and the creation of the simulation program. All of you have put in a tremendous amount of time and energy on this project; thank you.

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