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Reliable and Inexpensive Colorimetric Method for Determining Protein-Bound Tryptophan in Maize Kernels

Eric Nurit,[†] Axel Tiessen,[‡] Kevin V. Pixley,[†] and Natalia Palacios-Rojas*,[†]

[†]Global Maize Program, International Maize and Wheat Improvement Center (CIMMYT), Km. 45 via Mexico-Veracruz, Texcoco CP56120, Edo de México, México, and [‡]Departamento de Ingeniería Genética, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV), Irapuato CP 36821, México

Biofortification programs in maize have led to the development of quality protein maize (QPM) with increased contents of the essential amino acids lysine and tryptophan, and increased nutritional value for protein deficient populations where maize is a staple food. Because multiple genetic systems control and modify the protein quality of QPM, tryptophan or lysine monitoring is required to maximize genetic gain in breeding programs. The objective of this work was to develop an accurate, reliable, and inexpensive method for tryptophan analysis in whole-grain maize flour to support QPM research efforts around the world. Tryptophan reacts with glyoxylic acid in the presence of sulfuric acid and ferric chloride, producing a colored compound that absorbs at 560 nm. A series of experiments varying the reagent concentrations, hydrolysis time, and length of the colorimetric reaction resulted in an optimized protocol which uses 0.1 M glyoxylic acid in 7 N sulfuric acid and 1.8 mM ferric chloride, and 30 min reaction time. This method produced stable and reproducible results for tryptophan concentration in whole-grain maize flour and was validated by comparison with data obtained using an acetic acid-based colorimetric procedure (r^2 = 0.80) and high pressure liquid chromatography (HPLC) (r^2 = 0.71). We describe adaptations that permit high throughput application of this tryptophan analysis method using a microplate platform.

KEYWORDS: Biofortification; quality protein maize; tryptophan

INTRODUCTION

Quality protein maize (QPM) is the product of 40 years of research, which began mainly in USA, Mexico, and South Africa but recently includes scientists in countries around the globe (*1*). QPM is similar to all other maize, but it contains one naturally occurring mutant gene, the *opaque-2* (*o2*) gene, which results in increased concentrations in grain of the essential amino acids lysine and tryptophan (*2*,*3*). Thus, the nutritional value of QPM is superior to that of non-QPM because it contains about double the amounts of lysine and tryptophan, which are the most limiting amino acids in the protein of maize. It is commonly cited that the biological nutritional value of QPM protein is about 90%, whereas that of non-QPM is about 40% of that of the protein in milk (*4*). Several studies with children and adults have demonstrated nutritional benefits from eating QPM instead of non-QPM (*1*, *5*, *6*), and a recent meta-analysis of nine community-based studies indicated that consumption of QPM instead of non-QPM led to a 9% (95% confidence interval (CI): 6−15%) increase in rate of growth in height and a 12% (95% CI: 7−18%) increase in rate of growth in weight for young children with mild to moderate undernutrition from populations in which maize is a significant part of the diet (*7*). In addition to its value as human food, interest in QPM for animal feed, particularly for poultry and swine, is driven by the possibilities of cost savings in commercial production (by partially substituting other protein sources with QPM) and of enhancing productivity of small scale or household production that does not rely on purchased balanced feed rations (*4*).

QPM is the product of conventional breeding and does not involve transgenic or genetic engineering events (*8*). Breeding of QPM varieties requires manipulation of three genetic systems (*1*, *8*): First, the *o2* gene must be in its homozygous recessive form, thereby reducing the rate of transcription of genes encoding zein proteins, which contain very small quantities of lysine and tryptophan; second, modifier genes of the *o2* gene must be selected, to modify the undesirable soft and chalky (opaque) kernel features that are typical of *opaque-2* maize; and third, additional (non-*o2*) genes affecting lysine and tryptophan concentration in grain must be selected to ensure that concentrations of these amino acids are within the high end of the range of variation observed for maize. Molecular marker assisted selection is currently only possible for the first of these three genetic systems (*9*), and phenotypic selection is therefore essential to visually select genotypes with modified endosperm and to quantify and select for tryptophan concentration using laboratory assays (*1*, *3*). Tryptophan concentration in normal maize grains commonly ranges from 0.2 to 0.5% of the total protein content, in *o2* maize it typically varies from 0.5 to 1.1 (average 0.8%), whereas CIMMYT (the International Maize and Wheat

*To whom correspondence should be addressed. Phone: +52 (55) 4804 2004. Fax: +52 (55) 5804 7558. E-mail: n.palacios@cgiar.org.

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Improvement Center) defines QPM as containing >0.8% (*1*, *10*). Determination of tryptophan concentration is a necessary step to develop QPM, and every QPM breeding program must have access to a laboratory equipped to do tryptophan analysis.

A colorimetric method based on acetic acid (*11*) has been used to analyze tryptophan concentration in maize grains for more than 30 years at CIMMYT and several institutes with QPM breeding programs around the world (*10*). Unfortunately, the colorimetric method based on acetic acid is sometimes unreliable because the reaction varies with different batches of acetic acid; consequently, poor reproducibility of tryptophan data among different laboratories has been found (Dr. Miloje Denic, consultant of SG 2000, Mozambique, 2008, personal communication). Alternative semiquantitative methods using tryptophan mutant fungi or bacteria (*12*) have also been developed, but they are laborious and limited by microorganism growth rate. An HPLC method can be used (*13*), but basic protein hydrolysis under vacuum and at high temperatures is required prior to *o*-phthaldialdehyde (OPA) derivation, making the procedure time-consuming and too expensive to support a large scale breeding program.

Our objective was to develop and validate a rapid, inexpensive, and reliable laboratory assay for tryptophan concentration in maize grain. Such an assay is essential to facilitate QPM breeding, during which thousands of samples must be analyzed each year in laboratories worldwide. We revised the acetic acid method of Villegas et al. (*11*), conducted a series of studies evaluating the effect of changing reagent concentrations, hydrolysis time, and length of the colorimetric reaction, and describe herein the optimized, validated, and improved protocol for tryptophan analysis in maize grain.

MATERIALS AND METHODS

Chemicals and Reagents. Glyoxylic acid (G10601), ferric chloride (F2877), and papain (P3375) were obtained from Sigma/Aldrich (St. Louis, MO, USA), sodium acetate (3460) was from J.T. Baker (Phillipsburg, NJ, USA), and DL-tryptophan (1.08375.0025) and sulfuric acid (1.00731.2500) were from Merk (Whitehouse Station, NJ, USA). Papain solution (4 mg/mL) was prepared daily using 0.165 M sodium acetate at room temperature to ensure complete solubility. DL-Tryptophan was used as a standard and prepared in 0.1 M sodium acetate at pH 7. A stock of 30 N sulfuric acid was prepared by slowly adding 833.3 mL of sulfuric acid (96%) to 166.7 mL of distilled water in an Erlenmeyer flask on ice and under continuous stirring with a magnetic stirrer. After cooling to room temperature, distilled water was gradually added to complete the final volume of 1000 mL in a volumetric flask.

Four reagents were prepared for use in the tryptophan assay:

- Reagent A (0.1 M glyoxylic acid) was prepared daily in 7 N sulfuric acid. The 7 N sulfuric acid was also prepared daily, by mixing 35 mL of 30 N sulfuric acid with 115 mL of distilled water and adding additional distilled water, if necessary, to complete 150 mL volume.
- Reagent B was prepared daily and consisted of 1.8 mM $FeCl_3$-$6H_20$.
- Reagent C was 30 N sulfuric acid.
- Reagent D was prepared daily (1 h before the next step of the analysis) in a brown-glass bottle by mixing reagents B and C in equal proportions.

Maize (*Zea mays*) Samples. One normal (CML312) and one QPM (CML160) CIMMYT maize line (CML) were used as controls in all stages of the method development and validation. One hundred samples of maize grain, comprising 50 QPM and 50 normal maize genotypes from the tropical QPM breeding program at CIMMYT were used for cross-validation of the method. Whole-grain samples were used for all analyses (colorimetric assays and HPLC), and two technical replicates were performed for the colorimetric methods.

Extraction Procedure. Twenty to 30 seeds were milled for each maize sample using a cyclotec mill (manufactured by Tecator, Hoeganaes) with a 0.5 mm sieve. The milled flour was then defatted with hexane in a Soxhlet-type continuous extractor for 6 h. After hexane evaporation, 80 mg of powder was digested using 3 mL of 4 mg/mL papain. A blank with only papain solution was included as a control. The tubes were incubated at 65 °C for 16 h (shaken at least twice in the first hour of incubation), allowed to cool to room temperature, and centrifuged at 3600*g* for 10 min, ensuring a very clear supernatant.

Table 1. Optical Density (OD) at 560 nm Values of the Tryptophan Standard Curve[a]

tryptophan concentration (μg/mL)	OD readings at 560 nm
0	0
10	0.070−0.090
15	0.130−0.150
20	0.175−0.205
25	0.230−0.260
30	0.295−0.325

[a] Values were determined by comparing the results obtained in control samples (normal and QPM) and using different factor curves. Values of r^2 for the standard curve vary between 0.97 and 0.99.

Colorimetric Reaction. One milliliter of the hydrolysate (supernatant) was carefully transferred to a clean tube, and 3 mL of reagent D (colorimetric reagent) was added. Samples were thoroughly stirred (vortexed) and then incubated for 30 min at 65 °C. The samples were allowed to cool to room temperature before reading their optical density (OD) at 560 nm in a Beckman DU-6 UV−visible spectrophotometer.

Standard Curve. To determine the sensitivity of the reaction, we did one calibration curve ranging from 0.5 to 30 μg of tryptophan per mL. On the basis of the expected ranges of tryptophan in the maize kernels, the subsequent calibration curves were done from 10 to 30 μg of tryptophan per mL. A stock solution of 100 μg/mL of DL-tryptophan was prepared weekly in 0.165 M sodium acetate at pH 7 and stored at 4 °C. The solution was diluted in the same sodium acetate to 0, 10, 15, 20, 25, and 30 μg of tryptophan per mL to develop a standard curve. Colorimetric reaction of the standard curve was evaluated ensuring that the OD values at 560 nm were in the ranges shown in **Table 1**.

Percentage Recovery Experiments. Maize samples or the BSA sample spiked with 0, 5, 7.5, and 15 μg of tryptophan were extracted, and tryptophan was determined according to the glyoxylic acid method described herein.

Validation of the Glyoxylic Acid Method for Tryptophan Analysis. *Accuracy of the Method.* Tryptophan concentration of the 100 maize samples was also analyzed in our laboratory (CIMMYT, Mexico) by the acetic acid colorimetric method described by Villegas et al. (*11*) and by an independent laboratory using HPLC as described by Huang et al. (*13*).

Technical Reproducibility. Precision of our glyoxylic acid method and of the acetic acid method was assessed by analyzing and calculating the correlation coefficient between two technical replicates of 100 samples.

Sensitivity of the Method. The limit of detection (LOD), defined as three times the standard deviation of the blank ($p < 0.01$), was calculated from 10 repeated measurements of the papain blank. The LOD value was 0.018. Despite this theoretical LOD value, our practical experience is that more than 5 μg of tryptophan per mL can be detected reliably with the present method.

Calculations and Statistical Analysis. Percentage of tryptophan was obtained by multiplying the corrected OD at 560 nm ($OD_{560\ nm\ sample} - OD_{560\ nm\ average\ of\ papain\ blanks}$) by the factor (hydrolysate volume/(standard curve slope x sample weight). Percentage of recovery was calculated by dividing the micrograms of tryptophan measured during sample analysis by micrograms of tryptophan added to each sample, and multiplying by 100. The tryptophan analysis methods were compared using one factor analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Lysine and tryptophan concentration in maize kernels of agronomically advanced QPM lines are highly correlated (**Figure 1**). A 3:1 ratio of lysine to tryptophan has been reported

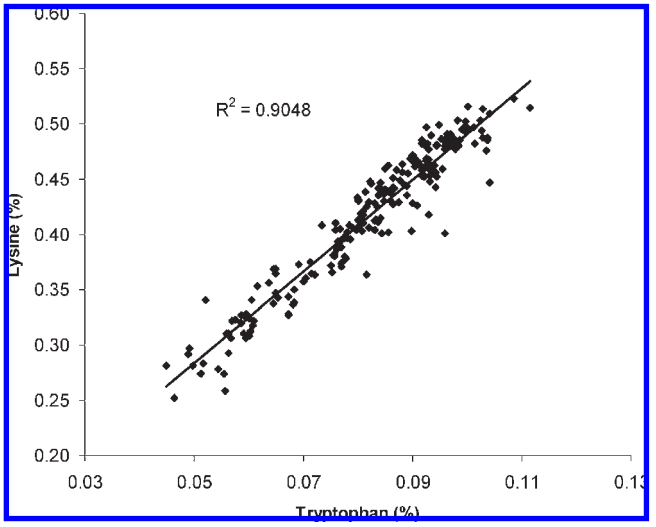

Figure 1. Correlation between percent tryptophan and percent lysine in whole grain of maize (n = 284 advanced QPM samples).

in normal and quality protein maize (*3*, *10*). Therefore, in a QPM breeding program only one of these amino acids is typically monitored, tryptophan being the more commonly measured of the two. At CIMMYT and several other laboratories, the colorimetric method based on acetic acid (*11*) has traditionally been used for this purpose, but the results obtained by this method are dependent on the source of acetic acid used (*10*), with some batches of acetic acid producing a good colorimetric reaction and others failing to produce a useful reaction (data not shown). This variability made it necessary to test each new batch of acetic acid to ascertain whether it was suitable for use in routine tryptophan analyses (*10*). Since this can be a cumbersome procedure, particularly in countries where it is difficult to obtain acetic acid and other reagents, we began experimenting to modify the method to make it independent from the batch, source, or supplier of acetic acid. The variability of the colorimetric reaction due to the batch of acetic acid suggested that an impurity present in the acetic acid solution could be the cause of the inconsistent results. The molecular formulas of some possible impurities of acetic acid, particularly some C2 carbon compounds that can react with the amine or imine groups of tryptophan, are shown in **Figure 2**. Hopkins and Cole (*14*) suggested that one molecule of glyoxylic acid can react with two molecules of tryptophan giving a violet color that absorbs at 560 nm. We therefore conducted some experiments in which we replaced acetic acid with glyoxylic acid and varied the concentrations of sulphuric acid and ferric chloride.

Effect of Glyoxylic Acid. We tested six concentrations of glyoxilic acid (0.01 to 0.1M) using 15 and 30 mg/L of tryptophan as standard and keeping the concentrations of sulfuric acid and ferric chloride constant. As can be seen in **Figure 3**, the reaction was very weak at low concentrations of glyoxilic acid, reached its maximum at 0.025M, and remained constant thereafter until 0.1M. Using 0.1 M glyoxilic acid produced more reproducible results for maize samples than the use of lower concentrations (data not shown); therefore, we recommend this concentration.

Our results are in agreement with observations by Hopkins and Cole (*14*), who suggested that the acetic acid method (*11*) relies on the amount of glyoxilic acid present as an impurity. This observation and our results indicate that using glyoxilic acid for the tryptophan assay is more reliable than the use of acetic acid. Before recommending the use of glyoxylic acid for routine analysis, however, we conducted a series of tests to optimize all

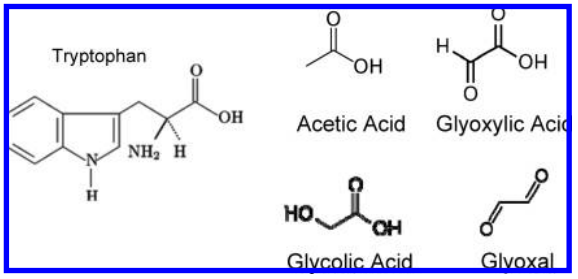

Figure 2. Chemical structure of L-tryptophan and possible impurities present in commercial preparations of acetic acid, such as glycolic acid, glyoxal, and glyoxylic acid.

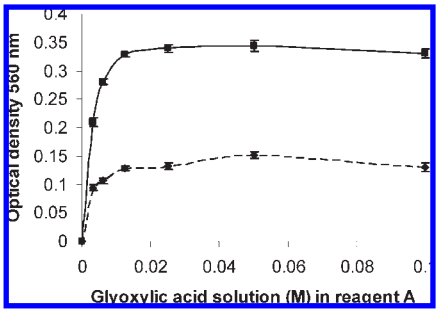

Figure 3. Effect of glyoxylic acid concentration on absorbance values (optical density ± standard error) of tryptophan standards (dashed line = 15 μg/mL, and continuous line = 30 μg/mL).

other assay parameters. We revised the extraction procedures and protocol steps, as well as the concentrations of all other reagents. The following experiments were conducted to check the robustness of the new method.

Effect of Papain Concentration and Incubation Time on Protein Hydrolysis. Protein hydrolysis can be achieved using acid, basic, or enzymatic hydrolysis. However, for tryptophan determination in maize kernels, the acidic hydrolysis leads to very low recoveries because destruction of tryptophan molecules can occur (*15*). Hydrolysis with 4 to 6 M sodium hydroxide leads to better recovery values for tryptophan, but jellification of starch can occur, and it requires a predigestion step, use of vacuum, and high temperatures (*16*−*18*), which can make the procedure more laborious and expensive. Piombo and Lozano (*15*) reported the optimization of basic hydrolysis using barium hydroxide to avoid jellification of starch; however, other compounds such as barium sulfate or barium carbonate can be formed and coprecipitate with tryptophan (*19*). Enzymatic hydrolysis is commonly used when amino acids are determined by colorimetry (*16*, *20*, *21*). Papain is a low cost, easily obtained enzyme that has been used successfully for protein hydrolysis in maize kernel samples (*11*, *21*).

The acetic acid method for tryptophan determination uses 4 mg/mL of papain for protein hydrolysis in the maize samples (*11*). With the objectives of optimizing the protocol and reducing costs of the analysis, we tested lesser amounts of papain (0.5 to 4 mg/mL) and found that the use of 1 mg/mL of papain produced results similar to those with 4 mg/mL of papain (data not shown). A Pearson correlation coefficient of 0.94 was obtained between tryptophan concentration determined for maize grain samples (n = 50) using these two concentrations of papain.

The incubation time for the hydrolysis reaction was evaluated using 4 to 24 h periods. Similar results were obtained when maize samples were hydrolyzed for more than 16 h, but shorter

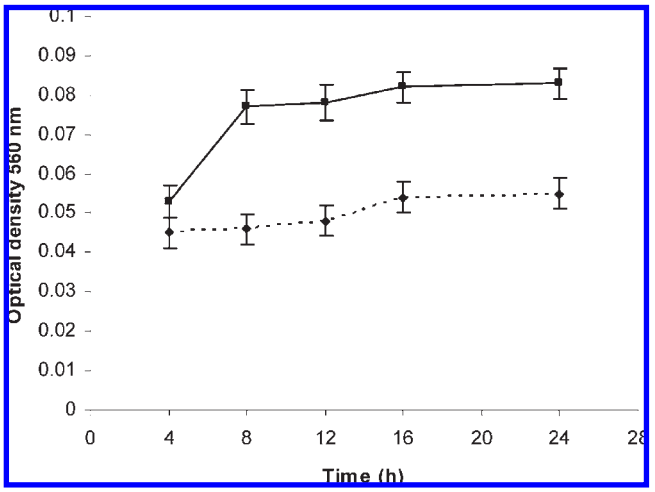

Figure 4. Effect of incubation time on protein hydrolysis (optical density ± standard error) using 1 mg/mL of papain (the dashed line is a normal sample, and the continuous line is a QPM sample).

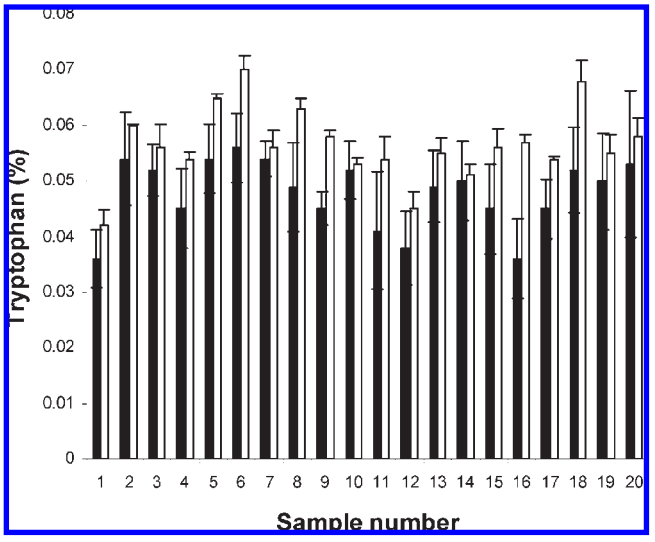

Figure 5. Percent of tryptophan (±standard error) in maize kernels analyzed with and without defatting. Black bars are for samples that were not defatted; white bars are for samples defatted for 6 h with hexane ($n = 4$).

incubation time led to underestimation of the tryptophan amount due to partial protein hydrolysis with 1 mg/mL of papain (**Figure 4**).

Effect of Defatting. Maize kernels typically contain between 3.5 and 7% crude fat, which is mainly located in the germ. The presence of fat can interfere with the quantification of other compounds in the grain. Extraction with hexane using a Soxhlet equipment is a useful process for defatting the flour of ground maize grain samples, but although it is a relatively inexpensive procedure, it is time-consuming. The acetic acid method for tryptophan determination requires defatting of the ground samples (*11*); therefore, we investigated whether time could be saved by avoiding defatting in our glyoxylic acid method. Unfortunately, we obtained variable results when samples were not defatted (**Figure 5**); the mean standard deviation of tryptophan estimates for nondefatted samples were three times larger than that for defatted samples. Variable results were also observed when the defatting process was shorter than 6 h (data not shown). On the basis of these results, we recommend defatting the samples for 6 h using hexane in a Soxhlet apparatus to ensure precision of the method.

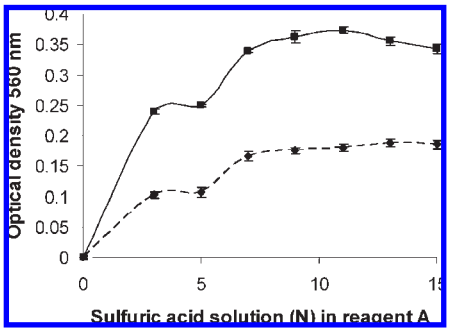

Figure 6. Effect of variation of sulfuric acid concentration on absorbance values (optical density ± standard errors) of tryptophan standards (the dashed line is for 15 μg/mL, and the continuous line is for 30 μg/mL).

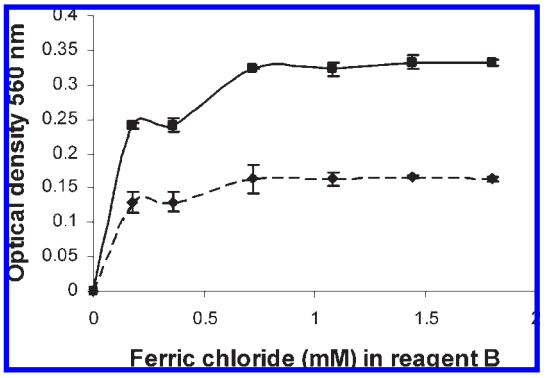

Figure 7. Effect of variation of ferric chloride concentration on absorbance values (optical density ± standard errors) of tryptophan standards (the dashed line is for 15 μg/mL, and the continuous line is for 30 μg/mL).

Effect of Sulfuric Acid. The stability of the reaction was evaluated using 15 and 30 μg/mL tryptophan and four different concentrations of sulfuric acid stock solution. An exothermic reaction occurs when diluting sulfuric acid; therefore, the dilutions and/or titration required to ensure the exact normality must be prepared on ice. Glyoxylic acid and ferric chloride concentrations were kept constant while varying the normality of sulfuric acid. As shown in **Figure 6**, color development stabilized when ≥7 N sulfuric acid was used, but ODs began to decrease for the higher tryptophan concentration (30 μg/mL) when sulfuric acid in the reaction was >10 N. The acetic acid method for tryptophan quantification uses 12 N sulfuric acid (*11*), which our results suggest could lead to underestimating tryptophan concentration for samples with highest tryptophan content. This may explain why the glyoxylic acid method produces higher values of tryptophan than the acetic acid method for some samples.

Effect of Ferric Chloride. Ferric chloride is a highly hygroscopic reagent and sometimes is very difficult to weigh accurately, especially in very humid environments (such as some of the tropical places where maize is grown and laboratories are located to support QPM breeding programs). We therefore tested the effect of different concentrations of ferric chloride on color development. Concentrations of ferric chloride ranging from 0 to 1.8 mM in reagent B were used, while other components of the reaction were kept constant. The experiment was performed using 15 and 30 μg/mL of tryptophan. As shown in **Figure 7**, the OD at 560 nm was stable when >0.72 mM $FeCl_3$ was used in reagent B, and this result was independent of the concentration of tryptophan.

Duration of the Colorimetric Reaction. Using two maize samples, one QPM and one non-QPM, we studied the effect of

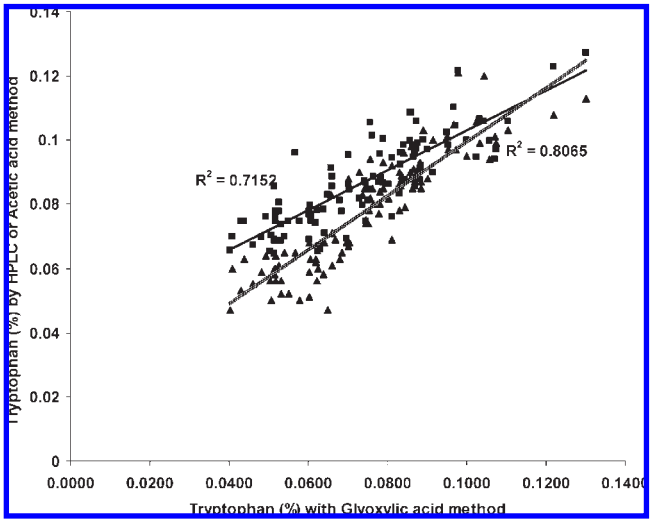

Figure 8. Comparison of percent tryptophan determined by the glyoxylic acid method with percent tryptophan values obtained by HPLC (■, continuous line) and the acetic acid colorimetric method (▲, dashed line).

varying the duration of the colorimetric reaction from 5 to 120 min at 65 °C. The OD at 560 nm did not vary after 30 min of incubation, but less time led to the underestimation of the amount of tryptophan. When the acetic acid method is used, the protocol requires that the colorimetric reaction is done for 15 min to obtain maximum color development (*11*). However, our results indicate that the time required for the reaction might be dependent on the amount of glyoxylic acid present as impurity in the acetic acid, and therefore, we speculate that longer incubation time may be required for certain batches of acetic acid to avoid underestimation of tryptophan.

Specificity of the Reaction. Given the nature of the reaction, it is likely that molecules very similar to tryptophan (such as indole groups with some substitution) could react with glyoxylic acid and confound the results; however, this has not occurred among the range of maize samples that we have analyzed, and we do not consider this an issue for tryptophan determination in maize seeds.

Comparison of Three Tryptophan Analysis Methods. One-hundred samples, 50 QPM and 50 non-QPM, were analyzed using three methodologies: the colorimetric assays based on acetic acid or glyoxylic acid, and HPLC. For the colorimetric assays, proteins were hydrolyzed using papain, but for HPLC, a basic hydrolysis under vacuum and at high temperature was performed (*13*). The coefficient of determination between tryptophan estimates using the colorimetric methods was 0.80; it was 0.75 for data by HPLC and the acetic acid colorimetric method, and 0.71 for HPLC data with our glyoxylic acid colorimetric method (**Figure 8**). Tryptophan values estimated by HPLC were significantly different from estimates obtained by the colorimetric methods (**Table 2**). We noted that the correlation among tryptophan estimates using the different methods was lower for non-QPM than for QPM genotypes, which may have been due to their small tryptophan concentrations (0.04 to 0.07) with relatively larger standard errors, making it difficult to accurately differentiate among values. It is also possible that the smaller tryptophan values were outside the detection limits of the HPLC; the smallest value of tryptophan determined by HPLC for any of the samples was 0.065%, whereas the minimum value was on average 0.043% ± 0.004 for both colorimetric procedures. Sastry and Tumuru (*18*) reported a spectrophotometric method for tryptophan determination in proteins which has high sensitivity (0.2 ppm of tryptophan in protein hydosylates) compared to that of HPLC or the method described here. However, to our knowledge,

Table 2. Comparison of Tryptophan Percentage in 100 Maize Samples Analyzed by HPLC, Acetic Acid, and Glyoxylic Acid Methods

method	*n*	mean	Tukey group[a]
HPLC	100	0.086892	A
colorimetric based on acetic acid	100	0.077710	B
colorimetric based on glyoxylic acid	100	0.074145	B

[a] Different letters indicate significant statistical difference ($p < 0.05$).

values of tryptophan in normal maize are no lower than 0.04%. The method reported by Sastry and Tumuru (*18*), however, was described only for pure proteins, and the cleanup procedures if using maize matrix could make the protocol more expensive and time-consuming than that originally presented by Sastry and Tumuru (*18*). In any case, tryptophan concentration in maize is typically no lower than 0.04%; therefore, additional low-end detection range below that of the glyoxylic method should not be necessary.

We obtained recoveries between 90 and 95% (CV of 3.09%) using the glyoxylic acid method for whole-grain maize flour samples and BSA (bovine serum albumin), which are very similar to the recoveries using the acetic acid colorimetric assay (between 92 and 96%) and demonstrates the high sensitivity of the new method presented here.

The glyoxylic acid method described herein has higher precision ($r^2 = 0.95$ between technical replicates) than the original acetic acid method ($r^2 = 0.82$ between technical replicates) and is well suited for use in laboratories in developing countries where QPM breeding activities require tryptophan analysis. The method uses a one cell spectrophotometer (560 nm) and allows the analysis of about 50 samples (in duplicate) per day per person. However, we have also established a high-throughput and low-cost adaptation of the procedure to perform and read the reaction using microplates. This modification is not possible with the acetic acid method because the large quantities of sulfuric acid and acetic acid required would rapidly damage the microplate reader. To adapt the new glyoxylic acid method to use microplates, we scaled down the method without changing the proportions of sample and reagents. Thus, the hydrolysis is made using 1.125 mL of papain in eppendorf tubes, and the colorimetric reaction is performed in 96-well plates in a total volume of 200 μL. This high-throughput method allows the analysis of 120 samples (in duplicate) per day per person, which is almost 2.5 times more than that with the acetic acid method (*10*, *11*) and with the method described by Piombo and Lozano (*15*).

Because QPM involves three genetic systems that influence tryptophan and lysine content, it is essential to monitor the levels of these amino acids in any QPM research program. On the basis of results using the glyoxilic acid method described herein, we recommend that breeders consider as QPM samples with values $>0.07\%$ for tryptophan in the sample and with quality index $\geq 0.7\%$ (quality index is the percent of tryptophan multiplied by 100 and divided by protein percent (*10*)). For elite and precommercial breeding material, we recommend analyzing tryptophan, lysine, and protein to ensure the superiority of the material. The colorimetric method for tryptophan analysis in flour of whole maize kernels presented here is a reliable, simple, and low-cost procedure to support QPM breeding activities.

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