A fluorimetric assay to determine variation of lysine in wheat gliadin proteins
by Jerry Dean Harris

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Agronomy
Montana State University

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Twenty fractions of wheat gliadins were collected and analyzed by the fluorimetric assay. Ten of these fractions were also analyzed for lysine content by an amino acid analyzer. Regression of lysine content by the two methods was linear ($R^2 = .75$) and lysine content varied from 0.6 to 1.4 percent of the protein.

Identification of high-lysine gliadin subunits could be implemented into a program of breeding for increased lysine in wheat. With the availability of an HPLC and a post column injection system, this fluorimetric assay and wheat gliadin separation could be adapted to a one-step procedure.

A FLUORIMETRIC ASSAY TO DETERMINE VARIATION OF LYSINE IN WHEAT GLIADIN PROTEINS

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APPROVAL

of a thesis submitted by

Jerry Dean Harris

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

A fluorimetric assay with o-phthalaldehyde (OPA) was used to determine the lysine content of wheat gliadin proteins. Wheat gliadin proteins were separated and collected by RP-HPLC. The protein content of the subunits was determined by UV absorbance. OPA reacts specifically with the ϵ-amino group of lysine in protein.

Twenty fractions of wheat gliadins were collected and analyzed by the fluorimetric assay. Ten of these fractions were also analyzed for lysine content by an amino acid analyzer. Regression of lysine content by the two methods was linear (R^2 = .75) and lysine content varied from 0.6 to 1.4 percent of the protein.

Identification of high-lysine gliadin subunits could be implemented into a program of breeding for increased lysine in wheat. With the availability of an HPLC and a post column injection system, this fluorimetric assay and wheat gliadin separation could be adapted to a one-step procedure.

INTRODUCTION

Amino acid composition is a major factor in determining the nutritive value of protein. Man and other monogastrics such as swine and poultry cannot synthesize certain amino acids. For proper growth their diets must contain sufficient quantities of these essential amino acids. The grain of most cereal crops is deficient in one or more of the essential amino acids lysine, tryptophan, threonine, isoleucine and methionine.

Wheat grain, which is nutritionally deficient in lysine, is the largest source of food in the world. Increasing the amount of lysine in wheat would increase its nutritional value substantially.

Selection for protein quality is necessary for improvement of the nutritional value of wheat. It has been shown that total wheat protein does not contain significant variation for lysine. Identification of high lysine protein subunits in wheat could be valuable as a selection tool for increasing lysine. Accurate techniques for lysine analysis exist but are time consuming and often difficult. The objectives of this study were to attempt to establish a

method for lysine analysis of the gliadin proteins that would be quick, simple and accurate, and determine the variation for lysine content of some gliadin proteins.

LITERATURE REVIEW

Increasing Lysine Content of Cereal Grains

The discovery of opaque-2 maize, with an improved amino acid balance (Mertz et al.,1964), greatly stimulated the investigation of genetic variation for amino acid composition in cereal grains. This discovery was followed by increased research in developing techniques, evaluating cereal strains and inducing variation for lysine content in cereals.

Techniques used for lysine determination were dye-binding capacity (DBC) (Mossberg 1969; Hagberg and Karlsson, 1969), Carpenter's method (Carpenter 1960), microbiological assays (Ford, 1966; Bell et al.,1977), ninhydrin method (Mertz et al.,1974), and amino acid analysis (Muntz et al.,1979).

In searching for existing variation for lysine content in cereals, Muntz et al. (1979) evaluated 8,000 wheat and 6,000 barley strains from the World Collection of Cultured Plants at Gatersleben. Johnson et al. (1979) evaluated 20,000 entries from the World Wheat Collection for protein and lysine content, and over 10,000 accessions of rice have been evaluated at the International Rice Research Institute.

Mutagenesis has been used to induce variation in lysine content in cereals. Ingversen et al. (1973) screened 6,000 mutagenically treated barleys, Gaul et al. (1973) screened 526 mutant barley strains and Sajo (1979) screened seeds of generations M6 through M8 of the rice variety Nucleoryza.

The above represents only a small sample of the many screenings that have been performed to select for increased lysine. These tests have identified high-lysine lines in barley (Munck et al.,1970; Doll, 1971), wheat (Johnson et al.,1985), sorghum, millet and maize. The lines identified as high lysine have had several drawbacks, such as: lower yields, small seed size, reduced weight, slowed drying and reduced resistance to pest damage. Of all the lines tested, "no cereal hybrid, synthetic, or cultivar with high lysine has been widely accepted for commercial production yet" (Bhatia and Rabson, 1987).

Wheat Gliadins

Lysine content as a percentage of whole grain wheat protein is about 3 percent. The content of white flour is less than 2 percent. The percentage recommended by FAO for young healthy adults is 5.5. This is nearly twice that found in wheat protein (Rakosky 1989).

The genetic component for lysine variation in total wheat protein is 0.5%, which is less than the 2.25%-2.50% needed

to increase lysine to the proper balance with the other essential amino acids (Johnson et al.,1985).

All previous tests for lysine content have been performed on total wheat protein. There is little genetic variation for lysine content in total protein, but there is obvious variation among different proteins. It seems feasible to test individual protein subunits for lysine content and, if variation in lysine content exists, then select for increased amounts of high-lysine subunits.

Thirty to 60 percent of wheat protein is extracted in alcohol-water mixtures and is defined as gliadins (Bhatia and Rabson, 1987). In most cases high-lysine is associated with or caused by a decrease in the major storage proteins, such as the gliadins, which are naturally low in lysine. This decrease in storage proteins is what causes the high-lysine lines to have poor agronomic and baking characteristics (Kasarda et al.,1974).

Huebner and Bietz (1986) and Al-Khawlani (1988) used RP-HPLC to separate wheat gliadin subunits and demonstrated that certain subunits were correlated with grain quality, some positively and some negatively. It seems possible then that one could breed for increased quality by selecting for specific subunits as "quality markers". If a high lysine subunit could be identified it could also be used as a "quality marker". Since gliadins represent a major portion of wheat protein, increasing the proportion of gliadin

subunits that are high in lysine and decreasing the subunits low in lysine could significantly increase the lysine content of wheat protein.

Lysine analyses are time consuming and expensive. An accurate, quick and simple method for estimating lysine content in cereal protein subunits would greatly assist in the development of wheat cultivars with greater potential nutritional value.

Lysine Determination

Spectroscopic methods can be used for estimation of tyrosine and tryptophan content in peptides but unfortunately these methods do not work for distinguishing lysine from other amino acids.

Existing techniques for lysine analysis are accurate, but involve carefully controlled hydrolysis procedures which must be followed by one of a number of types of chromatographic separations. A quicker chemical method for lysine quantification of proteins was recently developed (Goodno et al.,1981). This method involves the reaction of o-phtalaldehyde (OPA) and 2-mercaptoethanol with primary amines, forming a highly fluorescent isoindole ring (Figure 1). o-Phtalaldehyde is five to ten times more sensitive then fluorescamine and is soluble in aqueous buffers (Benson and Hare,1975). In studies using OPA for peptide detection

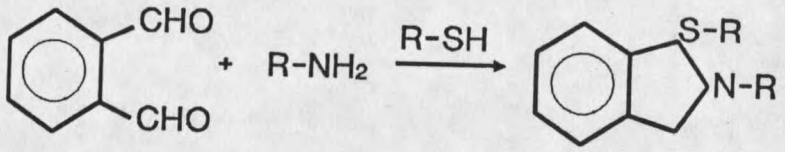


Figure 1. Reaction of OPA and primary amines in the presence of mercaptoethanol, forming a highly fluorescent isoindole ring.

it was found to be unreactive with the primary amino terminus, but reactive with peptidyl lysine groups (Joys and Kim, 1979).

Fluorescence yields vary depending on reaction temperature (Cronin et al.,1979), thiol or solvent used (Cronin et al.,1979; Skaaden and Greibrokk, 1982; Simons and Johnson, 1978), and time between initial reaction and fluorescence reading (Simons and Johnson, 1978). Half lives of the adduct were reported to be as short as ten minutes and as long as 33 hours, depending on conditions.

In comparison to the methods generally used for analysis of lysine, the OPA method could save considerable time and effort as well as working on sample sizes less than 1 μg without sacrificing accuracy.

MATERIALS AND METHODS

Preparation of Protein Standards

Bovine serum albumin (BSA), carbonic anhydrase and lysozyme were chosen as protein standards because of their availability, purity and range in lysine content (Table 1). Stock solutions were prepared from lyophilized proteins and dissolved in 0.1 M sodium phosphate (pH 7) and 5 mM sodium azide.

Table 1. Lysine content of protein standards.

Protein	Molecular Weight	Lysine Residues	Total Residues	% Lys Residues
Lysozyme[a]	14,314	6	129	4.65
Carbonic Anhydrase[b]	30,000	23	273	8.42
BSA[a]	66,296	59	582	10.14

[a] Molecular weights and residue information are from Dayhoff (1972,1976).
[b] Molecular weights and residue information are from Kirschenbaum, D.M. 1971.

Quantification of Protein Standards

Protein standards were quantified by using two different methods; ultraviolet (UV) absorbance on the basis of peptide bond absorbance at 210 nm (Ahokas, 1978) and the Coomassie blue protein assay (Bradford 1976). UV absorbance was measured using a flow injection analysis (FIA) system which consisted of a high performance liquid chromatography (HPLC) pump, an automatic sample injector and a UV variable wavelength absorbance detector (Figure 2). Reagents for the Coomassie blue protein assay were obtained from a commercial kit (Bio-Rad).

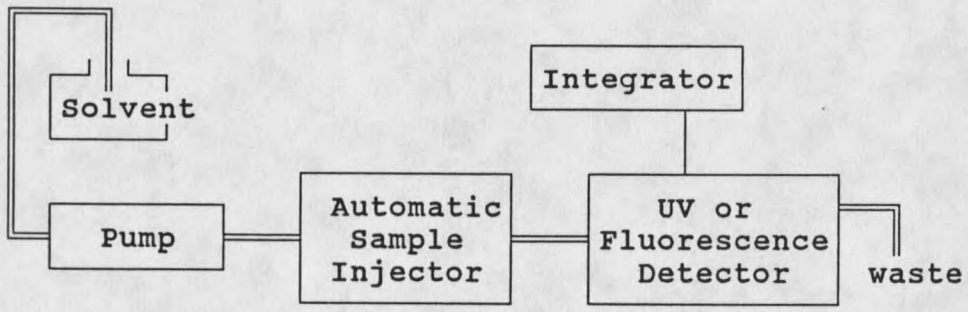


Figure 2. Flow Injection Analysis (FIA) system used for protein quantification and fluorimetric determinations.

Gliadin Extraction

12.5 mg of flour from winter wheat cultivar Redwin, CI 17844, were suspended in 1 ml of a 70 percent ethanol solution. Suspension was continuously agitated at room temperature for 1 hour and then centrifuged at 20,000 g for 15 minutes. Supernatant was then removed, filtered through a .45 micron millipore filter and stored at 4° C until analysis.

HPLC Analysis of Gliadins

Reversed-phase high performance liquid chromatography (RP-HPLC) was used to separate gliadin subunits. RP-HPLC conditions were similar to those described by Huebner and Bietz (1987). The HPLC system consisted of an HPLC solvent delivery pump, gradient former, UV variable wavelength absorbance detector, integrator, and a 250 X 4.5 mm SynChropak RP-P (C18) column (Figure 3).

Column temperature was maintained at 70° C with an HPLC water jacket and circulating water bath. All solvents used were filtered through either a .2 or .45 micron filter. Solvent flow rate was 1 ml/min. Solvent A was HPLC grade water containing .06% trifluoroacetic acid (TFA), and Solvent B was HPLC grade acetonitrile containing .05% TFA. Gliadin subunits were eluted with a linear gradient of 23-

45% acetonitrile over 40 minutes. An additional linear gradient of 45-90% acetonitrile over five minutes with a five minute isocratic hold at 90% acetonitrile was used to remove any non-soluble materials. 15 minutes were allowed for system to return to initial conditions, making a total run time of 65 minutes. Samples were injected into a 2 ml loop using a sample injection pump. Subunits were detected at 210 nm and 20 fractions were collected individually throughout the run.

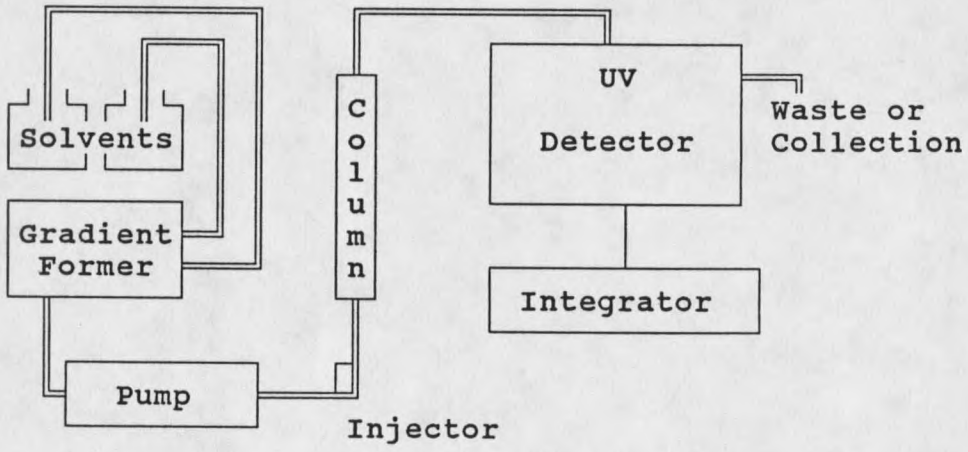


Figure 3. High Performance Liquid Chromatography system used for separation of wheat gliadins.

Quantification of Gliadin Fractions

A 200 ul sample of each of the 20 fractions collected was injected into the HPLC system with similar run conditions to determine purity and protein content. Solvent gradient was linear at 23 to 45% acetonitrile over 40 minutes with a five

minute hold at 45% and then 15 minutes allowed for return to initial conditions making a total run time of 60 minutes. UV absorption at 210 nm was used for protein detection and peak areas were calculated by an integrator and translated into micrograms by comparison of peak areas obtained from analysis of known amounts of BSA.

Electrophoretic Analysis of Gliadins

SDS Polyacrylamide gel electrophoresis was performed on each peak to determine molecular weights and to compare HPLC separations with electrophoretic separations.

Fluorimetric Determination of Protein Standards

The o-phtalaldehyde (OPA) reagent was obtained from Pierce Chemical Company in a "ready to use" solution called Fluoraldehyde. The Fluoraldehyde reagent solution contains a highly purified solution of OPA, Brij-35 detergent, and mercaptoethanol in a borate buffer.

Peptidyl lysine groups were estimated by reacting equal volumes of the OPA reagent solution with the protein solution. The mixture was vortexed for 10 seconds at room temperature, held at room temperature for 50 seconds and then injected immediately into a FIA system. The FIA system consisted of an HPLC pump, an auto-injector and a fluorescence detector (Figure 2). Excitation and emission filters used to detect the OPA reaction were 340 nm and 418

nm, respectively. The solvent was methanol and water (1:1) with a flow rate of 1.5 ml/min. A 20 ul sample was injected with a run time of 60 seconds.

Fluorescence readings of standards were then divided by amount of protein and a standard curve made of relative fluorescence versus percent lysine to determine accuracy of technique.

Fluorimetric Determination of Gliadins

Fluorimetric determination of gliadins used the same procedure as for protein standards except that the excitation filter was set at 229 nm for an increase in sensitivity (Hill et al.,1979). Six fluorimetric determinations were made for each of the 20 gliadin peaks. Fluorescence was then divided by protein concentration to obtain a value of relative fluorescence.

Lysine Determination

To correlate the results of the fluorimetric assay with actual lysine content, samples of ten of the 20 peaks were sent to the Department of Biochemistry, University of California-Davis for amino acid analysis by an Amino Acid Analyzer (AAA).

Effect of Reaction Conditions on Fluorimetric Determination

The linear response of fluorescence to sample size was studied by ranging the concentration of BSA from 0.25 μg/ml to 10.0 μg/ml.

The response of fluorescence to reaction time was examined to determine the optimum time for injection.

The relationship of concentration of OPA to fluorescence was examined by varying the concentration of OPA.

RESULTS AND DISCUSSION

Protein Standards

Relative fluorescence was determined by taking the fluorescence reading obtained from reacting OPA with the protein standards and dividing this by the amount of protein according to either the Coomassie Blue Protein Assay or UV absorption at 210 nm.

The protein standards used in this study ranged in lysine content from 4.7 to 10.1 percent. A high correlation was found between relative fluorescence, as obtained from the fluorimetric assay, and the known lysine content of the protein standards (Figure 4).

Gliadins

HPLC separated the gliadins into 35 to 40 major peaks. Of these peaks, 20 fractions were collected with some fractions containing more than one peak (Figure 5). Total volume of each of the fractions collected, from the 50 injections, ranged from 5 to 25 mls and protein content ranged from 3.78 to 35.96 μg/ml (Table 2).

An injection of 200 ul of each of the 20 fractions into the HPLC system was used for quantification as well as an estimate of purity. Percent purity was calculated by

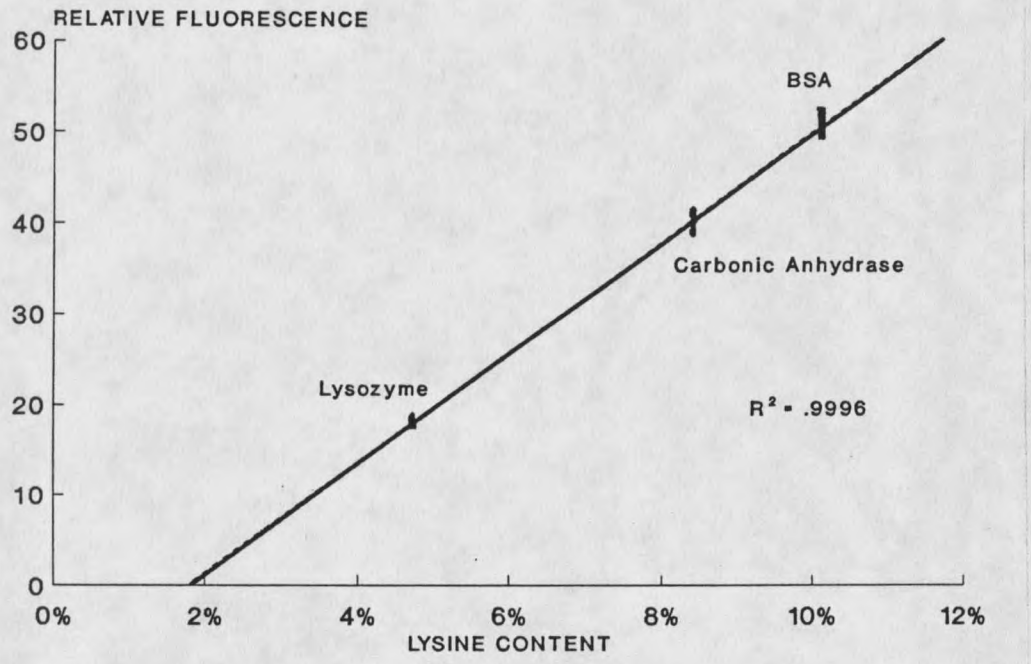


Figure 4. Correlation between the relative fluorescence as obtained from the fluorimetric assay and the percent lysine of commercially available proteins of known amino acid content.

dividing total peak areas of the run by the area of the peaks corresponding to the fraction injected (Table 2).

Running each of the fractions on SDS gel electrophoresis showed, in some cases, existence of more proteins than was apparent with HPLC separations (Figure 6). It was also used for molecular weight determination of the fractions (Table 2).

Ten of the 20 gliadin fractions collected were analyzed for lysine content by an amino acid analyzer (AAA) by the Department of Biochemistry, University of California-Davis. Correlation of the results from the AAA for lysine content of the ten fractions with the relative fluorescence from

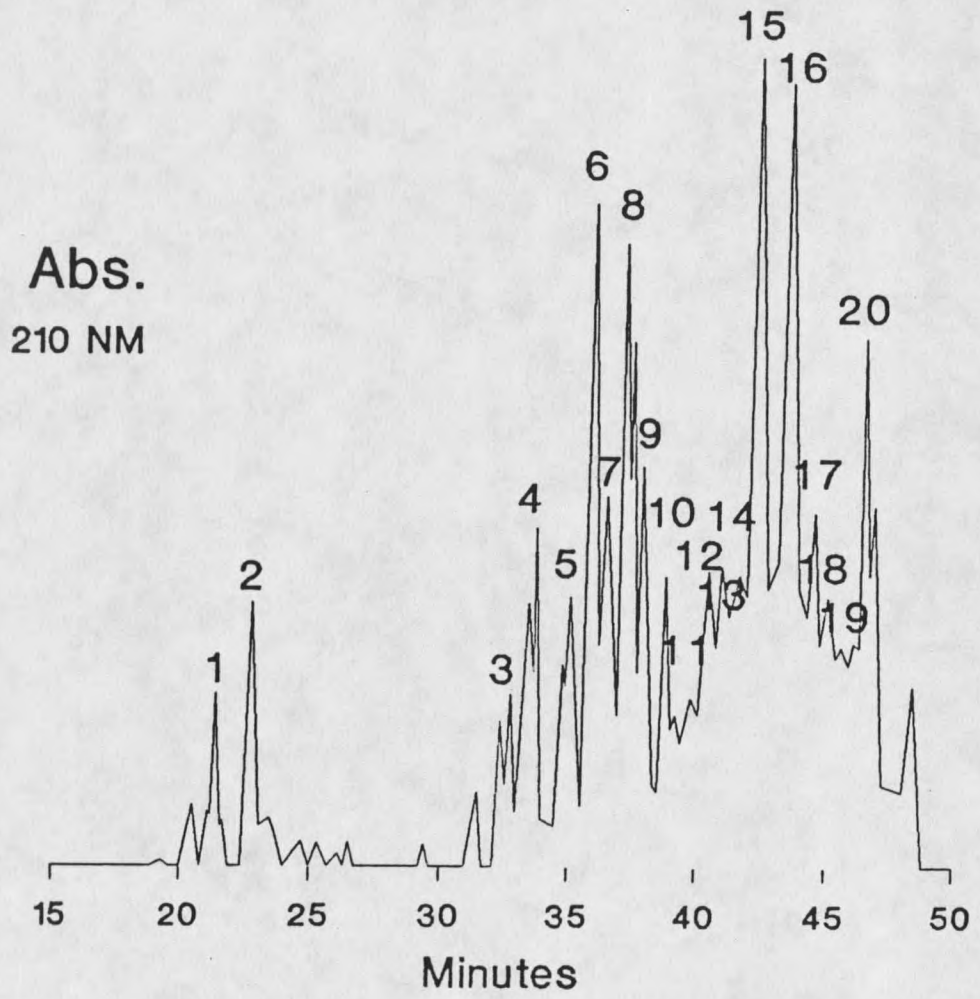


Figure 5. HPLC chromatogram of wheat gliadin separation as detected by UV absorption at 210 nm at elution times between 15 and 50 minutes. The numbered peaks (1 to 20) correspond to the fractions collected for fluorimetric determinations.

Table 2. Molecular weight, percent purity, protein concentration, relative fluorescence, and percent lysine of the 20 wheat gliadin fractions.

Gliadin Peak	M.W. x 10^{-3}	Percent Purity	µg/ml Protein	Fl/unit Protein	Percent Lysine
1	65	89	3.78	1570	1.43
2	65	93	6.56	904	1.07
3	40	96	8.42	610	0.91
4	37	97	13.71	492	0.85
5	45	98	14.59	780	1.00
6	38	99	35.96	207	0.69
7	27	99	21.92	339	0.76
8	36	97	35.01	248	0.71
9	37	93	14.46	214	0.70
10	35	98	14.42	65	0.61
11	[a]	99	9.08	298	0.74
12	38	99	14.17	195	0.69
13	33	99	14.74	533	0.87
14	[a]	99	15.89	178	0.68
15	35	96	34.05	358	0.77
16	39	97	35.06	287	0.74
17	33	97	22.48	532	0.87
18	[a]	99	16.24	796	1.01
19	32	99	11.16	460	0.83
20	38	99	24.49	374	0.78

[a] represents data missing because too many bands existed on SDS gels.

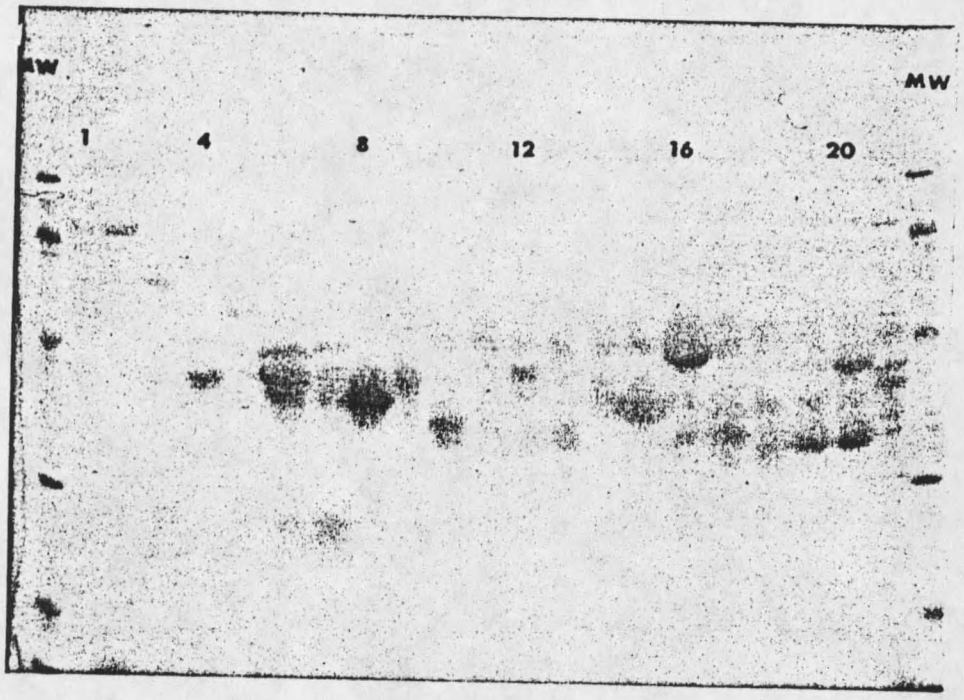


Figure 6. SDS-polyacrylamide gel electrophoresis of the 20 fractions of wheat gliadins. Track on far left and one on far right are molecular weight standards.

the fluorimetric determination produce an $R^2 = 0.75$, which is significant at the .01 level of significance (Figure 7a).

Of the five highest lysine containing fractions according to the AAA, four were also rated highest by the fluorimetric determination (Figure 7b). Over all, the two methods ordered the fractions quite similarly according to lysine content. If three of the ten fractions were omitted, the two methods ordered the remaining fractions exactly the same (Figure 7b).

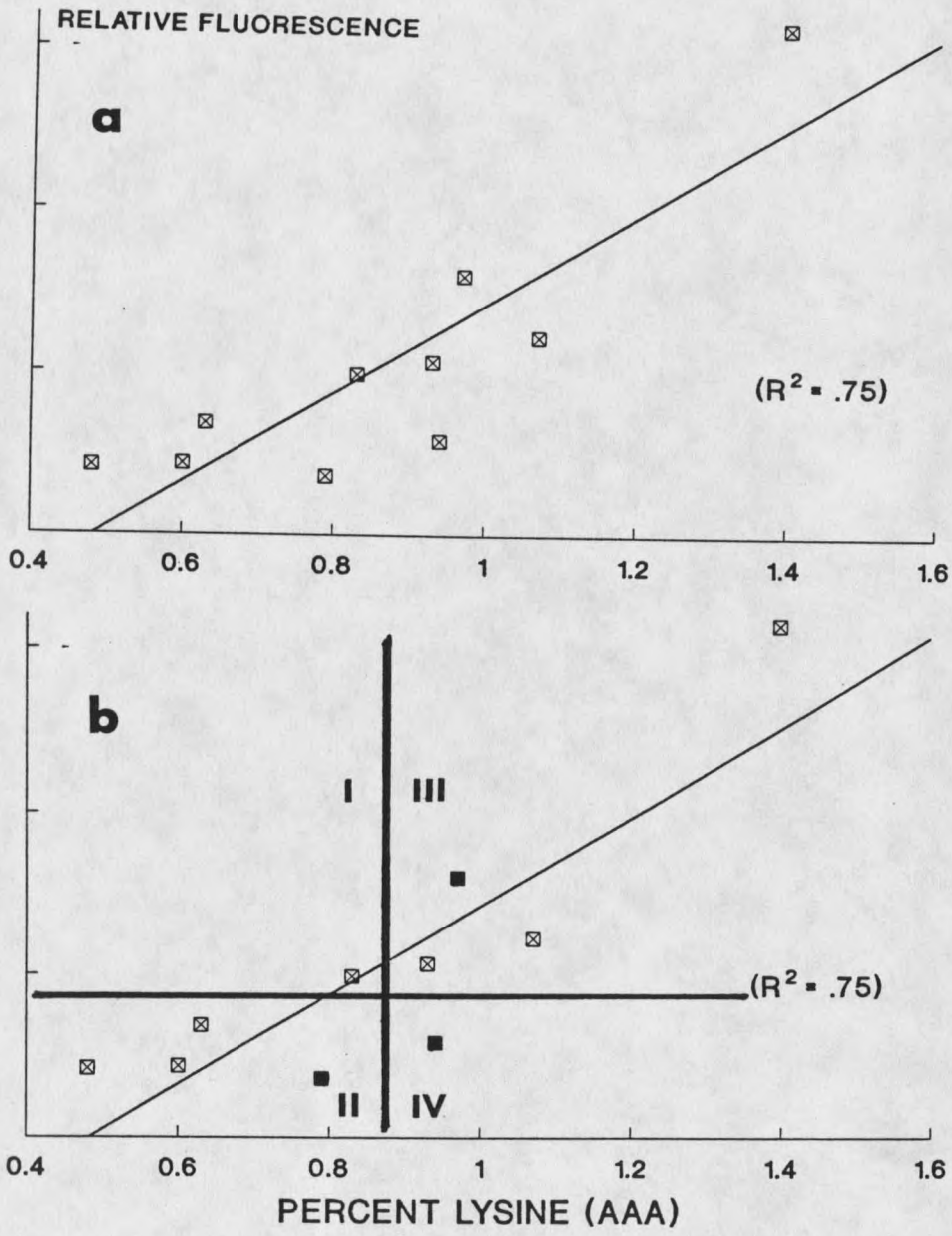


Figure 7. a) Correlation of the percent lysine as obtained from the AAA on ten selected wheat gliadin fractions with the corresponding relative fluorescence as obtained from the fluorimetric assay ($y = 0.579 + .0005x$). b) Overall rating of the fractions between the two methods. Points in quandrant I and III correspond to the fractions with highest fluorescence readings. Points in quadrants III and IV correspond to fractions with highest lysine percent. Points in quadrant III correspond to fractions rated highest by both methods. Note! if solid points were omitted, remaining points would be ordered identically by both methods.

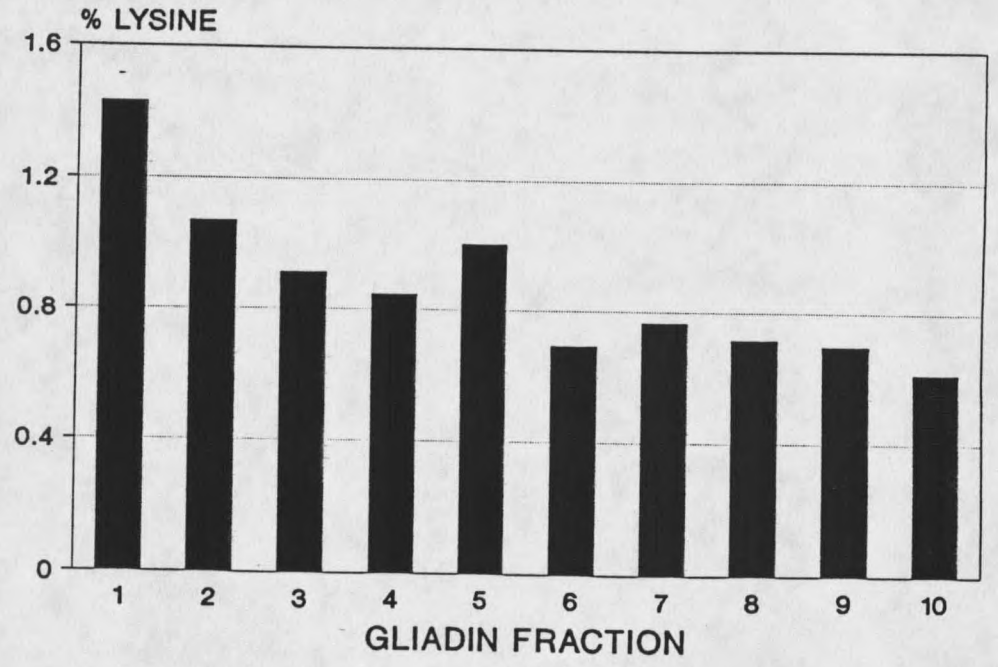
Lysine Variation

Lysine content of all 20 fractions was calculated by use of the regression equation in Figure 7. The gliadin fractions varied in estimated lysine content from 0.6 to 1.4 percent of protein, a more than two-fold difference in lysine content (Figure 8).

The mean lysine content of the 20 fractions is 0.79 percent. If the protein content of all fractions greater than the mean were increased by 50 percent and all the fractions less than the mean were decreased by 50 percent, the result would be a six percent increase in the lysine content of the total gliadin fractions (0.79% to 0.84%). This may not be a substantial increase in lysine, but if the same procedure was performed on the other classes of wheat proteins, total lysine could possibly be increased by a nutritionally significant amount.

Reaction Conditions

The time allowed for the reaction to occur had an effect on the amount of fluorescence. Peak fluorescence was experienced shortly after one minute (Figure 9). All samples were reacted for 60 seconds and then injected directly into the FIA system to obtain uniform readings and peak fluorescence each time.



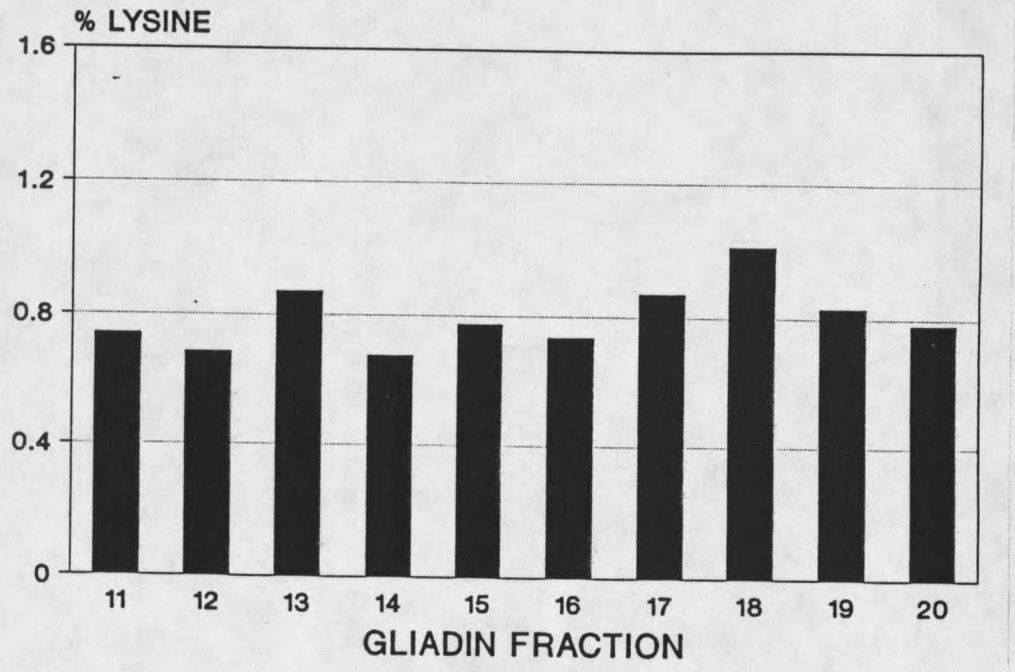


Figure 8. Percent lysine of the 20 wheat gliadin fractions as calculated by the equation in figure 7. Note! greater than 2-fold difference between wheat gliadin fraction 1 and 10.

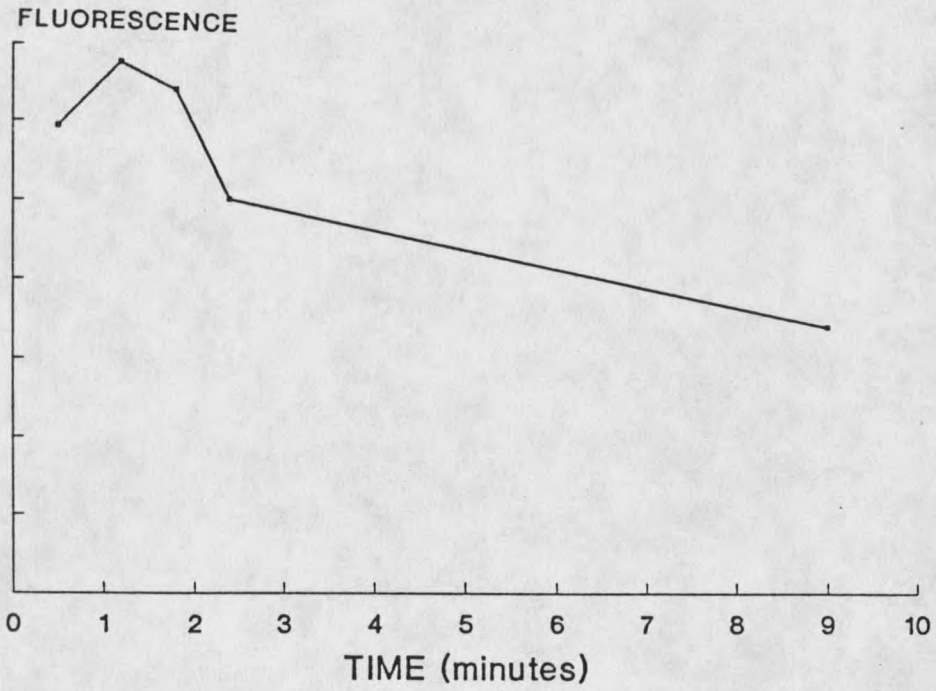
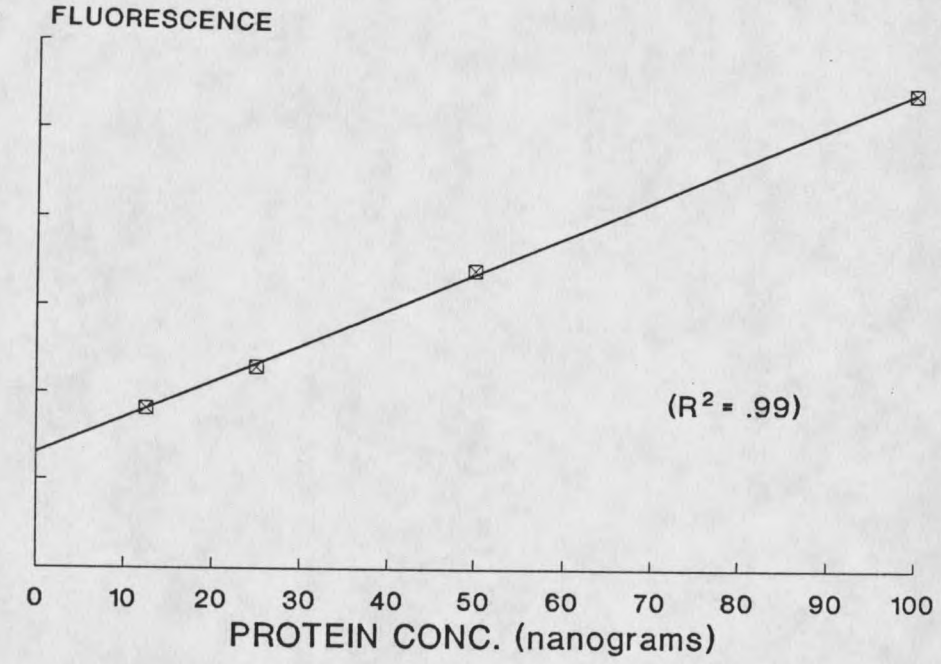


Figure 9. Fluorescence as obtained from fluorimetric determinations on BSA as effected by reaction time in minutes. Peak fluorescence was obtained shortly after one minute of reaction time.

Varying the sample size of protein produced a linear response to fluorescence over the entire range of protein covered. R^2 values were 0.99 for the ranges of 12.5 ng to 100 ng and 1.25 μg to 10 μg (Figure 10). Best results were obtained at protein levels of 15 ng or more.

Not much OPA was required to reach peak fluorescence, but extra was added so as to buffer the reaction and maintain peak fluorescence for a longer time. Increasing concentration of OPA and holding protein concentrations constant showed a negative exponential type curve with



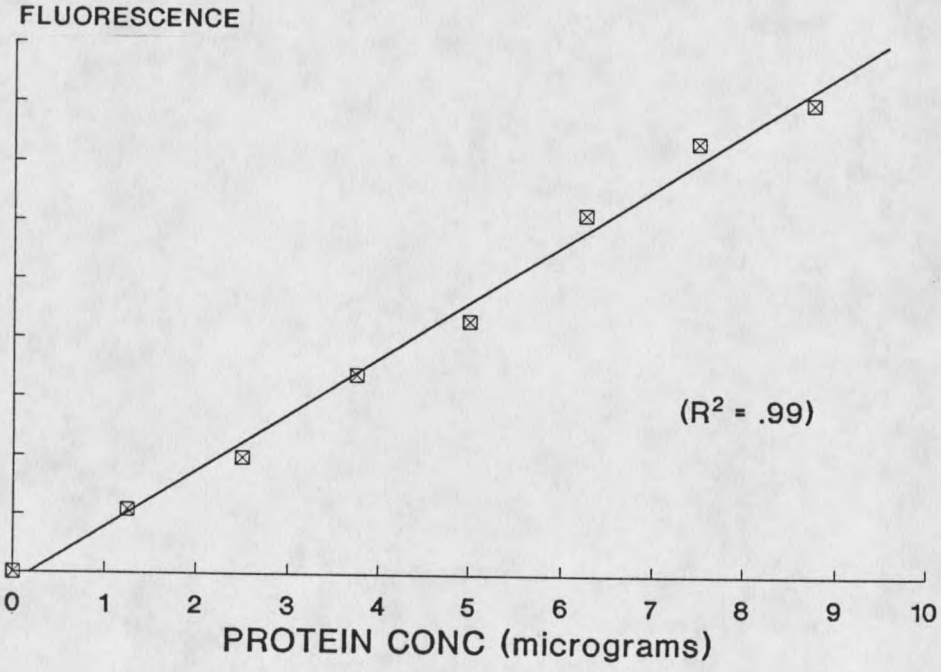


Figure 10. Fluorescence as obtained from the fluorimetric determination of protein BSA at varying concentrations.

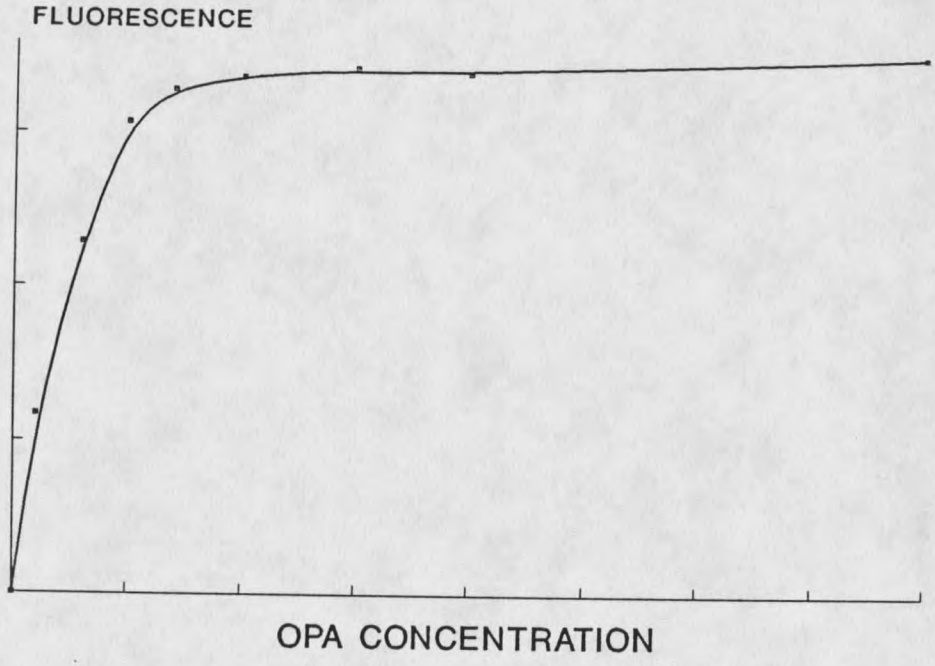


Figure 11. Fluorescence as obtained from the fluorimetric determination by holding the concentraion of BSA constant and increasing the concentration of OPA.

fluorescence (Figure 11). The slope varied depending on the age of the Fluoraldehyde solution. Solutions older than 3 months showed decreased slopes so every 2 months the old solution was discarded and a new one purchased.

Use of a post-column injection system along with the HPLC used for separating gliadins would allow this procedure to be performed in one step. Separation and detection of gliadins would be followed by injection of OPA, reaction with available amino groups of lysine and fluorescence detection. This would eliminate the need for collecting gliadin fractions and replicating protein quantification and

fluorimetric determinations (Figure 12). This one-step procedure would also reduce sources of error associated with handling samples many times, such as; sample contamination, improper measurements, and protein solubility problems.

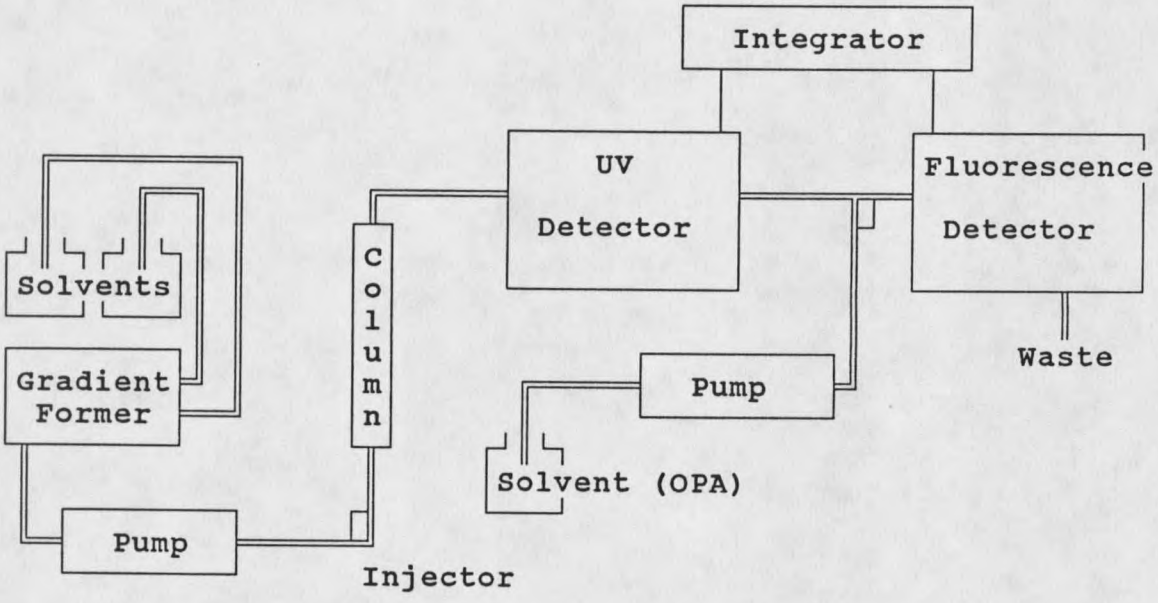


Figure 12. Proposed post-column injection system to determine lysine content of wheat protein by a one-step method. Post-column injection would allow OPA to be inserted into system and react with the available amino groups of lysine in protein. Reaction would occur after protein separation and quantification and before fluorescence detection.

SUMMARY

The results of this study show that OPA can be used with a fluorimetric assay to determine the lysine content of wheat gliadins. The method is quick, simple and relatively accurate. By implementation of a one-step procedure, the accuracy of this method could be increased even more.

Better than a two-fold difference in lysine content was found to exist between wheat gliadin fractions one and ten. The total lysine content of wheat gliadins could be increased by selecting for lines with increased amounts of high-lysine gliadin fractions and decreased amounts of low-lysine fractions.

The OPA fluorimetric assay could also be performed on the other three wheat protein classes; glutenins, albumins and globulins. By combining the selection for increased lysine in the different classes it should be possible to significantly increase total lysine in wheat grains.

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APPENDIX

Table 3. Percent lysine and fluorescence values of the ten chosen wheat gliadin fractions.

Fraction	% Lysine (AAA)	Fluorescence /ug protein	% Lysine (regr.)
1[a]	1.31/1.55[b]	1570[c]	1.43[d]
3	1.07/1/09	610	0.91
4	0.85/0.81	492	0.85
6	0.36/0.60	207	0.69
7	0.65/0.61	339	0.76
9	0.60/0.61	214	0.70
14	0.80/0.78	178	0.68
16	1.09/0.97	287	0.74
17	1.06/0.79	532	0.87
18	0.79/NA	796	1.01

[a] Fractions chosen for amino acid analysis.
[b] Percent lysine according to amino acid analysis (rep1/rep2).
[c] Fluorescence as obtained from the fluorimetric determinations.
[d] Percent lysine derived from the regression of percent lysine and fluorescence/ug protein.

Table 4. Mean fluorescence and standard error values for the 20 collected fractions of wheat gliadin.

Fraction	Fl/ug	SE	Fraction	Fl/ug	SE
1	1570[a]	36[b]	11	298[a]	23[b]
2	904	24	12	195	11
3	610	37	13	533	27
4	192	11	14	178	05
5	180	22	15	358	11
6	207	11	16	287	10
7	339	08	17	532	13
8	248	15	18	796	17
9	214	04	19	460	24
10	65	09	20	374	04

[a] Mean value from six fluorimetric determinations divided by micrograms of protein.
[b] Standard Error of the six fluorimetric determinations.