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# Development of a Method to Quantify Lysine in Small Amount of Rice Grain

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## Abstract

A lysine determination method for low quantity of rice was modified from the original Dye-Binding Lysine (DBL) method used in the national standard in China [GB 4801-84, 1984]. By making use of the property that lysine does not bind to the crocein orange G dye after treated with propionic anhydride, the amount of lysine in rice samples could be determined directly by calculating the difference between the absorbancesof the treated and the untreated samples. Various commercial rice samples were purchased from market and evaluated. Several methods were tested by varying both the sizes of the samples and the concentrations of the dye solutions. Results showed that when using 1.284 mM of crocein orange G dye solution and 15.5 mg of sample, the results were most reproducible. The corresponding lysine content in sample were 3.36 ± 0.09 mg/g and 3.35 ± 0.19 mg/g by traditional method and modified method, respectively. Statistically, there was no significant difference between the results ($p>0.05$).

**Key words** : lysine, rice, propionic anhydride, methodology

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## INTRODUCTION

Lysine is one of eight essential amino acids for human [1]. Different methods were developed to evaluate the lysine content in different samples, in which liquid chromatography, reversed-phase high performance liquid chromatography and biosensors, etc. are often involved [1, 2, 3, 4, 5]. For example, in the biosenors, most investigators use the oxidase produced by the *Trichoderma viride* and immobilized the enzyme in a solid phase such as polyurththane hydrogel [6], silica gel [7], etc. Oxidation of L-lysine by L-lysine alpha-oxidase would lead to the production of hydrogen peroxide which is the compound to be monitored and measured to reflect the quantity of lysine present in a food. Different detectors were reported to be used in the detection of the hydrogen peroxide. Some of these detectors include the platinum typed electrode, fiber-optic hydrogen peroxide detector and gold-poly(m-phenylenediamine) electrode[8,9]. However, these methods are often very complex and require expensive equipments. The traditional Dye-binding Lysine (DBL) method [10]used to determine the lysine content in rice was economic and easy to perform. However, the sample size required for the determination is relatively high (1.2 g for each trial) when compared with the yield from rice breeding experiment (about 3 g). For samples with only limited quantity from a breeding experiment, the method may not be suitable. Therefore, the objective of this investigation was to develop a reliable lysine determination method comparable to the DBL method for rice in small quantity.

## MATERIAL AND METHODS

***Samples***

Commercial rice (Calrose rice) samples (sample A - G) were purchased from local market. The head rice milled into powder (KM800 and A941, Kenwood Limited, Britain) and was screened through a sieve (250 m, No. 60, VWR Scientific, West Chester).

***Moisture Content Determination.***

The percentage moisture of each sample was determined using a Mettler LJ16 moisture analyzer (Mettler-Toledo, Swizerland).

***Reagents.***

Preparation of reagents was based on the protocol suggested by the National Standard in China (GB 4801-84, 1984) [11].

**Buffer solution:** A buffer solution was prepared by dissolving 3.4 g potassium dihydrogen orthophosphates and 20 g oxalic acid dehydrate in 100-mlhot distilled water in a 1000-ml volumetric flask. Subsequently, 1.7 ml of phosphoric acid, 60ml of glacial acetic acid and 1 ml of propanoic acid were added. Distilled water was used to add up to the mark.

**Acid orange 12 (dye) solution:** To prepare 1000 ml of 3.89 mM acid orange 12 dye solution, 1.9471 g Crocein Orange G (70% purity) was

dissolved in the afore mentioned buffer solution in a 1000-ml volumetric flask. Similarly, to prepare 1000 ml of 1.284 mM acid orange 12 dye solution, 0.6427 g Crocein Orange G (70% purity) was dissolved in buffer solution in a 1000-ml volumetric flask.

**16% sodium acetate:** 16.0 g of sodium acetate was dissolved in distilled water and made up to 100 ml in volume.

Propionic anhydride

***Steps involved in the Dye-binding Lysine (DBL) Methods (Original and Modified)***

Overall, six methods were used to evaluate the lysine content in a rice sample (sample A) including two original methods and four modified methods. The lysine content of the sample was firstly determined by following the original method (Ori) suggested by the National Standard in China (GB 4801-84, 1984). In the method, 3.89 mM acid orange 12 dye solution was used. Then, an alternative low quantity method (L) suggested in the same method using a lower concentration of acid orange 12 dye solution (1.284 mM) and lower sample weight than the original method was carried out. In order to achieve the objective, four modified methods (L1/5, L1/10, L1/20 and L1/30) were set up to reduce both the sample weight volume of reagents proportionally. Method L1/5 is equivalent to decrease the weight of samples and the volume of reagents by a factor 1/5 based on method L. Similarly, method L1/10 decreases by a factor of 1/10 of method L, etc.

Major steps involved in these methods were shown in Fig. 1.

***Construction of standard curves for dye***

The standard curve was prepared for the original method (Ori) as shown in Table 1 (GB 4801-84, 1984).

Table 1. Solutions prepared for construction of standard curve for the original method (Ori).

| *Concentration of dye* | *Preparation* |
|---|---|
| 2.20mM | 2.00ml 3.89mM dye and 1.536ml buffer |
| 2.10mM | 2.00ml 3.89mM dye and 1.704ml buffer |
| 2.00mM | 2.00ml 3.89mM dye and 1.890ml buffer |
| 1.90mM | 2.00ml 3.89mM dye and 2.094ml buffer |
| 1.80mM | 2.00ml 3.89mM dye and 2.322ml buffer |
| 1.70mM | 2.00ml 3.89mM dye and 2.576ml buffer |
| 1.60mM | 2.00ml 3.89mM dye and 2.862ml buffer |
| 1.50mM | 2.00ml 3.89mM dye and 3.186ml buffer |
| 1.40mM | 1.00ml 3.89mM dye and 1.779ml buffer |
| 1.30mM | 1.00ml 3.89mM dye and 1.992ml buffer |
| 1.20mM | 1.00ml 3.89mM dye and 2.242ml buffer |

Similarly, Table 2 shows the solutions prepared of standard curve (GB 4801-84, 1984) [7] for the modified methods (L, L1/5, L1/10, L1/20 and L1/30).

The solutions listed above were prepared and their absorbances were measured at 482nm. Standard curves with absorbance against concentration of dye were established for each of the methods

Table 2. Solutions prepared for construction of standard curve for the modified method (L, L1/5, L1/10,L1/20 and L1/30).

| *Concentration of dye* | *Preparation* |
|---|---|
| 0.50mM | 1.00ml 1.284mM dye + 1.565ml buffer |
| 0.55mM | 1.00ml 1.284mM dye + 1.335ml buffer |
| 0.60mM | 1.00ml 1.284mM dye + 1.140ml buffer |
| 0.65mM | 1.00ml 1.284mM dye + 0.975ml buffer |
| 0.70mM | 1.00ml 1.284mM dye + 0.834ml buffer |
| 0.80mM | 1.00ml 1.284mM dye + 0.605ml buffer |
| 0.90mM | 1.00ml 1.284mM dye + 0.427ml buffer |

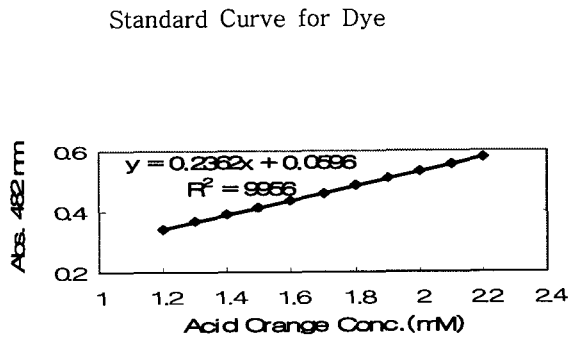
*Major difference between the methods*

Major differences between the methods were shown in Table 3.

Table 3. Details of the methods (Ori, L, L1/5, L1/10, L1/20, L1/30).

| Method | Ori | L | L1/5 | L1/10 | L1/20 | L1/30 |
|---|---|---|---|---|---|---|
| Container | 50ml tube | 50 ml tube | 50 ml tube | 50 ml tube | 1.5 ml tube | 1.5 ml tube |
| Sample weight [a] | P: 0.7000 g NP: 0.5000 g | P: 0.1800 g NP: 0.1300 g | 0.0360 g NP: 0.0260 g | P:0.0180 g NP: 0.0130 g | P: 0.0090 g NP: 0.0065 g | P: 0.0060 g NP: 0.0043 g |
| 16% Sodium acetate | 2.000 ml | 2.000 ml | 0.400 ml | 0.200 ml | 0.100 ml | 0.067 ml |
| Propionic Anhydride(P)/ Buffer (NP) | 0.200 ml | 0.200 ml | 0.040 ml | 0.020 ml | 0.010 ml | 0.007 ml |
| Acid Orange 12 | 20 ml | 20 ml | 4 ml | 2 ml | 1 ml | 0.667 ml |

[a]P: propionic anhydride treated sample; NP: non-propionic treated sample



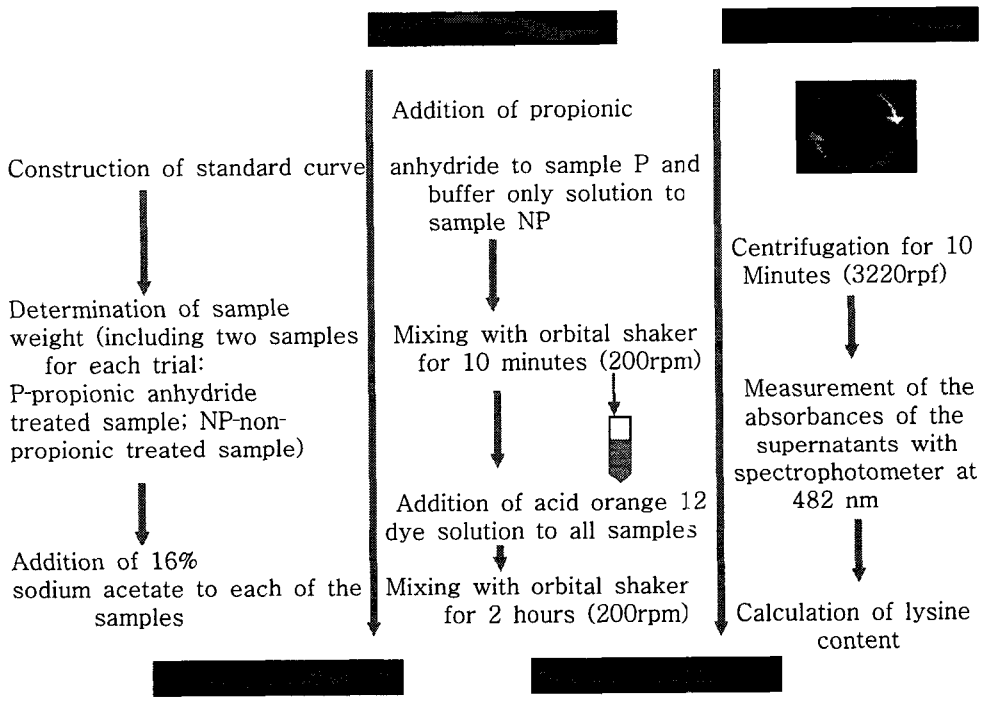




**Fig. 1. Major steps involved in Dye-Binding Lysine methods.**

### *Calculation*

The concentrations of dye remained in a solution ($C_{NP}$ and $C_P$) was calculated from absorbances of the two supernatants according to the standard curve obtained. The lysine contents in the samples were calculated by the following equations.

$$\text{No. of mole of dye reacted with lysine (mmole / g of dried sample)} = \frac{\text{Concentration of dye reacted with lysine (mM)}}{\text{Dry weight of sample (g)}} \times \text{Volume of dye added} \quad \text{(equation 1)}$$

Where

$$\frac{\text{Concentration of dye reacted with lysine (mM)}}{\text{Dry weight of sample (g)}}$$

$$= \frac{\text{Conc. of dye bind to untreated sample}}{\text{Dry weight of untreated sample}} - \frac{\text{Conc. of dye bind to treated sample}}{\text{Dry weight of treated sample}}$$

$$= \frac{\text{Conc. of original dye added}^{a} - \text{Corrected conc. of dye left in supernatant originally containing rice sample}^{bc}}{\text{Dry weight of untreated sample}^{d}} - \frac{\text{Conc. of original dye added}^{a} - \text{Corrected conc. of dye left in supernatant originally containing rice sample and propionic anhydride}^{bc}}{\text{Dry weight of treated sample}^{d}}$$

$$= \frac{C_{dye}{}^{a} - 1.11^{b}\ C_P{}^{c}}{W_{NP}{}^{d}} - \frac{C_{dye}{}^{a} - 1.11^{b}\ C_{NP}{}^{c}}{W_P{}^{d}}$$

[a] $C_{dye}$ = Concentration of original dye added (3.89 mM for methanol Ori; 1.284 mM for the other methods).

[b] 1.11= 1/dilution factor of the solutions.

[c] $C_{NP}$ and $C_P$ = the concentration of dye (mM) in the supernatant of samples w/o & w/propionic anhydride, respectively calculated from absorbances.

[d] $W_{NP}$ and $W_P$ = the dry weight (g) of sample without & with propionic anhydride, respectively.

Volume of dye added in L (refer to table 3)

Binding ratio of dye : lysine is 1:1,

No. of mole of dye reacted = No. of mole of lysine

Lysine content (mg / g dry weight)
= No. of mole of lysine (mmole / g dry weight) molar mass of lysine (equation 2)
Where Molar mass of lysine = 146.2 g / mole

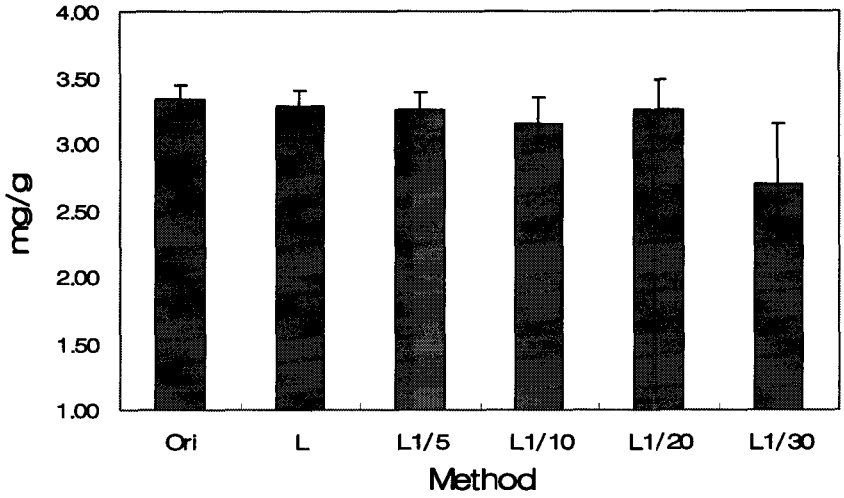



Fig. 2. Mean (±SD) lysine control of rice sample A determined by different methods (n=9). Lysine contents in the sample determined by different methods marked with different alphabets (A and B) are statistically significant different (Tukey, $p<0.05$).

## RESULTS AND DISCUSSION

The mean lysine content of rice sample A determined by the original methods (Ori & L) and the modified methods (L1/5, L1/10, L1/20, L1/30) with reduced sample quantity are shown in Fig. 2. The values of lysine content of sample A determined by difference methods were compared by one-way ANOVA test (Tukey). It was shown that value determined by method L1/30 was significantly different from those of the others ($p<0.05$). Thus, method L1/30 was not suitable to determine the lysine content.

The coefficients of variations (CV) among the methods are shown in Fig. 3. The CV of method Ori was the lowest and was the most reproducible. The highest CV was obtained from the method L1/30. Since CV at level about 5% or less are considered as acceptable. Methods L1/5 and L1/20 are considered to be reproducible ones. High CV in method L1/30 might be due to the limitation on weighing low quantity of sample accurately (P: 0.0060, NP: 0.0043 g). Also, the value of lysine content was significantly different from the others ($p<0.05$) (Fig. 2). This method is not suitable for determination of lysine content.

Method L1/20 was considered to be the most suitable method to determine lysine quantity in small amount of samples due to several reasons. Firstly, the quantity of sample required was the lowest (P: 0.0090, NP: 0.0065 g) among the other methods except

method L1/30 which was rejected. Secondly, there was no significantly difference ($p$>0.05) between the value of lysine content determined in sample A by this method (3.35±0.19 mg/g) and that determined by method Ori (3.36±0.09 mg/g). Finally, the reproducibility of this method was high. Thus method L1/20 was selected and tested on other rice samples (sample B - G). The results are shown in Fig. 4.

The values of lysine determined by both methods Ori and L1/20 for each sample were compared by the t-tests. In all samples, the lysine contents determined by method L1/20 were notsignificantly different from that of the method Ori ($p$>0.05). Besides, these two methods were compared by paired t-test on the values of lysine contents in different samples. Results showed that these were no significant difference between them ($p$>0.05).

One weakness of method L1/20 was that its CV was slightly bigger than the method Ori. Its average CV was 5.68% where for method Ori was 3.85%. However, this method is useful to determine the lysine content in the presence of low quantity of rice, especially for samples obtained from breeding experiments.

In conclusion, Method L1/20 with representative and reproducible results was shown to be suitable as an alternative of the Dye-Binding Lysine method for small quantity of rice. The sample weight of rice required for each trial is only 0.0155 g, which is much lower than the original method (1.2000 g) by 77 times.

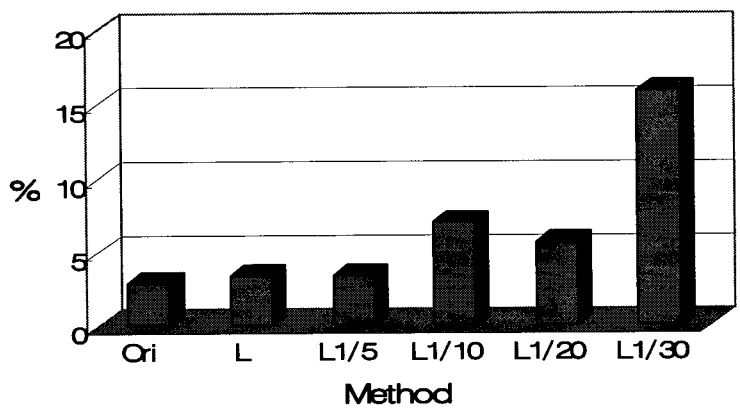



Fig. 3. Coefficient of variations of each of the methods (n=9)

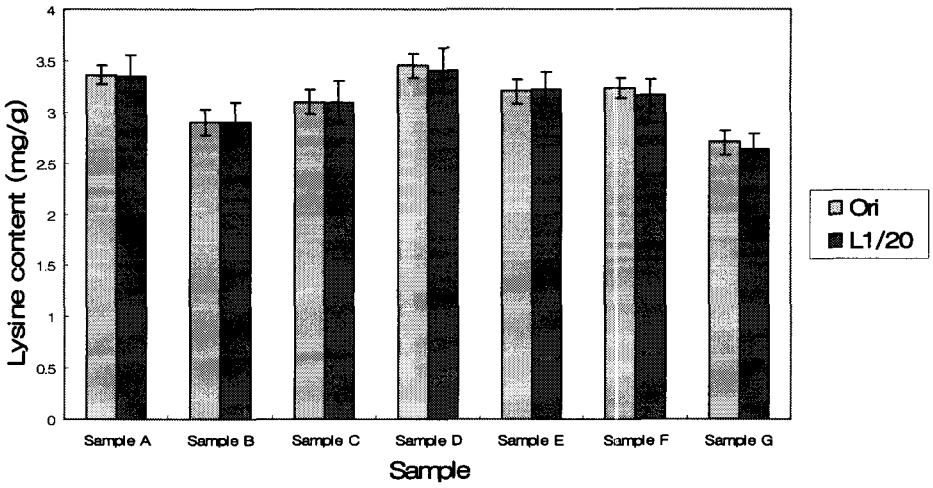



**Fig. 4. Lysine contents in the rice sample determined by the original and the modified DBL methods (n=9).**

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