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Analytical Methods

A practical method for extending the biuret assay to protein determination of corn-based products

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ABSTRACT

A modified biuret method suitable for protein determination of corn-based products was developed by introducing a combination of an alkaline reagent with sodium dodecyl sulfate (reagent A) and heat treatments. The method was tested on seven corn-based samples. The results showed mostly good agreement ($P > 0.05$) as compared to the Kjeldahl values. The proposed method was found to enhance the accuracy of prediction on zein content using bovine serum albumin as standard. Reagent A and sample treatment were proved to effectively improve protein solubilization for the thermally-dried corn-based products, e.g. corn gluten meal. The absorbance was stable for at least 1-h. Moreover, the whole measurement of protein content only needs 15–20 min more than the traditional biuret assay, and can be performed in batches. The findings suggest that the proposed method could be a timesaving alternative for routine protein analyses in corn processing factories.

1. Introduction

Corn is one of the most important crop produced worldwide. According to the latest statistics, China's corn production hit a record 224.58 million tons, and nearly one-quarter is processed to produce various products, such as starch, sweeteners, organic acids, fuels and feeds (Wang, 2016). The feed products including corn gluten meal, corn germ meal, distiller's dried grains and solubles, accounting for about 25 percent of the corn products, are utilized as important supplemental protein sources in animal diets. Protein determination of intermediate and end products as required by quality control management is a laborious routine work. Kjeldahl method and Dumas method are the international standard methods for the quantitative determination of crude protein in feed and food products based on digestion-titration and combustion, respectively. However, conventional Kjeldahl digestion is time-consuming (at least 1–2 h), and the Dumas has a high initial cost and the small sample size makes it difficult to obtain a representative sample. Furthermore, another disadvantage for both methods is a suitable nitrogen conversion factor depending on the type of protein is required (Dakin & Dudley, 1914; Figenschou, Marais, & De Figueiredo, 2000; Finete, Gouvêa, Marques, & Netto, 2013).

The biuret method, as a classical colorimetric method, is rapid and simple to carry out, and does not depend on the amino acid composition of the protein with a fine sensitivity. However, the accuracy achieved is sometimes not satisfactory for the thermally-dried corn-based products. Because drying with superheated steam or hot air may cause strong protein hydrophobic interactions and aggregations in the overheated portion, resulting in significant decreases of protein solubility and extraction efficiency (Malumba, Vanderghem, Deroanne, & Béra, 2008). Sodium dodecyl sulfate (SDS) can disrupt the interactions, but because SDS associates with potassium to cause a precipitation, it is not compatible with the traditional biuret reagent (Moore, DeVries, Lipp, Griffiths, & Abernethy, 2010; Watters, 1978). Therefore, sodium potassium tartrate in the biuret reagent could replace with sodium tartrate to improve the method, and extend the range of application (Watters, 1978). This modification was also adopted by the Markwell (a modified Lowry) and bicinchoninic acid (BCA) methods, which were based on biuret reaction but with higher sensitivity (Markwell, Haas, Tolbert, & Bieber, 1981; Walker, 2002). However, in our previous study, the low efficiency of protein extraction, and starch, fiber, oil and other nonprotein substances relating to a turbid assay solution were found to impede the application of the colorimetric methods to corn-based products. Furthermore, when compared with the biuret reaction, the Markwell and BCA reactions are dependent on the amino acid composition, and chromophores are unstable resulting in a large derivation in batch measurements. The biuret assay is thereby chosen and fur-

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ther developed in the present study to provide an alternative rapid protein determination method for corn or corn-based products.

2. Materials and methods

2.1. Materials

Seven samples of corn and corn coproduct were randomly collected from starch and ethanol plants. Thermally-dried corn gluten meal (CGlM), corn germ meal (CGeM) were obtained from COFCO Biochemical Industry Co., Ltd. Corn flour (CF) and distiller's dried grains and solubles (DDGS) were obtained from COFCO Bioenergy Co., Ltd. Cornflakes was obtained from C. Hahne Mühlenwerke GmbH & Co. KG. Food-grade powdery zein was obtained from Freeman Industries LLC. Bovine serum albumin (BSA) was obtained from Amresco (No. 0332). Other chemicals were of reagent grade obtained from Sinopharm Chemical Reagent Co., Ltd.

2.2. Preparation of reagents

A solution containing 2.0% SDS and 0.8% sodium hydroxide was prepared and named as sample treatment reagent (or reagent A). A modified biuret reagent containing 0.15% cupric sulfate pentahydrate, 0.49% sodium tartrate dihydrate and 0.75% sodium hydroxide was prepared according to Watters (1978) and named as Watters reagent (or reagent B) (Watters, 1978). The reagents can be stable for at least 3-month when stored in plastic bottles at room or refrigerator temperature.

2.3. Sample treatment

Samples (except for zein and CF) were ground with a pulverizer (Great Wall GW-400, Shanghai, China) at a speed of 28000 rpm for 0.5–1 min followed by passing through a 60-mesh (or smaller) sieve. Then, the sieved samples were dispersed in distilled water to obtain suspensions containing approximate 0.2–16 mg/mL protein. Before being subjected to protein assay, the particle size of sample granules in solution could be optionally further reduced by a homogenizer (IKA T25) at 6500 rpm for 1 min. Aliquots (1 mL) of the well-mixed suspensions were transferred into screw-top test tubes (with cap), then equal volumes of sample treatment reagent were added to reach about 0.1–8 mg/mL protein. The mixtures were heated in a boiling water bath for 10–15 min. During heating, vigorous vortexing was applied at 3 min intervals for 5–10 s. The heated mixtures with colloidal appearance (Fig. 1) were obtained and quickly cooled down to room temperature in water.

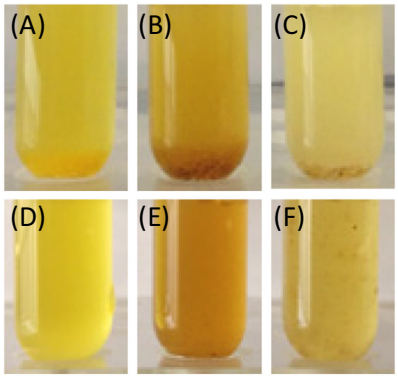

Fig. 1. Typical samples in reagent A before and after heat treatment. Samples of corn gluten meal (CGlM) (panel A and D), distiller's dried grain with solubles (DDGS) (panel B and E) and corn flour (CF) (panel C and F) were selected to present after mixed well with sample treatment reagent (A)–(C) and subsequentially heated in a boiling water bath for 10 min (D)–(F).

2.4. Protein assay

Aliquots (0.5 mL) of the cooled samples were mixed with 2.5 mL of Watters reagent. Because the use of detergent was reported to slow down the rate of color development (Watters, 1978), the incubation was carried out at 60 °C for 25 min. The colored samples were cooled to room temperature using a water or ice bath, and centrifuged at 1500*g* for 5 min to remove the insolubles. In the present method, when compared to Watters (1978), the concentration of SDS in the colored solution was reduced to 0.16% (about 6 mM) to give a better clarity, and corn proteins including zein were still soluble (Deo, Jockusch, Turro, & Somasundaran, 2003). The supernatants were filtered with 0.45 μm poly(ether sulfone) (PES) membrane, and the absorbances of filtrates were measured on an Unico Model 2800 UV/VIS spectrophotometer at 540 nm against a blank containing 0.5 ml of 1% SDS/0.4% sodium hydroxide and 2.5 ml of Watters reagent. In addition, because lipid could yield a cloudy reaction mixture, this substance was removed by addition of at least 0.6 ml petroleum ether and with thorough mixing before the centrifugation step, then the aqueous phase was subjected to filtration. To prepare standard curve, BSA, in amounts from 0.05 to 4 mg were dissolved in 0.5 ml of solution containing 1% SDS/0.4% NaOH, i.e. two-fold dilution of sample treatment reagent. Then the absorbance readings were obtained as described above. The protein concentration of each unknown sample was calculated by the standard curve with dilution factor.

When compared with the classical biuret assay, the proposed method introduced a preliminary protein extraction of the investigated materials with the Watters reagent-compatible solution (reagent A) and simultaneous heating treatment to effectively improve the solubility of proteins in the thermally processed corn products. Moreover, the incubation temperature during color development was increased to 60 °C. These modifications also brought about an enhanced accuracy of prediction on zein content using BSA as standard. The features were proved and elaborated below.

Kjeldahl nitrogen values were determined by the method of the AOAC. (2006). Protein contents were converted from the Kjeldahl nitrogen by the factor 6.24.

2.5. Evaluation of sample treatment in absorbance response

BSA (a standard protein) and zein (the major protein in corn) were individually dissolved in reagent A in the range of about 0.1–8 mg/mL with or without (as controls) heating, and the protein concentrations were determined by the proposed biuret method. In addition, the color stability of the proposed method was compared with that of bicinchoninic acid (BCA) method.

2.6. Evaluation of protein extraction

CGlM samples (with protein content of ~60%) were used as representative samples to investigate the effect of the proposed protocol on protein extraction. The samples were dispersed in distilled water at different protein concentrations of about 1 mg/mL, 4 mg/mL and 16 mg/mL. The following procedures were performed as described in Sections 2.3 and 2.4 until transparent filtrates were obtained, except that cupric sulfate pentahydrate was removed from Watters reagent. The protein concentrations were determined by the proposed method. Protein yields were calculated by dividing the biuret protein contents of filtrates by that of the solutions before centrifugation.

2.7. Statistical analysis

Unless specified otherwise, a minimum of three independent trials (n = 3) with triplicate sample analyses were performed. Data were subjected to analysis of variance using the general linear model's procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL). Tukey HSD all-pairwise multiple comparisons were performed to identify significant differences between individual means.

3. Results and discussion

3.1. Comparison between the proposed biuret and Kjeldahl methods

As seen in Table 1, the protein values by the proposed and Kjeldahl methods varied in a wide range of about 7–90%. The interassay coefficients of variations (CV) were calculated to evaluate the repeatability of the proposed method, the CV values were below 10% showing the method is reliable. Furthermore, the biuret proteins were normalized to the corresponding Kjeldahl proteins (the controls), which were given percentage values, and data were shown relative to controls. The variation ranged from approximate 98% to 112%. There was no significant difference ($P > 0.05$) observed except for corn steep liquor (CSL) ($P < 0.05$). For this sample, the biuret protein was 20% less than the Kjeldahl value, because oligopeptides and amino acids in CSL, as fermentation products in corn-steeping, lacked response to biuret reagent and diminished the chromophores (Foda, Salem, Hegazi, & Badr-Eldin, 1973). In addition, normalized data showed that the difference between both methods in protein contents of CF and cornflakes samples were slightly larger than that in the other samples, probably due to the interference by higher proportions of starch (Mitsuda & Mitsunaga, 1974). The regression line was fit in Fig. 2 to show relation between the two methods. The correlation coefficient (R = 0.997) was highly significant for the seven samples with the standard error of the estimate (SEE) 1.848. When CSL was remove from the fitting model, the value of R and SEE was improved to 0.999 and 1.382, respectively.

Additionally, it is common to evaluate the measurement system capability for quality control in manufacturing using the precision to tolerance ratio (P/T ratio). The P/T ratio is calculated as:

$$P/T = \frac{6SD}{USL - LSL}$$

where SD is the standard deviation of the measurement; USL and LSL are the upper and lower specification limits, respectively; USL–LSL denotes the specified tolerance. If the P/T ratio is low, the impact on product quality of variation due to the measurement system is small. For a particular product (with the same specified tolerance), P/T is positively correlated to SD. In the present study, the standard deviation of the proposed method was about 1.3–7.5 times higher than that of the Kjeldahl, showing much better performance for protein determination of CGlM and zein. Furthermore, if the proposed method would be applied to the product and process quality control in manufacturing, the removal of interfering substances in some products and the technical skills of operators should be further improved in order to reduce the SD.

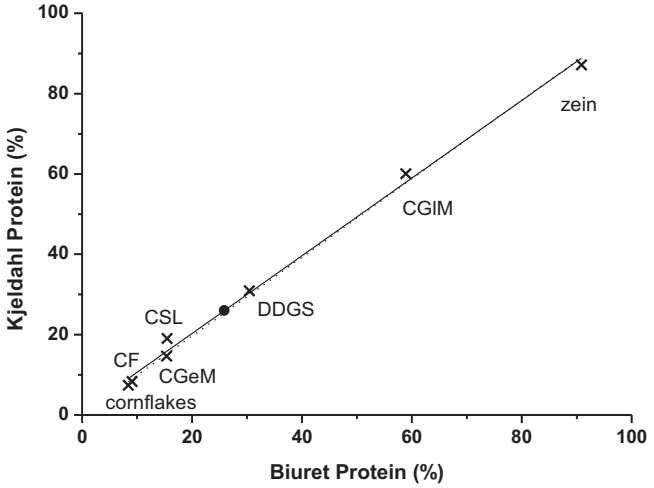

Fig. 2. Relation between biuret protein and Kjeldahl protein for corn-based products. Solid line: regression line for all samples (Y = 0.97X + 1.01, R = 0.997); dash line: regression line for the samples when CSL was removed from the model (Y = 0.98X + 0.08, R = 0.999).

3.2. Effect of sample treatment on protein determination

As seen in Fig. 3, without sample treatment the biuret absorbance value of zein was higher than that of BSA at the same protein concentration level, which was in agreement with the literature (Gornall, Bardawill, & David, 1949). Linear regression was used to obtain the correlation between the measures of zein and BSA with the same treatment. The correlation coefficient (R) of model was 0.988, and SEE was 0.363. When the sample treatment was performed, there was no evident alteration found in the response of BSA, whereas the absorbance of zein was interestingly decreased and approached to BSA. Moreover, the R-value of model was increased to 0.999, and the SEE of model was decreased to 0.078 indicating a significant improvement in the accuracy of corn protein determination when BSA standard was used. This phenomenon was probably due to the synergistic effect of SDS and heating on destabilizing secondary and tertiary structure of zein, leading to a modulated accessibility of some peptide bonds to

Table 1
The protein content in various corn products determined using the Kjeldahl and the proposed method.

Samples	Kjeldahl		Proposed method		Normalized values[b]
	Protein value[a]	CV	Protein value	CV	
CGlM	60.10 ± 1.07% (a)	1.78%	58.89 ± 1.64% (a)	2.78%	97.99%
CGeM	14.70 ± 0.43% (a)	2.94%	15.37 ± 1.05% (a)	6.80%	104.54%
CSL	19.10 ± 0.23% (a)	1.23%	15.46 ± 0.82% (b)	5.32%	80.92%
DDGS	30.97 ± 0.39% (a)	1.27%	30.42 ± 2.92% (a)	9.61%	98.21%
CF	8.39 ± 0.18% (a)	2.11%	9.08 ± 0.80% (a)	8.83%	108.18%
Cornflakes	7.43 ± 0.19% (a)	2.50%	8.38 ± 0.60% (a)	7.18%	112.78%
Zein	87.21 ± 2.15% (a)	2.47%	90.88 ± 2.80% (a)	3.08%	104.21%

[a] The values are expressed as the means ± standard deviations. Means with different letters in the same row differ significantly ($P < 0.05$).
[b] The normalized value was derived from the ratio of the mean biuret protein to the corresponding mean Kjeldahl protein.

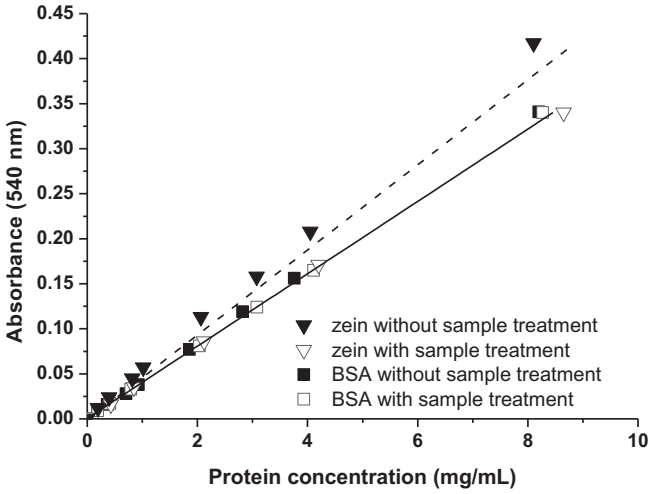

Fig. 3. Scatterplots of Biuret protein for a series of diluted BSA and zein (in reagent A) with or without sample treatment procedure. Linear regression was establish between protein content and absorbance for the samples within the same treatment. Dash line: regression line for BSA and zein without sample treatment (Y = 21.16X + 0.03, R = 0.988), Solid line: linear regression for BSA and zein with sample treatment (Y = 24.89X - 0.01, R = 0.999).

cupric ion during color development (Deo et al., 2003; Emmambux & Taylor, 2009). In addition, a trace amount of lipids associated with zein could also be released and removed by degreasing process, which resulted in an enhanced transparency of solution.

3.3. Effect of sample treatment on protein extraction

CGlM samples were employed to show the heating and dilution effects on protein yield. In Fig. 4, protein extractions were found to be time-dependent and reached a maximum in 10 min, and decreases occurred after 15 min, which were likely due to a heat-induced degradation intensified by SDS (Hodges & Hirata, 1984). Moreover, All these data together with the corresponding protein contents in Table 1, strongly suggested that the dense protein matrix of CGlM should be effectively broken down and exposed almost all proteins to the biuret reagents. In addition, the sample treatment could also help to release the starch granule-bound proteins by gelatinization, including the surface-localized and internalized proteins ranging from 0.3 to 1.0% (Mu-Forster et al., 1996) which may cause underestimation of biuret protein. Fig. 4 also demonstrated the differences in protein yields of the samples extracted at the same time were no more than 2% before 15 min, suggesting the dilution ratio did not significantly affect protein extraction.

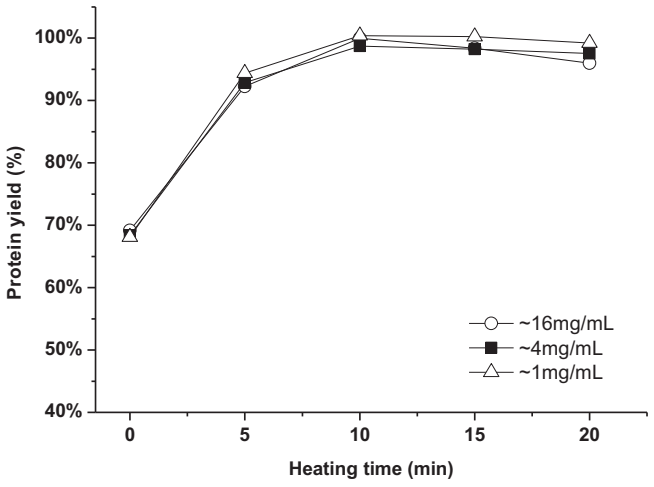

Fig. 4. Effect of heating time and dilution on protein yield for CGlM.

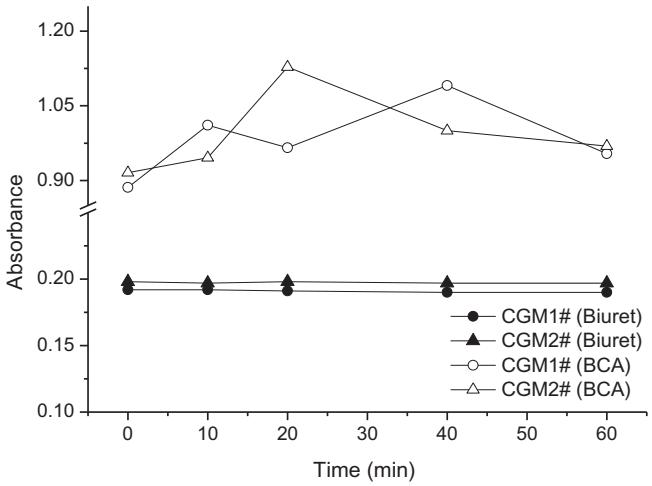

Fig. 5. Color stability of CGlM determined by the modified biuret method (540 nm) and BCA (562 nm) after color development.

3.4. Color stability

CGlM samples were used to further illustrate the color stability of the proposed method. The absorbances were occasionally recorded after color development (Fig. 5). The colors were found fairly stable for at least one hour when compared to the initial value (CV < 0.55%), thus it allows enough time to read the absorbance for a large quantity of samples. In addition, in our previous study, an attempt was also made to investigate alternative method such as the bicinchoninic acid (BCA) assay according to Walker (2002). However, the absorbances could only be stable in minutes and led to a large derivation between batches (CV > 8%) (Fig. 5).

4. Conclusion

On the basis of the experimental results, it can be concluded that the sample treatment is finely compatible with the modified biuret assay in protein determination for the most common corn based products. However, the proposed method could cause more deviation for such samples containing large proportion of amino acids, e.g. corn steep liquor because of the inherent deficiency of the biuret method. Moreover, the present method is relatively simple and rapid, and only requires conventional laboratory equipment, which could facilitate the protein determination for larger scale routine sampling inspection in corn processing factories.

Conflict of interest

The authors declare that there is no conflict of interest in the study and in the report described in the paper.

Acknowledgments

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