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SIMULTANEOUS DETERMINATION OF PROTEIN AND ESSENTIAL AMINO ACIDS IN BIOLOGICAL SAMPLES

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Abstract: The utilization of spectrophotometric methods for the determination and analysis of proteins is of great interest as they are generally simple, rapid and with high sensitivity. These protein assays have many uses in the food, plant breeding, pharmaceutical and biomedical industries, as well as in forensic science and a variety of other research fields. Among these, the biuret method is highly sensitive and is widely used in clinical and biological laboratories. However, the use of copper sulphate in the traditional biuret method of protein identification can lead to substantial interference from copper ions. In this framework, we modified the traditional biuret method by using an alkaline-alcohol reagent and insoluble copper phosphate; the protein-copper complex could also be evaluated at 545 nm. In addition, aliquots of the same solution are treated with specific reagents to determine two essential amino acids; tryptophan reacts with glyoxylic acid and lysine is treated with a ninhydrin-based reagent. Calibration curves must also be made at three wavelengths, with absorbance values in relation to the concentration of each component measured by standard methods. The advantage of this new approach is that it requires only 50 mg of ground seed meal to quantify three different components in each sample.

***Keywords*:** Protein determination; Tryptophan; Lysine; Ninhydrin; Spectrophotometry

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Introduction

Protein determination of foods and crop seeds is important for a variety of reasons.[1] For protein determination, spectrophotometric methods have advantages over other methods in terms of simplicity, speed, accuracy, specificity and sensitivity.[2] Thus, the benchmark assay of crude protein determinations, the micro-Kjeldahl method, is time-consuming.[3] Among the spectrophotometric techniques, the biuret method is very accurate and simple, and useful during protein separation procedures since there are fewer salt interference reactions than other procedures.[1,4,5] However, the measurement of the nutritive quality of food samples also require the normalization of essential amino acid content to that of protein. First, it is necessary to quantify the total (crude) protein content of food and seeds.[6] In addition, most of cereal species have low protein quality due to their small amounts of lysine and tryptophan.[7] Lysine and tryptophan can be determined by various methods. Generally, lysine is colorimetrically determined with 2-chloro-3-5-dinitro-pyridine,[8] and tryptophan using 0.1 M glyoxylic acid in 7 N sulfuric acid and 1.8 mM ferric chloride, the reaction time being 30 min.[9]

Since weighing ground seed samples is time-consuming and the sample quantity can be very small, the objective of this work is to develop an accurate, reliable, and inexpensive method for the analysis of plant nutritive quality. The assay is based on the simultaneous determination of protein, tryptophan and lysine in ground seeds. This is a combined method for nutritive quality evaluation based on the extraction of proteins with an alkaline alcohol solution under ultrasonic conditions, followed by the biuret absorbance measurement and the determination of essential amino acids, tryptophan and/or lysine, in the extract with suitable colour reagents.

Results and Discussion

The new version of the biuret micro-method. First, proteins in the seed flours were extracted by alkaline alcohol solutions and reacted with copper ions mobilized from insoluble copper phosphate powder to form the biuret complex. Second, volumes of biuret were treated with specific reagents for tryptophan and lysine and the absorbance of resulted coloured solutions measured in a spectrophotometer. Third, the content of protein and these two essential amino acids was calculated using calibration curves made with BSA and tryptophan or with some known values for tryptophan, lysine and pure or crude protein, determined by classical methods of analysis.

Absorbance at 545 nm of the biuret supernatants was proportional to the protein content of the samples (Figure 1). A close correlation was found between biuret absorbance of protein supernatants and BSA concentration, $r = 0.995^{***}$. The calibration curve was linear in the range 0-10 mg mL^{-1}, for which the following regression equation was calculated: $A_{545} = 0.1988 \cdot C + 0.032$, where A_{545} is the absorbance value and C, BSA concentration. BSA can be used as a standard for proteins extracted in biuret supernatants. However, the total protein content (crude protein) of 30 samples of maize (*Zea mays* ssp.) and 30 samples of beans (*Phaseolus vulgaris* L.) samples was measured by both the micro-Kjeldahl method and this new biuret method.

From the 30 maize samples with crude protein content values in the range 7.72% - 15.97%, determined by the micro-Kjeldahl method, several samples with low, medium and high biuret absorbance values were selected for the calibration curve (Figure 2). Although the correlation coefficient $r = 0.964^{***}$ was lower than for protein determination using BSA as a

standard, the results were more accurate because crude protein means pure protein plus free amino acids plus inorganic nitrogen and other nitrogen compounds in the samples.

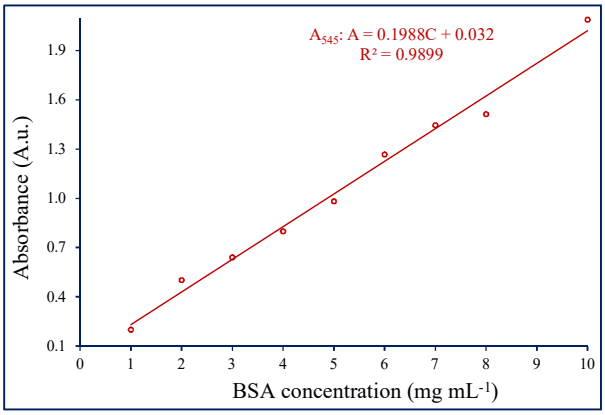

Figure 1. Calibration curve for protein determination using the new biuret variant.

In addition, the correlation coefficient decreases when the range of values is small (only 7.72-15.97%) and, in this case, the difference between samples is large. Of the 30 maize samples, 17 samples were QPM (Quality Protein Maize, containing *opaque-2* gene which increases the lysine content and decreases that of zein, a prolamin protein), 2 double hybrids, 5 trilinear hybrids and the rest inbred lines.

Real samples. We determined the absorbance of several opaque-2 and normal corn hybrid flours and found a high correlation between total protein (crude protein as determined by micro-Kjeldahl method) and the biuret absorbance values. The crude protein content of the 30 maize samples analysed by the biuret method proposed in this paper was positively correlated with lysine and tryptophan content (Figure 3). However, QPM varieties had a higher lysine and tryptophan content. Determination of the two essential amino acids in maize using biuret from protein analysis may allow identification of high quality maize varieties in breeding work.

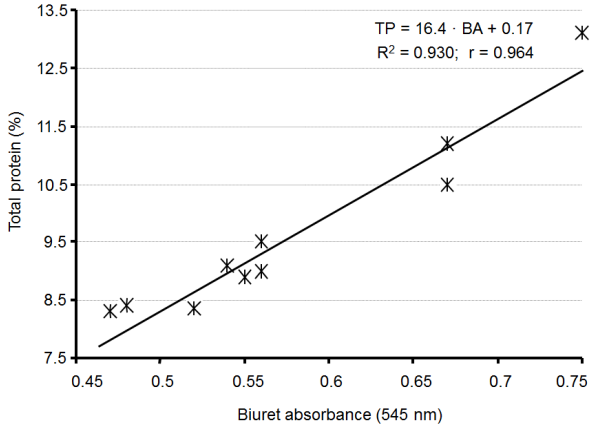

Figure 2. Relationship between the total/crude protein (TP, micro-Kjeldahl) content of some maize samples and the biuret absorbance (BA).

When using the Barnstein method for the determination of pure proteins (precipitation of pure proteins with copper sulphate in alkaline medium, removal of non-protein compounds and determination of protein concentration by micro-Kjeldahl), only the protein content is measured. We then compared pure protein values with biuret absorbance of the same samples. We found a better correlation between biuret absorbance and pure protein content in maize (not shown). We also examined 30 bean samples and assayed them by classical methods (micro-Kjeldahl, hydrolysis with papain followed by lysine determination with 2,4-dinitrochlorpyridine and tryptophan with glyoxylic acid)[8,9] and by the proposed combined method. To demonstrate the feasibility of our proposed method, we selected 10 biuret solutions with absorbance values between 0.465 and 0.795 A.U. and compared them with the protein content of these samples (23.00-30.90% crude protein), which was determined by micro-Kjeldahl procedure (Figure 4). Then, 1 mL of biuret reacted with the reagent for tryptophan and another one with the ninhydrin reagent for lysine. The results were compared with those obtained by classical methods (Figure 5).

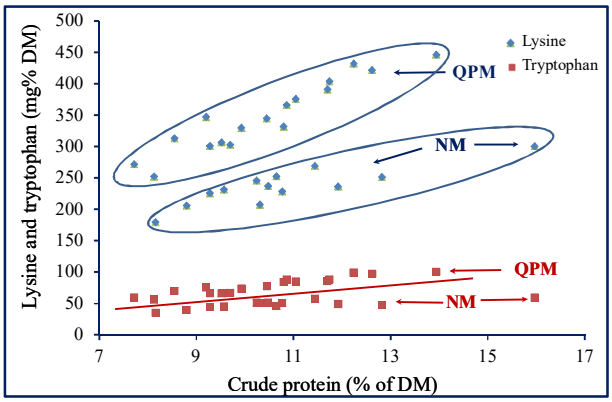

Figure 3. Relationship between crude protein and lysine as well as tryptophan in 30 maize samples.

Tryptophan and lysine determination. In general, there are highly significant correlations between lysine and tryptophan and a negative one between the content of the two amino acids found in proteins and that of crude protein. However, when reported the essential amino acids on a dry matter basis, samples containing higher amounts of proteins had higher levels of these two amino acids (Figure 3). In addition, the method indicated different values for tryptophan in *opaque-2* proteins from the normal maize proteins.

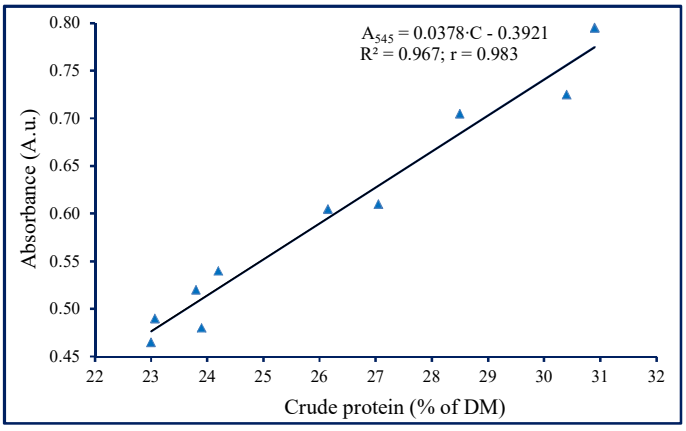

Figure 4. Calibration curve for the determination of protein in beans using values obtained by the micro-Kjeldahl method.

A close correlation was found between lysine and ninhydrin absorbance in bean samples (Figure 5a). However, a closer correlation was observed for tryptophan (Figure 5b). Thus, we found that proteins and amino acids in biuret react with ninhydrin to form red colored compounds, the absorbance of which is proportional to the lysine content of the seeds. Regression equations of the form $A_{515} = 0.0012 \cdot L - 0.5077$ and $A_{557} = 0.0012 \cdot T - 0.0229$ can be applied for lysine and tryptophan determination ($r = 0.963$ and $r = 0.970$, respectively). A very significant correlation was seen between lysine content of dried seeds of bean and that of tryptophan ($r = 0.983$). The regression equation was: $L = 1.1295 \cdot T + 697.7$, where L and T are the content of the two amino acids expressed as mg in 100 g of DM. Therefore, only tryptophan can be determined to evaluate the quality of bean, considering that it is dependent on these essential amino acids.

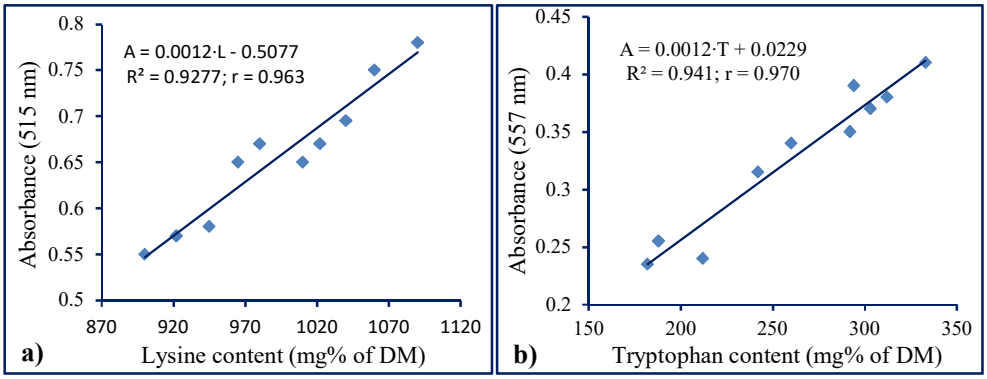

Figure 5. Calibration curves for the determination of a) lysine and b) tryptophan content of beans based on values obtained by classical methods.[8,9]

The best calibration curves for all the measured compounds were obtained using the values of a few representative samples, which had previously been analyzed with standard methods from literature. For example, a calibration curve is plotted for each series on a given day, followed by calculation of the protein content of each sample determined by

biuret. If the seed samples are defatted and stored for a longer period of time, then standard samples with representative values can be used which are introduced into each daily series of determinations for calibration.

A similar procedure is used for tryptophan and lysine determinations. Tryptophan can also be determined using a calibration curve with tryptophan solved in an alkaline alcohol solution in the concentration range 0-50 μg mL^{-1}.

Discussion. Recently, the biuret is the most recommended method for samples with high salt concentrations.[10] BSA assures metrological traceability to the biuret method for urine and serum total protein.[11] Nevertheless, in all experiments, the biuret reagent was prepared using 1.5 g of copper sulphate and 6 g of sodium potassium tartrate.[12] Thus, the total soluble protein content can be determined by a modified biuret method.[13] Glutelin content was also tested using the biuret method.[14] However, by using copper phosphate instead of copper sulphate, a highly selective and sensitive protein analysis based on biuret absorption in the ultraviolet region can be achieved.[15]

Experimental

Chemicals. All reagents used in this work were of analytic purity and the solutions were prepared using deionized water (18.2 MΩ cm) from a MilliQ system (Millipore, Bedford, MA). Bovine serum albumin (BSA, used as standard), tryptophan and lysine were purchased from Sigma–Aldrich (Saint Louis, Missouri, USA). Solvents used for extraction and degreasing of maize flours such as ethyl alcohol, acetone, petroleum ether, as well as reagents (copper sulphate, potassium hydroxide, sodium hydroxide) were purchased from Merck (Germany), and used without further purification. Insoluble copper phosphate was prepared by mixing equimolecular amounts of copper phosphate and dipotassium phosphate,

followed by washing the resulting precipitate with water for three times and drying to dryness. The biuret reagent (BR) consisted of an alkaline alcohol solution (AAS) and insoluble copper phosphate (CuP). The AAS was prepared by dissolving 20 g of KOH in approximately 100-200 mL of milliQ-grade water, adding 450 mL of absolute ethanol or 475 mL of 95% (v/v) ethanol and making up to 1 L with water. The reagent used for the determination of tryptophan (TR) in the biuret supernatant contained 270 mg FeCl3, 370 g pure sulphuric acid and 7.5 g glyoxylic acid dissolved in one litre of distilled water. The ninhydrin reagent (NR) used for the determination of lysine in the biuret supernatant was as follows: 400 mg ninhydrin and 400 mg of cadmium chloride were dissolved in 25 ml acetate buffer (pH 5.5) and made up to 100 ml with ethylene glycol.

Instruments. Plant kernels were ground to the desired granulation using a laboratory electric cereal mill (SAMAP Mod F100, Andolsheim, France) with adjustable millstones. Various particle-sized maize flours were screened with an analytical vibratory sieve shaker (Retsch, Germany). Ultrasonic extraction of proteins using the biuret reagent and the reaction of the extracted proteins with copper ions from copper phosphate powder were performed on an ultrasound bath cleaner (J.P. Selecta Ultrasons system, 40 kHz; Barcelona, Spain). Defatted maize flours (DMF) were obtained by Soxhlet Traditional Solvent Extraction (TSE) using petroleum ether as a solvent.[16] Centrifugation of eppendorf vials was performed at 10,000-18,000 rpm using a Hettich Mikro 22R centrifuge (Tuttlingen, Germany). The absorption measurements were performed with a Biochrom Libra S35 PC UV-visible spectrophotometer (Cambridge, England) in quartz cuvettes of 10 mm in the range from 200 to 700 nm. Absorbance was expressed as absorbance units (A.U.).

Biological material. Biological material consisted of seeds maize and been with approximately 12% moisture from the collection of Suceava Plant Gene Bank, Romania and Fundulea Plant Institute, Romania. Both *opaque-2* (QPM) and normal maize varieties have been analyzed.

Procedure. The plant seeds were ground to obtain fine flours and duplicates of 50 mg each of such flours were mixed with about 50 mg of copper phosphate powder. Next, 5 ml of alkaline alcohol solution was pipetted into each test tube. The mixtures were ultrasonicated for 30 minutes, followed by centrifugation at 15000 rpm for 5 minutes. The absorbance of the resulting supernatants was read at 545 nm and was proportional to the protein content of the samples. Tryptophan was determined by treating the biuret solution collected from protein determination with TR reagent, followed by absorbance reading at 557 nm; lysine content was measured at 515 nm, were the ninhydrin-amino acid dye absorbs.

Statistics. Results of triplicate measurements were reported on a dry matter or crude protein basis. The standard deviation (s), standard deviation of the mean (s_x), t parameters and the coefficient of variation, CV %, were calculated in order to compare the methods.

Conclusions

A new micro-method for the simultaneous determination of protein, lysine and tryptophan in food and ground plant seeds has been advanced. The traditional biuret reagent has been modified by replacing copper sulphate and Seignette salt with an alkaline alcohol solution and insoluble copper phosphate. The advantage of this new approach is that it requires only about 50 mg of biological material to quantify three different components in each sample. A highly significant correlation between lysine

and tryptophan was found in plant seeds. Although albumin, lysine or tryptophan can be used as standards, the best calibration curves for all measured compounds were obtained using the values of a few representative samples. The method has multiple advantages, being simple, costless, rapid and accurate; a single sample is weighed to determine both protein and essential amino acids (lysine and tryptophan) in the same biuret supernatant.

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