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Quantitative Determination of the Protein Content of Citrus Leaf Extracts: A Comparative Study

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Three spectrophotometric methods for the quantitative determination of proteins were compared taking as a reference the method of Kjeldahl. The abnormally high protein values obtained when these methods were applied to soluble extracts were decreased by using protective and precipitating agents. The method of M. M. Bradford (*Anal. Biochem.* **72**, 248–254 (1976)) gave values identical to the method of Kjeldahl when ethylenediaminetetracetic acid, 2-mercaptoethanol, and polyvinylpolypyrrolidone were added to the extraction medium and the soluble proteins were precipitated with trichloroacetic acid prior to protein determination.

The quantitative estimation of the protein content in plant materials is a daily problem at the plant sciences laboratories. Garfield (1) has shown recently in a bibliometric study dealing with the more-cited papers in the area of botany, including plant physiology and biochemistry, that the method of Lowry *et al.* (2) for the measurement of protein content has become the most-cited article during the last 7 years. However, it is well known that this and other currently used methods give abnormally high values of protein when applied to extracts from plant materials (3,4). This is due to interference produced directly by secondary products as well as to the interactions produced between proteins and several compounds such as vacuolar acids, carbohydrates, and especially phenolic compounds during the process of tissue homogenization (5–8).

Our aim has been to carry out a comparative study of three spectrophotometric methods, those of Lowry *et al.* (2), Bradford (9), and Flores (10), for the quantitative determination of proteins in soluble extracts of citrus leaves. We have used the method of Kjeldahl as a reference. We have also studied the effects produced on the amount of protein determined when we added to the extraction medium several protective agents: EDTA, EtSH,[2] and PVPP.

Citrus leaves have been selected because we observed in previous studies (11,12) the difficulties in making quantitative measurements of proteins in this plant material, probably due to their very high content of phenolic compounds. The results obtained could apply to other plants rich in phenolic substances.

MATERIALS AND METHODS

Plant Material

Leaves of citrus trees (*Citrus sinensis* L. Osbeck), 15 years old were selected from trees free of any apparent disease symptoms.

Chemicals

Coomassie blue G, PVPP, and BSA were purchased from Sigma. Bromphenol blue

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[2] Abbreviations used: EtSH, 2-mercaptoethanol; PVPP, insoluble polyvinylpolypyrrolidone; BSA, bovine serum albumin; TCA, trichloroacetic acid.

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and EDTA were from U.C.B. (Belgium), and tris(hydroxymethyl)aminomethane and EtSH from Fluka. All other products were of the highest purity available.

Extraction

Leaves of the penultimate flush were homogenized in a cold mortar with 19 ml/g of 50 mM Tris–HCl, pH 7.5. When indicated, the following additions to the extraction medium were made: 2 mM EDTA, 10 mM ETSH, and 2 g PVPP/g of fresh wt. Homogenates were centrifuged at 1000*g* for 5 min. The pellets were discarded and the supernatants centrifuged at 30,000*g* for 10 min. The supernatant of the last centrifugation was used as the soluble extract. All operations were performed at 4°C.

Precipitation of Soluble Extracts

Precipitation of proteins of the soluble extracts was accomplished by one of the following procedures. To 100-μl aliquots of soluble extract, either 800 μl of 10% TCA at 4°C or 200 μl of 0.75% uranyl acetate at 4°C was added. Alternatively, the soluble extract was boiled at 100°C for 2 min and then cooled to 4°C. In the three cases, the precipitate obtained after 15 min at 4°C was collected by centrifugation at 1000*g* for 10 min. The supernatant was discarded and 10 μl of 0.1 N NaOH was added to the pellet to facilitate its solubilization in the appropriate extraction medium.

Protein Determinations

Protein contents of soluble extracts were determined by the methods described by Lowry *et al.* (2), Bradford (9), and Flores (10). We used the method of Kjeldahl with some modifications (13,14) as the reference method. All the methods used gave very reproducible results as described previously by the authors. BSA was used as standard protein. We adjusted its concentration spectrophotometrically at 280 nm according to $\epsilon_{280}^{1\%} = 6.6$ (9,15).

Standard Assay of the Modified Method of Kjeldahl

To a 5-ml aliquot of soluble extract, 40 ml of 10% TCA was added. This mixture was shaken and kept at 4°C for 2 h and then centrifuged at 1000*g* for 10 min. The supernatant was discarded and the pellet washed with 15 ml of 10% TCA and centrifuged again as described above. The supernatant was discarded and the sediment dissolved with 1.5 ml of 0.1 N NaOH and diluted to 5 ml with distilled water. For the *digestion* of the 5 ml of solution obtained, 2 g of potassium sulfate, 0.4 g of potassium oxalate, and 5 ml of 97% H_2SO_4 N-free, were added. The mixture was heated until it was transparent. At this point, the temperature was raised to the boiling point of the mixture and heated for 30 min to complete the digestion. The solid residue was cooled to room temperature and dissolved with 2 ml of distilled water. Then 25 ml of 40% NaOH was added and the solution was distilled by means of a standard Kjeldahl apparatus. One hundred milliliters of distillate was collected over 15 ml of the solution of boric *acid–indicators* (see below). Titration was carried out with 0.01 N H_2SO_4. From the nitrogen content, the protein content of soluble extract is calculated by the use of the factor 6.25 (16).

Solution of indicators. A 0.33-g amount of Bromocresol Green and 0.165 g of Methyl Red were dissolved in 500 ml of ethanol.

Solution of boric acid–indicators. Forty grams of boric acid was dissolved in 800 ml of distilled water. Then, 20 ml of *the solution of indicators* was added, the pH was adjusted to 5 with NaOH, and the volume was completed to 1 liter with distilled water.

RESULTS AND DISCUSSION

Effect of Precipitating Agents on the Amount of Protein Determined

Three precipitating procedures were used: by means of TCA or uranyl acetate and boiling the extracts at 100°C for 2 min. Their effectiveness, judged by the absorbance at

TABLE 1

EFFECT OF PRECIPITATING AGENTS ON THE AMOUNT OF PROTEIN DETERMINED IN CITRUS LEAF EXTRACTS

Method	Without precipitation	Precipitating agent		
		TCA	Boiling	Uranyl acetate
Lowry[a]	164.16	37.50	38.42	53.08
Flores[a]	35.92	18.68	18.65	15.79
Bradford[a]	24.27	9.99	6.53	10.54

[a] Values are given in micrograms equivalent to BSA per 50 μl of citrus leaf extract and are the means of three experiments with very similar results.

280 nm, was 95.4, 94.1, and 87%, respectively, using BSA as standard protein.

Large differences were observed between the apparent content of protein determined before and after precipitation with the three methods as shown in Table 1. None of the methods was adequate for the determination of protein content in soluble extracts. The method of Lowry showed the largest interference. In this respect, we agree with Loomis (17), who concluded that the determination of protein content in plant extracts that are rich in phenolic compounds is greatly distorted if the Lowry method is used. Attempts to overcome this problem working with preparations from citrus fruits have been done by Potty (4). This author improved the results obtained with the Lowry method by measuring the phenols in the absence of copper. However, the effectiveness of the proposed method is limited to tests containing less than 40 μg of phenol. To apply his method when phenol contents are higher than 40 μg, he proposes to precipitate the proteins prior to their determination with 67% ethanol, a concentration that proves to be effective when tested with ovalbumin as standard protein. A more extensive study would be necessary to establish the validity of this method for complex mixtures of proteins from plants. Our results also agree with those of Robinson (18) working with the method of Bradford. It seems that the interference produced by

TABLE 2

EFFECT OF PROTECTIVE AND PRECIPITATING AGENTS ON THE AMOUNT OF PROTEIN DETERMINED IN CITRUS LEAF EXTRACTS

Method	Treatment	Without additions	With additions				
			EDTA	EtSH	PVPP	EDTA EtSH	EDTA EtSH PVPP
Lowry[a]	Without precipitation	6.96	6.46	7.12	6.56	6.68	6.57
	Precipitated with TCA	1.55	1.39	1.57	1.52	1.42	1.32
Flores[a]	Without precipitation	1.63	1.45	1.36	1.32	1.16	1.10
	Precipitated with TCA	0.81	0.74	0.70	0.71	0.61	0.57
Bradford[a]	Without precipitation	0.93	0.77	0.80	0.61	0.60	0.50
	Precipitated with TCA	0.37	0.33	0.34	0.28	0.29	0.27
Kjeldahl[a,b]	Without precipitation	0.38	—	—	—	—	—
	Precipitated with TCA	0.27	—	—	—	—	—

[a] Values are given as gram equivalent to BSA per 100 g of fresh leaves and are the means of three experiments with very similar results.

[b] Adequate controls to show if homogenization with EDTA and insoluble PVPP may contribute TCA-precipitable nitrogen have been carried out. Values of protein content of samples extracted in absence or presence of EDTA and PVPP are practically identical.

phenolic compounds using this method is smaller than that observed with the Lowry method (19,20).

Effect of Protective and Precipitating Agents

A decrease was observed in the abnormally high amount of protein measured by the three spectrophotometric methods if protective agents such as EDTA, EtSH, or PVPP were added to the extraction medium. The decrease of the interference was maximum when EDTA, EtSH, and PVPP were used together, although the presence in the extraction medium of the mentioned agents was not enough to avoid the interference. On the other hand, procedures such as filtration of the extracts under vacuum (Millipore membrane immersible CX, exclusion pore 10,000 daltons) or dialysis were similarly ineffective in totally avoiding the interference.

Using the method of Kjeldahl as a method of reference, we found (Table 2) that a combination of a precipitating agent (10% TCA) and protective agents (2 mM EDTA, 10 mM EtSH, and 2 g of PVPP/g of fresh wt) was necessary in order to avoid the interference in the determination of the protein content in citrus leaf extracts. This was accomplished by using the method of Bradford. In this sense our results expand on the previous recommendation of Robinson (18) to use the method of Bradford especially in the case of woody plants that are rich in phenolics. We think that such a procedure could be useful in quantitatively measuring protein contents in plant materials when the main problem is the presence of high amounts of phenolic compounds.

With similar procedures the methods of Flores and Lowry still gave protein contents 2.1 and 4.9 times those of the method of Kjeldahl.

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