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Determination of copper in different chloroplast preparations

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Abstract

The determination of total Cu is not often correlated with states of deficiency in plant material. This fact makes it necessary to look for biologically active Cu. Suspensions of thylakoid membranes and photosystem II particles, properly diluted with 13 mM nitric acid, were used for this purpose. The presence of a minute quantity of an antifoaming agent, such as 1-octanol, is essential when an aliquot of the slurry is injected into the graphite furnace of the atomic absorption spectrophotometer. Good agreement was obtained between our results and those obtained by a classical dry combustion method. Reproducibility was better than 5% when expressed as relative standard deviation.

Introduction

Copper is an essential microelement for the development of algae and higher plants (Barón and Sandmann, 1988), yet analytical results for total foliar copper are rarely correlated with Cu deficiency in higher plants. This points to the existence of biologically inactive Cu in leaves .It is therefore necessary to determine biologically active Cu to overcome the problems of the analytical diagnosis of its deficiency (López Gorgé et al., 1985).

The total concentration of leaf Cu ranges between 1 and 15 μg^{-1} dry weight. The main pool of active Cu is located in the chloroplast, with 50% linked to plastocyanin, a blue Cu containing protein which plays a role in photosynthetic electron transport as the electron donor of photosystem I (PS I). However, total chloroplast Cu content was found to be two to four times higher than that of plastocyanin (Droppa and Horvath, 1990). The physiological role of this 'extra Cu' is not known, and some authors consider it to be associated with photosystem II (PS II) (Barón et al., 1990, 1992; Sibbald and Green, 1987)

The goal of our study was to search for Cu- binding sites related to PS II in chloroplasts. The Cu content of thylakoid membranes and PS II enriched particles was determined to analyze the contribution of the different pools of active Cu in the chloroplast and to use this parameter for the diagnosis of Cu deficiency in higher plants. From the analytical point of view two main problems were encountered: The very small quantity of material available for analysis and the need to simplify sample preparation to avoid contamination. Both problems could be solved by the use of flameless atomic absorption spectrometry, which allows the injection of a sample suspension (slurry) into the graphite tube. The use of these slurries in electrothermal atomic absorption spectrometry (ETAAS) has been widely reported since its description for the determination of lead (Brady et al., 1974). We have used it previously for the determination of Cu in plant materials (Lachica and Mingorance, 1984), since it has the advantage of direct samples injection into the graphite tube by conventional procedures (Stephen et al., 1985).

Materials and methods

A Perkin-Elmer model 503 atomic absorption spectrophotometer equipped with a HGA-400 graphite furnace was used.

All the reagents were of analytical-reagent grade. High-purity deionized water in quartz was used. Working standard solutions were obtained by diluting a standard stock solution of Titrisol from Merck (1.000 g Cu L^{-1} in water). All the labware was washed in detergent solution, rinsed with water, soaked in 25% (v/v) HNO_3 and thoroughly rinsed with deionized water. Treatments in detergent and nitric acid solutions were carried out by sonication for 30 min.

Plants

Pea *(Pisum sativum* L., cv. Lincoln) plants were grown hydroponically in a Hewitt full nutrient solution in a growth chamber for 4 weeks (Barón and Sandmann, 1988).

Spinach *(Spinacea oleraceae* L.) was obtained from a local market. Thylakoid membranes and PS II particles were isolated according to Berthold et al. (1981) as modified by Ford and Evans (1983). Leaves were ground in 50mM sodium phosphate pH 7.4, 5 mM $MgC1_2$ and 300mM sucrose at 0°C for 15 s up top speed (Sorvall Omnimixer), the homogenate was filtered, and after 4 washes followed by centrifugation, a thylakoid pellet was obtained. This pellet was resuspended in a stock buffer (50 mM Mes pH 6.5, 400mM sucrose, 5 mM $MgC1_2$ and 15 mM NaCI) and used for the measurements of the Cu content of thylakoid membranes. For the isolation of PS II particles, Triton X-100 in a ratio 25:1 (detergent/chlorophyll, w:w) was added, and the suspension was stirred for 25 min and

centrifuged at 40 000 g for 25 min. The resulting pellet was suspended in the stock buffer as described, and represented the PS II particles used.

Sample preparation

The slurry was shaken vigorously in a Vortex mixer for 1 min. Aliquots of 25 µL were then pipetted into a 2 mL test tube. A known volume of 13mM HNO_3, adapted to the sample Cu concentration, as well as 5 µL 1-octanol were added to the slurry and mixed in a Vortex mixer. Thylakoid membranes were previously treated with a tissue homogenizer.

A modification of the Comité Inter-Institus d'Etude des Techniques Analytiques de Diagnostic Foliaire procedure (C.I.I., 1969) was used as reference for the determination of the accuracy of the proposed method: 50µL slurry were poured into a platinum microcrucible, dried at 105°C and ashed at 450°C for two hours, cooled and the residue solubilized in diluted HCL.

Instrumental analysis

The instrumental parameters used are given in Table 1. Manually inject 20-µL well agitated (with Vortex mixer) blank, standard or sample into the graphite furnace. Perform at least 2 measurements for each blank, standard and sample.

Table1. Instrumental parameters for electrothermal atomic absorption spectrometry.

Atomic absorption spectrophotometer				
Analitical wavelength	324.7 nm			
Slit width	0.7 nm			
Deuterium background correction	yes			
Mode	peak height			
Graphite tube	normal			
Gas	nitrogen			
Sample injection volume	20 µL			
Graphite furnace program				
step	Temp.(ºC)	Ramp (s)	Hold (s)	Gas flow (mLs^{-1})
Dry	100	20	10	300
Dry	130	10	10	300
Char	900	10	10	300
Atomize	2250	0	3	stop
Cleaning	2650	1	3	300

Results and discussion

Sample preparation

Previous homogenization of thylakoid membrane samples

The difference between PS II particles and thylakoid membranes made a pretreatment of the latter necessary due to their non-uniform particle dispersion even after mechanical shaking and especially after having been frozen. After defrosting, the very coarse thylakoid membranes may give rise to volumetric errors from pipetting and to variations in the size and number of particles sampled. Occasionally, the micropipet was clogged. Some of the problems related to slurry heterogeneity may be overcome by treating the whole sample with a tissue homogenizer for about one minute. The errors can thus be minimised by working with smaller particle sizes and narrower particle-size distributions, as pointed out by Holcome and Majidi (1989). The results presented in Table 2, for homogenized and non-homogenized samples, showed a significant difference between the 2 blocks and a higher relative standard deviation (RSD) for the non-homogenized samples.

Table2. Comparison between homogenized and non-homogenized spinach thylakoid membranes[a].

	Thylakoid (ng mL^{-1})
Homogenized	1139 – 1217 – 1178 – 1148 – 1178 - 1227 – 1227 – 1286 – 1109 – 1276 - 1129
	Mean = 1192 RSD = 4.96 %
Non-homogenized	1453 – 1513 – 1513 – 1266 – 1227 – 1306 – 1306 – 1217 – 1257 – 1276 – 1286 - 1207
	Mean = 1319 RSD = 8.39 %

[a] Each result is the average of 3 determinations.

Addition of 1-octanol

When slurries of well-homogenized thylakoid membranes or PS II particles diluted with 13mM

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HNO_3 were used, reproducibility was very poor. For some duplicate injections, there was either a very low signal or no signal at all. However, there were no problems with the

synthetic solutions used for calibration. This differential behaviour between samples and standards indicated that the problem came from the composition of the samples. In fact, the simple observation of the graphite tube port could account for the situation when a very low signal was obtained. Due to the surface tension of the slurry the droplet remained suspended in the inner part of the port. On starting the furnace program, the gas flow ejected part of the sample and a very low signal or even no signal was recorded. The high surface tension of the slurry was related to the nature of the material and also to the presence of a wetting agent added during the preparation of the material for analysis. In addition, some foam was produced, introducing air bubbles and making very difficult a reproducible sampling of the slurry (Fuller and Thompson, 1977). A similar difficulty had been described by Temminghoff (1990) when sample plus a matrix modifier, separated by a small amount of air, are injected together. To overcome these difficulties, a very small amount of 1-octanol was added to decrease the surface tension of the sample. The data presented in Table 3 show an almost doubled RSD for sub- samples without 1-octanol. This problem had previously pointed out by Hutchinson et al. (1986). Two subsamples were prepared from a unique suspension of thylakoid membranes and each subsample was used for 12 different injections. The concentration of 1-octanol was not critical, since it was shown that foam and air bubbles disappeared with its mere presence

Table3. Effect of the presence of 1-octanol on pea PS II particles.

	Thylakoid (ng mL^{-1})
Whithout 1-octanol	0.116 – 0.120 – 0.121 – 0.110 – 0.127 – 0.130 – 0.117 – 0.117 - 0.105 – 0.123 – 0.114 – 0.102 Mean = 0.117 RSD = 7.10 %
With 1-octanol	0.119 – 0.122 – 0.126 – 0.125 – 0.133 – 0.124 – 0.121 – 0.125 - 0.124 – 0.122 – 0.130 – 0.118 Mean = 0.124 RSD = 3.44 %

Addition of nitric acid

The use of HNO_3 not only facilitated the extraction of Cu into the slurry medium (Bendicho and de Loos-Vollebregt, 1991), but it was also useful as a matrix modifier (Miller-Ihli, 1992). A highly dilute acid solution was used to protect the graphite tube against rapid deterioration.

Evaluation of the method

The reliability of the method was assessed by determining Cu in thylakoid membranes with the proposed method and with that recommended by the C.I.I. (1969) for plant materials, without removing silica since it is not present in this material. The results (Table 4) showed that the agreement between the two methods was better than 90%.

Table4. comparison of methods using pea thylakoid membranes.

Method	ng mL^{-1}[a]
C.I.I.	187 ± 5
Proposed	170 ± 8

[a]Mean of 4 results

The reproducibility of the method was tested by analyzing six replicates of a PS II particle sample. Statistical analysis of individual results (576; 554; 523; 584; 526; and 561ng mL^{-1}) gave 4.55% for the RSD.

The method proposed is thus quite reliable, in addition to its simplicity and rapidity. From the biochemical and physiological points of view, the localization of new Cu-binding sites in the chloroplasts could allow the correlation between analytical results (pools of "active Cu" in photosynthesis), biochemical parameters (activity of electron transport) and physiological aspects (diagnosis of a Cu-deficiency or toxicity status of the plant). We have demonstrated previously (López Gorgé et al., 1985) a close relationship between the pattern of Cu content of thylakoids and the PS I-dependent electron transport rate, as a function of Cu concentration in the medium. Similar experiments in relation to PS II are in progress.

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