Amperometric Titration Largely Overestimates Chloride Concentrations in Chloroplast Extracts

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Determination of chloride contents in aqueously isolated chloroplasts from spinach by amperometric titration indicated chloride concentrations of 60 to 100 mmol l^{-1} (based on a chloroplast volume of 25 µl mg^{-1} chlorophyll). However, when analyzed by anion chromatography, chloride contents in chloroplasts were much lower (1–8 mmol l^{-1}).

In leaf extracts chloride concentrations obtained with both methods were rather similar. Boiling of chloroplast extracts prior to chloride titration reduced indicated chloride contents by a factor of three or four, but did not change results obtained with anion chromatography. It is concluded that chloroplasts contain large amounts of silver complexing agents giving rise to a drastic overestimation of chloroplast chloride concentrations when measured by amperometric titration. Boiling and centrifugation of extracts apparently precipitates these compounds only insufficiently.

Introduction

Knowledge of anion concentrations, and especially of chloride concentrations in extracts from plant material is of large interest to plant physiologists in general, but especially to those working in the field of salinity research. Whereas atomic absorption spectroscopy provides a simple, specific and reliable procedure for cation analysis, until now anions have been measured by a number of different amperometric or colorimetric procedures, which are laborious and rather insensitive. In earlier work on ion distribution between chloroplasts and extrachloroplastic space in spinach leaves, we and others have originally reported chloroplastic chloride concentrations of up to 80 mmol l^{-1} compared to about 10 mmol l^{-1} in leaf extracts [1–3].

This puzzling result was obtained by amperometric chloride titration, and prompted us to assume a chloride accumulation mechanism at the chloroplast envelope or at the mesophyll tonoplast [1]. The result was the more surprising, since under salinity leaf chloride contents increased drastically up to several hundred millimolar, whereas chloroplastic chloride concentrations remained more or less constant [1, 3 4]. Meanwhile we have intensively applied anion chromatography as an excellent tool for anion analysis in plant extracts. Surprisingly we found now, that chloride contents in chloroplasts appeared to be at least one order of magnitude lower than indicated by amperometric titration, and thus quite in the range of chloride concentrations in whole leaf extracts. This discrepancy was investigated in more detail, and results are presented below.

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Materials and Methods

Plant material

Spinach *(Spinacia oleracea* v. "Yates") was grown in soil culture in a green house with additional light sources (HQi-lamps 400 w, Schreder, Winterbach, FRG) at an average light intensity of 130 w m^{-2}, with a day/night cycle of 11:13 h. Daytime temperatures were 20 to 25 °C, night temperatures 10 to 15 °C, with 30% to 75% relative air humidity.

Leaf extracts

Freshly harvested leaves were washed with distilled water and blotted with filter paper. After weighing, leaves were frozen in liquid nitrogen and ground to a fine powder. Water soluble compounds were usually extracted by boiling about 1 g of leaf material in 5 ml of distilled water. For some experiments, extracts were used unboiled. After centrifugation at high speed, aliquots of the clear supernatant (1 ml) were filtered through 0.45 µm micro-membrane filters (Acro LC3A). After suitable dilution they were used for analysis of anions by isocratic anion chromatography (IC 1000, fitted with a conductivity (BT 0330) and UV-detector (BT 3030), automatic sample injector (BT 7041; Biotronik, Maintal, FRG) and integrator (C-R1B; Shimadzu)). When plant ex-

tracts with large contents of phenolic compounds were to be analyzed (e.g. from pine needles), extracts had to be pretreated with purified insoluble polyvinylpyrrolidon in order to prevent irreversible damage of separation columns. Further, it proved necessary to use small precolumns filled with a mixture of neutral resin and anion exchange resin. Precolumns were changed after about 50 to 150 samples. Amperometric titration was carried out with an Aminco solide state chloride titrator (Aminco, Silver Springs, USA).

Chloroplast were isolated by a fast single step density gradient centrifugation method as previously described [5]. After determination of the chlorophyll content [6] and of intactness [7], the chloroplasts were lysed by addition of 4 ml of distilled water to 1 ml of the isotonic, "salt-free" suspension. After spinning down the chloroplasts, the clear supernatant was used after proper dilution for anion analysis as described above. For comparison, parts of the extracts were boiled in a water bath for about 10 min, and after spinning down the precipitate at $16000 \times g$ (5 min), the supernatant was again used for analysis.

Results

Until now, anion chromatography has been rarely used for analyzing plant extracts (compare [8, 5]). Therefore we will first present some original chromatogramms from a standard and from spinach leaf and chloroplast extracts (Fig. 1, A–C) and discuss some basic methodical problems. Plant extracts are very complex mixtures of organic and inorganic solutes. Major soluble anions which are present in significant amounts and which can be well separated by anion chromatography are chloride, nitrate, phosphate and sulfate as inorganic anions, and hexosemonophosphate, succinate, malate, malonate, tartrate, oxalate, fumarate and ascorbic acid. Malonate elutes closely after malate, but this is not shown in Fig. 1. Citrate usually elutes only very slowly and does not interfere with determination of other anions. Acidic amino acids are not separated. Fumarate and ascorbic acid usually co-chromatograph with oxalic acid. In such a case, a coupling of UV-detection and conductivity detection systems is advantageous, since fumarate, but not oxalic acid strongly absorbs at 210 nm. The same holds for NO_3, which can be well measured by its absorption on 210 nm, even in presence of large amounts of malate, which at high malate/nitrate concentration ratios can interfere with conductivity measurement. A further problem is posed by the fact that aqueous leaf and chloroplast extracts always contain a group of compounds eluting right at the beginning of the chromatogram within the range of the fluoride peak (Fig. 1, B, C). In contrast to fluoride, these compounds (usually about 3 to 5 separate peaks) were highly UV-absorbing. They consist most probably of aromatic compounds. Due to their short retention time, they pre-

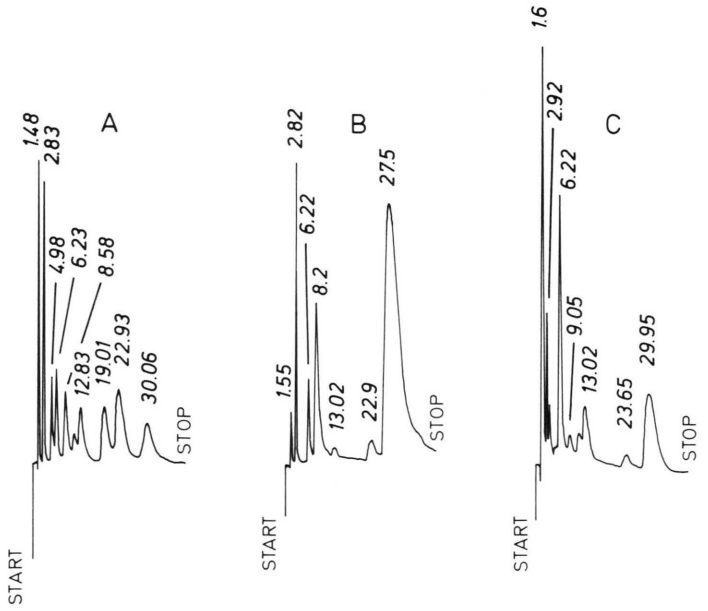

Fig. 1. Original chromatogramms from a standard (A), from a spinach leaf extract (B) and from a chloroplast extract (C) measured by ion chromatography. The following anions were separated and identified, and are listed according to their elution sequence (retention times in min. are shown in the figure as numbers on the peaks and are given here in brackets after each ion): F^- (1.49); Cl^- (2.83); hexose-p^- (4.99); PO_4^{2-} (6.23); NO_3^- (8.58); succinate (11.16); malate (12.83); tartrate (19.01); SO_4^{2-} (22.93); oxalic acid (30.06). In the standard chromatogramm, all ions were 0.1 mmol l^{-1} each.

vent a reasonable determination of F^- in plant extracts. Despite of these problems anion chromatography is excellently suited for measuring anion concentrations in aqueous plant extracts.

Chloride determination by anion chromatography and by conventional amperometric titration lead to very different results in some cases. In Table I the chloride content of unboiled and boiled spinach leaf extracts as obtained with amperometric titration is compared with chloride measurements by anion chromatography in the same extracts. Generally, chloride contents in leaves were about 12 to 15 μmol g^{-1} FW. Results obtained with both methods were rather similar, and boiling of extracts caused only a minor reduction of indicated chloride contents.

With chloroplast extracts, the situation was very different: In unboiled extracts, amperometric titration gave chloride contents in the range from 39 to 64 μmol g^{-1} FW. Boiling of extracts decreased these values drastically by a factor of 4 or 5. An internal chloride standard (1 mmol l^{-1}) was not changed by boiling and protein precipitation (Table II). Also, a calibration curve obtained by amperometric titration with the same procedure was perfectly linear in the range from 0.01 to 1 mmol l^{-1}, when KCl was added to a chloroplast extract (not shown here).

Chloride contents obtained by anion chromatography of unboiled extracts were lower by a factor of 10 or 15, compared with amperometric titration results. Boiling caused only minor changes in chloride contents obtained by anion chromatography. Thus, the low values of 2 to 7 μmol chloride g^{-1} FW appear to reflect the real chloride concentration existing in spinach chloroplasts. This is much less then published earlier by us and others [1–3].

Table I. Chloride concentration in spinach leaf extracts (μmol g^{-1} FW) and chloroplast extracts (mmol l^{-1}) as determined by amperometric titration or ion chromatography. Chloride concentrations in chloroplasts are corrected for intactness and based on an average chloroplast volume of 25 μl per mg chlorophyll.

Exp. No.	Unboiled Amperometric	IC	Boiled Amperometric	IC
		Leaf extracts		
1	14.60	14.96	14.65	12.21
2	14.02	12.84	13.49	12.98
3	14.17	13.63	11.66	13.63
		Chloroplast extracts		
1	38.68	7.09	9.34	6.12
2	62.80	3.97	11.72	6.03
3	63.16	3.98	16.21	4.22

Table II. Detection of an internal standard (1 mmol l^{-1} chloride) in a chloroplast extract (means from two determinations).

	Cl^- (mmol l^{-1}) Amperometric	IC
unboiled extract	1.04	1.08
boiled extract	0.92	1.10

The reason for the overestimation of chloroplastic chloride contents by amperometric titration is yet unclear. Apparently, chloroplast extracts contain compounds complexing or reducing silver ions which will deceive high chloride contents. The nature of these compounds is still unknown. Bovine serum albumine (BSA) at concentrations of up to 50 mg ml^{-1} (which is about tenfold the protein concentration in chloroplast extracts) gave only a small chloride signal at the chloride titrator. The signal was not changed by boiling of the protein solution. Thus, it presumably reflected a contamination of BSA with chloride (not shown). It is known that chloroplasts contain large amounts of acidic amino acids, mainly aspartate and glutamate [9], which are potentially complexing agents for divalent metal ions. However, even at concentrations exceeding those in chloroplast extracts by a factor of 1000, glutamate and aspartate didn't give rise to a chloride signal at the titrator. Chloroplasts also contain considerable amounts of reducing compounds such as ascorbate or glutathion. Ascorbic acid at concentrations of up to 10 mmol l^{-1} gave no signal at the chloride titrator. Glutathion, however, was indicated with a similar sensitivity as chloride in the concentration range from 0.1 to 5 mmol l^{-1} (not shown). Thus, it is believed that compounds carrying -SH-groups are present in chloroplast extracts in concentrations giving rise to completely erroneous chloride determinations.

Conclusions

Our results demonstrate that conventional amperometric tritration is not suited for determining chloride contents in chloroplast extracts. Possibly, this holds also for other biological material. Therefore, data obtained in this way should be carefully reexamined.

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