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Simultaneous Determination of Ascorbic Acid and Dehydroascorbic Acid in Plant Materials by High Performance Liquid Chromatography

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The adaptation of an isocratic reversed-phase ion-pairing high performance liquid chromatographic method in combination with derivatization of dehydroascorbic acid by 1,2-phenylenediamine prior to injection for simultaneous determination of ascorbic acid and dehydroascorbic acid in plant materials is presented. Extraction was carried out in meta-phosphoric acid, and hexadecyltrimethylammonium bromide was used as the ion-pairing reagent. Ascorbic acid was detected directly at 248 nm whilst dehydroascorbic acid was simultaneously detected as the 1,2-phenylenediamine derivative at 348 nm. Resolution and accuracy were shown to be satisfactory for naturally occurring amounts in plant materials as demonstrated with extracts of Norway spruce (*Picea abies* (L.) Karst.) needles.

Keywords: ascorbic acid; dehydroascorbic acid; high performance liquid chromatography; *Picea abies*.

INTRODUCTION

In studying stress physiology in plants, much attention has been paid to the biochemistry of free radical formation and detoxification (Elstner, 1982; Salin, 1988). In plant cells metabolic radical-quenching pathways exist for the removal of toxic oxygen species (Alscher and Amthor, 1988). Within the radical scavenging systems in plant cells, ascorbic acid (AA) plays an important role as an antioxidant or as a participant in enzymic detoxification cycles (Foyer and Halliwell, 1976). The pool sizes of AA, as well as corresponding enzyme activities, are commonly used as markers of plant responses to oxidative stresses (see, for example, Tausz *et al.*, 1994; Schmieden and Wild, 1994). In this respect, the native redox status of antioxidants in the cells would be of great interest as Schmieden *et al.*, (1993) worked out for the antioxidant glutathione.

Since the determination of AA and its oxidized form, dehydroascorbic acid (DAA), in biological samples is still a current topic in scientific work, many different methods have been discussed in the literature (for reviews see Helsper, 1987; Polesello and Rizzolo, 1990; Rizzolo and Polesello, 1992; Washko *et al.*, 1992). Despite numerous procedures for the determination of AA and DAA in food stuffs and medical samples, their simultaneous determination in leaf material is still an unresolved problem. Researchers dealing with ecophysiological questions either confine themselves to the measurement of total AA (AA+DAA after reduction of the latter) or of AA alone, or they determine DAA as the difference between AA and total AA. Unfortunately, this approach suffers from a lack of accuracy if the samples contain a five to ten-fold excess of AA over DAA (Esterbauer *et al.*, 1980). Furthermore, for ecological and plant physiological studies, a determination procedure must be quick and accurate enough to allow the processing of large sample numbers prepared from limited amounts of leaf material. Such methods are not currently available in this field.

Owing to the presence of interfering substances (e.g. phenols), leaf materials require specific extraction and determination procedures especially developed for and tested on them. A modification of a high performance liquid chromatographic (HPLC) method developed by Kneifel and Sommer (1985) for the determination of AA in milk was used previously to assay spruce needles (Tausz *et al.*, 1994). In this work extraction with meta-phosphoric acid was used in combination with an isocratic reversed-phase ion-pairing HPLC method. For the simultaneous determination of AA and DAA we followed suggestions of Keating and Haddad (1982) who, for orange juice samples, described the derivatization of DAA with 1,2-phenylenediamine prior to HPLC analysis. A very similar method was later published by Zapata and Dufour (1992) who tested it on several foods and beverages.

The objective of the present study was to develop a convenient, quick and sensitive method for the simultaneous determination of AA and DAA in plant materials, the results of which would be of high plant physiological relevance.

EXPERIMENTAL

Chemicals. Hexadecyltrimethylammonium bromide was purchased from Fluka (Büchs, Switzerland), DAA and 1,2-phenylenediamine were from Sigma (St Louis, MO, USA), and all other reagents from Merck (Darmstadt, Germany). All chemicals were of analysis grade: HPLC-grade water was prepared by two-step distillation.

Standard solutions of AA and DAA were prepared in 1.5% (w/v) meta-phosphoric acid immediately before use.

Plant material and preparation. Analyses were carried out on needle material of Norway spruce trees (*Picea abies* (L.) Karst.). Needles were cut from the branches and shock-

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frozen immediately in liquid nitrogen. The frozen needles were freeze-dried in a Hetosic CD4 freeze-drier (Heto, Allerød, Denmark) for at least four days, and ground under liquid nitrogen in a dismembrator (Braun Mikro-Dismembrator II, Braun, Maria Enzersdorf, Austria). The resulting powder was stored in humidity proof plastic vials at −25°C until required for extraction.

Extraction. Aliquots (0.2–0.5 g) of powdered needle material were homogenized in 5 mL 1.5% (w/v) metaphosphoric acid containing 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate in an Ultraturrax T25 (Janke and Kunkel, Staufen, Germany) mixer for 1 min and then sucked through a paper filter (589/1 Black Ribbon; Schleicher and Schuell, Dassel, Germany). All extraction steps were carried out in an ice bath.

Sample preparation. The obtained extracts could be analysed directly in order to determine AA alone or were derivatized in order to carry out simultaneous determination of both the oxidized and the reduced form as follows. An aliquot (700 μL) of the sample extract was adjusted to neutral pH by adding 450 μl of 0.2 M Tris (in water) in a brown Eppendorf (Hamburg, Germany) test tube. Ten μL of a freshly prepared solution of 1,2-phenylenediamine dichloride (10 mg in 10 mL water) were added. The sample was mixed by vortex for 15 s and then allowed to incubate for 25 min at 25°C in the dark. The reaction was stopped by the addition of 85% ortho-phosphoric acid (10 μL) and by cooling the tube in ice. Samples were centrifuged at 17,500 × *g* in a cooled (4°C) centrifuge (Beckman Avant 30—Fullerton, CA, USA) for 20 min and then transferred to brown autosampler glass vials.

HPLC analysis. The HPLC system consisted of a cooled (4°C) Hewlett Packard HP 1050 auto sampler, an LKB 2150 solvent pump, an HP 1040M Series II diode array detector, and an HP ChemStation PC integrator. The stationary phase was Spherisorb S5 ODS2 (250 × 4.6 mm i.d.; particle size 5 μm) with a Spherisorb S5 ODS2 precolumn (50 − 4.6 mm i.d.). The mobile phase was methanol:water (1:3; v/v) containing 1 mM hexadecyltrimethylammonium bromide and 0.05% (w/v) sodium dihydrogen phosphate monohydrate. The pH was adjusted to 3.6 by adding 85% (w/v) ortho-phosphoric acid. The flow rate was 1 mL/min.

An aliquot (20 μL) of the sample solution was injected by the autosampler. Detection was performed at two wavelengths simultaneously, namely at 348 and at 248 nm, and analysis time was set to 20 min.

Statistics. Statistical analysis was carried out using a Statistica (StatSoft, Tulsa, OK, USA) computer program. The linearity of the detector response based on standard concentrations of sample was calculated by linear regression; the Pearson's correlation coefficient and its significance level were calculated. Sample variations were determined as standard deviations of replicates. The loss of AA due to the derivatization procedure was checked by comparison of underivatized with derivatized extracts and evaluated by paired *t*-test after verifying normal distribution of the compared groups. Differences between sample groups were calculated by two sample *t*-test when data was normally distributed.

RESULTS AND DISCUSSION

A typical HPLC chromatogram of an extract of spruce needles is shown in Fig. 1. AA was detected at 248 nm and eluted after about 7 min, which is sufficient to separate it from interfering peaks. DAA was detected as the 1,2-phenylenediamine derivative (DFQ) at 348 nm and eluted just after the peak of underivatized 1,2-phenylenediamine. No interfering peaks occurred in needle extacts at this wavelength, and underivatized needle extracts showed no peaks at this wavelength except the injection peak. Peak purity was checked by wavelength scans of the peaks in sample

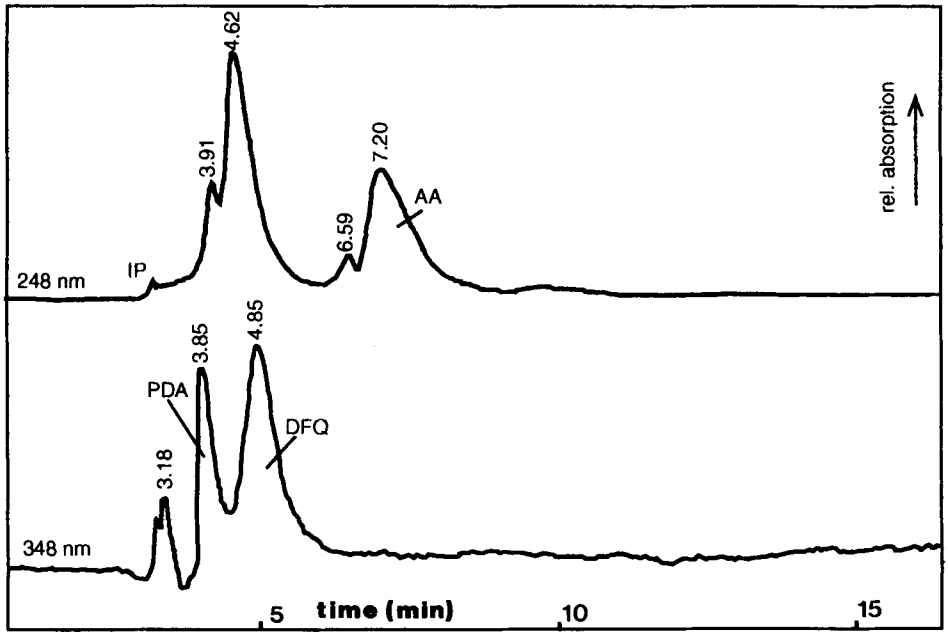

Figure 1. HPLC chromatogram of an extract of spruce needles of the current year. Ascorbic acid (AA) was detected at 248 nm, the 1,2-phenylenediamine-derivative of dehydroascorbic acid (DFQ) at 348 nm: retention times (min) of the eluting substances are shown above the peaks. AA and DFQ are well resolved from underivatized 1,2-phenylenediamine (PDA) and from interfering peaks (not marked) as well as from the injection peak (IP).

Table 1. Recovery of ascorbic acid and dehydroascorbic acid from spiked needle extracts

Ascorbic acid			Dehydroascorbic acid		
Extract content (μg/mL)	Addition (μg/mL)	Recovery[a] (%)	Extract content (μg/mL)	Addition (μg/mL)	Recovery[a] (%)
83–158	77	103.7±5.7	47 to 98	62	101.6±6.7
58–164	142	102.2±5.2	0 to 70	154	102.6±7.7

[a]Mean±standard deviations of five replicate experiments carried out on different needle extracts.

extracts and comparing them to the respective pure standard peaks.

Linearity of peak area response of standards prepared in concentrations corresponding to those occurring in the samples was good. For AA over the range 15 to 700 μg/mL the correlation coefficient between concentrations and peak-area was 0.9998 ($n=9$; $P<0.05$), and for DAA over the range 10 to 150 μg/mL the correlation coefficient was 0.9905 ($n=9$; $P<0.05$). Within these ranges standard deviations of repeated injections within 3 h amounted to less than 5% of the means for the lower concentrations and under 1% of the means for the higher concentrations. Calculations of the contents in the samples were performed by linear regression from standards.

Recovery studies were carried out on different needle extracts spiked with two different amounts of AA and DAA (Table 1). The standard deviations of the recoveries were slightly greater for DAA which is probably due to smaller peak-areas and to small amounts of DAA in some of the needle extracts. Nevertheless, recovery percentages and variations proved that the extraction and derivatization procedure was sufficiently reproducible.

The repeatability of the method was tested by repeated analysis of different extracts of the same needle material. Standard deviations amounted to 7.3% of the means for DAA (sample means, 0.77 mg/g dry weight) and 4.3% of the means for AA (sample means, 5.49 mg/g dry weight; $n=15$).

Determination of the optimal derivatization time for DFQ formation produced results similar to those reported by Zapata and Dufour (1992). Standard solutions of DAA were derivatized for different times and injected immediately. Derivatization times of greater than 20 min did not produce any further increase in area of the resulting DFQ peak and so the derivatization time was set to 25 min.

In order to determine the loss of AA due to the derivatization step, underivatized samples of the same needle extracts were compared with derivatized ones. Paired t-test results revealed no differences between the detected amounts of AA ($n=35$; $P>0.40$).

Table 2. Ascorbic acid and dehydroascorbic acid content of two different clones of spruce trees grown in the botanical garden in Graz

	Ascorbic acid (mg/g dw)	Dehydroascorbic acid (mg/g dw)
Clone A[a]	4.51±1.04a[b]	0.54±0.14
Clone B[c]	3.40±0.88b	0.50±0.16

[a] FBVA Vienna 85119023.
[b] Different letters represent significant differences between the clones at significance level $P<0.01$ (Two sample t-test)
[c] FBVA Vienna 85119040.

Stability tests of AA samples in meta-phosphoric acid have been carried out and reported previously (Bradley *et al.*, 1973, Margolis *et al.*, 1990). Stability tests of DFQ reported by Keating and Haddad (1982) revealed multiple peaks for unstable DFQ in aqueous solution. Using our protocol multiple peaks did not occur, suggesting that the stability of DFQ is sufficient for convenient analysis within 1 h as shown by the variations of DAA standards (standard deviations below 5% of the means for concentrations above 10 μg/mL). On the other hand, long-term chain analyses using an autosampler were seriously disturbed by stability problems of the derivative, as peak areas began to increase slowly after 1 h.

As an example of the application of the technique described, Table 2 shows the results of determinations of AA and DAA carried out on two clones of spruce trees grown in the botanical garden in Graz. Needles from the current year were harvested at the beginning of September, repetitive samples being taken from ten individual trees per clone. The concentration ranges of AA and of DAA are highly comparable with data for Norway spruce trees published previously (e.g. Esterbauer *et al.*, 1980; Schmieden and Wild, 1994; Tausz *et al.*, 1994). The results show that the application of the described method to spruce needle samples is accurate and reliable enough for use in the study of stress-physiological problems. A particular advantage of this method is the simultaneous determination of DAA in the presence of a surplus of AA in one step, and this is of great interest with respect to questions of the redox status of the AA system in plant cells.

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