

DETERMINATION OF ASCORBIC ACID IN SHOOTS FROM DIFFERENT CONIFEROUS SPECIES BY HPLC

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Abstract

The stability of ascorbic acid (AA) in different aqueous solutions was studied for the development of optimal methods and procedures for the HPLC analysis of this compound in young shoots with needles of conifers. The best extraction solvent that offered the maximum stability of AA was aqueous 0.5% acid oxalic. The chromatographic separation was carried out on Lichrosorb RP-18 column using as mobile phase: 95% NaH₂PO₄ 0.05 M (aqueous solution) adjusted to pH 3.6 and 5% methanol. The method proposed was applied to the analysis of AA in shoots samples of five coniferous species: silver fir (*Abies alba* Mill.), Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco), European larch (*Larix decidua* Mill. ssp. *carpathica* (Dom.) Siman), Norway spruce (*Picea abies* L. Karst.), and black pine (*Pinus nigra* Arn. ssp. *nigra*). In order to evaluate the dynamics of AA accumulation in young shoots with needle during the growing season, from buds sprouting in spring until the end of growing period in autumn, the samples were harvested in three distinct moments: on May the 17th, July the 11th and October the 7th (2011). For four of the five species studied the highest content of AA was determined in samples collected in October: 301.5 mg in *Picea abies*, 275.1 mg in *Pseudotsuga menziesii*, 231.3 mg in *Pinus nigra* and 158.8 mg in *Abies alba* (per 100 g dried weight).

Rezumat

S-a studiat stabilitatea acidului ascorbic (AA) în diferite soluții apoase pentru dezvoltarea unei metode optime de analiză a acestui compus în lujeri tineri de conifere prin cromatografie de lichide la presiune ridicată. Cel mai bun solvent de extracție, care a oferit o stabilitate maximă a acidului ascorbic, a fost soluția apoasă de acid oxalic 0,5%. Separarea cromatografică s-a făcut pe o coloană LiChrosorb RP-18, utilizând ca fază mobilă soluție apoasă 0,05 M NaH₂PO₄ 95% (pH 3,6) și metanol 5%. Metoda propusă s-a folosit pentru determinarea AA din lujerii a cinci specii de conifere: brad (*Abies alba* Mill.), duglas (*Pseudotsuga menziesii* (Mirbel) Franco), larice (*Larix decidua* Mill. ssp. *carpathica* (Dom.) Siman), molid (*Picea abies* L. Karst.) și pin negru (*Pinus nigra* Arn. ssp. *Nigra*). În scopul evaluării dinamicii de acumulare a acidului ascorbic în lujerii de rășinoase de-a lungul sezonului de vegetație s-au prelevat probe în diferite perioade ale anului 2011: 17 mai, 11 iulie și 7 octombrie. Pentru patru din cele cinci specii studiate, cel mai mare

conținut de acid ascorbic s-a evidențiat în probele recoltate în luna octombrie: 301,5 mg în *Picea abies*, 275,1 mg în *Pseudotsuga menziesii*, 231,3 mg în *Pinus nigra* și 158,8 mg în *Abies alba* (la 100 g substanță uscată).

Keywords: Ascorbic Acid, HPLC, coniferous shoots.

Introduction

Ascorbic acid (vitamin C) is a water soluble vitamin that can be synthesized by plants and many mammals, but not by humans [5]. It is considered that a human adult, on average, requires about 50 mg of ascorbic acid per day [4,18]. Fruits and vegetables are the main source of vitamin C for the human body. The content of ascorbic acid in oranges and lemons (50 mg/100g) is exceeded by that in the needles and bark of several coniferous species [1,3,14]. For the identification and quantification of ascorbic acid in various vegetable resources (e.g. fruits, leaves, shoots, bark etc.) HPLC techniques were used by many authors [5,7,10,12,13,15-17]. It is well known that ascorbic acid is unstable in aqueous based solutions, to heat and air oxidation, but stable in solid state. Therefore, the development of optimal methods for the determination of ascorbic acid requires the selection of the conditions for its maximum stability. There are many papers dealing with the stability of ascorbic acid in different extraction systems and eluting solvents in HPLC [2,6,8,11].

The objectives of this study were the development of an optimal method for the determination of ascorbic acid, which requires the selection of experimental conditions that ensure its maximum stability, and the utilisation of this method for ascorbic acid analysis in shoots samples from different species of conifers.

Materials and Methods

Reagents and solvents. Ascorbic acid (AA), potassium dihydrogen phosphate, orto-phosphoric acid 85% p.a., oxalic acid p.a., citric acid p.a. and the solvents for HPLC analysis (water and methanol) were purchased from Merck (Darmstadt, Germany) and lactic acid Ph Eur was provided by Fluka-Chemicals (Dorset, England).

Stock solutions 50 mg/100 mL of ascorbic acid were prepared in water and in aqueous 0.5% oxalic acid, 0.5% citric acid, 0.5% lactic acid and 0.05% phosphoric acid. Working solutions of lower concentrations were prepared by appropriate dilution with adequate solvent. For the calibration curve, ascorbic acid solutions with concentrations in the range 12.5 – 250 ng/ μ L, using 0.5% oxalic acid aqueous solution as dilution solvent were prepared. All these solutions were prepared daily in dark volumetric flasks.

Plant material. The samples of young shoots with needles (around 1000 g) of silver fir (*Abies alba*), Douglas-fir (*Pseudotsuga menziesii*), European larch (*Larix decidua ssp. Carpathica*), Norway spruce (*Picea abies*) and black pine (*Pinus nigra ssp. nigra*) were collected from an intensive plantation located in the tree nursery of Forest Research and Management Institute (Ilfov, Romania), 10 km north-east of Bucharest. For each species, the samples were harvested from ten individual 6 years old trees. The samples were stored at 4°C and analysed no longer than two days from the harvest time. Five grams samples were weighed in order to determinate the fresh samples humidity (expressed as mean of three replicates).

Extraction procedures. In order to identify the optimal extraction solvent of ascorbic acid from the resinous shoots a study of stability of ascorbic acid was made. For this purpose solutions of ascorbic acid of 100 ng/ μ L were prepared in the following aqueous solutions: pure water, 0.5% oxalic acid, 0.5% citric acid, 0.5% lactic acid and 0.05% phosphoric acid. In these solutions, stored at room temperature, the content of AA was measured immediately, after three, four, seven and ten days from preparation.

The samples were grounded in a Waring LBC15 blender for 2 minutes and then 10 g of finely grounded shoots were mixed with 50 mL of 0.5% oxalic acid aqueous solution in an Erlenmeyer flask. The flask was then placed in an Elma Transsonic 460/H ultrasonic bath for 30 minutes. The mixture was filtrated through a 0.45 μ m filter and 20 μ L of filtrate was injected in the HPLC apparatus.

HPLC analysis. The HPLC system used was a Beckman with solvents Module 126, UV Gold 166 detector and Rheodyne injector system. The separation was performed by Lichrosorb RP-18 column (15 cm x 4.6 mm, 5 μ m d. p.) from Merck (Darmstadt, Germany). A mobile phase composition of 95% aqueous NaH_2PO_4 (0.05 M) adjusted to pH 3.6 by adding 85% phosphoric acid, and 5% methanol in isocratic mode was used. The wavelength detection was set at 242 nm. The flow rate of the mobile phase was 0.7 mL/min and the volume injected was 20 μ L. For the experimental parameters control, data acquisition and processing the "Gold software" was used. All data are presented as mean \pm standard deviation of triplicate analysis.

Results and Discussion

The first step of our study was the optimization of HPLC conditions. The column, mobile phase and flow rate were chosen in order to obtain a short retention time for the ascorbic acid. The extraction solvent was chosen

based on a stability test of AA in different solutions. The results of this test are presented in Figure 1 and Table I.

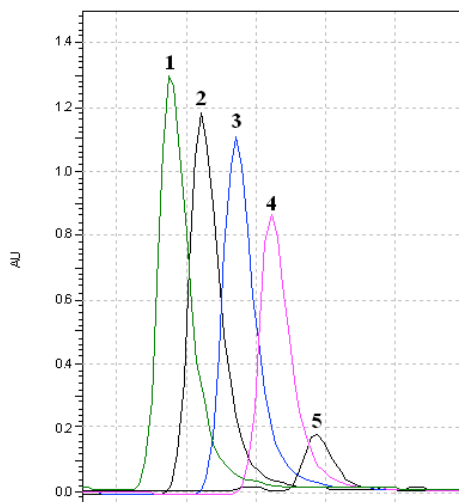


Figure 1

Chromatograms of ascorbic acid, 72 h after preparation, in solutions of: **1** - 0.5% oxalic acid, **2** - 0.5% lactic acid, **3** - 0.5% citric acid, **4** - 0.05% phosphoric acid, **5** - water.

Table I

The amount of ascorbic acid found after 3 – 10 days of preparation of the solutions (kept at room temperature).

Time (days)	Amount of AA found (%)				
	Water	0.05% Phosphoric acid	0.5% Oxalic acid	0.5% Citric acid	0.5% Lactic acid
3	12.13	58.91	84.21	76.13	77.41
4	-	46.39	74.84	53.64	49.59
7	-	37.17	64.68	38.90	38.61
10	-	28.02	54.15	26.53	28.92

Three days after the preparation of the solutions, only 12.13% of the initial amount of AA was found in water. Thus, we can see that using water as a solvent for working solutions can affect essentially the analytical results. The best stability of AA was found using an aqueous solution of oxalic acid as extraction solvent.

The chromatogram of ascorbic acid in 0.5% oxalic acid aqueous solution shows a peak at a retention time of 3.42 minutes for oxalic acid and at 4.18 minutes for AA. No interfering peaks occurred in shoots extracts chromatograms in chosen experimental conditions (Figure 2).

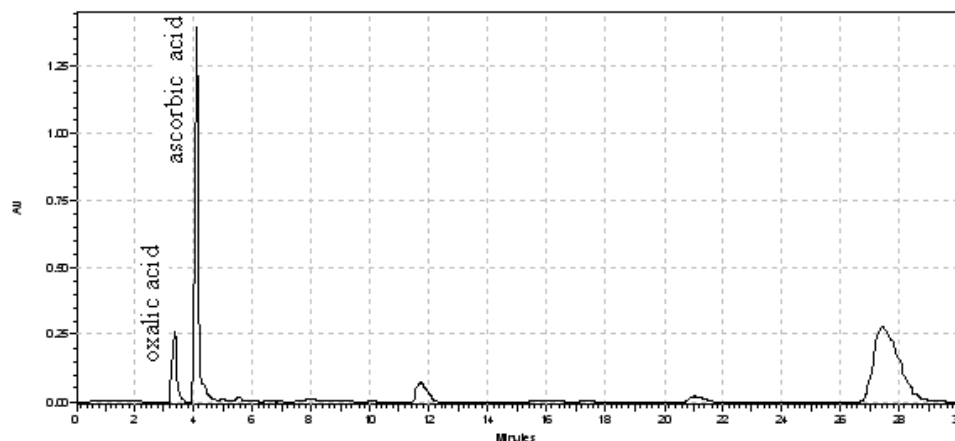


Figure 2

Chromatogram of *Picea abies* extract.

The data concerning validation parameters of the method are shown in table II. The linearity of the method was studied in the range 12.5 – 250 ng/ μ L ascorbic acid concentrations. The linear calibration curve was constructed by least squares regression of chromatographic signal (area) *versus* concentration (ng/ μ L). Good linearity was found, the coefficient R^2 being 0.9996. Detection (LODs) and quantification (LQDs) limits of AA were determined at the lowest concentration levels that yielded a signal-to-noise ratio (S/N) of 3.

Table II

Summary of the validation parameters of HPLC method.

Validation criteria	Parameter	Results
Linearity	Regression parameters	$Y = 0.0777 X + 0.3727$
	Range	12.5 – 250 ng/ μ L
	Correlation coefficient	0.9996
Precision	Repeatability*	RSD% = 3.44 (peak area = 56.40 ± 1.94)
		RSD% = 1.03 (retention time: 4.18 ± 0.04)
Recovery**		RSD% = 0.99 97.66 ± 0.97 %
	Detection limit (response standard deviation and slope)	3.15 ng/ μ L
	Quantification limit (response standard deviation and slope)	9.55 ng/ μ L

* 5 consecutive injections of ascorbic acid 50 ng/ μ L.

** 5 replicates of spiked shoots with ascorbic acid (added 200 mg/100g dw).

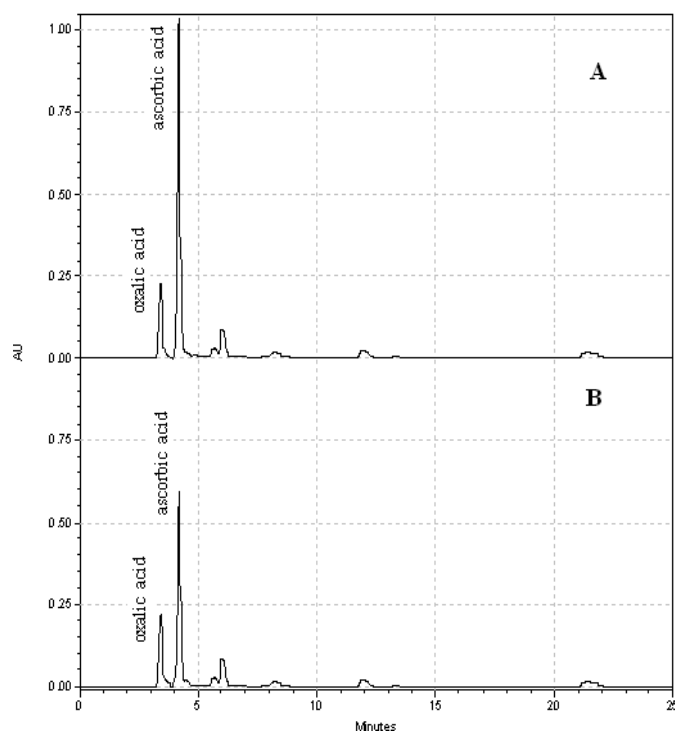


Figure 3

Chromatograms of extracts of *Pinus Nigra*: **A** – fortified shoots sample with AA (200 mg/100g dw), **B** – unfortified shoots sample.

The recovery rate of ascorbic acid was evaluated in order to assess the extraction efficiency of the proposed method. For this purpose about 110g of shoots from *Pinus nigra* harvested in May were finely grounded. Five samples from this material were analysed with the proposed method. From the finely grounded material 50 g were spiked at a concentration level of ascorbic acid of 2 mg/g (relative to dried material). Five samples from spiked material were also analysed with the same method (Figure 3).

Recoveries (R_1 - R_5) were calculated as follows: $R(\%) = \frac{C_{1-5} - C_0}{C_s} \times 100$, where: C_0 is the average content of AA in unfortified samples; C_s is the added content of AA (200 mg/100g); C_{1-5} is the total content of AA determined in fortified samples.

The results (Table III) show that the highest content of AA is present in shoots of *Picea abies* 194.5 mg/100g dw for samples harvested in May and 301.5 mg/100g dw for those harvested in October.

Table III
Ascorbic acid content in different shoots samples

Sample	Ascorbic acid (mg/100g dw)								
	May			July			October		
		Mean conc. \pm SD	CV %		Mean conc. \pm SD	CV %		Mean conc. \pm SD	CV %
<i>Abies alba</i>	161.4	159.1 \pm 2.5	\pm 1.586	154.3	157.4 \pm 2.7	\pm 1.741	156.7	158.8 \pm 1.9	\pm 1.180
	156.4			159.5			159.4		
	159.5			158.4			160.3		
<i>Picea abies</i>	195.7	194.5 \pm 2.6	\pm 1.345	243.8	244.0 \pm 1.8	\pm 0.741	304.3	301.5 \pm 3.5	\pm 1.174
	196.3			242.3			297.5		
	191.5			245.9			302.6		
<i>Larix decidua</i>	42.5	42.3 \pm 0.8	\pm 1.935	24.9	24.5 \pm 0.5	\pm 1.926	8.2	8.1 \pm 0.1	\pm 1.240
	41.4			24.0			7.9		
	43.0			24.7			8.1		
<i>Pseudotsuga menziesii</i>	59.2	59.5 \pm 0.9	\pm 1.494	78.9	80.0 \pm 1.1	\pm 1.377	278.9	275.1 \pm 4.4	\pm 1.598
	58.8			81.1			270.3		
	60.5			79.9			276.2		
<i>Pinus nigra</i> *	114.5	114.1 \pm 1.2	\pm 1.026	121.0	122.4 \pm 1.4	\pm 1.143	234.5	231.3 \pm 3.1	\pm 1.342
	112.6			123.9			231.1		
	115.3			122.4			228.3		
	113.1								
	114.9								

*5 replicates were analysed and the mean is C_0 .

On the other hand, the lowest content of vitamin C was detected in *Larix decidua* species with values between 8.1 – 42.3 mg/100g dw. *Larix deciduas*, a deciduous conifer, is the only of the five species studied in which the AA content is decreasing from spring to autumn. In shoots of *Picea abies*, *Pseudotsuga menziesii* and *Pinus nigra* it can be observed a significant increase of AA content from spring to autumn. The AA content in shoots of *Abies alba* does not differ markedly from May to October.

The amount of AA detected in shoots samples of *Picea abies* is comparable to that reported by Tausz et al. [16] in needle samples of two clones of spruce trees grown in the botanical garden in Gratz. The amount of AA detected in some shoots samples is higher than the amount detected in citrus fruits [13] and *Rosa canina* [2].

It is interesting to observe that in the case of evergreen coniferous species (*Abies alba*, *Picea abies*, *Pseudotsuga menziesii*, *Pinus nigra*) there is a trend of increasing the content of AA during the growing season, from sprouting of buds, in spring, until the end of the growing period, in autumn. For these four species the highest content of AA was detected in shoots samples collected in October.

Based on data obtained in our study we can say that these coniferous shoots could be an alternative and potential source of natural vitamin C known as a compound with strong antioxidant activity [9,12].

Conclusions

This paper is a contribution to the development of a rapid and precise HPLC method for the analysis of vitamin C in shoots of conifers. The results on ascorbic acid content in the shoots samples suggest that coniferous shoots may be a natural source of vitamin C, an easily accessible source for a long period of the year.

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