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Note

Isocratic reversed-phase high-performance liquid chromatographic analysis of pigments in Norway spruce

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The analysis of plant pigments has been one of the major subjects of research in plant science, especially plant physiology and biochemistry, for many years. The degradation of plant pigments due to pollution has been investigated by many groups. Discoloration of leaves, especially yellowing on the sunny upper sides of leaves, has been found to occur in forest trees in various parts of West Germany, most prevalent in Bavarian Forests at high altitudes. The mechanisms of yellowing are still unclear. It has been suggested that the yellowing of needles or leaves may be due to magnesium deficiency[1], photo-oxidation by ozone[2] or the accumulation of UV-light-absorbing growth-inhibiting substances such as *p*-hydroxyacetophenone (*p*-HAP) and its β-D-glucopyranoside (*p*-HAPG), especially in the needle epidermis[3–5]. In numerous physiological and biochemical investigations, improved methods of analysis of plant pigments, especially for forest trees, have been urgently required to handle a large number of samples.

Until now, plant pigment analysis has been mainly carried out by thin-layer chromatography and/or high-performance adsorption or high-performance gradient reversed-phase liquid chromatography (HPLC)[6–8]. Although high-performance adsorption chromatography gives good separations of plant pigments, losses of samples due to irreversible adsorption and/or frequent cleaning of the adsorption columns or re-packing of the columns with new adsorption material are major obstacles to its efficient and reliable use. Gradient reversed-phase HPLC suffers from problems due to baseline drift and longer durations of equilibrium in comparison with isocratic reversed-phase HPLC. Therefore, a quantitative method based on isocratic reversed-phase HPLC for plant pigment analysis was investigated.

This paper describes the isocratic reversed-phase HPLC determination of various pigments in needles of Norway spruce plants exposed to ozone, and treated with *p*-HAP + UV light in the nanogram range of pigment analysis.

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EXPERIMENTAL

Plant materials

Needles were detached from current year shoots of Norway spruce plants, *Picea abies* (L.) Karst. (experiment 1, 3.5 years old, clone no. 1027, supplied by Pflanzgarten Laufen, harvest September 1986; experiment 2, 4 years old, clone no. 14, supplied by Pflanzgarten Laufen, fumigated with different concentrations of ozone, harvest April and December 1986).

Authentic samples

Authentic samples of β-carotene (HPLC purity, 86.67%), chlorophyll *a* (HPLC purity, 100%), chlorophyll *b* (HPLC purity, 84.29%), lutein (HPLC purity, 97.49%), phaeophytins *a* and *b* and zeaxanthin (HPLC purity, 70.15%) were obtained from C. Roth (Karlsruhe, F.R.G.) and Serva (Heidelberg, F.R.G.).

Treatment of plants with p-HAP + UV light and long-term low-level ozone

p-HAP + UV light. The current-year shoots of 3-year-old healthy Norway spruce plants were cut (three shoots per treatment), 20 mg of *p*-HAP per shoot were dissolved in water and the dissolved *p*-HAP was fed to each shoot through the cut surfaces for *ca.* 7 h (40 klux, 30°C, relative humidity 45%). The amount of substance left after feeding was dissolved in 200 μl of ethanol and injected into the cut surfaces of each shoot. The control shoots were fed simultaneously only with water and subsequently injected with 200 μl of ethanol. The shoots treated with *p*-HAP were simultaneously exposed to UV light (λ = 254 nm, 8 W) for *ca.* 69 h.

Long-term low-level ozone exposure. Long-term low-level ozone exposure of Norway spruce plants was performed in the continuous fumigation chambers of the MAGL Project, GSF, Munich, F.R.G.

High-performance liquid chromatography with ultraviolet detection

The analysis was performed on a Kontron HPLC system (T-414 HPLC pump, Uvikon 722LC detector) and a Beckman HPLC system (114M HPLC pump solvent delivery module, absorbance detector).

For the separation of Norway spruce pigments using the Kontron HPLC system two Kontron Spherisorb ODS-2 columns connected in series (each 250 mm × 5 mm I.D., 5 μm particle size) were connected with a Vydac 201 SC precolumn (50 mm × 5 mm I.D., 30–40μm particle size) and the absorbance was monitored at 400 nm (experiment 1). Under these conditions, the detection limit of chlorophyll *a* and *b* was *ca.* 30 ng.

Norway spruce pigments were separated further using the Beckman HPLC system with two Altex Ultrasphere ODS columns (each 250 mm × 4.6 mm I.D., 5 μm particle size) connected with an MN cartridge (50 mm × 5 mm I.D., 30–40 μm particle size) and the absorbance was monitored at 430 nm (experiment 2). Under these conditions, the detection limit of chlorophyll *a* and *b* was *ca.* 10 ng. The data were acquired and processed by a Basic-programmable Shimadzu integrator Chromatopack C-R3A). The chromatographic conditions are given in the figure legends.

Preparation of plant samples for measurement by HPLC

The plant samples (0.2–0.5 g, needles from current year's shoots, experiment 1) or 9–10 needles (0.007–0.03 g, needles from 1-year-old main shoot, experiment 2) were finely homogenized in water and extracted with cold (−18°C) 100% acetone [+ 100 ppm butylated hydroxytoluene (BHT) as antioxidant, experiment 1] or homogenized in 100 μl of eluting solvent using a micro-grinder at room temperature each time prior to injection (experiment 2). The extracts were filtered, dried *in vacuo* at −18°C (experiment 1) or dried under a stream of nitrogen at room temperature (experiment 2). The prepared samples were diluted in the ratio 1:200 to 1:100, equivalent to *ca.* 1–5 mg fresh weight of needles (experiment 1), or in the ratio 1:15 to 1:10, equivalent to *ca.* 0.5–3 mg fresh weight of needles (experiment 2), isooctane being added to the dissolving eluting solvent in the ratio 1:3. Further, 10 μl of isooctane were added to the sample for injection in order to ensure a rapid on-line delivery of non-polar pigment samples from the precolumns (experiment 2). No influence of BHT could be detected in the analysis under the conditions described (experiment 1).

RESULTS AND DISCUSSION

β-Carotene, chlorophyll *a* and *b*, lutein and zeaxanthin could be detected at 400 (experiment 1) and 430 nm (experiment 2). Owing to the non-availability of an absorbance detector system for 445 nm in our laboratory, we used the above wavelengths for the quantification of various pigments. In our work on the levels of β-carotene, chlorophyll *a* and *b*, lutein and zeaxanthin, we observed no significant

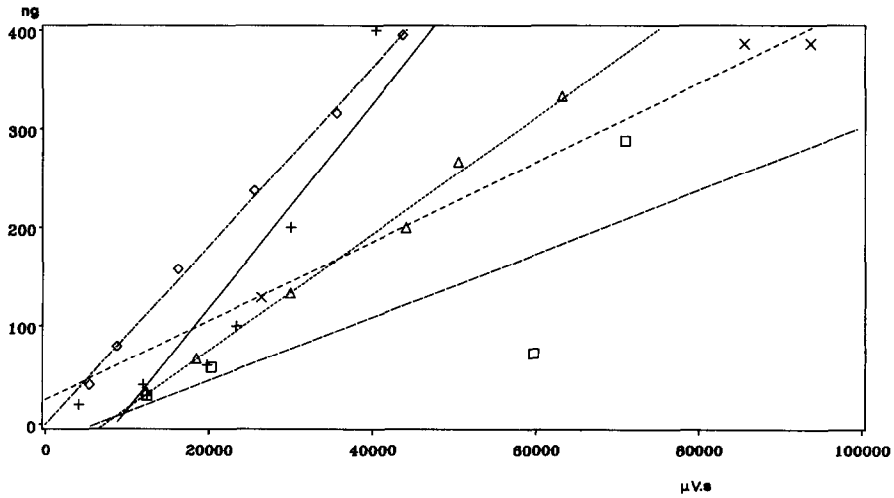

Fig. 1. Test of linearity of the measurement of β-carotene (×), chlorophyll *a* (+), chlorophyll *b* (△), lutein (□) and zeaxanthin (◇) by isocratic HPLC [flow-rate, 1 ml/min; λ = 430 nm; t = 0.2 s; mobile phase, methanol–isooctane–acetonitrile (9:1:0.05)]. The linearity of the measurements of β-carotene, chlorophyll *a*, chlorophyll *b*, lutein and zeaxanthin can be described by the regression lines $y = 24.76 + 0.0040224x$ (r^2 = 0.99), $y = -84.97 + 0.0102688x$ (r^2 = 0.85), $y = -42.97 + 0.0059128x$ (r^2 = 0.99), $y = -22.03 + 0.0032962x$ (r^2 = 0.63), and $y = 0.03 + 0.0090737x$ (r^2 = 1.0), respectively, where y = ng of substance and x = μV s.

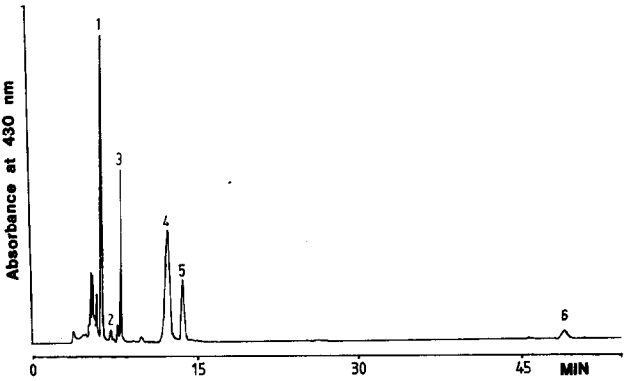

Fig. 2. Separation of authentic samples of lutein (1; 301 ng), zeaxanthin (2; 119 ng), chlorophyll *b* (3; 139 ng), chlorophyll *a* (4; 8 ng), phaeophytins (5; 3.84 μg) and β-carotene (6; 386 ng).

TABLE I

RETENTION TIMES OF PIGMENTS

Compound	*Retention time (min)*
Lutein	5.9
Zeaxanthin	6.7
Chlorophyll *b*	7.7
Chlorophyll *a*	13.5
β-Carotene	47.7

TABLE II

PRECISION AND ACCURACY OF THE HPLC MEASUREMENT OF β-CAROTENE, CHLOROPHYLL *a* AND *b*, LUTEIN AND ZEAXANTHIN BY HPLC IN NEEDLES OF NORWAY SPRUCE

Sample	*Sample size (n)*	*Substance*	*Average ($\bar{x}$) (μg/g fresh weight)*§	*Standard deviation (μg/g fresh weight)*	*Precision** *(%)*	*Accuracy*** *(%)*
Authentic samples	3	β-Carotene	128.79	0.08	0.06	0.18
	4	Chlorophyll *a*	100.00	2.43	2.43	4.47
	5	Chlorophyll *b*	112.38	11.71	10.42	14.46
	5	Lutein	100.22	0.81	0.80	1.11
	5	Zeaxanthin	39.63	0.92	2.32	3.22
Plant samples***	3	β-Carotene	200	10	0.05	0.15
	4	Chlorophyll *a*	740	30	3.53	6.49
	4	Chlorophyll *b*	390	20	4.65	8.55
	5	Lutein	6650	10	0.12	0.17
	4	Zeaxanthin	50	10	21.28	39.14

* Precision = coefficient of variation (C.V., %) = standard deviation × 100)/$\bar{x}$.

** Accuracy ($S\bar{x}$, %) = (C.V. × t)/$(n-1)^{\frac{1}{2}}$, where t = t-value of two-tailed Student's t-test at p = 0.05 and $n-1$ degrees of freedom.

*** Samples from experiment 2 (fumigated with 40 ppb of ozone).

§ Authentic samples (in μg).

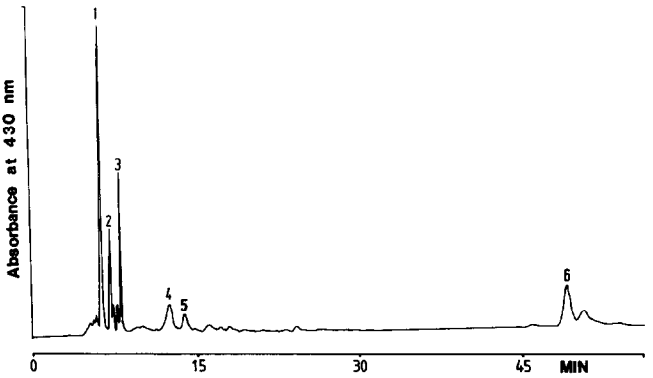

Fig. 3. Analysis of lutein (1), zeaxanthin (2), chlorophyll *b* (3), chlorophyll *a* (4), phaeophytins (5) and β-carotene (6). An aliquot of the diluted extract equivalent to 1.45 mg fresh weight was injected for measurement. The chromatogram shown was obtained from needles of an ozone-exposed Norway spruce plant (fumigated with 40 ppb of ozone for 118 days).

interferences at these wavelengths from contaminants in the 100% acetone or eluting solvent extracts of Norway spruce needles.

Fig. 1 shows the linearity of the measurements of β-carotene, chlorophyll *a* and *b*, lutein and zeaxanthin by HPLC and Fig. 2 illustrates the separation of the authentic compounds. The separation of these substances from plant material is depicted in Fig. 3. The pigments were eluted in the order lutein, zeaxanthin, chlorophyll *b*, chlorophyll *a*, β-carotene (Table I).

Quantification was performed using the external standard method and the results are summarized in Tables I–III. The precision and accuracy of the HPLC measurements shown in Table II demonstrate the good reliability of the method. Table III illustrates the changes in contents of chlorophyll *a* and *b* in needles of current year's shoots in response to treatment with *p*-HAP + UV light (experiment 1) and the changes in the contents of β-carotene, chlorophyll *a* and *b*, lutein and zeaxanthin in the needles in relation to low-level ozone exposure (experiment 2).

CONCLUSIONS

The results show that the determination of β-carotene, chlorophyll *a* and *b*, lutein and zeaxanthin can be conveniently carried out by isocratic reversed-phase HPLC. The merits of the method are convenient, rapid measurement (8–10 samples per day), stable baseline, rapid cleaning of the column system and short equilibrium period, sensitive measurement in the nanogram range, low cost per measurement and measurement of different pigments in the same run. Re-filling of precolumns is usually required after about 40 measurements in order to ensure high reproducibility and reliability. A further increase in sensitivity for the quantitative measurement of plant pigments could be achieved by using an absorbance detector system at 445 nm.

TABLE III

CONTENTS OF LUTEIN, ZEAXANTHIN, CHLOROPHYLL *a* AND *b* AND β-CAROTENE IN NEEDLES DETERMINED BY HPLC

Treatment	*Concentration (ppb)*	*Exposure (days)*	*Plant No.*	*Units**	*Lutein*	*Zeaxanthin*	*Chlorophyll a*	*Chlorophyll b*	*β-Carotene*
Ozone	20	118	1	a	237.44	23.65	970.33	19.45	0
	20		1	b	0.41	0.04	1.64	0.03	0
	20		2	a	166.23	15.25	938.14	22.14	8.58
	20		2	b	0.56	0.05	3.16	0.07	0.03
	40		3	a	39.10	0	633.72	82.29	–
	40		3	b	0.06	0	0.98	0.13	–
	40		4	a	29.35	13.25	374.98	291.77	–
	40		4	b	0.73	0.03	0.93	7.21	0.03
	20	238	1	a	321.47	17.45	455.76	267.70	14.60
	20		1	b	0.29	12.24	0.42	0.24	0.01
	20		2	a	146.84	11.71	103.51	122.51	89.71
	20		2	b	0.24	0.02	0.17	0.20	0.15
	40		3	a	191.65	1.82	249.96	179.57	89.72
	40		3	b	0.24	0.002	0.32	0.23	0.11
	40		4	a	192.55	9.62	241.88	154.64	34.57
	40		4	b	0.34	0.02	0.43	0.27	0.06
p-HAP + UV light	0**		1	a			161.67	16.90	
			2	a			19.17	0.51	
			3	a			17.43	–	
	20**		4	a			42.42	1.93	
			5	a			34.68	0.19	
			6	a			20.15	0.38	

* a, μg/g fresh weight; b, μg per needle.
** mg *p*-HAP per current year's shoot.

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