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Note

Rapid extraction and high speed liquid chromatography of *Nicotiana tabacum* leaf pigments

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The analysis of plant pigments using column liquid chromatography lies at the foundation of the chromatographic sciences. Since the classic work of Tswett[1,2], the separation and analysis of chlorophylls, carotenoids, xanthophylls and related pigments have undergone considerable refinement. Today, numerous methods exist for the analysis of plant pigments[3–8]. Prime criteria for modern methods of plant pigment analysis are that they be rapid, avoid degradation of the pigments, and be amenable to automation. This paper reports a method which we have found to meet these criteria for the isolation and rapid analysis of pigments from leaf tissues.

The isolation of pigments from plant tissues usually involves maceration of the tissue followed by repeated extractions with organic solvents such as acetone, methanol, ethanol, dimethyl sulfoxide or aqueous solutions of these solvents. More recently, N,N-dimethylformamide has been applied to the extraction of plant leaf materials without the need for maceration of the tissues[3,9–11]. This technique presents obvious advantages when it is necessary to extract large numbers of samples. Of equal significance, Bergweiler and Lutz[3] have observed that pigments extracted and stored in this solvent remain stable for extended periods.

Following extraction, the other critical step in the determination of plant pigment samples is that of quantitative analysis. A number of recent papers have reported methods for quantitative chromatographic analysis of plant pigments (for a review see Schwartz and von Elbe[12]). Typically, these methods require an analysis time of approximately 30 min or more, including the time required for re-equilibration of the initial mobile phase. The advent of 'high speed' liquid chromatographic techniques[13] has led to considerable savings in time and solvent consumption in analyses of a wide range of materials. We report here the application of this approach to the analysis of *Nicotiana tabacum* green leaf pigments.

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EXPERIMENTAL

General

Samples were kept on ice and exposed to a minimum of light and oxygen (air) during field sampling, transport, and storage prior to and after extraction. Low actinic glass volumetric flasks were used as containers for the extracted pigments which were stored at −78°C under nitrogen until analysed.

All solvents used were high-performance liquid chromatography (HPLC) grade obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Solvents, standard solutions, and samples for HPLC were filtered through 0.45-μm nylon 66 membrane filters prior to use. Authentic standards were obtained from the following sources: chlorophyll *a*, chlorophyll *b*, β-carotene —Sigma (St. Louis, MO, U.S.A.); xanthophyll and lutein —Atomergic (Farmington, NY, U.S.A.); β-apo-8′-carotenal —Fluka (Ronkonkoma, NY, U.S.A.).

Pigment extraction and storage

Twenty disks (1.7 cm diameter) were removed from leaves of tobacco at a point approximately two-thirds of the way from the base of the leaf and approximately half the distance from the midvein to the leaf margin. Disks were transferred immediately to plastic bags, sealed, and stored on dry ice in a darkened container for transport to the laboratory.

At the laboratory, ten frozen disks were taken from the total of twenty and placed in scintillation vials, 4.0 ml of N,N-dimethylformamide (DMF) [containing 0.1% (v/v) butylated hydroxytoluene (BHT) as an antioxidant] were added, and a nitrogen atmosphere introduced. The vials were then capped, covered with aluminum foil, and placed on a reciprocating shaker operated at 120 cycles per min. The samples were extracted for 24 h at the end of which time the pigment extracts were pipetted into 10.00-ml low-actinic glass volumetric flasks. The volumetric flasks were then stored at −78°C under nitrogen. An additional 4.0 ml of fresh DMF were added to the scintillation vials containing the samples, the vials were recapped under nitrogen and extracted as before for a second 24-h period. The second solvent extract was transferred to the corresponding 10.00-ml volumetric flask and the vial was then rinsed with a small (approximately 1 ml) amount of fresh solvent. A 1.00-ml volume of an internal standard solution of β-apo-8′-carotenal (containing 0.1%, v/v, of BHT) was added to each volumetric and the volume was then adjusted to 10.0 ml with DMF as needed.

Samples were stored at −78°C under a nitrogen atmosphere and removed as needed for analysis by HPLC. During analysis, samples were maintained at 0°C in the dark by immersion in an ice bath equipped with an opaque cover.

The remaining ten leaf disks from each sample were transferred to a tared scintillation vial and weighed in order to obtain fresh weight of leaf tissue per sample. The vials and disks were then lyophilized, reweighed in order to determine dry weights of the leaf tissue samples, and discarded.

HPLC analysis

Pigment analyses were carried out on a Perkin-Elmer Series 4 liquid chromatograph equipped with an LC 85B variable-wavelength detector set at 440 nm. The

column was a Supelcosil C_{18}-DB (3.3 × 0.46 cm I.D.) high-speed analytical column (Supelco, Bellefonte, PA, U.S.A.) containing 3-μm particles. The sample was applied to the column using a Rheodyne 7125 injector equipped with a 50-μl sample loop. Flow-rate was 2.0 ml/min. The mobile phase consisted of a 4-min linear gradient from 70% aqueous methanol (solvent A) to a 40:60 mixture of solvent A and ethyl acetate (solvent B) followed by a 4-min isocratic elution at the final solvent composition. The solvent program was returned to initial conditions over 2 min and re-equilibrated for 4 min prior to injection of the next sample.

Chromatographic resolution of complex samples was improved by dilution of the DMF-sample aliquot 1:1 with HPLC-grade water. Such measures were, however, typically not needed for the majority of samples analysed.

Date analysis was carried out using an IBM System 9000 computer running the CAP 2.0 version of the chromatographic data analysis software. An internal standard method was used throughout the experiment with β-apo-8'-carotenal as internal standard. Standards were prepared and calibration curves were determined routinely for the following components: chlorophyll *a*, chlorophyll *b*, β-carotene, lutein, and β-apo-8'-carotenal.

RESULTS AND DISCUSSION

Leaf disk samples were removed from the leaf lamina of fully expanded tobacco leaves in such a way that the disks were representative of the whole leaf sample. Prior work in our laboratory and elsewhere has shown that a number of leaf constituents vary as a function of position within the leaf[14,15].

The solvent extraction procedure utilizing DMF proved to be an efficient and reproducible method for the extraction of pigments from large numbers of samples. Extraction of leaf disks was complete after 48 h in all cases (data not shown). The method employed allowed the efficient workup of fifty samples a day. Larger numbers of samples could readily be accommodated without excessive increases in time. Work in our laboratories with other plants (soybean, peanut and barley leaves) indicates that this extraction technique is applicable to these plant materials as well.

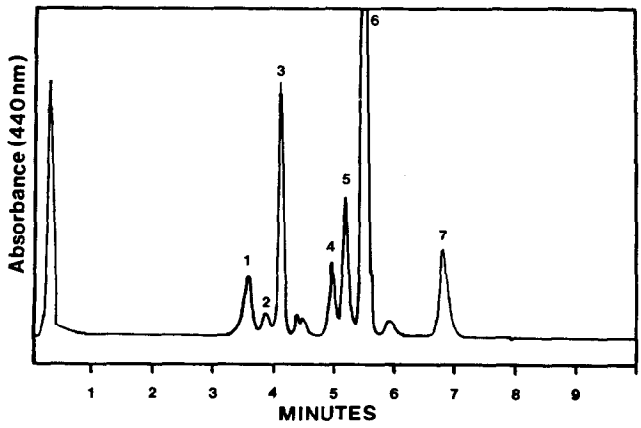

Fig. 1. A typical chromatogram of the analysis of *Nicotiana tabacum* green leaf pigments. For peak identification, see Table I.

TABLE I

IDENTIFICATION AND RELATIVE RETENTION TIMES OF *NICOTIANA TABACUM* LEAF PIGMENTS

Average of five determinations selected at random.

Peak No.	*Compound*	*Average retention time (min)*	*Relative retention time*
1	Neoxanthin	3.45 ± 0.03	0.72
2	Violoxanthin	3.83 ± 0.03	0.78
3	Lutein	4.09 ± 0.03	0.83
4	β-Apocarotenal[a]	4.94 ± 0.03	1.00
5	Chlorophyll *b*	5.15 ± 0.03	1.04
6	Chlorophyll *a*	5.44 ± 0.03	1.10
7	β-Carotene	6.80 ± 0.09	1.38

[a] Internal standard.

The use of sub-samples for fresh and dry weight determinations allows the expression of results to be made on either a leaf area, fresh weight or a dry weight basis. These determinations add to the overall time of analysis, but the actual time required is small and inclusion of these steps is required only if results are to be expressed on a weight basis. The use of leaf punches of known area eliminates the need to measure leaf area directly.

A typical chromatogram obtained in the course of these experiments is shown in Fig. 1. The time of analysis was 14 min from injection to injection. The use of a high-speed column proved to be of great value in improving the speed of the analysis. Prior attempts in our laboratory to optimize analysis time using normal analytical columns (*i.e.* 25 × 0.46 cm I.D. C_{18} columns) resulted in analysis times of approximately 25 min at best, with little increase in resolution. Thus, the use of the high-speed column allows roughly a two-fold increase in sample through-put with virtually no loss in efficiency and also results in savings in solvent and column costs.

Table I gives the identification and relative retention data for the peaks identified in Fig. 1. Identification was based on comparison with known standards, known

TABLE II

RELATIVE ERROR OF QUANTITATION OF *NICOTIANA TABACUM* LEAF PIGMENTS

Pigment	*Relative error (%) (n = 3)*				
	Mass of pigment injected (μg)				
	0.25	*0.50*	*1.25*	*2.50*	*25.0*
Lutein	2.3	1.7	0.4	0.4	0.3
β-Apocarotenal	5.3	0.9	0.6	0.3	0.7
Chlorophyll *a*	9.8	1.7	1.3	1.0	0.7
Chlorophyll *b*	1.2	1.9	0.9	0.6	0.2
β-Carotene	6.2	0.8	1.1	1.9	0.4

elution order from C_{18} columns, and spectral analysis of eluting peaks. Apocarotenal proved to be good choice for internal standard as it eluted in a time window which was devoid of other peaks. In addition, apocarotenal has not been reported to be a component of tobacco pigments.

Table II presents data on the reproducibility of the analytical method. These results indicate that the method yields quantitative results for most pigments analysed at most masses. Data were semi-quantitative for β-apo-8'-carotenal, chlorophyll *b*, and β-carotene at the lowest mass tested, 0.25 μg. This was well below the mass range normally encountered during our analyses.

CONCLUSION

The combination of the DMF-extraction technique and the high-speed HPLC method results in a system which is particularly amenable to the analysis of large numbers of samples. The use of DMF as an extracting solvent has proven to be a reliable and efficient means for the isolation of plant pigments. The stability of the DMF-pigment solution is particularly advantageous when samples cannot be immediately analysed. Samples in DMF stored for up to a week showed no sign of degradation, in agreement with the earlier report of Bergweiler and Lutz[3]. The addition of a small amount of antioxidant (0.1% BHT) to the extracting solvent and internal standard solutions may have enhanced this stability further. Storage of the solutions at -78°C under an inert atmosphere (nitrogen) also is advisable for maximal sample stability.

The high-speed HPLC method reported results in a least a two-fold improvement in time of analysis compared with existing pigment methods. The column in these experiments has been used for the analysis of over 300 samples with no significant loss of resolution.

In conclusion the overall method described here results in a significant savings in time and labor over existing techniques. The method should be of interest to researchers involved in repetitive analyses of large numbers of plant pigment samples. The savings derived from use of the less expensive high-speed liquid chromatography columns and the reduction in solvent consumption and resulting wastes are also positive considerations.

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REFERENCES

1 M. S. Tswett, *Ber. Dtsch Bot. Ges.*, 24 (1906) 316.
2 M. S. Tswett, *Ber. Dtsch Bot. Ges.*, 24 (1906) 384.

3 P. Bergweiler and C. Lutz, *Environ. Exp. Bot.*, 26 (1986) 207.
4 Y. Shioi, R. Fukae and T. Sasa, *Biochim. Biophys. Acta*, 722 (1983) 72.
5 K. Eskins, C. R. Scholfield and H. J. Dutton, *J. Chromatogr.*, 135 (1977) 217.
6 P. S. Stewart. P. A. Bailey and J. L. Beven, *J. Chromatogr.*, 282 (1983) 589.
7 S. Takagi, *Agric. Biol. Chem.*, 49 (1985) 211.
8 W. A. Court and J. G. Hendel, *Tob. Sci.*, 26 (1982) 103.
9 R. Moran and D. Porath, *Plant Physiol.*, 65 (1980) 478.
10 R. Moran, *Plant Physiol.*, 69 (1982) 1376-1381.
11 J. D. Hiscox and G. F. Israelstam, *Can. J. Bot.*, 57 (1979) 1332.
12 S. J. Schwartz and J. H. von Elbe, *J. Liq. Chromatogr.*, 5, (1982) Suppl. 1, 43.
13 J. L. DiCesare, M. W. Dong and L. S. Ettre, *Introduction to High Speed Liquid Chromatography*, Perkin-Elmer, Norwalk, CT, 1981, p. 106.
14 D. A. Danehower, *Tob. Sci.*, 31 (1987) 32.
15 R. W. Jenkins, H. J. Grubbs, R. H. Newman, R. T. Bass, J. S. Brenizer, D. C. Jones, T. G. Williamson, D. A. Danehower and R. C. Long. *J. Radioanal. Nucl. Chem.*, 113 (1987) 477.