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High-performance liquid chromatography–diode-array detection of photosynthetic pigments

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ABSTRACT

High-performance liquid chromatography and photodiode-array detection were applied simultaneously for the separation and identification of the photosynthetic pigments of a plant extract. Using a photodiode-array detector permitted simultaneous recording of the chromatographic analysis at different wavelengths, the contour plots of the chromatograms and the spectra of the pigments separated by high-performance liquid chromatography. Employing these techniques, and on the basis of their spectroscopic characteristics, the following compounds were identified: β-carotene, lutein, taraxanthin, violaxanthin, neoxanthin and chlorophylls *a* and *b* and their respective epimers, chlorophylls *a'* and *b'*. The rapidity, specificity, sensitivity and reproducibility of this technique make it particularly suitable for plant pigment analysis.

INTRODUCTION

High-performance liquid chromatographic (HPLC) methods are extensively used nowadays in the analysis of photosynthetic pigments [1–5]. This technique combines the ability to separate the pigments with the spectroscopic features of sensitive, selective identification through peak scanning and quantification. The identification of the peaks in a chromatogram is easy when known standards are available, but in the case of plant pigments only chlorophylls and some carotenes can be obtained commercially. Most pigments must be prepared by means of semipreparative chromatographic techniques, but this procedure is rather tedious and is not exempt from possible alterations [3].

At present, detectors based on photodiode-array spectroscopy permit the immediate identification of the components of a mixture by using their spectral characteristics. Moreover, photodiode-array detection (PAD) provides the spectrum, the absorbance ratio and the criteria for assessing the purity of the peaks.

In this paper we describe the separation and spectrophotometric identification of foliar photosynthetic pigments, using normal-phase HPLC with PAD.

EXPERIMENTAL

Apparatus

HPLC separation was carried out using as Shimadzu liquid chromatographic system (Shimadzu, Kyoto, Japan) equipped with two LC-6A pumps, an SCL-6A controller and an SPD-M6A photodiode-array detector. The data were stored and processed by a 386 SX personal computer (Olivetti, Ivrea, Italy) provided with chromatographic software (Shimadzu).

The absorption spectra of isolated pigments were recorded on a Hitachi U-3200 spectrophotometer (Hitachi, Tokyo, Japan), with double monochromator and wavelength(λ) accuracy of 0.3 nm. The spectrophotometric software provided,among other things, the automatic detection of λ-maxima.

Columns

Analytical separation was performed on a stainless-steel column (25 cm × 4.6 mm I.D.) of Spherisorb (Phase Separations, Norwalk, CT, USA)

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(5-μm spherical particles). Semipreparative separation was carried out on a stainless-steel column (30 cm × 7.8 mm I.D.) of μPorasil (Waters, Milford, MA, USA) (10-μm irregular particles).

Pigment extraction

A uniform sample of *Citrus limon* leaves was chopped into small pieces, and 10-g subsamples were extracted with 100% acetone in a Sorvall omni-mixer (Sorvall, Norwalk, CT, USA) at low temperature. The homogenate was filtered through a sintered-glass funnel and the residue was re-extracted until it was colourless. The filtrates were combined and made up to 250 ml with the same solvent. For HPCL analysis a 5-ml aliquot was dried in a nitrogen stream and the residue was dissolved in 500 μl of methanol and passed through a 0.45-μm nylon filter (Lida, Kenosha, WI, USA) prior to injection. The extract (20 or 200 μl for analytical or semipreparative separation, respectively) was injected into the chromatograph. The reagents employed were HPLC quality (Romil Chemicals, Loughborough, UK).

Chromatography

The pigments were eluted using an initial solvent mixture of light petroleum (LP) (b.p. 40–60°C) and ethanol (97:3, v/v). After 7 min, a gradient was initiated, changing the LP concentration linearly to 85% in 3 min. This composition was maintained until the pigments were completely eluted. The flow-rate for the analytical separation was 1 ml/min. Semipreparative separation was performed with the same gradient with the flow-rate increased to 4 ml/min.

Identification of pigments

Isolated pigments were identified according to their visible absorption characteristics compared with those in the literature [5–14]. The presence of epoxide groups was shown by their hypsochromic shift after treatment with hydrochloric acid [3].

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of a methanol extract obtained from *Citrus limon* leaves. Complete separation of all pigments was obtained in less than 12 min, showing that the stepwise gradient elution minimizes elution time without loss of resolution. Thus, with this chromatographic programme in normal-phase, we achieved a much sorter separation time than is usual with reversed-phase chromatography. The quality of the separation was evaluated by means of separation selectivity and resolution of the peaks was calculated according to Kirkland [15] (Table I).

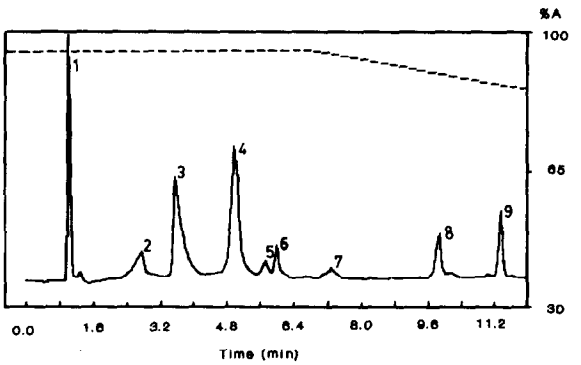

Fig. 1. Chromatogram of a foliar extract recorded at 430 nm. The linear gradient of light petroleum (A)–ethanol (B) is indicated by the dotted line. Analytical conditions are described in the text.

PAD is a powerful tool for the identification of different pigments present in a leaf extract; it permits a spectral analysis in real time without stopping the flow. The post-analysis treatment of data

TABLE I

CHROMATOGRAPHIC PARAMETERS CORRESPONDING TO THE CHROMATOGRAM SHOWN IN FIG. 1

Symbols: t' = reduced retention time ($t' = t_R - t_0$); t_0 (dead time) = 0.80 min; ω = band width (min); α = separation factor; R_s = resolution.

Peak No.	t'	ω	α	R_s
1	0.28	0.09	–	–
2	1.97	0.47	7.03	6.04
3	2.77	0.19	1.41	2.42
4	4.21	0.19	1.52	7.58
5	4.95	0.09	1.18	5.29
6	5.20	0.14	1.05	2.17
7	6.49	0.19	1.25	7.82
8	9.07	0.09	1.40	18.43
9	10.57	0.09	1.17	16.66

employing chromatographic software provides spectra, contour plots, three-dimensional representations, maxima detection and peak purity. The spectra obtained can be overlaid on a standard stored in a computer-aided library to help subsequent identification.

Table II summarizes the identification of the chromatographic peaks according to spectral data provided by PAD and data obtained from pure pigments isolated by semipreparative chromatography, subsequently purified in an analytical column and characterized in a typical solvent. These last data agreed with those referred to previously by us [5]. Data were compared with those presented in the references.

The chromatographic system made possible the identification of nine pigments. Five of them were carotenoids: β-carotene, lutein, taraxanthin, violaxanthin and neoxanthin. The others were chlorophylls and derivatives. We wish to emphasize the resolution of the epimers chlorophyll *a'* and chlorophyll *b'* in relation to chlorophylls *a* and *b*, respectively.

The contour plot, which is a pseudo-tridimensional representation of the absorbance, wavelength and time data (isogram), has proved to be very useful for the identification of plant pigments. The isograms summarize the chromatographic information and can aid in the selection of an optimal detection wavelength; they also give information on the spec-

TABLE II

IDENTITIES, RETENTION TIMES (t_R), PURITY AND SPECTRAL DATA FOR PIGMENTS SHOWN IN FIG. 1

Peak No.	t_R (min)	Pigment	Spectra maxima of the PAD (nm)			Purity	Solvent	Published maxima (nm)			Ref.
1	1.08	β-carotene	421	446	472	0.999	Eluent				
							Hexane	425	449	476	5
							Hexane	427	450	476	6
							Hexane	425	450	477	7
2	2.77	Chlorophyll *a'*	429	614	663	0.999	Eluent				
							Diethyl ether	428	614	663	8
3	3.57	Chlorophyl *a*	429	616	663	0.999	Eluent				
							Diethyl ether	430	615	661	5
							Diethyl ether	430	614	662	9
							Diethyl ether	430	615	662	10
4	5.01	Lutein	420	446	472	0.999	Eluent				
							Hexane	420	447	472	5
							Hexane	420	445	474	11
							Hexane	420	445	475	7
5	5.75	Chlorophyll *b'*	456	643		0.999	Eluent				
6	6.00	Chlorophyll *b*	456	645		0.999	Eluent				
							Diethyl ether	452	642		5
							Diethyl ether	453	643		9
							Diethyl ether	455	644		10
7	7.29	Taraxanthin	421	446	472	0.996	Eluent				
							Hexane	420	444	470	5
							Hexane	419	442	470	12
							Hexane	420	445	470	7
8	9.87	Violaxanthin	419	442	470	0.999	Eluent				
							Hexane	420	444	473	5
							Hexane	416	441	471	13
							Hexane	414	436	468	14
9	11.37	Neoxanthin	415	437	466	0.997	Eluent				
							Hexane	412	435	465	5
							Hexane	412	436	465	12
							Hexane	408	432	462	14

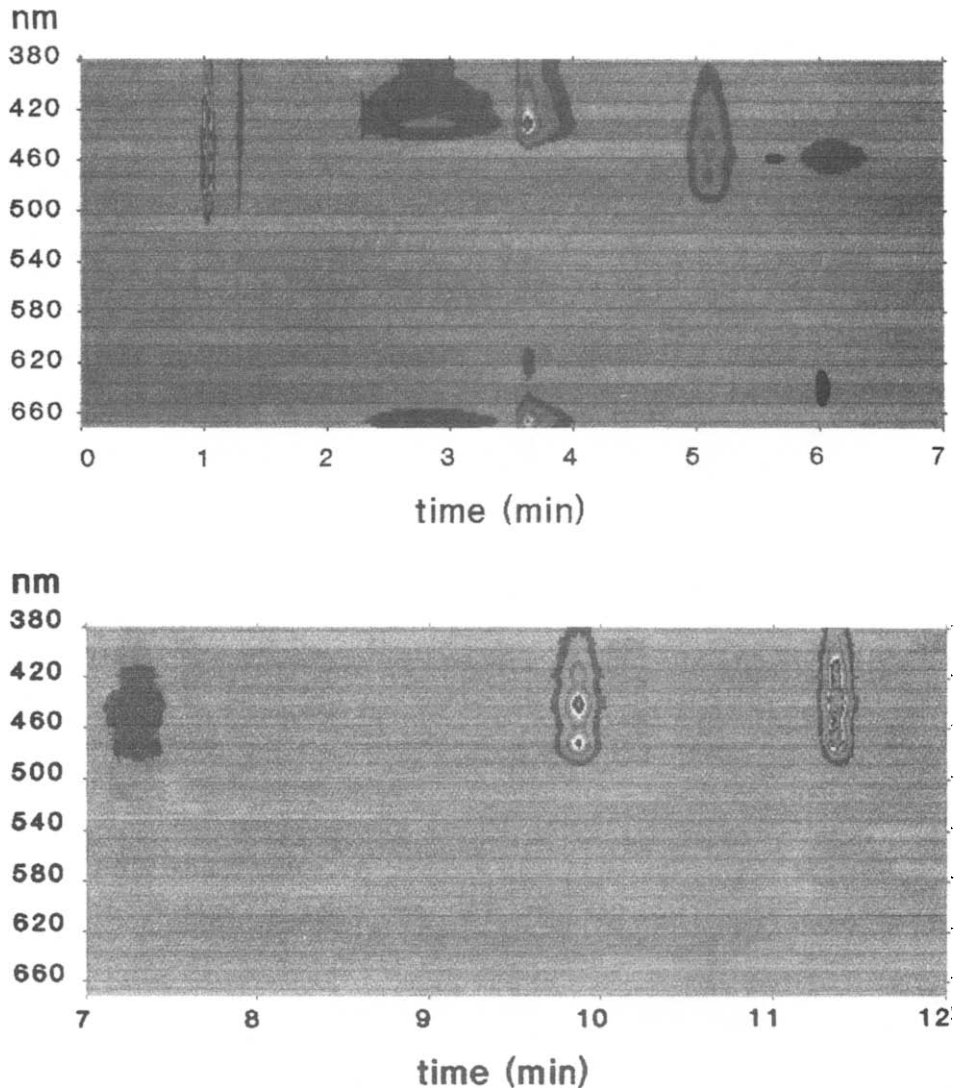

Fig. 2. Isogram of the chromatographic separation shown in Fig. 1.

tral maxima of the peaks. Fig. 2 shows the isogram of the chromatographic separation displayed in Fig. 1.

While the contour plots of the carotenoids show a high degree of symmetry, those of the chlorophylls present signs of tailing (poor chromatographic symmetry); nevertheless, their purity is proved by the symmetry in the wavelength dimension.

In addition, the isogram permits the detection of co-eluting peaks, particularly when their absorption maxima are sufficiently separated. Fig. 3 shows an isogram corresponding to a chromatogram where lutein and chlorophyll *b* co-eluted at the same time (*ca.* 3 min) in a seemingly symmetric peak. Nevertheless, the isogram shows a distortion between 2.7 and 3.3 min and, also, a contour plot is displayed around 645 nm; both effects are the result of the presence of chlorophyll *b*. When this chromatogram is run at 446 and 645 nm simultaneously, a

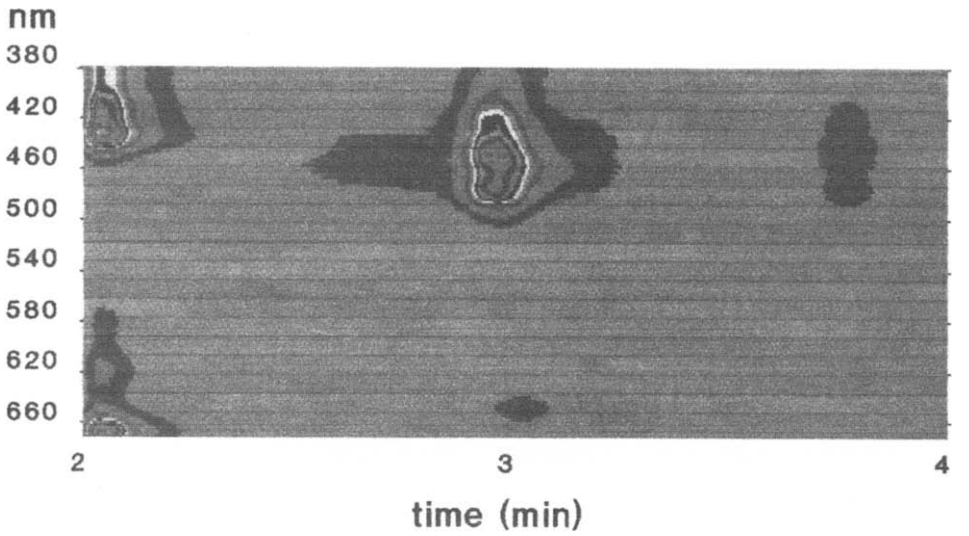

Fig. 3. Isogram of a poor chromatographic separation in which lutein and chlorophyll *b* co-eluted at the same time.

slight displacement in the retention time of the peaks eluted around 3 min can be observed. It is attributed to the fact that in each case a different pigment is detected. Thus, isograms may prove useful in setting up the conditions to optimize the elution programme and to obtain a rapid overview of the quality of the separation.

In conclusion, the joint use of HPLC and PAD is very useful in separating and identifying plant pigments as absorption spectra are obtained within fractions of a second. PAD also permits simultaneous chromatographic analyses at different wavelengths. In this way, each pigment can be detected at its maximum absorption wavelength, which provides a higher sensitivity. Even in the case of co-eluting peaks, the use of factor analysis and peak suppression techniques permits the mathematical resolution of overlapping peaks.

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