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HIGH-SPEED VIDEO-DENSITOMETRIC DETERMINATION OF CHLOROPHYLLS AND CAROTENES SEPARATED BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The quantitative degradation of chlorophyll *a* and *b* into pheophytin *a* and *b* during thin-layer chromatographic (TLC) separation on silica gel plates containing 10% of ammonium sulphate suggests an indirect method for determining the original chlorophyll *a* and *b* contents in addition to the total carotenes in plant extracts. Such chromatograms, showing the three well separated spots of carotenes and pheophytin *a* and *b* above the less well resolved zones of accompanying carotenoid pigments can be evaluated by the recently developed high-speed video-densitometric technique, originally used for the large-scale quantitative TLC analysis of amino acids. The sensitivity and reproducibility of the video-densitometric pigment determinations were similar to those of spectrophotometric measurements, but the former technique has several advantages.

INTRODUCTION

The quantitative analysis of the main photosynthetic pigments chlorophyll (chl) *a*, chl *b* and carotenes is often required for the characterization of plant materials in ecology, plant breeding, food industry, etc. The usual spectrophotometric methods for the determination of chlorophylls[1], however, result in a relative error of as much as 20%[2] caused especially by the degradation of the chlorophylls into the corresponding pheophytins (phe) during the sample preparation processes. To solve this problem, some workers have proposed the spectrophotometric determination of chlorophylls after their acidic degradation into pheophytins[3] or after removing the magnesium from chlorophylls by cation-exchange chromatography[4]. These indirect methods, however, have not become generally accepted because the resulting preparation contains some pigment impurities in addition to pheophytins[5]. Further, the spectrophotometric determination of carotenes (mainly β-carotene) in the presence of chlorophylls and their derivatives in the same extract is also difficult because of overlapping of the absorption bands[6]. Further purification (*e.g.*, saponification of chlorophylls) is needed in order to overcome this problem, which makes the determi-

nation very time consuming. To our knowledge, no reliable method for the determination of photosynthetic pigments on thin-layer chromatographic (TLC) plates after their separation has been suggested.

Recently, a reflection densitometer employing an electronically controlled television (TV) camera (Telechrom automatic video-densitometer, Type OE-976, marketed by EuroLab, Munich, G.F.R.) has been used for high-speed quantitative evaluations of chromatosheets, electropherograms and various photographic plates[7–10]. This apparatus is suitable for the densitometric determination of the visible spots of one-dimensionally developed thin-layer chromatograms in either the manual or automatic scanning mode according to a pre-set programme. If chromatograms are uniformly developed, up to six spots of nine different samples can be evaluated automatically on a single plate. The measurement of one spot takes only about 4 sec, including the printing of the relevant density and quantitative values. Such a rapid determination, however, requires geometrically uniform chromatograms consisting of well separated spots of the components to be measured.

Unfortunately, most of the available TLC procedures that have been suggested for the separation of photosynthetic pigments[11,12] are unsuitable for obtaining such well resolved chromatograms. In these instances only the single spot of total carotenes is located separately near the solvent front but the following spots of chl *a* and *b* cannot be well separated from each other and those of polar carotenoids. Chromatograms on silica gel plates also show readily distinguishable spots of phe *a* and *b*, which are the mild acidic degradation products of the corresponding chlorophylls, formed during the TLC separation or during the preceding extraction steps[13].

In this paper we describe a modified TLC method for the indirect video-densitometric determination of chl *a* and *b* in the form of phe *a* and *b*, respectively, in the presence of the extracted carotenes after the chlorophylls have been degraded quantitatively to the readily separable pheophytins during the chromatographic development.

EXPERIMENTAL

Leaf extract and pigments

About 20 g of fresh spinach leaves were extracted by a common method using boiling water before the extraction step[14] instead of the simultaneous disruption of leaves. This simple method led to the quantitative extraction of chlorophylls with a light petroleum (b.p. 00–00°)–methanol mixture, resulting in colourless leaf residues. On washing the extract with water an essential part of carotenoid pigments remained in the water–methanol phase. After evaporation of the light petroleum solution in a vacuum evaporator the dry extract was dissolved either in 1 ml of diethyl ether for storage or in 1 ml of chloroform for application of the sample on to the chromatoplates.

Pure chl *a* and *b* were prepared chromatographically on powdered sugar columns[14] from spinach leaf extract dissolved in light petroleum (b.p. 30–40°). The extinction ratios of the Soret and red absorption bands of the pigments obtained were 1.30 and 2.81 for chl *a* and *b*, respectively, in diethyl ether. Commercial β-carotene (Sigma, St. Louis, Mo., U.S.A.) was used without further purification.

TLC plates

For preparing TLC plates, 25 g of silica gel G (Reanal, Budapest, Hungary) or silica gel HF_{254} (Merck, Darmstadt, G.F.R.) were suspended in 60 ml of water and the resulting slurry was spread on the surface of 20 × 20 cm glass plates to a thickness of 0.25 mm with a Desaga spreader. The wet plates were air dried and activated at 120° for 1 h before use.

Plates containing ammonium sulphate were prepared in the same way except that the appropriate amount of ammonium sulphate was added to the silica gel before mixing it with water (*e.g.*, 2.5 g of ammonium sulphate added to 25 g of silica gel gives a 10% ammonium sulphate content expressed as a percentage of the silica gel used). The pre-coated 20 × 20 cm silica gel 60 plates (Merck) were impregnated by the ascending technique with 10% ammonium sulphate solution in a chromatographic chamber before heat activation.

TLC separation

The chloroform solutions of leaf extracts and pure pigments were applied to the activated plates with a Hamilton microsyringe as a short band located 2.5 cm from the lower edge of the layers. The solvent system used for development[12,15] was isooctane–acetone–diethyl ether (3:1:1). The ascending separations were carried out in a temperature-controlled box to a height of 16 cm. This process took about 45–50 min.

Pure chl *a*, chl *b* and β-carotene were used for the identification of the main pigment spots. For spectrophotometric measurements the spots were scraped off the plates and the pheophytins and carotenes were extracted from the gel with diethyl ether or chloroform, respectively, and the extracts were filtered through Whatman glass-fibre paper. The absorption spectra of pigments were recorded on a Specord UV–VIS spectrophotometer (Carl Zeiss, Jena, G.D.R.).

For video-densitometric measurements 1–10 μl of a standard mixture of 2 mg of chl *a*, 1 mg of chl *b* and 1 mg of β-carotene dissolved in 1 ml of chloroform were applied at the various sample positions on silica gel plates containing 10% of ammonium sulphate. For the determination of the amounts of pigment in an unknown sample 5 μl of a chloroform solution of spinach leaf extract were applied to the chromatoplates next to 2- and 8-μl samples of a standard mixture.

Video-densitometric measurements

The chromatograms were evaluated with a Telechrom automatic video-densitometer, Type OE-976 [7–10], by placing them so that the sample components have a horizontal arrangement in the sample holder instead of the usual vertical arrangement. During these horizontal measurements the black "electronic window" measuring the total density could cover the same coloured spots of the different samples applied on the same chromatoplate. In this way, the difficulties arising from the colour dependence of the vidicon signal and from changes in the total density values in samples with different compositions could be ignored.

RESULTS AND DISCUSSION

The TLC separation of chlorophylls and carotenes on silica gel plates, either self-prepared or pre-coated, does not result in a satisfactory resolution of the indi-

vidual pigment zones. The spots of chl *a* and *b* overlapping each other and those of polar carotenoid pigments on a pre-coated silica gel 60 plate are shown in Fig. 1A. The presence of an appropriate amount of ammonium sulphate in silica gel layers of different types, however, leads to the quantitative degradation of chlorophylls, resulting in the disappearance of tailing spots and the appearance of well separated greyish spots of the pheophytin degradation products. Such a chromatogram developed on a pre-coated silica gel 60 plate which was impregnated with 10% ammonium sulphate solution before sample application is shown in Fig. 1B.

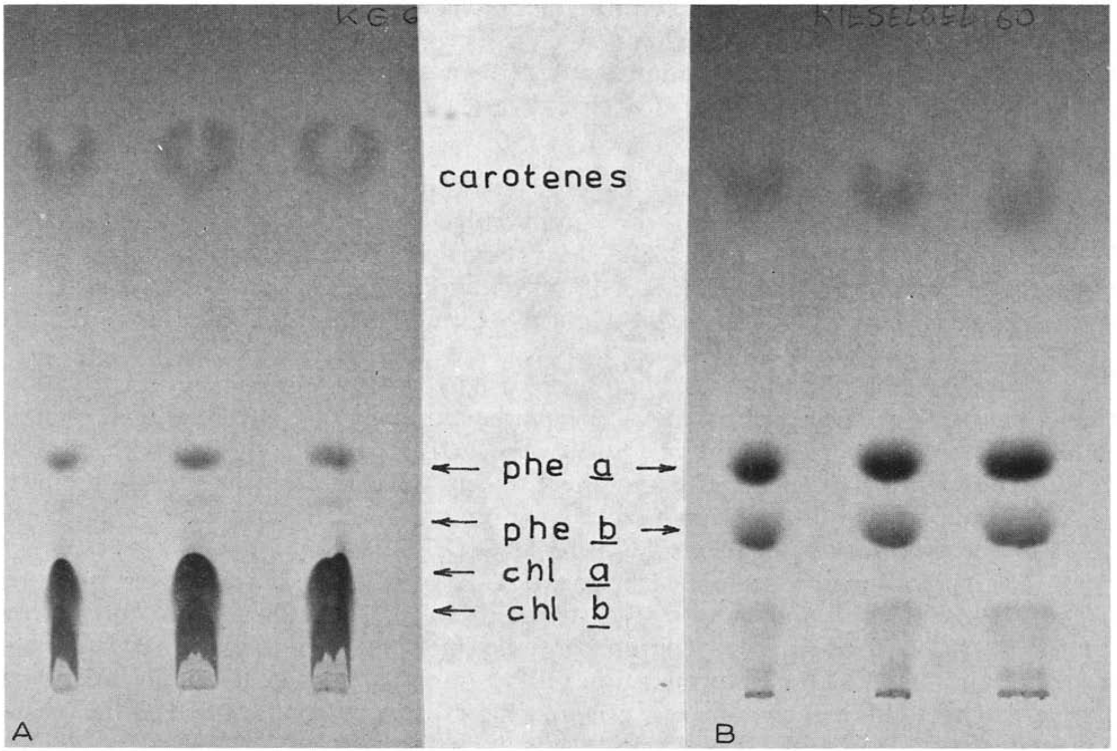

Fig. 1. Thin-layer chromatograms of a spinach leaf extract. The chromatograms were developed on pre-coated silica gel 60 plates not treated with ammonium sulphate solution (A) and impregnated with 10% ammonium sulphate solution before sample application (B). The pale spots covered by chlorophylls on plate A or observed under the phe b spot on plate B are those of lutein and xanthophylls. The volumes of the chloroform solution of the extract applied (2.5 mg of chlorophyll/ml of chloroform) were 3, 4 and 5 μl from the left.

Tests with various amounts of ammonium sulphate in self-prepared chromatographic plates indicated that apparently quantitative degradation of chlorophylls can be accomplished with about a 10% ammonium sulphate content. At higher levels the salt tends to form crystals in the drying layers, which interfere with the uniform separation of pigment spots. On developing the chromatograms at different temperatures, an increasing effects on the separation of the spots was observed at higher temperatures, but it is not reasonable to heat them to more than 30° on account of the high vapour pressure of the solvent system used. The absorption spectra of the

grey pigments eluted from the chromatograms developed on layers containing 10% ammonium sulphate agree well with those of phe *a* and *b* published by French[16]. In addition, no change in the absorption of carotenes running together near the solvent front was observed during the TLC separation, even on the plates containing ammonium sulphate.

The effect of ammonium sulphate on the degradation of chlorophylls can be explained by the generation of sulphuric acid from ammonium sulphate during the heat activation of the chromatoplates before sample application. Such a partial heat decomposition of ammonium sulphate to sulphuric acid is generally used for charring colourless organic substances by the heat treatment of thin-layer chromatograms[17,18]. Indeed, after heat activation, the colour changes characteristic of the pH shift toward the acidic range can be observed by the ascending technique using pH indicator solutions (bromophenol blue and methyl orange) in the layers containing ammonium sulphate. Such colour changes of the pH indicator solutions are not observed before heat activation of plates prepared with ammonium sulphate or on plates that do not contain ammonium sulphate, even after heat treatment. Using other salts, such as magnesium sulphate and calcium sulphate (the latter is the binding material in silica gel G), we did not observe any detectable effects on the degradation of chlorophylls.

Chromatograms developed under the optimal conditions (10% of ammonium sulphate, temperature 30°) contain well separated pigment spots as required for the high speed video-densitometric determinations. The sensitivity of video-densitometric measurements studied with a standard mixture of chl *a*, chl *b* and *β*-carotene is similar to that found for amino acids stained with ninhydrin[10,19]. A linear relationship between the measured relative density and the applied amounts of the different pigments is observed in the approximate 1–10 μg (Fig. 2). At higher pigment levels the chromatograms become overloaded, which leads to the unsatisfactory separation of the individual pigments and interference with the video-densitometric measurements. Fading of the separated pigment spots in light was negligible for 15 min under the conditions of the densitometric measurements.

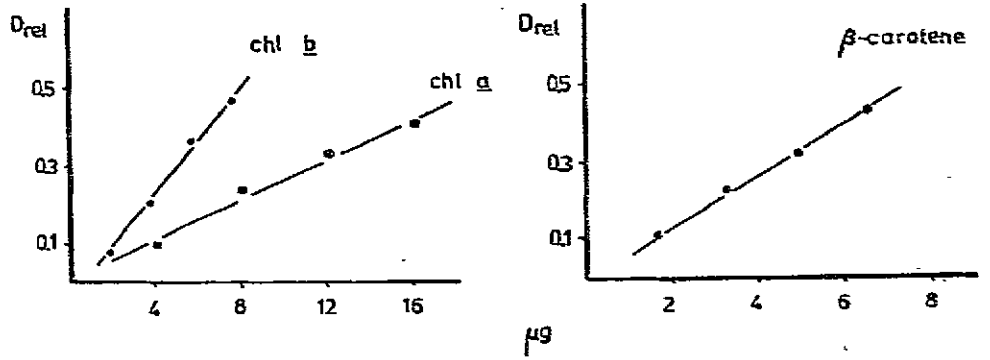

Fig. 2. Sensitivity curves showing the relative densities (D_{rel}) obtained by video-densitometric measurements and plotted against the amounts of pigment applied on the chromatoplates. The mean values plotted are the average of four separate measurements carried out on different chromatoplates on which 2, 4, 6 and 8-μl volumes of the standard mixture (2.02 mg of chl *a*, 0.95 mg of chl *b* and 0.82 mg of *β*-carotene dissolved in 1 ml of chloroform) at different sample positions were applied.

The amount (q) of a well separated sample component can be calculated from the equation

$$q = \frac{Qd_{rel}}{D_{rel}}$$

where Q is the amount of standard material applied and D_{rel} and d_{rel} are the measured relative densities of the standard and the unknown component, respectively.

The amounts of chl *a* and *b* determined by spectrophotometric and video-densitometric measurements were in good agreement (Table I). According to the standard deviations of the mean values, the reproducibility of the video-densitometric pigment determinations is about ± 5–10% in each instance.

TABLE I

COMPARISON OF chl *a* AND *b* CONCENTRATIONS DETERMINED BY SPECTROPHOTOMETRIC AND VIDEO-DENSITOMETRIC MEASUREMENTS

In both instances the results of three separate measurements and the mean values ± standard deviations are shown.

Substance	*Concentration (μg/μl)*							
	*Spectrophotometry**				*Video-densitometry*			
	1	*2*	*3*	*Mean ± S.D.*	*1*	*2*	*3*	*Mean ± S.D.*
Chl *a*	2.01	1.91	1.84	1.92 ± 0.09	1.70	1.88	1.86	1.81 ± 0.10
Chl *b*	0.75	0.71	0.63	0.69 ± 0.06	0.66	0.68	0.59	0.64 ± 0.05

* Solutions in diethyl ether.

These findings suggests that the chl *a*, chl *b* and total carotene contents of plant materials can be determined by the proposed rapid video-densitometric technique using thin-layer chromatograms and involving the conversion of chlorophylls into the corresponding pheophytins during the separation of extracted pigments. The method has the following advantages:

(a) It is insensitive to the conversion of chlorophylls to pheophytins which usually occurs during the sample preparation;

(b) Determination of the total carotene content in the presence of chl *a* and *b* in the same extract is possible;

(c) Several hundred samples can be analysed per day, and the error is no higher than that in the spectrophotometric measurements, considering the variations among the chromatograms measured.

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