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Fluorgraphic Technique for Detecting and Recording Chlorophyll and its Derivatives on Paper and Thin-Layer Chromatograms

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FLUOROGRAPHIC TECHNIQUE FOR DETECTING AND RECORDING CHLOROPHYLL AND ITS DERIVATIVES ON PAPER AND THIN-LAYER CHROMATOGRAMS

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ABSTRACT

Light from a blacklight lamp (General Electric BLB) is restricted to a waveband range 360 - 470 nm by cut-off filters consisting of 4 cm 1 M aqueous $CuSO_4$ and acrylic plastic sheets. This filtered lamp emission is used to excite the characteristic red fluorescence of chlorophyll and its derivatives on paper or thin-layer chromatograms. Fluorescent spots are detected and recorded by exposure of red-sensitive panchromatic photographic printing paper (Kodak Panalure) to the fluorescing chromatogram. A thin yellow filter interposed between the chromatogram and the photoprint paper restricts the detected fluorescence to wavelengths greater than about 500 nm. Standard development of the photoprint yields grey to black spots on a white background. Intensity and size of the recorded spot is proportional to the amount of a single chlorophyll derivative on the chromatogram over a limited range of pigment applied to the chromatogram. A one-minute exposure with filtered light from an 8 watt GE BLB lamp at a 10-cm distance will record 0.3 nanomole (270 nanogram) chlorophyll *a* on Whatman No. 1 or on Whatman 3 MM paper chromatograms. Detection of chlorophyll derivatives by this technique is at least 10-fold more sensitive than visualization of the pigment spots on the chromatograms by their green color. This fluorographic technique can be a useful adjunct to chromatographic analyses of porphyrins in general.

INTRODUCTION

Chlorophylls and other porphyrin derivatives emit characteristic orange to deep red fluorescence when irradiated with ultraviolet (UV) light or with blue-violet light. This fluorescence allows visual detection of these substances on paper or thin-layer (TL) chromatograms with greater sensitivity than visualization of the pigment spots by their reflected colors in white light (Falk, 1964; Šesták, 1963). Depending on the color of the compound and the background, spots containing a few micrograms of some porphyrins are visible by reflected light. However, with fluorescence using 365 nm excitation, detection can be increased 100-fold, and with special fluorescent enhancement techniques as little as 0.005 μg of some porphyrins can be detected on paper chromatograms (Blumer, 1956).

Location of fluorescent spots on chromatograms can be recorded by outlining the spots while the chromatogram is viewed under UV illumination (Udenfriend, 1962) or by photography (Jackson, 1965; Milton, 1962; Sievers and Hynninen, 1977). Many of the chlorophyll derivatives are unstable under such conditions, i.e. UV light adsorption causes bleaching apparently via photo-oxidation processes (Falk, 1964). Thus, recording of fluorescent spots of chlorophyll derivatives on paper or TL chromatograms requiring prolonged or repetitive exposure to UV light may lead to incomplete registering because of spot disappearance.

Contact-photoprinting of fluorescence from paper chromatograms was first reported for recording nucleic acid spots (Markham and Smith, 1949). By flooding the developed chromatogram with a fluorescent compound, the UV-adsorbing nucleic acid spots appeared as dark areas on a fluorescing background when the chromatogram was viewed under UV (265 nm) light. When a contact photoprint was made of the fluorescing chromatogram, nucleic acids were recorded as white spots on a dark background. Abelson (1960) developed a contact printing process for recording fluorescent spots of paper chromatograms directly. This technique involves use of a near-UV light source (ca. 365 nm) for excitation. The fluorescence exposes a sheet of Kodabromide printing paper. A Kodak Wratten 2A filter interposed between the chromatogram and the photographic paper prevents spurious UV light from being recorded. Modifications of these basic fluorographic techniques have been used to record several different kinds of fluorescent substances or UV-absorbing substances on chromatograms (e.g., Abelson and Rosenfeld, 1962; Bush, 1952; Jones, 1965). However, application of fluorographic techniques for recording spots of porphyrin derivatives, in particular chlorophyll derivatives, has yet to be reported.

A fluorographic method based generally on the technique of Abelson (1960) has been developed for chlorophyll and its derivatives. Fluorescence of the pigment spots is excited with blue-violet light and is then recorded by contact printing on photographic printing paper having a panchromatic emulsion.

METHODS AND MATERIALS

Fluorographic Apparatus and Technique.

Arrangement of components in the apparatus is depicted in Figure 1. The excitation light source for fluorescence is an 8 watt blacklight lamp with an integral filter (General Electric BLB lamp) which emits light maximally ca. 365 nm. This light source emits UV which causes significant, rapid bleaching of chlorophyll derivative spots on chromatograms. The destructive UV wavelengths shorter than 350 nm are removed by inclusion of three 2.5 mm-thick acrylic plastic sheets (Flex-O-Glaze; Warp Brothers, Chicago, IL) in the light path. This filter system yields uniform 65% transmittance from 400 to 800 nm with less than 2% transmittance at wavelengths less than 350 nm. A liquid filter of 1 M aqueous $CuSO_4$ is also required to eliminate the small amount of lamp-emitted red light which causes fogging of the photographic paper during exposure. Although the container for the liquid filter shown in Figure 1 was a Pyrex glass tray, a container can also be made from acrylic plastic to combine the two kinds of cut-off filters that must be used with the blacklight source. The emission of this filtered light source, calculated from the transmission curves of the filter systems and from the spectral emission curve for a GE BLB lamp (Jackson, 1965) has the major emission band from 360 to 470

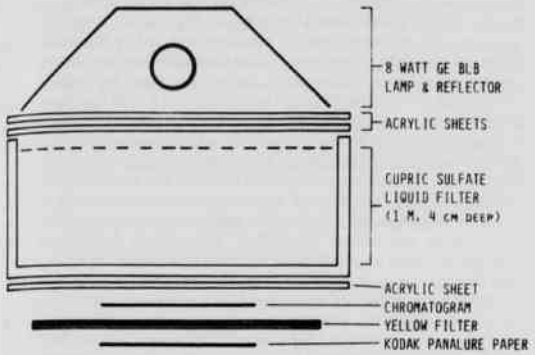

Figure 1. Arrangement of light source, filters, chromatogram, and photographic paper during fluorography.

nm with a peak near 390 nm. A secondary filter interposed between the chromatogram and the photographic paper is used to restrict the fluorescence detected by the photographic paper to wavelengths greater than ca. 520 nm. Any yellow or orange filter materials can be used, provided they transmit strongly from 550 to 750 nm but have less than 1% transmittance from 350 to 480 nm. A useful secondary filter is composed of four layers of commercial yellow cellophane sandwiched between 5 mil-thick, rigid plastic sheets. It is important that this secondary filter be as thin as possible for maximum fluorescence detection and minimum spot magnification during exposure of the photographic paper. Transmission characteristics of all the filters in the system were determined by measurement with a Perkin-Elmer model 202 spectrophotometer.

The critical element in the fluorographic apparatus is the panchromatic photographic paper which must be sensitive to light in the range 600 to 750 nm where prophyrin derivatives fluoresce (Falk, 1965). Kodak Panalure photographic printing paper has a spectral sensitivity from 400 to 680 nm (Eastman Kodak Co., personal communication). It is readily obtained and is sensitive to chlorophyll *a* fluorescence.

The blacklight lamp with reflector and excitation light filter system are mounted in a box that has a stop-aperature to minimize internal light scattering. A sliding panel in the light path is located below the liquid filter and above the chromatogram and is used as a shutter for controlled exposure periods. Before exposure, the yellow filter is placed over a sheet of photoprinting paper, and the developed chromatogram is placed over the yellow filter. With TL chromatograms having sucrose or cellulose adsorbent layers, the adsorbent surface of the developed chromatogram is covered with a protective 1 mil polyethylene film before placement, adsorbent surface down, on the yellow filter. Thus, the excitation light passes through the glass support of the chromatogram, and the fluorescing spots are close to the photographic emulsion. The chromatogram is held in close contact with the yellow filter and photographic paper by a piece of plate glass or in a photographic enlarging easel. This assembly is inserted into the box along guides which provide precise registry of the chromatogram in the light field at a 10-cm distance from the lamp. The operation must be conducted in a darkroom illuminated only with dim yellow-green or amber safelight because of the broad spectral sensitivity of the Kodak Panalure paper emulsion. Exposure times of 45 to 90 seconds are routinely used. Chromatograms on Whatman No. 1 paper require generally shorter exposures, while Whatman 3 MM and sucrose TL chromatograms require longer exposures for similar spot recording. The exposed photographic paper is then developed as recommended by the manufacturer.

Preparation of Chlorophyll Pigment Solutions.

To demonstrate the effectiveness of the fluorographic technique in analysis for pigment purity, fresh and aged extracts of henbit (*Lamium amplexicaule* L.) leaves were chromatographed, then fluorographed. One leaf sample was extracted by maceration with 80% aqueous acetone, then the brei was centrifuged free of debris. This extract solution was aged by exposure to room light and air for 5 hrs before chromatographic analysis. A second leaf sample was similarly extracted, but the extract was chromatographed within 15 min after extraction. Equivalent amounts of the two extracts (measured on the basis of their light absorption at 665 nm via spectrophotometry) were applied to the same chromatogram.

Sensitivity of detection by the fluorographic method was tested by chromatographing a mixture of chlorophyll *a* and pheophytin *a*. For this test, a chromatographically-pure mixture of the pigments from leaves of mung bean (*Vigna radiata* [L.] Wilczek) was prepared as follows: an acetone extract of the tissue was mixed with petroleum ether; the pigments were transferred into the ether layer by addition of water; the epiphase was partitioned with an equal volume of 90% aqueous methanol to extract the xanthophylls; then the epiphasic petroleum ether solution was chromatographed on a column of powdered sugar according to Smith and Benitez (1955). After development, the blue-green zone and part of the more mobile grey-green zone were removed from the column, and the pigments were eluted from the adsorbent with diethyl ether. The absorption spectrum of this solution was measured, and the amounts of chlorophyll *a* and pheophytin *a* were calculated from the absorbance values at 410 nm and 430 nm and the molar absorptivities of these pigments (Smith and Benitez, 1955). The molar ratio of chlorophyll *a* to pheophytin *a* in the solution was 1.8:1. Amounts of total pigment from 0.5 to 5.0 nmol (0.29 to 2.9 μg and 0.16 to 1.6 μg of chlorophyll *a* and pheophytin *a*, respectively) were applied to the chromatogram.

Paper Chromatography.

Pigment solutions were applied as spots near one end of 5 × 17 cm sheets of Whatman No. 1 or Whatman 3 MM chromatography paper. Pigment applications were limited to minimize overloading of the chromatograms (Strain, Sherma, and Grandoflo, 1968). Ascending development of the chromatograms with mixtures of acetone, benzene, and petroleum ether (b.p. 60-100 C) gave resolution of the chlorophyll derivatives as distinct spots within a solvent migration of ca. 13 cm which required 25 to 30 min at room temperature. All solvents were reagent grade. Routinely, a developing solvent of acetone-benzene-petroleum ether, 1:5:5 (v/v), gave sufficient resolution of the chlorophyll derivatives, and the spots remained stable for several exposures to light during fluorography. Solvent systems containing more polar components, e.g. 1-propanol, contributed to the rapid bleaching of some of the red-fluorescing spots during fluorography. All manipulations for developing and preparing the chromatogram for fluorography were conducted in dim room light or darkness to minimize degradation of the pigments. The developed chromatograms were air-dried before fluorography. No special techniques were used to enhance the fluorescence of spots on the chromatogram. When needed for future reference, the origin line and solvent front line on the developed chromatogram were marked with a small spot of 0.3% (w/v) aqueous Eosin Y solution near each end of the lines. Fluorescence of the dye in the spots was recorded during fluorography and allowed precise orientation of the chromatogram and fluorogram during subsequent analysis.

RESULTS AND DISCUSSION

The fluorographic technique reported here, in combination with paper and TL chromatographic analyses, has been useful for assessing the purity of chlorophyll derivatives, for estimating the extent of pigment degradation in leaf extracts and chlorophyll preparations,

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and for detecting unsuspected chlorophyll derivatives in certain pigment preparations. These aspects are illustrated in the fluorograms shown in Figure 2.

Figure 2A shows a fluorogram of the simultaneous chromatographic analysis of two extracts of *Lamium* leaves. Chromatographic separation of pigments in the fresh extract (Fig. 2A.i.) shows the presence of chlorophylls *a* and *b* and some pheophytin *a* (near the solvent front). Similar analysis of the pigments in an extract that was exposed to room light and air for 5 hrs before chromatography (Fig. 2B.ii.) shows the effect of destructive "aging" on the chlorophyll pigments; more pheophytin *a* was present, as well as two additional red-fluorescing pigments near the origin. Based on R_f characteristics in the chromatographic system used, the pigment at the origin was probably "oxidized chlorophyll" and that of $R_f = 0.07$ was probably chlorophyllide *a* which was produced by enzyme-catalyzed hydrolytic cleavage of the phytyl group from the chlorophyll *a*. The spots of both these low R_f compounds could not be detected on the chromatogram by their color in white light.

Sensitivity of the fluorographic technique is illustrated in Figure 2B. In this chromatographic analysis of various mixtures of chlorophyll *a* and pheophytin *a*, the lower limits of detection by fluorography are indicated as 0.18 nmol (0.16 μg) pheophytin *a* and 0.65 nmol (0.58 μg) chlorophyll *a*. (Chlorophyll *a* was recorded at 0.32 nmol on the original fluorogram, but the spot was lost during photographic reproduction for publication.) By comparison, only 1.1 nmol pheophytin *a* and 1.9 nmol chlorophyll *a* could be detected by the color of the spots in white light. These results also illustrate that detection is more sensitive for some chlorophyll derivatives than for others, e.g. pheophytin *a* could be detected at levels 2- to 5-fold less than could chlorophyll *a*. As suggested by the evidence in Figure 2B, the fluorographic method can be semi-quantitative for detection of chlorophyll derivatives on chromatograms. However, self-absorption of chlorophyll *a* fluorescence probably limits not only the sensitivity of detection but also the reliability of quantitative measurement of this pigment by fluorography. Therefore, application of this technique to quantitative analysis of individual chlorophyll derivatives (and porphyrins) must be established for each compound using standardized chromatographic and fluorographic methods.

This fluorographic technique may be a useful adjunct to paper or TL chromatographic analysis of porphyrin derivatives in general. Any fluorescence-exciting light source that emits light at 380 to 450 nm, the range of Soret band light absorption of the porphyrins (Falk, 1964), can be employed. The secondary filter interposed between the chromatogram and the photographic paper must be selected for transmission characteristics to eliminate any light from the exciting source that will cause background fogging of the photographic paper. This filter should also be highly transparent to the fluorescence emitted by the spots on the chromatogram. Although only one photographic printing paper (Kodak Panalure) has been used for fluorography in this study, any photoprinting paper with a red-sensitized, panchromatic emulsion should be adaptable to the fluorography of porphyrin spots on chromatograms. Usefulness of the technique can be extended by modifications of the chromatogram before fluorography. For example, enhancement of the fluorescence by pretreatment of the chromatogram with iso-octane (Blumer, 1956) should increase the sensitivity of detection of trace components in a porphyrin mixture. Also, since some metal chelates of porphyrins do not fluoresce or fluoresce only weakly (Falk, 1964), their presence on chromatograms could be detected by their Soret band absorption. In this case the developed chromatogram should be sprayed with a solution of a fluorescent substance (Blumer, 1956), dried, then fluorographed without the secondary filter in place so that the blue-violet light absorbing spots would be recorded as white spots on a dark background.

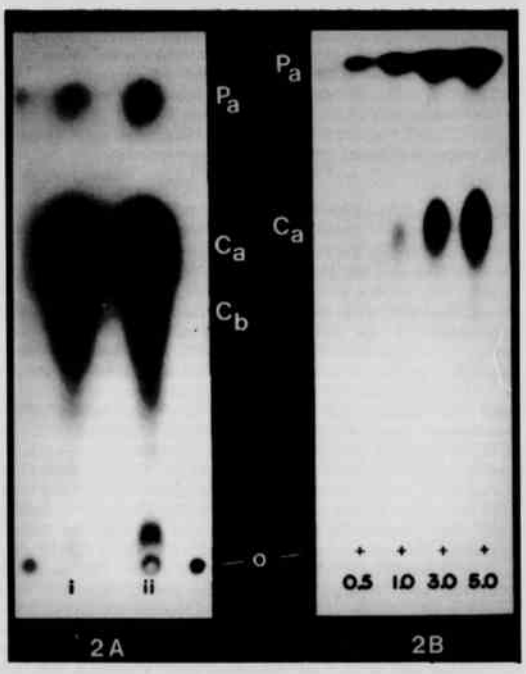

Figure 2. Fluorograms of paper chromatographic separations of chlorophyll derivatives. Ca = chlorophyll *a*, Cb = chlorophyll *b*, Pa = pheophytin *a*, o = origin.

Figure 2A: Chromatographic separation of pigments present in a fresh *Lamium* leaf extract (i) and of those present in an aged *Lamium* leaf extract (ii).

Figure 2B: Chromatographic separation of various amounts of a mixture containing chlorophyll *a* and pheophytin *a* (ratio of chlorophyll to pheophytin = 1.8:1); numbers below origin indicate the nanomoles total pigment applied at each spot.

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