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Note

Thin-layer assay of chlorophyll and analogous dry-column separation with effluent fluorometry

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In many analytical situations, a "dry"-column procedure can be derived from a thin-layer (TLC) technique[1]. A packed "dry" column before development contains no eluent fluid, and can achieve separations of the same quality as that of the TLC plate. It is time-saving when a solvent–sorbent scheme can be first piloted on TLC plates. Once this scheme is proven, a corresponding column can be packed. With continuous elution[2] from the column, good quantitation is obtained by a detection device monitoring the effluent. Finally, it may be practical to regenerate the column *in situ* rather than repacking for each analysis.

It was found that a fair separation of plant pigments is accomplished on silica gel plates with a mixture of petroleum ether and a halocarbon containing acetone and a polar solvent. As an example, Quantagram Q-4 plates (Quantum Industries, Fairfield, N.J., U.S.A.) and a solvent (No. 1), made by mixing 80 ml of petroleum ether (b.p. 30–60°), 20 ml of carbon tetrachloride, 15 ml of acetone (anhydrous) and 1 ml of methanol (absolute), were used. The Q-4 plates as received seemed to have a suitable degree of activation and were not treated. The above solvent mixture, however, appeared at times to produce double-banding, which was attributed to a phase break from local depletion of methanol near the solvent front. Therefore a similar solvent was searched for in which no constituent would be present in too small a fraction. This led to the present choice (No. 2) of mixing 22 ml of petroleum ether (b.p. 30–60°), 3 ml of dichloromethane, 3 ml of acetone (anhydrous) and 2 ml of ethyl acetate.

With this solvent combination and Q-4 plates, about a dozen well-isolated pigments were obtained from a typical green plant cytolysate.

Using solvent No. 2, the same quality of performance was then translated into a column operation. Adsorbosil-2 (Applied Science Labs., State College, Pa., U.S.A.) was selected for the silica packing. The column design is detailed in Fig. 1.

The sorption column is built from 3/8 in. O.D. (7.80 mm I.D.) stainless steel. The fluid feed line is connected to the column top through an adapter (A). The column top consists of a 3/8-in. Swagelok union (B), which is a semi-permanent part of the exchangeable column. The union holds a suitable hex-nut (C) pressing upon a loading spring (D), followed by a washer (E) and a 180-mesh screen disc (F). The silica gel packing (G) has a net length of 160 mm and weighs 4.5 g. The bottom end of the column contains a sequence of a fiberglass disc (H), a screen disc (I), and a PTFE bushing (J), which holds a 1/16-in. Swagelok union (K). The silica gel is easily

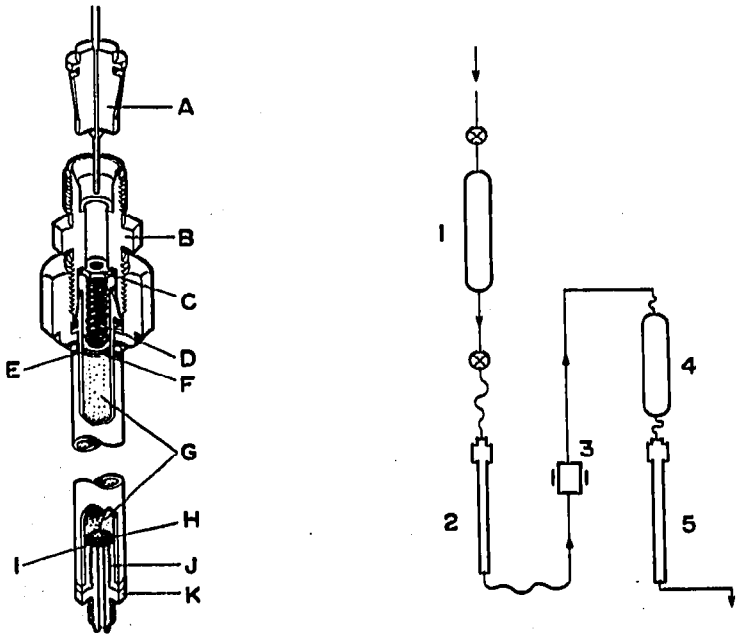

Fig. 1. Sorptive column and flow schematic. For explanation see text.

packed by applying suction from the bottom end. Because of the flow properties of silica gel, the spring-loading assures a firm packing at all times, and only after a number of runs will it become necessary to remove the first few millimeters of caked gel at the top, or to add a small amount of gel in order to restore good spring tension.

The analytical column is part of the setup described in the schematic of Fig. 1. The elution fluid reservoir is a steel flask (1) connected to a helium gas supply at the top and to the analytical column (2) below. The end of the analytical column is tied by a short link of 0.5-mm I.D. PTFE tubing to the fluorometer flow-cell (3), whose top end leads to a suppressor stage made up of a 50-ml steel reservoir (4) and a second silica column (5), which is a duplicate of the analytical column. The suppressor stage has the purpose of eliminating gas bubbles in the fluorometer cell by creating a flow resistance. The reservoir (4) and column (5) are periodically filled to the top with acetone. Small void spaces such as may develop cause no problems.

The fluorometer now used is a Turner Model 111 (G. K. Turner Ass., Palo Alto, Calif., U.S.A.) with an R-136 multiplier tube and a T-5 blue light source. A No. 47-B excitation filter and a No. 23-A emission filter were chosen. A faster circuit response was obtained by substituting smaller capacitances at three points. The door-function of the fluorometer was abandoned, and the incoming and outgoing fluid lines were led through small bores, observing proper light insulation. The PTFE fluid lines were wrapped in black tape to bar light-piping. The cylindrical flow-cell, drawn from quartz to a wall thickness of 0.5 mm, has an I.D. of 3.0 mm and an active volume of about 100 μl. Each end of the cell was shaped into a tip to which the 0.5-mm I.D. PTFE lines were pressfitted while warm. No adhesive was necessary although the lines have to withstand up to 25 p.s.i. of pressure.

The columns are designed for a three-stage programmed flow which can be automated. In preparation for a run, the fluorometer circuit must be nulled to a solvent blank. The empty cell gives a higher baseline from a scattering effect. After filling the suppressor stage with acetone, the following procedure is used.

The conditioned analytical column is installed. The feed line adapter is still kept disconnected while the pigment mixture, contained in 10–50 μl of a non-polar solvent (preferably petroleum ether), is deposited on the top screen (F) of the "dry" column. The elution solvent is first fed by gravity for 5 min. This is followed by a helium pressure-driven descent stage at 2 p.s.i. for 40 min. After that time, the pressure is raised to 25 p.s.i., and the record by the fluorometer detector is started. During this elution stage, the flow-rate is about 20 ml/h.

Three analyses are possible in a work day. An optional automatic shutoff stops all flow and the record after the last run.

A simple way of conditioning or regenerating the analytical silica columns is as follows. A freshly packed column, or one that has been used, is first purged with 10 ml of anhydrous acetone, driven through by a 20-p.s.i. pressure of dry helium which is applied for 20 min (the main portion of acetone permeates in less time). Subsequently, 10 ml of acetone containing 7 % water are driven through the column, again with helium at 20 p.s.i. After about 20 min, the gas pressure is raised to 100 p.s.i., and the column is blown to "dryness" at that pressure for 2.5 h. The gas flow-rate during this time amounts to about 150 ml/min (STP). It is important to allow the pressure at the column entrance to decay slowly before removing the column from the gas supply.

Fig. 2 shows sample chromatograms of the early-eluted red-fluorescent pigments from cytolyzed algae. Each of the chromatograms from a given preparation was com-

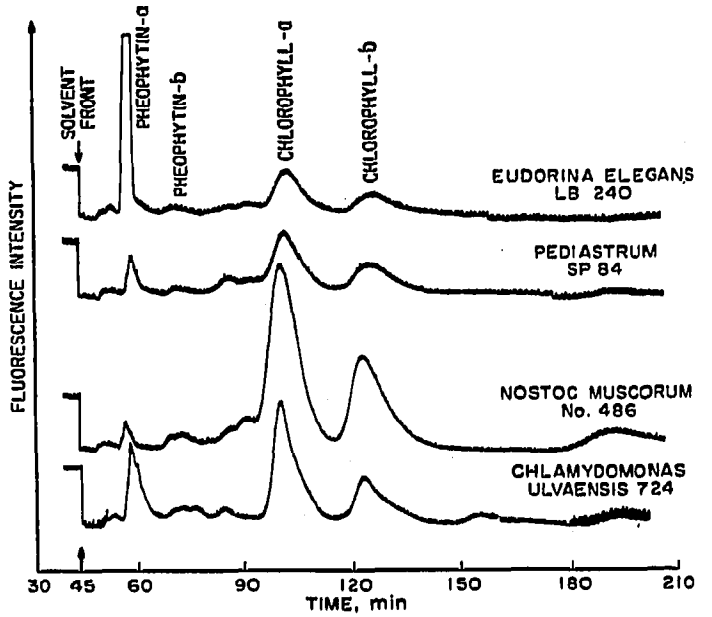

Fig. 2. Chromatograms of algal red-fluorescent pigments.

pletely reproducible regardless of the particular column used. The possibility of altering solvent No. 2 such as to stretch other regions of the elution sequence was examined. For example, a reduction in the amount of petroleum ether (and the resulting increase in polarity) caused all pigments to move faster. The pheophytins were now poorly resolved, while chlorophyll *c*, when present, became distinguishable behind chlorophyll *b*. Bearing in mind some reservations expressed by Strain and Svec[3] about possible alteration of pigment by silicic acid, a few purified chlorophylls were recycled on Q-4 plates. No evidence of degradation could be found, even when the acidity was deliberately increased by traces of acetic acid in the solvent.

The sensitivity of the complete chromatographic sequence for several fluorescent pigments was estimated by running aliquots of reference solutions, independently measured by procedures indicated by Holden[4], through the columns and detector. A signal amplitude of twice the average noise width was observed with the following amounts: pheophytin *a*, 10 ng; pheophytin *b*, 50 ng; chlorophyll *a*, 45 ng; and chlorophyll *b*, 35 ng.

The responses were found to be linear within the dynamic range of the fluorometer.

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