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Note

Separation of the neutral carotenoids of *Neurospora crassa* using concave gradient elution chromatography

A. H. GOLDIE and R. E. SUBDEN

Department of Botany and Genetics, University of Guelph, Guelph, Ontario (Canada)

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Neurospora crassa is an ideal organism for studying the genetics of carotenogenesis. It is easily cultured on synthetic media, and it is suitable for high resolution genetic recombination and complementation studies. The biochemical analysis of recently isolated *N. crassa* carotenoid mutants in this laboratory* led to the development of a chromatographic system for the separation of *N. crassa* carotenoids.

Previous attempts to study *N. crassa* carotenoids have made use of stepwise elution column chromatography[1–3] or thin-layer chromatography[4,5]. Although these methods have been successful in the chromatography of neutral carotenoids from other sources[6,7], they are not adequate for quantitative analysis of carotenoid intermediates of fungi.

Large amounts of sterols (*ca.* 1% of dry weight)[8], interfere with the chromatography of neutral carotenoids, which are present in smaller quantities (*ca.* 0.01% of dry weight)[9], in *N crassa*. Even after cold crystallization or digitonin precipitation[4] to remove steroids, difficulties arise if conventional chromatographic techniques are used. Columns or thin-layer plates often jam, R_F values are lower than for carotenoids from other sources, and resolution is poor. Excessive losses of carotenoids occur if fractions are scraped off thin-layer plates[4,5] or if columns are extruded and sliced[1,3] prior to re-chromatography.

Concave gradient elution, which has been used successfully to separate steroids[10], nucleotides[11], and carbohydrates[12], produced useful one-step separations of *N. crassa* carotenoids.

EXPERIMENTAL

N. crassa mycelium was grown for 6 days in 12-l aerated cultures at 25° with diffuse illumination. The mycelium was then harvested in cheesecloth and subjected to intense illumination under fluorescent tubes for 3 h in shallows trays of medium. It was then extracted as described elsewhere[9]. Steroids were removed by crystallization overnight in a small volume of light petroleum (b.p. 37–49°) at −17°, followed by digitonin precipitation of 3-β-hydroxysterols.

The carotenoid extract was dissolved in a small quantity of light petroleum and

* A paper describing the mutant strains, their carotenoid complements and the extraction procedure in detail has been submitted for publication (ref. 9).

layered on a column (30 × 2 cm) of deactivated alumina (80–120 mesh, 6% water) topped with a 1-cm layer of anhydrous Na_2SO_4. The column was developed with 300 ml of light petroleum followed by a concave elution gradient of diethyl ether in light petroleum and 260 fractions (5 ml) were collected. The gradient mixer apparatus described by Bock and Ling[13] was used, for which the following equation has been shown to apply:

$$C = C_1 - (C_2 - C_1)(1 - v/V)^{A_2/A_1}$$

where C is the gradient concentration, C_1 and C_2 are reservoir concentrations, A_1 and A_2 are the cross-sectional areas of the reservoirs, v is the elution volume, and V is the initial total volume in the two reservoirs.

RESULTS AND DISCUSSION

To check the accuracy of the gradient calculations, the gradient mixer was operated with 1.18 mg of β-carotene in the first reservoir. Using a flow-through cell in a Beckman Acta III spectrophotometer, the absorption at 450 nm was monitored for the mixer eluate, and was plotted versus the expected absorption (Fig. 1) calculated from the equation above.

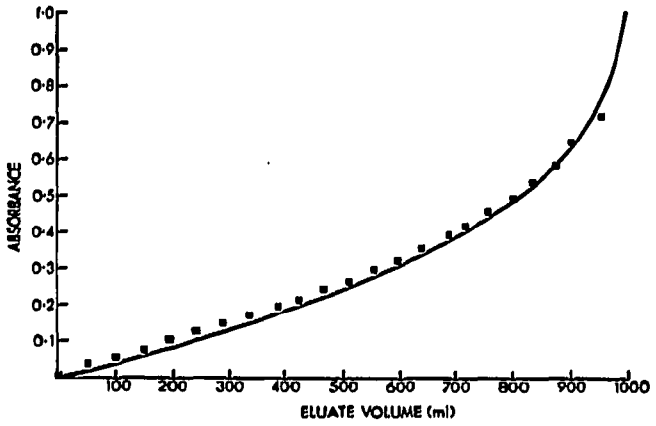

Fig. 1. Gradients for elution chromatography were produced by the apparatus described by Bock and Ling[13]. To check the validity of the gradient calculations, the apparatus was operated with 1.18 mg of β-carotene in the first reservoir. Absorbance determinations were made at 450 nm (points) and compared with absorbance calculated using $E^{1\%}_{1\,cm} = 2505$ for β-carotene at 450 nm (solid line).

Concave gradient elution chromatography produced good separations in which most fractions contained single carotenoids, suitable for absorption spectrum analysis in the spectrophotometer. Some fractions containing two carotenoids were resolved using Beer's Law for two component mixtures. Absorption maxima, $E^{1\%}_{1\,cm}$ values and absorptivities have been listed elsewhere[9].

The carotenoids eluted, along with the calculated diethyl ether concentrations are shown in Fig. 2.

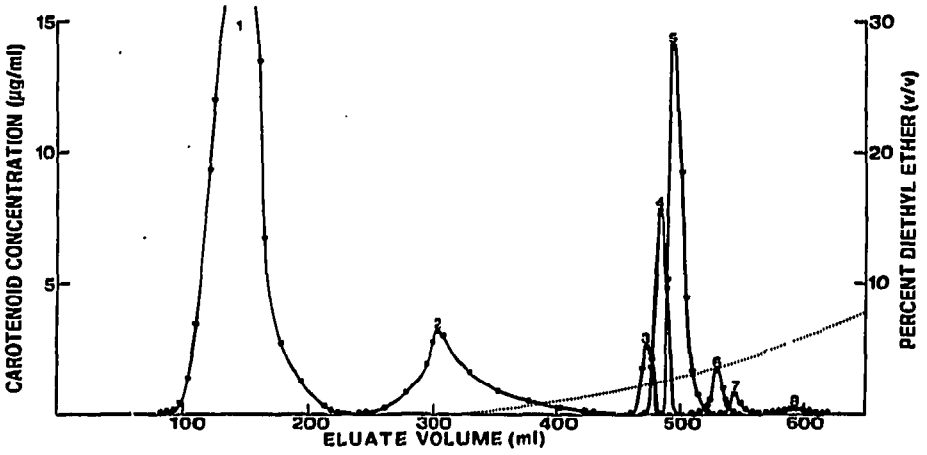

Fig. 2. Elution of *N. crassa* carotenoids from a (30 × 2 cm) alumina column (80–120 mesh, 6% water) was monitored by tracing absorbtion spectra for individual 5-ml fractions. The carotenoids eluted were as follows: 1 = phytoene, 2 = phytofluene, 3 = ζ-carotene, 4 = β-carotene, 5 = neurosporene, 6 = torulene, 7 = lycopene, 8 = 3,4-dehydrolycopene. Fractions containing two carotenoids were resolved using Beer's Law for two component mixtures[9]. The column was developed with light petroleum, followed by an increasing concave gradient of diethyl ether in light petroleum (dotted line).

Phytoene was eluted from the column along with ultraviolet absorbing impurities. Re-chromatography on a column (20 × 2 cm) of deactivated alumina (80–120 mesh, 2.5% water) produced phytoene free of ultraviolet absorbing impurities.

This chromatographic system is more rapid and produces higher recovery of carotenoids than other methods (stepwise elution or thin-layer chromatography of silica, alumina or magnesium oxide) presently used for separation of fungal carotenoids.

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