*Journal of Chromatography*, 310 (1984) 372—378
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2192

## Note

# Computer-optimized normal-phase high-performance liquid chromatographic separation of *Corynebacterium poinsettiae* carotenoids

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(First received December 8th, 1983; revised manuscript received May 14th, 1984)

Modern high-performance liquid chromatography (HPLC) owes much of its usefulness to the wide variety of mobile phase components available. Once the mobile phase components have been selected, one is left with the arduous task of simultaneously manipulating several variables, in order to arrive at a mobile phase composition which will produce an acceptable resolution of components. Therefore, a computer algorithm has been developed for optimizing a mobile phase composition to produce a satisfactory resolution of sample components according to a rigorously defined numerical evaluation of that resolution.

The optimization of the separation of a complex mixture, frequently encountered in biological samples, and typified by the carotenoid sample with which this communication is concerned, is correspondingly more difficult than simple two- or three-component samples. The difficulty stems in part from the fact that frequently the investigator does not know the exact number of components in the sample. In this paper we describe an approach to this problem which consists of first selecting a finite number of resolutions of major components of the sample such that they (1) represent apparently difficult

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0378-4347/84/$03.00 

separations and (2) are somewhat evenly spaced throughout the chromatogram. Secondly, the optimization algorithm is allowed to progress toward the ultimate goal of producing resolution factors of 1.5 for each selected resolution.

Carotenoids are produced by a variety of plants, animals and microorganisms where they most often exist as mixtures. Norgard et al. [1] detected nine pigments by thin-layer chromatography in extracts of *Corynebacterium poinsettiae*, the organism used in this study. The six major pigments were identified as a $C_{40}$ hydrocarbon, lycopene; a $C_{45}$ alcohol, C.p. 482 (2-isopentenyl-3,4-dehydrorodopin); a $C_{50}$ diol, C.p. 470 (3,4,3′,4′-tetrahydrobacteriorubrin); a $C_{50}$ diol, C.p. 496 (bisanhydrobacterioruberin); a $C_{50}$ diol, C.p. 473 [1′-hydroxy-1′2′-dihydro-2-isopentenyl-2′-(hydroxyisopentenyl) torulene]; and a $C_{50}$ diol, C.p. 450 [2-(dihydroxyisopentenyl)-2-isopentenyl-β-carotene]. They also partially characterized mono-ol alcohols, C.p. 450 and C.p. 473 and a pigment designated C.p. 435 by their absorption maxima in light petroleum or acetone. The C.p. 450 and C.p. 473 alcohols gave monoacetates after acetylation.

## EXPERIMENTAL

### *Materials*

HPLC grade hexanes, acetone, and methylene chloride (Baker, Phillipsburg, NJ, U.S.A.) were used in the mobile phase for HPLC. Solvents and reagents for extraction of pigments and thin-layer chromatography (TLC) were reagent grade.

### *Chromatography*

Separations were performed on a Waters Assoc. (Milford, MA, U.S.A.) μPorasil silica column (30 cm × 3.9 mm, 10 μm particle size). A Waters liquid chromatograph was equipped with an M6000A pump, a U6K syringe injector, and a Model 450 variable-wavelength ultraviolet (UV) detector. A Sargent (Dallas, TX, U.S.A.) Model SR strip-chart recorder was used. Injections (10 μl) of carotenoid sample dissolved in hexane were made throughout the optimization. The detector was set at 450 nm with a sensitivity of 0.04 a.u.f.s.

### *Optimization algorithm*

The simplex optimization algorithm first presented by Spindley et al. [2], and later modified by Nelder and Mead [3] was used with one major modification: quadratic interpolations, as suggested by Routh et al. [4] were implemented in our FORTRAN package as previously described [5].

The objective function which was used to evaluate the quality of the separation is shown in eqn. 1.

$$F_{obj} = \sum_{i=1}^{N-1} [10(1.5 - R_s)]^2 \qquad (1)$$

where the summation is over adjacent peaks, $N$ is the total number of peaks to be resolved, and $R_s$ is the resolution of the $i^{th}$ pair of peaks as conventionally

defined [6]. This particular objective function was designed as an empirical approach to a complex problem. The obvious non-Gaussian peak shapes, differing peak sizes, and similar elution times make the application of a more rigorous separation function of questionable value. While an exhaustive comparison of objective functions such as that carried out by Weyland et al. [7] would have been instructive, the simple, practical function shown in eqn. 1 was chosen in view of the complex sample mixture and the simple goals of this particular separation. The problem of peak-crossing would have been relatively easy to identify considering the differing peak sizes; however, we saw no evidence of it throughout the optimization. Considering the complexity of the carotenoid mixture, their existence at multiple optima is virtually inevitable.

The three-component mobile phase represented two degrees of freedom, so the parameters optimized were:

$$\alpha_1 = f_1 \tag{2}$$

$$\alpha_2 = \frac{f_2}{1 - f_1} \tag{3}$$

where $f_1$ was the volume fraction hexanes and $f_2$ was the volume fraction acetone. The volume fraction methylene chloride was calculated by difference (eqn. 4).

$$f_3 = 1 - f_1 - f_2 \tag{4}$$

Both parameters ($\alpha_i$) were constrained over the region (0, 1).

These three mobile phase components were selected because of their differing chemical properties (hydrocarbons and methylene chloride are recommended for normal-phase selectivity by Snyder et al. [8], carotenoid solubility and successful application to carotenoid separations by TLC [1].

*Organism*

*Corynebacterium poinsettiae* ATCC 9682, obtained from the American Type Culture Collection, was maintained on Tryptic Soy Agar (TSA) slants (Difco Laboratories, Detroit, MI, U.S.A.) and was subcultured every three weeks.

*Culture conditions*

Seed flasks, 50 ml of half-strength Trypticase Soy Broth (TSB) (Difco) per 250-ml flask, were inoculated from slant cultures and incubated for 24 h at 30°C on a rotary shaker (Eberbach) (160 rpm) with continous lighting from cool white fluorescent lamps positioned 1 m above the surface. Flasks (4 l) containing 1 l of half strength TSB were inoculated with 10 ml of the seed flask culture and incubated under the same conditions as the seed flasks. Cells were harvested by centrifugation at 7700 *g* for 15 min and washed once with water before extraction.

*Extraction of pigments*

Cells were extracted four to five times with methyl alcohol. The pooled extracts were saponified by adding an equal volume of 10% aqueous potassium

hydroxide and allowing the mixture to stand for 1 h at 22—24°C. Saponified pigments were then extracted with light petroleum (b.p. range 35—60°C) which was then washed free of alkali with water and dried over anhydrous sodium sulphate. The extract was then evaporated to dryness in a rotary vacuum evaporator and the residue redissolved in 1—3 ml of either light petroleum or hexane.

*Calculation of pigment levels*

Pigment levels in crude extracts were estimated assuming an $E^{1\%}_{cm}$ of 2500 at the absorption maximum of the extract, normally 478 nm. For C.p. 496 a molar extinction coefficient of 182,000 was used [6].

*TLC*

For identification of C.p. 496 resolved by HPLC, the pigments in the crude extract were first purified by TLC. A volume of 200 μl of crude pigment (0.3 μg/μl) in light petroleum was banded on silica gel G plates (0.25 mm thick) and resolved in light petroleum—acetone (80:20, v/v); before banding the plates were heat-activated at 100°C for 15 min. C.p. 496 ($R_F$ 0.50) was scraped off and the pigments eluted with methanol. The methanol was then evaporated to dryness and the residue redissolved in acetone or light petroleum. The pigment was identified by comparison of the absorption maxima in these solvents with those reported by Norgard et al. [6].

*HPLC*

Normally, 10 μl of crude pigment (0.3 μg/μl) in hexane were injected. The level used for C.p. 496 in determining linearity of response for quantitation was 0.025 μg/μl. The pigments were resolved with hexane—acetone—methylene chloride (11.35:1.73:1.00, v/v/v). Instrument conditions were: chart-speed, 2.54 cm/min; flow-rate, 2 ml/min; sensitivity, 0.04 a.u.f.s.; detection, absorption maximum of sample.

## RESULTS AND DISCUSSION

The particular objective function selected for this optimization was constructed so that the best possible cumulative separation would be represented by resolutions of 1.5 for each pair of adjacent peaks. The ten peaks whose separations were to be optimized were selected from the sixteen identifiably separable components of the sample so that the optimization process would concentrate on potentially difficult separations of prominent compounds more-or-less evenly distributed throughout the chromatogram.

Table I shows the mobile phase compositions used during the optimization process. The objective function values were calculated from elution data for ten of the peaks. Experiment C-10 represents the overall best resolution and the chromatogram is shown in Fig. 1. Resolution of adjacent peaks is clearly variable as would be expected from the fact that the information from nine simultaneous separations is accumulated in a single number. The separations are summarized in Table II. Mobile phase C-10 results in poor separations of peaks 2 and 3, peaks 6 and 7, and peaks 7 and 8. However, in each of these cases a major peak was being resolved from a minor shoulder.

TABLE I

OPTIMIZATION PROGRESS

See the Experimental section for sample injection details and definition of $F_{obj}$

| Point | Hexanes* | Acetone* | Methylene chloride* | $F_{obj}$ | Resulting from** |
|---|---|---|---|---|---|
| C-1 | 0.800 | 0.200 | — | 904 | Initial |
| C-2 | 0.750 | 0.100 | 0.150 | 444 | Initial |
| C-3 | 0.800 | 0.100 | 0.100 | 356 | Initial |
| C-4 | 0.763 | 0.043 | 0.194 | — | Short contraction |
| C-5 | 0.787 | 0.154 | 0.060 | 352 | Long contraction |
| C-6 | 0.837 | 0.134 | 0.029 | 232 | Reflection |
| C-7 | 0.818 | 0.165 | 0.017 | 432 | Short contraction |
| C-8 | 0.806 | 0.123 | 0.071 | 251 | Long contraction |
| C-9 | 0.856 | 0.106 | 0.038 | 592 | Reflection |
| C-10 | 0.804 | 0.142 | 0.054 | 202 | Long contraction |

*All values are expressed as volume functions uncorrected for molar volumes.
**See ref. 4 for a description of this application of the simplex algorithm.

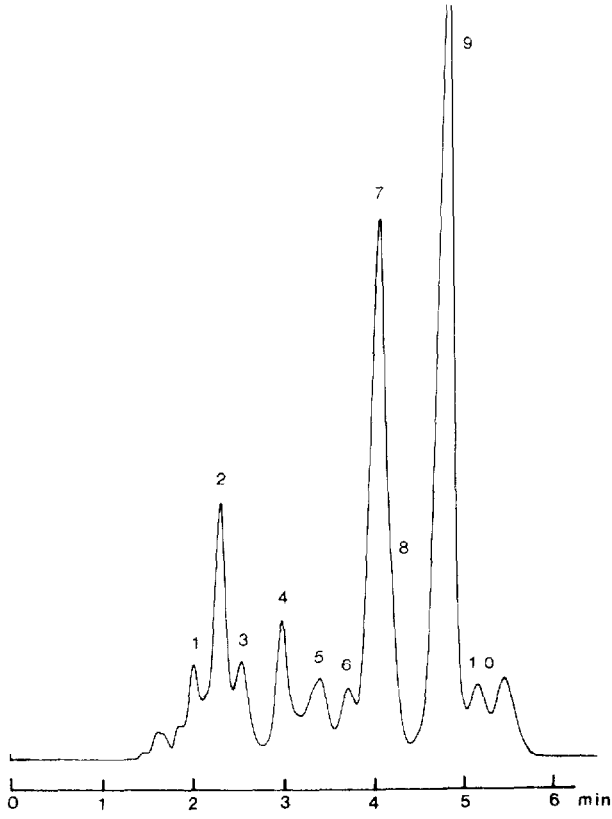


Fig. 1. Resolution of *C. poinsettiae* pigments using mobile phase C-10 (see Table I). The peaks used for the optimization correspond to the peak numbers in Table II.

TABLE II

INDIVIDUAL PEAK ELUTION DATA FOR OPTIMIZED MOBILE PHASES

See the Experimental section for sample injection details and definition of $R_s$; $t_R$ = retention time; mobile phase compositions (C-10 and C-9) are defined in Table I.

| Peak | C-10 | | C-9 | |
|---|---|---|---|---|
| | $t_R$ (min) | $R_s$ | $t_R$ (min) | $R_s$ |
| 1 | 2.02 | | 2.47 | |
| | | 1.34 | | 1.85 |
| 2 | 2.39 | | 3.08 | |
| | | 0.74 | | 1.03 |
| 3 | 2.62 | | 3.48 | |
| | | 1.64 | | 2.19 |
| 4 | 3.17 | | 4.43 | |
| | | 1.37 | | 3.43 |
| 5 | 3.71 | | 6.10 | |
| | | 1.05 | | 1.29 |
| 6 | 4.14 | | 6.91 | |
| | | 0.78 | | 1.19 |
| 7 | 4.43 | | 7.66 | |
| | | 0.77 | | 1.10 |
| 8 | 4.75 | | 8.50 | |
| | | 1.86 | | 1.88 |
| 9 | 5.52 | | 10.01 | |
| | | 1.41 | | 2.47 |
| 10 | 6.14 | | 11.91 | |

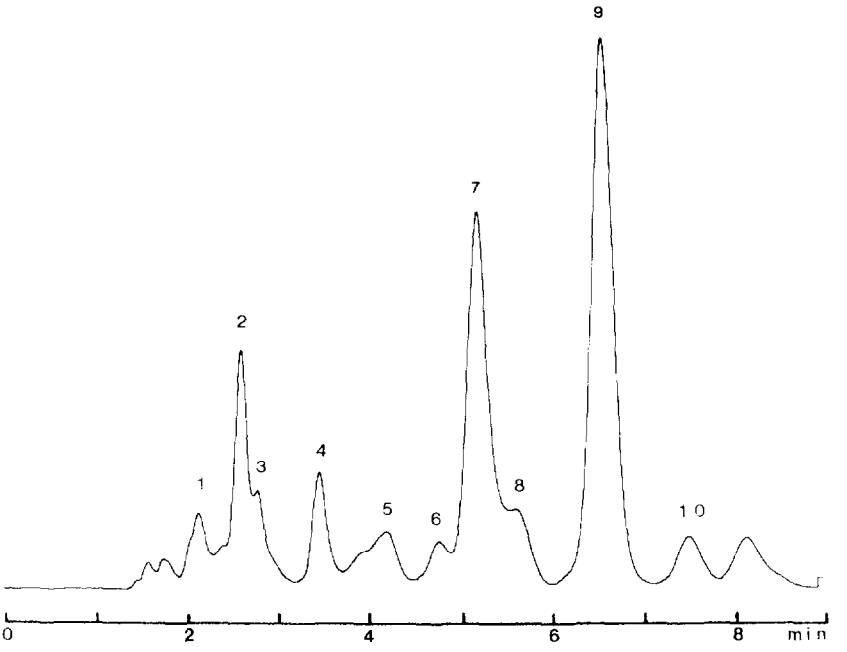


Fig. 2. Resolution of *C. poinsettiae* pigments using mobile phase C-9 (see Table I). The peaks used for the optimization correspond to the peak numbers in Table II.

Mobile phase C-9 shown in Table I succeeded in spreading the peaks to a greater extent than any of the other mobile phases. In particular the first part of the chromatogram was better resolved as shown by the individual $R_s$ values listed in Table II. The chromatogram itself is reproduced as Fig. 2. The reason for the unsatisfactory objective function was that while resolution of the three difficult pairs improved over that obtained with mobile phase C-10, five of the other seven resolutions were significantly greater than the optimal 1.5 and

therefore added to the value of the objective function. This particular mobile phase (predictably) resulted in almost double the elution times produced by mobile phase C-10. It is also noteworthy that peak 1 was resolved into two peaks and a new peak appeared between peaks 7 and 8. Of course, it is quite possible that certain uses to which carotenoid separations are applied might require the resolution provided by mobile phase C-9 and the increased analysis time prove inconsequential. This is, therefore, offered as an alternative mobile phase.

Pigment C.p. 496 was purified by TLC ($R_F$ 0.50) as described in Experimental. With mobile phase C-10, peak 7 eluted at the same time as this pigment. Peak height can be used to quantitate this pigment over the range 0.1—0.7 $\mu$g. Reproducibility was found to be better than ± 1.5%.

ACKNOWLEDGEMENTS

The authors would like to express their appreciation to Ms. Karen Oray for preliminary HPLC experiments, to Dr. D.L. Dunn for valuable HPLC procedural discussions as well the suggestion to apply eqn. 1, and to Mr. Tyler Tull (Waters Assoc.) for allowing the use of his liquid chromatograph for this work. Funding for this project was provided in part by NTSU Faculty Research.

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