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Note

Use of dual-wavelength detection in high-pressure liquid chromatography for the quantitative determination of unresolved or partially resolved compounds

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Dual-wavelength detection in high-pressure liquid chromatography (HPLC) is easily accomplished by connecting two variable-wavelength detectors in series. This not only increases the scope of the detection system but, by selecting appropriate wavelengths, provides sufficient information to quantitate unresolved or partially resolved peaks. The measurement of cannabinol in the presence of unresolved cannabigerolic acid has been described[1] and this paper extends the method to the more general case of partially resolved compounds. A useful feature is that one compound can be determined in the presence of another when all that is known about the second compound is the ratio of its detector responses relative to an internal standard at the two wavelengths used.

THEORY

Assume that two compounds, A and B, are partially resolved on HPLC and are detected at two wavelengths, 1 and 2. Compound A is to be measured quantitatively. A third compound in the mixture acts as the internal standard.

Let C_A and C_B be the concentrations of A and B, respectively, and let F_{1A}, F_{2A}, F_{1B} and F_{2B} be the calibration factors for A and B at wavelengths 1 and 2, *i.e.*

$$F_{1A} = C_A \text{ when } \frac{\text{Peak height of A at wavelength 1}}{\text{Internal standard peak height at wavelength 1}} = 1$$

The HPLC peaks are approximately Gaussian and, since A and B are only partially resolved, it is assumed that the shapes of their peaks are identical and independent of wavelength. Let the retention distances of A and B be x_A and x_B. Then, at wavelength 1, the peaks due to A and B are defined by the equations

$$y = \frac{C_A}{F_{1A}} \cdot e^{-(x-x_A)^2/2\sigma^2}$$

and

$$y = \frac{C_B}{F_{1B}} \cdot e^{-(x-x_B)^2/2\sigma^2}$$

where σ is related to the width of the peaks. Similar expressions will apply for wavelength 2.

When A is chromatographed in the absence of B at wavelengths 1 and 2, the peak heights relative to the internal standard are C_A/F_{1A} and C_A/F_{2A}. When the mixture of A and B is chromatographed, the peak heights relative to the internal standard are measured at the retention distance of A (x_A) and will be greater than C_A/F_{1A} and C_A/F_{2A} due to the overlap of the peaks. Denote these relative heights by R_1 and R_2. Now

$$R_1 = \frac{C_A}{F_{1A}} + \frac{C_B}{F_{1B}} \cdot e^{-(x_A - x_B)^2/2\sigma^2}$$

and

$$R_2 = \frac{C_A}{F_{2A}} + \frac{C_B}{F_{2B}} \cdot e^{-(x_A - x_B)^2/2\sigma^2}$$

Rearranging and cross-multiplying these equations eliminates the exponential terms and gives

$$C_A = \frac{\frac{F_{1B}}{F_{2B}} \cdot R_1 - R_2}{\frac{F_{1B}}{F_{2B}} \cdot \frac{1}{F_{1A}} - \frac{1}{F_{2A}}} \tag{1}$$

This is the same equation that was derived in the simpler case where A and B are exactly coincident[1]. In practice, the only difference in applying the method to coincident or to partially resolved peaks is that in the latter case the peak heights are measured at the retention distance of A and not at the maxima of the peaks which have been displaced due to the presence of B.

R_1 and R_2 are calculated from peak height measurements and F_{1A} and F_{2A} are found from calibration curves. The ratio F_{1B}/F_{2B} is calculated from peak height measurements on running a pure sample of B mixed with internal standard since

$$\frac{F_{1B}}{F_{2B}} = \frac{\frac{H_{2B}}{S_2}}{\frac{H_{1B}}{S_1}}$$

where H_{1B} and H_{2B} are the peak heights of B and S_1 and S_2 are the internal standard peak heights at wavelengths 1 and 2. It is not necessary therefore to calibrate for B in order to measure A.

The method is not applicable to compounds whose extinction coefficients change by the same relative amount on changing wavelength, for such compounds would be indistinguishable.

EXPERIMENTAL

A simple HPLC system was used to test the method in which components A and B were fluoranthene and pyrene, the internal standard was 1,2-benzanthracene, and wavelengths 1 and 2 were 250 and 270 nm, respectively.

The chromatographic system was essentially that described previously[1]. A short column (7 cm) and a high solvent flow-rate (3.5 ml/min) were used to reduce the efficiency of the separation until there was considerable overlap between the fluoranthene and pyrene peaks. The eluting solvent was methanol–water (4:1) and the injections (1 μl) were made in chloroform solution. The chart speed was 6 cm/min.

Standard and test solutions were prepared from a bulk solution of 0.33 mg/ml 1,2-benzanthracene in chloroform so that the internal standard concentration was constant throughout. Fluoranthene standards containing 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml were chromatographed in triplicate. The calibrations were linear at both wavelengths. A pyrene standard of arbitrary concentration was chromatographed ten times to determine an average value for F_{1B}/F_{2B}.

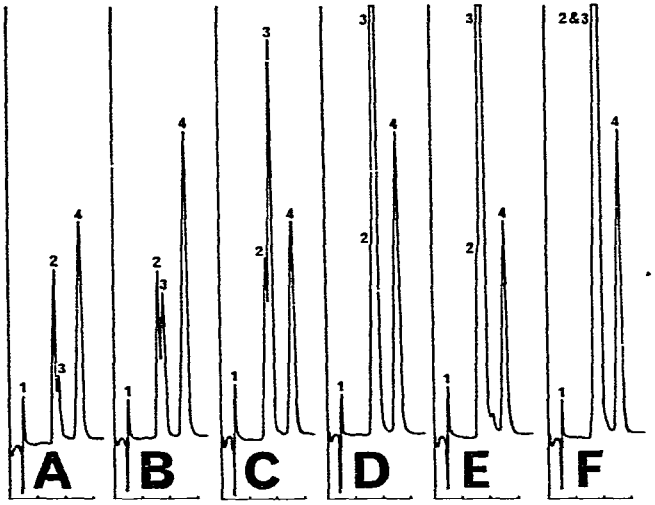

Fig. 1. Liquid chromatograms of test solutions I, II, and III (*cf.* Table I). (A) I at 250 nm; (B) I at 270 nm; (C) II at 250 nm; (D) II at 270 nm; (E) III at 250 nm; (F) III at 270 nm. Chromatographic conditions: 1-μl injections in chloroform (stop-flow injection) on to a 7 cm × 4.9 mm I.D. stainless-steel column packed with silica-C_{18} (medium load of C_{18} on Partisil 5); eluting solvent, methanol–water (4:1) flow-rate, 3.5 ml/min at *ca.* 1500 p.s.i.; detectors, Cecil 212 variable-wavelength, absorbance 0.2; chart speed, 1 cm/min (for quantitative determinations a chart speed of 6 cm/min was used to facilitate measurement of the peaks). Scale graduations represent 1-min intervals. 1 = Solvent (chloroform); 2 = fluoranthene; 3 = pyrene; 4 = 1,2-benzanthracene (internal standard).

Three test solutions containing 0.40 mg/ml fluoranthene and varying amounts of pyrene were used (Fig. 1) and each was chromatographed twenty times. A portion of the same fluoranthene solution with no added pyrene was chromatographed ten times before running the test solutions, and then once after every fifth chromatogram of a test solution. This confirmed the fluoranthene concentration (average of all runs, 0.40 mg/ml) and enabled the retention of fluoranthene relative to 1,2-benzanthracene to be determined.

The fluoranthene retention was calculated for each chromatogram and the peak height at this distance was measured relative to the internal standard. The

calculated values of R_1 and R_2 were then used to determine C_A from eqn. 1, which, when numerical values were substituted for the constants, became

$$C_A = \frac{1.435R_1 - R_2}{1.704} \text{ mg/ml}$$

RESULTS AND DISCUSSION

Mean results and coefficients of variation for the fluoranthene concentrations in the test solutions are given in Table I. The values obtained using eqn. 1 are compared, where possible, with those found by measuring the maxima of the fluoranthene peaks relative to the internal standard and referring to the calibration graphs. As expected, the latter results were erroneously high. When eqn. 1 was applied to pyrene solutions of varying concentrations, the calculated fluoranthene concentrations were zero to well within the standard deviation of the method.

TABLE I

FLUORANTHENE CONCENTRATIONS CALCULATED USING EQN. 1 COMPARED, WHERE POSSIBLE, WITH CONCENTRATIONS CALCULATED FROM PEAK HEIGHTS

Test solution (0.40 mg/ml fluoranthene)	*Calculated fluoranthene concentration (mg/ml)**		
	From eqn. 1	*From peak heights at*	
		250 nm	*270 nm*
I (Low pyrene concentration)	0.40 (2.9)	0.42 (2.2)	0.45 (5.7)
II (Medium pyrene concentration)	0.40 (4.1)	0.43 (3.5)	—**
III (High pyrene concentration)	0.42 (11.4)	—**	—**

* Each result is the mean of twenty analyses followed by the coefficient of variation in parentheses.

** Fluorenthene maximum obscured by pyrene peak.

Table I shows that valid results are given by eqn. 1 even when the fluoranthene peak is virtually obscured by pyrene, though the coefficient of variation increases with the pyrene concentration, as might be predicted, since at high pyrene concentrations the fluoranthene peak heights are measured on the sides of steeply sloping peaks.

As well as quantitating coincident or partially resolved peaks, the method described in this paper provides a simple way of checking the purity of a single peak in a liquid chromatogram. Additionally, in routine pharmaceutical or industrial applications of HPLC, it may be simpler and less expensive to apply the method rather than devise a more efficient separation, which may be complex and time-consuming. Although the method was developed specifically for HPLC, it is likely that the principle could be applied to any chromatographic system employing dual detectors that give differing responses.

REFERENCE

1 R. N. Smith and C. G. Vaughan, *J. Chromatogr.*, 129 (1976) 347.