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APPLICATION OF DERIVATIVE SPECTROSCOPY TO THE DETERMINATION OF CHROMATOGRAPHIC PEAK PURITY*

ANNE GRANT* and PRANAB K. BHATTACHARYYA
Pharmaceutical Research Products Section, Quality Control Department, Hoffmann-La Roche Inc., 340 Kingsland Street, Nutley, NJ 07110 (U.S.A.)
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SUMMARY

Derivative spectroscopy was used in the interpretation and exploitation of data obtained from rapid-scan UV–VIS absorbance detectors for ascertaining the purity of chromatographic peaks. Two types of derivatives were examined. The derivative of the elution curve with respect to the specific wavelength at which the major compound has a zero derivative (referred to as the spectral derivative null technique) proved to be rapid and useful for the determination of co-eluting impurity peaks which might form in solution or during chromatography from compounds otherwise known to be pure. The derivatives of the spectral curves obtained during chromatography (referred to as the derivative spectral mapping technique) were also examined both by computer simulation and experimentally and found to have the potential for universal applications in screening compounds for possible overlapping impurities in high-performance liquid chromatographic scans. A novel approach using the derivative spectral mapping technique is described to relate impurity detection limits to chromatographic and spectral resolutions of closely absorbing species which are incompletely resolved chromatographically ($R_c < 0.5$). Using the above techniques, it was possible to detect, under suitable conditions, as little as 0.1% impurity which co-eluted chromatographically with the major compound.

INTRODUCTION

The recent advent of rapid-scanning UV–VIS absorbance detectors in liquid chromatography has made it possible to acquire much more information about a given chromatographic peak. In a sense another dimension has been added to the chromatographic process. In addition to the usual separation on the chromatographic column as a function of time it now becomes possible to observe spectral separation of any given chromatographic peak as a function of wavelength. The question then becomes one of deriving the maximum use from this information. One way of doing

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this is by performing a mathematical manipulation on the data to express it in terms which are readily interpretable in the context of chromatographic peak purity.

The method which was chosen for this study was the one of derivative spectroscopy. There are really two aspects to our approach. The first is one in which the compound under study is of known purity but the processes to which the compound is subject during the chromatographic run are unknown and the formation of impurities which may co-elute with the main band is a possibility. It is necessary to study this problem since the presence of an unresolved component will affect the quantitativeness of our measure of the purity of the main peak. Many workers have used the derivative of the elution curve for various applications[1,2]. In this case, we have monitored the derivative of the elution curve at the wavelength of maximum absorption. The derivative should be ideally zero at this point so that nullification of the main peak is obtained and the presence of other constituents with differing absorption properties is revealed. This procedure will be referred to as the spectral derivative null technique. Two applications of this technique are discussed in the section Applications of the spectral derivative null technique.

The second aspect of the study is the estimation of intrinsic purity of the peak in those cases in which the purity of the compound is not known. This is a question which has occupied many workers recently[3,4], no doubt due to the fairly recent advent of instrumentation which has made it possible to study the problem in greater depth. We chose to approach the problem with the use of complete derivative spectra acquired along the elution curve. Derivative spectroscopy has been applied to the quantitative analysis of mixtures both experimentally and theoretically using computer simulation[5]. The derivative of the spectral curve during chromatography has been studied[6]. Additionally, a slope analysis of the chromatographic peak revealed certain relationships between percent impurity, chromatographic resolution and the ratios of derivative extrema[7]. In this paper, we will combine the various aspects of the problem (including analysis of mixtures, behavior of the derivative, and the changes that take place during chromatography) to develop some measurable parameters which can serve as criteria for peak purity. The development and applications of this technique, which will be referred to as the derivative spectral mapping, are discussed in the section Derivative spectral mapping technique.

EXPERIMENTAL

Reagents and materials

All of the compounds in this study were synthesized at Hoffmann-La Roche and characterized by NMR, IR, mass spectrometry (MS) and UV. Solvents used for liquid chromatography were high-performance liquid chromatographic (HPLC) grade (Fisher).

Equipment

Chromatography was done using a DuPont 870 pump and Waters UK-6 injector. The detector used for peak purity determinations consisted of a Hewlett-Packard (HP) 8450A rapid-scanning UV–VIS spectrophotometer having four cell positions. One of these positions was dedicated as a chromatographic detector and fitted with an adjustable cell holder which held an 8-μl Hellma flow cell that had input

connections for a chromatographic column. The HP8450A was interfaced to an HP82901M disc drive for storage of data as well as an HP7245A plotter. To extend the data processing capabilities of the instrument, it was also interfaced to an HP85A computer.

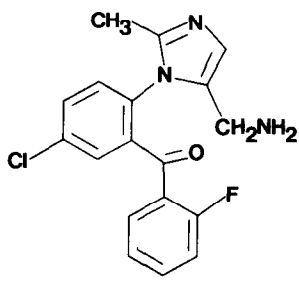
The HP1040A became available for use as a detector. The HP1040A was also interfaced to the HP85A computer as well as the disc drive and plotter.

Software for simulating first and second derivative spectra and their behavior during chromatography was written on the HP85A computer in BASIC.

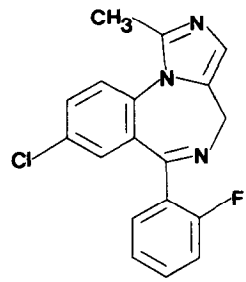
APPLICATIONS OF THE SPECTRAL DERIVATIVE NULL TECHNIQUE

The formation of breakdown products from an otherwise pure compound sometimes occurs during chromatography. The reasons for this may be instability in a solvent system otherwise necessary for dissolution or separation. The compound may be unstable on the column itself. A small number of compounds may be rendered unstable during the photochemical process of detection. Often these changes are revealed by an additional peak in the chromatogram or by tailing of the main component. It is possible however to conceive of a situation in which the breakdown product or products formed are not resolved from the main component. In this situation two sources of error are introduced which might affect the validity of the chromatographic measurement. One source of error is that impurities are present which are masked by the main component. In such situations, the presence of one single chromatographic peak indicates purity that is not actually there. The second source of error is that since quantitative determinations are made at a particular wavelength *versus* a standard, unless the standard is subjected to exactly the same conditions as the sample the presence of other species with different absorption characteristics will affect this determination. The sample may be subjected to different conditions from that of the standard simply by virtue of the fact that it is in a different matrix or that it is analyzed at a different time.

To illustrate the procedure of using the derivative of the elution curve to reveal co-eluting impurities, we give two examples. In both cases, compounds of known purity were made to undergo known chemical reactions and the products formed artificially induced to co-elute with their parent compounds. The first illustration is that of the reversible equilibrium reaction between 8-chloro-6-(2-fluorophenyl)-1-



I



II

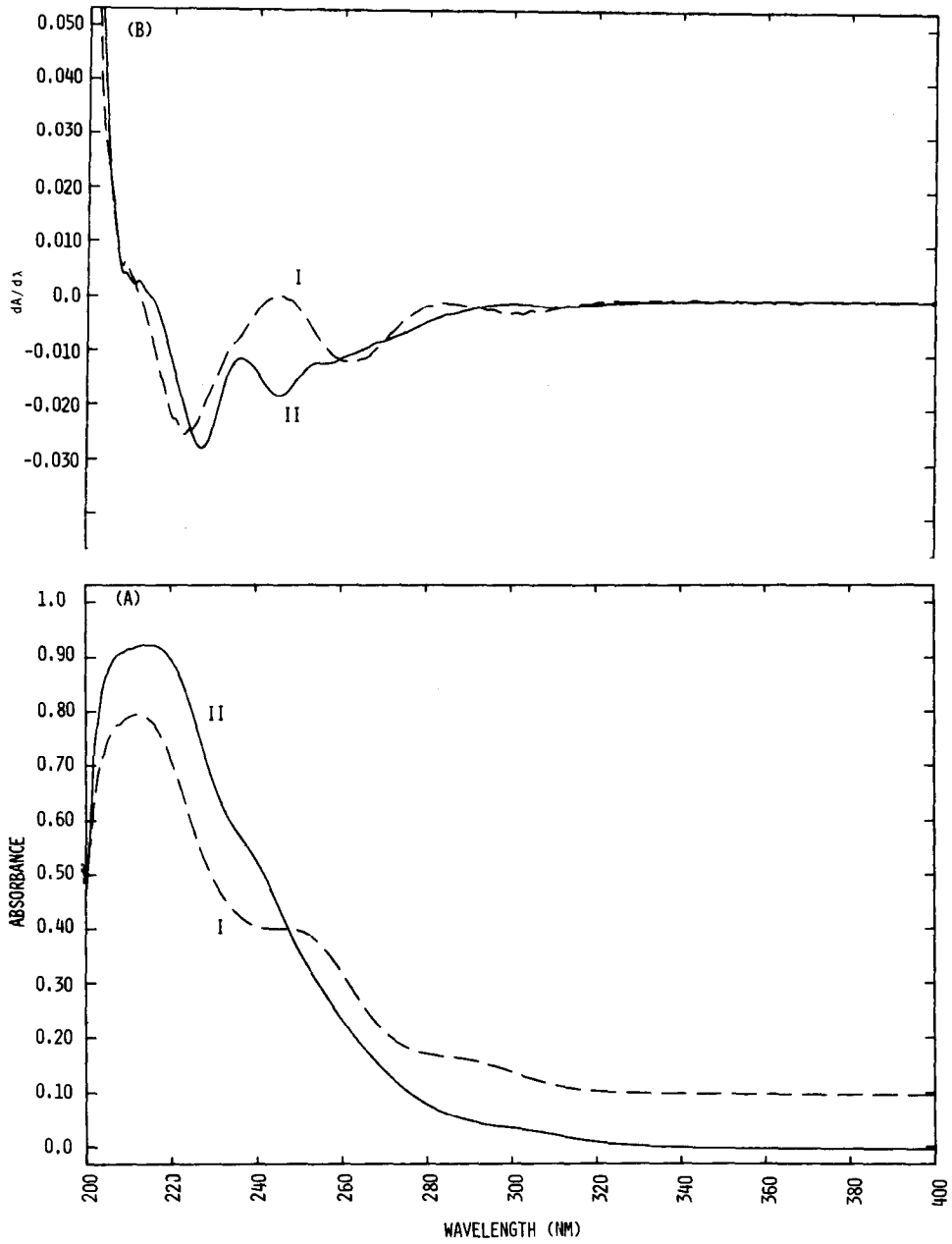


Fig. 1. (A) Absorption spectra and (B) first derivative spectra of 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-*a*][1,4]benzodiazepine (II) and its benzophenone form (I).

methyl-4H-imidazo[1,5-*a*][1,4]benzodiazepine (II) and its benzophenone form (I). A comparison of the first derivative spectral curves of the two compounds (Fig. 1) indicates that, at 245 nm, the derivative of I is approaching zero absorbance while

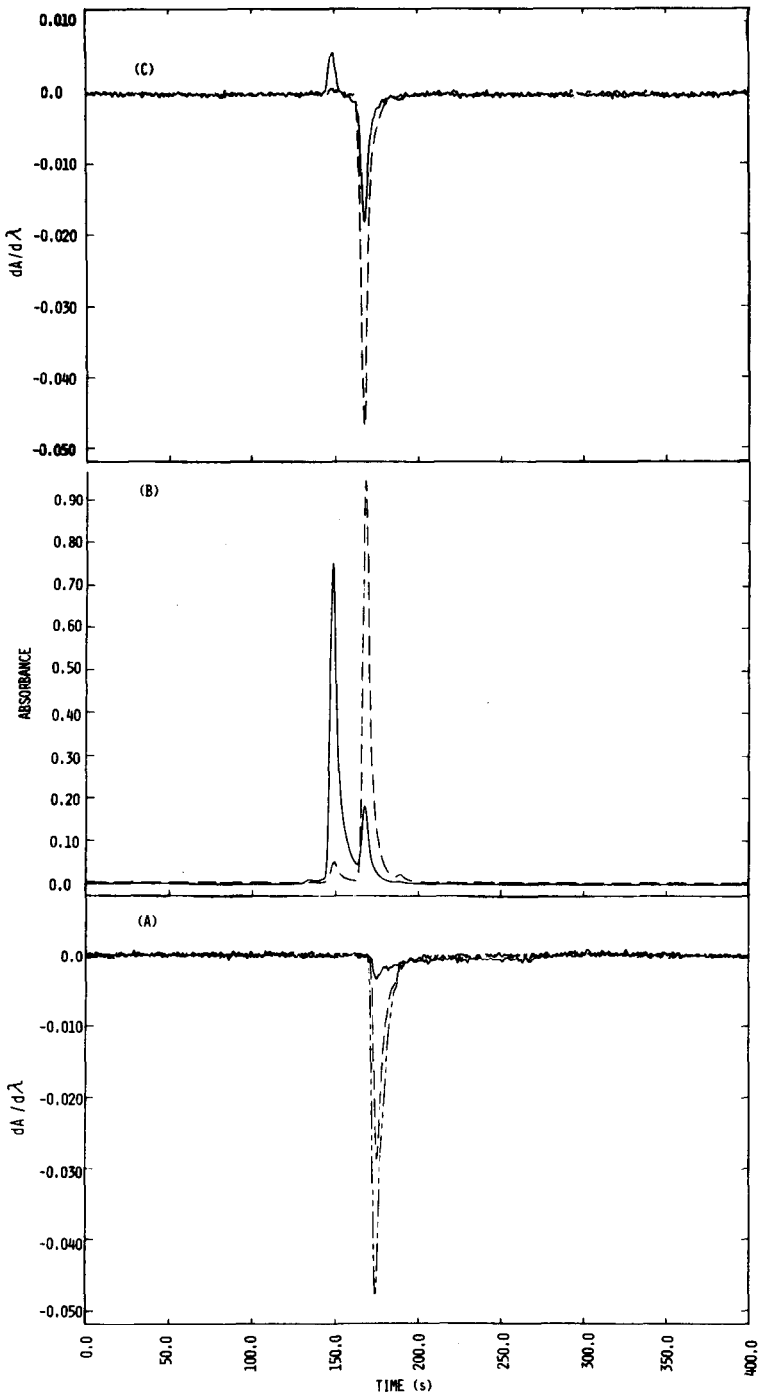
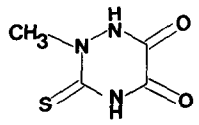


Fig. 2. (A) Spectral derivative of elution curve at 245 nm for the chromatographic system in which compounds I and II co-elute; (B) Elution profile at 245 nm for the system in which I and II are chromatographically separated; (C) Spectral derivative of elution curve for case B. The various curves are identified as follows: zero time (–); 0.5 h (--); final (———).

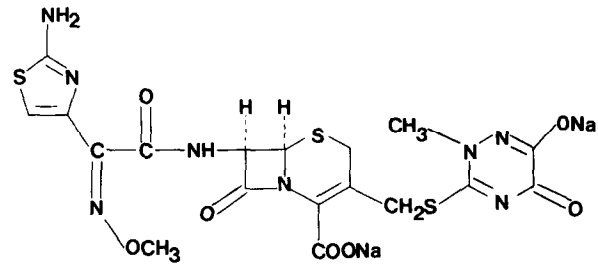
the derivative of II is at a maximum (negative quantity). It can be seen from the zeroth derivative curves that no such differentiation is possible since the curves overlap in all regions of the spectrum. If the derivative of the elution curve is monitored at 245 nm, the formation of II from I can be followed without the necessity for a separation. This situation can be artificially induced to occur by choosing chromatographic conditions such that I and II coelute. Using a reversed-phase column (Ultrasphere 5 μm, 25 cm $\times$ 4.6 mm) and methanol as a mobile phase, the formation of I in methanol solution was followed by making chromatographic injections of a solution of I at various time intervals over an 8-h period and monitoring the chromatographic peak of the first derivative at 245 nm. By measuring peak height at these intervals, the half-life of the combined reactions occurring in solution and on the column is seen to be about 0.5 h (Fig. 2A). Use of the derivative of the elution curve may also yield information about very fast processes which are happening on the chromatographic column. This is observed (Fig. 2B) during the chromatography of I in a mobile phase which is composed of a mixture of phosphate buffer (pH 7)–methanol (1:10).

Fig. 2B shows the formation of II from I at various intervals. This reaction occurs more rapidly than the one in methanol, and monitoring the derivative at 245 nm (see Fig. 2C) reveals a positive inflection in the first peak (compound I) which diminishes with time because of the formation of II (second peak). The most likely interpretation of this observation is that compound I has a slightly positive derivative which is not ordinarily detectable because of the rapid equilibrium of I $\leftrightarrow$ II under most conditions of measurement. It is apparent that this type of technique can be used to reveal other anomalies of a given chromatographic peak, such as tailing.

The second example was chosen to monitor thiotriazinone (III) in the presence of ceftriaxone disodium salt (IV) and is an illustration of the linearity and detection limits possible using the technique of the derivative of the elution curve at a chosen wavelength. From a comparison of the first derivative curves (Fig. 3), it can be seen that monitoring the first derivative of the elution curve at 271 nm should result in a positive inflection for thiotriazinone while the signal for ceftriaxone will be effectively blanked out in an HPLC system in which the two compounds coelute. The result of making various dilutions of thiotriazinone in ceftriaxone and monitoring their chromatography at the derivative of the elution curve at 271 nm is shown in Fig. 4A. In this case, a detection limit as low as 0.1% was achieved (Fig. 4B).



III



IV

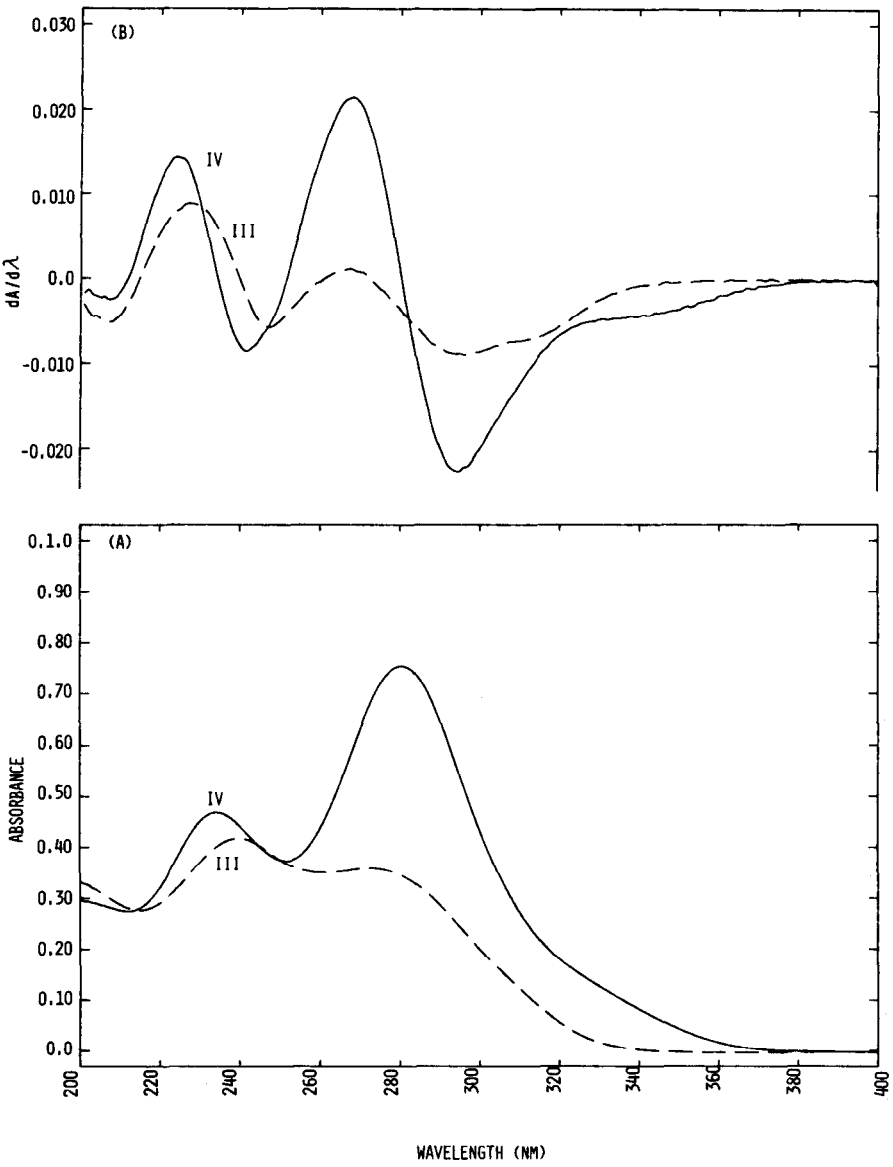


Fig. 3. (A) Absorption spectra and (B) first derivative spectra of thiotriazinone (III) and ceftriaxone disodium salt (IV).

DERIVATIVE SPECTRAL MAPPING TECHNIQUE

Theory

We have arbitrarily imposed certain limits in the development of this technique. We discuss particular cases in which the spectral curve of the suspected impurity falls within the bandwidth of the spectral curve of the main compound. In

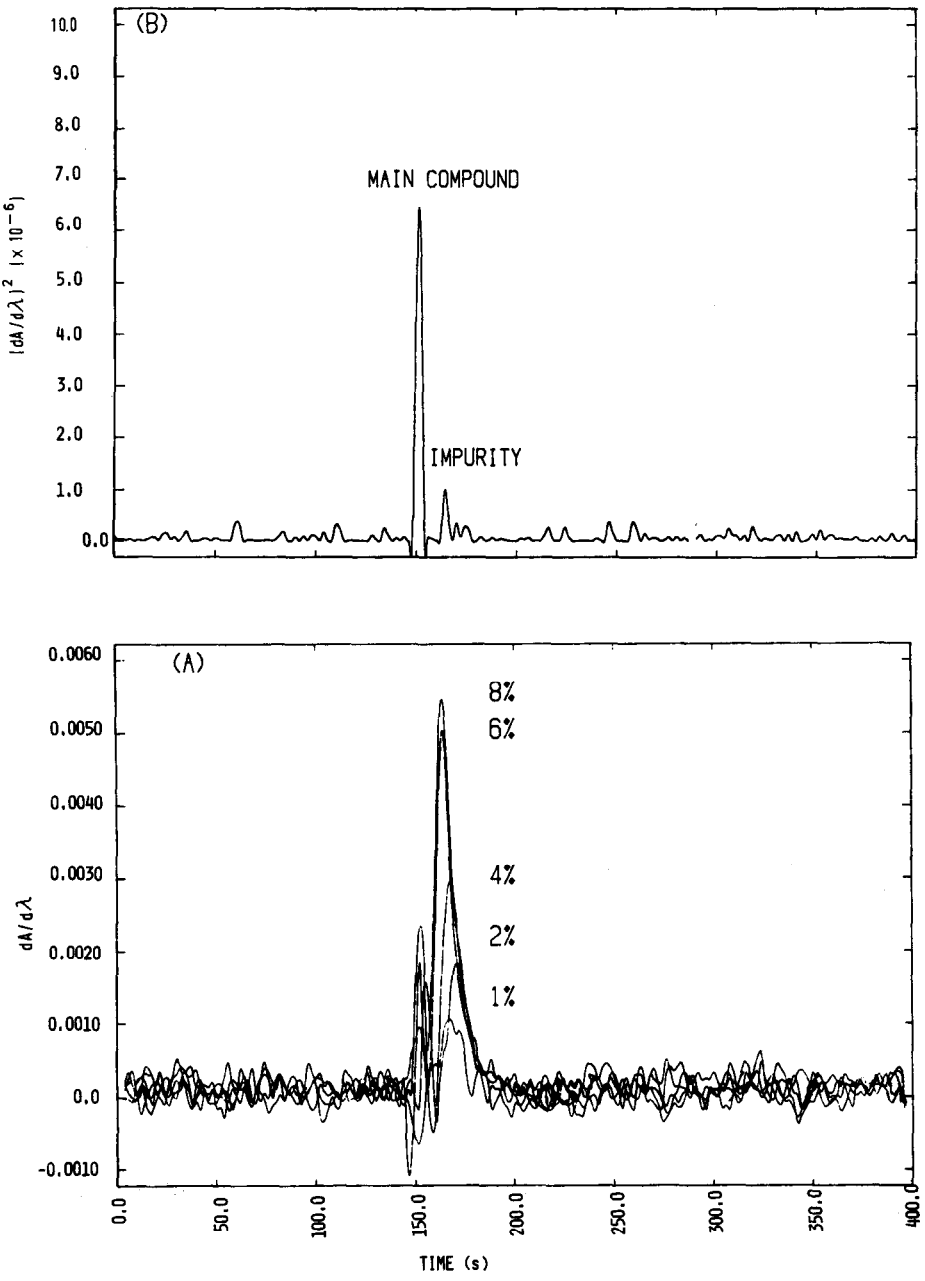


Fig. 4. (A) Investigation of linearity and (B) detection limit (impurity level 0.1%) for the chromatographic system in which the impurity (III) and compound (IV) overlap. The square of spectral derivative is plotted in B for increased sensitivity.

addition, we assume some spectral and chromatographic disparity between the two components. These procedures, while not appearing to be very general, allow us to draw some valuable quantitative relationships between peak purity and spectral and chromatographic resolutions and, in fact, may be applied in a number of real situations.

The equation for a given spectral curve may be expressed in terms of a Gaussian equation:

$$y = A \exp\left[\frac{-(\lambda-C)^2}{2B^2}\right] \tag{1}$$

where y is the absorbance, λ the wavelength, C the wavelength of maximum absorption, A the absorptivity and B the half-bandwidth. The first and second derivatives of this expression are

$$\begin{aligned}\frac{dy}{d\lambda} &= -\frac{A(\lambda-C)}{B^2}\exp\left[\frac{-(\lambda-C)^2}{2B^2}\right] \\ \frac{d^2y}{d\lambda^2} &= \frac{A}{B^2}\left[\frac{(\lambda-C)^2}{B^2}-1\right]\exp\left[\frac{-(\lambda-C)^2}{2B^2}\right]\end{aligned} \tag{2}$$

The equations for the composite curves are also given:

$$\begin{aligned}Y &= A\exp\left[\frac{-(\lambda-C)^2}{2B^2}\right] + A_1\exp\left[\frac{-(\lambda-C_1)^2}{2B_1^2}\right] \\ \frac{dY}{d\lambda} &= -\frac{A(\lambda-C)}{B^2}\exp\left[\frac{-(\lambda-C)^2}{2B^2}\right] - \frac{A_1(\lambda-C_1)}{B_1^2}\exp\left[\frac{-(\lambda-C_1)^2}{2B_1^2}\right] \\ \frac{d^2Y}{d\lambda^2} &= \frac{A}{B^2}\left[\frac{(\lambda-C)^2}{B^2}-1\right]\exp\left[\frac{-(\lambda-C)^2}{2B^2}\right] + \\ &\quad \frac{A_1}{B_1^2}\left[\frac{(\lambda-C_1)^2}{B_1^2}-1\right]\exp\left[\frac{-(\lambda-C_1)^2}{2B_1^2}\right]\end{aligned} \tag{3}$$

where Y is the absorbance of composite curve, C_1 the wavelength of maximum absorption of added component, A_1 the absorptivity of added component and B_1 the half-bandwidth of added component.

Fig. 5 shows the first and second derivative simulated curves for 1:1 mixtures of a compound with a UV maximum at 360 nm with various other compounds absorbing at longer and shorter wavelengths, all with equal absorptivities. This diagram indicates that the effect of the addition of such impurities will be to shift the zero-crossing wavelength, which is the wavelength where the derivative curve crosses through zero value on the wavelength axis, either to the right or the left of the original curve.

During chromatography, of course, the relative proportions of the two com-

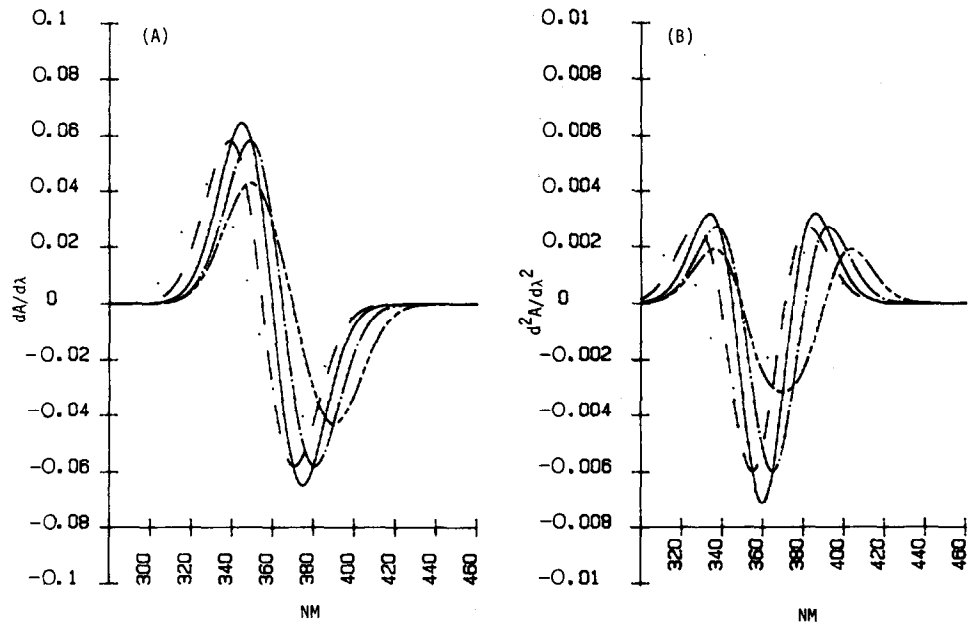


Fig. 5. (A) Simulated first derivative of composite spectra and (B) simulated second derivative of composite spectra of species added in the ratio of 1:1. The mixtures of species with the given UV maxima are identified as follows: 340 and 360 nm (· · · · ·); 350 and 360 nm (----); 360 nm only (——); 360 and 370 nm (– · – · – ·); and 360 and 380 nm (——).

ponents are constantly changing. To represent this situation, an expression is derived for the change of the derivative of the composite spectral curve with respect to time.

The equation of the chromatographic curve may also be approximated by a Gaussian function:

$$z(\lambda,T) = H(\lambda) \exp [-(T - T_0)^2/2D^2] \tag{4}$$

where T is the time, T_0 the retention time, D the bandwidth and $H(\lambda)$ the peak height. Here, $H(\lambda)$ is a function of λ since it is apparent that the magnitude of the chromatographic peak will vary depending on which wavelength we choose to monitor the chromatography. Thus, it will be dependent on the nature of the spectral curve. Therefore, the general expression for the chromatographic curve at any wavelength is given by

$$z(\lambda,T) = A_m \exp (w) \tag{5}$$

where $w = -\frac{(\lambda-C)^2}{2B^2} - \frac{(T-T_0)^2}{2D^2}$ and A_m is the maximum absorbance for all λ and T values. It would be the absorbance at the peak of the elution curve at λ_{max}.

Finally the expressions for the changes of the derivative spectral curves during

chromatography, *i.e.*, the corresponding derivative chromatographies, would be

$$\frac{dz}{d\lambda} = -\frac{A_m}{B^2}(\lambda - C)\exp(w)$$
$$\frac{d^2z}{d\lambda^2} = \frac{A_m}{B^2}\left[\frac{(\lambda-C)^2}{B^2} - 1\right]\exp(w) \quad (6)$$

The expressions for two co-eluting compounds would be

$$\frac{dZ}{d\lambda} = -\frac{A_m}{B^2}(\lambda - C)\exp(w) - \frac{A_{1m}}{B_1^2}(\lambda - C_1)\exp(w_1)$$
$$\frac{d^2Z}{d\lambda^2} = \frac{A_m}{B^2}\left[\frac{(\lambda-C)^2}{B^2} - 1\right]\exp(w) + \frac{A_{1m}}{B_1^2}\left[\frac{(\lambda-C_1)^2}{B_1^2} - 1\right]\exp(w_1) \quad (7)$$

where w_1 is the corresponding value of w for the added component. Based on the equations derived above, algorithms were developed for programs to be used to simulate the behavior of the first and second derivatives during chromatography and, by using these programs, various simulations were made for the first and second derivative chromatography of two compounds absorbing at 348 and 360 nm respectively (Figs. 6–8). Here, it can be seen that the effect of the added component during chromatography is to change the point of zero-crossing of the derivative spectral curve. The magnitude of this change would depend upon the ratio of the two com-

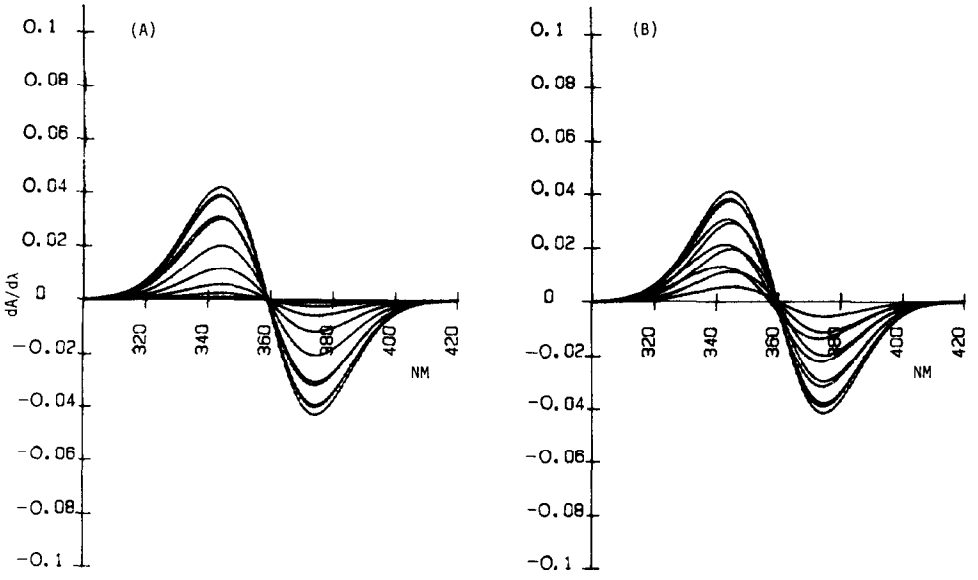


Fig. 6. Simulated first derivative spectra of the elution profile of 1:10 mixture of two chromatographically overlapping compounds absorbing maximally at 348 and 360 nm, respectively, with the chromatographic resolutions (R_c) of 0.1 (A) and 0.3 (B). The various first derivative spectra are shown during the chromatographic elution of the composite peak with a bandwidth at half-height of *ca.* 50 s.

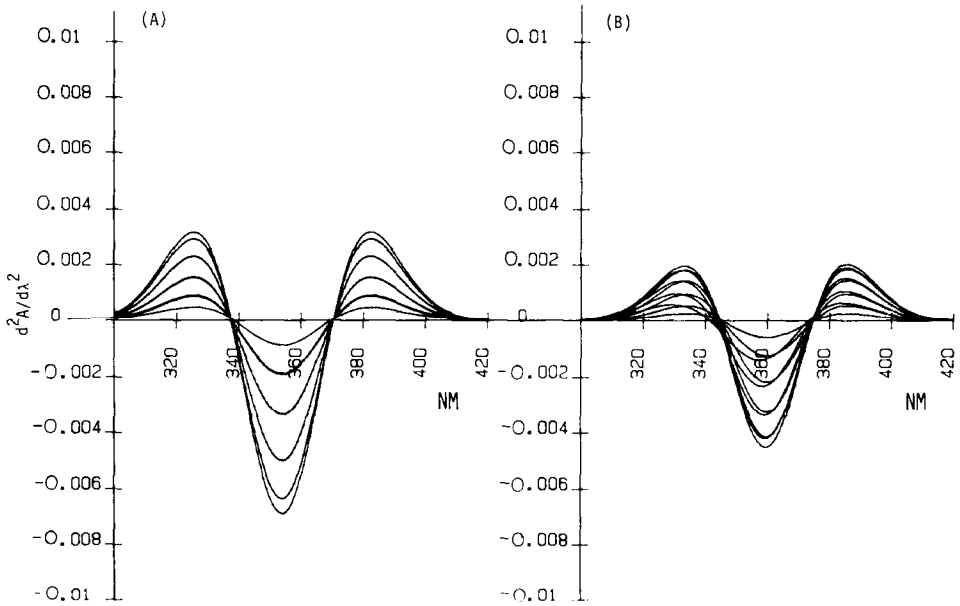


Fig. 7. Simulated second derivative spectra of the elution profile of two compounds absorbing maximally at 348 and 360 nm, respectively, for (A) 1:1 mixture with no chromatographic resolution and (B) 1:10 mixture with a chromatographic resolution of 0.3. The various second derivative spectra are shown during the elution of the composite peak with a bandwidth at half-height of *ca.* 50 s.

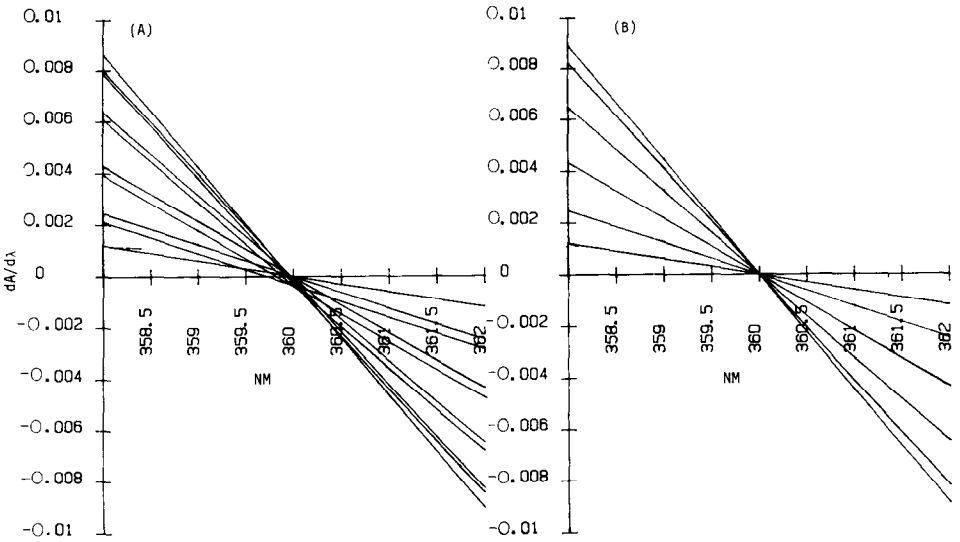


Fig. 8. Zero-crossings in the simulated first derivative spectra of the elution of (A) 1:100 mixture of compounds with the respective UV maxima at 348 and 360 nm (expanded scales); (B) pure compound with the UV maximum at 360 nm.

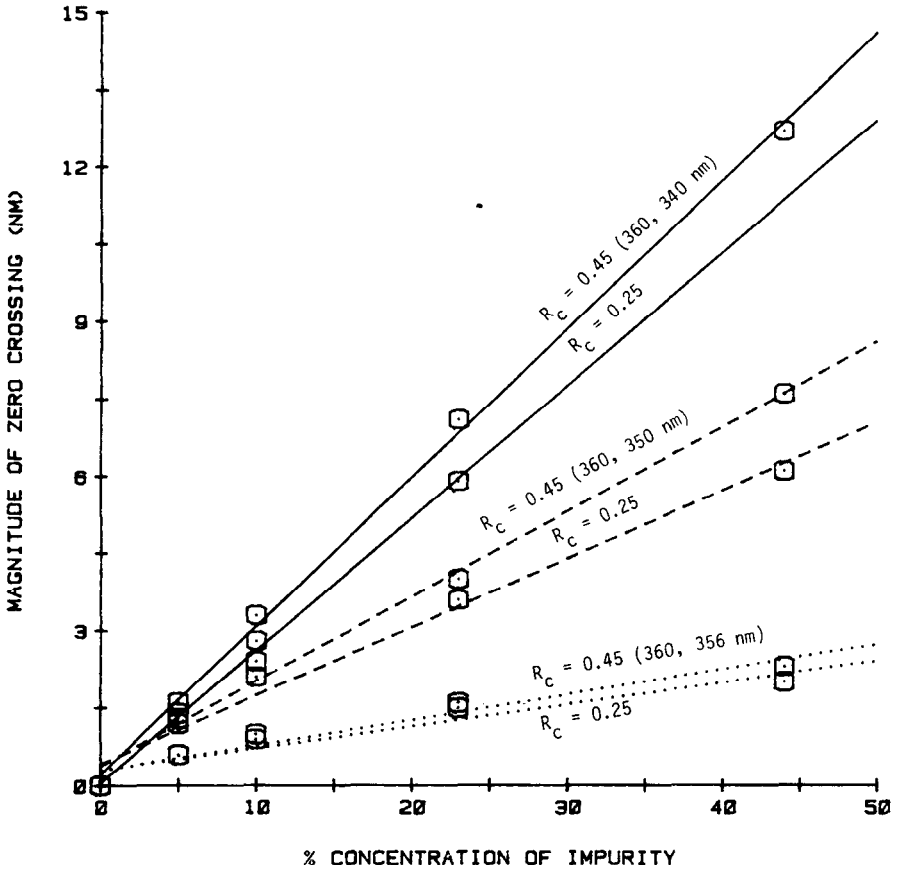


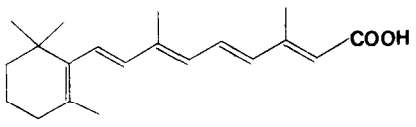
Fig. 9. Computer simulation of the magnitudes of zero-crossings of the first derivative spectra of a major compound (UV maximum at 360 nm) containing various concentrations of impurity as shown. The pairs of lines correspond to different chromatographic resolutions (R_c) between the major compound and impurity with different UV maxima for the latter, as indicated.

ponents and the spectral and chromatographic resolutions between them. These relationships are shown in Fig. 9.

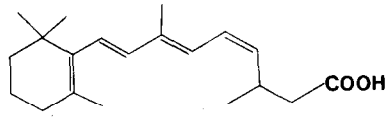
Results and discussion

To test the results obtained by computer simulation, shown in the previous figure, an experiment was performed. The compounds used in this case were all-*trans*-retinoic acid (V) and 11,13-di*cis* isomer of retinoic acid (VI).

The spectra of the two compounds are shown in Fig. 10. The two compounds



V



VI

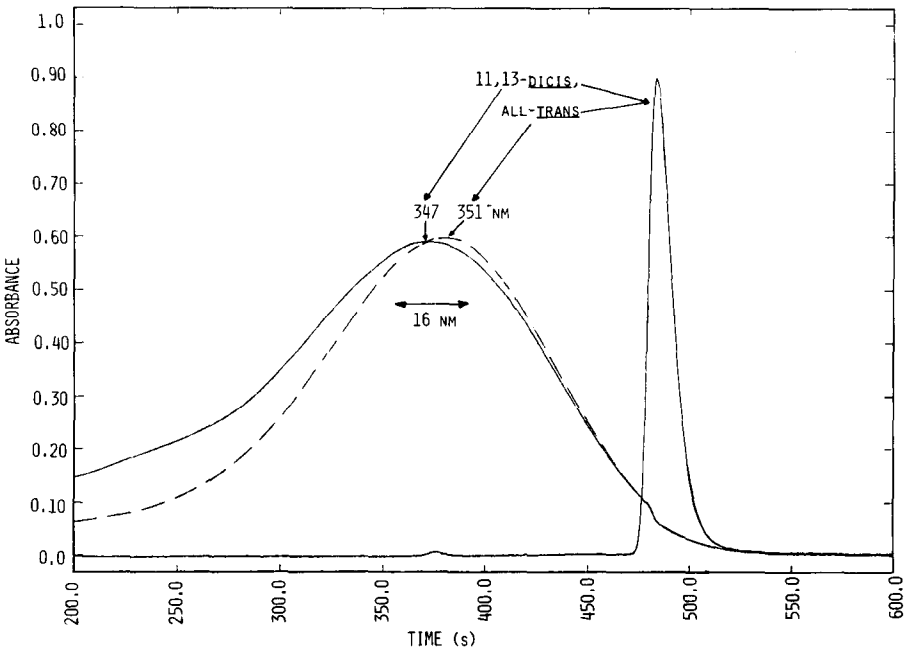


Fig. 10. The HPLC peaks from 1:10 mixture of 11,13-di-*cis*- and all-*trans*-retinoic acid are unresolved (R_c = 0.25) in the system. Corresponding spectral curves are shown to indicate wavelengths for maximum absorptions.

were made to co-elute with two different solvent compositions to give two different chromatographic resolutions. In the first case, a Zorbax Sil column was used with a mixture of ethyl acetate–hexane (5:95) containing 0.1% acetic acid to give a chromatographic resolution (R_c) of 0.45, which was determined by measuring the retention time and bandwidth of each individual chromatographic peak from two separate injections and computing the resolution from the formula:

$$R_c = \frac{T_2 - T_1}{2(\sigma_2 + \sigma_1)} \tag{8}$$

where T_2 and T_1 are the retention times of the two peaks and σ_2 and σ_1, the respective half-bandwidths at the inflection points. (Technically, two peaks with $R_c < 0.5$ are unresolved chromatographically.) By increasing the amount of ethyl acetate in the solvent system to 6%, a resolution of 0.25 was obtained. The chromatogram at this resolution is shown in Fig. 10. At each of these resolutions, various dilutions of the 11,13-di*cis*-retinoic acid in all-*trans*-retinoic acid were made and chromatography was done. Spectra were taken across the entire length of the chromatographic peak at 10-s intervals (the spectra were accumulated for a 10-s period to obtain a better signal-to-noise ratio) and the derivatives of these spectral curves examined. The magnitude of zero-crossing for each experiment was obtained by taking the difference in nanometers between the zero-crossing wavelengths of the derivatives of the first spec-

trum taken and the last spectrum taken. The results of these experiments for a series of dilutions are shown in Fig. 11.

Let us now examine the above illustration of overlapping peaks in which $R_c = 0.25$ and the spectral peaks of the major compound and impurity differ by 4 nm. In this case, using Figs. 9 and 11, it is seen that 5% of the impurity can be detected in the presence of the major compound with which it overlaps. The simulated zero-crossings of composite curves (Fig. 9) were obtained for different compounds having similar absorptivities. Greater sensitivity might be obtained in those situations in which there were greater separation between spectral peaks and the impurity had a greater absorptivity than the main compound. For example, in the exceptional cases in which the spectral peaks of the major compound and impurity are 20 nm apart and the two compounds are just barely unresolved chromatographically ($R_c = 0.45$), it should be possible to see as little as 0.4% impurity for comparable absorptivities. The detection limit drops to 0.1% if the absorptivity of the impurity is four times higher than that of the major compound.

It should be realized that simulating the zeroth derivative spectra of a multi-

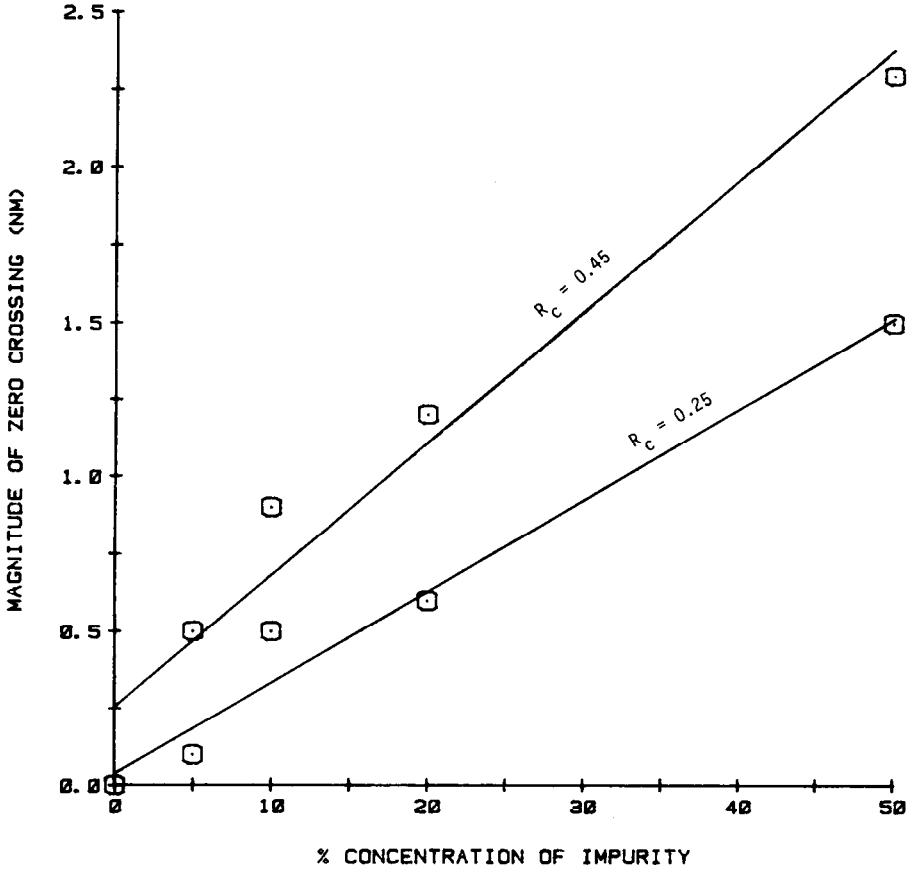


Fig. 11. Experimental determinations of zero-crossing magnitudes of all-*trans*-retinoic acid (tretinoin) doped with various amounts of 11,13-di-*cis* retinoic acid (as impurity) at two different chromatographic resolutions as shown.

component mixture during chromatography should also serve to indicate the effect of an impurity on the composite spectra obtained. There were, however, in spite of lower signal-to-noise ratio of the first derivative relative to that of the zeroth derivative, certain advantages in choosing the first derivative to perform the simulations. One advantage was that choosing the first derivative allowed the point of reference to be zero, which is somewhat less ambiguous than finding the exact wavelength of maximum absorption of a composite, both from an experimental and a theoretical point of view. Experimentally, it is often difficult to choose one single wavelength of maximum absorption on a curve since there may be a number of isoabsorbance points. Additionally, the wavelength may only be determined to 1 nm on the HP8450 and 2 nm on the HP1040, and the peak maximum may be at some intermediate point between these limits. By displacing the maximum through zero with the use of the derivative, one point and one point only is determined which need not be an integral wavelength. Theoretically, it seemed that an easier task was to define the zero-crossing magnitudes with the derivative equations previously given than to locate the composite peak maximum using the Gaussian spectral equations since, in order to do the latter, one would have to compute the resultant bandwidth of the composite curve to characterize the peak maximum properly.

A comparison of the two lower curves of Fig. 9 to that of Fig. 11 shows agreement between experimental and theoretical results in that the magnitude of zero-crossing of the experimental derivative spectra falls within the general range predicted for closely absorbing species at the chromatographic resolution chosen. Exact agreement would depend upon how closely experimental curves conform to ideal Gaussians. Even though this would probably be the exception rather than the rule, the important consideration in terms of peak purity is that a pure compound should have no spread in the magnitude of zero-crossing during chromatography; whereas, an impure compound may exhibit a change depending upon whether some chromatographic and spectral resolutions are present. However, in reality, this statement must be qualified since there is a certain spread in the magnitude of zero-crossing even for a pure compound because of wavelength reproducibility (which is, *e.g.*, 0.05 nm for HP8450), concentration dependence of spectral curves and experimental errors caused by signal-to-noise ratios of derivative spectra obtainable in particular situations. For example, using the chromatographic system of retinoic acid as a model, it was determined that the spread in the zero-crossing of a pure chromatographic peak ranged from 0.3 nm for the HP1040 HPLC detector to 0.5 nm for the HP8450 UV–VIS spectrophotometer. Such spreads in zero-crossing of a pure chromatographic peak would be the limiting factor for the lowest limit of detection of a co-eluting impurity.

CONCLUSION

It was demonstrated that, in very favorable cases, impurities which chromatographically co-eluted with the major compound could be detected at levels as low as 0.1%, essentially by the suppression of the signal from the major compound. For this purpose, the two techniques of spectral derivative null and derivative spectral mapping were described and utilized. The spectral derivative null technique was found to be especially rapid and useful in the detection of certain chromatographi-

cally overlapping products which could form from pure compounds because of their degradation or instability under certain conditions. It was realized that the spectral derivative mapping technique was much more general and powerful, even though slightly slower, in disclosing the presence of impurities which might overlap in HPLC scans with the major compound of unknown purity. Because of the potential of the latter technique for universal applications such as screening compounds for undetected impurities or decomposition products in HPLC studies, its advantages must be fully exploited.

In our work, conclusions have been drawn based on results obtained by the technique employing ideal systems in which the chromatographic peak maximum of an impurity falls within the bandwidth of the main component. These conclusions should also be valid for non-ideal systems in which the absorption spectrum of the major compound consists of several bands which combine to form the composite spectrum. In such cases, it is possible to have multiple zero-crossings in the derivative spectrum of a pure compound. All of these zero-crossings can then be exploited to our advantage to optimize the determination of the chromatographic peak purity, utilizing techniques similar to that discussed in this paper.

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