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Cross-Correlation Analysis of Molecular Fluorescence Spectra

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A new computational method for the identification of fluorescence spectra has been developed utilizing cross-correlation analysis of molecular fluorescence spectra. The spectra are obtained with conventional instrumentation and then transformed to relative time-space by using fast Fourier transform procedures. This method is applicable to a wide variety of species with overlapping fluorescence spectra. The procedure has been tested upon a representative series of polycyclic aromatic hydrocarbons and has proven to be successful for the identification of both isolated and overlapped multicomponent mixtures.

Polycyclic aromatic hydrocarbons (PAH) have been established to be a serious health threat due to their carcinogenic and mutagenic properties. They are found primarily in the residue of various combustion products (soot, coal tar, and some smoke condensates) and materials derived from petroleum and coal. The composition of these mixtures may include 100 to 200 unique PAH species. The separation and identification of such mixtures have been carried out by using reversed-phase high-performance liquid chromatography (HPLC) in conjunction with direct insertion probe mass spectrometry (*1*).

Molecular fluorescence is well-suited to the identification of the components of a mixture of PAH's due to its selectivity and high sensitivity. The major impediment to this approach is the inherent broad-band nature of conventional ambient temperature fluorescence spectroscopy, which results in nonoptimal differentiation among similar compounds and frequently to severe overlap in multicomponent systems. Several techniques have been proposed as solutions to this problem. The Shpol'skii effect utilizes an *n*-alkane matrix at temperatures below 80 K in order to reduce the spectral linewidth to that of the vibrational levels in the ground state (*2*). O'Haver and co-workers have employed derivative spectroscopy to enhance minor spectral features in the emission spectrum (*3*).

Christian and co-workers (*4*) have published extensively on the use of videofluorimeters to identify fluorescence spectra. Despite the high information density of the resulting excitation–emission spectra, unique identification of mixture components using principal component and factor analysis remains elusive when high interspecies correlation is present. Faulkner and co-workers (*5*) have relied upon analysis of conventional fluorescence spectra to compare selected features of an unknown spectrum against the first 1000 fluorescence spectra in the Sadtler collection. Identification was based upon an index of similarity, incorporating parameters such as peak locations, intensities, and excitation maximum. Another approach subjected a digitized spectrum to a fast Fourier transform algorithm (*6*) and utilized a dissimilarity index which reflected differences in the real and imaginary parts of the Fourier component of the excitation and emission spectra of both an unknown and a reference spectrum. Gold and co-workers explored the use of decomposition analysis to identify electronic spectra, particularly of PAH (*7*). While overcoming the necessity to use specialized equipment, decomposition analysis has difficulty differentiating among highly correlated spectra; this was recently contrasted with several principal component analysis procedures (*8*).

While each of these approaches can be made to work in particular cases, none of them has achieved a level of universality. The ideal electronic spectral characterization procedure would produce a compact and unique set of numerical parameters for each species. Further, the characterization procedure would not be affected by overlapped spectra when mixtures were considered.

Computer file-searching algorithms generally subject an unknown digitized spectrum to an encoding process and directly compare the resulting parameters to a collection of known compounds stored in an encoded library file. Statistically significant matches are reported, but loss of spectral information during the encoding process is a common limitation.

An IR search system utilizing direct comparison of interferograms of an unknown chromatographic peak produced by a GC/FTIR against a library of known compounds was reported by Isenhour (*9*). Powell and Hieftje (*10*) developed an IR search system based upon cross-correlation in which the complete cross-correlation was calculated between each unknown and every member of the library file.

In the present study, a computer file-search procedure is introduced which identifies the digitized fluorescence spectrum of an unknown species (or of a mixture) with the aid of the Cooley-Tukey fast Fourier transform (FFT) algorithm (*11*). The program performs a preliminary search which isolates reference spectra which possess a high degree of similarity to the unknown species. Final identification utilizes cross-correlation of the unknown with those reference compounds isolated in the initial test. The details of this procedure and

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its application to a library of some 200 fluorescence spectra are discussed below.

EXPERIMENTAL SECTION

A Hitachi MPF-44B fluorescence spectrometer was used to obtain excitation and emission spectra. Spectral quality cyclohexane (MCB) was used as the solvent for all of the polycyclic aromatic hydrocarbon species (all reagent grade or better) that were studied.

Computational Procedure. The cross-correlation of two functions $A(f)$ and $B(f)$ may be represented as $A(f)^*B(f)$ and is defined mathematically by the integral (*12*)

$$C_{AB}(\nu) = A(f)^*B(f) = \lim_{F\to\infty} \frac{1}{2F}\int_{-F}^{+F} A(f)B(f \pm \nu)\, df \qquad (1)$$

where ν represents the relative displacement between the two wave forms being compared. Implementation as a computer algorithm has been described previously (*13*).

For convenience, the Cooley–Tukey FFT algorithm was used to compute the discrete FT of the digitized spectrum. The FFTRC and FFTCC subroutines of the International Mathematical and Statistical Library (IMSL) were employed to perform forward and inverse FT operations in this study. Details of the general cross-correlation procedure are discussed by Horlick and Hieftje (*12*) and graphically depicted by Isenhour and co-workers (*14*).

This procedure is incorporated in a FORTRAN program entitled SIPS (spectral identification program system), which is used to determine the extent to which an unknown and a reference spectrum are correlated. The unknown and reference spectra are properly termed "power spectra" since they encode data as optical power per unit bandwidth (*15*). The power spectrum (or spectral density) $I_A(\omega)$ is defined as (*16*)

$$I_A(\omega) \equiv \frac{1}{2\pi}\int_{-F}^{+F} dt\; e^{-i\omega t}\langle A^*(0)A(t)\rangle. \qquad (2)$$

Fourier inversion of (2) leads to an expression for the so-called time-correlation function in terms of the power spectrum (*16*)

$$\langle A^*(0)A(t)\rangle = \int_{-F}^{+F} d\omega\; e^{+i\omega t} I_A(\omega) \qquad (3)$$

The time-correlation function is the first-order electric-field autocorrelation function and may properly be referred to as an interferogram. Popular usage is to refer to the time-correlation function as the "time-domain spectrum" and to its Fourier pair, the power spectrum, as the "frequency-domain spectrum" (*14*).

Examination of eq 1 shows that if two spectra are identical, the largest value of $A(f)^*B(f)$ will be obtained when $\nu = 0$, that is, when every point is multiplied by itself. This is a special case of cross-correlation and is termed autocorrelation. If $A(f)$ and $B(f)$ are not identical, the maximum correlation value may be shifted some distance (in ν units) from $\nu = 0$. The criterion of a match is therefore the absolute magnitude of $A(f)^*B(f)$ at $\nu = 0$.

SIPS normalizes the value at $\nu = 0$ to the largest point in the cross-correlation function. A value of 1.000 is obtained if perfect correlation exists between the unknown and a particular reference spectrum. Compounds that fluoresce in the same wavelength region will often exhibit a high degree of correlation when judged utilizing this criterion. A second criterion employs the absolute value of the function at $\nu = 0$ for further discrimination. The input reference spectrum is first normalized and the intensity at each point is squared. The sum of these squares (SUMSQ), computed over the interval from 12 800 to 38 500 cm^{-1}, for each spectrum is stored in the library as an additional parameter. Whenever an unknown spectrum contains a component that matches a reference spectrum, the absolute value at $\nu = 0$ divided by the number of discrete digitized points, N, will be equal to SUMSQ. Perfect correlation will result in this (viz. $C_{AB}(0)/N$) dividend being equal to 1.000.

The calculation of a complete cross-correlation of the unknown against each reference spectrum in the library would require a large amount of computer time. Therefore, a partial cross-correlation (PCC) is initially used to determine the degree of similarity prior to calculating the complete cross-correlation (CCC). The data for the PCC calculation are contained in the complex array that results from implementing the simple product $\bar{A}(\tau)\cdot B^*(\tau)$ where the bars indicate Fourier transformation. If the FT of the unknown spectrum is multiplied by the complex conjugate of itself, the real components of the resulting complex array contain *only* positively signed components. As the reference spectrum becomes increasingly unlike the unknown spectrum, the negative component increases in magnitude. The presence of negative components at this point is caused by spectral dissimilarities between the unknown and a particular reference species. A rejection factor empirically based upon the magnitude of this negative component limits the computational time required for the analysis since the CCC is carried out only for those reference spectra which give PCC values more positive than this rejection factor.

Search Implementation. The 200 PAH reference spectra were originally collected at the Argonne National Laboratory, stored on magnetic tape, and subsequently published by Berlman (*17*). A magnetic tape copy of the library, containing excitation and emission spectra, was obtained from Berlam for use. Due to systematic errors in the tape records, some data reconstruction was required. The emission spectra were separated from the excitation spectra and the two halves stored in two library files. SIPS presently utilizes the emisson library exclusively.

Both library files contain aromatic compounds that vary in complexity. Each spectrum was digitized at a constant 100-cm^{-1} interval. When required, spectra in wavelength units were converted to cm^{-1} (an energy unit) with SPECSOLV, a FORTRAN program previously described (*18*). Distinct library spectra were coadded to generate pseudounknown bicomponent mixtures for use in this study. In each instance, the resulting spectrum was adjusted to fill a sampling window from 12 800 to 38 350 cm^{-1}. Regions having zero intensity were assigned a base line value of 1.000×10^{-6}. Spectra acquired in this laboratory were processed in an identical manner.

When a discrete CCC is performed on the unknown and one of the reference spectra, both of equal length, an artificial period is imposed upon each. This period is constant since it depends upon the length of the data record. The correlation function can be distorted by "end effects" which arise from the improper overlap of one period with another when one spectrum is shifted with respect to the other.

Bendat and Piersol have shown that end effects do not significantly influence the correlation values near zero displacement ($\nu = 0$), provided that the correlation function decays rapidly (*19*). Powell and Hieftje (*10*) ignored the use of zero filling, without affecting the reliability of a search system used to identify infrared spectra using CCC values near zero displacement. SIPS relies primarily upon CCC values near $\nu = 0$, so zero filling has not been employed, although partial zero filling has been used so that each spectrum spans the same wavenumber region and is represented by 265 data points. A two-point linear interpolation doubles the point density prior to transformation to the complementary time domain. The sequential steps of SIPS are illustrated in Figure 1.

RESULTS AND DISCUSSION

Fluorescence spectra of three common PAH's (anthracene, naphthalene, and 3,4-benzophenanthrene) are shown in Figure 2. The partial cross-correlation (PCC) of anthracene with itself is shown in Figure 3A. A key feature of Figure 3A is the absence of any negative components. In contrast, Figure 3B shows the characteristic negative component that results from the PCC of anthracene with naphthalene. The magnitude of the negative component in this PCC is a reflection of the differences that exist in the anthracene and naphthalene spectra. This conclusion is easily reached by looking specifically at the input spectra of these species. If the spectrum of anthracene is compared with a spectrum that fluoresces in the same wavelength region and has similar spectral features (that is, a more highly correlated spectrum), the result is a decrease in the magnitude of the negative component. This "magnitude" refers to the negative values of a PCC which are summed and eventually compared to a rejection factor established in SIPS. As the unknown is compared sequentially with the reference spectra stored in the library file, SIPS will calculate the complete cross-correlation only if the magnitude

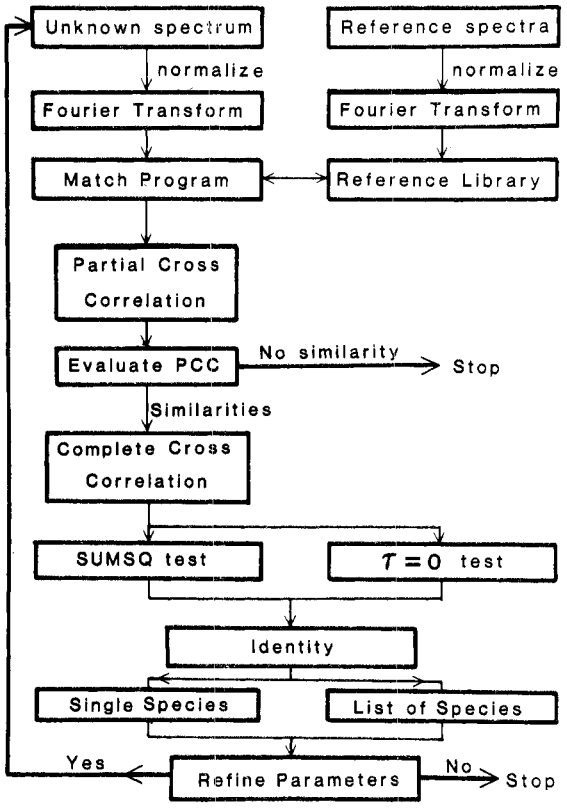

Figure 1. Block diagram of the operation of SIPS (spectral identification program system).

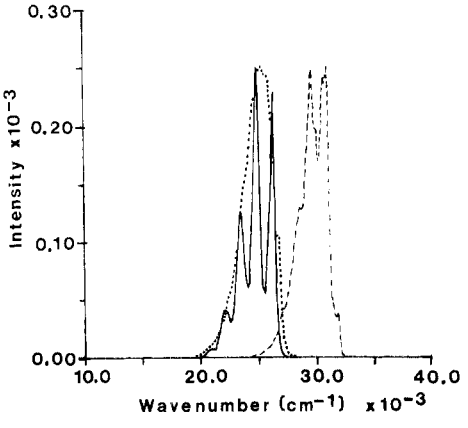

Figure 2. Input fluorescence spectra: anthracene (—), naphthalene (- - -), and 3,4-benzophenanthrene (···) after digitization at constant wavenumber interval.

of the negative component is smaller than the rejection factor. The illustration in Figure 4 graphically depicts complete cross-correlation.

Figure 4A shows anthracene cross-correlated with naphthalene. Note that the curve is asymmetric and that the maximum is shifted away from $\nu = 0$. This asymmetry and the displacement from $\nu = 0$ clearly demonstrate that these two spectra are not identical. If anthracene is cross-correlated with itself, as shown in Figure 4B, the curve is symmetric with a maximum at $\nu = 0$. Using a species that fluoresces in the same wavelength region as anthracene results in the maximum approaching $\nu = 0$, but the asymmetry of the curve is still apparent. SIPS does not currently utilize the symmetry information contained in these plots since this feature is ambiguous when studying multicomponent systems whose component spectral features overlap.

The second criterion relies upon the fact that the value at $\nu = 0$ divided by N should equal SUMSQ. Table I presents

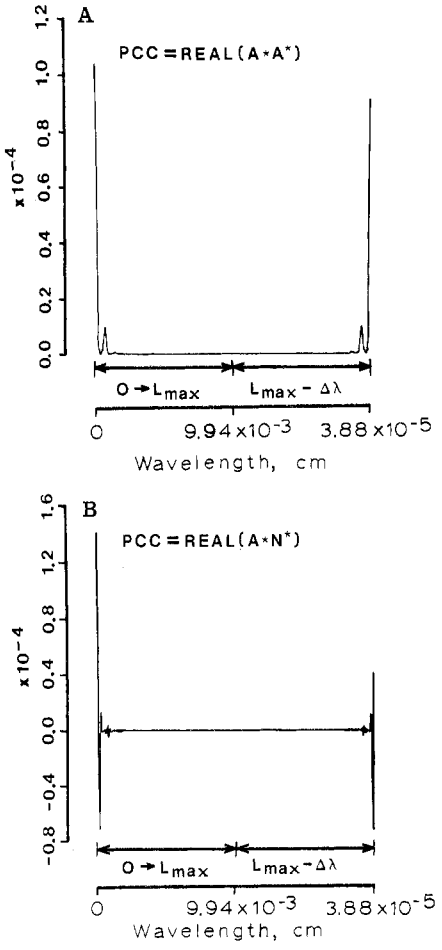

Figure 3. Results of the partial cross-correlation (PCC) of (A) anthracene with anthracene and (B) anthracene with naphthalene. The left portion of the abscissa to the midpoint (inclusive) extends from 0 to L_{max} where $L_{max} = \{(N-1)/[2(E_{max} - E_{min})]\}$. The right portion of the abscissa extends from $(L_{max} - \Delta\lambda)$ to $\Delta\lambda$ where $\Delta\lambda = \{(N-1)/[N(E_{max} - E_{min})]\}$. E_{max} and E_{min} have units of energy in the context of this study.

Table I. Illustration of the Result of Complete Cross-Correlation (CCC) of a Pseudounknown with a Small Set of Library Spectra, the Unknown Was Anthracene

	correlation parameter	
library species	CR1	CR2
anthracene	1.0000	1.0000
3,4-benzophen-anthrene	0.9987	0.6084
naphthalene	0.0274	0.0197

data for the correlation of anthracene with itself, with 3,4-benzophenanthrene, and with naphthalene. The value obtained at $\nu = 0$ relative to the maximum in the complete cross-correlation function is reported as criterion 1 (CR1), while the comparison based on the value at $\nu = 0$ is referred to as criterion 2 (CR2). With these three spectra as a small library file, anthracene is clearly the correct match.

When anthracene is compared to a library file containing 200 reference spectra, the number of potential matches increases. Table II lists the most probable spectral matches in the order that they were found. When one looks at the correlation values CR1 and CR2, anthracene is the best choice for a match. Deuterated anthracene has very similar spectral features and emission wavelength region, so that the relatively high correlation values which were obtained are not unexpected.

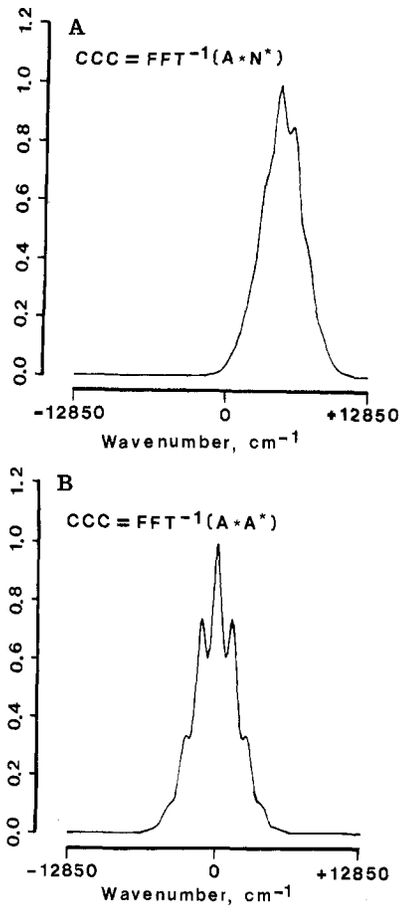

Figure 4. *Plots of the complete cross-correlation (CCC) of* (A) anthracene with naphthalene and (B) anthracene with anthracene.

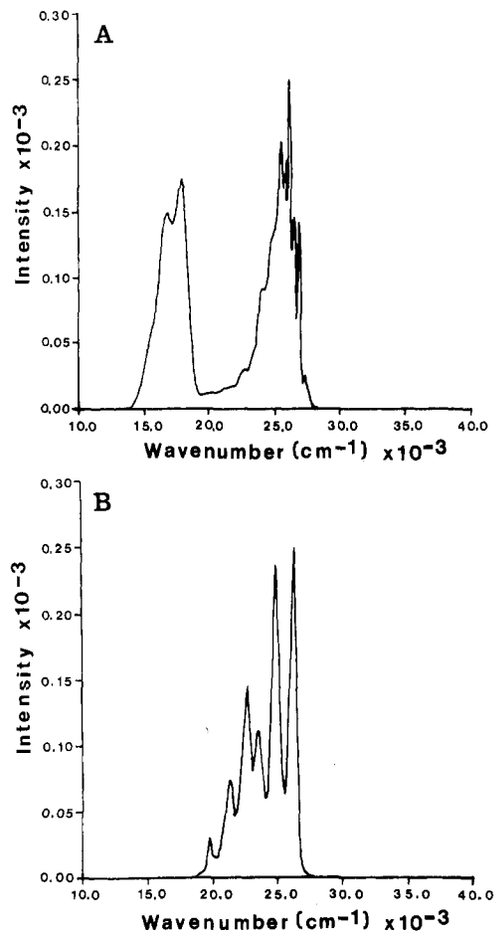

Figure 5. Input fluorescence spectra of binary mixtures of fluorescent species: (A) pyrene/rubrene and (B) anthracene/perylene.

Table II. Results of a Complete Library Search (200 Species) for the Identity of an Unknown Compound (Anthracene), the Most Probable Matches Are Listed Below

	reported correlation parameters	
library species	CR1	CR2
anthracene	1.0000	1.0000
deuterated anthracene	0.9918	1.0000
3,4-benzophenanthrene	0.9987	0.6084
benzidine	0.9995	0.5971
sodium salicylate	0.9832	0.5369
tetramethyl-*p*-octaphenyl	0.9811	0.5455
tetramethylphenylene-diamine	0.9557	0.5640

The identification of a bicomponent mixture is easily carried out if the spectrum has minimal or, of course, zero overlap. This is the case for anthracene/perylene and rubrene/pyrene, respectively. The input spectra for these mixtures are shown in Figure 5. Note that the fluorescence intensities of the components in each mixture are not identical.

Table III contains the output correlation values for both mixtures. Each component of the mixture is identified individually and sequentially, although the initial analysis may reveal the identity of each component. The CR1 and CR2 values are used to select a particular reference spectrum as the "best" match. This reference spectrum is then subtracted from the input spectrum and the resultant spectrum is reevaluated as a unique unknown. The identity of the second component is confirmed by this process.

Table III. Results of a Library Search for the Identity of Components of Binary Mixtures of Fluorescent Species[a]

	first search			second search		
		correlation parameter			correlation parameter	
unknown mixture	library species found	CR1	CR2	library species found	CR1	CR2
pyrene/rubrene	pyrene[b]	1.000	1.011	rubrene	1.000	1.001
	3,4-benzophenanthrene	0.9783	0.6619	rubicene	0.9983	0.8829
	deuterated anthracene	0.9549	0.8818			
anthracene/perylene	perylene[b]	0.9984	1.0000	anthracene	1.000	0.9785
	anthracene	0.9715	1.022	deuterated anthracene	0.9924	0.9820
	deuterated anthracene	0.9598	1.026	3,4-benzophenanthrene	0.9963	0.5934
	2-aminoanthracene	0.9526	0.6946	pyrene	0.9525	0.8713

[a] The most probable identities of the unknowns are shown initially and after mathematical removal of one species (second search). [b] Library species selected as match following first search. This species was mathematically subtracted prior to second search.

Table IV. Results of a Library Search for the Identity of the Components of a Binary Mixture of Fluorescent Species[a]

	first search			second search		
		correlation parameter			correlation parameter	
unknown mixture	library species found	CR1	CR2	library species found	CR1	CR2
pyrene/chrysene	chrysene[b]	0.9978	1.141	3,4-benzophenanthrene	1.000	0.6477
	pyrene	0.9934	1.009	pyrene	0.9910	0.9411
	2-phenylphenanthrene	0.9943	0.7733	9-methylanthracene	0.9588	0.8007
	4,4-di(*n*-butoxy)-1,1-binaphthyl	0.9904	0.6185			
	1,4-diphenylnaphthalene	0.9706	0.5950			

[a] The most probable identities of the unknowns are shown initially and after mathematical removal of one species (second search). This table illustrates the case of severe overlap. [b] Library species selected as match following first search. This species was mathematically subtracted prior to second search.

In the case of the rubrene/pyrene mixture, pyrene is clearly the best match. Pyrene is also the major fluorescent component of the mixture. The second search easily identifies rubrene. In the anthracene/perylene mixture, perylene is identified as the best match, but anthracene also appears as a potential component. After subtraction of perylene, anthracene is confirmed as the other component in the second search. Deviations of the CR1 and CR2 values from unity result from the slight overlap between the two individual spectra, but this does not prohibit successful identification.

A bicomponent mixture whose superimposed spectra have a high degree of overlap is considered next. As shown in Figure 6, chrysene and pyrene fluoresce in essentially the same region. With increasing overlap, the number of potential matches increases and the correlation values CR1 and CR2 deviate from unity. This arises since the relative intensities of the component peaks in the unknown spectrum are altered, as a result of overlapping spectral bands, with respect to the individual species in the reference library. In addition, the unknown bicomponent spectrum may have aggregate features similar to unique single component reference spectra stored in the library.

SIPS lists the reference spectra which have CR1 values greater than 0.9500 and then relies upon the user to assist in the evaluation of the data. On utilization of the chrysene/pyrene mixture as a representative unknown, the reference spectrum that had the largest CR1 value was chrysene (0.9978). The correlation values for 2-phenylphenanthrene, pyrene, and 4,4-di(*n*-butyl)-1,1-binaphthyl were 0.9943, 0.9934, and 0.9904, respectively. These values indicate that the spectral features of the unknown have the largest degree of similarity when compared with the reference spectrum of chrysene. The unknown might indeed be thought to contain pyrene, 2-phenylphenanthrene, or 4,4-di(*n*-butyl)-1,1-binaphthyl since the CR1 values for these compounds are high. Reliance upon the CR2 parameter in this case must be done carefully since the large degree of spectral overlap for this mixture causes the peaks to merge and to change intensity, thereby causing the absolute magnitude at $\nu = 0$ to increase. Consequently in the case of highly overlapped spectra, the ratio between the value at $\nu = 0$ and variable SUMSQ, corresponding to a reference spectrum that is a component of the unknown mixture, will be greater than unity.

Chrysene and pyrene both have CR2 values greater than unity, while 2-phenylphenanthrene and 4,4-di-*n*-butyl-1,1-binaphthyl do not. This evidence strongly suggests that both chrysene and pyrene are present in the unknown mixture. If chrysene is chosen as a match, a second search which subtracts the chrysene spectrum and reanalyzes the resultant spectrum as a unique unknown is carried out. The CR1 and CR2 values tabulated in Table IV confirm the presence of pyrene in the sample. Although 3,4-benzophenanthrene has an exceptionally

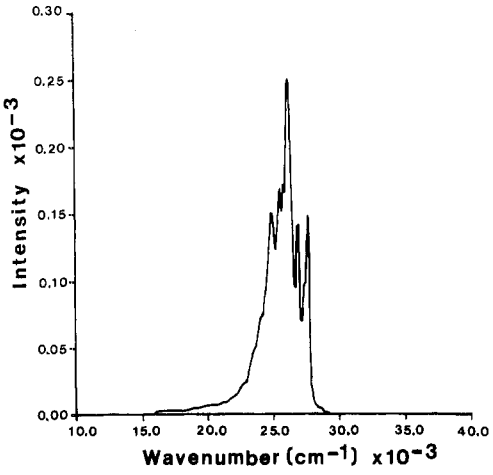

Figure 6. Input fluorescence spectrum of a mixture of pyrene and chrysene.

good CR1 value of 1.000, the CR2 value of 0.6477 is much smaller than 1.00 and therefore allows this species to be eliminated from consideration.

Spectra acquired in this laboratory were satisfactorily identified by use of SIPS when the unknown and reference spectra were well-behaved in the sense of not exhibiting concentration quenching. Mixtures were also identified properly when all species were individually well-behaved. Under these conditions, identification of real mixtures resulted in search patterns essentially unchanged from those shown for the synthetic mixtures of Tables III and IV. In some cases (substituted naphthalenes for instance), the results using emission spectra are unsatisfactory; work is ongoing to incorporate excitation spectra into the search scheme in an attempt to reduce the number of spurious identifications.

Identification of conventional molecular fluorescence spectra via cross-correlation benefits from an encoding process which allows for full data retention. Previous work (*6*) has shown that the higher frequency harmonics present in the transformed spectra can be removed without any substantive loss of spectral information, and ongoing work in this laboratory seeks to incorporate such data compression.

File searching of fluorescence spectra is plagued by the irreproducibility of fluorescence spectra from one instrument to another. Data obtained in various laboratories (*17, 20*) indicate that spectra cannot be adequately corrected by reliance upon a series of standard spectra. Yim and Faulkner (*6, 21*) have shown that point by point division of an unknown spectrum by the spectrum of a standard material can be beneficial.

The lack of comparability among spectra collected on different instruments has hindered the development of

fluorescence file searching routines. Conversely, this absence has diminished the need to develop standard protocols for fluorescence instrumentation. This interface between the requirements of the collection and identification of fluorescence spectra promises to be a challenging area for future investigation.

The FORTRAN code for SIPS compiles and runs under the TOPS-10 operating system on the University of Delaware Computation Center DEC-10 system. Source listings are available from the author. Portability is restricted by the IMSL subroutine calls and by use of DELPLOT for hard-copy graphics output.

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Registry No. Anthracene, 120-12-7; 3,4-benzophenanthrene, 195-19-7; naphthalene, 91-20-3; deuterated anthracene, 54261-80-2; benzidine, 92-87-5; sodium salicylate, 54-21-7; tetramethyl-*p*-octaphenyl, 83399-67-1; tetramethylphenylenediamine, 27215-51-6; pyrene, 129-00-0; perylene, 198-55-0; 2-aminoanthracene, 613-13-8; rubrene, 517-51-1; chrysene, 218-01-9; 2-phenylphenanthrene, 4325-77-3; 4,4-di(*n*-butoxy)-1,1-binaphthyl, 4499-67-6; 1,4-diphenylnaphthalene, 796-30-5.

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Determination of Salicylic Acid in Aspirin Powder by Second Derivative Ultraviolet Spectrometry

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The second derivative spectrum of salicylic acid showed a trough at 292 nm and a satellite peak at 316 nm. When large amounts of aspirin coexisted, the trough disappeared but the height of the satellite peak (D_L) was not altered even at an aspirin concentration 20 000 times that of salicylic acid (corresponding to salicylic acid content of 0.005%). A plot of 25 sets of D_L values and salicylic acid concentrations (1.00–10.02 μg/mL) gave a straight line (correlation coefficient = 0.9999) and relative standard deviation ($s/\bar{x}$) for a slope of 1.2%. A typical assay result for commercial aspirin powder was that the content of salicylic acid was 0.0361 ± 0.0005% (at the 95% confidence limit) with $s/\bar{x}$ of 1.2% for five measurements.

It has been shown that the application of derivative techniques to spectrophotometry is very useful when there exists signal overlapping or interferences (*1–3*). Moreover, the experimental procedure is simple and time-saving. Despite these advantages, however, few applications of derivative spectrophotometry have been published (*4–7*).

This paper describes an application of second derivative UV spectrometry to permit a simple and rapid assay of salicylic acid in aspirin powder. As salicylic acid, the major decomposition product of aspirin, irritates the digestive system, the limit of salicylic acid content in aspirin powder is prescribed to be 0.1% by the Pharmacopeas (*8*, *9*). But the assay for aspirin powder described in them is qualitative. There have been some reports on the assay for salicylic acid of 0.1% content level in aspirin by gas–liquid chromatography (GLC) (*10*), liquid chromatography (*11*), and high-performance liquid chromatography (*12*). Although the retention times of salicylic acid were within 10 min in every one of these chromatographic methods (*10–12*), time-consuming chromatographic conditioning was essential for all of them and chemical derivatization was necessary for the GLC method (*10*).

EXPERIMENTAL SECTION

Solvent and Chemicals. A 1% chloroacetic acid–ethanol solution was used as solvent. Salicylic acid was twice recrystallized from hexane, and standard salicylic acid solutions of various concentrations were prepared by dilution of a stock solution with the solvent. Salicylic acid free aspirin was obtained by twice recrystallization of aspirin from acetone.

Preparation of Assay Solutions. Assay solutions were prepared immediately before measurement. A 150-mg sample of aspirin powder was dissolved in the solvent to bring the mixture to 25.0 mL.

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