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The SPEX Speaker

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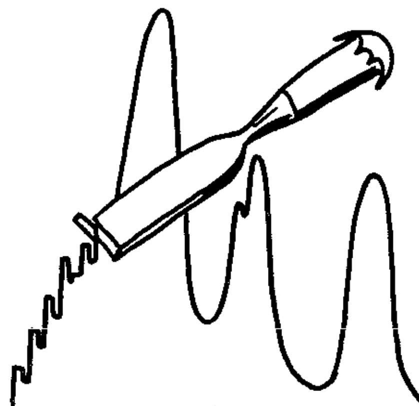
SPECTRAL SCULPTURES FROM RAW DATA WITH THE SC-31 PROGRAMMABLE DATA HANDLER

"Creating a work of art is easy," said one critic. "You simply start with a block of marble and chip away everything that isn't a statue."

With a deft twist of the metaphor, that idea can also be applied to spectroscopy. Experienced researchers have learned that generating the best possible spectra often takes a considerable amount of artistry. Before anything at all can be coaxed out of a system, slit widths, operating voltages, and scanning speeds must all be skillfully adjusted and tuned. Still, despite these flourishes of talent, the resulting spectra may very well emerge in an essentially raw form. Contributions from the sample may be mixed with those of the solvent, distorted by components in the spectrometer system, and all of this may be overlaid with a thick coating of random noise. To expose the elegant spectral sculpture concealed somewhere inside this rough-hewn block of data, the spectroscopist must chip away the excess—everything that isn't the true spectrum of the sample.

Data polishing of this sort—background subtraction, radiometric correction, signal averaging, etc.—was once the unchallenged province of massive, extravagant computers that had to be fed data for processing off-line. Later, the advent of the mini-computer created a minor revolution for, when harnessed to the spectrometer system, it could ingest and record data directly while automating wavelength scanning. That these "Mini's" were potent and effective is evident from the imposing numbers that have been awarded prominent positions in research and industrial laboratories. Yet, the scope of their applications has proved to be limited by the initial expense, the time and effort required to become familiar with such complicated devices, and the painstaking debugging so often demanded. Also, their widely praised virtue of high speed is often illusory in spectroscopy, since the actual regulator of data acquisition is usually the rate at which the analytical instrument can accumulate information.



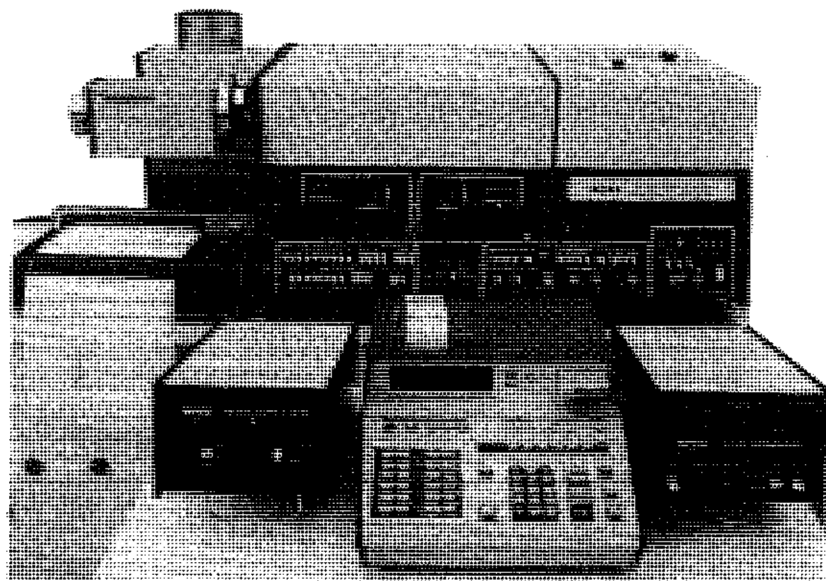
Unplagued by most of these flaws, the microprocessor has lately found a nesting place in spectroscopy. Compact, economical, and simple to operate, its comparatively slow speed is compatible with existing instrumentation. Yet, the microprocessor is a strictly dedicated module, designed and hardwired to fulfill a given set of functions and no more. In this sense, it is an inflexible drone; obedient, though obstinate, it can serve only one master.

Another approach, with a largely untapped potential, involves interfacing a key-programmable calculator to the system both to drive the spectrometers and manage the data. This arrangement retains the advantages of moderate cost, ease of operation and a computational speed that easily keeps pace with real-time analysis, while remaining malleable enough to accept command modifications instantly or to function independently as an off-line calculator.

With this in mind, we have explored the implications such a calculator-based system might have on the methods of spectroscopy and have gone on to push that concept a few steps further.

The Ground Rules

Initially, it is realistic to expect that any data handling system designed specifically for spectroscopy should minimize all the distortions introduced by the hardware or technique. Before it can be recorded, the true spectrum sought by a spectroscopist is unavoidably adulterated by contributions



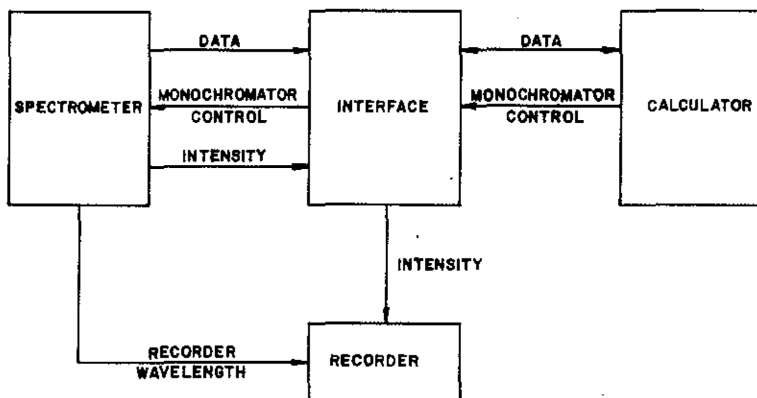


Fig 1 In the SC-31 data handling system scanning of one or two monochromators is controlled by the calculator though the interface which also passes data from the spectrometer to the calculator where appropriate correction is applied and the values stored. Data to be plotted is sent back to a digital-to-analog converter in the interface, and then to the recorder. The chart feed of the recorder is controlled directly by the spectrometer. If a direct output from the spectrometer to the recorder is desired, the interface can be made transparent to the spectrometer.

from many sources, as shown in the equation below.

$$\text{Signal}(\lambda)_{measured} = [\text{Signal}(\lambda)_{true}] \times R_1(\lambda) + R_2(\lambda) + N$$

The measured signal that eventually becomes the spectrum can be deformed by wavelength-dependent factors, such as the varying response (R_1) of the system over the spectral region, and signals from the solvent and impurities (R_2) may also be included. These are both reproducible distortions in the sense that they will always retain the same values for a given wavelength irrespective of when the signal is measured. Factors that effectively cancel out changes in instrument response lead to radiometric corrections, while the other reproducible distortions can be eliminated with background subtraction.

The term N embraces all the non-reproducible contributions which are usually identified as noise. Signal averaging is one of the most potent eradicators of these distortions.

Not until an undistorted spectrum has been unveiled can demands be extended to make the data-handling system capable of extracting specific information from the results. Yet meeting even these primary demands with an answer both economical and tractable is no trivial assignment.

Assembling the System

The Tektronix 31 is a desktop, key-programmable calculator, possessing an extensive set of arithmetic operations, a generous memory capacity of 2048 programmable steps with 1000 data registers, 24 user-definable keys, a magnetic tape recorder, an optional printer and a seductive price tag. It therefore seemed the ideal executive for the system we had envisioned.

But to bend such a device to our will, we first had to develop an interface to act as a go-between for the calculator and SPEX spectrometer systems. Once this was accomplished, we set about devising software to take fullest advantage of the calculator's endowments. A diagram of the ultimate sytem, identified as the SC-31 Programmable Data Handler, is shown in Fig 1.

The interface forms the hub of the configuration and, in this sense, it is a moderator through which all data and commands must be routed. After the operator calibrates the system by keying the position of the monochromators and the desired scanning region into the calculator, the software loads the interface encoders which drive the monochromators to the intial position in the scan. Now the interface accepts a digital signal from the spectrometer on a 16-bit, BCD line and converts this to a 4-bit serial BCD output to the calculator where this data is corrected, stored, printed, or passed back to the interface to be converted to an analog signal for the recorder. Then the software loads the interface encoders with the next point in the scan. This sequence continues until the end of the scanning range is reached. The chart feed of the recorder is driven directly by the spectrometer and, should the need arise, the interface can be made transparent and the data fed directly to the recorder without having to pass through the calculator.

The software, stored on a single magnetic tape cassette for easy initiallization, is composed of two distinct programs. The main program controls the mechanics of spectrometer scanning and all the details of data acquisition and processing, including the summing of repeated scans, subraction of background, multiplication or division of data, and radiometric corrections in accordance with arrays of wavelength-dependent factors. Generating these correction factors is the responsibility of the second program. To completely understand just how this is accomplished, it is helpful to become familiar with the nature of radiometric corrections.

Radiometric Correction of Reproducible Distortions

The interpretation of spectra from two different instruments (or even the same instrument operated under different conditions) can be hopelessly complicated by variations in the intensity and width of their peaks since it is not immediately obvious whether the equipment, the conditions, or the samples are at fault. The allurement of radiometric correction is its power to weed out the incidential variables and restore the correspondence between such spectra. An emission spectrum is said to be radiometrically correct if the signal that forms it is proportional at all wavelengths to the spectral irradiance from the sample. Spectral irradiance, in turn, is the radiant flux per unit interval of the spectrum that is incident on a unit area of surface a given distance away. Since radiant flux may be expressed in terms of energy or the number of photons, and the spectral interval may be measured in units of wavelength or wavenumber, there are four separate representations to choose from. This choice will dictate what form the emission spectrum will take and, consequently, the conclusions that can be drawn from it (1). In spectrofluorometry, spectral irradiance is usually gauged in units of photons per second per wavelength in order to achieve a representation in which the area beneath a peak reflects the relative number of photons emitted from the excited state of the sample. In Raman spectroscopy, on the other hand, emitted power per wavenumber interval is the preferred representation.

As noted, any spectrometer system, left to its own devices, will not produce radiometrically correct emission spectra. This is not necessarily a fault of the instrument design. Rather, it is a consequence of the essential nature of the measuring process and the failure of real-world instrument components to ever compare with what is theoretically ideal. So the overall response of the system must be determined as a function of wavelength. This can be done by scanning the spectrum of a standard, calibrated source and comparing the results with the known spectral irradiance of that source. Ratios generated from these measurements provide a series of wavelength-contingent factors from which a radiometrically correct emission spectrum can be calculated for future scans from this equation:

$$\text{Sample}(\lambda)_{correct} = \text{Sample}(\lambda)_{measured} \times \frac{\text{Standard}(\lambda)_{calibrated}}{\text{Standard}(\lambda)_{measured}} \quad (2)$$

For this relation to hold, two constraints must be imposed. First, the bandpass (or spectral "bite") of the instrument must be

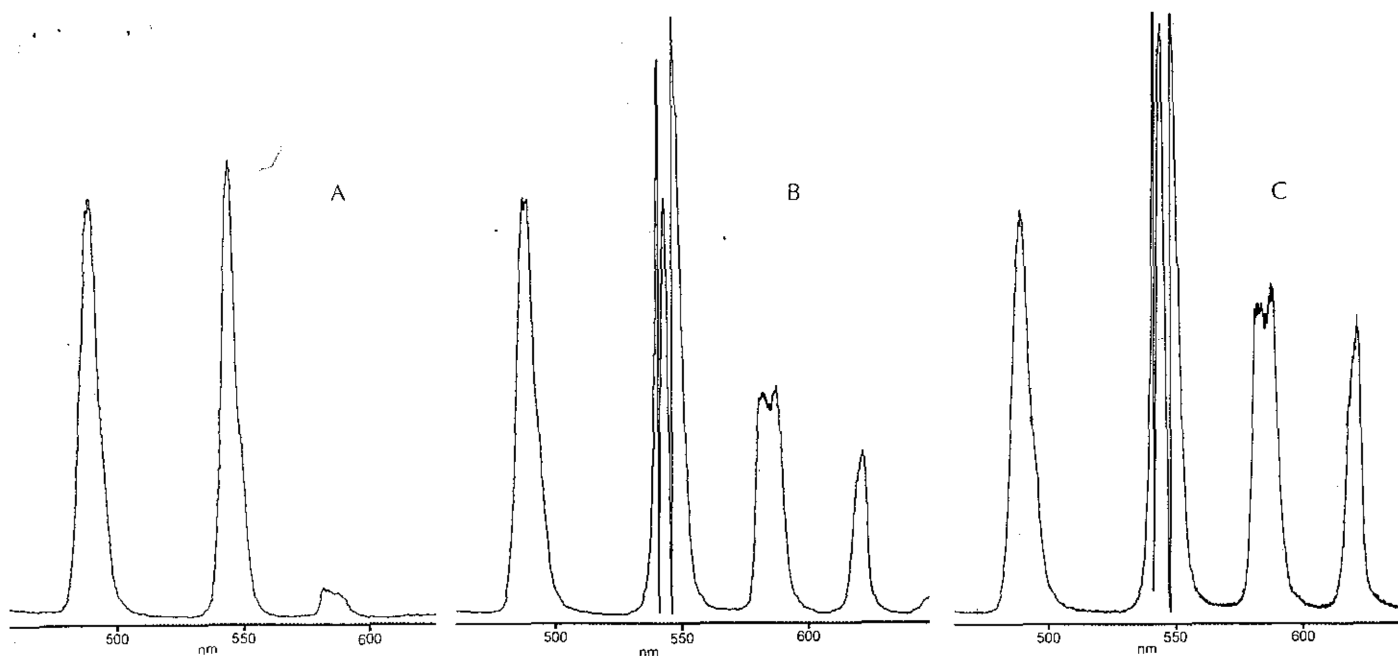


Fig 2 The uncorrected spectra of terbium chloride, when detected with a gallium arsenide phototube (B) and a bialkali phototube (A), differ substantially from that of the radiometrically correct spectrum (C), even though recorded on the same spectrometer. (The peaks at 490 nm have been normalized to the same intensity.)

sufficiently narrow during the scans so that the intensity of the signal varies linearly across the slit. Second, the conditions under which the sample and source were scanned must be essentially equivalent. As long as the response function implicit in the two measured quantities remains the same (to within a constant of proportionality), it is effectively erased from the final spectrum.

To fully appreciate the impact these radiometric corrections can have on a fluorescence spectrum, refer to Fig 2. The plot in A is an uncorrected emission scan of terbium chloride taken on the SPEX FLUOROLOG spectrofluorometer with a bialkali photomultiplier tube that responds poorly in the red, or longer wavelengths. Compare this with B, taken with a gallium arsenide PMT that has a relatively flat response over the region, to see how the spectrum has been distorted. The peak at 625 nm doesn't even appear in the spectrum taken with the bialkali tube and the peak at 590 nm is barely discernable. Furthermore, note how drastically different are the ratios of the peaks at 500 and 550 nm. C is the same spectrum corrected by the SC-31 and the relative intensities of the peaks demonstrate that even the gallium arsenide PMT does not faithfully reproduce the luminescence spectrum of the sample. And, since the peak heights indicate the number of photons emitted, only the corrected spectrum validly reflects the population of the terbium excited states and their coupling to the ground state. The peak at 490 nm was normalized in all three instances to serve as a convenient reference. When spectra generated on the same spectrometer can vary so greatly, it is easy to understand how difficult it can be to compare those generated on two different spectrometers—unless radiometric corrections have been applied!

Obviously, the quantum efficiency of PMTs is not the sole system parameter that alters with wavelength. Optical components, source output, even the spectral bandpass, are all susceptible to unique, though inherently similar, afflictions. The best way to keep these inconsistencies from intruding into the data is to flatten out the response function of the instrument and cancel this wavelength dependence.

Adjustments for source output are especially crucial when an excitation spectrum is generated in spectrofluorometry since the number of photons that reach the sample will not remain constant while the monochromator scans. On the FLUOROLOG, the bulk of this excitation correction is accomplished by selecting the ratio (Em/R) mode in which the signal from the emission detector is scaled by the signal from a rhodamine B detector that references the excitation radiation. Though the quantum efficiency of rhodamine B is reasonably flat down to 200 nm, the separate optical paths leading to the sample and the reference detectors make additional correction beneficial to the final spectrum in the region below 300 nm.

Under user control, the SC-31 will generate radiometric correction factors for both the primary emission and residual excitation corrections. Values of the spectral irradiance of a standard quartz-halogen source, at uniform wavelength intervals, are intially loaded into memory. Then a scan of that source is performed over a matching interval with the appropriate monochromator. Now the SC-31 calculates the ratio of the known irradiance of the standard source to the measured output at the same wavelength, divides all the factors by the smallest result in the array, then stores these normalized factors on the same magnetic tape cassette that holds the operating programs. Later, when a radiometric correction of emission, excitation, or a synchronized scan of both monochromators is requested, the SC-31 reads these arrays back into memory and interpolates to find the proper correction factor for each data point. After multiplying these two values together, the SC-31 simultaneously outputs the corrected data to the recorder and stores it in memory. All this is accomplished without sacrificiing time.

Background Subtraction

The hardware cannot be blamed for all reproducible distortions that disfigure the measured spectrum. The other interferences of this type are often grouped together as background, whether they originate in the solvent, the sample cell, the dark signal of the PMT, or arise from an additional form of luminescence from the sample itself that competes with the emission under study. Some commercial spectrofluorometers handle these background contributions with a double-beam arrangement that alternately excites the sample and a blank while the emission is recorded. Though this technique is certainly convenient, it is only valid if the two optical paths are identical—an assumption rarely justified.

The FLUOROLOG/SC-31 combination easily avoids this drawback by scanning the sample and the blank separately in the same position, with the same optical path. Once the blank spectrum is subtracted out of the sample spectrum, all that is left is the emission under investigation.

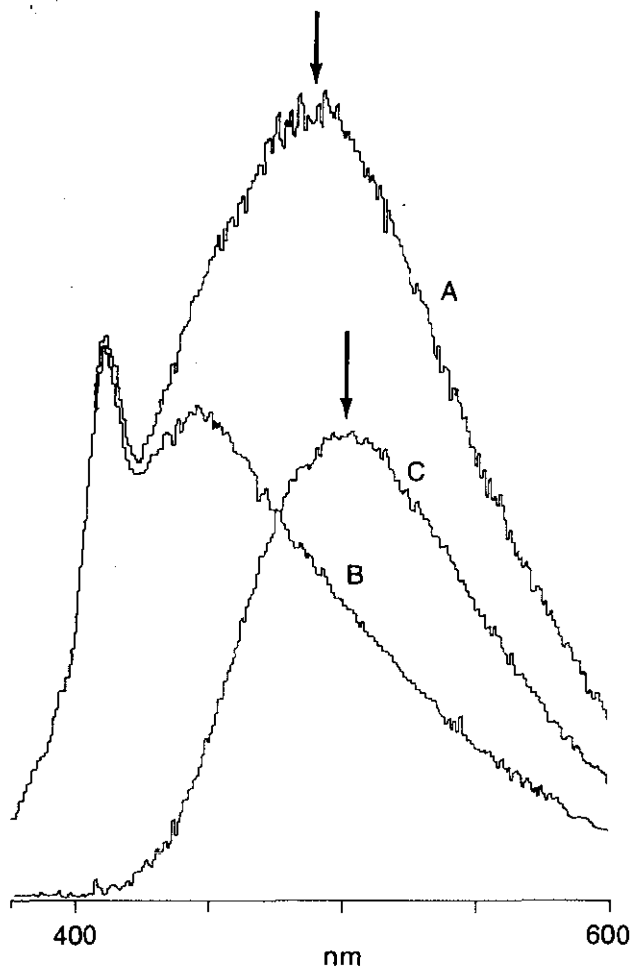


Fig 3 Spectrum A shows the fluorescence of ribosomes (RNA decoders), including the signal from the fluorescence tag, dansyl chloride. B is the unlabeled ribosomes. The peak at the far left is Raman scattering from water. The second peak in B is the unlabeled ribosome fluorescence. In the difference spectrum C, the SC-31 subtracted B from A. Only the signal from the fluorescence tag remains and, without the contribution of the background, the peak is positioned at a lower energy than indicated by the uncorrected spectrum. The peak position of a fluorophor is an indicator of the chemical envionment at the point of its attachment to the molecule.

In Fig 3, the SC-31 has performed such a subtraction on an aqueous solution of ribosomes that has been tagged with dansyl chloride. A is the fluorescence spectrum of the ribosomes including the tag, while B is the untagged spectrum. The peak to the far left, at 410 nm, is Raman scattering from water and the faithful reproduction of this feature in both scans verifies the duplication of conditions in both runs. The other peak in the blank spectrum is the natural fluorescence of the ribosomes and when both these contributions are subtracted away, only the dansyl chloride fluorescence remains in C. This peak is clearly displaced some 15 nm from its apparent location in A, revealing that the spacing in the dansyl chloride energy levels is smaller than might have been assumed. Since the position of this peak indicates the strength of the hydrophobic force at the point of the tag's attachment to the ribosome (which, in turn, contributes to the overall shape of the ribosome molecule), the spectrum in C provides information too obscured in A to be accurately evaluated.

This background subtraction expertise of the SC-31 can also aid research into the nature of induced cancer. Currently, a number of theories on chemical carcinogenesis are focusing on DNA modification by suspect agents such as polycyclic aromatic hydrocarbon (PAH). Ultra-sensitive techniques are essential to identify and characterize these modifications since the extent of binding of the agents to DNA is only about one part in 10^5. Also, fluorescence of these covalently bound complexes is strongly quenched at room temperature. The combination of these two factors has, until recently, kept the phenomenon inaccessible to conventional, commercially available fluorescence instrumentation (2).

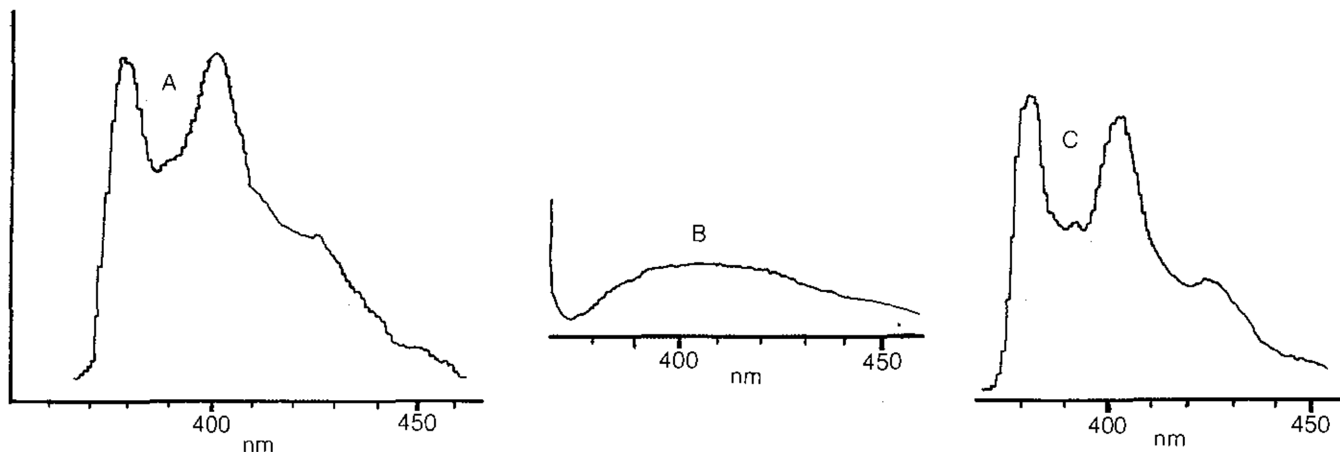


Fig 4 Fluorescence spectrum of a sample of DNA complexed by one part in 10^5 of benzo(a)pyrene (A). The sample was cooled to 120°K. B is the spectrum of uncomplexed DNA and C is the difference spectrum with only the fluorescence specific to the DNA-BP complex remaining.

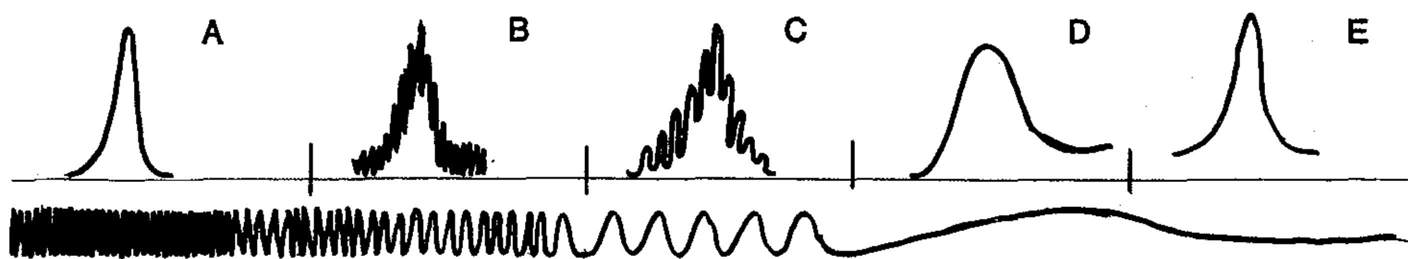


Fig 5 The effects of very high-frequency noise tend to be ignored by the system (A). High-frequency contributions are minimized by increasing the sampling time or repeated scanning (B). By randomizing the occurrence of intermediate-frequency noise (C), repeated scanning diminishes the distortion of the spectrum. Low-frequency effects (D) are attenuated by multiple scans, exacerbated by increasing the sampling time. Finally, (E) very low-frequency contributions appear as a baseline effect that can be subtracted out of the spectrum by the SC-31.

BP, benzo(a)pyrene (a constituent of cigarette smoke), is known to be one such carcinogenic agent. Fig 4A is the fluorescence spectrum of a DNA-BP conjugate taken on the FLUOROLOG spectrofluorometer with the excitation wavelength from a 450W xenon lamp set to 345 nm where the absorption of DNA is minimal and that of the DNA-BP at a maximum. To reduce quenching, the sample was cooled to 120°K. Fig 4B is the fluorescence spectrum of DNA. In Fig 4C the SC-31 Programmable Data Handler has subtracted out this DNA background, leaving only the fluorescence specific to the DNA-BP complex. Hidden structural features are now observed at 390 and 435 nm.

Thus, with the FLUOROLOG/SC-31 combination, fluorescence becomes a viable technique to plumb the mechanisms of carcinogenic modifications. And now that everything from artificial sweeteners to the once-sacred hamburger is under suspicion of harboring active agents, such a technique is vital. In fact, it is difficult to conceive of an area of spectrofluorometry that would not benefit from background subtraction.

Signal Averaging

All non-reproducible fluctuations in a signal are usually referred to as noise. But whether or not they actually inflict a distortion on a recorded spectrum depends almost entirely on their frequency. As seen in Fig 5, if the noise period is very much shorter than the sampling time of the spectrometer system (the interval over which data is accumulated at a given wavelength) its contribution will be essentially averaged out (A). On the other hand, if the period of the noise is very much longer than the scanning time (the total time required to obtain the spectrum) it will be seen only as a baseline offset (E). Within these extremes it is possible to distinguish high-frequency noise (with a period comparable to the sampling time), low-frequency noise (a period comparable to the scanning time), and intermediate-frequency noise which is an inconvenient gap-filler between the other two.

A principal source of the high-frequency species of non-reproducible distortions is the random variation in the intensity of a light source or sample that is directly traceable to the quantum nature of the individual events responsible for the emission. Intermediate-frequency noise may be due to a drop in input voltage caused by the cycling of an air conditioner or some other electrical device in the area, while low-frequency noise could be a result of instrument drift. The crucial point to notice in these definitions is that they are relative to the choice of sampling and scanning times. Increasing the total time consumed by a scan will expand the field open to intermediate and low-frequency interferences. Therefore, more sources of noise will be introduced into the spectrum. For example, the most common method for reducing the distortions of high-frequency noise is to average the signal over a sustained interval by extending the sampling time. The integrated signal itself will grow in proportion to the duration of sampling, while the noise which surrounds it will increase only as the square root of that value. Thus, lengthening the sampling time by a factor of nine will improve the signal-to-noise ratio by a factor of three. Yet this technique carries disadvantages along with it. Since the scanning time has been correspondingly lengthened, distortions with frequencies formerly too low to contribute significantly may now become prominent, and the effects of instrumental drift, negligible in shorter scans, may become pronounced.

An alternative method to perform signal averaging of this type is to preserve the original value of the sampling time and repeat several scans while summing the results. For the same total scanning time, this technique is just as effective in reducing high-frequency noise, yet it eludes the debilities that plague a single, long scan. The noise of intermediate frequency still contained within the scanning limits is averaged by this iteration scheme since the interval between successive measurements at the same wavelength tends to randomize their positions. And the low-frequency drift that seriously impairs a single, long scan will hardly affect the sum of numerous short scans. If, for instance, the signal from a spectrometer decreased by 1% per hour, a ten hour scan would differ in relative intensity by 10% from one end to the other. But the sum of ten, one hour scans would vary by only 1% along its length. Repeated scans can also be expected to reduce the blight an abrupt change in the output of a signal introduces into the spectrum when an instability appears in the light source.

Averaging several short scans offers other advantages. Light-sensitive materials that may be degraded during an unrelieved exposure to the exciting beam will exhibit far fewer aberrations if a new sample is prepared for each scan. Also, a single, long scan precludes an accurate estimate of the noise it contains.

Though all these benefits of signal averaging were recognized long before the computer appeared on the scene, running repeated scans manually demanded more stamina and endurance than most researchers could justify. Even if we ignore for the moment the tedium of resetting the spectrometer for each scan, the data from these scans must somehow be stored and summed. All this is done automatically by the SC-31. The operator has merely to enter the scanning parameters (start and end wavelengths, increment between steps, and number of scans), choose the type of scan (corrected or uncorrected) and the mode of storage (STORE; STORE+, to add more scans; STORE−, for subtraction; STORE X, for multiplication; or STORE÷, for ratioing). The Data Handler then carries on unattended and will contentedly run all night, if necessary, directing the spectrometer over the scanning range, resetting the monochromators (including backlash removal), and accumulating the successsive spectra in its memory. The data can also be examined periodically and the number of scans adjusted as the signal-to-noise ratio is driven within bounds.

Decisive testimony for the power of signal averaging with the SC-31 is supplied by 1-chloronaphthalene which exhibits an elusively faint room-temperature phosphorescence that is eclipsed by the tail of its own fluorescence throughout the region shown in Fig 6A. Though the SPEX 1934 Pulsed-lamp Phosphorimeter easily uncovered the phosphorescence (B) by inserting a delay after each excitation flash to insure the interfering fluorescence was extinguished before the emission was sampled, the low photon yield (about two photons per lamp flash), aggravated by a discordant amount of statistical noise, kept the luminescence disguised. Yet eight scans with the SC-31 (C) so reduced the noise that the true shape of the spectrum finally emerged.

The above sample was solubilized in isooctane. If, on the other hand, 1-chloronaphthalene is micellarly suspended in water, the already feeble room temperature phosphorescence contracts acutely. Twenty eight scans were needed in this instance (D) to reproduce the signal to noise of C. And, though the scanning time consumed a total of 12 hours, the effects of instrument drift are negligible.

In Raman spectroscopy signals from relevant vibrational modes are often weak, especially in low concentrations or when forced to compete against noise from a high fluorescence background. Applying the technique of signal averaging to such a sample can extend the limits of detectibility as shown in Fig 7. On the SPEX RAMALOG laser-Raman system, a single scan (A) of the Raman spectrum of an aqueous solution of 0.01% nitrate and sulphate anions produced bands that barely manage to peak through the noise from the water background. When five scans are summed (B), however, the signal-to-noise ratio is more than doubled and it is no longer a problem to gauge the positions of the bands. For a short scanning range, 100 repetitions should be feasible, effecting an improvement in detectibility by a factor of 10.

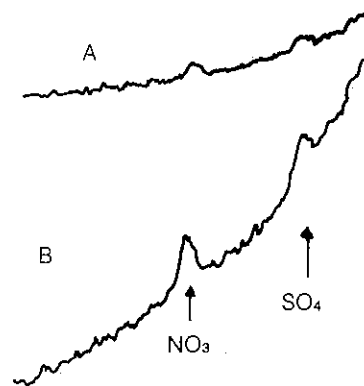
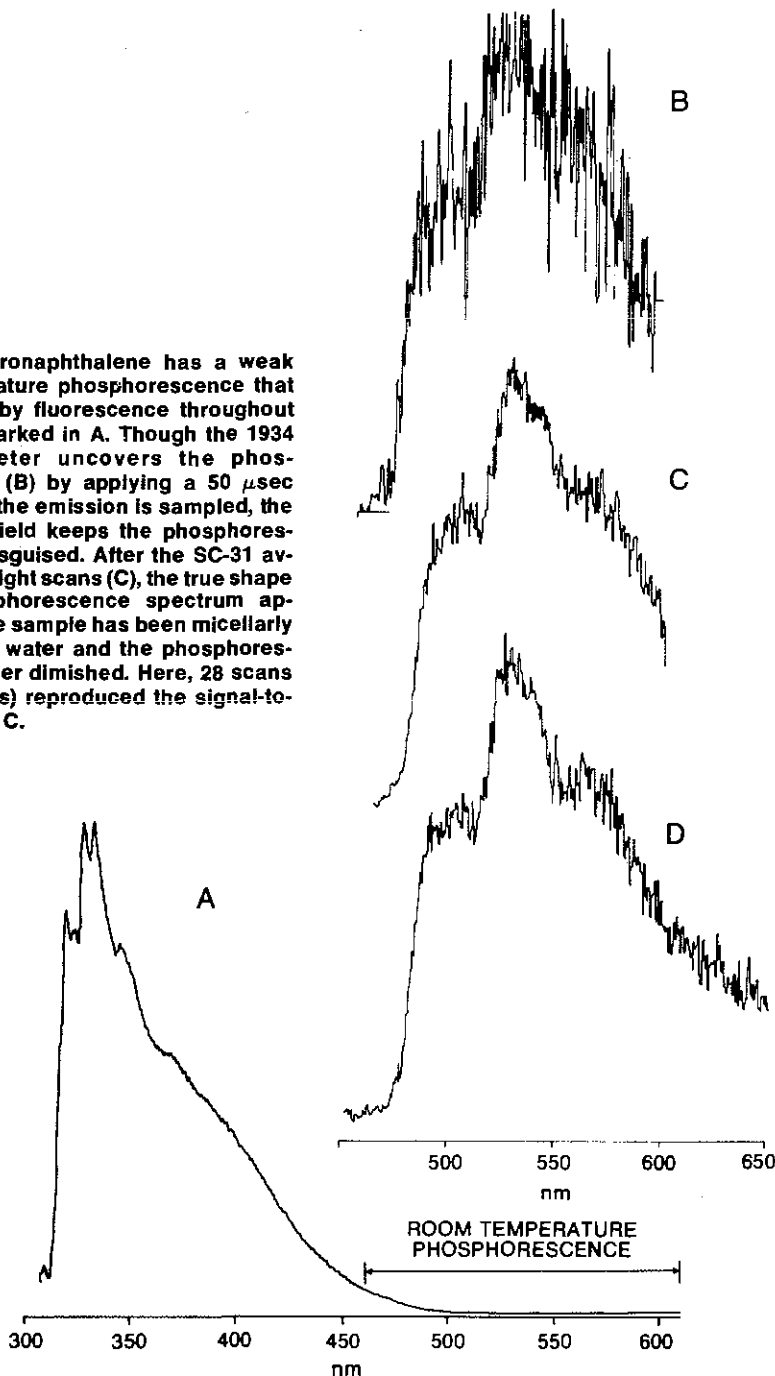


Fig 7 Signal averaging can extend the limit of detectibility of a sample. A shows one scan of the Raman spectrum of a 0.01% solution of NO_3 and SO_4 anions; B shows the sum of five such scans, with the signal-to-noise ration improved by more than a factor of two.

Fig 6 1-chloronaphthalene has a weak room-temperature phosphorescence that is swamped by fluorescence throughout the region marked in A. Though the 1934 Phosphorimeter uncovers the phosphorescence (B) by applying a 50 μsec delay before the emission is sampled, the low photon yield keeps the phosphorescence well disguised. After the SC-31 averages over eight scans (C), the true shape of the phosphorescence spectrum appears. In D the sample has been micellarly solubilized in water and the phosphorescence is further dimished. Here, 28 scans (over 12 hours) reproduced the signal-to-noise ratio of C.



The Future

Gathering radiometric corrections, background subtraction, and noise reduction through signal averaging into a single, inexpensive unit that automates the spectrometer and can stand alone as a powerful scientific calculator, is an efficient answer to the primary demands of Raman and fluorescence spectroscopy. But as long as the sciences continue to evolve, no such answer can ever be complete. Routines to locate and catalogue peaks, to determine the area under bands, to calculate derivatives, as well as quadratic smoothing and curve fitting would all be ambitious projects to consider. Though these and similar manipulations are not yet a part of the SC-31 repertoire, they may well become a part of its future.

Visitors to our Applications Laboratory have been unanimous in the opinion that the SC-31, in its present form, offers many of the advantages of a mini-computer having several times its price and complexity, and yields spectra that are elegant enough for display beside other masterworks of spectroscopic artistry.

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