Looking Ahead

Chromatography seems to work best in combination with other techniques. For instance, nonredundant (that is, dissimilar) combinations of selectivity effects have the potential to make LC more powerful than any currently known limit. Examples include the use of varied LC interactions, known as column switching (*41*, *42*), or combinations of detection principles, known as hyphenated methodology. Automation has begun to build reliability into and to take the drudgery out of repetitive experiments.

A simpler and more reasoned implementation of chemical selectivity is being developed. There is a relation between LC control, its dependence on analogs, and the role of selectivity. The instrumentation in LC is suited to user-programmed operation. The column and liquid incorporate programming in the form of chemical software, interactive chemical codes that carry out a sorting operation on other codes borne by the sample chemicals. McCorduck's quotation (*43*) from physicist Donald MacKay seems appropriate: "Being an analog man myself . . . I started thinking what sort of general mechanisms one could conceive of, artificial mechanisms, which would handle information in a more general sense than a digital computer does. For instance, a digital computer is unable to represent the concept of in between." LC represents that concept surprisingly well.

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Multichannel Detection in High-Performance Liquid Chromatography

James C. Miller, Stephan A. George, Barry G. Willis

High-performance liquid chromatography (HPLC) has been the fastest growing technology in the analytical field since the early 1970's (*1*). Some of the more notable advances in HPLC have been improvements in detector technology. With the advent of integrated circuits and inexpensive but powerful microprocessors, HPLC detectors have become so refined that they are now commonly the most sophisticated equipment in an HPLC system. Yet even with major advances in detector technology, the technique of HPLC has suffered from lack of a universal detector that provides optimum sensitivity and flexibility.

Currently, the most popular detectors in HPLC are photometers that measure absorbed ultraviolet and visible radiation. Photometric detection is highly sensitive for many compounds, but the technique has been somewhat limited by the technology available in commercial detectors. In this article we describe the development, design, and applications of a new commercially available photometric detector that uses photodiode array technology and computer network concepts to overcome technological limitations. Although it is still limited to compounds that absorb ultraviolet and visible light, this detector allows simultaneous acquisition of light intensity data at all wavelengths between 190 and 600 nanometers and is a powerful addition to liquid chromatographic detection.

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Development

The first HPLC photometric detectors were fixed-wavelength ultraviolet detectors using the strong 254-nanometer emission line of low-pressure mercury lamps. The low cost and relative universality of these detectors have made them the most common type on the market today. However, these detectors have the severe limitation that they are committed entirely to one wavelength. This

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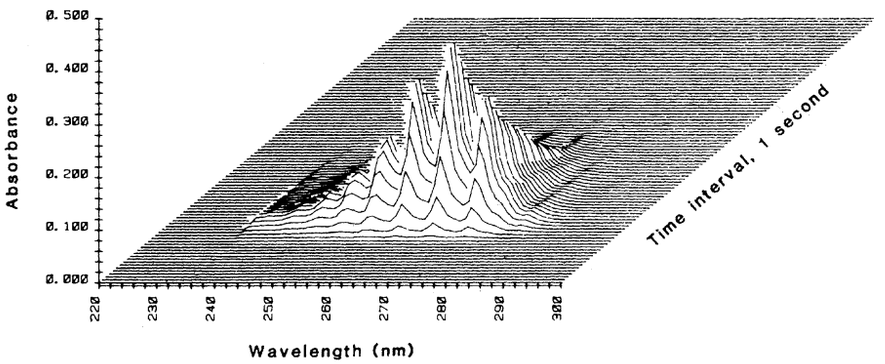


Fig. 1. Multiwavelength monitoring of benzene in acetonitrile as it elutes from an HPLC column.

is a significant disadvantage when compounds show little or no absorbance at 254 nm, or when the presence of interfering substances makes accurate quantitation at that wavelength impossible.

This limitation led to the development of selective filter photometers that could monitor a limited number of wavelengths, and to variable wavelength detectors offering a wide selection of ultraviolet and visible wavelengths. With variable wavelength detectors the chromatographer can change the detection wavelength during an analysis, thereby optimizing it for each compound as it elutes from the column. The chromatographer can choose the wavelength of maximum absorbance to gain sensitivity, or the wavelength providing the largest signal relative to possible interfering substances to gain selectivity.

The ability to change wavelengths during a chromatographic run greatly enhances the applicability of photometric HPLC detection, but some advanced knowledge of the absorbance characteristics and retention times of individual compounds is necessary to properly select the wavelengths to be monitored. Spectrophotometric detectors—sophisticated variable wavelength detectors analogous to commercial ultraviolet-visible spectrophotometers—provide the needed information by scanning the wavelengths in the ultraviolet-visible range to produce complete absorbance spectra. Conventional instruments use forward optics techniques to achieve spectral scans. The flow cell is placed after a monochromator in the optical path, and one wavelength impinges on the cell at a time. The monochromator serially scans across the spectral region of interest, while a photodetector, usually a photomultiplier tube, monitors the changing light intensity. The data on absorbance versus wavelength are sent to a recorder and an absorbance spectrum results.

Summary. A linear photodiode array has been used as the photodetector element in a new ultraviolet-visible detection system for high-performance liquid chromatography. This array allows simultaneous acquisition of light intensity data at all wavelengths between 190 and 600 nanometers. By use of a computer network concept in the electronics, this detection system can process eight different chromatographic signals simultaneously in real time and acquire spectra manually or automatically. Detector response times are variable and can be as low as 0.040 second, and bandwidth selection is variable from 4 to 400 nanometers. These characteristics permit fast chromatographic techniques and user-selectable signal-to-noise ratio enhancement. Spectra can be acquired in 10 milliseconds, permitting qualitative characterization at several different points on a single peak without destroying chromatographic signal integrity. Examples illustrate applications in fast high-performance liquid chromatography peak purity determination, and postanalysis data reduction.

Spectrophotometric detectors are probably the most useful and versatile HPLC detectors on the market today, but there are several problems with the conventional technology. The serial measurement process is slow, taking many seconds and in some cases minutes, so that scans must be made under stopped-flow conditions. When a peak is detected as compounds elute from the column, the chromatographic flow system is stopped to trap the eluting compound inside the flow cell while a scan is taken. Once the scan is complete, the flow system is started again and allowed to continue until the next peak appears. Aside from being time-consuming, this process may introduce a source of chromatographic band broadening that precludes quantitation for practical purposes.

Reversing the optical arrangement to pass the source light through the sample cell before wavelength selection allows the use of multiple detectors. Simultaneous detection of a sample absorption band and an unabsorbed wavelength provides a means of signal correction including both overall source and flow system variations. This gives an improved signal-to-noise ratio at accepted data rates, or higher data rates at a constant signal-to-noise ratio. Optical compensation allows a dynamic reference and true spectral ratio measurement. Reverse optics improves the speed and versatility of photometric HPLC detection, but there are still several drawbacks. Scans still have to be made under stopped-flow conditions, and wavelength changes can only be made when there is sufficient time between peaks to ensure quantitative repeatability. Another factor to be considered is the trend in HPLC technology toward high-efficiency columns with smaller bores. These columns can dramatically decrease chromatographic peak widths, thereby shortening analysis times. In the near future, columns will regularly produce peaks with widths as low as 1 second; peaks now are on the order of 5 seconds or more. If detectors are to keep pace with the higher analysis speeds, they must show a corresponding increase in measurement speed. On the basis of commonly accepted assumptions regarding noise and required precision, a detector should be able to take at least 25 data points across a peak to allow precise quantitation. This means that the detector response time, the speed at which chromatographic data are taken, must be 0.20 second if 5-second peaks are to be quantitated and 0.04 second for 1-second peaks. Currently, most detectors have response times on the order of 0.1 to 0.2 second.

The most desirable feature in spectrophotometric HPLC detection is an instrument that can continuously monitor the entire ultraviolet-visible spectral range throughout a chromatographic run (*2*). The potential for such a detector has been appreciated for some time; however, until recently instrumentation approaching this has existed only as experimental models and has not been avail-

able commercially (*3–7*). In May 1979 Hewlett-Packard introduced its model 8450A Diode Array UV/VIS Spectrophotometer (*8*), which can make absorbance measurements from 200 to 800 nm in as little as 1 second. It uses a reverse optics system to transmit broadband light through the sample, but instead of having a single photodetector that is mechanically scanned across the spectrum, the 8450A has two linear photodiode arrays that detect the light at all wavelengths in the spectrum simultaneously. The photodiode arrays, silicon integrated circuits containing 211 individual light-sensitive photodiodes on a common substrate, are the result of a research program that yielded a proprietary process for minimizing noise and degradation of the arrays by long-term exposure to ultraviolet light (*9*). Ultraviolet degradation of silicon integrated circuits has in the past limited the use of photodiode arrays in analytical spectrophotometry (*10*).

When interfaced to a liquid chromatograph, the 8450A demonstrates the possibilities for photodiode array technology in HPLC detection (*11*). The pseudo-isometric presentation in Fig. 1 illustrates the data acquired by the 8450A as benzene in acetonitrile elutes from an HPLC column. Parallel access to all wavelengths in the ultraviolet and visible spectrum allows the chromatographic dynamics of a system to be observed both temporally and spectrally. The increase in useful data is a function of the wavelengths employed during analysis and provides greater productivity in both qualitative and quantitative tests. Chromatographic signals of absorbance versus time can be obtained at many different wavelengths simultaneously to enhance quantitation, while qualitative spectral information can be extracted at any time without destroying chromatographic signal integrity.

Spectral acquisition at 1-second intervals is extremely fast for an analytical spectrophotometer, but with the new fast HPLC technology, where peaks have widths as narrow as 1 second, the time constant is too slow for high-precision quantitation. This prompted an extensive research program to develop a photodiode array instrument made specifically for fast HPLC detection. The result of this effort, the Hewlett-Packard model 1040A, is a system that can acquire, process, display, and store chromatographic data in both dimensions faster than any other spectrophotometric detector currently available. It can process as many as eight different chromatographic signals simultaneously in real time. Detector response times are variable and can be as short as 0.040 second, and full absorbance spectra from 190 to 600 nm can be acquired in 10 milliseconds either manually or automatically, thereby improving the match between measurement requirements and capability.

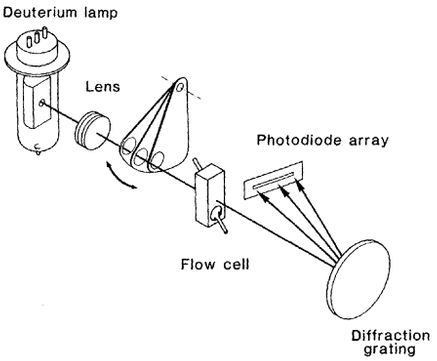


Fig. 2. Single-beam reverse optical system.

Design

In designing an instrument compatible with current and anticipated future HPLC technology, the optical system was a major consideration. Small-bore HPLC column technology requires a flow cell with a very low volume to prevent peak broadening due to mixing within the cell. Therefore, a flow cell with a volume of 4.5 microliters was constructed by ultrasonically drilling a conical hole in a solid piece of quartz glass. Two holes were drilled in the long axis for inlet and outlet capillaries, with internal diameters of 0.125 and 0.25 millimeter, respectively. The chromatographically determined requirements for the flow cell defined the overall optical configuration. A single-beam reverse optics system, illustrated in Fig. 2, was chosen to maximize the amount of light transmitted through the flow cell. Broadband radiation emitted from a deuterium discharge lamp is focused onto the aperture of the flow cell by an achromatic lens system. The emergent radiation is diffracted into its component wavelengths by a holographically recorded grating and focused onto a single photodiode array. The array is positioned to pick up the positive first-order diffraction. A shutter between the lens system and the flow cell is used to cut off the radiation for dark current compensation or to interpose a holmium oxide filter for diagnostic purposes.

The photodiode array contains 211 photosensitive diodes, 205 of which are used to monitor the incident radiation from 190 to 600 nm; the remaining diodes are used for mechanical tolerances in alignment of the optical system. Each of the 211 photodiodes has a storage capacitor connected in parallel. Photocurrent resulting from light striking the photodiode causes the capacitor to discharge toward zero potential. The extent to which the capacitor is discharged is a measure of the amount of light that fell on the photodiode during the integration time.

Serial readout of the array is accomplished by means of a digital shift register designed into the photodiode array chip. Each photodiode is read in 44.5 microseconds, and the entire array readout process is repeated every 10 msec to prevent saturation of the photodiodes resulting from high incident light intensities. The result is an analog signal containing information on the state of all 211 photodiodes.

The array signal is fed to the input of a programmable gain amplifier for selective gain amplification. The selective amplification is a function of wavelength and compensates for variation in the output of the lamp, the efficiency of the grating, and the spectral response of the photodiodes. The output of the gain amplifier is coupled to a ground reference stage for noise compensation and sent to an analog-to-digital converter (ADC) for digitization. The output of the ADC is a stream of digital data processed at a rate of 22,500 data points per second.

In computer terms, each datum may represent 2 bytes of information. Since the cost of storing 45,000 bytes of information per second becomes prohibitive with most available technology, a high-speed computer network was designed to process the data in real time and reduce it to a size manageable by a low-cost storage system. This computer network, illustrated in Fig. 3, distributes the major tasks among three individual microprocessors. Two of these microprocessors, the data acquisition processor (DAP) and the communications processor (COM), reside in the mainframe of the detector. The third microprocessor, an HP 85 personal computer, is external to the mainframe and interconnected through a Hewlett-Packard interface bus (HP-IB), a version of the IEEE-488 industry standard interface. The DAP acquires and processes data from the photodiode array, the COM is responsible for system control and communications, and the HP 85 acts as the user interface and system master computer.

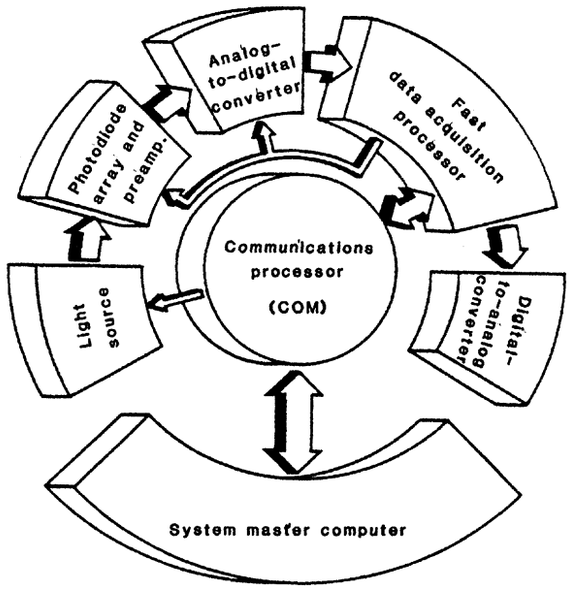
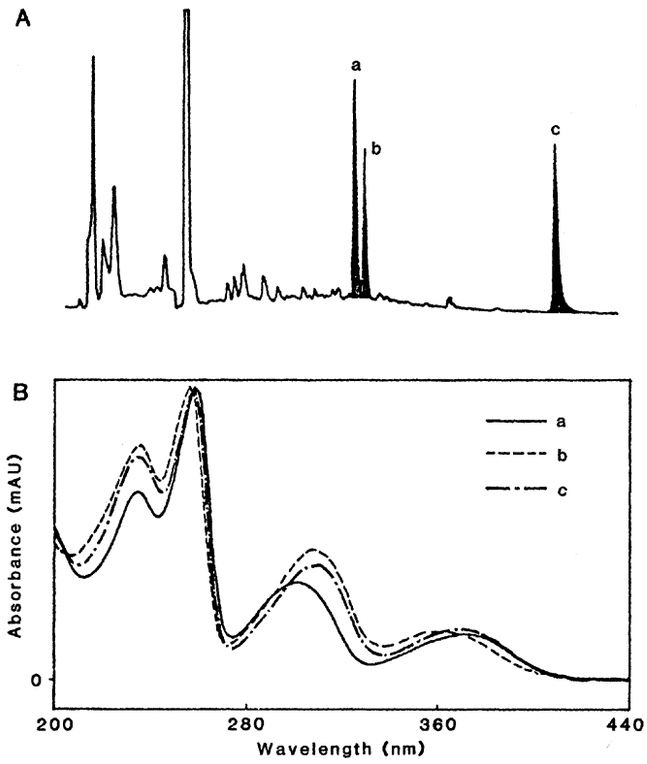


Fig. 3. Computer network system used for high-speed data processing.

The DAP forms the heart of the computer network. It is responsible for acquiring the 45,000 bytes of raw data every second and reducing the data to a few hundred bytes of useful data in the form of chromatographic signals and spectra. During processing, the data go through dark current, gain level, and ADC offset compensation, balancing, logarithmic conversion, data reduction, and filtering. In all, more than 270,000 operations are performed each second. With conventional microprocessor technology, a single-chip processor cannot attain this speed, so a bit-slice processor was developed that consists of four individual processors connected in parallel. The 16-bit digital values from the ADC are split into four 4-bit segments and processed in a parallel fashion. This high-speed technique provides conversion times on the order of 40 μsec per datum to keep pace with the stream of data originating from the photodiode array.

Since the DAP is so busy, it cannot afford to be interrupted to communicate with the relatively slow outside world. The COM acts as a go-between for the DAP and the HP 85, intercepting, interpreting, and distributing commands from the analyst. The COM also ensures correct data transfer throughout the system and controls slower hardware operations.

As a user interface and system master computer, the HP 85 is used to manipulate, store, or display the data as programmed by the analyst. The software for the master computer can be divided into three modules. The preanalysis software sets up an interactive dialogue with the user to permit configuration of the detector for signal acquisition and storage, spectral acquisition and storage, and any time-dependent functions that are desired. From the information obtained during the dialogue, the computer takes over control of the computer network for the course of the analysis. During the analysis, interaction with the user is minimal. Signal acquisition and spectral acquisition occur automatically; however, manual entry through the keyboard may be used to override the settings configured in preanalysis. At the conclusion of the analysis, software again sets up a dialogue with the operator to manipulate the data obtained. Programs can be automatically or manually called from storage for manipulation of the data as desired.



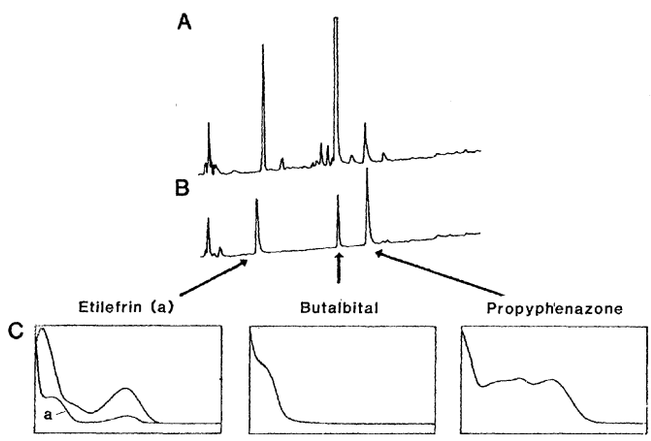


Fig. 4 (left). (A) Chromatographic output from plant extract analysis. (B) Spectra of shaded peaks in (A) (*AU*, absorbance units). The similarity of the spectra indicates that the three substituents belong to the same class of compounds, in this case flavonoids. Fig. 5 (right). (A) Chromatogram of serum sample from a comatose patient. (B) Chromatogram of drug standards made from tablets found near the patient. (C) Spectra acquired at the three major peaks in each chromatogram, normalized, and overlaid for comparison. The mismatch of spectra for etilefrin indicates its absence in the serum sample.

Applications

The fast response time and very rapid spectral acquisition capability of this new detection system for HPLC provide opportunities for investigations with both quantitative and qualitative aspects. There now exists a lower cost option than mass spectrometry for qualitative analysis of spectrally active sample substituents. Examples of applications in which these capabilities are used are given in the following discussion.

Peak identification: Sample survey. The analyst is often engaged in the separation and determination of complex mixtures. Some constituents may be extraneous to the analysis. In the past, the analyst relied on known standards or on fractionation and further testing to determine the compounds of interest. This labor-intensive approach can be sidestepped, for the most part, if spectral acquisition is used. Figure 4A shows a chromatogram of a complicated plant extract. Spectra of each peak were obtained automatically during the chromatographic analysis illustrated. For clarity, only three of these are shown (Fig. 4B). The many spectra taken were evaluated visually. The compounds of interest were found to elute at the locations of the three shaded peaks, and their similar spectra indicated that they were related flavonoids. The spectral shifts of the primary chromophores are due to structural dissimilarities. If the analyst considers that these results define his or her area of investigation, no further qualitative procedures are needed.

Peak identification: Blood sample analysis. There is no tool that is as efficient as mass spectrometry for providing qualitative information on chemical components. However, the complexity and cost factors of mass spectrometry often limit its efficacy in situations where operators do not have the level of training required for definitive results. In such situations, HPLC with the new multichannel detection system may be an excellent solution, as illustrated in the following example. Figure 5A shows a chromatogram of a serum sample from a comatose patient whose symptoms indicated a possible drug overdose. Tablets found at the side of the victim contained compounds that might have caused the poisoning; a chromatogram of these compounds is shown in Fig. 5B. Spectra were taken at the apex of the three major peaks in each chromatogram. These spectra were normalized to a maximum absorbance of 1 and were then overlaid. The results are illustrated in Fig. 5C. The normalized spectra for the second and third peaks in the two chromatograms completely matched, showing that butalbital and propyphenazone could be found in the blood of the victim. The spectra for the first peaks did not match. One might have suspected two different compounds here because of the difference in retention times of the supposedly matching peaks in the chromatograms. However, complex sample matrices can affect retention shifts. The suspicion is confirmed by spectral analysis. A complete mismatch of the normalized spectra is apparent and, in fact, the peak found in the serum sample has a spectrum similar to that of caffeine. A set of chromatograms and spectra can thus be used as the basis of treatment in this situation.

Peak purity determination. Identification of constituents is closely followed by the determination of their purity or impurity. Spectra can provide a means for the analysis of peak purity. If the compound is pure, spectra taken anywhere along the envelope of the peak should exhibit changes only in the amplitude of the response. Shifts in maxima or minima are an immediate indication of the presence of an impurity in a peak. The chromatogram in Fig. 6A represents another plant extract. The complexity of the sample is indicated by the baseline drift under the sharp peaks. The purity of the shaded peak in the chromatogram was questioned because of its apparent incomplete resolution from a later eluting peak. To examine the purity of this peak, four spectra were acquired over it: two on the upslope, one at the apex, and one on the downslope. The four spectra are shown in Fig. 6B. The differences between these spectra appear to be only in amplitude, not in maxima or minima. This observation is confirmed when the four spectra are normalized; all four completely match (Fig. 6C).

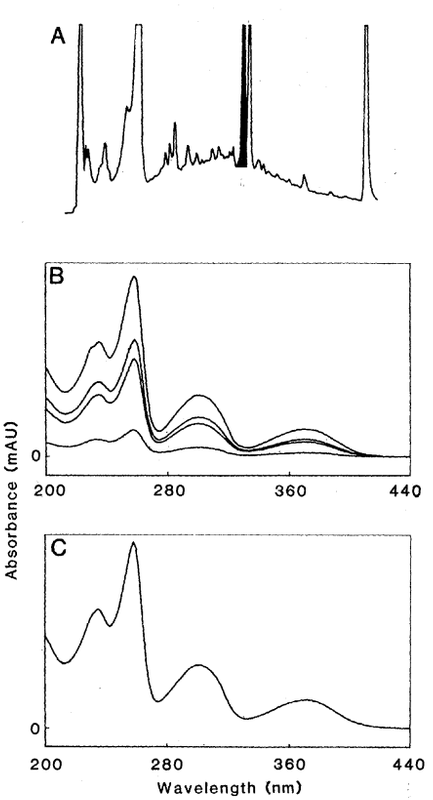


Fig. 6. (A) Chromatogram of plant extract; purity of the shaded peak was in doubt. (B) Four spectra taken over the shaded peak in (A) and overlaid for comparison; spectra appear different in amplitude only. (C) Normalized spectra; the complete match indicates purity of the shaded peak.

Impurity can also be determined. Figure 7 shows such an example where two preservatives, benzoic acid and sorbic acid, were not separated under typical chromatographic conditions, with an octyldecyl silane stationary phase and a water-methanol solution as the mobile phase. The acidity of the compounds caused peak tailing, as seen here, and therefore the peak could be integrated as a pure compound. Spectra acquired at the upslope, apex, and downslope of the peak immediately indicated coelution. The upslope spectrum is characteristic of sorbic acid, the downslope spectrum is that of benzoic acid, and the spectrum at the apex represents a composite of the two compounds.

Wavelength chromatography. The ability to simultaneously monitor many signal channels adds a dimension to chromatographic characterization that enhances the information content of any single run. We call this wavelength chromatography. The 1040A can simultaneously monitor eight different signals and obtain basic qualitative information. User selectability of wavelength, bandwidth, and a number of chromatographic signals permits rapid assessment of what may be happening in a given analysis. Figure 8 shows four signals obtained simultaneously at different wavelengths. It can be seen immediately that the major peak is not a pure compound. The retention time shifts indicate that spectral absorbance is not remaining constant, as it is in the first peak. If two peaks are not completely separated, but their spectra are sufficiently different, parallel integration of two analog signals will allow for quantitation of both peaks within the same analysis.

High-speed HPLC. As column dimensions and particle packing diameters are reduced, chromatographic peak widths

will decrease. Column technology will soon be able to resolve peaks whose baseline widths may be as small as 1 second. These improvements demand fast data rates or lower time constants. The 1040A meets those restrictions and also allows for spectral acquisition. Figure 9 shows a high-speed HPLC analysis resulting in five well-separated peaks. The 1040A provided an uninterrupted chromatogram at the rate of 25 data points per second and, independent of that process, automatically acquired three spectra for each peak, as shown.

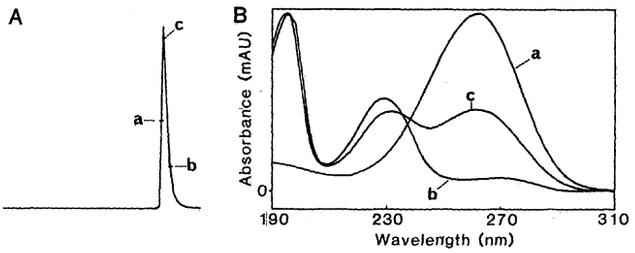


Fig. 7. HPLC analysis of benzoic and sorbic acids. (A) Chromatographic peak appears pure since the shape is normal for acidic compounds. (B) Spectra acquired automatically at the upslope, apex, and downslope of the peak are overlaid for comparison. The large mismatch confirms the presence of coeluting compounds.

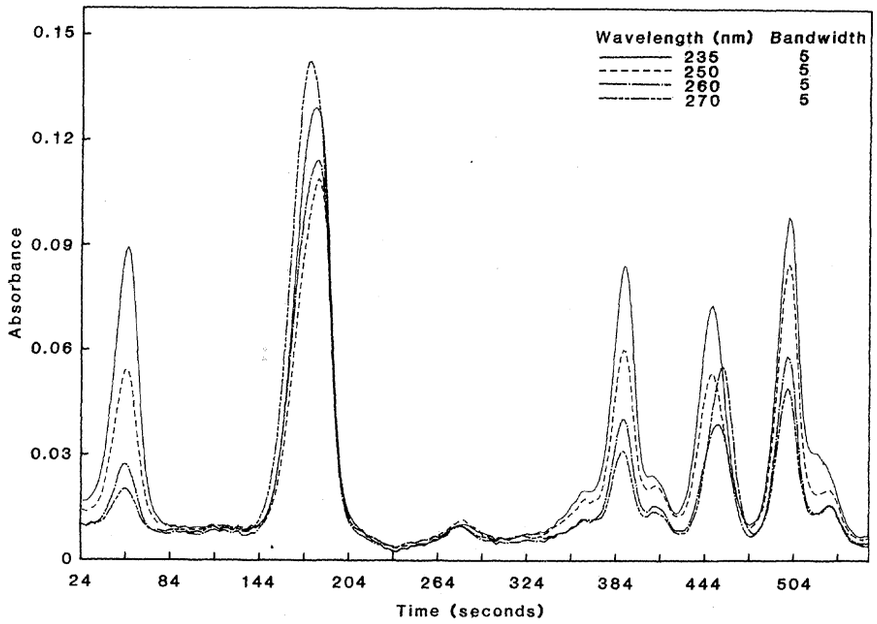


Fig. 8. Four chromatographic signals acquired at four wavelengths overlaid for comparison as a means of peak purity determination. Shifting retention times in the second and fourth peaks suggest coeluting compounds.

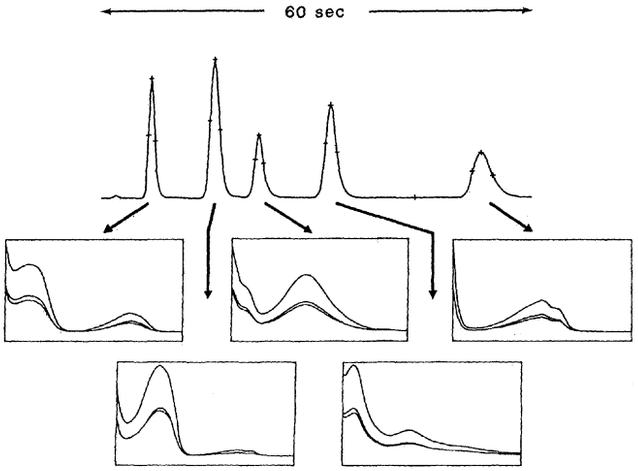


Fig. 9. Data acquired during a 60-second HPLC analysis of a sample containing five compounds. Spectra were acquired automatically at the upslope, apex, and downslope of each peak, as indicated by the tick marks on the chromatogram at the top, without interrupting the chromatographic signal. The three spectra for each peak are overlaid for peak purity determination.

Conclusion

Multiple parallel channel sensing, which has been used to advantage in several fields, is now available in a practical way in an ultraviolet-visible chromatographic detector. This adds a third dimension of information on difficult chemical mixtures. Chemical determinations that have depended on single-channel detection have often been limited in specificity because of the broadband absorption character of the chromophores. Specificity can be recovered by obtaining additional information about peak position in both the wavelength and time domains. Peaks can be identified and their purity confirmed with high levels of confidence. Identification of unknowns can be carried out concurrently with quantitation of known species in the same chromatogram. This allows maximum productivity in the analytical process. Increases in sensitivity are afforded by adaptability in both wavelength and time averaging. Theoretical analysis shows that significant increases in sensitivity are obtained by adapting the spectral bandwidth to the chromophore's natural bandwidth, just as time constants are adapted to the expected frequency bandwidth of the developing chromatogram.

In the future, we expect to add discrimination based on band shape as well as position. With this capability plus techniques such as factor analysis, we should be able to extract the spectral characteristics of materials that are poorly resolved in both domains of wavelength chromatography. Because of the enormous data reduction loads that an instrument like this must handle, a computer network with successive levels of information compression is necessary.

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