$\times 10^{-24}$ mole/sec. If 0.01 mole/l concentration of choline is initially formed in the synaptic gap during one cycle of nerve impulse transmission, the number of moles of choline wtihin the gap is 1.57×10^{-17}, if the dimension of the gap is assumed to be 2μ in diameter and 500A in width. Even with within the maximum rate of 3.69×10^{24} mole/sec., it will take 4.25×10^{6} sec. to take up all the molecules of choline ion. This means that the rate of diffusional outflow of choline is much larger than the uptake rate and there will be practically no choline uptake. This situation is, of course, untenable and it is obvious that a more accurate choline uptake rate should be obtained experimentally in order to be able to assess the role of chondroitin sulfate within the synaptic gap in affecting the uptake efficiency of choline.

Acknowledgement. The financial support of the Korea Science and Engineering Foundation is gratefully appreciated.

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Microprocessor Based Laser Induced Fluorometry
I. Development of System and Application to Liquid Chromatography

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An analytical applicability of the fluorescence detection with an optical multichannel analyzer to organic dyes was studied in this work. Continuous acquisition of spectra was possible with the use of a microcomputer. The maximum acquisition rate of a spectrum with 70-point average was about 3 seconds. Floppy discs were used to store data for later use in processing. Laser induced fluorescence detector in High Performance Liquid Chromatography was chosen for an application. Fluorescein below 10^{-15}g was detected satisfactorily using this system. With the help of microcomputer, three dimensional chromatograms of time-wavelength-intensity were obtained.

Introduction

Optical Multichannel Analyzer (OMA) which is consisted of a parallel optoelectronic image detector array and a digital signal accumulation system has merits of both spectrograph and spectrometer. So, its use in spectroscopy has been increased over the past several years[1-6].

In fluorescence spectroscopy, although a large variety of spectrofluorometers are commercially available now, the detection system used is entirely based on the use of photomultiplier tube (PMT) .The use of OMA as fluorescence detector is superior to the conventional detector because of its ability of recording entire spectrum simultaneously even though it has lower light detectability[3]. Therefore, it has unique applicabilities to transient spectrofluorometry, such as real time peak detection in liquid chromatography or other time resolved spectroscopic studies in kinetic application.

In chromatographic application, it gives the combination of coventional separation and molecular fingerprinting technique, and it offers the most powerful means of analyzing the complex material[7]. In the case where the components to be analyzed lack sufficient volatility for gas chromatography, liquid chromatography combined with fingerprint technique such as mass spectrometry or rapid scanning UV-Vis absorption spectroscopy offers a path to analysis[8]. But difficulties arise in the corresponding interface to a mass spectrometer and poor sensitivity of UV-Vis absorption spectroscopy, OMA detection of fluorescence spectra in

High Performance Liquid chromatography (HPLC) effluents has been reported[9-11].

The use of LASER as an excitation source gives many advantages in fluorescence spectroscopy. Due to its power and monochromaticity, ultra trace amount of fluorescing molecules can be detected[12-16]. Especially, in HPLC application, collimation proterty of LASER beam reduces detector volume, so it is possible to have specific cell designs for better sensitivity[15-18].

In this study, a fluorescence detection system using OMA was constructed by minor changes of a commercial spectrofluorometer, and applied it as a detector to HPLC effluents to demonstrate its usefulness. Argon ion LASER was replaced as excitation source to improve the detection limit. Because there were a large amount of data during experiments and it was necessary for control of above system, computer and data storage device were necessary[8, 19, 20, 29]. We also made an interface system between OMA and a commercial microcomputer for acquisition and processing of data.

Instrumentation

Instruments used in this study were Princeton Applied Research's Optical Multichannel Analyzer with Vidicon detector (1205A and B), Waters Associates' HPLC, model 164-09 Argon ion laser by Spectra Physics, Farrand MK-1 spectrofluorometer, and Apple II plus personal computer with Epson FT80 printer.

Overall block diagram of the experimental set-up is shown in Figure 1.

(*i*) *HPLC-Flow Cell.* Column effluent from HPLC was directly connected to a 10 μl flow cell *via* stainless-steel tubing of 0.009'' ID. A small volume flow cell was necessary to have a good resolution.

(*ii*) *Optical Arrangement.* In the first part of work where argon ion laser was not needed, an original xenon arc lamp was used in fluorometer. The monochromatic 488 nm line output from argon ion laser as an excitation source, however, was replaced for the excitation part of the spectrofluorometer in the trace analysis and characterization of the system. The laser beam was focused on the flow cell with a condensing lens. Fluorescence emission was gathered at 90 degree angle by an objective lens in order to broaden solid angle.

The standard 28,000 grooves per inch grating in the emission monochromator was replaced with a 15,000 grooves per inch grating to increase the spectral range of the detector. The spectral coverage was calculated from the linear disperson of grating, the focal length of monochromator, and the target size of detector[21] and it was about 100 nm. The relative aperture of this system was calculated to be f/4.9 from the reduced grating size (30×30 mm)[22].

The focal plane of monochromator must be in the position of exit slit which was removed in this work to accomodate the vidicon array detector and since this detector sits 18.5 mm beyond the exit slit, a concave lens of focal length 140 mm has to be utilized in front of the exit slit[23]. This brings the focal plane just onto the target of the detector.

Since the vidicon detector of the OMA is designed electronically to substract the signals originating from the upper 0.2 inch portion from those in the lower portion, dark current can be easily substracted from each channel if the upper target area is illuminated[24]. Because the inverted image of the entrance slit is formed at the focal plane in Czerny-Turner mounting, the lower portion of entrance slit was masked with black tape in order to prevent the spectrum from falling on the upper portion of the target[4].

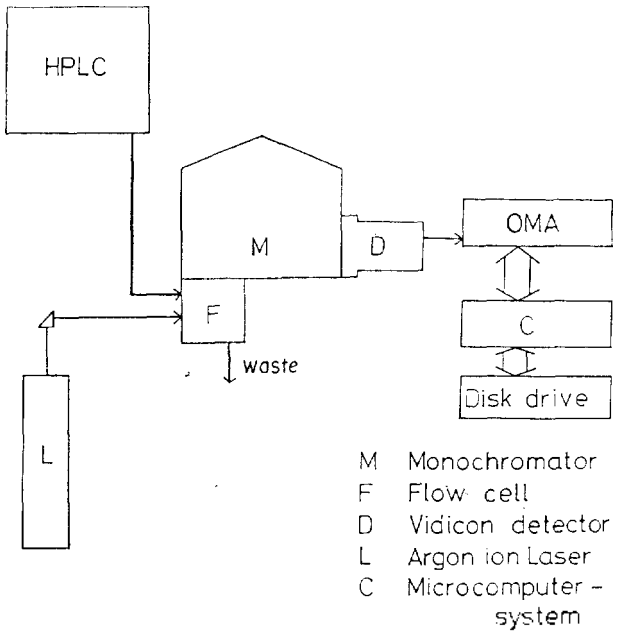

Figure 1. Block diagram of overall system.

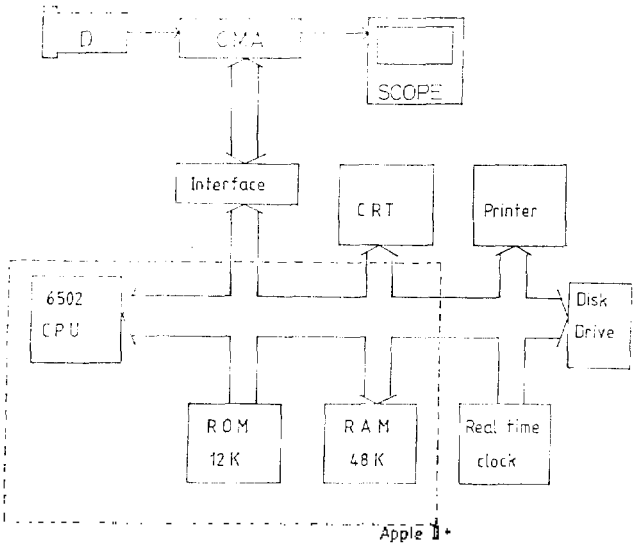

Figure 2. Block diagram of data system.

(*iii*) *Data System and Computer Interface.* The signal from 500 linear array of parallel detectors can be scanned by OMA in less than 33 msec., then they are digitized and stored to either one of 21 bit 500 words in OMA memories A or B[24]. This digitized data can be displayed on the oscilloscope and transferred to a microcomputer for processing through an interface circuit built in this laboratory. An Apple II plus (Apple Computer, Inc.) microcomputer with 48K Random Access Memory (RAM) was used for that purpose. Also the microprocessor is needed to handle the

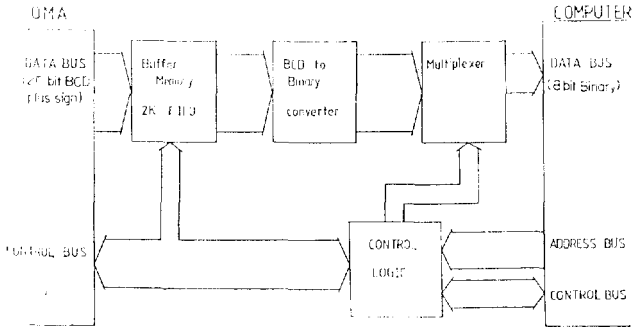

Figure 3. Block diagram of interface circuit.

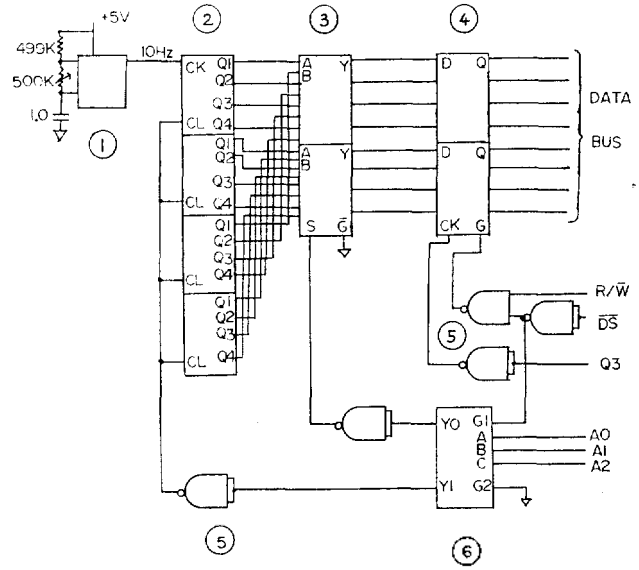

Figure 4. Circuit diagram of real time clock. 1;555 Timer, 2;74LS93 (binary ripple counter), 3;74LS257(tri-state 2 of 1 multiplexer), 4;74LS374 (tri-state latch), 5;74LS00 (NAND gate), 6;74LS138 (3 of 8 decoder).

huge amount of raw data in order from the OMA. Since 1.5K bytes of 8-bit memory were required per one OMA spectrum, about 20 spectra (30K) can be stored at once in the RAM of the computer. The remaining 18K memory was used for the system program. In order to increase the number of spectra to be stored in the experiment, a floppy disc drive was used. It turned out that a 5.25 inch diskette was able to store about 80 spectra. The whole data system in blocks is shown in Figure 2.

A home made real time clock was incorporated to computer in order to know the exact time interval among spectra. A cathod ray tube monitor was employed to monitor the program and graphic display of the data. Hard copies of data were obtained by the use of printer.

An interface between OMA and computer was constructed with Transistor-Transistor Logic (TTL) devices and is shown as functional block diagram in Figure 3. The output of OMA data is consisted of 5-digit (20 bit) Binary Coded Decimal (BCD) and one bit for the sign. Even though the available rate for data is the same as the line scan rate of the OMA's 500 sequential channels which is 32.8 msec. (64 μsec. per channel) in fast transfer mode, the transfer reat of data to RAM which is controlled by 6502 microprocessor in the computer exceeds 100 μsec per channel. In order to solve this dismatch in time domain, buffer memory was constructed using C-MOS RAM which could store the data from OMA at 64 μsec per channel using hand shake signal of DATA REQUEST and DATA READY which were in the back pannel terminal of the OMA.

The sequence of getting new data was as following. One of the J-K flip-flop (74LS76) in interface circuit generated a flag for DATA REQUEST. Then DATA READY flag was detected by the computer data bus in address $ C0sC(see the appendix for all addresses used in this work and thier functions) and it caused buffer to write mode and a 21 bit BCD datum was stored to buffer memory. After a datum was written in buffer, channel advance clock was generated by the computer using address bus and decoder IC($ C0s3) and it also caused address counter of buffer (74LS161, binary synchronous counter) increased. By repeating the above cycle 500 times, OMA data of one spectrum was stored to buffer memory in 32.8msec.

BCD to binary converter was constructed by cascade connection of 19×74184 (BCD to binary converter)[25] and resulting 8-bit binary information lines were connected to the computer through a bus transceiver (74LS245). A bus transceiver instead of uni-directional tri-state buffer was used in order to expand the capability of the interface using bidirectional bus[26].

A/B accumulation of OMA could be activated by soft switches $ C0s7 and $ C0s8 and deactivated by the DELINH signal which indicated the end of input cycle of the OMA. DELINH signal also activated IRQ flag and $C0sA reset the IRQ flag, The IRQ flag was connected to computer to interupt the computer at that point.

The real time clock is shown in Figure 4. A 555 timer generated 10 Hz pulses and counted in 16 bits by the cascade connection of binary counter. A 16 bit datum was then multiplexed twice and connected to computer data bus.

(iv) Data Storage into Magnetic Diskette. Computer memory (RAM) was found to be able to store only up to 20 spectral data. In order to expand this capacity, disc storage process should be included without disturbing the data transfer from OMA to computer while the experiment was being in progress. For the purpose of this uninterrupted dara storage, a disc storage software was developed. Figure 5 shows the schematic diagram of the process.

RAM memories in the computer which were available for the data storage was divided into two blocks (each one contained about 15K or space enough for 10 spectra). While computer spent most of its time to store the selected block of memories to disc, OMA could accumulate signals from detector for a given period of time. Every time the input mode of OMA was completed, IRQ flag was activated and it caused the computer halt its operation and jumped to the interrupt routine. During the interrupt routine, data from OMA were transferred to another block. Upon the completion of the data transfer, computer read time from the interface and had OMA start for next generation of data from

detector[27]. Then computer returned from the interrupt routine to the main program, and continued its operation of the storage of data to disc. If the storage of one block to disc was completed and another block was full, then block select switch which was controlled by software was alternating.

The maximum acquisition rate of spectral data was limited by disc storage time of the computer. Therefore storaging one block of data into disc should be finished before the completion of another block. Because the storage time of a disc was fixed, transfer rate of data from OMA to computer (*i.e.* interrupt rate) should be adjusted. During the period of interrupt interval, OMA spent its whole time for accumulation of signals from the detector. The maximum spectral acquisition rate used by the above scheme was found to be 3 sec., and in terms of accumulation number, it was 70 points accumulation. The software flow chart is shown in Figure 6.

Experimentals

488nm line of Argon ion laser was used in single mode condition as excitation source with 500mW output. E.P. grade fluorescein and rhodamine 6G were used as test sample without further purification. Triply distilled water and HPLC grade methanol were used as solvent. Reverse phase HPLC was carried out with μ-Bondapak C_{18} column and 70 %(v/v) methanol was used as eluent. Solvent was degassed thoroughly in ultra sonic bath. In the calibration curve and the study of fluorescein, Xe-lamp was used as excitation source, conventional cell holder was used instead of flow cell chamber. PH of sample was adjusted to 11 with NaOH.

Results and Discussion

(*i*) *Wavelength Calibration.* Calibration of channel was made by Rayleigh scattering peak using Xe arc lamp. Excitation monochromator was used with 1 nm band pass slit. From wavelengths of excitation monochromator and the highest scattering peak channel of OMA, we found that the relationship between channel number and wavelength was linear. Resolution of detector was found to be 0.2nm which is the wavelength difference in each adjacent channel total spectral coverage was 100nm. The conversion formula and the between wavelength and channel number was as follows.

$$Y = -0.2\ X + (15/28) \cdot (M + 864.4)$$

where Y is wavelength in nm, X is the channel number, and M is the emission monochromator reading in nm. Also fluorescence spectrum was used to confirm the wavelength calibration.

Due to the fact that the focal plane at the face of detector was not flat as the detector was, so the beam was not focused exactly on the detector face especially at the both ends of the detector. This might give some diffused effect on the intensity, however, the wavelength turned out to be within the limit of resolution of the detector.

(*ii*) *Chromatogram of Fluorescein and Rhodamine 6G.* In order to characterize this system, we chose two typical fluorescent dyes, fluorescein and rhodamine 6G, which had

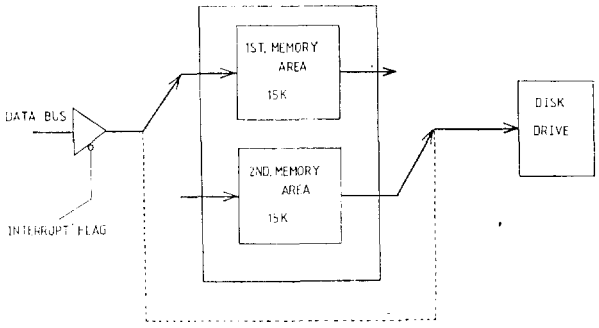

Figure 5. Software scheme for disc storage.

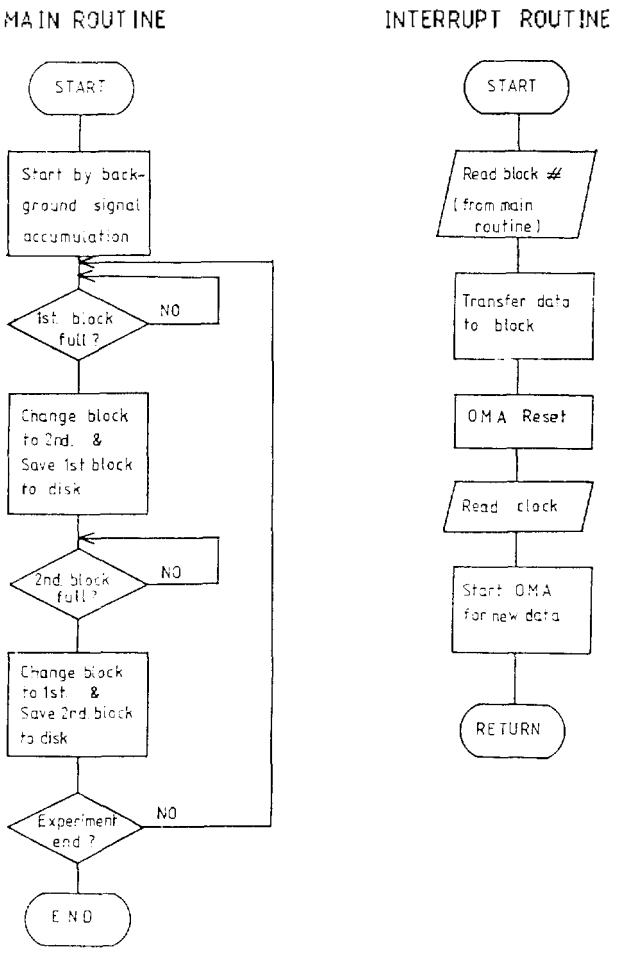

Figure 6. Software flow chart.

absorption bands near 488nm of argon ion laser. Even though rhodamine 6G absorbed only a little at this wavelength, high intensity of the excitation source could give a good fluorescence intensity. Figure 7 shows a three dimensional chromatogram of fluorescein and rhodamine 6G with the flow rate of 2m*l* per minute. BASIC language and high resolution graphic of the computer were used to obtain such a chromatogram. From this data, cross sectional views, such as intensity vs. time (chromatogram) at any wavelength and intensity vs. wavelength at any desired retention time (spectrum) can be obtained in seconds.

(*iii*) *Effect of Spectral Acquisition Rate on Chromatographic Peak Shape.* Assuming chromatogaphic peak is as Gaussian, it can be shown[8,28] that the sampling interval, s(t), as function of band eluting time, t, is given by

$$s(t)=0.271t/N^{1/2}$$

where N is the number of plates. In this study, $s(t)$ was fixed at 3 sec. and we do not want to change the conditions of HPLC part, so the detector part of the system should be improved. Since OMA can acquire a spectrum as fast as 33 msec., therefore, the capacity of computer memory must be increased in order not to use disc storage device during the separation if peak shape requires faster sampling rate such as species of short retention time (*e.g.* fluorescein peak of Figure 7). But in the real case, no chromatographic system requires sampling time as fast as 33 msec (which is more than 30 spectra in one second, and 1800 spectra in a minute).

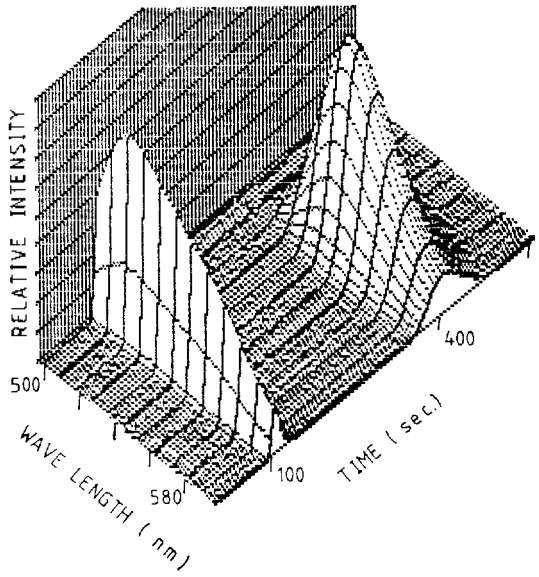

Figure 7. Three dimensional chromatogram of fluorescein and rhodamine 6G.

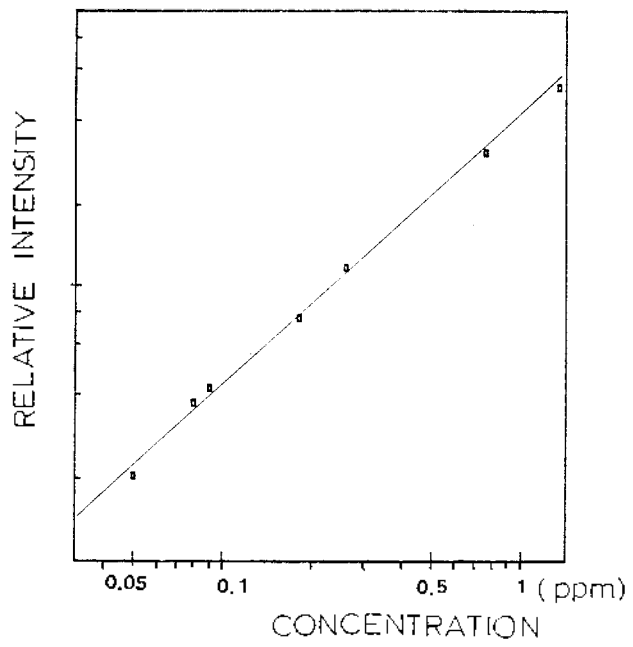

Figure 8. Analytical curve of fluorescein with 100 channel summation.

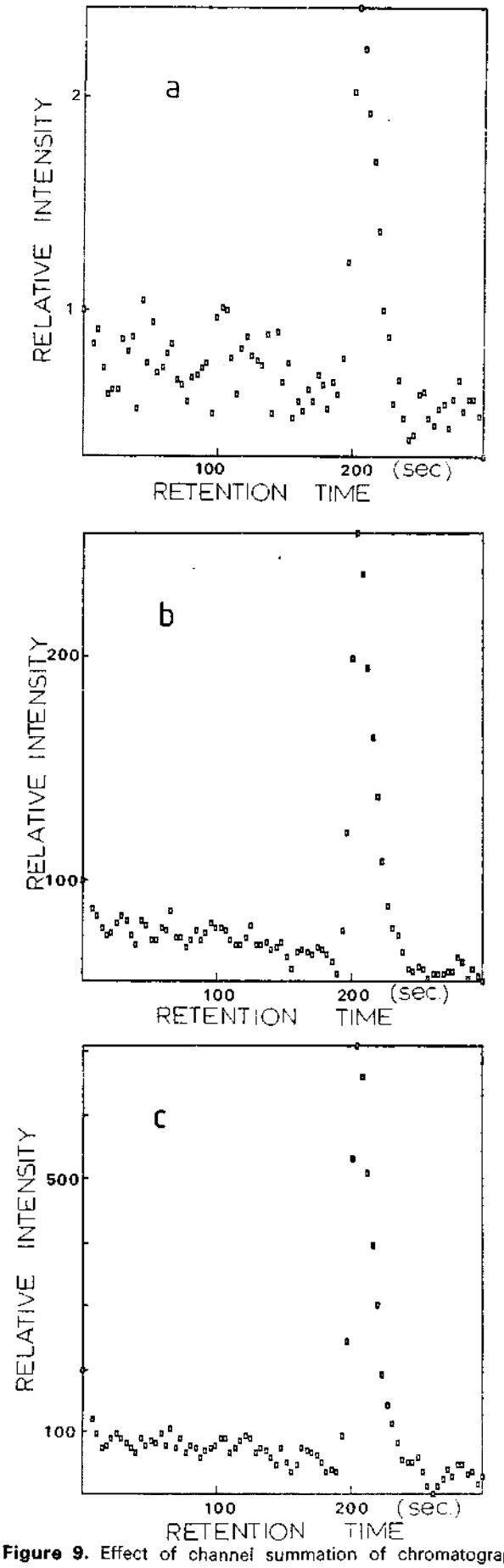

Figure 9. Effect of channel summation of chromatographic eluent of 10^{-15}g fluorescein, a; 510nm(single channel), b; 500–520 nm (100 channels), c; 497–597 nm (500 channels), Excitation; Argon ion laser at 500 mW(488 nm), Column; μ-Bondapak C-18, Solvent; 70 % MeOH–H_2O (1 ml/min).

The value we chosen as 3 seconds which was mainly due to the use of storage device was fast enough for routine chromatographic separation. It still can give 20 whole spectral information in one minute.

Three second accumulation corresponded to the summation of about 70 spectra. This had another advantage in terms of signal averaging. Since the noise is random and not synchronized with the experimental output, the random noise fluctuations in the individual waveforms will begin to cancel by summation. The signal to noise ratio, in fact, should increase proportionally to the square root of the number of averaging cycles[30].

An application in trace analysis was demonstrated in Figure 8. It gave good linearity in sub-ppm concentration range. The straight line did not go through the origin mainly due to the method of background correction. In this work, only electronic noise of the detector was automatically substracted from the signal[24] and thereby no real background correction such as stray light and emission by other substances were made. Another major difference in this working curve was the relative intensity was not one measured at a specific single wavelength as most of the conventional work had been made. Rather the intensities of 20 nm-wide fluorescence band were integrated not only to improve the detection limit but also to minimize the effect of noise which appeared randomly in terms of time and wavelength. Effect of channel summation on correlation coefficient of linearity was shown in Table 1. Here all coefficients were acceptable no matter how many channels were used for integration. We chose 20 nm which was the width at its half maximum.

For the signal to noise ratio, effect of channel summation was shown in Figure 9. The background and the shape of spectral band were definitely getting clear as the number of channels used for integration increased from one to 500.

Conclusions

A fluorescence detection system for liquid chromatographic effluent was developed using an array detector and microprocessor with a disc storage device. Argon ion laser was employed as the excitation source to improve the fluorescence intensity. A mixed sample of two fluorescent dyes was separated and detected to 10^{-15} g using channel integration technique. This integration also improved signal to noise ratio.

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TABLE 1: Effect of Channel Summation on Linearity of Analytical Curve of Fluorescein

Number of channels	Correlation coefficient
1	0.999756
10	0.999768
20	0.999774
50	0.999777
100	0.999780
200	0.999775
500	0.999771

Appendix. List of Addresses and Their Functions

Address*	Function
$ C0s0	First 8 bits of data are stored in this memory location. If $C0sB is ON, data are from buffer. If $C0sD is ON, data are from OMA.
$ C0s1	Second 8 bits of data stored in this memory location.
$ C0s2	Third 8 bits of data are stored. If its value is larger than $10, then negative flag is ON.
$ C0s3	Channel number of OMA is increased by 1 using this address.
$ C0s4	Channel number of OMA to 000
$ C0s5	Erase the OMA A memory.
$ C0s6	Erase the OMA B memory.
$ C0s7	Start the OMA A accumulation.
$ C0s8	Start the OMA B accumulation.
$ C0s9	Reset the OMA preset hold switch.
$ C0sA	Reset the IRQ flag.
$ C0sB	Off the tri-state buffer and make buffer to read mode.
$ C0sC	In this memory location DATA READY flag is stored, if its value is $FF, then DATA READY flag is ON.
$ C0sD	On the tri-state buffer and make buffer to wrtie mode.
$ C0sE	The DATA REQUEST flag is activated using this switch.
$ C0sF	Reset the DATA REQUEST flag.

* $ means that the address is a hexadeximal number and s means the slot unmber plus 8.

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