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MULTI-COLUMN LIQUID CHROMATOGRAPHY

IV. COMPUTERIZED ON-LINE SPECTROPHOTOMETRIC QUANTITATION SYSTEM FOR MULTI-COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

A system for fast high-capacity quantitation of liquid chromatograms has been built. Chromatographic samples in multi-cuvettes are moved by a transporter past a light beam that is sent from a monochromator through the multi-cuvette to a photomultiplier tube. The optical data are sampled and processed by a minicomputer and results plotted on-line as chromatograms with a simultaneous output of calculated peak values on an electrostatic printer/plotter.

INTRODUCTION

For the last seven years we have made almost daily use of the previously described[1] system for quantitation by colorimetry of chromatograms produced by multi-column chromatography. The system has worked well for routine purposes but it became clear that a much simpler and more productive system could be devised if it was possible to abandon the flow-cell approach to the colorimetry.

As the central component in the new system we therefore developed the multi-cuvette[2] which opened the way for a new integrated modular system for multi-column chromatography with new techniques for the chromatography itself[3] and a new type multi-collector[4]. The most important part of this system is the quantitation system. This is greatly improved compared with our previous system[1]. It uses spectrophotometry instead of the filter colorimeters used by us previously. It has higher capacity for less investment in instrumentation. It is much simpler in mechanical construction with less maintenance. It uses a dedicated minicomputer for direct on-line treatment of data and gives a direct print-out on an electrostatic printer/plotter of recorded chromatograms and calculated quantitated values instead of the punched tape output we

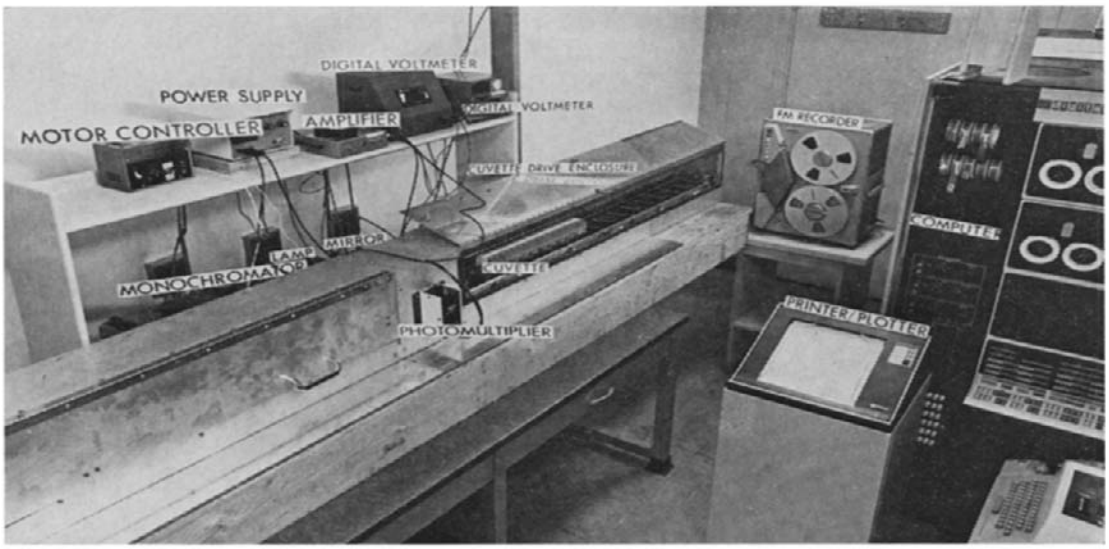

Fig. 1. The complete system for computerized quantitation. The optical system with the cuvette transportation system and the electronic interface to the computer is to the left. The computer, the electrostatic printer/plotter and a backup FM recorder are to the right.

have had to fall back on for routine work in the older system with the delay in data processing necessitated by the separate data acquisition and data processing procedures.

DETAILS OF CONSTRUCTION

An overview of the total system is given in Fig. 1. The multi-cuvettes are transported by a chain drive resembling an assembly line inside a metal enclosure with large doors in the front. The cuvettes pass a light beam directed via a 45° mirror from a monochromator behind the cuvette drive to a photomultiplier tube located in front of the enclosure (for double-beam operation another photomultiplier tube is positioned behind the enclosure). The electrical signal from the photomultiplier tube which has its own high-voltage power supply is amplified to proper voltage by an amplifier and then sent to the computer. The signal is transformed to optical density equivalents in the computer and quantitation is performed by the computer. Chromatograms and calculated values are then sent from the computer to the electrostatic printer/plotter for print-out. As one of several back-up components a tape recorder is kept on hand for temporary storage of data in case of computer breakdown.

The transportation system

The power for the drive comes from a variable speed 1/8-h.p. motor (Heller, No. 54 RL) with a solid-state variable-speed controller (Heller, No. S-20) transmitted through a chain to a drive wheel (Fig. 2) of a double-chain drive (Fig. 3) which pulls the cuvettes over a number of rollers past the light source. The variable-speed motor is chosen so that three cuvettes can be driven past the light source in as little as 10

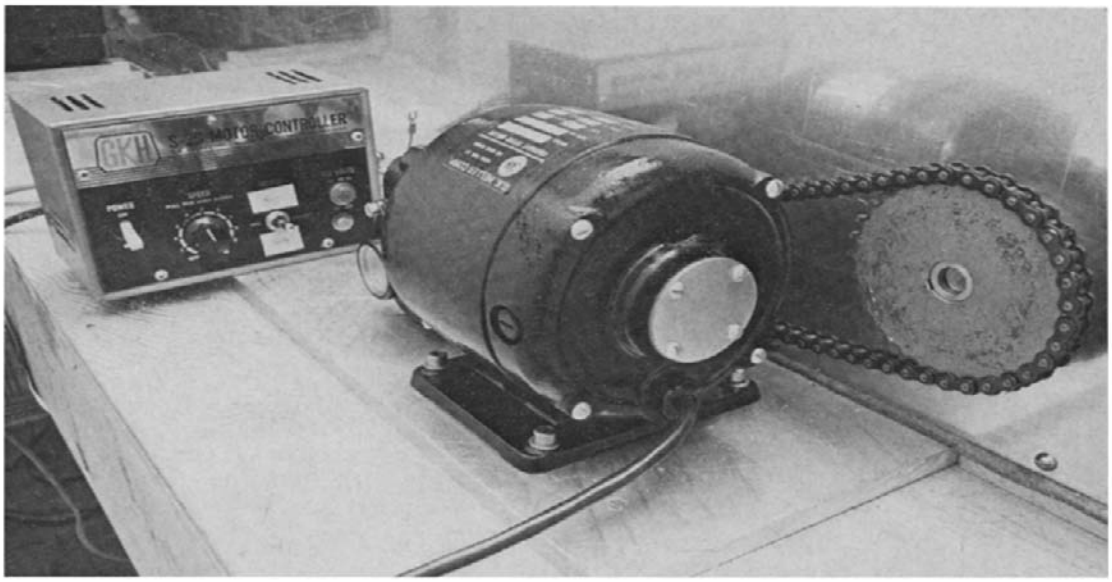

Fig. 2. The variable-speed drive for the cuvette transportation system. Power from a variable-speed motor controlled by a solid-state controller is transferred by a chain to the assembly-line drive inside the cuvette enclosure.

sec or as slowly as over a 10-min period. The motor is electrically reversible so that the cuvettes can be returned to the start position or shuttled back and forth for continuous readings at short intervals. To prevent damage to the cuvette cut-off switches are installed at each end of the cuvette enclosure. They ensure that the current to the drive motor is cut when the cuvette nears the ends of the enclosure.

The optical system

The optical system as viewed from above (Fig. 4) shows the monochromator

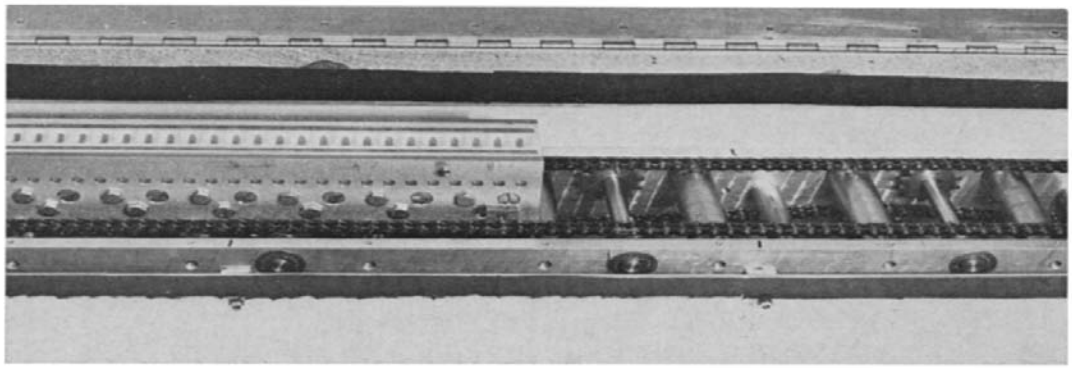

Fig. 3. The double-chain drive that pulls the multi-cuvettes past the light beam in the optical system. The cuvettes glide on rollers.

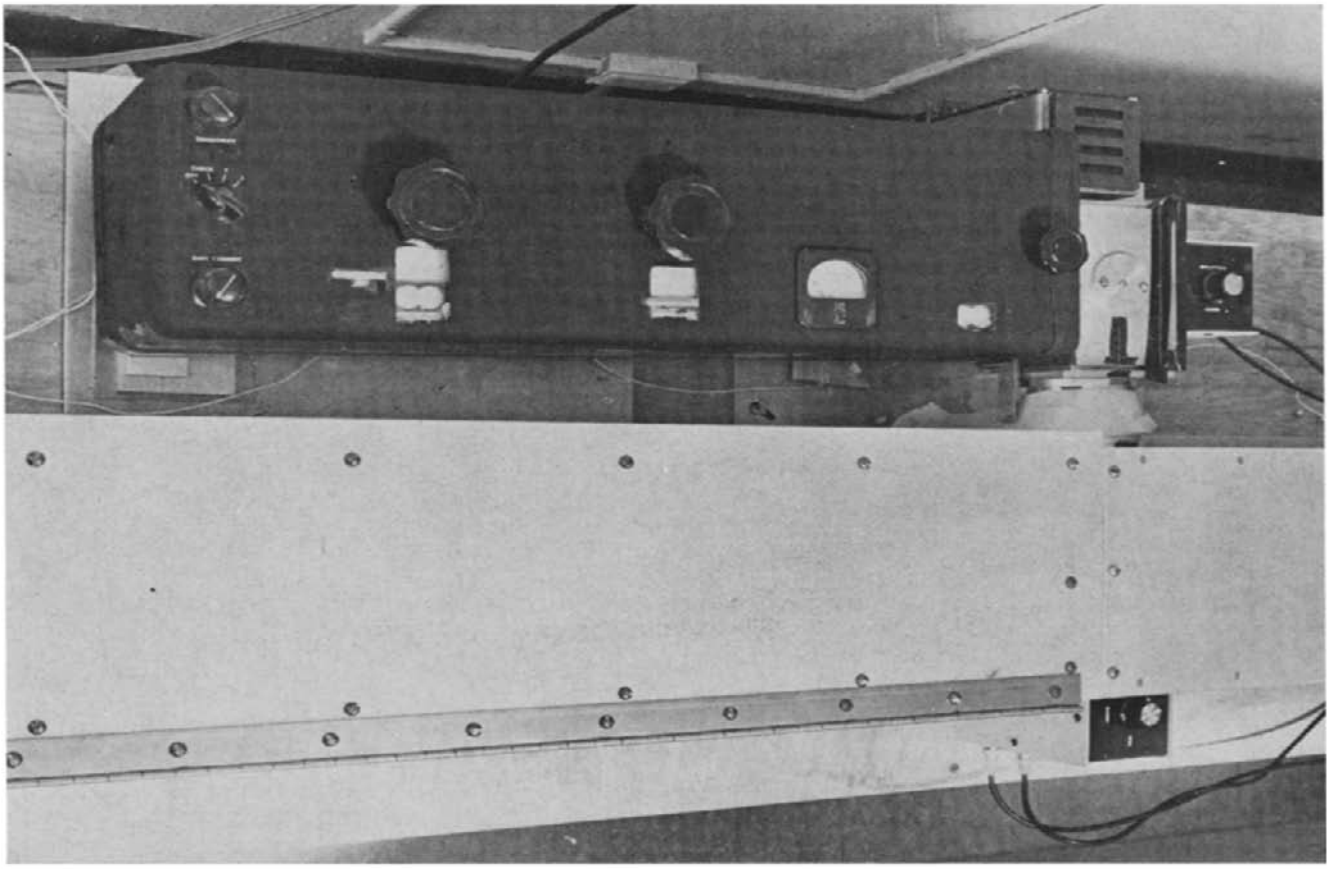

Fig. 4. The optical system viewed from above. Lamp housing, monochromator, mirror housing and the double-beam phototube is at the top of the cuvette enclosure. The single-beam phototube is at the bottom.

with its lamp, mirror housing and double-beam photomultiplier housing (Heath, EU-701-93) at the top of the cuvette enclosure and the single-beam photomultiplier housing at the bottom of the enclosure. A twenty-year old Beckman Model DU monochromator was used in the construction. The mirror housing contains a mirror oriented at a 45° angle to the long axis of the monochromator and that of the multi-cuvettes. In double-beam operation a partially transmitting mirror sending part of the light beam through to the phototube in the back of the enclosure is used. For standard single-beam operation as used in all our routine quantitation a solid mirror sends the full light beam through the multi-cuvette windows to the photomultiplier tube in front of the cuvette enclosure. The photomultiplier tubes used (RCA, No. 1P28A) have their own power supply (Heath, EU-42A).

A sketch of the total optical arrangement is given in Fig. 5. The light source in the system is a standard tungsten lamp as used in the older Beckman Model DU systems. It draws its power from a heavy-duty 6-V automobile battery that has its own battery power supply (Beckman, No. 14500).

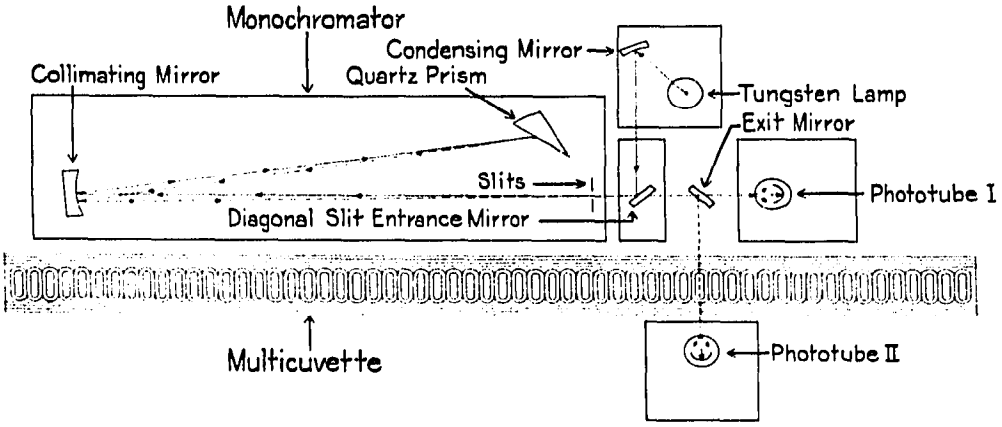

Fig. 5. A schematic representation of the optical system showing the path of the light beam through prism, mirrors and multi-cuvette to phototube.

Electronic components and circuitry

The rapid advances in electronics in recent years have greatly simplified the construction of the electronic circuitry needed to interface the optical system with the minicomputer and with the back-up recording equipment. Off-the-shelf solid-state electronic components have been used throughout to ensure easy serviceability and inexpensive construction.

Photomultiplier–minicomputer interface

The signal from the photomultiplier must be properly amplified before it is compatible with the analog input section of the minicomputer. To this end we have constructed an interfacing amplifier. It consists of an operational amplifier (Teledyne/Philbrick, SQ 10A) with its own power supply (Teledyne/Philbrick, No. 2203) and a high-frequency filter.

Photomultiplier–oscillographic recorder interface

The original signal from the photomultiplier must be logarithmically transformed to give a voltage linearly proportional to optical density and concentration. This is accomplished in the main operating mode by a section of the computer program. It can also be done electronically, however, and a signal proportional to optical density can be obtained this way for recording on a fast oscillographic recorder. We have for this interface used a logarithmic amplifier (Analog Devices, No. 755 P) with a power supply from the same firm (No. 915). An operational amplifier similar to the one used in the computer interface is used as an inverting amplifier in front of the logarithmic amplifier.

Back-up recording options

In two years of routine operation we have not used the back-up recording equipment, but have felt it necessary to have it ready in case of prolonged computer failure. Sooner or later a more than 24-h computer "down time" is likely to occur or a computer breakdown may occur in the middle of a run.

For these contingencies we have on stand-by two inexpensive systems that will make it possible to carry on without a routine load of chromatograms piling up or chromatograms in progress being lost. For the simplest of these systems we use an analog tape recorder. We at first used a multispeed Ampex (No. FR-1300) FM tape recorder as illustrated in Fig. 1. Experiments have shown that an inexpensive casette FM recorder (Vetter, No. C-2) performs adequately and this recorder is now our standard back-up tape recorder. We have also made provisions for simultaneous computer processing and tape recording when a permanent record of the analog data are desired.

A very inexpensive oscillographic recorder (Astro Med, No. 202-100) allows a direct recording of the logarithmically transformed signal that is proportional to optical density. This recorder has low resolution (chart width, 100 mm) but a direct outputting of the chromatograms on such a recorder shows adequately whether the chromatograms are acceptable and ready to be recorded on the tape recorder in case of computer breakdown. Approximate quantitation can also in an emergency be performed using values read off the chart from the oscillographic recording.

DATA PROCESSING

Computer hardware

The computer used was the Digital Equipment Corporation (DEC) PDP-12 already present in our laboratory. The configuration includes 8K of core memory, dual LINC tape transports (TU55), CRT display (VR12) and teletypewriter with paper tape (ASR33). The 10-bit analog to digital converter (AD12) has a resolution of 2 mV. An electrostatic printer/plotter (Versatec 1100A) was added to provide both graphic and printed output with a resolution of 100 points/in. at a rate of 600 lines/min.

While the PDP-12 has a dual instruction set (PDP8 and LINC), our programs are almost entirely in the PDP8 instruction set. Our program could therefore easily be modified to run on a more modest configuration of a PDP8E. The printer/plotter could also be eliminated if one were satisfied with a cruder chromatogram produced on the teletypewriter and would accept the slower output. Current trends in small computers and output devices lead us to expect that a cost of $10,000 for a computer configuration with greater capabilities than ours is a realistic expectation for the near future.

Computer program

The current program is still being improved, although currently adequate for routine chromatography. The program has four sections: data sampling, peak location and identification, quantitation, and output.

Data sampling

The program begins sampling data from a fraction by sensing the change from zero transmission (when the light beam is interrupted by the solid sections between windows) to transmission above a threshold level when the light beam passes through a window section. The data signal is a linear function of the transmittance since it is the amplified photomultiplier current. Absorbance is calculated in the computer.

Each value of the transmittance used by succeeding parts of the program is an average of 256 data samples taken over 1/60 sec. This averaging procedure reduces both random fluctuations and AC pick-up.

Successive values of transmittance are compared, and the largest value is stored when the computer determines that the transmittance has again fallen below threshold. The value used is therefore the minimum absorbance value for that window. This prevents a speck of dust or scratch on a window from resulting in an incorrect larger absorbance.

Peak location and identification

The computer locates peaks in the recorded data by examining five fractions centered at each successive fraction. A measure of convexity is calculated by taking the average absorbance for the center three fractions and subtracting the average absorbance of the outer two fractions. Two successive positive values are required, in order to suppress fluctuations in the data, after which the fraction at which the largest measure of convexity occurs is recorded as a peak center. The upper and lower limits of the peak are determined by scanning values of absorbance away from the peak center until the absorbance no longer decreases or one exceeds a maximum allowed half width of a peak. The size of each of the above regions is controlled by adjustable parameters and can be increased for wider peaks.

Peaks are identified by calculating the difference between the peak center and the location of each standard previously entered into computer memory. A possible identification is accepted if this difference is less than a pre-set limit (currently 6). The value of this difference in position is printed as well as the component name, and values calculated on the basis of that identification. Experience will show if it is possible to reduce the limit to such a value that only one identification will be printed. Occasionally a poorly packed column or technician's error will result in a sample chromatogram being shifted from that of the standards. To compensate for such an eventuality, an internal-standard option has been provided. For the internal standard to be useful it must appear as an isolated peak, or be large enough so that it can be identified by size alone. If the internal-standard option has been chosen, the program compares the previously entered value of its position with the location of each peak found in the sample which is also larger than a predetermined minimum area. The closest such peak is identified as the internal standard and the difference used to determine the offset of the sample chromatogram from the standard chromatogram. This offset is subtracted from the differences of position between the unknown peaks and the standard peaks so as to correct for the shift. These corrected values are printed with the output. Usually the correct identification is printed with location differences of 0 or 1. In those cases where an uncorrectable shift has occurred, one can cross out the misleading identification, leaving the correct results.

Quantitation

The first step in quantitation for individual peaks is a summation of absorbance values over the individual fractions within the peak and a subtraction of baseline absorption. The baseline is calculated as a straight line between points representing an average of the lowest four absorbance values near the beginning of the chromatogram and the average of the four lowest values near the end of the chromatogram. As in

almost all types of chromatography, a subtraction of such baseline values is of considerable importance for the precision and the reproducibility of the assay.

The calculation of absolute values for individual fractions is now done by comparison with total absorbance for the similar peak in the chromatogram of the standards. We have also, in all our routine work, included a direct calculation from standards placed in front of the chromatogram and not passed through the chromatograph to constantly monitor the performance and recovery of the system.

Output

The form of our chromatogram plots has varied with different program versions in that we have used both semi-logarithmic and linear absorbance scales. The semi-logarithmic scale expands the low-absorbance region and is particularly useful when low readings are expected and the highest sensitivity of the chromatographic system is desired since it gives a better visual display of this part of the chromatogram. Our usual display is a chromatogram using a scale linear in absorbance, since it reflects directly the differences in concentration between fractions.

The printed output beside the chromatogram gives the corresponding cuvette fraction number, absorbance for that fraction, and cumulative summed absorbance. The cumulative value is provided for manually determining peak areas by subtracting values at the beginning and end of the peak. Labels, previously entered into computer memory, are printed at the top of each chromatogram. Following the chromatogram the program prints output for each peak it located, giving peak position, area, baseline correction and net total absorbance, followed by each trial identification for that peak and values calculated on the basis of the identification, including both absolute values and amounts per 24 h (volume per sample and volume per 24 h having been entered previously).

OPERATION

The practical performance of the quantitation system is closely connected to the development of a few special devices that tie the system together with the multi-cuvette design[2], the special multi-collector[4] and the newly developed systems for multi-column chromatography[3].

Transfer of samples to the multi-cuvette

In many cases direct chromatography into multi-cuvettes is possible, but for large-capacity multi-column chromatography it becomes too costly to build the large number of multi-cuvettes that would be necessary for direct chromatography. Instead in high-capacity multi-column chromatography the collection is performed in the special forty-compartment PTFE collector modules and the samples are transferred to the multi-cuvette either directly or after reaction with appropriate reagents from the collector modules. These, therefore, can serve either as straight collectors or as compartmented reaction vessels. For transfer from the collector module to the multi-cuvette several approaches have been tried. A special "tipper" has been built. It holds a collector module firmly while the contents are tipped into the multi-cuvette. Experience has shown, however, that an even simpler device works adequately and with proper practice works faster than the more complicated "tipper" device. The transfer

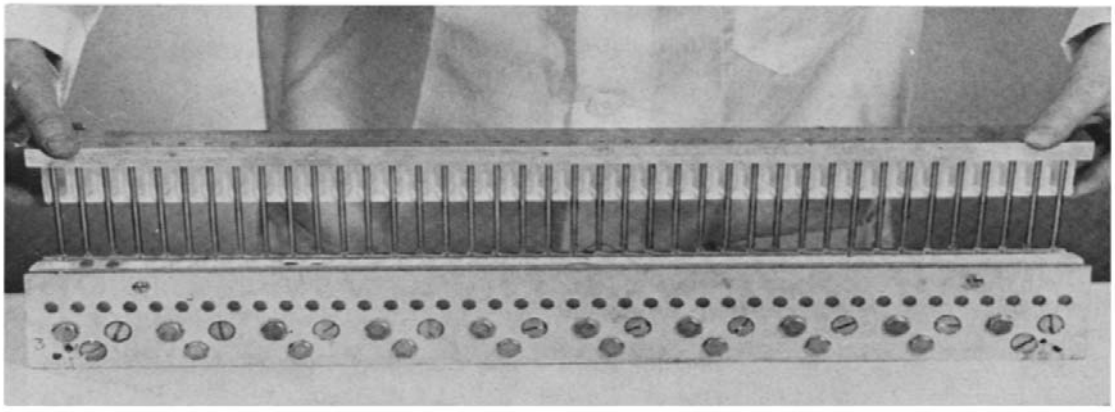

Fig. 6. The transfer device showing the simultaneous transfer of forty fractions from PTFE collector module to multi-cuvette.

in routine work is now performed exclusively with a number of comb-like structures as shown in Fig. 6. This device consists of forty pieces of stainless-steel rod threaded into a stainless-steel bar at intervals equidistant to the width of each section in the multi-cuvette and the collector modules.

When transferring samples from collector to cuvette the tool is first properly positioned with a stainless-steel rod centered over each compartment in the collector and during transfer pointed towards the center of a corresponding multi-cuvette compartment. The collector is now tipped and all fourty samples are transferred in a few seconds to the multi-cuvette.

Evacuation of the multi-cuvettes

Equally important for the practical use of the quantitation system in high-capacity work is the special tool built for the simultaneous fast evacuation of samples from all forty compartments in the multi-cuvette. This device (Fig. 7) consists of a central circular suction component connected through a large Erlenmeyer vacuum flask with sidearm to a vacuum source. Vacuum is distributed through forty outlet ports using flexible PTFE tubing to fourty pieces of stainless-steel tubing arranged in a row at intervals equidistant to the interval between multi-cuvette and collector module compartments. For cleaning the multi-cuvette after use it is convenient to insert a three-way valve in the line connecting the suction device to the vacuum bottle. The third outlet in this valve is connected to a bottle containing either acetone (for water-containing reagents) or ether (for organic solvents). In practical operation one first sucks the multi-cuvette empty by opening the vacuum. Next the valve is switched for a few seconds to the organic solvent that by gravity fills the compartments in the multi-cuvette and the valve is then switched back to vacuum to empty the cleaning solvent from the multi-cuvette. A hot air blower in the fume hood in which these operations are performed will blow the remnants of acetone or ether out of the cleaned cuvette in a very short time. The whole operation with practice takes about half a minute per cuvette. It can obviously be automated if very large scale multi-column chromatography is contemplated.

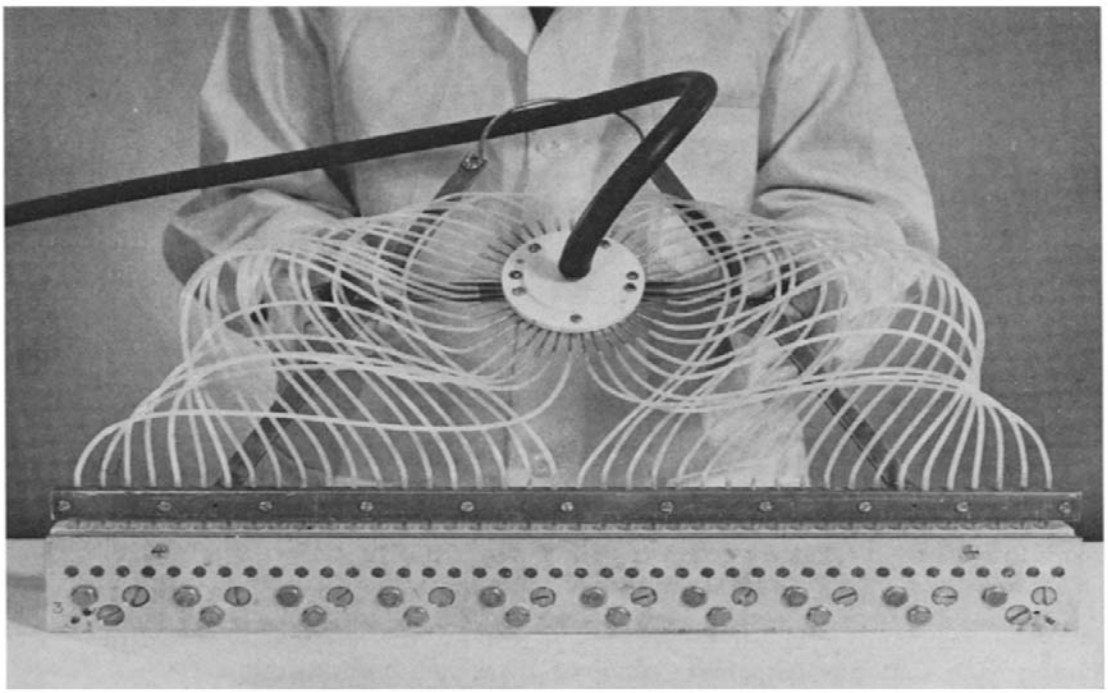

Fig. 7. The suction device that allows simultaneous emptying of the forty compartments of a multi-cuvette or PTFE multi-collector module.

Operational procedures

With the help of the transfer and suction devices practical operation of the quantitation system is performed in the following way. As a first step the appropriate computer program is entered into the minicomputer. Proper factors are also entered if values in the chromatography are to be expressed in concentration per liter or total values per 24 h. In the latter case individual factors for each chromatogram will have to be entered into the computer and later an analog signal is sent to the computer during the operation as each chromatogram is run to identify the chromatogram to the computer.

If not collected directly in the multi-cuvettes, samples are now transferred to the cuvettes using the transfer device. The cuvettes are placed in the cuvette enclosure and a push-button switch simultaneously starts the chain drive moving the cuvettes past the light beam and activates the computer program for data acquisition. The front end of the first multi-cuvette hits the cut-off switches at the end of the cuvette enclosure. This cuts off the current to the motor drive and the multi-cuvettes can be removed for cleaning using the special suction device followed by re-filling of the cuvettes or by reversing electrically the direction of the motor returned to the start position.

The read-out procedure takes approximately one minute per 120-fraction multi-cuvette run in our standard routine arrangement. As data are calculated the

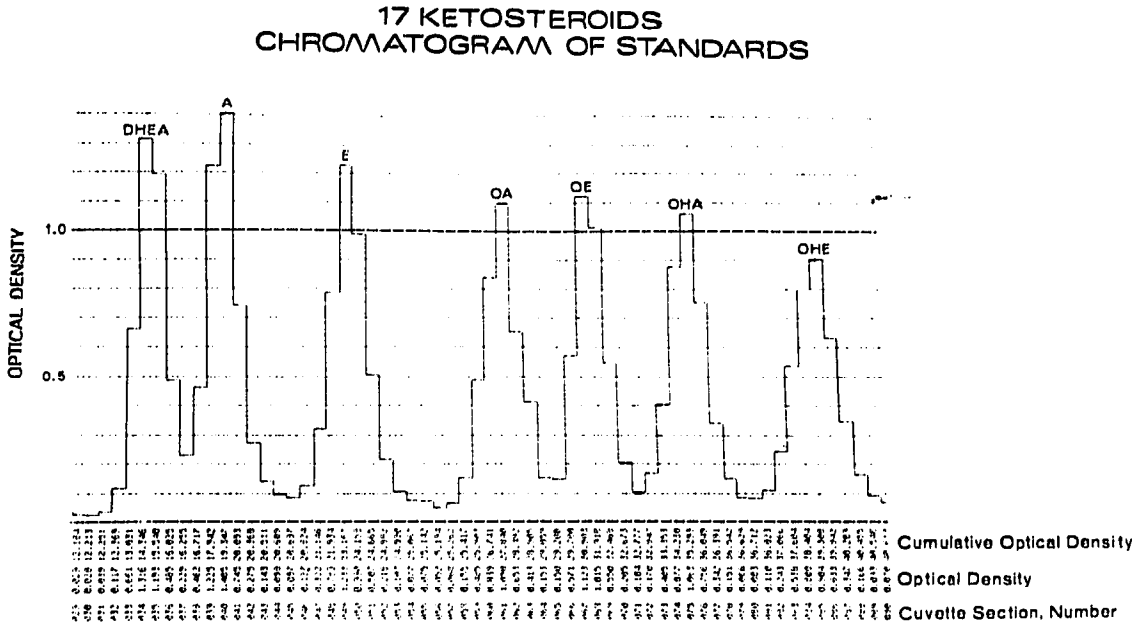

Fig. 8. A chromatogram of standards as it is included in each multi-column run for comparison with the biological samples. In this case the seven 17-ketosteroids as used in our previous systems[1,6] have been run in amounts of 50 μg each. Abbreviations: Dehydroisoandrosterone (DHEA), androsterone (A), etiocholanolone (E), 11-keto-androsterone (OA), 11-keto-etiocholanolone (OE), 11-hydroxy-androsterone (OHA) and 11-hydroxy-etiocholoanolone (OHE). The optical-density output format was used. The bottom lines show cuvette section number, optical-density values for each fraction and the summed optical-density values.

computer sends them to the printer/plotter for graphic display and print-out of calculated data.

PERFORMANCE

The output in the new quantitation system is illustrated in Fig. 8 which shows the seven 17-ketosteroids commonly present in human urine. These steroids have been used to make comparison possible with the presentation in our older system[1].

A recording from a biological sample, a recent run of urinary 17-ketosteroids from a pooled urine similar to such samples run in our older systems[1,6] is shown in Fig. 9. An internal standard, 11-ketoandrosterone (OA), is run in concentrations higher than that of expected steroids in the chromatogram and is used in the computer calculations. The quantitation part of the computer output (Fig. 10) from the chromatogram in Fig. 9 gives information about the location of the chromatographic peaks found by the computer and shows peak number, peak center and beginning and end of each peak in the four columns to the left. The next three columns summarize optical-density calculations for the peaks giving crude optical-density summations, background subtraction and the corrected optical-density values. The next two columns give peak-identification information. The peak nearest to the peak in the chromato-

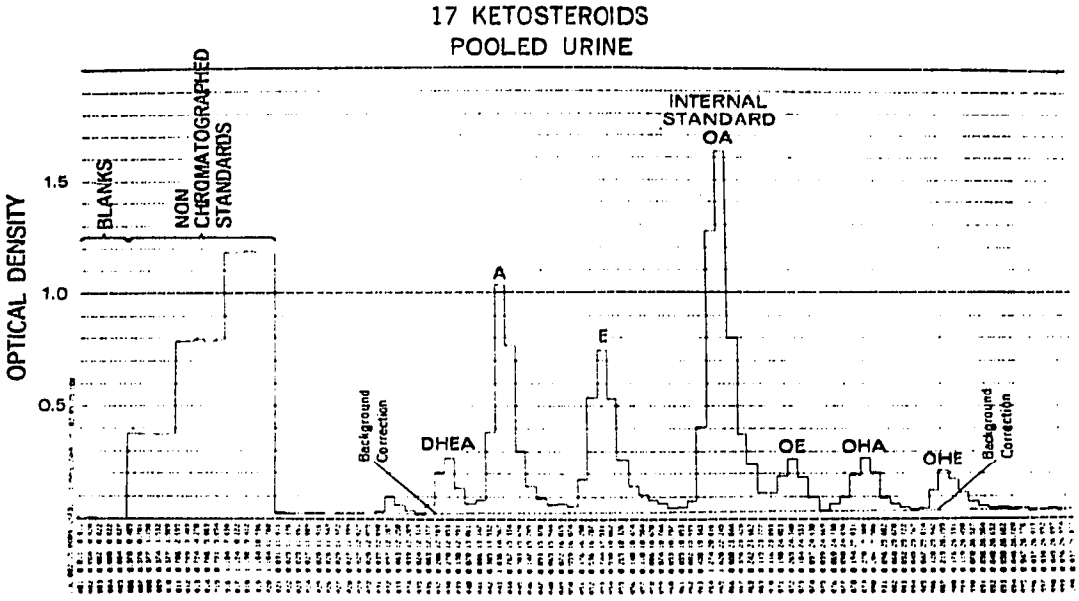

Fig. 9. A chromatogram of the steroids in an extract from human pooled urine. Abbreviations as in Fig. 8. A set of blanks and non-chromatographed standards are run in front of the chromatogram. An internal standard, 11-ketoandrosterone (OA), has been added to the mixture. The line for background corrections runs across the bottom of the chromatogram.

gram of standards run together with the sample chromatogram in a multi-column run is printed out with that label together with any peak within six fractions of this peak. In the sample shown the "true" peaks are in agreement within two fractions with the chromatograms of standards and all peaks are labelled correctly as peaks nearest to the peak in the chromatogram of standards, as is always checked by visual inspection. Computer selection by comparison with the chromatogram of standards using the OA internal standard as a fixpoint is correct in more than 95 % of cases. An occasional chromatogram may be off by one peak position and very occasionally (if unusual chromatograms with unknown peaks, gross impurities or errors in procedures are encountered) hand calculations may be necessary. The summed optical densities available make such calculations easy.

The first two of the last four columns in the calculation give the absolute amounts of steroid present in micrograms relative to the standards run in front of the chromatogram. This has been found to be useful as an internal control of procedures in routine work. The last two columns give the actual final output, *viz.*, the values calculated relative to the set of standards chromatographed with the sample in the multi-column run.

It should be emphasized that our computer programs are at a preliminary stage and we hope as they get developed to improve the procedure for the identification of peaks. It will always be prudent, however, to include a visual inspection check of the chromatogram to preclude computer error if unusual chromatograms occur.

PEAK NUMBER	PEAK CENTER	PEAK BEGINS	PEAK ENDS	SUMMED OPTICAL DENSITY	BACKGROUND CORRECTION	CORRECTED OPTICAL DENSITY	PEAK COMPARISON FACTOR	PEAK CHOICE	PEAK RELATIVE TO STANDARDS	IN MG/LITER	PEAK RELATIVE TO CHROMATOGRAM OF STANDARDS	IN MG/LITER
002	033	029	035	0.288	0.165	0.123						
003	038	035	040	0.724	0.144	0.581						
							001	DHEA	7.412	0.494	9.359	0.624
004	043	040	048	2.885	0.241	2.643						
							004	DHEA	33.731	2.249	42.591	2.839
							002	A	33.731	2.249	34.771	2.318
005	053	050	060	2.726	0.327	2.399						
							001	E	29.695	1.979	31.235	2.082
006	065	060	069	4.948	0.313	4.635						
							000	OA	59.149	3.943	72.375	4.825
007	072	069	075	0.930	0.219	0.711						
							002	OE	9.439	0.629	11.453	0.763
008	079	075	084	1.095	0.344	0.751						
							005	OE	9.966	0.664	12.093	0.806
							001	OHA	10.062	0.671	12.093	0.806
009	087	084	092	0.863	0.321	0.542						
							001	OHE	6.922	0.461	8.090	0.539

Fig. 10. The quantitation part of the computer output from the chromatogram in Fig. 9. The four columns to the left show data related to peak position. Then follow three columns summarizing optical-density calculations for the peaks. After that come the peak-selection data and the last column gives the final quantitation data in mg/l relative to the (chromatogram of) standards run simultaneous with the sample in the multi-column run.

Reproducibility

The most important performance testing of a system primarily intended for high-capacity multi-column chromatography is an actual test of practical performance when applied to multi-column chromatography. And one of the most crucial tests for the practical usefulness of a chromatographic system is its reproducibility. To make a direct comparison possible with our older system we have in this newer system (as previously in the older system[1]) run in parallel a series of twelve chromatograms of the same seven 17-ketosteroids as shown in Fig. 8. Approximately 50 μg of each steroid was used and standard deviations given as coefficients of variations (or standard deviations in per cent of average amount of steroid determined) were calculated as done in our previous work[1]. Nitrogen pressure was used in both systems to move the gradient through the capillary columns.

Table I compares the results from the new and the older system. As can be seen from this table the new system in practical multi-column runs using mixtures of pure steroids performs at the same level of reproducibility as did our previous system with a coefficient of variation of about 2%. This variability reflects the combined variability of the chromatography which still has a number of manual steps and the quantitation procedures which probably in both systems contribute less than the manual steps to the variability observed. Improvement of the reproducibility will have to await further improvements in the multi-column chromatographic procedures with full automatization of these procedures as the ultimate goal.

TABLE I

COMPARISON OF FLOW-CELL SYSTEM WITH MULTI-CUVETTE SYSTEM

Averages with coefficients of variations for two 12-column runs of batches of seven 17-ketosteroids. Nitrogen systems. Flow-cell values from previous publication[1]. Approximately 50-μg amounts were used. Values refer to a DHEA standard and are uncorrected for differences in chromogenicity. Coefficient of variation is the standard deviation in per cent of the mean. For abbreviations of 17-ketosteroids, see legend of Fig. 8.

Steroid	*Multi-cuvette system*		*Flow-cell system*	
	Average (μg)	*Coefficient of variation (%)*	*Average (μg)*	*Coefficient of variation (%)*
DHEA	49.5	2.6	48.5	1.6
A	50.4	2.1	50.2	2.4
E	49.8	1.7	47.7	2.1
OA	46.4	2.0	44.9	2.5
OE	47.8	2.2	46.2	2.6
OHA	47.9	2.3	47.2	2.6
OHE	43.4	2.0	43.4	2.3
Average		2.1		2.3

Capacity

The potential capacity of this new system for quantitation of liquid chromatograms is very great. We are currently running 120-fraction chromatograms at a speed of approximately one chromatogram per minute in our routine work but preliminary results indicate that chromatograms can be recorded at double or treble speed, so potentially about a thousand chromatograms can be read in an 8-h working day.

A great deal of additional construction of automated transfer and evacuation equipment, fast automatic pipetting devices, a re-design of the cuvette transportation system and re-programming of the computer output will be needed, however, before this potential is realized. At this time about one hundred chromatograms per day is realistically the capacity of the system considering the manual operations necessary.

Sensitivity

For any given compound this will be determined by the molecular absorptivity of the compound under investigation and the cuvette dimensions. The current configuration has a path-length of 25.4 mm and a minimum volume of about 1 ml is needed to fill the cell for proper reading. Most of our routine work has been with 17-ketosteroids and for these compounds we have found that the minimum amount that can be distinguished from the background absorption is approximately 0.2 μg using the Zimmermann reaction in pyridine[5]. This sensitivity can possibly be improved somewhat. It seems probable that narrower and shallower cuvettes can be made to work perhaps at some sacrifice of speed. At best an improvement by a factor of about 3 can be hoped for by modifications in cuvette design. For higher sensitivity work we hope that we will be able to develop a fluorometric accessory to the current system.

Because of the 60% longer light path in this system compared with our older system and the fact that 1 ml sample per fraction is sufficient in this system whereas 2 ml were needed in the older system, this new system for routine operation has about

three times the sensitivity of the old system. In biological work this generally gives a great saving in reagents and solvents, since less of both will be needed in the assays.

Practicality

One of the real advantages this system has compared with our older system[1] is that it is much simpler in mechanical construction and therefore will need less maintenance. Although we have kept the older system going for seven years a fair amount of maintenance and repair has been necessary because of the relative complexity of the system with its many mechanical parts. The advantage on this point has been obvious over two years of simultaneous operation of the two systems while we were phasing the old system out. Maintenance and repair time has been about 1:8 in favor of the new system. Even considering the age of the older system there can be no doubt of the considerable superiority of the new system on this point.

On-line mini-computer system

We have in our previous quantitation systems for multi-column chromatography[1,6] used either off-line processing of punched tape or an on-line system where data processing was done on a large IBM computer. We were entirely dependent on time being available on the big computer if we wanted to run on-line in this system and it proved of little value for our daily routine work since we could rarely get time assigned when it was most convenient for us to run the chromatograms. This was largely due to the necessity of dedicating the large computer to our work when we wanted to go on-line. Other institutions may have large computers that will allow simultaneous time sharing of a number of programs in which case an on-line large computer program may be available at all times and in that case the large computer may be the best choice. In our situation, however, unquestionably the dedicated mini-computer available at all times when needed has proven superior. A necessity for high-capacity on-line work is the electrostatic printer/plotter with its high output of graphic and printed data. The superiority of the true on-line system over the off-line data processing we previously had to rely on most of the time has been obvious. The complete chromatogram has been available for inspection while a re-run has still been possible so that for example dilution of fractions with very high optical-density readings has been possible, thereby saving re-runs of chromatograms.

DISCUSSION

Most systems for automated chromatography today are continuous-flow systems. Such systems offer advantages relative to our multi-cuvette system in some areas. They are for example, generally speaking, easier to automate completely from sample introduction to final quantitation and in low-capacity work results from a few analyses can usually be obtained somewhat faster in a continuous-flow system because of the time lag involved in transfer of collected fractions to multi-cuvettes for quantitation.

The main advantage of the proposed multi-cuvette system compared with continuous-flow systems is its potential for high capacity. Continuous-flow systems are inherently slow systems because the speed of such systems is set by their slowest component, *viz.*, the chromatographic system. In even the fastest high-pressure liquid

chromatographs today approx. 10 min are needed for the running of a complex chromatogram to which must be added the time it takes to re-condition the column for the next run. This gives a maximum capacity of about fifty chromatograms in an 8-h day. In contrast our basic strategy in the multi-cuvette system is not to let the speed of the entire system be set by the slowest phase *viz.* the chromatography. By separating quantitation and chromatography we can achieve higher overall capacities since a number of multi-column collectors can feed the same fast quantitation system and much higher capacities can be reached this way with a reasonable investment in instrumentation.

If, as we believe we will, we succeed in adapting our multi-column systems to high-pressure techniques, two 25-column systems, run in tandem twice per hour with 10 min for the chromatography and the rest of the time for re-conditioning and start-up, would allow us to feed one hundred chromatograms per hour to the quantitation system, something it can unquestionably be built to handle with the further development of the multi-cuvette system. The multi-cuvette system at this point in its development is limited to a colorimetric endpoint and a comparison with continuous-flow systems is currently only possible in this area.

Another advantage of the multi-cuvette *versus* the continuous-flow system in spectrophotometric analyses is the greater versatility and flexibility of the colorimetric endpoint reactions that can be used in the system.

When for example organic solvents are used for elution in the chromatography, evaporation of the solvents before addition of reagents allows the establishment of optimal conditions for the color reaction, whereas in a continuous-flow analysis the adaptation of a color reaction to continuous-flow conditions not necessarily yields optimal conditions and sometimes currently available color reactions may be difficult or impossible to adapt to a continuous-flow analysis.

A further point in favor of the multi-cuvette system is the considerable flexibility of the system. Different chromatographies can be performed simultaneously in the same multi-collector run, different reactions can then be performed for each chromatogram and quantitation can be done fast, sequentially, in the quantitation system by switching from wavelength to wavelength on the monochromator. A further comparison with continuous-flow systems must await the development of our ultra-violet systems and our systems for quantitation by fluorimetry and from thin-layer plates. We believe that this new quantitation system for high-capacity spectrophotometric quantitation of liquid chromatograms represents a significant step forward compared with our previous system[1]. The new system is much simpler in construction, which means lower cost of acquisition and less maintenance and repair which again means lower operating costs. The potential capacity of the system is higher by a factor of about three compared with the older system and at a much lower cost in components and construction. The optical system using a monochromator with a photomultiplier tube as the detector has several advantages over the older colorimeter with the flow-cell system. The use of a monochromator–photomultiplier with narrow slit width is a superior combination in optical performance giving strict agreement with Beer–Lambert's law. The monochromator has also the advantage that costly filters are not needed and that any wavelength within the visible area can be selected. We have used an inexpensive grating monochromator (Schoeffel, No. GM100) in a recently built back-up system instead of the Beckman quartz-prism monochromator. This

monochromator together with the photomultiplier cell and its power supply costs less than one of the colorimeters of which we used three in the older system. It performs satisfactorily in routine use. The dedicated minicomputer approach to data processing has in our laboratory proven clearly superior to a time-sharing system using a large computer. A truly on-line system with results available as the chromatogram is being quantitated has been an important advantage in daily routine work.

Overall then we feel that we have developed a greatly improved system for high-capacity quantitation by spectrophotometry of liquid chromatograms.

As a first step towards very high capacity systems the transportation system would have to be re-designed by the addition of magazine towers as used in our older system[1] with lightlocks and located on each side of the light beam so that a continuous flow of cuvettes past the lightbeam can be maintained. The techniques for multi-column chromatography must be further simplified so that the necessary number of chromatograms can be produced in a day. Small-particle, high-pressure liquid chromatography that will yield complete chromatograms in minutes rather than hours is the logical solution. We are currently working on such systems adapted for multi-column chromatography.

Another important development that would greatly increase the potential of the combined multi-column and high-capacity quantitation systems is the construction of relatively inexpensive multi-cuvettes using PTFE film windows instead of glass. Initial experiments with such cuvettes look promising. They can only be used in combination with a computer for high-capacity work since the optical qualities of the plastic films are not nearly as good as glass. But we have found that —at least in our preliminary experimental work— such cuvettes can be used if "blank" runs are performed on each cuvette and the data for each cuvette stored in the computer for subtraction when the cuvette is used in chromatography later. If this idea proves viable we believe that indeed in a few years we will be able to realize a capacity of a thousand liquid chromatograms a day for this type of system.

It will probably first be reached by direct chromatography in plastic film cuvettes of colored derivatives that can be read directly in the quantitation system. We are also working on an accessory system for quantitation by spectrofluorimetry. This system is in its infancy and it is too early to tell whether a practical system can be developed or not.

One advantage of the plastic film cuvette, if we succeed in getting this design from the experimental to the routine stage, is that it will make most of the ultraviolet area accessible for quantitation, which will further enhance the usefulness of the system.

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