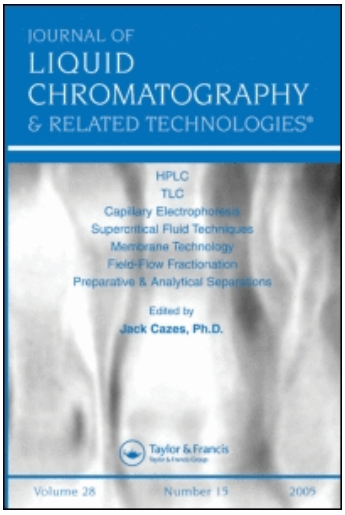

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Computer Assisted Data Acquisition and Analysis for Size Exclusion Chromatography

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COMPUTER ASSISTED DATA ACQUISITION AND ANALYSIS FOR SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

Two programs, ACEACQ and ACEANN, provide data acquisition and analysis for Size Exclusion Chromatography (SEC). ACEACQ is used for data logging, real-time smoothing and data reduction by boxcar averaging. ACEACQ requires that traditional SEC hardware be coupled with a Hewlett Packard data logger and microcomputer. A Mettler electronic balance is connected to the computer in a novel fashion to continuously monitor the mass of solvent eluted during an experiment. ACEANN provides analysis for the data collected by ACEACQ. Calculations include molecular weight averages, molecular hydrodynamic diameter averages and column separation efficiency parameters. Significant reductions in analysis time have been realized along with improvements in accuracy of data acquisition, analysis and experimental reproducibility.

INTRODUCTION

The field of size exclusion chromatography (SEC) is experiencing considerable growth, both in the breadth of its practical applications and in the basic research

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directed toward a more complete understanding and utilization. SEC is a useful tool for determination of molecular size and size distribution of polymers. Due to the ease with which data are obtained, it is unsurpassed with respect to size distribution characterization.

SEC has found varied application in qualitative work and separation of components in mixtures. However, its quantitative usefulness has been limited by several factors, poorly understood separation theory, measurement inaccuracy and labor intensive reduction and analysis of raw data.

Recent advances in equipment design, SEC separation theory and reduced costs of electronic equipment have generated considerable interest in computer interfacing with SEC for both data acquisition and data analysis.

The objective of the work reported in this paper was to construct an inexpensive system which interfaced an SEC apparatus with a computer for data logging and analysis. This system is called the SEC Automated Chromatography Evaluation system, or SECACE. The SECACE system was intended to:

a) increase reliability over conventional SEC;

b) reduce analysis time;

c) measure and record solvent flow by using an analytical balance;

d) increase accuracy of molecular size and size distribution calculation by methods including: realtime boxcar averaging, realtime spike rejection and linear baseline subtraction.

e) use readily available computer hardware and traditional SEC pumps, columns and detector converter (scanner).

f) use BASIC as the programming language.

BACKGROUND

The ultimate computer interface systems are the so-called "turnkey" systems. Turnkey systems allow for closed loop sampling, injection, data acquisition and analysis. They are usable by an operator with little or no theoretical knowledge. Ideally, they require no human interaction, except to activate the system. In usual practice, however, accuracy demands human participation. The usefulness of the data acquisition and analysis portions of the turnkey systems is clear. The autosampling and injection portions become important when many samples must be injected frequently.

Most laboratory instrument manufacturers offer data logging and analysis systems (1 - 5). Data loggers for interface with popular BASIC microcomputers have recently been introduced by both Nelson and Isaac. The Nelson system combines a Nelson data logger with an HP-85 microcomputer (6). The Isaac instrument uses an Isaac data logger and the Apple II computer (7).

Also, the literature contains individual efforts to construct an inexpensive, specific-use data acquisition and analysis system (8 - 12). The "hardwired" nature of such systems sacrifices universality. Low cost in hardware can be realized, however the electronic construction of these

systems require a significant development effort which is usually expensive and time consuming.

All of the above systems improve SEC experimentation but tend to be inadequate in one or more of the following areas: 1) inaccuracy of raw data collection and correction, 2) use of a simple program language, 3) cost, 4) system flexibity.

Determining the actual progress of flow in SEC has not recieved the consideration warranted. The methods in wide use are of questionable reliability and accuracy. The most widely used method considers that the SEC flow rate is constant, so chart length or time is used as the measure of flow progress. However, experience has shown that significant variations in the flow rates of SEC pumps occur. It has also been shown that both short and long term flow-rate drift can account for considerable uncertainty in molecular weight calculations. (13)

The other major method used to measure flow progress employs a syphon which fills to a constant, pre-calibrated volume of solvent and then spills the solvent past a photocell which records an event mark on a strip chart. Both temperature fluctuations and use of aqueous solvents, which do not wet the glass vessel, can result in measurement error in the flow progress. In addition the eluate will occasionally stop in front of the photocell, masking several syphon drainings.

A novel and useful approach to improve measurement of flow progress has been described by Van Kreveld and Van Den

Hoed (14). They drained the eluate onto a balance and occasionally manually marked the strip-chart with the solvent mass. This bypasses inconsistancies in flow rate and temperature. Evaporation was assumed constant, and a correction factor was multiplied into the flow progress.

Use of a modern electronic balance could provide flow progress information easily and more accurately, but no such attempts have been reported in the literature.

SYSTEM CONSTRUCTION

System construction was roughly divided into two work areas: hardware configuration and interface; and software construction.

Hardware

The SECACE system was a mating of hardware used in traditional SEC with data logging and computing equipment. Figure 1 is a diagram of the system configuration and Table 1 is a description of the hardware components.
The computer hardware has delivered exceptional service and the overall dependability exceeds that of the SEC hardware. The programming language is a subset of BASIC and is certainly one of the most complete available. The error recovery ability of the computer saved time in de-bugging and in fact made some routines possible which would otherwise be possible only by using a machine language.

As expected sizable down-times have been experienced with the SEC hardware. The principle reasons were

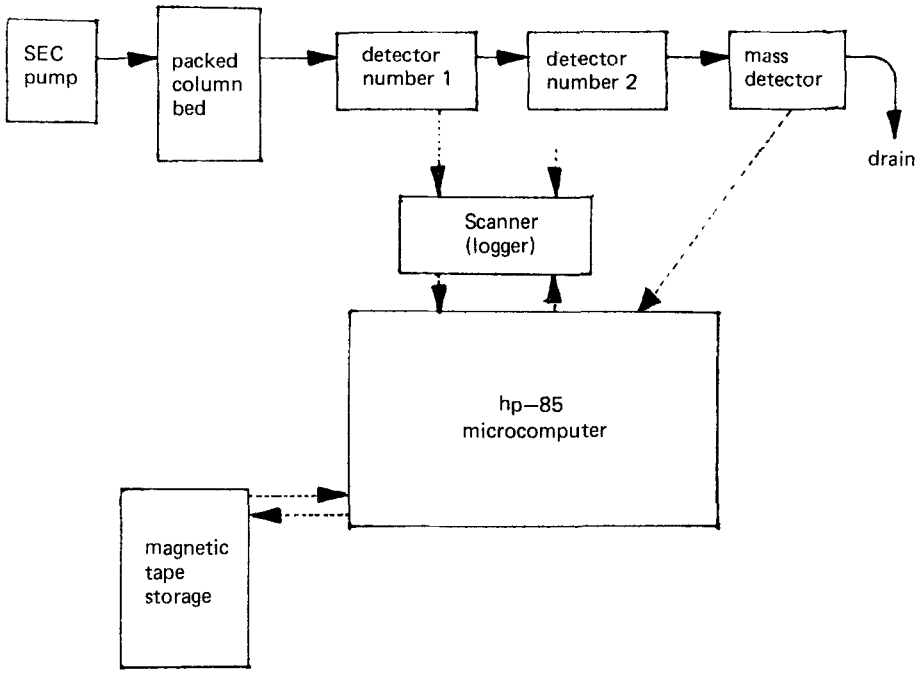

Figure 1: The SECACE system; solid line represents the flow of liquid and dotted line represents the transfer of digital or analog information

TABLE 1

SECACE Hardware:

1) HP-85 computer containing (15):

   Matrix ROM--to perform certain specialized calculations rapidly on data in arrays
   Input/Output ROM--allows the computer to communicate with other devices
   Advanced programming ROM--allows subprogram operation
   Printer Plotter ROM--provides additional programming commands for advanced plotting
   16K Memory Module--doubles the computer's memory
   IEEE-488 (HP-IB) bus--to interface the scanner with the computer
   RS-232 (HP-82939A Option 001) bus--to interface the balance with the computer
2) HP-3497A data logger (scanner)--to read the detector signals and convert them to digits for the computer analysis (16); it contains a twenty channel multiplexer
3) SEC pump, injector and columns (17)
4) Detectors (refractive index, ultraviolet etc.) (18)
5) Mettler PC-400-03 electronic balance (19)

recalibration or adjustment of detectors and the need for pump maintenance.

Interfacing the analog output detectors was quite easy using the instructions given in the data logger manuals. The high (+), low (-) and ground (guard) of each detector were connected by shielded wire to the appropriate channel terminals in the analog-to-digital (A/D) converter card in the scanner.

The interface of the electronic balance required connecting the RS-232 serial bus to the balance. Some interfacing ploblems resulted from the inability of the balance to completely "handshake" with the HP85 microcomputer. However these problems are adequately handled with software as described below.

SECACE Software

The operations of the SECACE system is divided into data reduction with logging and data analysis. The logging and reduction are done "real-time" (that is, while the SEC is being run). All other analysis is done "off-line" (that is, after the chromatogram data is collected and stored on magnetic tape). There is a program package for each job, ACEACQ is for logging and reduction and ACEANN performs the analysis duties.

ACEACQ Software

ACEACQ is intended to read the SEC data, reduce the quantity of data, remove random noise spikes and store the results along with information which describes the SEC

experimental operating conditions. Figure 2 shows the logical flow of ACEACQ.

The intent in writing ACEACQ was to use as much universal software as possible, thereby making the SECACE system applicable to many situations. Hewlett Packard Level III data acquisition software was selected for this purpose. Level III is a collection of subroutines designed to be controlled by a user-designed "main-frame" program. Each subroutine performs a specific task in data acquisition. In addition to being developed specifically for the hardware, it was initially expected that the programmer would not need to become proficient in input-output (I/O) operations, since the Level III software was expected to perform the I/O tasks. However, due to the complicated nature of the package, it became apparent that I/O operations must be understood to efficiently use Level III.

By definition, a generalized system lacks specificity. In computer systems this translates into slowness and a certain amount of bulkiness. Specifically, Level III software is intended for a multi detector system where all inputs go directly to the scanner. So long as the scan interval is relatively long and a small quantity of data is held in the computer at any one time, the Level III package works well. However, Level III is not intended to be used in conjunction with "real-time" data manipulations. Our system uses the scanner plus the serial interface (for the balance), performs a real time calculation (data

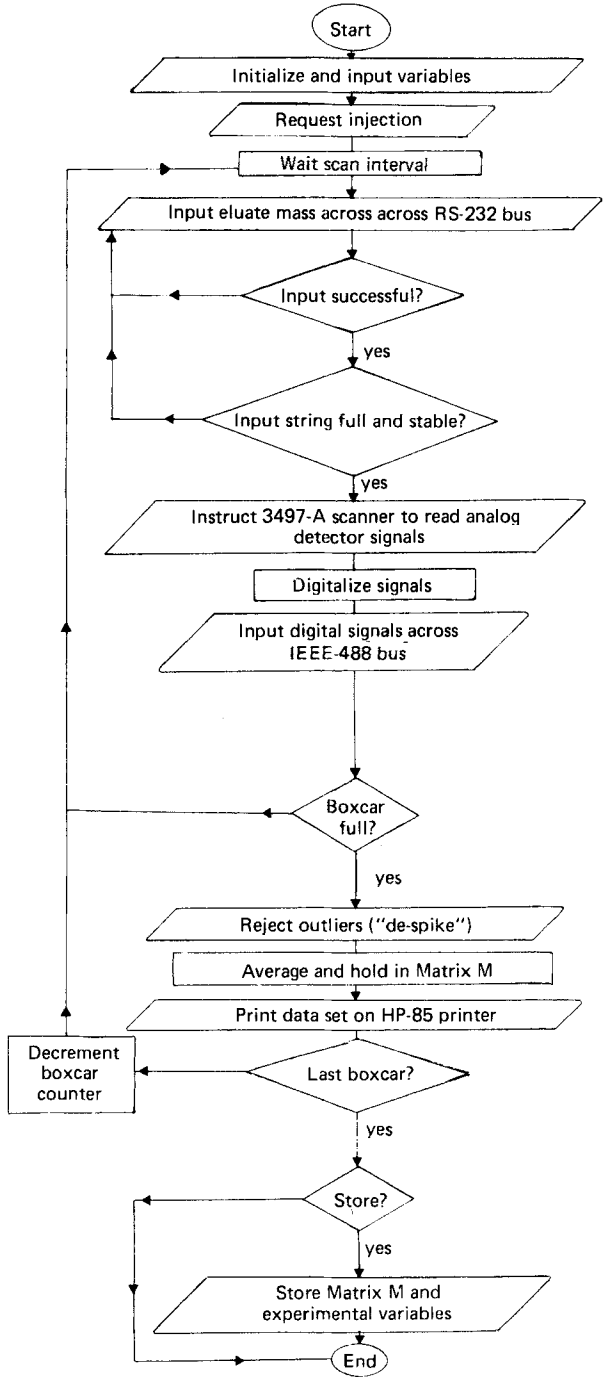

Figure 2: ACEACQ Flowchart

reduction), uses only two channels, and scans about every two seconds. Therefore, ACEACQ did not need to have extremely fast program execution. Also because it was written in BASIC, it is easy to understand and modify.

Mass Inputs From the Balance

The only real problem in software construction resulted from inclusion of the balance on the RS-232 interface. The balance was not capable of signaling the computer when it was ready with data or understanding when the computer wanted a data reading. This data transfer protocol is called "handshaking" and is a standard to most RS-232 devises, but not this balance. Therefore, inputting the mass from the balance, which was supposed to be a simple I/O operation, required two loops to access the signal. This portion of ACEACQ is shown in Figure 3. The first loop was constructed so that when an input error occured, the computer simply accepted a new string. This process continued as many times as required to get an intelligable string. Usually only two tries were required to obtain a string. Since there is no two-way communication between computer and balance, the input string may contain too few characters in the string. This would not cause an input error, but the string would be useless to ACEACQ. Also, the balance sends out a special character to indicate instability. Instability can occur when the balance is first turned on or after it is accidentally bumped. The second loop determined if the reading was stable and that all characters are present. If necessary, a

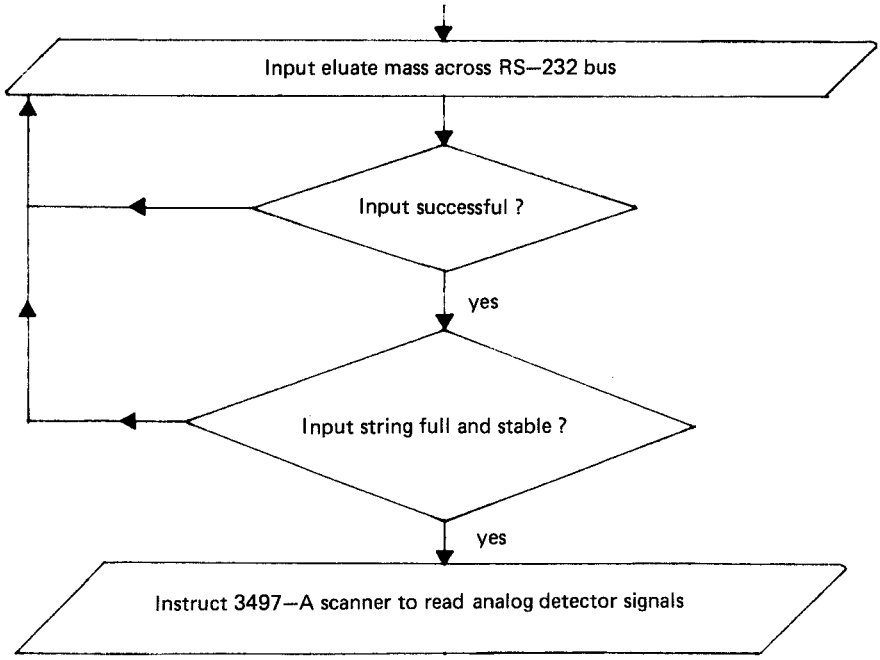

Figure 3: Mass Inputs From The Balance

new input is taken until the balance is stable and all string characters are present. This process usually requires another two scans, and up to nine scans were occasionally needed. On the average, between half a second and one second are required to get a good balance input. After a balance input the mass was extracted from the string and stored.

Data Manipulation in ACEACQ

Only two real-time operations are performed on the data by ACEACQ. Only Boxcar averaging for data reduction

and spike removal are performed on line, and all other calculations are performed off-line.

ACEACQ differs from most other chromatographic data logging systems because it stores a large quantity of data for analysis. Traditionally, twenty data points per SEC chromatogram peak are considered adequate. However, analysis of more data reduces errors caused by random noise. Therefore, ACEACQ is intended to provide analysis for one to three data points per minute for each two to three hour experiment. Up to 480 data points per detector may thus be captured. At low SEC flow rates, such data sampling rates should capture even the most minute changes in detector signals.

To be sure that these points are as free from random noise and spikes as possible, the technique of boxcar averaging was employed. The operator selects the boxcar size, which is the number of scans that will be averaged to form a single reduced data point. Since SEC flow rates are small, about 0.5 ml per minute, the mass and detector changes occuring between scans is very small then the corresponding analog signal changes are usually less than a micro-volt. Therefore each data point in the boxcar should have approximately the same value, unless electronic noise has altered some data points.

Before actually averaging the data in the boxcar, the data from each detector and the balance are checked for noise spikes. This is accomplished by calculating the average and standard deviation of the data in the boxcar.

Spikes are defined as data points which deviate from the mean by the product of the standard deviation and the despike parameter, S2. Statistically, if S2 is set equal to 1.96, then ninety five percent of the data will be accepted (20). Thus, data points in the boxcar which are extremely different from the mean are rejected. Thereafter, the mean of the acceptable boxcar data is calculated and saved as a final, "reduced" data point.

The final real time action taken is to print on the HP-85 thermal printer the reduced data points from each set of scans in a strip chart fashion. The scale of the paper strip is set by an operator input. The scale is automatically increased if the data exceeds the input scale. Thus off scale strip chart chromatogram traces are not experienced, due to the auto-scaling capabilities of the software.

Ending ACEACQ

While scanning and logging are proceeding, all HP85 computer and scanner keys except one, k6, are inoperable. After the data has been collected or the data collection has been manually terminated by pressing K6, the operator may elect to store the collected data. If desired by the operator, the chromatography data and the parameters which describe the SEC conditions associated with the SEC experiment are stored on magnetic tape.

ACEANN Software

ACEANN is truly a software "package." It consists of a mainframe program and ten subprograms. A subprogram is a

small program which is called from a tape or disk into computer memory by a mainframe program for a short time, then purged from computer memory. This allows large programs to be run on small computers. ACEANN, for example is about 50,000 bytes, and the HP-85 will only hold about 30,000 at any given time. Figure 4 is the flow chart of ACEANN. The mainframe is menu driven, giving the operator eight analysis options. Table 2 is a list of the menu options. The names in parentheses are the subprograms used by each menu option. Discussions of each menu option follow.

Subprogram SETUP

This subprogram prompts the operator to define the elution limits where chromatograms were obtained. Then baseline drift is subtracted from the data. Thereafter, excess baseline data before the first peak and after the last peak is purged. The above procedures eliminate unnecessary data points and therefore future calculations will be less time consuming.

Subprogram SC

This subprogram is used to read chromatographic data from tape and to construct the global variables and initialize the data for analysis. The global variables are those available to both the mainframe program and any of the subprograms. In contrast, local variables are used only within a single subprogram.

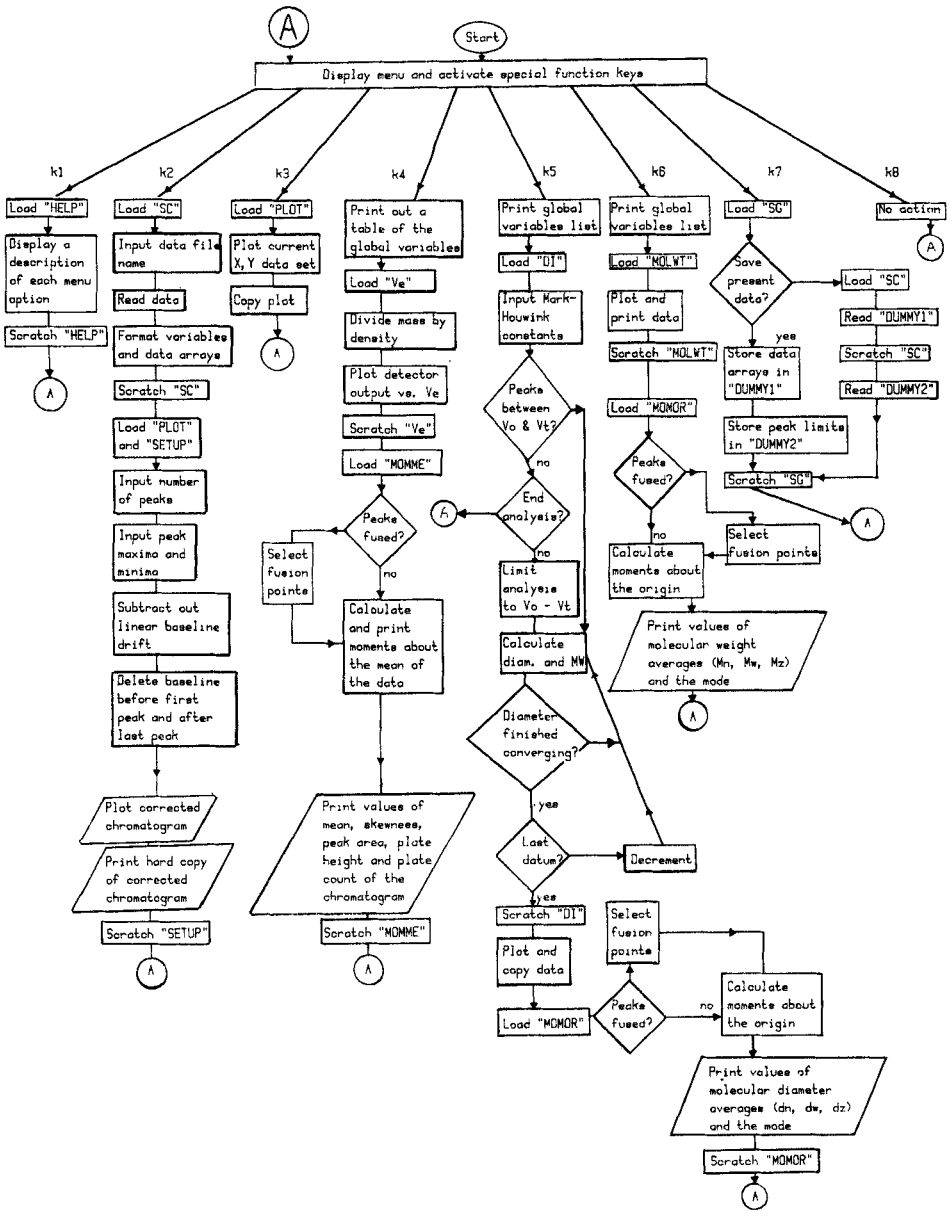

Figure 4: ACEANN Flowchart

Table 2: The Menu Options

k1, "HELP":	Describes the softkey functions. (HELP)
k2, "SETUP":	Sets the global variables and defines the peak limits. (SC, SETUP and PLOT)
k3, "PLOT":	Plots the current data. (PLOT)
k4, "Ve":	Plots Ve vs detector signal and calculated area, skewness, variance, plate height and peak elution volume for each peak. (Ve, MOMME and PLOT)
k5, "DIAM":	Plots diameter vs number density and calculates molecular diameter averages. (DIAM, PLOT and MOMOR)
k6, "MOLWT":	Plots molecular weight vs number density and calculates molecular weight averages. (MOLWT, PLOT and MOMOR)
k7, "SAVGET":	Stores or recalls scaled down data. (SG)
k8, "MODIFY":	Future expansion.

After setting up the global variables, the experimental data is scaled down from the maximum-dimensioned size. The maximum-dimensioned size was calculated in ACEACQ from SEC operator inputs. A maximum of 480 reduced data points from each detector is allowed. If the experiment actually required fewer data points, then SC reduces the array dimensions so that standard Matrix ROM operations can be used on the data. The process of scaling uses the fact that the mass data constantly increases. The row number of the largest mass is the data point to which all three arrays (mass and two detectors) are scaled. This "scaled-down" data and the global variables are returned to ACEANN for further analysis.

Subprogram PLOT

This plotting routine is much like others in the HP-85 manuals (15) or HP Level III software (16). However,

PLOT is an improved version because it will plot data of any order of magnitude with scientific notation axis labels. Thus, plots of large magnitude numbers can be constructed on the small HP-85 CRT screen.

Subprogram Ve

Subprogram Ve simply divides all mass (abscissa) values by the solvent density and places the results in working array X. Thus after this operation elution volume is in array X. Array Y is filled with the data for the detector selected by the operator. This data setup is passed to MOMME for moments calculation.

Subprogram MOMME

MOMME calculates moments about the mean, peak area, plate height and elution volume at peak maximum for each peak in the X-Y set from subprogram Ve. The algorythm employs the conventional mathematical techniques (21). For each peak, the operator is asked if the peak is fused and if so, instructed to move the cursor to the point where the peaks meet. A perpendicular is dropped from the cursor position to separate the peaks.

Subprogram DI

DI sets up the data in a molecular diameter versus relative number density format. Array X is used for elution diameter and array Y holds relative number density. Since the algorythm uses an itarative sort of each data point, array Z is filled with molecular weight to save sorting time later. The operator is prompted to input Mark-Houwink K and a values for calculation of the molecular wieght.

DI uses the Southern Calibration Method (22) to calculate molecular diameter from elution mass data. This requires prior calculation for each of N packing materials. The void volume, Vo; total permeation volume, Vt; the mean pore diameter, D; and the exponent n for each packing material must be input. Molecular hydrodynamic diameter is calculated for each point.

Subprogram MOLWT

MOLWT will move the molecular weight data in array Z to the abscissa array, X. Then the relative number density is placed in the ordinate array Y. Since the molecular weights are actually calculated by DI, DI must be executed before MOLWT.

Subprogram MOMOR

MOMOR calculates the moments about the origin and uses those moments to calculate weight average, number average, Z-average and dispersities of the X-Y data from DIAM or MOLWT. Dispersity Q1 is defined as the ratio of number average to weight average and dispersity Q2 is defined as the ratio of Z average to number average (29).

Subprogram SG

SG serves the dual functions of storing whatever data is in memory or retrieving files created by a previous execution of SG.

The "save" option will store the chromatographic data in temporary storage files. The peak data with the global

variables are stored in the same format as the chromatogram file created by ACEACQ.The file created by SG differs from the one created by ACEACQ in that the SG file contains the data after SETUP, which has been corrected and scaled down. Thus, if SETUP is completed and time does not permit a complete analysis, the data can be saved and analyzed later.

Subprogram HELP

HELP is several pages of text which explain the menu options. It is arranged in a menu identical to the main menu for clarity and continuity. No other operations are performed.

SYSTEM PERFORMANCE

ACEACQ

The acquisition software has been in continuous use and has successfully logged over 200 SEC experiments. During this time only minor software bugs were encountered which were easily corrected.

It is important to determine what part of the ACEACQ scanning took the most time. This information is neccessary to establish the optimum data collection conditions. Since an exact method to measure the elapsed time of a routine was unavailable, approximations were used. The routines which read the balance, scan the detectors, de-spike, boxcar average and the routine which simulates a strip-chart recorder on the printer were timed using the

HP-85 clock. Since this method also includes the time to read and assess time, the results are not completely accurate elapsed times, but should have significance in their relative magnitudes.

The results were unexpected. We assumed that the amount of time required to perform the program steps used by the Level III software to read the detectors and the time required for the computer to obtain a good reading from the balance would be similarly slow and that all other routines would be rather fast. However, the balance and the strip chart routines are fast. The despike routine is slower, followed by the scanner routine. The relative order for the four is roughly 1:1:3:10. Thus the scanner routine is the major bottleneck to collecting SEC data at a faster rate.

The possibility that solvent evaporation loss on the balance was significant was considered. At room conditions, approximately 0.1 grams of Tetrahydrofurane evaporated per hour. This translates into a loss of 0.00003 grams per second, well below our ability to detect. Further, such a small loss is probably on the order of the electronic stability of the balance. Therefore, no evaporation loss corrections were neccessary.

The ability of ACEACQ to automatically scale itself to any of several input voltage ranges from -100 volts to +100 volts has been a great advantage during data collection. This advantage frees the operator from scaling consideration, and insures that an experiment will never be

discarded due to and off-scale strip-chart trace. So long as the sample does not cause a full scale reading from the detector, the experiment can be collected and analyzed accurately. A full scale reading from the detector shows up as a peak with a flat top. In comparison when using only a strip-chart to record SEC data, approximately 20% of the experiments had to be repeated due to an off-scale chromatogram trace.Thus computer data collection has significantly reduced SEC analysis failures while simultaniously improving the precision of the data collected.

ACEANN

In order to insure that the system was accurately performing its assigned calculations, test data of three types were analyzed: two artificial data sets and one literature report of SEC data (21). In all cases, ACEANN results have agreed with the expected values.

Several rather subjective observations should be made here. It is intuitivly obvious that, as in manual SEC data analysis, defining the peaks incorrectly will introduce error into the analysis. The magnitude of this error is not so obvious. Since data are collected at equal increments with respect to time and nearly equal with respect to mass, these are the only two equal increment scales. Once SEC data have been converted to molecular diameter or weight, the data on the plot is no longer separated by equal increments. At low molecular weights, each time-increment represents a small molecular size change and a large

relative number density change. At high molecular weights, each time-increment represents a large molecular size change and a small relative number density change. Thus, incorrectly defining the end of the chromatogram at the large molecular size end (small Ve) results in skewed calculations of the second and third moments about the mean. Thus, error is introduced into the weight average and Z-average molecular weights.

A result of under-defining the chromatogram limits is error in area and plate height calculation. By reducing the variance of the data, plate height calculations will show small plate height (greater number of plates.) The chromatogram area also would be artificially reduced. Overdefining the chromatogram limits would also produce error by increasing area and plate height.

The reduction in analysis time resulting from the SECACE system is very significant. Manual collection and calculation of data from a strip-chart recorder usually consumes over thirty minutes if the trace is noisy or baseline drift is severe. Perhaps as many as three hours would be required to manually calculate the moments on a chromatogram containing only twenty data points. ACEANN does this job in a matter of minutes. The only time consuming operations in ACEANN for the operator are in the selection of the chromatogram limits and performing the menu selections. Still, a full ACEANN analysis may be performed in about fifteen minutes.

CONCLUSIONS

The SECACE system represents a technological advancement in SEC practice. Perhaps the greatest tribute to the applicability of the SECACE system is the fact that it consistently works. Portions of it have been in use for over a year and the full package for six months.

Using an electronic balance to measure the flow progress as a function of mass of eluate has resulted in increased SEC analysis reproducibility. The use of readily available SEC and Hewlett Packard computing hardware makes the system easy to assemble and run. Configuration of software in BASIC from original and commercially available programs makes future software modification possible. Such modifications are easy for ACEANN, but if I/O modifications are desired in ACEACQ, more programming skills are required. The data logger has already been centralized in our lab and interfaced with three independent SEC units and will soon be interfaced with a fourth.

The configured system offers improved hardware reliability and increased data accuracy. Data logging procedes without operator interaction after injection. Thus, operation overnight is comon practice. No maintenance problems have been experienced after a full year of operation. The dependability of the system in collecting data is much better than conventional or manual methods.

Conventional or manual data collection is good for only about one to one and one-half digits accuracy at best.

The SECACE is capable of five and one-half digits accuracy but is limited by the accuracy of the incoming data which is usually three to four digits. Since the balance is specified for three and one-half digits and since boxcar averaging is performed on a number of points, the overall accuracy of logging is probably about four digits. Some of this accuracy is lost in the analysis, due to inaccuracy in SEC calibration, peak definition and baseline drift. The SECACE is probably accurate to about two and one-half to three digits.The accuracy and dependability of the SECACE is greater than that of traditional collection and manual analysis of SEC data. Not only does it more accurately collect and analyze data, it also significantly reduces the amount of time and effort required to perform SEC experiments and analyze the results.

The logging of such a large quantity of data makes possible calculations that would otherwise be too time consuming. Such calculations might include flow rate over the whole run, and calculation of how area relates to quantity of sample injected in order to determine how much sample was retained by the column set.

For the future, a wide range of analysis upgrades are possible. Currently, routines for inclusion into ACEANN for band-broadening correction are being tested (23). This will result in higher accuracy of peak analysis. The accuracy with which peaks are defined could be enhanced with some routines to assist the operator. A routine to select chromatogram limits based on multi-parameter considerations

would reduce operator interaction and thus improve the speed with which peaks are analyzed. Future considerations would also include modifications to compensate for non-linear drift, change in detector signal slope and amount of material injected compared to peak area. A routine to separate fused peaks by a tangent skim as well as a perpendicular division, based on an automatic selection of the fusion point would further enhance the package. All of these routines would need to clearly indicate what parameters influenced the calculations. Operator over-ride capability would be essential and reanalysis based on operator over-ride could improve analysis accuracy.

With all the fine things that the SECACE and other computer interface systems can do, they will never take the place of human beings. The complex judgment of the chemist cannot be completely quantified and therefore cannot be programmed. However, the computer can be used to spare chemist from the drudgery of routine SEC calculations.

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AVAILABILITY

An Operator's Manual is available upon request from the authors. Included are specific information and details as well as all printouts

which are part of public domain. Taped copies of the program are also available.

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