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A STUDY OF PRECISION IN MODERN LIQUID CHROMATOGRAPHY USING A DEDICATED COMPUTER

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SUMMARY

The on-line coupling of a small digital computer to a liquid chromatograph is described. A new real-time language, MIRACL II, is used for programming. The experimental set-up is presented and a new automatic sample loop valve is described. The system has been studied in terms of the precision in peak area, peak height and retention attainable with commercial liquid chromatographic equipment. Precision in peak area and height is found to be significantly improved with the sampling valve relative to syringe injection. The influence of several pumping systems on precision was also studied and marked differences in the relative standard deviations of peak area and height were found. The relationship of the relative standard deviation of the peak area to sample size has also been studied. In addition, the important role of the flow-rate of the mobile phase on detection limits and precision is shown. Finally, some results are presented on the reproducibility of retention over short and long time periods.

INTRODUCTION

Significant advances have been made over the past few years in the development of column liquid chromatography as a separation tool. Apart from a few studies[1–4], not much work has been carried out on the precision and accuracy of retention and quantitation. Many workers believe that, in general, retention can be controlled to $\pm 0.5\%$ (relative standard deviation) and peak area to ± 0.5 to 1% if an internal standard is used. However, these results are based on the conditions of the experiment. Little attempt has been made to understand and control those factors which can influence precision and accuracy in liquid chromatography. The overall purpose of the work is to provide such an understanding.

It is well known from studies in gas chromatography[5–10] that in terms of precision and accuracy, computer evaluation of data should be used. Relative to manual

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or electronic methods, a computer offers convenience, flexibility and improved accuracy and precision. Therefore, we have coupled a small computer (PDP11/20, 8K memory) on-line to a home-built apparatus. In addition, we have used a new real-time computer language, MIRACL II, for simplified programming[11].

To date, the application of an on-line computer in liquid chromatography has been limited. Most efforts have been made in data reduction, especially with complex chromatograms. Computers have found use in amino acid analysis[12,13] and in the analysis of body fluids[14–18].

A number of studies on the accuracy and reproducibility of area and retention measurements have been made in gas chromatography (*e.g.*, refs. 19–24). Recently, Chesler and Cram[25,26] and Goedert and Guiochon[27–30] have explored in detail errors that may occur from the chromatograph and computer. Many of the conclusions from these studies are directly applicable to liquid chromatography.

As this is our first paper on the study of precision and accuracy in liquid chromatography, some attention is devoted to the salient features of the online computer liquid chromatographic system. The interfacing of the computer with the apparatus and the program developed to store and analyze the chromatographic data are described. The computer–chromatographic system is illustrated by examining the precision under typical liquid chromatographic conditions, using several injection methods and pumping systems. No attempt has been made to study accuracy.

EXPERIMENTAL

Chromatographic system

The high-pressure liquid chromatographic apparatus is shown in Fig. 1. The pump (P) was connected to a reservoir (R) consisting of a 2-liter flask thermostatted to 25.0 ± 0.1° using a Haake constant-temperature circulator bath (Polyscience Corp., Evanston, Ill., Model NBS). The two pumps used were a three-head, sinusoidal drive reciprocating pump (Orlita, G.F.R., Model M3S4/4) and a two-head special drive reciprocating pump with feedback for correction of fluid compressibility (Waters Associates, Framingham, Mass., Model M/6000). The Orlita pump was used with a

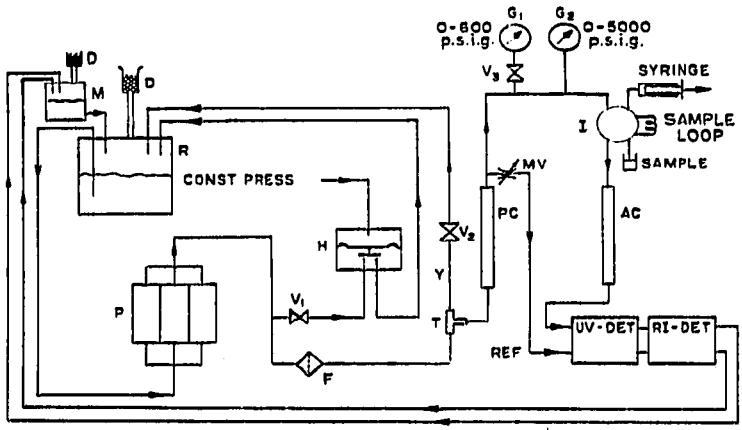


Fig. 1. Diagram of the general liquid chromatographic apparatus. Symbols are defined in the text.

pressure controller (H). As described previously[31], the liquid pressure was adjusted by applying a back pressure to the controller from a nitrogen tank with a high-pressure regulator (Harris Co., Cleveland, Ohio, Model 87-2500A). Using this configuration, it was possible to maintain a constant pressure to better than ± 1% (ref. 31). The controller also served as a pulsation dampener and safety valve.

A micro-metering valve (MV) (Whitey Research Tool Co., Emeryville, Calif., Model 21RS4) was used to by-pass the solvent stream into the reference cell of a UV detector (Laboratory Data Control, Riviera Beach, Fla., Model 1205, UV monitor-254) and a differential refractometer (Waters Associates, Model 401). The flow-rate of the reference stream was maintained at half the flow-rate of the sample stream. The refractometer was placed in series after the UV detector and was thermostatted using the Haake constant-temperature bath. Both the analytical and reference flows of the detector system were fed back to a pre-saturator (M) which helped to maintain the mobile phase saturated with stationary phase. This consisted of a heated (32°) flask with a side-tube which drained into the reservoir, filled with 5 ml of 3,3′-oxydipropionitrile (ODPN) (Eastman Organic Co.) and 50 ml of heptane. The signal outputs of both detectors were transmitted to the computer. In addition, a Hewlett-Packard strip-chart recorder (Model 7127A) was used to monitor the output of the UV detector.

A 5-μm PTFE filter (Millipore Corp., Bedford, Mass., LSWGO4700) in a high-pressure filter holder (F) (Millipore Corp., XX45 04700) was placed in the high-pressure supply line. For the Waters pump, it often proves useful to place a second filter ($<2\,\mu$m) before the pump in order to prevent damage to the check valves. To eliminate gas bubbles from entering the pre-column (PC) and analytical column (AC), a trap was installed, consisting of a two-way valve (V_2) attached to a 4-ft. length of glass tubing extended vertically from the supply line. During operation, bubbles could be trapped in this extension and removed by opening the valve. Both the analytical column and the pre-column were thermostatted with a water-jacket using the Haake constant-temperature circulating bath.

The pre-column was constructed from a 40 cm length of 4.6 mm I.D. stainless-steel tubing, containing 35% (w/w) of ODPN coated on 54–74 μm Porasil D (Waters Associates). The solid support used in the analytical column was Corasil I, 28–37 μm (Waters Associates), which was packed into a 49 cm stainless-steel column of 2.1 mm I.D. in the conventional manner. The Corasil was coated *in situ* by passing through the column 600–800 ml of the eluent, heptane (purified as described previously[32]) saturated with ODPN. This procedure gives a reproducible 1.1% (w/w) coating of ODPN[33].

Solutions were prepared from a 250-ml stock solution containing 0.50 ml of acetophenone, 1.00 ml of dimethyl phthalate, 4.40 ml of benzyl alcohol and 3.00 ml of *p*-cresol (previously distilled at 201°) in heptane. Subsequent solutions (Nos. 2–9) were made by successive two-fold dilutions, *e.g.*, 50, 25, 12.5 ml, etc., of the stock solution and 3 ml of hexane (used as the unretained component) with heptane to 100 ml. Injections of 5 μl were made with a syringe and of 20 μl with a sample loop injection valve.

Syringe injections were made using a commercial injector (Waters Associates, Model 904) with a 5-μl high-pressure syringe (Hamilton Co., Whittier, Calif., Model HP305SN) or a 10-μl Pressure-Lok syringe (Precision Sampling Co., Baton Rouge,

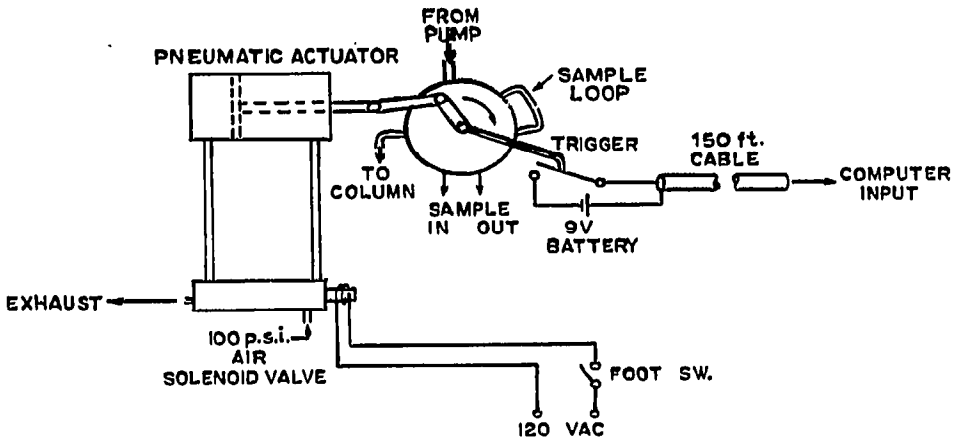


Fig. 2. Diagram to illustrate the automatic injection system.

La., Model 11022). Valve injections were made with the apparatus shown in Fig. 2. With the foot switch opened (Cramer Electronics, Newton, Mass., Model Linemaster 491D), a four-way solenoid valve (Automatic Switch Co., Florham, N.J., Model 8345C1) was de-energized, causing the pneumatic actuator (Chromatronix Inc., Berkeley, Calif.) to rotate the shaft of a high-pressure sampling valve (Chromatronix Inc., Model HPSV).

Computer equipment and interfacing

A PDP11/20 computer (Digital Equipment Corp., Maynard, Mass.) with 8K of 16-bit core memory was used in this work. The system included an ASR-33 teletype with control, a PC11 high-speed paper tape reader–punch, a KW11-L line frequency real-time clock and an AD01-D 10 bit analog-to-digital conversion subsystem (ADC) with two A124 four-channel modules providing an eight-channel multiplex capability (all of this equipment was obtained from Digital Equipment Corp.).

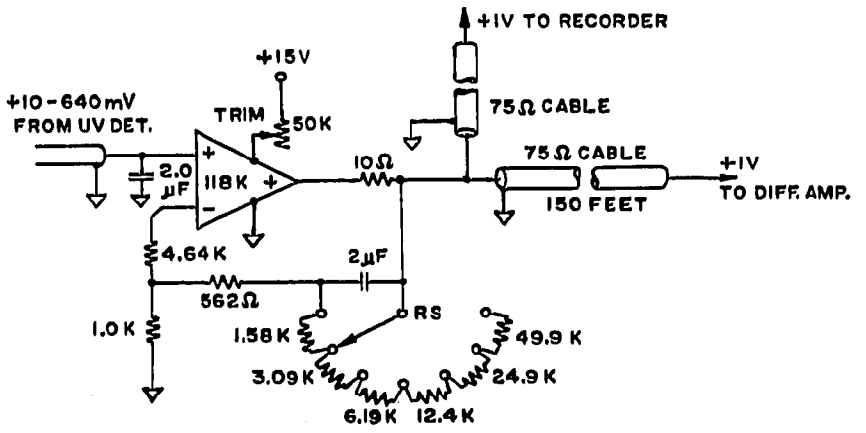


Fig. 3. Schematic of the amplifying circuit from the UV detector to the differential amplifier.

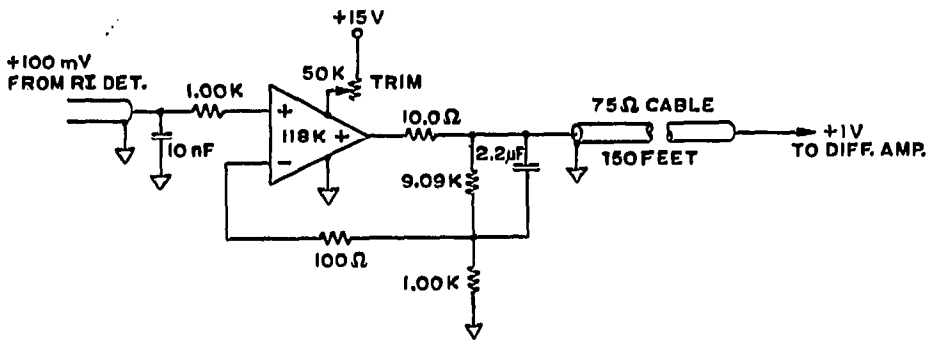


Fig. 4. Schematic of the amplifying circuit from the refractive index detector to the differential amplifier.

The circuit diagram of the UV amplifier-line driver is presented in Fig. 3. The ADC was located 150 ft. from the detector. To minimize noise, the UV detector signal was amplified 100-fold (from 0–10 mV to 0–1 V) prior to transmission in the line cable. Moreover, a special attenuating circuit was used because the one within the detector produced a different output impedance with each attenuation setting. The operational amplifier was obtained from Analog Devices Inc. (Norwood, Mass.), Model 118K.

Fig. 4 shows the refractive index detector amplifier-line driver. As the signal from this detector was 0–100 mV, only a 10-fold amplification factor was necessary. Moreover, as this detector has a low impedance operational amplifier output, the line driver did not require a variable gain.

The signals from both detectors were fed into differential amplifiers (Analog Devices, Model 118A), as shown in Fig. 5, in order to remove common mode pickup and to isolate the widely separated grounds. The differential amplifiers also performed an amplification of a factor of nine to bring the signal into the ADC in the correct 0–10 V range.

As high-frequency noise was known to be a problem (significant noise existed at the output of each detector), appropriate measures were taken to reduce its level. Capacitors were inserted into the feedback loop of the operational amplifiers (Figs. 3 and 4) in order to reduce their high-frequency gain. In addition, substantial filtering (2 μF) was added to the differential amplifiers (Fig. 5) to reduce high-frequency noise

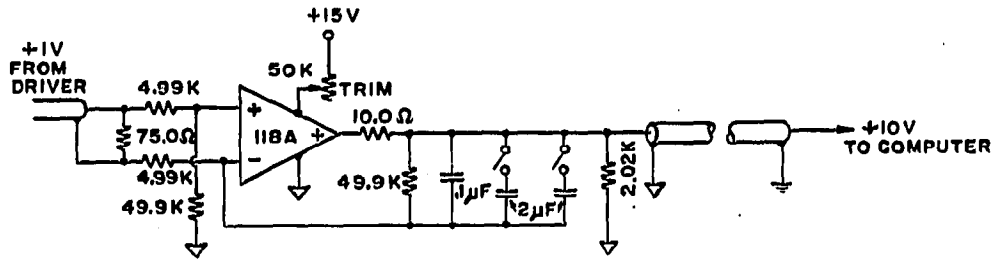


Fig. 5. Schematic of the RC filter and differential amplifier.

that might be electronically introduced into the circuits and also to dampen detector noise. While additional components would be required to reduce common-mode pickup (primarily 60 Hz), its magnitude proved small (see below). In most cases, the capacitors were switched into the circuit introducing a lag of 0.2 sec, but lags of this magnitude do not significantly distort the peak shapes found in this study[20]. Observed oscillographically, 60 Hz noise at the ADC input was about 1.0 mV (peak to peak) for both detectors. As the least significant bit was 10 mV for the 10-bit ADC, this noise level was deemed insignificant. Moreover, sampling was accomplished at sub-multiples of 60 Hz[34], rendering the effect of 60 Hz pickup small even for substantial amplitudes.

COMPUTER PROGRAM

The program, HSL/86, was written to acquire data from both the UV detector and refractometer, store the data in core memory and process the data when the chromatographic run is over. This program provides for on-line data acquisition and is flexible enough to be used for a variety of purposes in spite of the fact that the computer contains only 8K of core memory.

The choice of on-line *versus* off-line configuration to evaluate chromatograms depends on the type of analysis, the cost and availability of equipment, and the mode of desired chromatographic control —computer or manual. For on-stream analysis in processing plants, on-line computer systems are needed in order to give immediate results concerning the status of a particular process. Similarly, automated chromatographic systems must have updated results in order to interact with the computer. On the other hand, for routine analysis (*e.g.*, quality control), an on-line computer may not be necessary because immediate response is probably not required. As the hardware on which HSL/86 was run has inadequate peripheral data storage for the data rates and amounts encountered, we chose an on-line system for both data acquisition and processing.

The program has been written in MIRACL II (Macro Implemented Real-time Analytical Chemistry Language)[11], a language specifically designed for the computer control of analytical instrumentation. Implementation has been achieved using a macro-processor[35]. Descriptions of how the source language statements are to be translated into assembly language are written as macros that are input to the macro-processor. Each macro effectively acts as a subroutine during the translation process and can even call other macros, thus greatly simplifying translator programming. Moreover, programming for real-time operation becomes a relatively straightforward procedure, without the idiosyncracies of languages such as BASIC, FORTRAN or FOCAL when the source language has been designed for real-time.

A flow-chart of the overall program is shown in Fig. 6. With MIRACL II, timing using the 60 Hz clock in the computer was implemented as an integral part of the syntax. This was accomplished with subroutines called "AT TIME" blocks, which were executed when the time of the internal clock was equal to the value of the time variable found in the leading statement of a block. HSL/86 consisted of three AT TIME blocks, as indicated in Fig. 6. The first block, which was executed after initialization, instructs the computer to examine the trigger (input 2) every 66 msec in order to determine whether or not an injection has occurred (an injection was

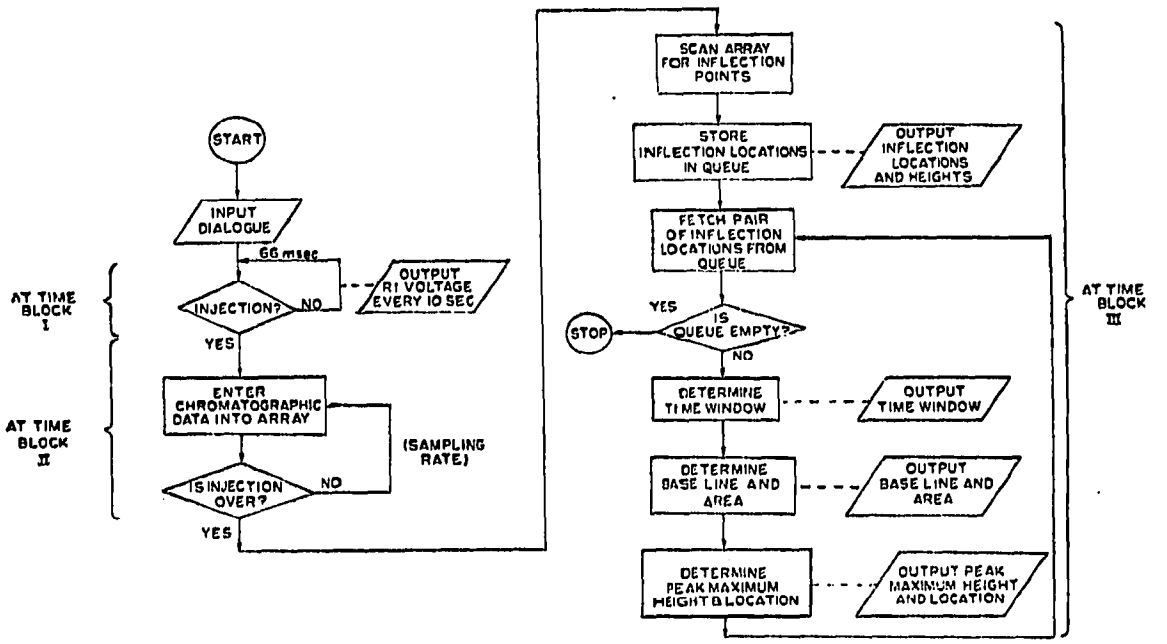


Fig. 6. Generalized flow chart of the HSL/86 program.

sensed if the voltage at input 2 exceeds 4 V). During this AT TIME block, the voltage of the refractometer (input 0) was also printed on the teletype every 10 sec in order that appropriate baseline adjustments could be made.

Once an injection had occurred, the second AT TIME block was activated. During its execution, digitized data taken at specified sampling rates were stored in an array. To help reduce noise and improve precision somewhat, four data points about 1 msec apart were summed and placed into the array as a single value. The array stored single word integers with a size of 1500 words for HSL/86 with 8K of memory.

It is frequently desirable to examine the signals from both the UV and refractive index detectors simultaneously in the analysis of real mixtures. With the limited storage capacity of our system (1500 locations) and the non-availability of a magnetic tape for storage, we were unable to achieve this combined operation. However, we were interested in using the refractometer in determining the retention of an unretained component (*e.g.*, hexane with an eluent of heptane) and in using the generally higher detectabilities of the UV detector for retained solutes. As a result, the computer was programmed to sense the output of the refractometer for a specified time and scan rate to determine the retention of hexane (a small time correction was applied to account for the time delay in traveling from the UV detector to the refractometer). After this time period, the computer then monitored the UV signal at another specified scan-rate for the rest of the chromatogram. The scan rates for both detectors and the length of time for monitoring of the refractometer were input to the computer.

As the refractive index of a solute can be greater or less than that of the mobile phase, peaks may be either negative or positive with respect to a given baseline.

The baseline of the refractometer was therefore adjusted to 5 V, which was the midpoint of the 0–10 V range of the ADC. The baseline of the UV detector was set between 0 to 1.5 V. To facilitate programming, the refractive index data were normalized, prior to being placed in the array, to 1 V.

When input 2 dropped below 4 V, the chromatographic run was over, and a flag was set in the array to mark the last location (should the array become overfilled, an error message will be printed). The third AT TIME block was then activated and data were processed. Note that this last block was entered only once. We shall now examine in detail our data processing procedures.

Peak sensing was accomplished by the determination of the inflection points of the bands. These points were located by taking a nine-point smoothed first derivative (cubic) of data points[36] that were above a threshold voltage. When a maximum or minimum of the first derivative was found, a parabola was fitted to the nine points in order to determine the height and location of the inflection point. This information was provided as output from the computer. The location was stored in a queue for processing. The queue, a first-in/first-out data structure, stores numbers in floating point form and has a capacity of 27 items or 13 peaks.

To help eliminate interference from baseline noise, a threshold of 1.5 V was chosen for both the UV and normalized refractive index signals. The threshold was chosen to be greater than the estimated baseline noise but lower than the peak inflections.

Several other precautions were taken to prevent the detection of false inflections caused by noise. First, if the location of an inflection point, as determined by the parabolic fit, was different by more than three data points from the location of the maximum (or minimum) of the first derivative, then it was rejected as a false inflection point, and an error message was printed. This indicated a poor parabolic fit to the nine points. Secondly, if the location of the second inflection point was within five data points of the first, it was also rejected.

One of the problems of interest in the use of computers for quantitative analysis in chromatography is where to begin and end peak integration. While sensing of slope changes and detection of threshold signals have been suggested as methods to define integration limits, we chose the use of time windows for higher accuracy and precision[20]. In this method, the locations of the two inflection points of a chromatographic band permitted the determination of the standard deviation (σ) of the band (in our case in time units). Integration was programmed to begin from a specified number of σ units before the first inflection point and to terminate after a specified number of σ units from the second inflection point. Note that the two limits on the front and back sides of the peak need not be equivalent, as shown in Fig. 7. Before the chromatographic run, the two σ units were input into the computer.

The selection of the number of σ units on the front and back sides of the peak over which to integrate will depend on the band shape and resolution of component pairs. Fig. 7 illustrates the former for a tailed peak in which a larger number of σ units are required on the back side of the band to encompass the whole area. Chesler and Cram[25] and Goedert and Guiochon[30] have considered this point in some detail. In terms of resolution, it is obvious that the time window ought not to overlap with the next peak. Thus, as the resolution becomes close to 1.0, problems begin to arise over the ability to isolate one band area from another. A somewhat different approach

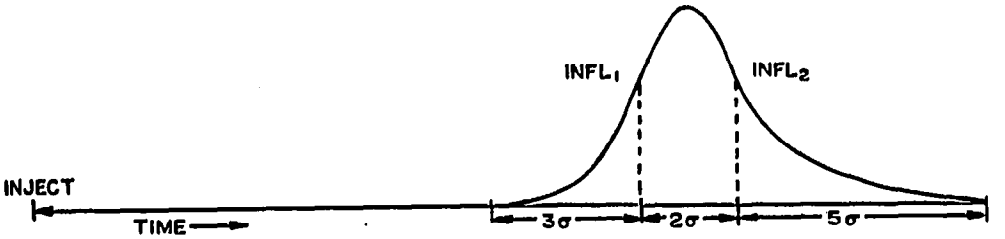


Fig. 7. Illustration of a 10σ time window for integration of a tailed peak.

to defining integration limits becomes necessary, such as dropping a perpendicular at the valley between two peaks and integrating the peaks according to the imposed limit.

For this initial study of precision in modern liquid chromatography, we have selected well resolved components ($R_s \geq 1.5$). Moreover, we input common front and back numbers of σ units for all bands. Later work will involve adjustable σ limits for each peak and analysis of overlapping bands. It is worth pointing out that it makes little sense to discuss high quantitative precision and accuracy when dealing with overlapped solutes. If the analysis is to be run repetitively (as in quality control), then extra efforts should be made to resolve bands in order to improve precision and accuracy.

Once a time window was set, an array pointer, which was used to access array locations, was placed eight data points before the beginning of the time window and a nine-point smooth was used to determine the baseline. The baseline was determined at the end of the time window in a similar fashion.

The peak was then integrated by the trapezoid method over the time window and corrected for the baseline with the following equation:

$$A_c = \left[A_u - \frac{U(F+I)}{2}\right] H \tag{1}$$

where A_c is the corrected area, A_u is the accumulated voltage, U is the number of data points taken over the integration interval, F and I are the final and initial baselines in volts and H is the number of seconds between data points. The digitized signals that were accumulated were not smoothed, because it can be shown (Appendix I) that smoothing an integral does not change the value of the integral, regardless of noise level.

After the area was determined, the time window was then scanned, using a nine-point smooth, for a peak maximum. Once found, a parabola was fitted through these nine points and the retention time and peak height were determined from the location of the maximum of the parabola. An appropriate correction in the peak height was made for the baseline at the band maximum. If the interval of these nine points covered a flat portion of the peak top, as indicated by a zero second derivative, an error message was printed warning that data points were taken too close together. The peak area, peak height and retention time were printed out from the computer.

RESULTS AND DISCUSSION

In this study, we have selected conditions typically encountered in modern

liquid chromatography in order to ascertain the precisions currently possible and in order to illustrate our on-line computer–liquid chromatographic system. In this light, a column of Corasil I coated with 1.1% (w/w) of stationary liquid phase (ODPN) was used. Moreover, the mobile phase (heptane saturated with ODPN) was recycled, as this mode is frequently used in practice. Finally, a synthetic mixture of four solutes was selected in which the resolution was greater than 1.5 in each case. While it is clear that precision (and accuracy) will decrease as the bands begin to overlap, the mixture did provide realistic peak shapes and retentions that are encountered in liquid chromatography. The results in this study serve as a basis upon which future improvements in precision can be compared.

Syringe injection

Our first set of experiments involved an examination of the precision in quantitation using syringe injection. Two types of syringes were examined —a 5-μl Hamilton high-pressure syringe and a 10-μl Pressure-Lok precision sampling syringe. Low-bleed septa (Hewlett-Packard Corp., Avondale, Pa.) were used. The average lifetime of a septum was 10 injections, by which point portions of septa were broken off. If these pieces of septa were allowed to build up at the top of the column, the flow-rate would decrease using a constant-pressure pump. Also, changes in peak shape and area (as well as column performance) might take place, affecting precision in quantitation. In order to prevent the build-up of material, a cloth disc was placed at the top of the packing and was changed periodically. For this work, the Orlita pump with the pressure controller was used. A microswitch, used to trigger the computer, was manually pressed with each injection.

The computer was programmed to sample the refractometer four times per second during the first 58 sec, after which time the sampling rate was decreased to one data point per second for the UV monitor. As mentioned previously, time windows for all peaks were 10 σ units (4 on the front side and 6 on the back side of the peak). Data rates of 4 points per σ for acetophenone up to 24 points per σ for *p*-cresol resulted. While the influence of data rate was not studied in this work, it is worth pointing out that this factor can have some influence on the accuracy and precision of the peak parameters[25,30,37]. However, at the rates selected, the effect on peak area should be minor.

Tables I and II present the results obtained with the Hamilton and precision sampling syringes, respectively. It should first be noted that the smaller peak areas and heights in Table I as compared with Table II were due to the different sample sizes and velocities used in each case. Correction for these two effects makes the two areas equivalent (see later).

The relative standard deviation in peak area was comparatively poor for the Hamilton syringe (Table I) at *ca.* 3.8%. Some improvement was shown when the precision sampling syringe was used, *ca.* 1.7%. The poorer performance of the Hamilton syringe was undoubtedly due to leakage of sample between the plunger and the barrel as the syringe was inserted into the high-pressure zone (*ca.* 200 p.s.i.) at the top of the column. Presumably higher inlet pressures would create even poorer precision. The precision sampling syringe was designed to overcome this problem somewhat through the use of a Teflon plug and washer, and hence improved precision was found.

The use of dimethyl phthalate as an internal standard significantly improved

TABLE I

PRECISION IN AREA AND PEAK HEIGHT IN LIQUID CHROMATOGRAPHY

Conditions: Syringe injection, 4 μl of solution No. 1, Orlita pump with pressure controller, UV attenuation × 64, velocity = 1.1 cm/sec (each value is the average of 6 runs). Hamilton high-pressure syringe.

	Acetophenone	*Dimethyl phthalate*	*Benzyl alcohol*	*p-Cresol*
Area				
Volt sec*	52.8 ± 1.8	109.8 ± 4.2	105.3 ± 4.0	119.7 ± 4.8
Relative standard deviation (%)	3.4	3.8	3.8	4.0
Relative standard deviation with internal standard (dimethyl phthalate) (%)	0.94		0.93	1.7
Height				
Volt*	5.97 ± 0.24	4.30 ± 0.16	2.69 ± 0.13	1.99 ± 0.10
Relative standard deviation (%)	4.1	3.7	4.9	5.0
Relative standard deviation with internal standard (dimethyl phthalate) (%)	1.3		0.73	1.3

* ± Standard deviation.

TABLE II

PRECISION IN AREA AND PEAK HEIGHT IN LIQUID CHROMATOGRAPHY

Conditions: Syringe injection, 5 μl of solution No. 1, Orlita pump with pressure controller, UV attenuation × 64, velocity = 1.2 cm/sec (each value is the average of 6 runs). Precision sampling syringe.

	Acetophenone	*Dimethyl phthalate*	*Benzyl alcohol*	*p-Cresol*
Area				
Volt sec*	61.6 ± 0.9	127.0 ± 2.4	120.4 ± 2.0	133.0 ± 2.5
Relative standard deviation (%)	1.5	1.9	1.7	1.9
Relative standard deviation with internal standard (dimethyl phthalate) (%)	1.1		0.6	0.7
Height				
Volt*	6.70 ± 0.06	4.45 ± 0.06	2.72 ± 0.04	1.95 ± 0.02
Relative standard deviation (%)	1.0	1.3	1.4	1.0
Relative standard deviation with internal standard (dimethyl phthalate) (%)	1.3		0.6	0.9

* ± Standard deviation.

the precision in both cases. Error in area measurements with the Hamilton syringe was reduced to around 1% while that of the precision sampling syringe was even lower. In a similar fashion, the precision in peak height measurements showed some improvement when the internal standard method was used. This method should therefore be used when injections are made by a syringe, as is well known. Representing peak areas or heights relative to an internal standard generally overcomes the problems of sample leakage and nonreproducible injection volumes[38,39].

Loop injection

In order to reduce the injection error, the injection valve with a 20-μl sample loop (see Fig. 2) was used. Solution No. 4 (1/8 dilution of solution No. 1) with the UV monitor attenuated at $\times 32$ was used. The sampling rates and time windows were the same as those with the syringe experiments.

The results of the precision study using the Orlita pump and pressure controller are shown in Table III. A comparison of areas with those in Tables I and II reveals a constant volume error of about 3.5 μl. It was probable that the injection valve plus loop volume was 23.5 μl rather than 20 μl (communication from the manufacturer). As we were interested in precision, rather than accuracy, no effort was made to calibrate the loop. However, for absolute measurements, it is necessary to determine the injection volume for each loop[40].

Relative standard deviations of areas ranged from 1.2 to 2.1% and in peak height from 1.0 to 1.3%. These values were similar to those obtained with the precision sampling syringe. The interesting point was that the internal standard did not significantly reduce the error (in fact, the error increased for *p*-cresol), suggesting that

TABLE III

PRECISION IN AREA AND PEAK HEIGHT IN LIQUID CHROMATOGRAPHY

Conditions: Sample valve injection, 23.5 μl of solution No. 4, Orlita pump with pressure controller, UV attenuation $\times$ 32, velocity = 1.1 cm/sec (each value is the average of 6 injections).

	Acetophenone	*Dimethyl phthalate*	*Benzyl alcohol*	*p-Cresol*
Area				
Volt sec*	77.5 ± 1.0	162.2 ± 2.2	162.0 ± 2.0	182.3 ± 3.8
Relative standard deviation (%)	1.3	1.3	1.2	2.1
Relative standard deviation with internal standard (dimethyl phthalate) (%)	1.4		1.1	2.7
Height				
Volt*	7.68 ± 0.08	5.48 ± 0.05	3.34 ± 0.04	2.43 ± 0.03
Relative standard deviation (%)	1.0	1.0	1.3	1.3
Relative standard deviation with internal standard (dimethyl phthalate) (%)	1.1		1.2	2.2

* ± Standard deviation.

the injection variation was not the major cause of error with the loop. The larger values in relative standard deviation found in Table III with the use of the internal standard as compared to those using a syringe (especially in Table II) is due to the lower signal-to-noise ratio (S/N) in the experiments with the loop injector (see later). If we define S as the signal at the peak maximum and N as the peak-to-peak noise level (frequency = 0.01–0.1 Hz), then for the conditions in Table II, $S/N = 200$ for dimethyl phthalate and 87 for *p*-cresol. In Table III, $S/N = 122$ for dimethyl phthalate and 54 for *p*-cresol. It is well known that as the baseline noise becomes a significant fraction of the total area or peak height, precision of quantitation is markedly affected[1].

If flow pulsations in the pumping system contribute to the noise levels obtained for the conditions in Table III, then from the above arguments one should observe changes in relative standard deviation in peak area or peak height as a function of pump type (especially at low signal-to-noise ratios). To test this, we selected a Waters Associates M6000 pump, which is known to produce fewer pulsations than the Orlita pump with a pressure controller (because of the low flow-rates used, we operated the Waters pump in the Hi Sensitivity Noise Filter Mode). Except for a slight increase in mobile phase velocity, all experimental conditions were the same as in Table III. The results of the precision study are shown in Table IV.

TABLE IV

PRECISION IN AREA AND PEAK HEIGHT IN LIQUID CHROMATOGRAPHY

Conditions: Sample valve injection, 23.5 μl of solution No. 4, Waters pump, UV attenuation × 32, velocity = 1.2 cm/sec (each value is the average of 6 injections).

	Acetophenone	*Dimethyl phthalate*	*Benzyl alcohol*	*p-Cresol*
Area				
Volt sec*	77.7 ± 0.6	150.9 ± 0.9	143.2 ± 0.7	157.6 ± 1.6
Relative standard deviation (%)	0.8	0.6	0.5	1.0
Relative standard deviation with internal standard (dimethyl phthalate) (%)	0.7		0.4	1.0
Height				
Volt*	7.91 ± 0.08	5.39 ± 0.02	3.28 ± 0.01	2.27 ± 0.01
Relative standard deviation (%)	1.0	0.3	0.4	0.5
Relative standard deviation with internal standard (dimethyl phthalate) (%)	1.0		0.2	0.5

* ± Standard deviation.

Relative standard deviations in area measurements ranged from 0.5 to 1.0% and were thus much better than those obtained with the Orlita pump for the same sample size. With the use of an internal standard, there was no significant reduction in the standard deviation, indicating negligible injection error with the sampling valve. The enhanced S/N relative to Table III was caused almost entirely by a lower noise level of the pumping system. S/N for dimethyl phthalate was 190 in Table IV *versus*

122 in Table III, and S/N for *p*-cresol was 80 in Table IV *versus* 54 in Table III.

It is interesting to note that for the higher velocity in Table IV, the peak heights are slightly lower than those in Table III. The UV monitor is a concentration-sensitive device for which the peak height should be independent of flow-rate; however, this presupposes no change in HETP. The slight increase in HETP with velocity for this column[33] was the cause of the change in peak height from Table III to Table IV. Excluding acetophenone, relative standard deviations in peak height were about 0.4% compared with 1.2% for the Orlita pumping system. As expected, the use of an internal standard did not significantly lower these errors.

The relatively large error in peak height for acetophenone was caused by the interference of a small peak of the unretained component (*n*-hexane) with the baseline determination. While hexane is not active in the UV region, it does have a different refractive index than heptane, which could be detected in the small cells of the UV detector. This peak becomes more prominent on a relative basis as the amount of hexane to the other sample components increases to a large extent (see Fig. 13). It was difficult to eliminate this peak from having an effect on acetophenone peak ($k' = 0.8$), especially in terms of the definition of the baseline. This was not seen with the Orlita pump (Table III), as the higher noise level generated by this pump was superimposed on the baseline measurement.

Influence of short-term noise on precision

Noise (*i.e.*, short-term noise) will influence precision in both peak area and peak height in two ways: (1) uncertainty in the baseline and (2) uncertainty in the magnitude of the signal. We will now present some relationships to help understand these effects; a more detailed error analysis will be presented in a later paper.

Consider first the uncertainty in the baseline. The standard deviation in the measurement of peak height due to noise affecting the determination of the baseline, σ'_h, is

$$\sigma'_h = \frac{\sigma_N}{\sqrt{2}} \tag{2}$$

while that for the peak area can be shown to be

$$\sigma'_A = \frac{t\sigma_N}{\sqrt{2}} \tag{3}$$

where t = time interval for integration and σ_N is the standard deviation of the short-term noise (note that this is related to the previously defined N by a constant).

Consider next the uncertainty in the magnitude of the signal. The error due to noise on peak height, σ''_h, is

$$\sigma''_h = \sigma_N \tag{4}$$

and that due to peak area is

$$\sigma''_A = \frac{t\sigma_N}{\sqrt{n}} \tag{5}$$

where n = number of data points taken across the peak. Eqn. 5 comes directly from

the principles used in radioactive counting[41]. Note further that the assumption is made that the same value of σ_N is used in eqns. 2–5. This assumption may not be completely valid in all cases, *e.g.*, if different smoothing routines are used to find the peak height and the baseline.

The total error due to random noise is then simply the square root of the sum of the variances. Thus

$$\sigma_h = \left(\frac{\sigma_N^2}{2} + \sigma_N^2\right)^{\frac{1}{2}} = \left(\frac{3}{2}\right)^{\frac{1}{2}} \sigma_N \tag{6}$$

and

$$\sigma_A = \left(\frac{\sigma_N^2 t^2}{2} + \frac{\sigma_N^2 t^2}{n}\right)^{\frac{1}{2}} = \left(\frac{1}{n} + \frac{1}{2}\right)^{\frac{1}{2}} \sigma_N t \tag{7}$$

It is to be noted that σ_h is independent of the number of data points across the peak, as expected. Moreover, σ_A will also be independent of n, provided that $n \gg 2$.

The relative standard deviation in peak height and peak area (with $n \gg 2$) can be written as

$$\frac{\sigma_h}{h} = \left(\frac{3}{2}\right)^{\frac{1}{2}} \frac{\sigma_N}{h} \tag{8}$$

and

$$\frac{\sigma_A}{A} = \frac{\sigma_A}{h\sigma_p\sqrt{2\pi}} = \frac{1}{2\sqrt{\pi}} \left(\frac{\sigma_N}{h}\right) \left(\frac{t}{\sigma_p}\right) \tag{9}$$

where σ_p = standard deviation of the band in time units. (We assume here that the peak is Gaussian.) Eqns. 8 and 9 show the importance of the signal-to-noise ratio on quantitation (equating h with S and σ_N with N). It is easily seen that the larger is h/σ_N the smaller will be the relative error in peak height and peak area. By noting that the time window, W, in σ_p units is equal to (t/σ_p), eqn. 9 can be written as

$$\frac{\sigma_A}{A} = \frac{W}{2\sqrt{\pi}} \frac{\sigma_N}{h} \tag{10}$$

where in our experiments W is a constant. Eqn. 10 indicates that the smaller is the time window at a given signal-to-noise ratio, the more precise will be the peak area. This conclusion is simply due to the fact that the wider are the integration limits, the larger will be the contribution to the error from baseline uncertainty. Of course, accuracy can decrease if the time window is not wide enough to include all of the peak. Thus a compromise must be made in setting the time windows.

It is interesting to combine eqns. 8 and 10 in order to examine the precision possible from peak height measurements relative to that from peak area. Again, this comparison is based on the assumption that noise is the major contributor to error in both cases. The ratio of errors can be written as

$$\frac{\sigma_A/A}{\sigma_h/h} = 0.23W \tag{11}$$

We reach the interesting conclusion that the precision difference in peak area and peak height is proportional only to the time window for integration. As $W = 10$ in our

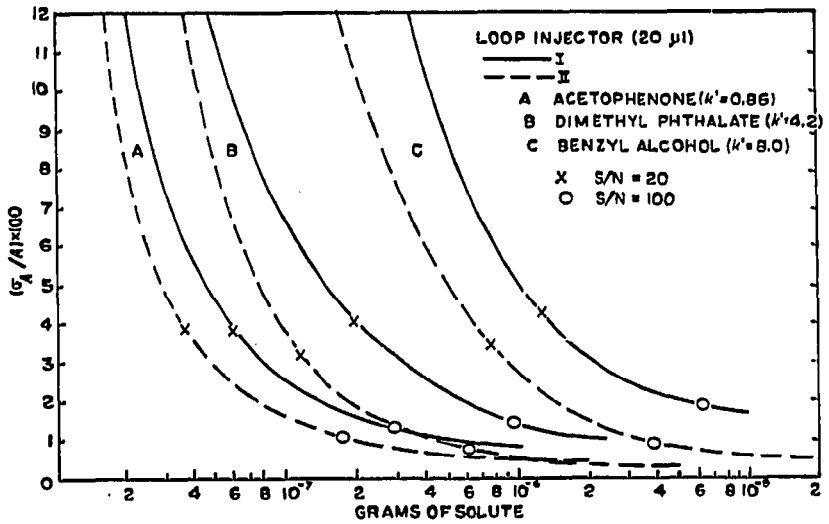


Fig. 8. Relative standard deviation of area measurements *versus* grams of injected solute using the Orlita pump with pressure controller (I) and Waters pump (II).

experiments, we predict a factor of 2.3 better precision for peak height. We shall now explore some results in light of the above arguments.

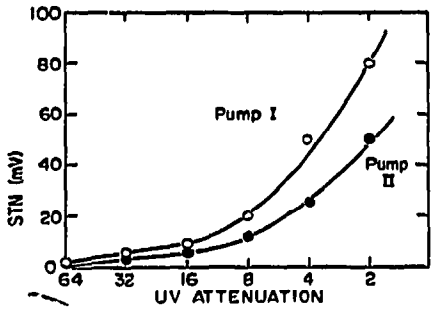
The relative standard deviation in area was determined as a function of the amount of each solute injected (grams) for both pumps using the loop injector. The results are presented in Fig. 8 for three of the solutes; *p*-cresol, which has the same trend, is omitted for the sake of clarity.

We first note that for a given amount of solute and a given pumping system, the relative standard deviation was lower, the smaller was the capacity factor. This is a reflection of the higher signal-to-noise ratio for the smaller k' solute. It can be concluded that at a given precision level, a more sensitive analysis can generally be made the smaller is the k' value, other factors being equal (response factors, for example, can alter the relationship between detection limits and k').

The errors obtained for both pumping systems at S/N values of 20 and 100 are also shown in Fig. 8. It is interesting to note that the relative standard deviation for all solutes is *ca.* 3.8% for $S/N = 20$ and *ca.* 1.2% for $S/N = 100$. The constancy of relative standard deviation for a given S/N with different pumping systems and different solutes is expected from eqn. 10, if baseline noise is the major cause of error in these cases. It is clear that when chromatographic conditions are compared in terms of their effect on quantitation, some specification concerning S/N is necessary in order to provide meaningful conclusions.

The trends in Fig. 8 reveal that if S/N is sufficiently high and if the Waters pump and the loop injector are used, relative standard deviations in peak area less than 0.5% are possible. Note that these results were obtained on an absolute basis, *i.e.*, no internal standard was used. It is worth pointing out that for dimethyl phthalate a precision as low as 0.2% was found.

A comparison of the two pumping systems revealed that for any given amount of solute, the Waters pumping system provided roughly twice the precision of the Or-



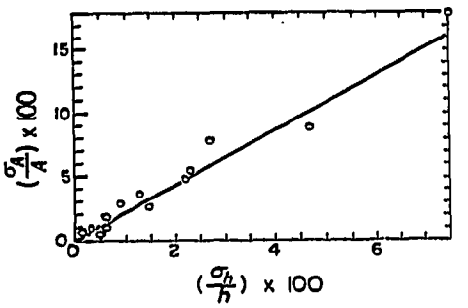


Fig. 9. Short-term noise (STN) (peak-to-peak, 0.01–0.1 Hz) of the UV detector using the Orlita pump with pressure controller (I) and the Waters pump (II). The noise is determined on a recorder from the signal with 100 × amplification (see Fig. 3).

Fig. 10. Relative standard deviation of peak area *versus* peak height for dimethyl phthalate. The points represent different concentrations or chromatographic conditions.

lita system. Fig. 9 shows that this result was due to the differences in short-term noise (0.01–0.1 Hz) produced from the two pumps. In Fig. 9, noise (peak-to-peak, mV) as a function of pump type and attenuation is plotted. The experiment involved flowing mobile phase through the column and detector at a velocity of 1.2 cm/sec and averaging the output of the first amplifier (× 100), Fig. 3, which was displayed on a recorder over a 20 min time interval. We see that the noise level for the Orlita pump was about twice that for the Waters pump (in agreement with the trend in precision in Fig. 8); moreover, the noise in both cases was found to be linear with attenuation.

Short-term noise resulted mainly from flow variations from the pumping systems and temperature variations in the cell. These fluctuations result in small changes in refractive index of the mobile phase in various regions of the detector cell. As a result of these refractive index changes, the source radiation is somewhat scattered as it travels through the cell, leading to variations in the amount of light reaching the photocell (Schlieren effects) and hence producing noise. There is evidence in the literature that these effects can be minimized by careful thermostatting of the detector cell[42]. This modification might substantially reduce not only the noise due to the pumping systems, but might also change the differences in noise levels between the systems.

From eqn. 7, it is seen that the absolute standard deviation in peak area is proportional to the time interval for integration, t, and hence σ_A will increase as peak width becomes wider. Our results are in general agreement with this conclusion. From Fig. 8 and the data for *p*-cresol, σ_A values were determined for dimethyl phthalate, benzyl alcohol and *p*-cresol with both pumping systems at given noise levels. In all cases, the ratios of σ_A values for two solutes were found to be in the ratios of the time intervals. The value of σ_A for acetophenone was higher than that predicted from its time window. This result is related to the previously discussed problem of defining the baseline, because of the close proximity of the hexane peak. It may also be noted that in Tables III and IV the σ_A values for benzyl alcohol were lower than predicted. This discrepancy is probably a reflection of the scatter in σ_A values. When smoothed data from a number of σ_A values are used (Fig. 8), the absolute standard deviations were proportional to the time windows.

Consider next the precision of peak area *versus* peak height. Eqn. 11 with $W = 10$ leads to the conclusion that peak height should be 2.3 times as precise as the corresponding peak area. Fig. 10 shows a plot of σ_A/A *versus* σ_h/h for dimethyl phthalate at different sample sizes with both pumping systems. The slope of the straight line was 2; benzyl alcohol and *p*-cresol gave similar plots with slopes of 1.7 and 1.9, respectively. This is in close agreement with results from eqn. 11, although several assumptions were made, including a Gaussian distribution for the peak.

The conclusion can be made that peak height provides improved precision for quantitative analysis in the application of liquid chromatography, when noise is a limiting factor on precision. This presupposes that no peak overlapping and no column overloading occur. Moreover, as peak height is more dependent on chromatographic conditions (*e.g.*, column temperature, phase ratio) than peak area, more frequent calibration will be necessary. Goedert and Guiochon[19] have dealt with these points in detail.

Flow-rate effects

As the UV detector is concentration sensitive, a decrease in flow-rate results in an increase in peak area[43]. This was confirmed in Fig. 11, which is a plot of area *versus* the reciprocal of the flow-rate. A lower detection limit was therefore possible at lower flow-rates because of the increased area and higher efficiency[33].

The flow-rate can also influence the noise level and hence precision and detection limit. This is shown in Fig. 12, which is a plot of peak-to-peak short-term noise (0.01–0.1 Hz) measured on a recorder after the 100× amplifier (Fig. 3). For both pumps, the noise increased as the flow-rate increased. Note that for the Orlita pumping

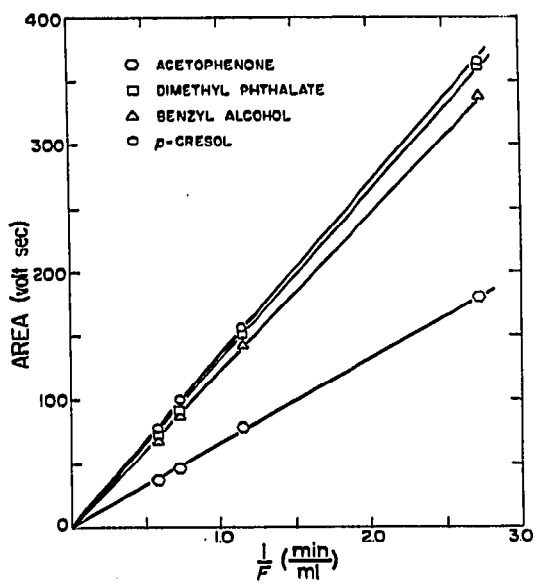


Fig. 11. Peak area *versus* reciprocal of flow-rate for the four test solutes.

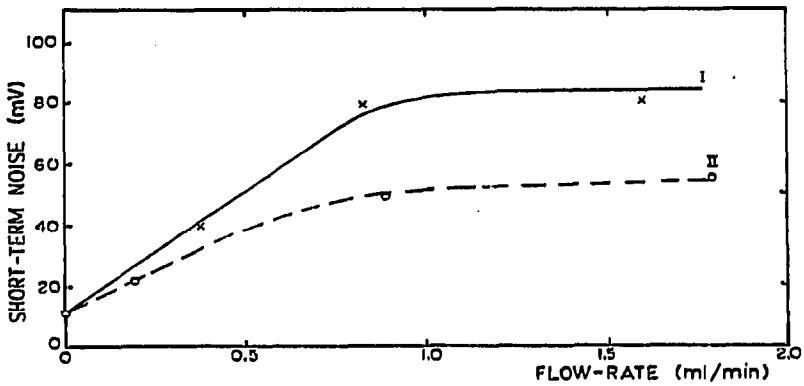


Fig. 12. Short-term noise (peak-to-peak, 0.01–0.1 Hz) by the recorder from the UV detector with 100× amplification (see Fig. 3) *versus* flow-rate for the Orlita pump with pressure controller (I) and the Waters pump (II).

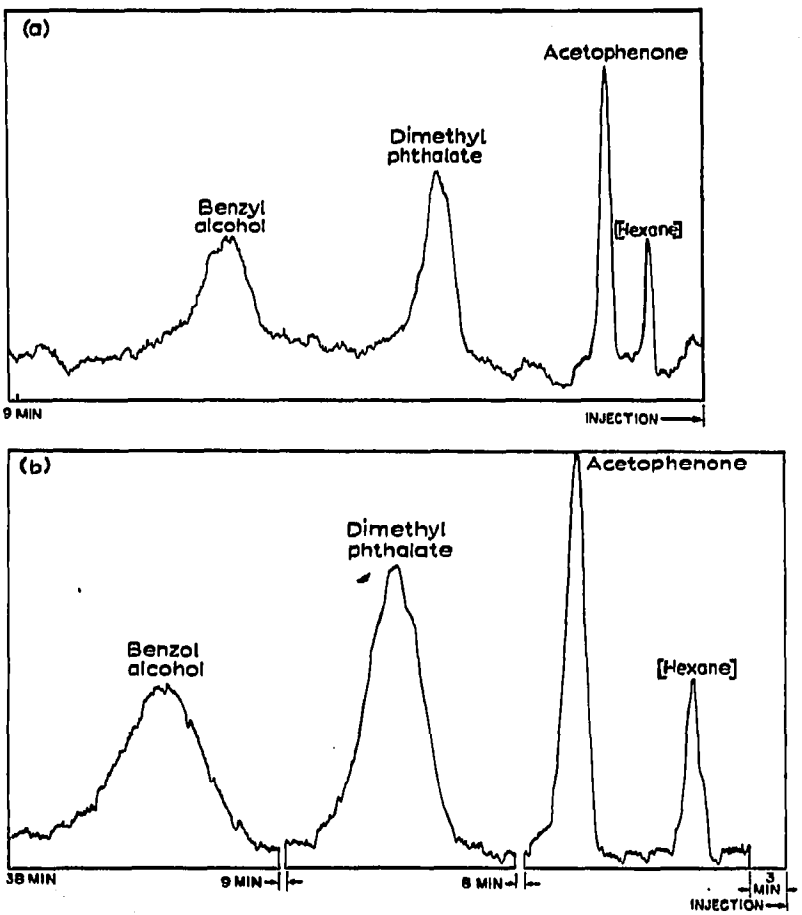


Fig. 13. Chromatograms to illustrate the importance of flow-rate on detection limit and noise. (a) Separation of acetophenone, dimethyl phthalate and benzyl alcohol using the Waters pump at v = 1.2 cm/sec. Attenuation = ×2. (b) Separation of the same mixture and sample size at v = 0.22 cm/sec. Attenuation = ×2.

system the shortterm noise level increased by a factor of 8. The noise seemed to level off at *ca.* 1 ml/min. Superimposed on the short-term noise was a higher-frequency component (2 Hz) with an amplitude of about 12 mV. As the flow-rate decreased, the noise levels from both pumping systems approached this value. Independent measurements indicated that this noise was electronic in origin. The results in Fig. 12 indicate a further gain in detection limit as flow-rate is decreased.

To demonstrate the influence of flow-rate, Fig. 13a (0.9 ml/min) and 13b (0.2 ml/min) present the chromatograms of acetophenone, dimethyl phthalate and benzyl alcohol (128× lower concentrations than solution No. 1) using the UV detector at ×2 attenuation with the Waters pump and the loop injector. It can be seen that a much higher signal-to-noise ratio was obtained at the lower flow-rate. This higher value of S/N can be translated either into a more precise analysis at a given sample size or a lower detection limit at a given precision level.

Retention reproducibility

Using the sample loop injector and the Waters pump, we undertook an examination of the repeatability of retention and capacity factors, and the results are presented in Table V. Retentions were determined from the times of peak maxima which were sensed as described in the computer program section. Relative standard deviations in absolute retention times ranged from 0.1% for acetophenone to 0.3% for *p*-cresol. These results illustrate the excellent repeatability possible with modern liquid chromatographic equipment. The capacity factors are seen to range from 0.25% for dimethyl phthalate to 0.5% for acetophenone. At these precision levels for capacty factors, the accuracy of the retention time of the unretained component becomes critical.

TABLE V

PRECISION OF RETENTION TIMES AND CAPACITY FACTORS

Conditions: Sample valve injection, 23.5 µl of solution No. 4, Waters pump, UV attenuation × 32, velocity = 1.2 cm/sec (each value is the average of 6 injections).

Variable	*n-Hexane*	*Acetophenone*	*Dimethyl phthalate*	*Benzyl alcohol*	*p-Cresol*
t_R (sec)*	44.25 ± 0.11	80.64 ± 0.08	223.03 ± 0.28	396.4 ± 1.0	640.6 ± 2.0
k'*		0.822 ± 0.004	4.04 ± 0.01	7.96 ± 0.02	13.48 ± 0.04

± Standard deviation.

Goedert and Guiochon[28] presented a detailed error analysis of retention time measurements in gas chromatography. There are four potential contributions to random error: (1) flow-rate variations, (2) column-temperature variations, (3) injection-time sensing variations and (4) data processing. At the flow constancy levels of this work, it was not possible to measure volumetrically the flow-rate variation. However, the results in Table V for *n*-hexane indicate that the flow-rate was constant to better than 0.25% relative standard deviation for the conditions of our experiments. It is

TABLE VI

EFFECT OF RECYCLING TIME ON CAPACITY FACTORS

Conditions: Hamilton syringe injection, 4 μl of solution No. 2, Orlita pump with pressure controller, UV attenuation $\times$ 32, velocity = 1.1 cm/sec (each value is the average of 6 injections). Values given are $\pm$ standard deviation.

Days	*Acetophenone*	*Dimethyl phthalate*	*Benzyl alcohol*	*p-Cresol*
1	0.829 $\pm$ 0.007	3.97 $\pm$ 0.03	7.87 $\pm$ 0.08	13.2 $\pm$ 0.1
2	0.819 $\pm$ 0.006	3.97 $\pm$ 0.03	7.88 $\pm$ 0.04	13.26 $\pm$ 0.06
3	0.831 $\pm$ 0.007	4.06 $\pm$ 0.02	7.89 $\pm$ 0.05	13.34 $\pm$ 0.08
4	0.850 $\pm$ 0.002	4.27 $\pm$ 0.02	8.03 $\pm$ 0.02	13.58 $\pm$ 0.03
8	0.858 $\pm$ 0.009	4.20 $\pm$ 0.02	8.01 $\pm$ 0.01	13.74 $\pm$ 0.02

to be noted that the constancy in retention can be no better than the constancy in flow-rate.

The second error can be attributed to slight temperature variations (note that the column temperature was controlled by a water-jacket to $\pm$ 0.1°). These variations can lead to a change in distribution coefficients (and secondarily flow-rate), which can affect retention[21]. It is expected that temperature fluctuations may have a greater effect on longer retained components, as seen in Table V. For the third error, the propagated standard deviation for injection sensing was 1.7×10^{-2} sec, arising from the fact that the computer was programmed to examine the input of the trigger every $6.6 \cdot 10^{-2}$ sec. This random systematic (within a chromatographic run) error was negligible in comparison with the error found in Table V. For the final error, the peak maximum was determined by a nine-point smoothed parabolic fit of the data, using a scan-rate of one point per second. As will be discussed in a later paper, these conditions lead to an error of about $1 \cdot 10^{-2}$ sec, which again is negligible in comparison with the experimental errors found. Hence it would appear that flow-rate and temperature variations are the major causes of errors in Table V. However, it needs to be noted that the results in this study are adequate for routine analysis.

The long-term stability of retention in terms of capacity factors was next examined (Table VI). In this study we used the Orlita pump with a pressure controller and a Hamilton high-pressure syringe. After the third day, a slight but significant increase in k' was noticed. After 8 days of operation, the k' values increased by 1.8 to 5.8%. The changes were most likely due to the recycling of the solvent. During the 8-day period, about 100 injections were made, representing the addition of about 10 mg of solute to about 1500 ml of mobile phase. In addition to these impurities, the presence of dissolved oxygen can affect ODPN[3].

As an indication of the importance of impurities in the system, fresh mobile phase was used at the end of the 8-day period and the capacity factors are presented in Table V. These values were close to those obtained at the beginning of the eight day study (Table VI). The results indicate that for long-term reproducibility of retention times, the mobile phase should not be recycled.

APPENDIX I

Proof that smoothing has no significant effect on the determination of the area of a chromatographic band

Smoothing by convolution involves multiplication of a sequence of data points, generally equally spaced, by fixed coefficients to obtain a smoothed value for one point. If there are $2p+1$ points being used to obtain the smoothed value, then the smoothed point, x_n', can be expressed in terms of the coefficients, c_i, and the unsmoothed points x_i, as

$$x_n' = \sum_{i=-p}^{p} c_i x_{n+i}$$

It is a further condition that the sum of the coefficients must be unity so that no change in magnitude will occur on application of the convolution to a linear (smooth) set of points. Mathematically stated,

$$\sum_{i=-p}^{p} c_i = 1$$

We wish to demonstrate that the sum over a region bounded by flat portions of approximately equal magnitude will not be significantly affected by a convolution method of smoothing. That is,

$$\sum_{n=0}^{N} x_n' \approx \sum_{n=0}^{N} x_n$$

Proof.

$$\sum_{n=0}^{N} x_n' = \sum_{n=0}^{N} \sum_{i=-p}^{p} c_i x_{n+i} = \sum_{n=0}^{N} \sum_{m=n-p}^{n+p} c_{m-n} x_m$$

$$= \sum_{m=p}^{N-p} x_m \sum_{n=m-p}^{m+p} c_{m-n} + \sum_{m=-p}^{p-1} x_m \sum_{n=0}^{m+p} c_{m-n} + \sum_{m=N-p+1}^{N+p} x_m \sum_{n=m-p}^{N} c_{m-n}$$

$$= \sum_{m=p}^{N-p} x_m \sum_{n=-p}^{p} c_n + \sum_{m=-p}^{p-1} x_m \sum_{n=0}^{m+p} c_{m-n} + \sum_{m=N-p+1}^{N+p} x_m \sum_{n=m-p}^{N} c_{m-n}$$

$$= \sum_{n=0}^{N} x_n + \Bigg(\sum_{m=-p}^{p-1} x_m \sum_{n=0}^{m+p} c_{m-n} - \sum_{m=0}^{p-1} x_m + \sum_{m=N-p+1}^{N+p} x_m \sum_{n=m-p}^{N} c_{m-n} - \sum_{m=N-p+1}^{N} x_m \Bigg)$$

The portions in parentheses are zero if x_m in the ranges $-p \leqslant m \leqslant p-1$ and $N-p+1 \leqslant m \leqslant N+p$ are constant and equal.

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