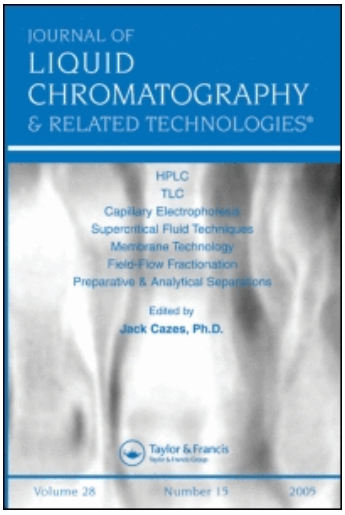

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Software for Microprocessor Controlled HPLC: Peak Area Integration

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SOFTWARE FOR MICROPROCESSOR CONTROLLED HPLC: PEAK AREA INTEGRATION

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ABSTRACT

Programs are described for determination of peak areas and peak retention times from the chromatographic data obtained by a dual-microprocessor data handling microcomputer (DHC). The programs provide the necessary equipment testing and calibration routines for an accurate reproduction of a recorded chromatogram, and they are written to be merged with the data acquisition programs to provide a true "real-time" integrator. The integration is perform-

*This Series of publications is dedicated with gratitude and admiration to Dr. Waldo E. Cohn, Oak Ridge National Laboratory on the occasion of his 76th birthday for his pioneering work in liquid chromatography, chemistry, and nomenclature of nucleic acids.

Address all correspondence to RPS. Inquire RPS for availability of complete program listings on floppy disk or hard copy (Tel: 316-689-3120). Program listings are also described in the dissertation of D. B. Smoll, it can be loaned from Ablah Library, The Wichita State University, Wichita, KS 67208. This is the fourth paper in this series (see ref. 1 through 3 for first three papers).

Abbreviations: A/D, analog-to-digital; D/A, digital-to-analog; HPLC, Hplc, High-Performance Liquid Chromatography; DHC, data handling microcomputer; see text for other abbreviations.

Note: Apple is a registered trademark of Apple Computer, Inc., and Explorer-85 is a registered trademark of Newtronics Research and Development, Ltd.

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ed with baseline stabilization and automatic peak splitting. These features make the integrator applicable to gradient elution chromatography, as well as for the integration of complex chromatograms with overlapping peaks. The integrated chromatogram can be displayed with the limits of integration for each peak. Results of peak area integration of simple and complex chromatograms demonstrate satisfactorily accurate and consistant results that are independent of chromatographic conditions and shape of the peaks.

INTRODUCTION

Our previous publications have dealt with the hardware and interfacing of microcomputers with conventional Hplc equipment, a software operating system for the microcomputers, and software controlled chromatographic data acquisition (1-3). The data collected by the microcomputer equipment is a digital representation of the chromatogram produced by the Hplc equipment. It is a series of A/D conversions of the UV monitor analog outputs (acquired at the rate of one second); when played back at the same rate, they produce a true reproduction of the chromatogram. The application program described here makes use of this data to integrate the peaks and determine peak retention times of the chromatogram. The integration program is written in order to allow merging with the data acquisition programs to produce a true "real time" integrator.

Many of the commercially available integrators are written in a "deferred time" general format (4,5). The chromatographic data is acquired through a "real-time" format, and prior to integration, additional information concerning baseline position and peak location is furnished to the microcomputer by the investigator. True "real-time" integrators have difficulty integrating chromatograms with unpredictable baselines accurately and predictably. These in-

tegrators generally resort to a fixed threshold and integrate all area above this arbitrary baseline without considering fluctuations in the actual baseline (6,7); hence, they cannot be used satisfactorily for gradient elution, where the baseline can fluctuate throughout the chromatography. The DHC-integrator described here, on the other hand, can be employed easily for gradient elution of Hplc. The baseline is stabilized by compensating drifts and shifts in the baseline without the use of a fixed integration threshold.

Conventional manual determination of peak area is an approximation at best. The oldest and earliest manual method involves weighing of a cutout peak. A more sophisticated method of approximation is the use of a theoretical guassian distribution curve [$A = (2\pi)^{\frac{1}{2}}\sigma H$](8), and the relationship of the width at 50 % of the maximum peak height to the distribution coefficient [$W_{\frac{1}{2}} = (2\pi)^{\frac{1}{2}}\sigma/1.065$] to determine the area (9,10). A simpler approximation method, though restricted to specific chromatography conditions, has been developed (11). The latter method takes advantage of the constant relationship between the distribution constant (Kd) and the distribution coefficient (σ), and relates peak height to peak area with an experimentally determined standard curve. Accuracy of a manual integration method depends on a theoretical fit of the peak to a guassian curve; and as a result it can yield erroneous results for overlapping and skewed peaks.

To avoid these problems, the DHC integrator employs numerical quadrature integration methods (12). These integration methods are inherently more accurate than the manual methods since approxima-

tions are confined to smaller units (subintervals), and they are not based on any assumptions concerning the shape or size of the peak. Three basic quadrature algorithms are commonly used for numerical integration: the Retangular Rule, the Trapezoidal Rule, and Simpson's Rule. Of these three the Rectangular Rule is the least accurate, especially for steep or complex curves, since it cannot approximate inclines accurately. In contrast, Simpson's Rule is the most accurate for nonlinear data but is the most difficult to program. For our application, we have used the simplest method which can provide satisfactory results to conserve processing time. Here, a comparison of peak areas is made between the microcomputer method (DHC integration) and several conventional manual methods. Satisfactory performance of the software programs is demonstrated by comparison of the DHC-integrated Hplc data with those determined by other methods available to us.

MATERIALS AND METHODS

A. Computer and Hplc Equipment

The computer and Hplc equipment were the same as described earlier (1-3). Samples were applied to a RP-LC column with a calibrated sample loop. The water jacketed reversed-phase (C-18) Hplc column was maintained at 40°C with a constant temperature water bath. The effluent was monitored with UV monitors which were connected to the A/D converters of the Explorer microcontroller. The chromatographic data was collected and displayed on a strip-chart recorder using a data acquisition program in the Explorer. The data was transferred to an Apple II computer for storage on floppy disk after completion of the chromatography.

TABLE I. ELUTION GRADIENT PROGRAMS for Hplc PUMP

Gradient Program A

SECTOR	TIME (min)	FLOW RATE (ml/min)	RESERVOIRS(%) A	B	C	GRADIENT
1	15.0	1.5	95.0	3.0	2.0	concave
2	15.0	1.5	85.0	9.0	6.0	concave
3	10.0	1.5	75.0	15.0	10.0	linear
4	10.0	1.5	25.0	45.0	30.0	linear
5	10.0	1.5	0.0	60.0	40.0	linear
6	5.0	1.5	0.0	60.0	40.0	

Reservoirs: A; 0.25 M Ammonium Acetate, pH 6.00;
B; Distilled Water, C; Acetonitrile

Gradient Program B

SECTOR	TIME (min)	FLOW RATE (ml/min)	RESERVOIRS(%) A	B	GRADIENT
1	9.0	1.0	97.5	2.5	
2	10.0	1.0	90.0	10.0	linear
3	15.0	1.0	80.0	20.0	step

Reservoirs: A; 10.0 mM Ammonium Phosphate, pH 5.30
B; Methanol

B. Gradient Programs

Elution gradients for the chromatography were produced using a microprocessor-controlled liquid chromatography pump (Perkin-Elmer, model Series 4). The elution programs used for this work are shown in Table I. The RP-LC columns were equilibrated and stored overnight in a mixture of 75.0 % methanol and 25.0 % water.

C. Calculations

1. Peak Area Integration

The peak area calculated by Manual Method A is based on a gaussian curve approximation for a peak. The basic equation for

the area is derived from the area equation for a theoretical gaussian curve and the relationship of the width at 50 % maximum peak height to the distribution coefficient (see Introduction). Values for the maximum peak height in absorbance (H) and the width at 50 % peak height ($W_{\frac{1}{2}}$) are substituted directly into the resulting equation: $A = 1.065(W_{\frac{1}{2}})(H)$. The calculated peak area is then corrected for difference in flow cell volume and the sample loop volume by multiplying by a unitless "flow-cell-dilution factor": Dil. Factor = flow-cell volume/sample loop volume. The final peak area value is a zero-dilution area expressed in absorbance units.

2. Percent Mole Fraction

The percent mole fraction calculations used in Method C uses a peak area determined from a linear standard curve of peak height versus peak area (11). The peak area in zero-dilution absorbance units is converted to moles by dividing by the molar extinction for each nucleoside corrected for absorbance at 254 nm. The final percent mole fractions are then calculated by the normal procedure.

DESIGN OF SOFTWARE FOR PEAK INTEGRATION

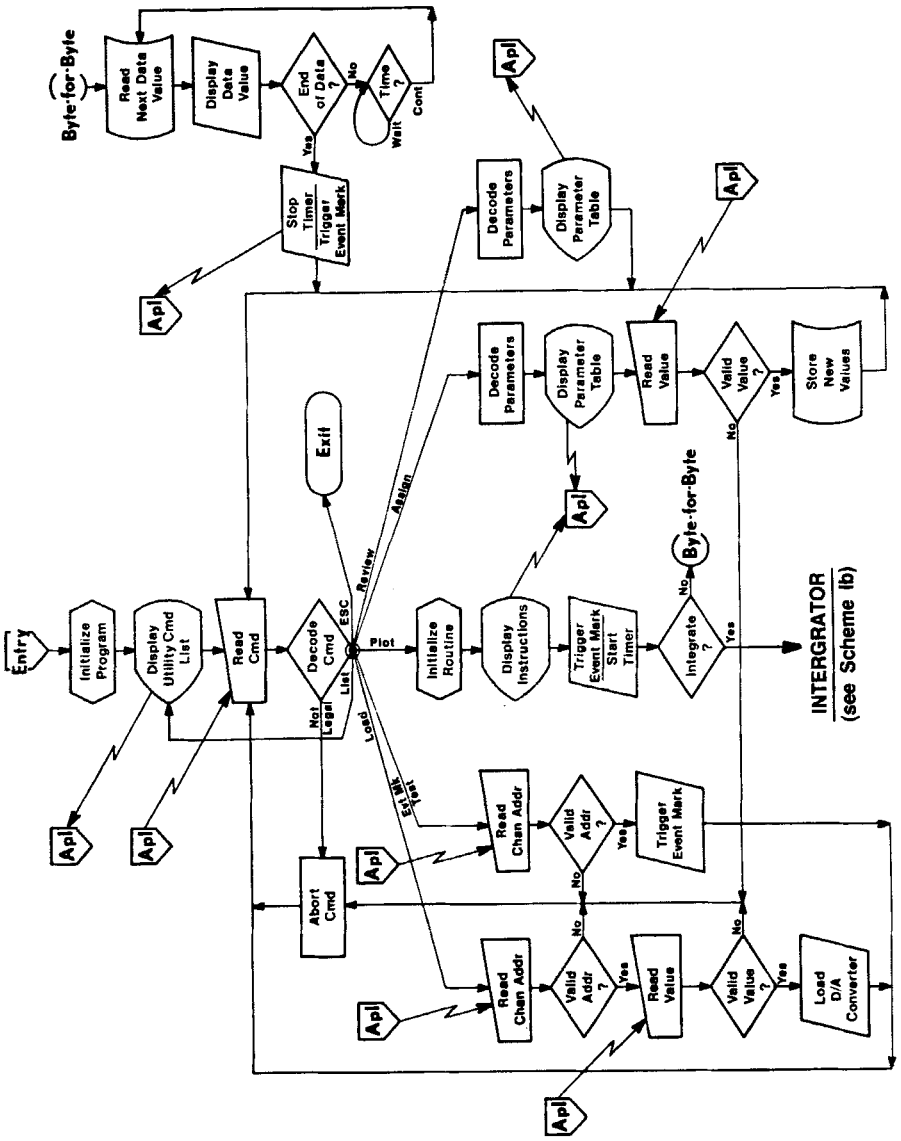
The microcomputer operating system software was essentially the same as that described in earlier publications (2,3). The Turnkey Program -- operated from the Apple II -- provided the operator interface for the Explorer microcontroller programs. The Turnkey automatically loaded the System Supervisor Operating Program as well as the "patched" version of the Apple II disk operating system (DOS 3.3) used by the system. The initialization of the

Explorer, loading and running of the Explorer programs, and storage and retrieval of the chromatographic data were achieved with the help of these programs.

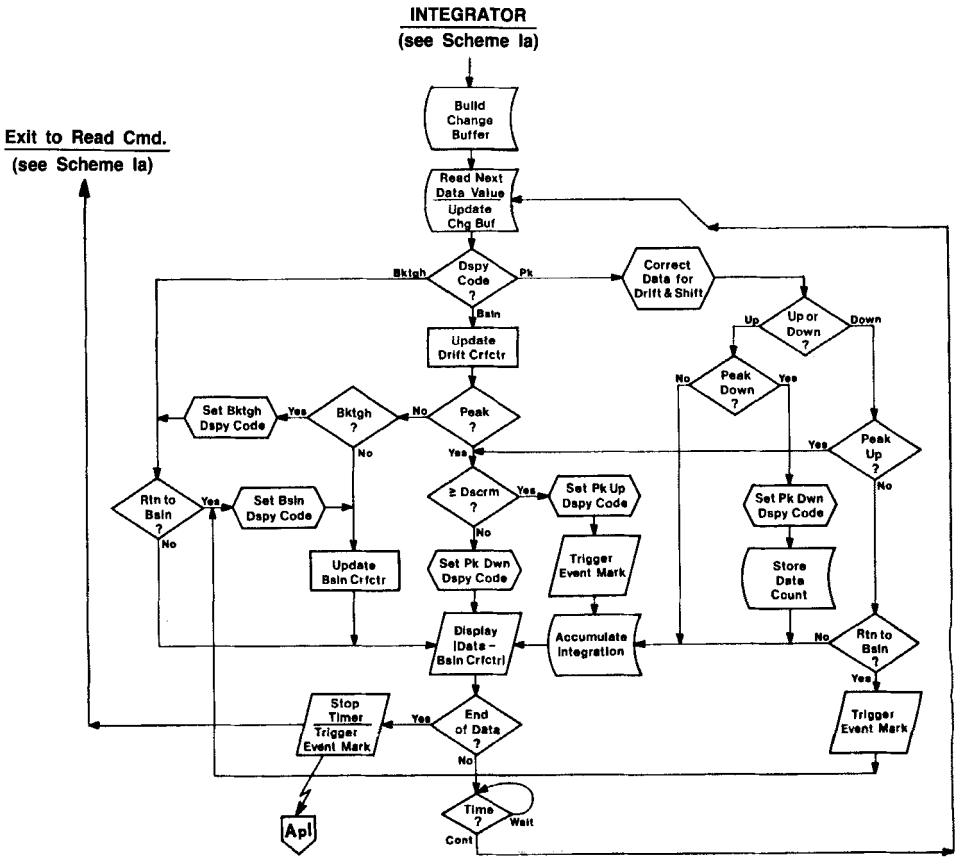
A. Description of Peak Area Integration Programs

A machine-language program, called "CHART REPRO", was used by the Explorer to integrate the chromatograms. The program-flow diagram is shown in Scheme I. This program accepted data and pointers produced by two data acquisition programs described in an ealier publication, and used the same storage buffers ($0F00 - $2FFF) for data stroage (3). The data values in these storage buffers were not altered during the integration process, but were corrected for baseline drift and baseline shifts prior to their display on a strip-chart recorder. The integration was computed on the basis of this corrected version using the Trapezoidal Rule. Each integration value was an accumulation of the sum of the value displayed minus the "baseline-correction factor", and one-half the change from the last data value (i.e., previous minus present data value) over the limits for the peak. Units of integration were relative to the attenuation of the UV monitor used and were in (AUFS/255) X sec units. These values were converted to absorbance units after their transfer to the Apple computer.

The program integrated both monitor channels simultaneously (Channel 0 and Channel 1). Integration values were stored in two buffers, $0F00 to $0F7F for Channel 0, and $0F80 to $0FBF for Channel 1, as a 16-bit unsigned integers conforming to the rules for machine-language variables (i.e., LS-Byte first, immediately fol-



Scheme 1a. Flow diagram of Data Integration Program: Integration Utility Commands. Utility functions are provided for equipment calibration, testing, and entry into the data integration routines.



Scheme 1b. Flow Diagram of Data Integration Program: Integration Routine. The data integration routine is entered through the plot utility command. The principal function of the routine is to integrate the digitalized version of the chromatogram produced by the data acquisiton program (3).

lowed by MS-Byte). Integration values for each peak were stored in consecutive locations in the order the peaks were eluted. These hexadecimal values could have any value from $0000 to $FFFE. The value $FFFF was reserved to indicate an "Integration-Value Overflow" (i.e., when an integration value exceeded the range of a 16-bit binary number), but the value was otherwise meaningless. The maximum number of peaks that could be integrated per integration was limited to 32 due to the limited size of the integration buffers. If this number was exceeded, an "Integration-Buffer Overflow" message was displayed. Integration of chromatograms having more than 32 peaks required dividing the data into sections, each section containing no more than 32 peaks.

Data-count values for the maximum of peaks (related to peak retention factor, Rf) were stored in a buffer between $0FC0 and $0FFF in locations correlating with the integration value for the corresponding peak. The value of this count could be any hexadecimal number from $0001 to $1000 computed in units of seconds.

A "Chart Speed Multiplier" parameter was provided which specified the rate at which the data was output to the strip-chart recorder. This parameter accelerated the rate at which the integration was performed relative to the rate at which it was collected (e.g., a multiplier of four reproduced the chromatogram in one-fourth the time it took to collect it). The multiplier had a range from 1 to 10, limited by the response speed of the strip-chart recorder. The higher speeds were most commonly used as a matter of convenience.

An additional option of the program included reproducing the chromatogram without integration and baseline stabilization. This byte-for-byte reproduction of the chromatogram can be used to check the integrity of the data saved on a disk and to locate sections of the data using the Marking Buffer for examination. An accelerated rate of display for this option was also available.

1. Utility Commands

The necessary utility commands required to perform the integration were included in the integration software. A list of utility commands was displayed when the program was entered and could be recalled at any time. Commands were entered only in response to a prompt (">"). Consequently, old commands had to be terminated before a new command could be issued. Only legal commands were decoded and illegal entries resulted in a default action, designated by a question mark which always aborted the command.

Many of these commands were similar to those used in the data acquisition programs and a similar procedure was followed to conduct the integration (3). The strip-chart recorders were calibrated by loading values directly into the D/A converters. However, the calibration command involved in the calibration of the A/D converters were not included in this program since these converters were not used. After calibration was completed, integration parameters were assigned using a parameter assignment command. These parameters determined: (a) the data range and baseline value to be used for display, (b) the chart-speed multiplier, (c) whether the data was integrated and displayed with baseline stabilization or

reproduced byte-for-byte without integration, and (d) whether or not to display the limits of integration for each peak.

The chromatographic data was integrated and displayed as specified by the parameters when a plot command was issued. The limits of integration for each peak (i.e., the start and end of integration for a peak) were designated on the strip-chart recorder with an event mark if the integrator was instructed to do so. After completing the integration, the program was exited and the data was transferred to the Apple computer for further processing.

2. Marking Buffer

The data count was saved in a Marking Buffer when the "I" key of the Explorer was pressed. This command did not disrupt the integration or display process, although event-mark instructions were issued to the strip-chart recorder to mark the position of the data count. The Marking Buffer resided in memory from $0F20 to $0F2F and could save a maximum of eight marks. After filling this data buffer, the data count was no longer saved, but event-mark instructions were still issued. This procedure was used to determine the data range for different sections of the chromatogram.

3. Integration Overflow

Integration and peak-location buffers were large enough to hold data for a maximum of 32 peaks. When the integrator started integrating the 32nd peak, the message "Integration Overflow Alert" was transmitted to the Apple computer. Integration of this peak was completed; however, the message "Integration Overflow" was displayed if another peak was encountered. Plotting and integration

of the remainder of the data was aborted, and program control was transferred to the Explorer System Monitor.

B. Description of Turnkey Operation

The Turnkey Program provided the same control functions as described in the previous paper (3). The Explorer was initialized and all programs and data were loaded by it. However, the Turnkey program was modified to load "CHART REPRO", and an additional section was added to it specifically designed to process the integration data from the Explorer.

This new Turnkey routine was executed after the integration data was transferred to the Apple computer from the Explorer. The final peak area value was calculated as a zero-dilution area using the same "flow-cell-dilution factor" as in Method A (see Materials and Methods). However, the peak area was determined from the DHC integration value by multiplying it by monitor attenuation divided by 255 (AUFS/255) and the flow rate (ml/sec). On the other hand, the percent mole fractions of each component were calculated directly from the DHC integration value by dividing by the molar extinction coefficient without converting to absorbance units or moles:

$$\% \text{ mole} = \frac{(\text{integration value}/\varepsilon \text{ at } 254 \text{ nm}) \times 100\%}{\Sigma(\text{integration value}/\varepsilon \text{ at } 254 \text{ nm})}$$

These values along with K (retention time) and K' (retention factor) values -- derived from the peak retention time and void volume of the column -- were printed out. A new program or new data could then be loaded into the Explorer and the process repeated.

RESULTS

A. Accuracy of Peak Area Determination

1. Model Ribonucleoside Mixture

A mixture of cytidine and uridine of known concentration was applied to a reversed-phase column and data was collected by DHC at three different full-scale monitor expansions (attenuations). The results were displayed on a strip-chart recorder using different chart speeds and three samples derived from the same solution for each full-scale (monitor) expansion. Peak area of each component, reflecting concentration of the solute, was determined by the usual manual methods (8-10) and by the DHC. A comparison of the two integration methods is made in Table II. The results shown in absorbance units are corrected for flow-cell volume and are compared to the actual amount of each applied to the column. The manual integration method (Method A) produced 17 % and 19 % losses of uridine and cytidine, respectively, when the most sensitive scale (AUFS) and slowest chart speed were used. Similarly, about 10-13 % losses were observed when the least sensitive scale and fastest chart speed were used. In contrast, integration by DHC (Method B) yielded variation within ±2.0 % range for cytidine and from +0.9 to -2.7 % for uridine irrespective of monitor sensitivity or chart speed.

2. Separation of Nucleosides Derived from a tRNA

Three identical samples of pre-determined amounts of nucleosides derived from a tRNA sample using enzymatic hydrolysis were applied to a reversed-phase column and eluted with a programmed gradient (Program A) under identical conditions. DHC results were

TABLE II

COMPARISON OF PEAK AREA DETERMINATION BY TWO INTEGRATION METHODS FOR CHROMATOGRAPHY OF THREE IDENTICAL SAMPLES[a]

Substance resolved	Experimental conditions	Absorbance Units, 254 nm (x 10^3) Amount applied	Peak Integration Method A[b]	Peak Integration Method B[c]	Percent Deviation from amount applied Method A[b]	Percent Deviation from amount applied Method B[c]
Cytidine	d	102	83	104	-19	2.0
	e	102	87	100	-15	-2.0
	f	102	92	103	-10	1.0
Uridine	d	112	93	113	-17	0.9
	e	112	95	109	-15	-2.7
	f	112	97	111	-13	-0.9

[a]A mixture of cytidine and uridine in 15 µl was injected to a C-18 reversed-phase column (0.4 x 25 cm, Rainin Microsorb) and eluted with 0.25 M ammonium acetate, pH 6.0, at 45°C, and 1.5 ml/min; effluent monitored at different AUFS and chart recording speeds (see footnotes below).

[b,c]See Materials and Methods section for details of peak area integration by Method A (manual) and Method B (Data Handling System).

[d]AUFS, 0.5; chart speed 0.5 in./min.

[e]AUFS, 1.0; chart speed 1.0 in./min.

[f]AUFS, 2.0; chart speed 2.0 in./min.

TABLE III

COMPARISON OF PEAK AREA DETERMINATION BY TWO INTEGRATION METHODS FOR A tRNA ANALYSES[e]

Peak No.	Peak Area in Absorbance Units at 254 nm (x 10^2) Method A[b]	Method B[c]
1	0.84 (±0.07)[d]	0.90 (±0.04)
2	1.90 (±0.27)	2.36 (±0.12)
3	4.06 (±0.14)	4.60 (±0.10)
4	0.14 (±0.03)	0.14 (±0.02)
5	2.25 (±0.14)	2.16 (±0.09)
6	8.84 (±0.50)	9.15 (±0.31)
7	0.33 (±0.07)	0.40 (±0.04)
8	3.82 (±0.29)	3.94 (±0.17)
9	0.62 (±0.04)	0.89 (±0.03)
10	0.14 (±0.04)	0.21 (±0.02)
Total[e]	22.9 (±1.04)	24.7 (±0.74)

[a]Three 15 µl samples of a transfer RNA hydrolyzate were applied to a C-18 reversed-phase column (0.4 x 25 cm, Rainin Microsorb), and eluted with gradient program A at 45°C, and 1.5 ml/min; the effluent was monitored at 0.1 AUFS, 254 nm.

[b,c]See Methods and Material section for details of peak area integration by Method A (manual) or Method B (Data Handling System).

[d]Standard deviation of data from mean.

[e]Total absorbance units (at 254 nm) applied to the column was 0.249.

reproduced via strip-chart recorder and integrated by the manual method and the DHC method. The integrations of different peaks for each method are listed in Table III in the order of their elution. The results were consistantly low for the manual-integration method than those for the DHC method by as much as 35 % for peak 10 and as little as 3.4 % for peak 6. Only the integration value for peak 5

exceeded (4.2 %) the DHC value. The total recovery of material as determined by the manual-integration method was 8.0 % less than the total amount of solutes applied to the column (0.249 absorbance Units at 254 nm), while the DHC method showed a loss of only 0.8 %. Futhermore, the standard deviation of the DHC data was one-half of the standard deviation observed for the manual method. These results are consistant with those noted earlier for model compounds.

3. Model Deoxyribonucleoside Mixture

A mixture of five deoxyribonucleosides (amounts pre-determined) was applied to a reversed-phase column and eluted with a programmed gradient (Program B). The mole fraction of a nucleoside was determined by two methods (Table IV): (a) the peak-height-measurement method, the determination of concentration from peak height and a standard curve (concentration versus peak height)(11); and (b) the DHC integration method. Results of the DHC method are in complete agreement with the other method for peaks 1, 4, and 5, but slightly lower for peak 2 and higher for peak 3. Furthermore, the DHC method produced a more consistant analysis for the majority of the peaks than the other method.

B. Application of DHC for Chromatographic Analysis

1.Determination of Nucleoside Composition in a Model Mixture

A complex mixture of the four common and 14 modified ribonucleosides was applied to a reversed-phase column and eluted with gradient program A. The DHC was connected to the monitor, and the digitalized chromatogram was stored on a floppy disk. Integration values -- obtained from DHC in hexadecimal notation (third column)

TABLE IV

COMPARISON OF PEAK AREA DETERMINATION BY TWO INTEGRATION METHODS FOR THE SEPARATION OF A DEOXYNUCLEOSIDE MIXTURE[a]

Peak No.	Nucleosides	Nucleoside Composition in Mole Percent		
		Method C[c] Batch #1	Method C[c] Batch #2	Method B[b] Analysis by DHC
1	dCyd	29.9 (±0.81)[d]	29.0 (±0.14)	28.6 (±0.27)
2	5MedCyd	20.2 (±0.50)	18.9 (±0.35)	18.9 (±0.26)
3	dGuo	15.3 (±0.40)	15.2 (±0.28)	16.1 (±0.15)
4	dThd	23.5 (±0.47)	22.2 (±0.07)	22.8 (±0.06)
5	dAdo	14.2 (±0.23)	13.5 (±0.00)	13.6 (±0.15)

[a]Three 15 µl samples of a mixture of major deoxynucleosides and 5MedCyd were applied to a C-18 reversed-phase column (0.4 x 25 cm, Rainin Microsorb) and eluted with gradient program B at 50°C and 1.0 ml/min; effluent monitored at 0.1 AUFS, 254 nm, and 0.08 AUFS, 280 nm.

[b,c]See Methods and Materials section for details of peak area integration by Method B (Data Handling System) or by Method C (standard calibration curve method of peak heights).

[d]Standard deviation from the mean.

and converted to decimal equivalent (forth column) -- are listed in Table V in the order they were eluted from the column. Final calculation of peak area (in absorbance units, last column) is based on full-scale expansion of the detector (254 nm; 0.1 AUFS) and flow rate (1.5 ml/min), and is corrected for dilution of the sample (15 microliter) caused by the volume of the flow cell (19 microliter). The results indicate that the total absorbance (sum of all peak areas) determined by the DHC method was 1.4 % less than the amount applied to the column. The peak characterization of the nucleosides was based on our earlier work and those of others (13).

2.Determination of DNA Composition

An enzymatic hydrolyzate of a DNA sample was applied to a RP column and eluted with gradient program B. The chromatography was followed by DHC connected to the UV monitors (254 nm and 280 nm). The results expressed as percent mole (deoxynucleoside) composition were calculated from DHC integration values using the appropriate molar extinction coefficient derived at 254 nm for each deoxynucleoside. A second peak identified as deoxyinosine (dIno, an artifact produced by deaminase contaminant of the hydrolyzing enzyme, DNAse I) was established from the reference compound (14). Ratio of dCyd plus 5MedCyd to dGuo was 1.1, while the ratio of dThd to dAdo plus dIno was 1.01. The same material analyzed using different columns and using the peak-height-measurement method yielded slightly lower ratios (dCyd + 5MedCyd/dGuo: 1.05 and dThd/dAdo: 0.97; Singhal et al, 1986 unpublished results).

In Figure 1, an actual separation of this DNA hydrolyzate is shown. In this particular experiment, in addition to dIno, another

TABLE V

COMPOSITION OF A COMPLEX MIXTURE OF RIBONUCLEOSIDES[a]

Peak No.	Nucleosides	Peak Area Integration by DHC: Hexadecimal[b] (Explorer)	Peak Area Integration by DHC: Decimal[b] (Apple II)	Peak Area Integration by DHC: Converted into A_{254} Units[c] ($\times 10^2$)
1	Ψrd	0F0F	3855	5.1
2	Cyd	0D18	3352	4.4
3	Urd	1133	4403	5.7
4	5MeCyd	0DF9	3577	4.7
5	1MeAdo	019E	414	0.5
6	2'MeCyd	087C	2172	2.8
7	Guo + 5MeUrd	0B60	2912	3.9
8	7MeGuo	0A3F	2623	3.5
9	2'MeUrd	099A	2458	3.2
10	2'MeGuo + 1MeGuo	243D	9277	12.1
11	Ado	097F	2458	3.2
12	$2Me_2$Guo	085B	2139	2.8
13	2'MeAdo	01F2	498	0.7
14	2MeAdo	0751	1873	2.4
15	6MeAdo	1311	4881	6.4
16	$6Me_2$Ado	118A	4489	5.9
			Total	67.3

[a] A 15 μl sample of four major and 14 minor modified ribonucleosides were applied to a C-18 reversed-phase column (0.4 x 25 cm, Rainin Microsorb) and eluted with gradient program A at 45°C and 1.5 ml/min; the effluent monitored with 0.1 AUFS, 254 nm and the results recorded by DHC. The nucleoside mixture applied to the column contained a total of 0.685 A_{254} units.

[b] Integration values reported as obtained directly from DHC. The Explorer or the decimal equivalent of the hexadecimal value produced by the Apple II computer.

[c] Integration values converted to A_{254} units: Peak area (in A_{254} nm units) = [integration value] x [AUFS/255] x [elution flow rate (in ml/min)/60 sec] x [flow cell dilution factor (flow cell volume/sample volume)]. (See Materials and Methods.)

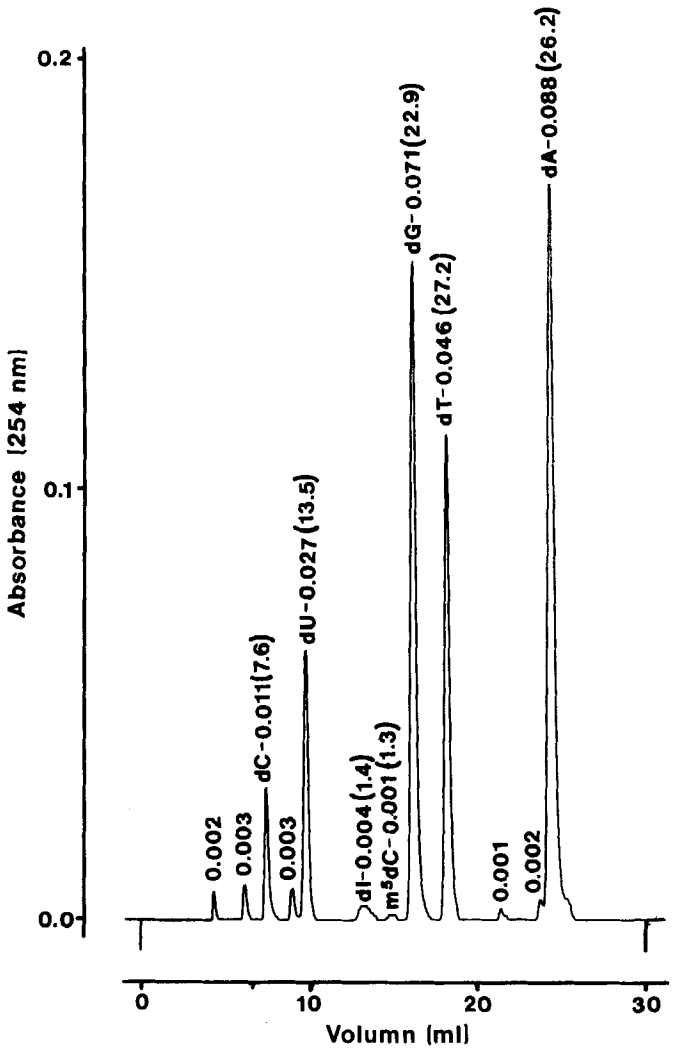


Figure 1. Integration of a Chromatography of DNA Hydrolyzate. An enzymatic DNA hydrolyzate (0.26 Abs. units at 254 nm) was applied to a pre-equilibrated C-18 RP-Hplc column (4.5 X 250 mm) and eluted with buffer A (10 mM ammonium phosphate, 2.5 % ethanol v/v, pH 5.3) for 5 min followed by elution with a linear gradient to 82.1 % buffer A and 17.9 % ethanol for 25 min. at 40°C. Integration values in absorbance units (at 254 nm) are shown above each peak with mole percent designated in parenthesis for appropriate peaks.

peak (peak 5) corresponding to deoxyuridine was found. (It is apparently derived from deamination of dCyd, peak 3.) Other peaks: 1, 2, 4, 10, and 11 were found to be due to salts and hydrolytic enzymes. All peaks were integrated and the results were converted to absorbance units. However, only DNA components were used to calculate the mole percent composition of the DNA sample. The results of three independent analyses gave ratios of: dCyd + dUrd + 5MedCyd/dGuo, 1.00 and dThd/dAdo + dIno, 0.99.

DISCUSSION

Liquid chromatography (LC) not only offers isolation and purification of the components in a complex mixture, but also furnishes quantitative and qualitative information about each constituent of the mixture. This can be achieved in a single chromatography provided peak monitoring is carefully linked to a data-acquistion and retrieval system. To simplify and facilitate data collection, storage, retrieval, quantitation, and characterization, we have interfaced a typical Hplc system to a dual-microprocessor controlled data-handling system (DHC).

The major goal of this work is to provide a convenient and practical means of peak integration for chromatograms of any complexity. The integration program ("CHART REPRO") of the Explorer is written for this purpose and can be merged with the data acquisition programs -- described earlier -- to produce a true "real-time" integrator. Baseline stabilization routines and automatic peak-splitting features are included in the integrator to simplify operations while maintaining a high degree of accuracy.

A. Peak Area Integration by Data-Handling Computers

1. Advantages

Evaluation of the data by DHC is undoubtedly superior to manual methods. For example, peak areas determined by the microcomputers are consistantly accurate as shown in Tables II and III due to the high precision used for acquistion of the chromatographic data by these computers. On the other hand, manual methods are dependent on subjective measurements and approximations, and consequently, the values determined by these methods are less reliable.

Integration by computers is convenient and fast. An average of five minutes is needed by this method to integrate ten chromatography peaks, including conversion of DHC integration units to absorbance units, and print-out of the results. Such a chromatogram required approximately three hours by the manual methods.

2. Overlapping Peaks

A major reason for this system's consistancy and accuracy is its ability to recognize and analyze irregularities in the chromatogram (such as peak-overlap and baseline drift). As shown in Figure 2, two overlapping peaks contain solute from the neighboring peak. Assumming two closely eluting peaks have common symmetry, the triangle produced by their overlap (ABC) should be approximately isosceles. Hence, by suspending peak integration of the first peak and starting integration of the second peak at the cross over point (B), the integration of each peak is compensated for the intrusion of the adjacent peak. Also, as a consequence, a smaller portion of the peak is required to determine its entire area then is required by the manual methods.

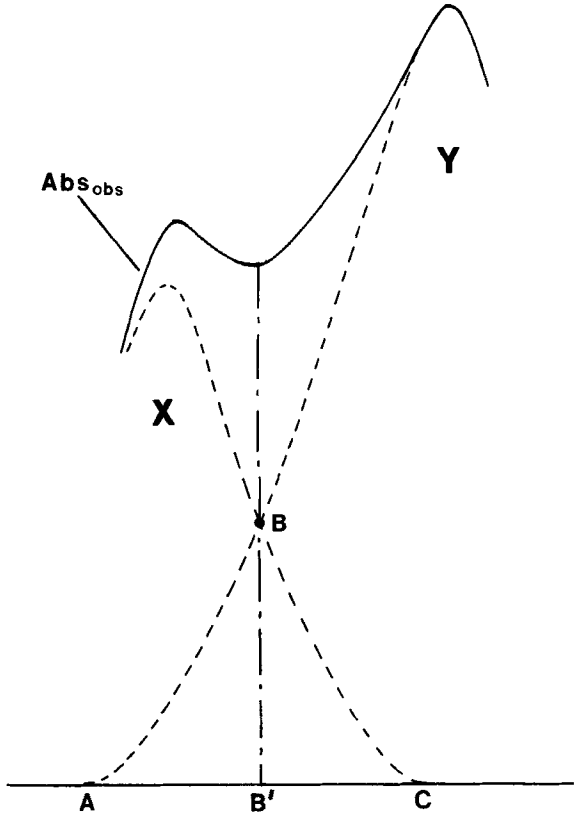


Figure 2. Representation of Two Hypothetical Overlapping Peaks, X and Y. The isosceles triangle, ABC, produced from the overlap of the two peaks is divided into two equal triangles, ABB' and BB'C, by the perpendicular line, BB' from the crossover point, B. The observed absorbance (Abs-obs) is the sum of the absorbance of each component in this region.

3. Baseline Fluctuations

Changes in the baseline involving a gradual loss ("drift") or an abrupt increase ("shift") from a constant value are the result of changes in the effluent absorbance. These baseline fluctuations often cause errors in the integration of especially smaller peaks.

To avoid or to minimize these errors, the DHC continuously subtracts a "baseline-correction factor" from all data points of a peak. This procedure essentially shifts the chromatogram to a constant baseline value and thus eliminates the baseline drift in the chromatogram. The "baseline-correction factor" at any time during the integration is based on the baseline value of the previous 32 seconds. The integrator, however, fails to correct immediately for baseline shifts. It initially tends to interpret a shift as a peak. If the baseline is not re-established within the next 64 seconds, a "shift factor" is included in the "baseline-correction factor" in order to accelerate return to the baseline. For peaks having a shift within their boundaries, the effect is the same as that expected from increasing the incline of the baseline. Peaks having no baseline shift and narrower than 64 seconds are not affected at all.

A baseline fluctuation is encountered when a column is eluted with one void volume. This "breakthrough" is the result of an abrupt solvent (refractive index) change in the flow cell resulting in a sudden absorbance change; however, unlike baseline shift, the absorbance returns to the initial baseline value. The integration program can normally handle a "breakthrough" baseline fluctuation. However, at lower monitor sensitivities, the absorbance may not change significantly enough for the program to detect a sudden drop due to the "breakthrough". Consequently, the program recalculates the "baseline-correction factor" to shift the baseline-date value down instead of jumping to the "breakthrough display" routine. The

integrator, as a result, interprets the subsequent return to baseline as the start of a new peak bearing an incorrect baseline. However, this problem was normally corrected by allowing the integrator to naturally re-establish the baseline via the "baseline-correction factor", or by manually erasing the "breakthrough" from the chromatographic data if it interfered with the integration.

4. Integration of Peak Area by Calibration Curve

Peak area determination using a calibration curve proposed by Singhal (1986, ms. submitted) improves accuracy and reproducibility over the other manual method, i.e., approximation by a gaussian curve -- a procedure used here as a reference for the integration method (9,10). A comparison of peak integration by DHC and the calibration curve method as shown in Table IV indicates that the results are in good agreement (with the exception of peak 2). The calibration curve method, however, is not a practical method since curves cannot be constructed for unknown solutes or used universally since the equilibrium constant (Kd) of the solute depends on LC conditions. The DHC integration method, however, is free of these limitations.

B. Application of Integration by DHC

A complex chromatogram is integrated in Table V. Here the DHC integrator identified and integrated 21 peaks, including one peak due to "breakthrough" and four peaks identified as contaminates/artifacts of the buffer and sample. Though extensive overlapping of peaks is encountered, total absorbance units of the solutes, as determined from the sum of the integrations by DHC, is 98.6 % of that applied.

CONCLUSION

Use of the integration value obtained directly from DHC simplifies the calculations for percent mole composition (for example see Tables IV and Figure 1). The agreement in the base pair ratios [(dCyd + 5MedCyd + dUrd)/(dGuo) and (dThd)/(dAdo + dIno)] with the expected value of 1.00 indicates consistancy of integration of the peaks regardless of peak size or geometry. These results demonstrate the ability of the LC-DHC system to detect and assay even minor, modified components present in very minute amounts in various RNA's and DNA's. In many instances, this degree of accuracy in data processing is not possible or practical by conventional manual methods.

The ability of DHC to record and integrate two detector channels simultaneously makes it feasible to redress errors inherent in the integration of the small peaks. This error, which is inversely proportional to the size of the peak, can be reduced by monitoring effluent simultaneously at two different detector sensitivities for a common wavelength, and by substituting the scaled-down integration value for the smaller peaks. Error in small peaks can thus be reduced by the ratio of the two detector attenuations.

To compensate for the baseline shifts and to discriminate for "breakthroughs" in the chromatogram is difficult since both events are unpredictable. An alternative, though, is to remove these irregularities from the chromatogram prior to the integration. As discussed earlier, baseline drift, baseline shift, and "breakthrough" are the result of effluent changes inherent to the gra-

dient program; hence, they are also present when a control blank is injected and the column is eluted as usual. This yields a "control" chromatogram which can be subtracted from the actual "experimental" chromatogram. Thus, correction of a chromatogram with a "control" can simplify baseline stabilization and improve the accuracy of integration. A "control" can also be used to delete peaks of artifacts found in the sample and buffer.

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REFERENCES

1. Smoll, D. B. and Singhal, R. P., Hardware for microprocessor controlled HPLC: Interfacing of an interval timer and interrupt controller to the S100 Bus System, J. Liquid Chromatogr., **6** (6), 2095, (1983).

2. Singhal, R. P. and Smoll, D. B., Hardware and Software for microprocessor controlled HPLC; Interfacing of converters (A/D and D/A) for data acquistion and display, J. Liquid Chromatogr., this volume, (1986).

3. Smoll, D. B. and Singhal, R. P., Software for microprocessor controlled HPLC: Data acquisition and display, J. Liquid Chromatogr., this volume, (1986).

4. Long, E. C., Managing laboratory data on a desktop personal computer: part three, Amer. Lab., **16** (10), 96, (1984).

5. Lisowski, G., Amer. Lab., **16** (2), 118, (1984).

6. Brenner, M. and Sims, C. W., Amer. Lab., **13** (10), 78, (1981).

7. Ogdin, C. A., in Microcomputer Design, Prentice-Hall, Inc., Englewood Cliffs, (1978), p. 128.

8. Cohn, W. E., The anion exchange separation of ribonucleosides, J. Amer. Chem. Soc., **72**, 1471, (1950).

9. Singhal, R. P. and Cohn, W. E., Analytical separation of nucleosides by anion-exchange chromatography: influence of pH, solvents, temperature, concentration, and flow rate, Anal. Biochem., **45**, 585, (1972).

10. Anderson, N. G., Green, J. G., Barber, M. L., and Ladd, F. C., Analytical techniques for cell fractions, nucleotides and related compounds, Anal. Biochem., **6**, 153, (1963).

11. Singhal, R. P., unpublished results.

12. Rogers, D. W. and Gratzer, W., Numerical integration using a personal computer, Amer. Lab., **16** (9), 78, (1984).

13. Buck, M., Connick, M., and Ames, B. N., Complete analysis of tRNA-modified nucleosides by High-Performance Liquid Chromatography, Anal. Biochem., **129**, 1, (1983).

14. Singhal, R. P., Separation and analysis of nucleic acids and their consituents by ion-exclusion and ion-exchange chromatography, in Separation and Purification Methods, Perry, E. S., van Oss, C. J., and Grushka, E., Ed., Mercel Dekker, Inc., New York, (1975), p. 339.