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VERSATILE MICROCOMPUTER-CONTROLLED, AUTOMATED GRADIENT ANALYTICAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM*

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SUMMARY

We have developed a computer-controlled high-performance liquid chromatography (HPLC) system which generates binary gradients for automated analyses of a large number of samples. The 64K RAM S-100 laboratory microcomputer was programmed to control Waters 6000A and M45 HPLC pumps while simultaneously gathering data from Waters 440 absorbance and 420 fluorescence detectors (2 A/D channels, ± 5 V, 12 bit resolution). The S-100 was interfaced with a Micromeretics 725 autoinjector. Data were stored on 8-in floppy disks, which have a capacity for storage of up to 128 HPLC runs. The operator establishes parameters from menu prompts; one of eight convex or concave gradients, or a linear gradient may be chosen. The software will automatically control HPLC gradient analyses of up to 64 samples, including return of columns to initial conditions before each injections. Data are collected during analyses from the detectors, stored on the disks, and may also be plotted on a printer during the actual analysis. Data may be retrieved and analyzed to prepare reports with peak retention times and peak areas.

INTRODUCTION

The use of autoinjectors with high-performance liquid chromatographs has greatly simplified the analysis of multiple samples. However, if one wishes to analyze multiple samples using a gradient controller for separations, an autoinjector does not offer any advantages over manual injection devices. To obtain automated, unattended gradient high-performance liquid chromatographic (HPLC) analysis of multiple samples requires expensive, dedicated computer-controlled systems. An alternative to these systems is to interface a less expensive microcomputer with a HPLC system. This is particularly attractive if it is not desirable to replace an existing HPLC system. A number of computer-HPLC controllers are available, but most only function as

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pump controllers; few are designed to handle pump control, data storage, and data processing. The most desirable approach is to design a laboratory computer system capable of simultaneously monitoring a series of detectors, gathering and storing data, and controlling at least two pumps during gradient separations. A further requirement is to interface such a system with an autoinjector to make multiple analyses possible.

We report here software developed by us to allow unattended, automated analyses of multiple samples using gradient HPLC separations. The system can be interfaced with existing HPLC detectors and pumps.

EXPERIMENTAL*

HPLC was carried out with two Waters pumps (6000A and M45, Waters Assoc., Milford, MA, U.S.A.), equipped with absorbance (Waters 440) and fluorescence (Waters 420) detectors and a Micromeretics 725 autoinjector (Micromeretics Instruments, Norcross, GA, U.S.A.). HPLC separations were achieved with a 25-cm Supelcosil 5 μm LC-18 column, preceded in line by a precolumn, containing a LC-18 cartridge (Supelco, Bellefonte, PA, U.S.A.). Output was recorded on a Varian A-25 strip-chart recorder (Varian, Palo Alto, CA, U.S.A.).

The laboratory computer system (Fig. 1) consisted of an S-100 General Purpose Computer (Centre Computer Consultants, State College, PA, U.S.A.) with a Z-80 4-MHz central processing unit (64K random access memory, internal clock accuracy ± 1 sec/month). The microcomputer was equipped with two double-sided/double-density floppy disk drives (1000 K per disk) and two serial and two parallel ports. The system is controlled with the CP/M-80 disk operating system. The computer was operated from a Televideo 910 (TeleVideo Systems, Sunnyvale, CA, U.S.A.) keyboard CRT monitor, and output went to either an IDS Prism 80 printer (Integral Data Systems, Milford, NH, U.S.A.) or a Zenith Model 121 graphics monitor (Zenith Radio, Chicago, IL, U.S.A.). The system was operated by a software package consisting of a HPLC controller (described in Results and Discussion), a peak integrator (PEKPKR), and graphics and curve fitting (GRAPH). The HPLC program was developed with a Fortran compiler and linker (Fortran-80, Microsoft, Bellevue, WA, U.S.A.).

The 6000A pressure monitor output and the two detector signal outputs were connected to three of eight S-100 A/D input channels (±5 V), 12 bit resolution, programmable gain). The "inject start" signal from the autoinjector was connected to one of eight S-100 DC volt on/off input channels (opto-isolated). The pumps were interfaced with the computer through a plug-in board containing a software-operated stepping motor speed controller for each pump.

Reagents and chemicals

The mobile phase was composed of 15 m*M* sodium phosphate buffer (pH 7.2) (solvent A) and 55% tetrahydrofuran (THF) in solvent A (solvent B). The amino-

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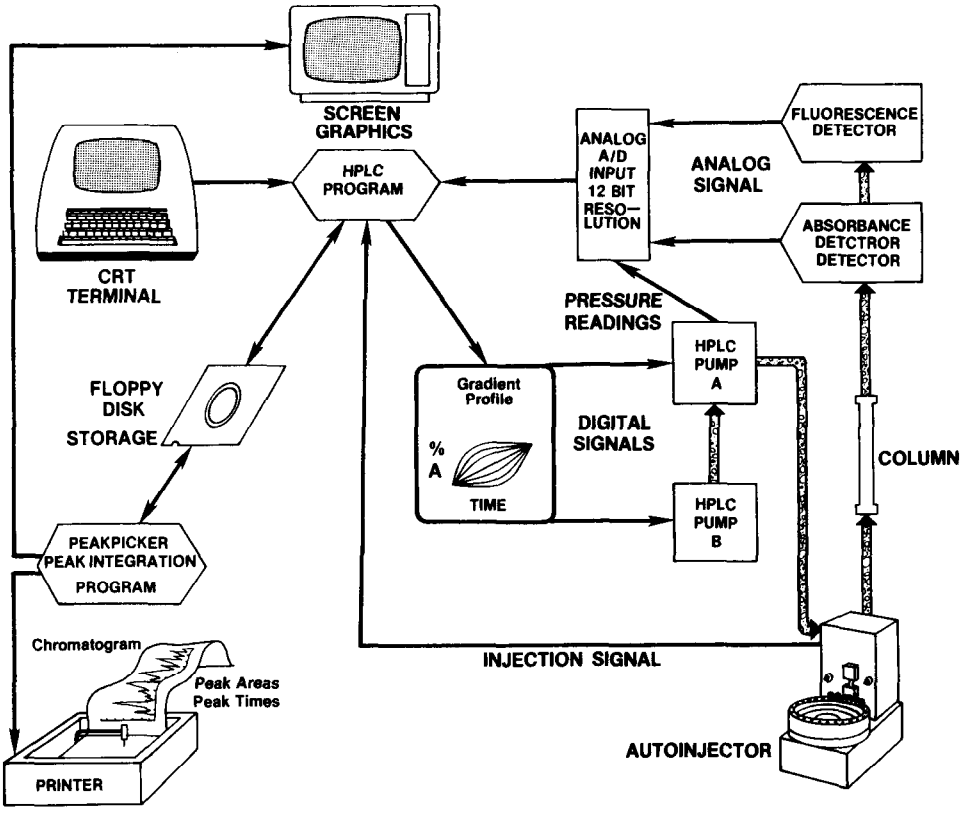


Fig. 1. Schematic diagram of the S-100 laboratory computer interfaced with HPLC equipment.

acid mixture was purchased from Pierce (Rockford, IL, U.S.A.) and *S*-methyl-L-cysteine sulfoxide (SMCO) was synthesized[1]. All reagents and organic solvents were HPLC grade; distilled water had a conductivity reading of at least 1.5 MΩ.

Derivatization

HPLC separation of *o*-phthalaldehyde (OPA) (Fluoropa, Pierce) was based on previous methods[2,3]. Derivatization reagent was prepared by dissolving 100 mg of OPA in 5 ml of absolute methanol; to this 0.1 ml of ethane thiol (Pierce Chemical) was added. Amino acids were derivatized by adding 0.1 ml of 0.5 *M* potassium borate buffer (pH 10.4) to 0.7 ml of methanol containing 1 nmole of each amino acid, followed by 0.1 ml of OPA solution. After mixing, the derivatized compounds were immediately transferred to an injection vial and subjected to gradient HPLC analysis (injection volume, 17 μl).

Chromatography

The column was equilibrated with 25% solvent B at a flow-rate of 0.8 ml/min. At injection, gradient No. 7 (Fig. 2) was initiated; the final condition of 75% solvent B was reached at 20 min and held for an additional 8 min before returning to initial conditions.

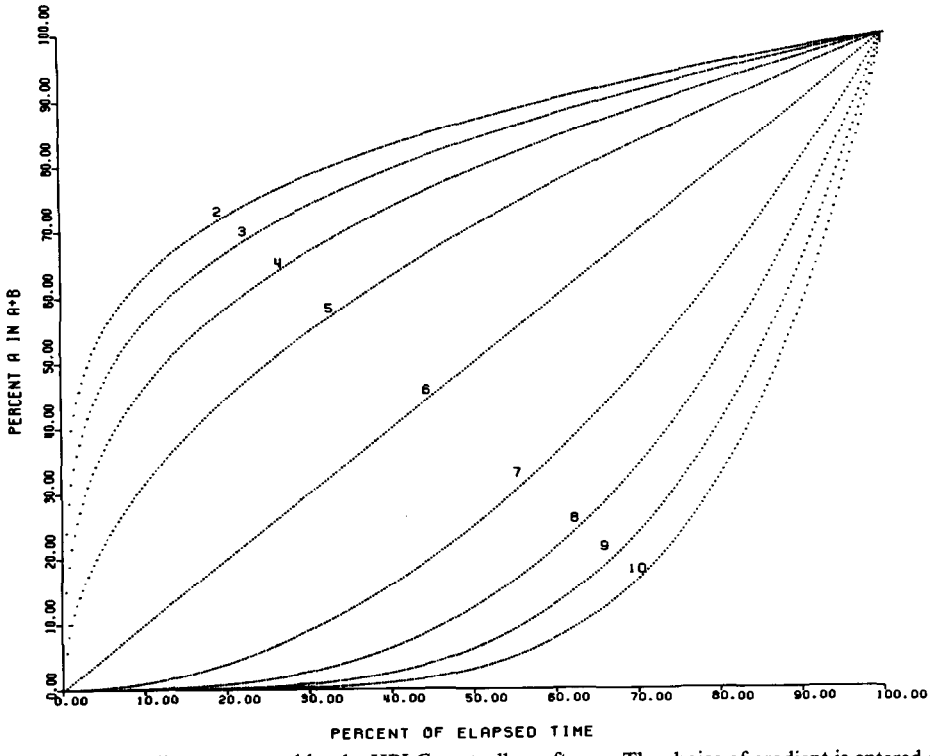


Fig. 2. Nine gradients generated by the HPLC controller software. The choice of gradient is entered after the prompt as shown in Fig. 4. The total time and initial and final percent of each solvent are chosen by the operator and entered in response to prompts.

RESULTS AND DISCUSSION

The HPLC controller and data collection and storage software was developed along the lines suggested by Salin[4] with Fortran-80. Extensive use was made of the CP/M 80 library linking capability. With the program running, HPLC analyses are handled from menus and prompts to make set-up and operation straightward. After the program is loaded and executed, the menu (Fig. 3) is displayed on the terminal screen. After entering CMD 1, the input parameters for the previous run are displayed (Fig. 4); at this point the operator may change any of the parameters by entering new data. In this way one can step through the parameters until they are satisfactory and then return to the menu upon a specific keystroke. Solvent composition, flow-rate, and run-time parameters are entered while in the set-up mode (Fig. 4). Note that data may be gathered both during the gradient time period and for a specified period of time after the gradient is complete; pumps may be returned to initial conditions after completion of the gradient or at the end of the post-gradient run. Following injection, initiation of the gradient profile may be delayed for a time period specified at set-up. This allows isocratic separation of low retention components before application of the gradient. Data is collected and stored on disk during this time. While setting up parameters, the operator may choose to gather data from

```
CMD            DESCRIPTION
 1   SETUP PARAMETERS FOR AN HPLC RUN
 2   GO TO INITIAL CONDITIONS
 3   SET AND GO TO INITIAL CONDITIONS
 4   START RUN
 5   SAVE SETUP PARAMETERS FOR NEXT PROGRAM RESTART
     CTRL C TO EXIT
```

Fig. 3. HPLC controller menu, as displayed on the CRT screen. This menu is shown after the S-100 has been successfully booted and the HPLC program loaded and executed.

```
INPUT PARAMETERS FOR RUN SETUP

DATE:  3-21-84
INITIALS:  DLG
DESCRIPTION:
SMCO
TOTAL FLOW RATE ML/MIN: 0.8
SOLVENT A:  PO4 BUFFER   SOLVENT B:  BUFFER/THF 45:55
INITIAL %B:  25 FINAL %B:  75 GRADIENT CHOICE NUMBER:  7
TIME OF RUN (MINUTES):  20.0
POST INJECTION TIME (MINUTES):0
CHANNEL 0 DETECTOR TYPE:F CHANNEL 1 DETECTOR TYPE:U GATHER DETECTOR:ZERO
INJECTION VOLUME (MICRO LITERS):  17.0
PRESSURE ALARM MINIMUM (PSI):  200
SAMPLING INTERVAL (SECONDS):  0.5
RETURN TO INITIAL AFTER GRADIENT:NO
POST GRADIENT RUN TIME (MINUTES):  5.0
GATHER DATA DURING POST GRADIENT RUN:YES
RETURN TO INITIAL COND AFTER POST GRD RUN:YES
NUMBER OF INJECTIONS:1
CHART LEFT SIDE MARGIN=  0.600E-01
CHART LEFT SIDE MARGIN=  0.00E+01
NUMBER OF CHART PAGES=  1
COMMENT:
```

Fig. 4. Run parameters displayed on the CRT screen. These are displayed after entering choice No. 1 from the HPLC controller menu (Fig. 2). Parameters are changed by entering the requested information; if a parameter does not need to be changed, it may be bypassed without changing it. F = Fluorescence detector; U = UV Detector. Chart margin values are used for printer plotting.

one or both detectors (at intervals of 0.5 sec or longer) and, if desired, the data can be plotted by the printer during the run. The HPLC controller can automatically control gradient elution and column equilibration for 1–64 samples. One of nine gradient curves (four convex, four concave and one linear) may be selected. Gradients are generated during runs as an exponential function of elapsed time:

$$\%A = \%A_i + (\%A_f - \%A_i)\,(TIME_c \div TIME_f)^{exp} \quad (1)$$

where $\%A_i$ is the initial percentage of solvent A, $\%A_f$ is the final percentage of solvent A, $TIME_c$ is the current time for %A, and $TIME_f$ is the time at which the gradient is complete. Exponent values are 1/5, 1/4, 1/3, 1/2, 1, 2, 3, 4 or 5 for gradient curves 2 through 10, illustrated in Fig. 2. Pump speeds are updated 1–20 times per second, depending on how many other computations and sensing operations are being handled at any given time. Since the minimum time for a gradient run is 5 min, there are at least 300 updates for pump motor speeds during a gradient run, thus the program provides smooth, reproducible gradients. To save the setup parameters on a disk file, CMD 5 is entered from the HPLC menu (Fig. 3).

To initiate analyses, CMD 4 is entered and a prompt is displayed requesting a six-character file name. As the samples are processed, a two-digit number is appended to the file name, beginning with 01 and increased by 1 for each run. The injection cycle time for the autoinjector is selected so that at the time of injection the column has had sufficient time to equilibrate at initial conditions. For the column conditions described here, the optimum equilibration time was 8 min. Data gathered from the fluorescence detector during separation of OPA-amino acids were stored on a floppy disk. Later, the data were analyzed with the PEKPKR program (Fig. 5).

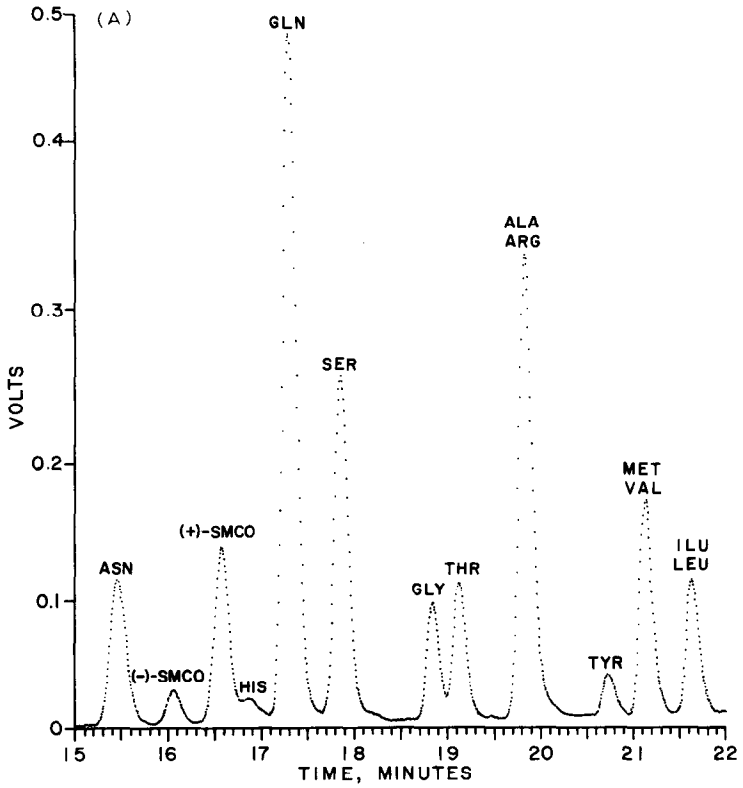
```
SMOOTHING      =      NO      THRESHOLD      =    0.2000000E+00
GATE           =      3       WIDTH FACTOR   =            1.00
START          =      0.0     STOP TIME      =           27.0

CMD            DESCRIPTION
 1   OUTPUT PEAK INFORMATION SUMMARY
 2   PLOT THE DATA
 3   DISPLAY VOLTAGES AT A SPECIFIED TIME
 4   SEARCH STANDARD FILE FOR TIME MATCHES
 5   SET SMOOTHING ON/OFF
 6   SET THRESHOLD VOLTAGE REQUIRED TO START PEAK
 7   SET "GATE" TO N POINTS IN A ROW DEFINE A RISE
 8   SET "WIDTH" PARAMETER TO DEFINE END OF A PEAK
 9   SET NEW ZERO TIME
10   CHANGE WINDOW TIMES FOR ZONE OF INTEREST
11   SET MANUAL BASELINE PARAMETERS
12   PICK PEAKS WITH LATEST PARAMETER SETTINGS
13   READ IN A NEW FILE FROM DISK
14   DUMP DATA
15   EXIT TO SYSTEM
```

Fig. 5. Peak picker menu. The parameters displayed at the top of the CRT screen can be changed by calling up any of the fifteen options shown on the lower part of the screen.

The menu displays the parameter settings for analyzing data from a chromatographic separation. The displayed commands are selected in order to reset parameters and to analyze the data. As part of another study, optimum conditions were determined for separation of SMCO diastereo isomers from each other and from amino acids in *Brassica* extracts. A portion of the data was analyzed by PEKPKR and plotted by the Prism 80 printer (Fig. 6A); peak locations and areas were computed and printed (Fig. 6B). To test gradient reproducibility, OPA derivatized SMCO was injected into a Supelcosil LC-18 column. The two diastereoisomers were separated with gradient No. 7 using the same column conditions as in Fig. 6A. Table I gives peak elution times of the SMCO isomers determined from runs on five separate days. The standard deviations (S.D.) were small for elution times determined on the same day, but the S.D. was high when elution times from different days were compared. Even though day-to-day variation for retention times was greater, peak areas were not affected, as evidenced by the low S.D. for peak areas over a time period of two weeks (31.4 pmoles (+)-SMCO; peak area 6.17 ± 0.33 units; $N = 7$). Generated gradients would not be expected to vary greatly, because the primary factor affecting reproducibility is the computer's internal clock which has a precision of 0.003% and accuracy of ± 1 sec/month. Thus, day-to-day variation was due to changes in solvent composition, pH, or temperature, and not to differences in the computer-generated gradient.

This computer-operated HPLC analysis system was designed to allow opera-



(B)

AAS13102HPO
Threshold: 0.002 Gate: 1 Width Test: 2.000 Smoothing On, Auto Baseline
Zero Time @ 0.000 Window Start @ 12.000 Window End @ 22.000

Start	Type	End	Type	Crest	Abs Hgt	Bas Hgt	Area	% Tot	Width
15.080	B	15.840	B	15.467	0.103	0.100	0.124653E+01	7.05	0.10
15.840	B	16.313	B	16.060	0.027	0.023	0.250726E+00	1.42	0.09
16.313	B	16.787	B	16.573	0.126	0.114	0.117638E+01	6.66	0.09
17.080	B	17.653	B	17.300	0.479	0.469	0.481441E+01	27.25	0.09
17.653	B	18.393	B	17.860	0.245	0.235	0.247184E+01	13.99	0.09
18.620	B	18.993	V	18.840	0.086	0.079	0.737026E+00	4.17	0.09
18.993	V	19.440	B	19.127	0.100	0.093	0.922497E+00	5.22	0.09
19.587	B	20.380	B	19.840	0.326	0.319	0.341526E+01	19.33	0.09
20.567	B	20.960	B	20.740	0.037	0.028	0.273283E+00	1.55	0.09
20.960	B	21.440	B	21.140	0.157	0.147	0.145899E+01	8.26	0.09
21.440	B	21.940	B	21.640	0.103	0.092	0.902773E+00	5.11	0.09

Fig. 6. (A) Separation of *Brassica* amino acids using HPLC controller software. An aqueous methanol extract (50 μl) of *Brassica rapus* L. was derivatized with OPA and injected onto a C_{18} column, equilibrated with 25% solvent B in solvent A. At injection, gradient No. 7 was initiated, with final conditions of 75% of solvent B in solvent A reached after 17 min. Final conditions were held for 5 min, and data were collected for the full 22 min. Data were analyzed and plotted with PEKPKR. (B) Retention times and peak areas of amino acids from PEKPKR analysis of data collected from the gradient separation in A.

TABLE I

RETENTION TIMES FOR OPA-(±)-*S*-METHYL-L-CYSTEINE SULFOXIDE DETERMINED FOR REPLICATE GRADIENT SEPARATIONS ON FIVE DIFFERENT DAYS

Amount of compounds injected, 60 pmol. Column and gradient conditions were the same as in Fig. 5.

Day	*Compound*	*Retention time (min)*	*N*	*S.D. (min)*
1	(−)-SMCO	17.94	8	0.12
	(+)-SMCO	18.39	8	0.11
2	(−)-SMCO	13.36	8	0.20
	(+)-SMCO	13.99	8	0.19
3	(−)-SMCO	15.06	7	0.05
	(+)-SMCO	15.62	7	0.04
4	(−)-SMCO	14.87	7	0.17
	(+)-SMCO	15.43	7	0.16
5	(−)-SMCO	16.09	9	0.14
	(+)-SMCO	16.63	9	0.14
1–5 combined	(−)-SMCO	15.46	5	1.69
	(+)-SMCO	16.01	5	1.63

tion of HPLC equipment in much the same way as one would carry out HPLC runs manually. This approach makes control of HPLC gradient separations a natural extension of normal manual operations, and training to use the computer is minimal. The greatest advantage of the system is that it frees the persons using it for other laboratory tasks while samples are being analyzed. The system gives excellent reproducibility of retention times as long as the analyses are performed on the same day, because the column conditions are accurately reproduced for each run. Excellent reproducibility is obtained for peak areas over weeks. Several safety features were also included to prevent column or pump damage and to alert the operator to conditions that will prevent a desired separation. When the operator sets up run parameters, he enters a minimum pressure to be tolerated (Fig. 4). If the pressure drops to the minimum, a warning message is sent to the screen. Thus, if a pump is not forcing solvent through the column or if the flow-rate is too low, the operator will have an indication of the problem. If the operator does not correct the problem within 25 to 60 sec, the computer will shut down the pumps. Conversely, if the pressure reaches 4000 p.s.i., the computer will shut down the pumps and the program, thus preventing use of the chromatograph until the operator corrects the blockage in the lines, and restarts the computer and HPLC program.

The system described in this communication is an excellent alternative to more expensive dedicated HPLC controllers. Its cost is intermediate between Apple II based systems and dedicated systems sold by HPLC manufacturers.

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