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COMPLETE COMPUTER AUTOMATION OF PREPARATIVE LIQUID CHROMATOGRAPHY THROUGH INTELLIGENT FRACTION COLLECTION, WITH UNLIMITED INJECTION VOLUME AND REPETITIVE COLLECTION OF SEPARATED SOLUTE PEAKS*

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SUMMARY

Most of the automatic high-performance liquid chromatography (HPLC) instruments, although comprising sophisticated injection and data systems, do not offer the possibility of achieving automatic preparative scale separations. To obtain the required capacity for unattended collection of the compounds separated, a programmable HPLC system controller (Waters 720) was interfaced between the detection system and a fraction collector. The computer program AUTOPREP, using BASIC language, was written to upgrade the standard 720 program, by automatically controlling the injection of large sample volumes and the collection of the peaks of solutes separated. Sample mixtures of unlimited volume could be injected through the pump head or, alternatively, small samples could be injected by the WISP autosampler. The peaks were defined by monitoring the first derivative of the detector signal, a process that has many advantages over the usual intensity threshold peak detection. A combination of continuously adjusted peak slope thresholds and time windows provided logic decisions during the chromatographic runs, which permitted to achieve various collection patterns. Finally, the model collection pattern generated by the program could be used for repetitive injections and collections. The practicability of the system is demonstrated with the automated isolation of compounds from sterol and phospholipid mixtures.

INTRODUCTION

By comparison to analytical applications of high-performance liquid chromatography (HPLC), in the past preparative HPLC has received only moderate attention from users and instrument manufacturers. Recent reviews[1,2] and symposia dedicated to this field have shown an increasing need to solve more difficult preparative separations and accordingly more interest has been shown for preparative instrumentation.

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To date, only a few automated preparative systems have been described[3–5]. However, automatization of HPLC in preparative applications is even more desirable than in analytical applications for the following reasons. First, the necessity of collecting pure separated compounds adds complexity to the chromatographic process. For instance, decisions for fraction switching must be taken in real time. Second, preparative throughput can be enhanced by a 24-h automatic working schedule. Third, preparative strategies often involve repetitive separation and collection patterns. Fourth, since emphasis on high sample capacity generally requires as a compromise longer separation times[6], a longer uninterrupted and more tedious schedule is created. Finally, the multiple tasks required simultaneously to evaluate the chromatogram —comparison with previous data, appropriate control of the fraction collector and reporting of these actions in chart form— often render the operator's action impracticable without some type of automation.

The lack of an automatic instrument fulfilling our needs prompted the present research. Our aim was to provide a team of chemists, either beginners or experienced in HPLC, working in the field of natural product structure elucidation, with unattended preparative HPLC and collection of separated compounds. Intended for research, this machine had to allow for maximal flexibility in the choice of the operating parameters. For instance, many of the marine sterol separations in our laboratory requiring automation are usually performed in a non-aqueous reversed-phase mode. This implied injection volumes of 5–10 ml (due to the poor solubility of the sterols), separation times of 2–3 h and refractive index (RI) detection[7,8]. Since most automatic injectors could not handle such large volumes and many commercial HPLC system controls were unable to deal with more than 2 h of running time, let alone an RI peak detection capability, we decided to develop a system tailored to our needs.

The design of an entirely new instrument, as was necessary in earlier pioneering studies[3,4], was not practically or economically feasible. Hence we considered primarily a modification of the software of a commercial system controlled by a microcomputer. Most systems available on the market in mid-1980 were of monolithic design, with a dedicated keyboard, and therefore were not suitable for a modification of software. Among the alphanumeric keyboard controlled modular systems, the Waters 720 System controller had some space left in its 32 Kbytes programmable memory for additional software written in BASIC.

This instrument also provided up to ten programmable relay contact closures and an easy communication with the system components (small volume autosampler, solvent delivery pumps and data acquisition/reduction computing integrator) through a two-way network linking their individual processors. The Waters 720 System controller was not originally distributed by its manufacturer to be used with programs generated or modified by the customers. However, it had all the desirable characteristics of a personal computer: an alphanumeric keyboard, a CRT for program editing and real-time display of chromatographic conditions, a printer-plotter and a cassette storage system.

Our approach to an automatic preparative system constitutes a step forward in narrowing the gap between dedicated set laboratory instruments (and related analytical technicians) and adaptable creative home computers (and related software programmers). It is an attempt by the analyst to avoid becoming the "victim of the ultimate black box"[9].

MATERIALS AND METHODS

Equipment

The majors components of the modular automated HPLC system are:

(i) A Waters (Milford, MA, U.S.A.) chromatographic system consisting of two Model M45 pumps, a WISP 710B auto-sampler (sample volume 1–2000 μl), a 730 data module, a 401 refractive index detector and a 450 variable-wavelength UV photometer.

(ii) A Waters 720 System controller (dual processor microcomputer, with 32K programmable memory) equipped with computer-controlled solenoids.

(iii) A Foxy (Isco, Lincoln, NE, U.S.A.) fraction collector externally driven by the System controller and equipped with either 200 tubes of 20 ml or 21 tubes of 500 ml or funnels connected to bottles for larger volumes.

(iv) Two pneumatically actuated Rheodyne (Cotati, CA, U.S.A.) valves: a 53-02 three-way valve and a 50-03 six-position valve connected to six sample reservoirs (typically of 100 ml each).

The specific columns, elution solvents and detection conditions used are described in the legend provided for each example of separation.

System design

Two different configurations of the automated system were used, depending on the sample volume injected. The design of the system in its large volume injection version is schematically illustrated in Fig. 1. Injection is achieved by the weak eluent

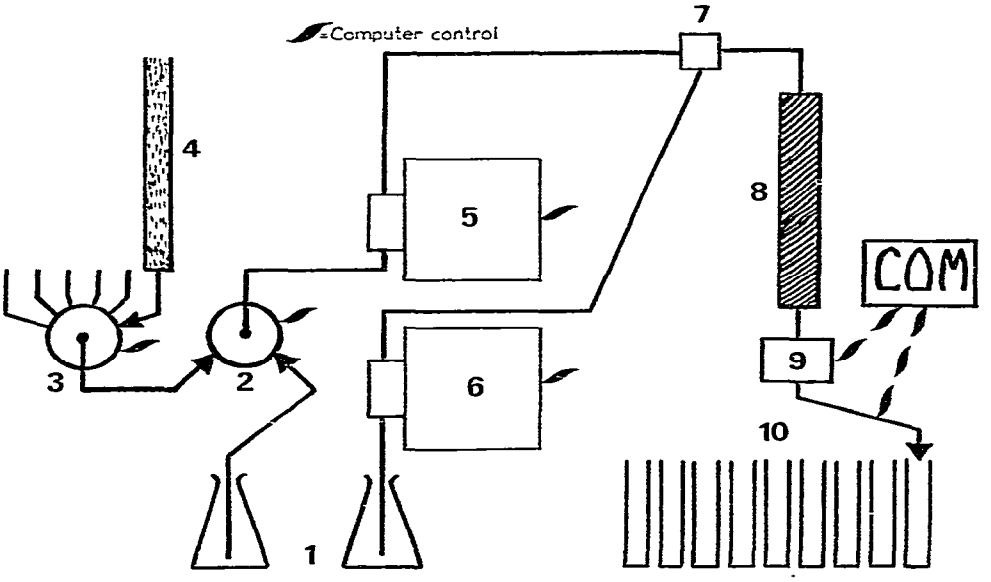

Fig. 1. Schematic diagram of the automated preparative HPLC system (large volume injection configuration). 1 = Solvent reservoir; 2 = three-way valve; 3 = six-ports valve; 4 = sample reservoir; 5 = HPLC pump for isocratic/weak eluent; 6 = HPLC pump for strong (gradient) eluent; 7 = mixing tee; 8 = HPLC column; 9 = detector; 10 = fraction collector.

pump (5), which moves the sample from one of the six sample reservoirs (4), through two valves (2 and 3). Selection of the scheduled sample is achieved by rotation of the six-port valve (3) under control of the computer. The injection volume, which can be different for each sample, is controlled by the operating time of the three-way valve (2) by switching the admission port of pump 5 from solvent line (rest position, spring loaded) to sample line (positive pneumatic action). The time necessary for an injection, equivalent to the ratio of sample volume to the flow-rate, is calculated and controlled by the computer.

For sample volumes smaller than 2 ml, a low-volume injection configuration of the system is used, with the WISP auto-sampler placed between the mixing tee (7) and the column (8).

The computer-controlled pumps 5 and 6 (a third optional pump may be installed) can be programmed to produce flow and solvent composition gradients. A maximum flow-rate of 20 ml/min can be achieved with two pumps.

The detection is carried out with unmodified analytical detectors that can be tuned to a low sensitivity whenever needed, using a variable UV or a refractometer. This permits the detector response to be kept within a non-saturated range, even with the high sample concentrations used in preparative separations. The signal of the detector is continuously digitized and plotted by the data module during chromatographic runs. Signal intensity data are transmitted to the system controller at regular intervals and fed into PEAKSRCH, a peak searching program routine based on a combination of peak slope, shape and related time windows.

The fraction collector is triggered by the computer at the beginning and end of peaks. For each change of fraction, the fraction number and time are reported on the chromatogram. The system managing program AUTOPREP decides, according to a maximum run time or the presence of peaks, to stop the current run and to start a new injection. AUTOPREP also controls the collection pattern: if samples from different vials are injected successively, the collection depends only on the PEAKSRCH routine. However, if re-injections of the same sample are programmed, the times at

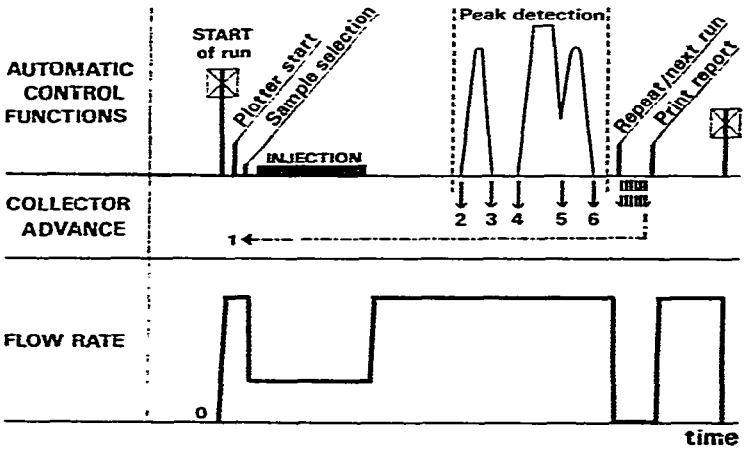

Fig. 2. Example of automatic run sequence for the large injection volume method. Nos. 1–6 are fraction numbers.

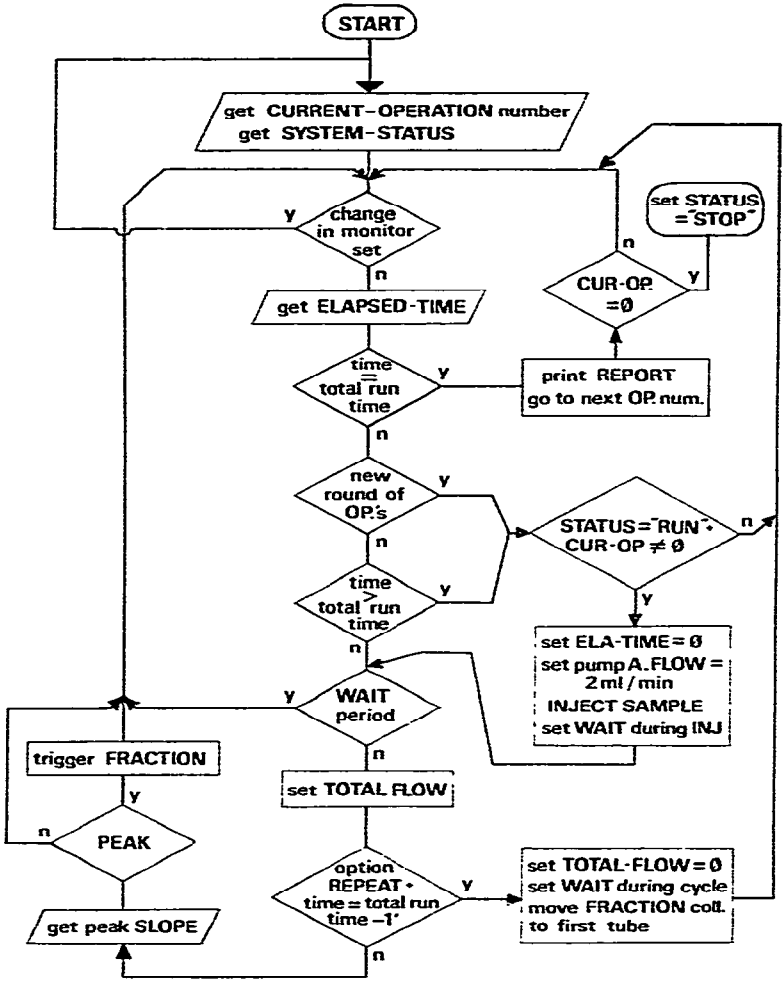

Fig. 3. Flow chart of the program AUTOPREP for automatic large volume injection and intelligent peak collection. y = Yes; n = no; OP. = operation; CUR-OP. = current operation; ELA-TIME = elapsed time; INJ = injection; coll. = collector; num. = number.

which the fraction collector was triggered during the first separation act as a main-frame adaptive pattern for the next re-separations. In this way, a single compound (or peak) separated in different successive chromatographic runs can be collected repetitively in a single vial.

Finally, after completion of all programmed operations, AUTOPREP turns off the pumps, the plotter and the fraction collector.

System managing program AUTOPREP

The sequence of operations performed by the different parts of the system under the command of the managing program AUTOPREP is shown in Fig. 2.

(i) After starting the pumps at the flow-rate used throughout the chromatographic run, all clocks are reset to 0.

(ii) The six-way sample valve rotates to the preselected sample reservoir while the flow-rate is decreased to 2 ml/min. Then, the three-way valve is switched to connect the pump with the sample reservoir during the time necessary to pump the preset sample volume.

(iii) The flow-rate returns to the original value and the peak detection is started. Depending on the compounds to be isolated, at the beginning of the chromatographic run the fraction collector can optionally stay on the first waste tube or advance at regular intervals of time.

(iv) After this period, switching of the collector is triggered according to the shape of the peaks.

(v) When the chromatographic run is completed, the fraction collector can be directed either to the next tube or back to the first tube.

(vi) After the printout of a report, the system is ready for another separation.

With the small volume injection version, the injection step (ii) is accomplished by the WISP auto-injector, under the command of the System controller. A modification of the module-linking software routines allows the selection of the different sample vials and injection volumes directly from the System controller. In this way, all commands needed to run the system are entered only through the keyboard of the System controller.

The flow chart of the system managing program AUTOPREP is given in Fig. 3.

Program commands

Even with the additional tasks of controlling an injector and a fraction collector, the instructions required from the operator to pre-program the functions of the automatic preparative chromatograph are kept at a minimum. Aside from the pump commands (pump set number, solvent composition and flow-rate) and the operations commands (operation number, run time, sample vial number, injection volume), six peak-collection commands must be given: the choice of fixed or continuously adjusted collection pattern, a positive and a negative slope threshold, a peak detection inhibition time, the volume of the collecting tubes and the number of the first tube. In most cases, the operator does not even have to modify the default value of these instructions (built into the program).

A re-injection option can be selected simply by choosing pump set numbers from 10 to 19. In this option, the fraction collector returns to the first tube after completion of a chromatographic run and follows the same collection pattern as in the previous chromatogram.

Since the switching of the fraction collector depends only on the real-time peak detection, it is not necessary to run first a master chromatogram, then measure the times at the beginning and the end of the peaks and, finally, to give to the system accordingly a long list of switching-time instructions.

An important feature of this system is that the operator is allowed to modify any parameter controlling the separation or collection during the performance of this separation. Unlike most single-microprocessor-controlled chromatographs, which must be stopped while the instructions are modified and then restarted, the Waters System controller has two memory-independent microprocessors, connected in a master–slave manner. When the master, controlled by the keyboard, is not giving orders to the peripherals, they act independently according to their preset instruc-

tions. Thus, the master can be reached at any time and provisions have been made in the program so that modification of an instruction does not interrupt the work of the peripherals.

Once the system has been programmed, it can work automatically for days until the last programmed operation has been completed. Once the separation conditions are standardized for a class of compounds, for example in the isolation of taxonomic markers from different families of living organisms, all the instructions needed can be stored on cassette tape. Resetting the automatic system to reproduce these particular conditions is then only a matter of inserting the cassette and pushing the RUN key.

Large volume injection

Unlike many loop injectors, the WISP has a variable injection volume, but its total volume is limited by the size of the sample vials to 3.5 ml. Furthermore, the slow maximum loop filling speed, 3.5 μl/sec, also precludes the injection of a large volume. However, for samples of low solubility, a 5–10 ml injection volume is highly desirable since this volume allows a preparative scale amount of sample (for sterols typically 10–50 mg), but well below mass overload of a 10 mm I.D. column. Furthermore, when the sample is injected in a solvent weaker than that used as eluent, the resulting concentration effect at the top of the column gives a narrower injection band.

In order to have a variable injection volume without size limitations, and to avoid loop injection, the sample is fed into the column with a high pressure pump. This is achieved by sucking the sample out of one of the sample reservoirs through a multiport selection valve. The volume of injection, which can be different for each sample, is controlled by the opening time of the three-way valve which switches the admission port of the pump from solvent line to sample line. The opening time, equivalent to the ratio of the sample volume to the flow-rate, is calculated and controlled by the computer.

Peak searching routine PEAKSRCH

The simplest way of defining a peak is to set a threshold at a desired value of detector signal intensity. The peak begins when this value is exceeded and ends when

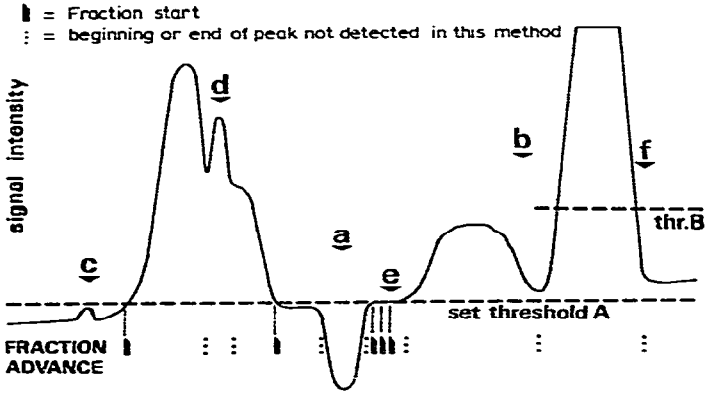

Fig. 4. Peak collection using intensity threshold detection. a–f = Points of the chromatogram where limitations are observed (see text). thr = Threshold.

it is in default. The severe limitations of this simplistic method are shown in Fig. 4.

(a) Negative peaks (generated by refractive index detection) are overlooked.

(b) A drifting baseline (generated in gradient elution) prevents peak detection for a part of the chromatogram.

(c) Minor peaks are overlooked.

(d) Peaks not resolved to baseline are not recognized as separated.

(e) Multiple triggering occurs due to signal noise, when a slowly changing signal is close to the threshold value.

(f) Only central (heart) cut of peaks can be achieved, but not the collection of a particular section of a peak or at the minimum between peaks.

Some of these problems (b and d) have been solved in an improved version of peak level detection, using an adjustable threshold[10]. However, this requires knowing beforehand the relative intensities and retention times of the chromatographic peaks. Therefore, a scouting chromatogram had first to be obtained, and then the different threshold values had to be entered manually in the collection program.

In order to achieve a completely automatic peak detection of chromatographic unknowns, the use of slope threshold rather than level threshold was examined. The signal of the detector is plotted by the data module, which also continuously digitizes its intensity; a full-scale deflection of the pen correponds to 999.999 units. The register containing this information is called at regular intervals by the System controller, under the command of the PEAKSRCH program. From this succession of points, the first derivative of the detector signal, *i.e.*, the slope, is calculated regardless of the signal intensity.

Two variables, the positive and the negative standard slope thresholds, valid for a whole chromatogram are entered manually into the "variables table" of PEAKSRCH. They permit rejection of baseline noise, handle drifting baselines and select cutting points on peak shoulders. PEAKSRCH also generates flags that help to characterize the peaks: the POSITIVE flag is generated when the slope values are above the positive threshold, the NEGATIVE flag corresponds to negative slope

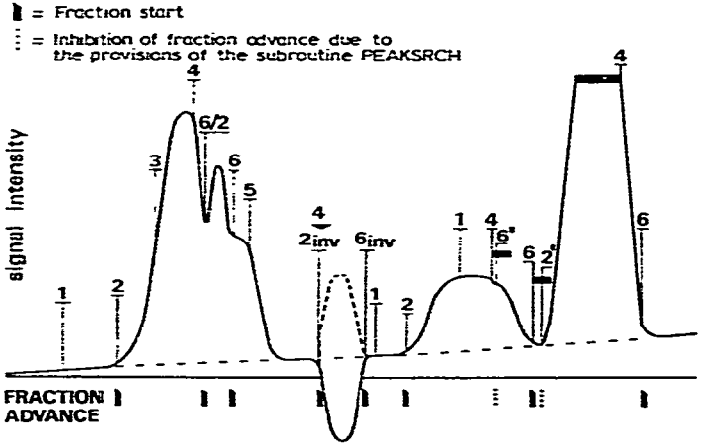

Fig. 5. Peak collection using slope threshold detection. 1–6 = Typical slope situations (see text).

values, the INVERSION flag is generated when the peaks are negative, the FUSED PEAKS flag is on during a period of time after triggering the fraction collector and the INHIBIT flag is set when peak detection is not wanted.

The different slope situations that can arise in a chromatogram are shown in Fig. 5.

(1) Before the first peak, the baseline is moving up and down within the limits of slope thresholds (background): the peak searcher is continuously watching the slope, but does not take any action. The same situation can be observed between peaks, on the baseline, or on flat peak shoulders.

(2) When the slope rises above the positive slope threshold, the POSITIVE flag is turned on. Simultaneously, the System controller generates a triggering signal for the fraction collector. This signal is not transmitted when a FUSED PEAKS flag is on (situation 2*); this prevents the collection of only a few drops of eluent in a separate tube when the detector signal does not stay on the baseline between two peaks.

(3) In the ascending part of a peak, when the POSITIVE flag is on, the peak sensor is watching for a negative slope (no action).

(4) When the slope decreases below the negative slope threshold, the NEGATIVE flag is turned on and a delay period (INHIBIT) is started. In this period, the negative slope can decrease to a larger negative value: this prevents a false end-of-peak diagnostic if the slope has decreased so slowly that detector noise brings the slope above the negative threshold (situation 6*). When the NEGATIVE flag is set on without having the POSITIVE flag already turned on and the intensity of the signal being close to the baseline value, a negative peak is diagnosed: the INVERSION flag is set on, the NEGATIVE flag is cleared and replaced by POSITIVE, the fraction collector is triggered and, finally, all slope values are reversed for the duration of the negative peak. Peak detection proceeds further, as for a positive peak.

(5) In the descending part of a peak, when the NEGATIVE flag is on, the peak sensor is watching for the slope to reach the negative threshold (no action).

(6) When the negative slope threshold is exceeded, the NEGATIVE flag is turned off. If the FUSED PEAKS flag is off, the fraction collector is triggered. The INVERSION flag is cleared at this point (situation 6_{inv} for a negative peak) and thus the peak sensor is ready to detect the inception of a positive or negative peak.

The flow chart of PEAKSRCH, the routine that allows intelligent decision-making for searching and collecting chromatographic peaks, is given in Fig. 6.

In this routine, the different slope situations 1–6, previously described, are only characterized after a succession of yes/no decisions. Like the remainder of the program, this routine is written in BASIC.

Although the slope threshold peak detection method generally solved all the problems encountered in level threshold detection, some improvements remained to be added to that method in order to adapt it to the particular conditions of chromatography. The isocratic chromatographic process causes the widening of the peaks and accordingly a decrease of their slopes as the separation proceeds. Therefore, the use of one fixed set of automatic peak detection parameters for the whole duration of a chromatogram does not provide optimal values for peaks having greatly different retention times.

Hence, the standard set of parameters was continuously adjusted by correction factors which depend on the retention time of the peaks. Assuming that the theoret-

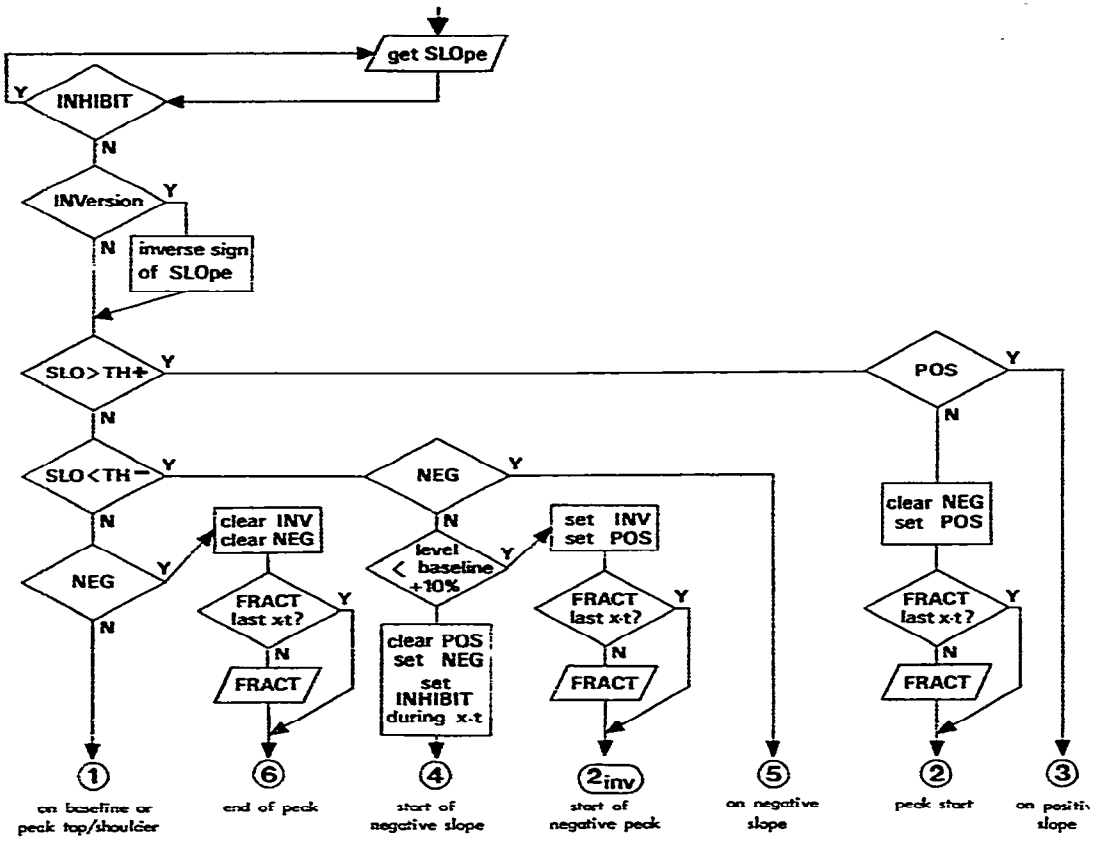

Fig. 6. Flow chart of the subroutine PEAKSRCH for intelligent peak search and collection. Y = Yes; N = No; TH = Threshold; FRACT = fraction.

ical plate number, N, of a column is constant for different peaks in a chromatogram, the peak width, t_w, will increase proportionately with the retention time, t_R (ref. 11). This proportionality can be expressed in terms of the equation:

$$t_w = t_R \cdot 4(1/N)^{1/2} \tag{1}$$

The corresponding reduction of peak height, h, for the later eluting peaks is accompanied by the reduction of their slopes, δ. The relative decrease of δ *versus* t_R can be established for compounds that show a constant peak area, S, at different t_R. Assuming that the peak shape is triangular, the area is:

$$S = t_w \cdot h/2 \tag{2}$$

and the slope is:

$$\delta = 2h/t_w \tag{3}$$

Consequently, by combining eqns. 2 and 3, the slope ratio of two peaks of equivalent area will be:

$$\delta_2/\delta_1 = (t_{w1}/t_{w2})^2 \quad (4)$$

This yields, after substitution of t_w by eqn. 1:

$$\delta_2 = \delta_1(t_{R1}/t_{R2})^2 \quad (5)$$

By considering δ_1 as a standard slope threshold at the standard retention time t_{R1}, the computer can update the actual positive and negative slope threshold values at any retention time, using eqn. 5. Similarly, the actual duration of the FUSED PEAKS and INHIBIT flags is continuously increased proportionately with t_R and their standard duration, using eqn. 1; this corrects the collection pattern for the increase of fraction volumes and the widening of peak tops. As an isocratic separation progresses, the detection signal to noise ratio becomes smaller, as do the slope thresholds. In order to avoid the interference of noise with the detection of peaks, more points are averaged to determine the slope as the retention times become larger.

All these dynamic features render PEAKSRCH particularly suitable for automatic peak search in the isocratic mode. In the gradient elution mode, a fixed set of peak detection parameters is generally effective for a whole chromatogram.

RESULTS

Examples of the individual modes of operation of the automated HPLC system are given hereafter. When these different functions are cooperating with the standard software of the 720 Waters System controller, both programs complete each other

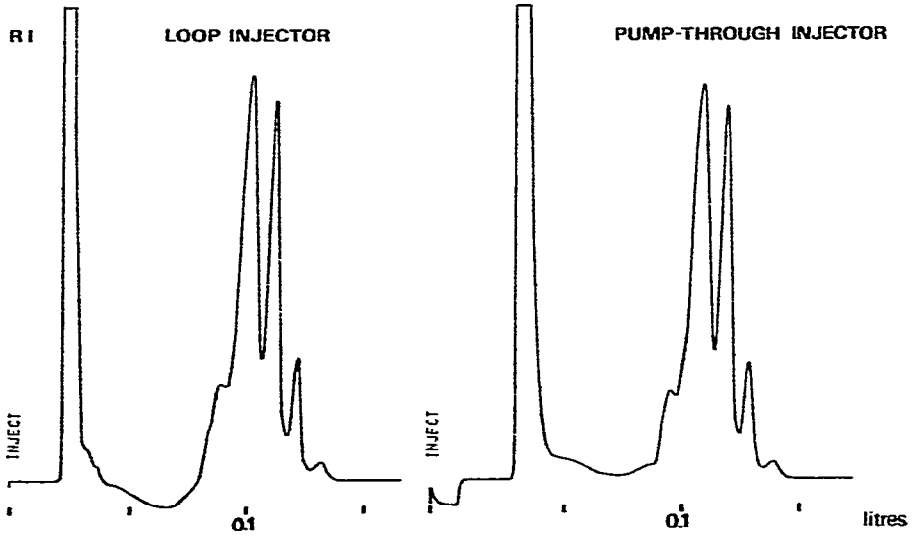

Fig. 7. Comparison of a 5-ml injection in two different injection techniques: loop injection (Rheodyne 7020, 5-ml sample loop) and sampling through the pump head. Sample: desmethylsterols from the tunicate *Ascidia nigra*. Column: Partisil 10 ODS-3 (Whatman) 9.4 mm I.D. × 50 cm. Eluent: 100% methanol, 25°C, flow-rate 5 ml/min. Detector: RI, attenuation × 16.

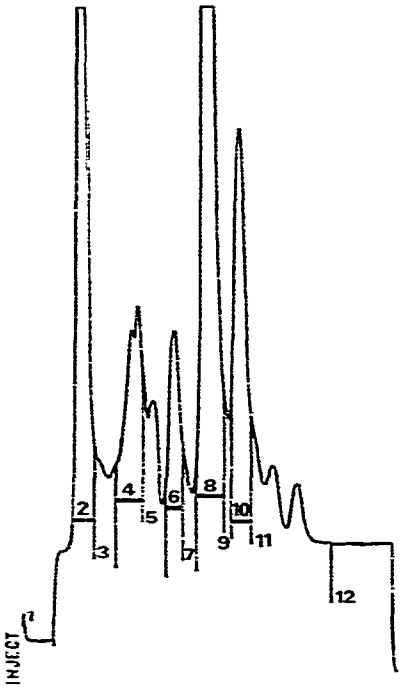

Fig. 8. Peak collection using high fixed slope thresholds (positive = 14,000; negative = −10,000). Sample: 5 ml (20 mg) methanol solution of sterols from the sponge *Calyx nicaensis*. Conditions as in Fig. 7 except for RI attenuation ×8. 1–12 = Fraction numbers.

to make a much more powerful system: from a mere executor and operator information device, AUTOPREP upgrades the system into an intelligent decision-making tool.

Large volume injection

A comparison of loop injection and high pressure pump injection is given in Fig. 7. A similar band broadening has been observed in each technique. Therefore, the successive passage of the sample in both pump heads apparently does not produce more "wall effects" than a large volume loop. A reproducibility of better than 98% has been measured for a 5-ml injection, the major error being attributed to the discontinuous admittance of the sample into the pump.

Peak detection

(i) High slope thresholds. Different values of slope thresholds have been examined in order to cut the peaks at different points. A chromatogram of a separation using high fixed slope thresholds is shown in Fig. 8. A heart cut of the peaks for the major compounds yields fractions with maximal purity. Minor compounds, detected as shoulders of major peaks, are automatically separated in different tubes, even before they can produce a visually detectable change of slope (tubes 5 and 11). This task would be practically impossible to achieve manually.

It is noteworthy that although the elution is isocratic, the peaks have nearly all

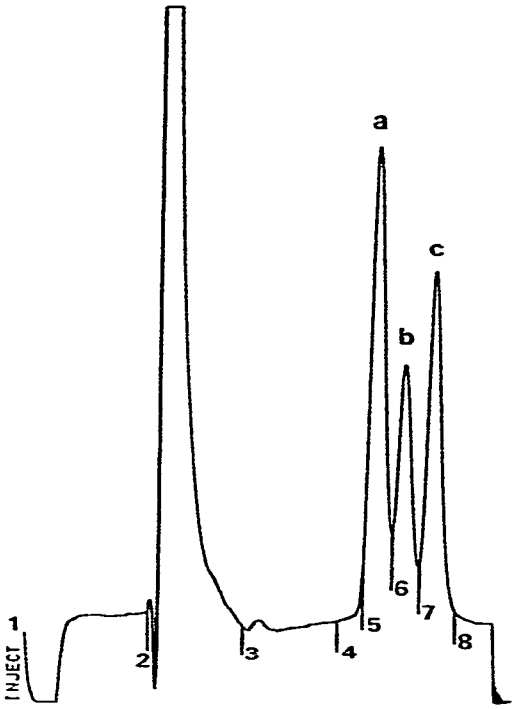

Fig. 9. Peak collection using low fixed slope thresholds (positive = 1500, negative −1200). Sample: 5 ml (10 mg) of a mixture of cholesterol (a), campesterol (b) and sitosterol (c). Conditions as in Fig. 7 except RI attenuation ×32. Fractions 2–4 were triggered by the maximum volume control (preset value 30 ml), fractions 5–8 by PEAKSRCH after a 20-min INHIBIT period.

the same band width; this can be attributed to the extra-column band broadening caused by the large volume of injection. The high fixed slope thresholds were therefore effective in the recognition of all major peaks. The minor last two peaks did not have slopes steep enough to be recognized.

One can expect that high slope thresholds (and heart cutting) will yield two kinds of fractions, those than contain pure major compounds, but represent only part of the injected quantity, and fractions containing the totality of the minor compounds injected, but mixed with tailing of the major compounds.

This is exactly what is desired in the preparative isolation of natural compounds from complex mixtures: after re-injection of the impure fractions, the minor compounds are separated as large peaks and recovered in a state of high purity. This procedure gives a high preparative throughput.

(ii) Low slope thresholds. A chromatogram of a separation using low fixed slope thresholds is shown in Fig. 9. The peaks were cut at the minimum point of the valleys between them. Peaks not resolved to the baseline contained impurities originating from the neighboring compounds.

Low slope thresholds are useful mostly for the collection of peaks resolved to the baseline, a situation generally encountered in analytical scale HPLC, whereas this type of peak cutting, similar to that of an integrator, is less useful in preparative scale HPLC, where the columns are overloaded for a maximal throughput.

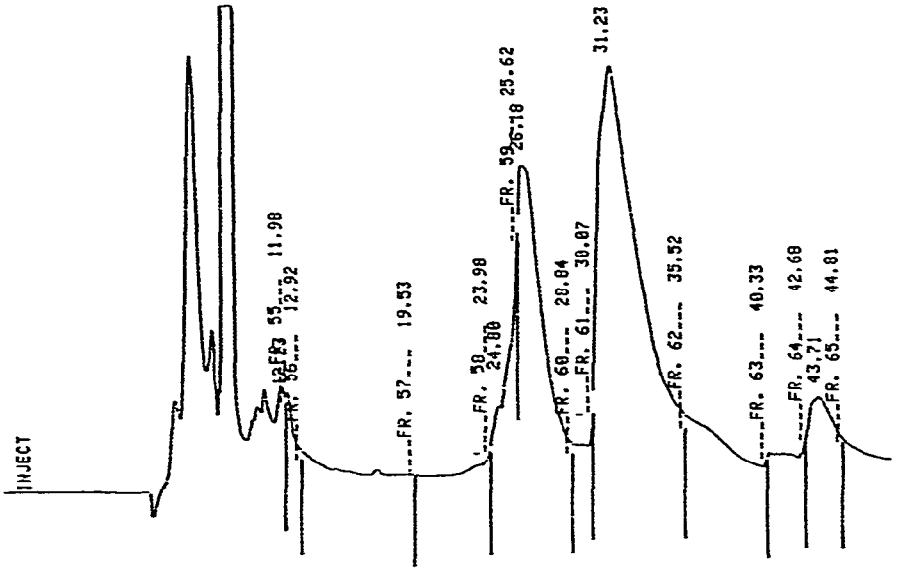

Fig. 10. Peak collection using continuously self-adjusted slope thresholds (standard positive = 6000, standard negative = −3000, at 20 min standard time). Sample: 100 μl (120 mg) egg lecithin. Column: Ultrasphere C_{18}, 5 μm, 25 cm × 10 mm I.D. (Altex, Berkeley, CA, U.S.A.). Eluent: 100% methanol, 25°C. 0–4 min, flow-rate 0.5 ml/min; 4–40 min, 3 ml/min; 40–50 min, 5 ml/min. Detector: RI, attenuation ×32. Fraction (FR) 55 corresponds to the first peak detected after the end of the INHIBIT period (11.9 min); fraction 57 was triggered by the tube overflow control (preset value: 20 ml) and fraction 63 was started by an increase of flow-rate at 40 min, which changed the baseline.

(iii) Continuously self-adjusted slope thresholds. A chromatogram of a separation using continuously self-adjusted slope thresholds is shown in Fig. 10. The resulting peak cuts permitted recovery of all peaks in separate fractions, although their widths and slopes varied considerably. Maximum slope thresholds before the standard time had to be selected, for the peaks of low retention, because the simple slope decrease in eqn. 5 (in Materials and Methods) does not take into account extra-column band broadening (due mainly to the injection).

(iv) Repetitive injections and collection. Representative chromatograms of a repetitive separation using fixed slope threshold peak detection are shown in Fig. 11. The separation was repeated 25 times (20 h) and the separated compounds were collected in only six large tubes, instead of the (25 × 7) fractions which would have been necessary with a conventional collector. Between the first and the last separations (a and b) a gradual change in chromatographic conditions (residual water content of methanol and temperature) shortened the retention times by 10%. Due to the flexibility of the PEAKSRCH program, this did not affect the peak recognition and collection.

DISCUSSION

Automation of preparative HPLC can only fulfill its purpose, *i.e.*, to increase the total sample throughput and reduce tedious and time-consuming work, if a completely unattended control and report of the operations performed is provided. The

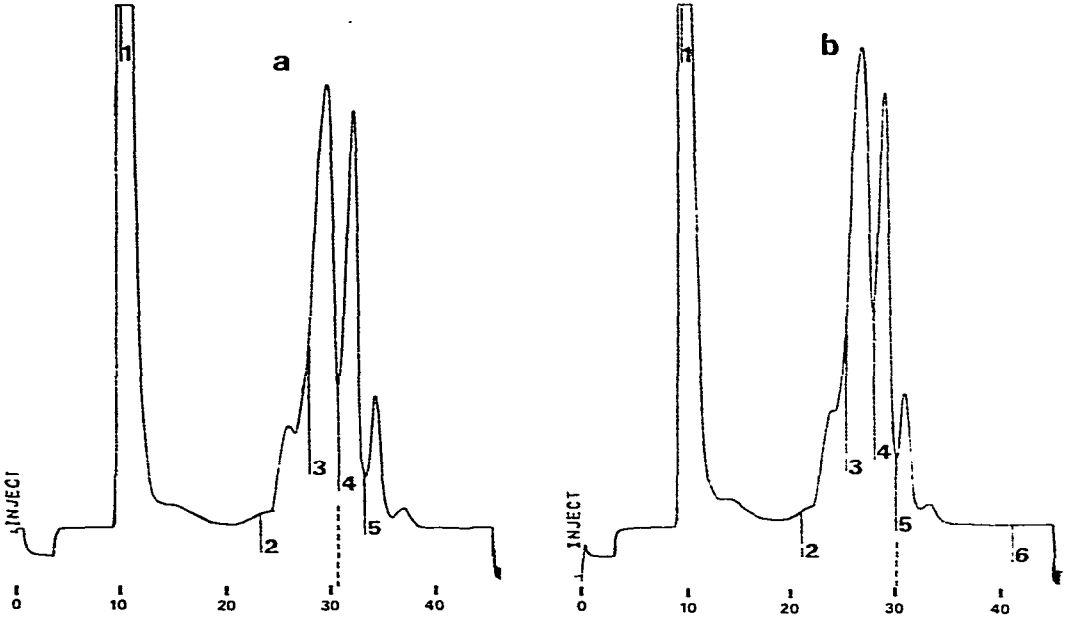

Fig. 11. Peak collection with repetitive injections and collection. a, First injection; b, 25th injection. Sample: 5 ml (12 mg) methanol solution of sterols from *A. nigra*. Column: Partisil 10 ODS-3 (Whatman), 50 cm × 9.4 mm I.D. Eluent: 100% methanol, 25°C, flow-rate 5 ml/min. Detector: RI, attenuation × 16.

use of an intelligent controlling and reporting system eases the task of the chromatographer before and during the separation stage as well as in the system instruction phase, and finally during recovery of the separated compounds.

Automation benefits during system programming

In the instruction phase, programming the modular elements of the system (auto-sampler, solvent supply, integrator, printer-plotter, fraction collector) requires many instructions to set up the independent microprocessors built into each module. Manual synchronization of the individual microprocessors into a completely automatic preparative system would put a difficult burden on the analyst, requiring an extensive training period before the successful mastering of the instrument.

However, with the AUTOPREP program, the analyst is prompted by simple English instructions on the CRT screen to insert the few necessary commands; if wanted, sample commands can be displayed. Fewer commands have to be placed because they reflect the logic of the events: the actual switching, printing or stopping times that formerly were deduced and manually entered as long listings are now calculated by the computer. Furthermore, the intelligent combination of information from different modules gives a unique logic-decision capability during a chromatographic run.

For example, once the volume of the collecting tubes has been given to the computer, the actual triggering times of the fraction collector needed to prevent solvent overflow are calculated by the computer, depending on the flow-rate delivered by the pumps. For the analyst, it is therefore as simple to handle flow programming as

to handle a constant flow-rate. Also if a tube change is triggered by the peak detector before the complete filling of this tube, the overflow protection triggering routine will be reset for the next tube.

Automation benefits during the chromatographic runs

In the separation phase, the calculation of valve switching times for large volume sample selection and injection, the determination of slope threshold points, the triggering of the fraction collector according to slope and tube volume and the reporting of all logic decisions taken in real time by the computer are accomplished without previous detailed instructions or any intervention from the analyst. Here also, only a few preprogrammed logic instructions are necessary, instead of the long listing of fixed cutting points generally found in programs that are only capable of post-run decisions[5]. Moreover, the computer can stop plotting after the last chromatogram and shut off the pumps within 15 min, without pre-instruction.

Automation benefits during results and sample recovery.

In the post-run phase, when the automatic peak collection has been completed, the evaluation of the chromatograms and, consequently, the recovery of the isolated compounds is facilitated considerably. After repetitive separations, all the fractions corresponding to the same peak in different runs are collected in a single large vessel. Also, following successive injections of different samples, the number of small tubes used to collect the successive peaks is efficiently reduced in comparison with collection based on constant time or number of drops. This more efficient use of a limited number of collection tubes considerably extends the autonomous run time capability of the chromatographic system.

However, with more fractions a higher demand is placed on the report of peak collection. Finding one among 200 tubes by counting the number of switching marks on the chromatograms soon becomes tedious and leads to a high probability of error. The automatic numbering of the fractions on the chromatogram, along with their retention times, has solved this problem. For more safety, a spike produced by the fraction collector confirms the effective tube change (see Fig. 10).

CONCLUSIONS

Automation means letting a machine perform tasks that were previously achieved under manual control. In this respect, the automation of our preparative HPLC system under the control of the program AUTOPREP went beyond this goal, offering a capability of decision and a speed of action that can hardly be duplicated manually.

The control of the chromatographic operations by a programmable microcomputer was a choice which permitted automation without major changes in hardware. Only commercially available pneumatic valves, for large volume injections, were added to the system. The more efficient use of a computerized Waters system through software modifications also only points out that the instrument can be adapted to the successive needs of different users (*e.g.*, small/large injection volume) and, in the future, to various new tasks. Therefore, it is unlikely to become quickly obsolete like monolithic instruments. Furthermore, this flexibility means that AUTOPREP can be

easily implemented in other instruments using the same (ICL 80) operating system. With some changes in input/output commands of the present program, any system that has a microcomputer equipped with an interpreter of BASIC could also be upgraded to achieve completely automatic preparative liquid chromatography.

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REFERENCES

1 M. Verzele and E. Geeraert, *J. Chromatogr. Sci.*, 18 (1980) 559.
2 P. A. Haywood and G. Munro, *Dev. Chromatogr.*, 2 (1980) 33.
3 W. H. Pirkle and R. W. Anderson, *J. Org. Chem.*, 39 (1974) 3901.
4 P. A. Bristow, *J. Chromatogr.*, 122 (1976) 277.
5 K. P. Hupe, H. H. Laurer and K. Zech, *Chromatographia*, 13 (1980) 413.
6 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979, Ch. 15, p. 616.
7 S. Popov, R. M. K. Carlson, A. Wegmann and C. Djerassi, *Steroids*, 28 (1976) 699.
8 U. Sjöstrand, J. M. Kornprobst and C. Djerassi, *Steroids*, 38 (1981) 355.
9 P. E. Field, *Amer. Lab. (Fairfield, Conn.)*, 14(1) (1982), 104.
10 M. Brenner and C. W. Sims, *Amer. Lab. (Fairfield, Conn.)*, 13 (10) (1981) 78.
11 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979, Ch. 2.