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CONVERSION OF AN ANALYTICAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM INTO AN AUTOMATED SEMI-PREPARATIVE UNIT AND ITS APPLICATION TO THE SEPARATION OF A MIXTURE OF BENZYL α- AND β-D-GLUCOFURANOSIDES AND GLUCOPYRANOSIDES

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SUMMARY

The conversion of an analytical high-performance liquid chromatograph to an automated semi-preparative unit capable of continuous 24-h operation is described. By the use of this technique a mixture of α- and β-D-glucofuranosides and glycopyranosides was separated into their individual components. The continuous operating capability of this system allowed 2.5–3 g of processed material to be collected over a 24-h period.

INTRODUCTION

When complex mixtures of closely related chemical structures are obtained as products of bacterial fermentation, plant extraction or chemical synthesis, isolation of pure components in the mg to g range is a prerequisite for conducting structural analysis, further chemical synthesis and the subsequent evaluation of biological properties. In many cases, conventional column chromatography is not an efficient approach to separating gram quantities of complex mixtures, due to the inherently poor resolution properties of closely related chemical structures. Significant use, therefore, is presently being made of semi-preparative high-performance liquid chromatography (HPLC) in a repetitive manual injection and collection mode to circumvent this problem[1–4]. Since this technique is time consuming, the automation of this procedure was clearly important. Such automated semi-preparative units have been described by Pirkle and Anderson[5], Bristow[6] and Hupe *et al.*[7]; however, these automated systems are relatively difficult to construct. For example, Bristow[6] employed a computer requiring complex software to control his system, while Pirkle and Anderson[5] used a specially constructed timer cycler. Furthermore, the fraction collecting devices described by Hupe *et al.*[7] and Bristow[6] were both specially constructed units, while Pirkle and Anderson[5] used a series of valves controlled by their timer to implement fraction collection.

The recent emergence of fraction collectors with remote input and microproc-

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essor controllers with programmable contact closure switches allows a commercially available analytical HPLC system to be readily converted into an automated semi-preparative chromatograph. In this report, we describe the construction of such an automated semi-preparative unit and its application to the separation of a mixture of benzyl α- and β-D-glucofuranosides and glucopyranosides.

EXPERIMENTAL

Synthesis of the four-component benzyl-D-glucoside mixture

A mixture of D-glucose (10 g), benzyl alcohol (50 ml) and AG 50W-X8 cation-exchange resin (0.1 g) was heated to 85°C for 24 h, whereupon the reaction mixture was cooled and then applied to a column of silica gel previously equilibrated with chloroform containing 1% (v/v) methanol. Elution was conducted initially with this solvent system to remove the bulk of the excess benzyl alcohol and then with chloroform–methanol (4:1, v/v) to elute the four component mixture shown in Fig. 1. By this preliminary purification process the majority of impurities were removed prior to HPLC application. The physical characteristics of the α- and β-D-glucofuranosides and glucopyranosides reported herein are as follows.

Benzyl α-D-glucofuranoside (I) was crystallized from ethyl acetate–light petroleum (b.p. 35–60°C) (1:2) m.p. 122–124°C, $[\alpha]_D^{23}$ + 82° (*c* 1, methanol). ^{1}H NMR (C^2HCl_3): δ 7.44 (s, 5H, $-C_6H_5$), 5.07 (s, 1H, H-1), 4.74 (d, 1H, $J_{H,H}$ –12.0 Hz, $-CH_2C_6H_5$), 4.66 (d, 1H, $-CH_2C_6H_5$), 4.26 (d, 1H, $J_{2,3}$ 5.0 Hz, H-2), 4.20–4.15 (m, 2H, H-3, H-4), 3.97 (m, 1H, H-5), 3.78 (dd, 1H, $J_{5,6}$ 2.8, $J_{6,6'}$ –12.1 Hz, H-6), 3.63 (dd, 1H, $J_{5,6'}$ 6.0 Hz, H-6′). The proton at C-6 in the spectra of compounds I and II giving the higher field signal was designated H-6′. Calc. for $C_{13}H_{18}O_6$: C, 57.78; H, 6.67%. Found: C, 57.52; H, 6.88%.

Benzyl β-D-glucofuranoside (II) was obtained as a syrupy product, $[\alpha]_D^{23}$ − 27° (*c* 1, methanol), ^{1}H NMR (C^2HCl_3): δ 7.45 (s, 5H, $-C^6H_5$), 5.28 (d, 1H, $J_{1,2}$ 4.3 Hz, H-1), 4.80 (d, 1H, $J_{H,H}$ − 12.0 Hz, $-CH_2C_6H_5$), 4.70 (d, 1H, $-CH_2C_6H_5$), 4.32 (dd, 1H, $J_{2,3}$ 3.5, $J_{3,4}$ 4.6 Hz, H-3), 4.16 (dd, 1H, H-2), 4.10 (dd, 1H, $J_{4,5}$ 8 Hz, H-4), 3.88 (m, 1H, H-5), 3.74 (d, 1H, $J_{5,6}$ 3.0, $J_{6,6'}$ − 12.0 Hz, H-6), 3.59 (d, 1H, $J_{5,6'}$ 6.6 Hz, H-6′). Calc. for $C_{13}H_{18}O_6$: C, 57.78; H, 6.67%. Found: C, 57.69; H, 6.92%.

Benzyl β-D-glucopyranoside (III), m.p. 122–123°C, $[\alpha]_D^{23}$ − 47° (*c* 1, water). Lit.[8]: m.p. 122°C, $[\alpha]_D^{23}$ − 56° (*c* 2, water); Lit.[9]: m.p. 122–124°C, $[\alpha]_D^{23}$ − 53° (*c* 1, water).

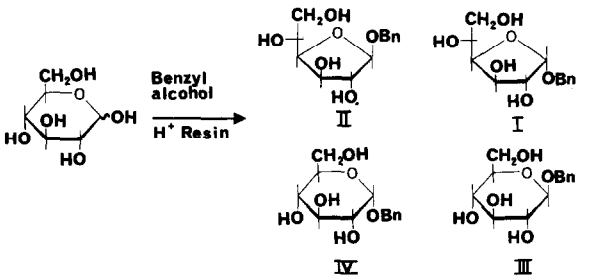

Fig. 1. Reaction scheme for the synthesis of benzyl α-D-glucofuranoside (I), benzyl β-D-glucofuranoside (II), β-D-glucopyranoside (III) and benzyl α-D-glucopyranoside (IV). Bn = benzyl.

Benzyl α-D-glucopyranoside (IV), m.p. 122–123°C, $[\alpha]_D^{23}$ + 117° (*c* 1, water). Lit.[8]: m.p. 122°C, $[\alpha]_D^{23}$ + 133° (*c* 2.5, water); Lit.[10]: m.p. 121–122°C, $[\alpha]_D^{23}$ + 133° (*c* 1, water).

Melting points were determined with a Thomas–Hoover capillary melting point apparatus and are uncorrected. Products were detected by thin-layer chromatography (TLC) which was performed on silica gel GF precoated thin-layer plates (Analtech) and developed by heating after the application of ethanol–5% sulfuric acid spray. ^{1}H NMR spectra were obtained with a 270-MHz Bruker HX-270 spectrometer. Elemental analyses and optical rotations were performed by Baron Consulting Co. (Orange, CT, U.S.A.).

Chemicals and related materials

Reagent grade D-glucose, ethyl acetate and chloroform were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Methanol and acetic acid were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.), while benzyl alcohol, 2,2′-dimethoxypropane and 3A molecular sieve were obtained from Aldrich (Milwaukee, WI, U.S.A.). AG 50W-X8 cation-exchange resin was supplied by Bio-Rad Labs. Nylon-66 filters (pore size 0.45 μm, diameter 47 mm) were obtained from Rainin Instruments Company. All solvents were dried over molecular sieve 3A for 48 h and then filtered through nylon-66 filters prior to use.

Liquid chromatography

High-performance liquid chromatography, including all scale up work, was conducted on a Whatman Partisil M9 (25 × 0.9 cm) column. Elution profiles were obtained by the application of 25-mg sample loads dissolved in either ethyl acetate–methanol (9:1) or chloroform–methanol (9:1) at a concentration of 50 mg/ml and were run at flow-rates of 2 ml/min. Continuous automated chromatography was conducted at flow-rates of 3 ml/min and approximately 50-mg sample loads in a volume of 1 ml were applied at each injection. Eluting products were detected by UV absorption at 254 nm, and all samples were processed at ambient column temperature. Reduction in the resolution properties of this column occurred periodically and were associated with the adsorption of water and other impurities. Column regeneration was achieved by washing with water until a uniform UV baseline was obtained, followed by chemical dehydration using a mixture of acetic acid and 2,2′-dimethoxypropane as described by Bredeweg *et al.*[11].

Chromatographic equipment

Automated semi-preparative chromatography was conducted on a system consisting of an Altex liquid chromatograph (Model 322) comprised of two Altex 110 pumps, a 2-ml mixing chamber and an Altex 420 microprocessor controller, which was equipped with an Altex pneumatic board (Model 420-40) (Fig. 2). The injection system components consisted of a 7030 column switching valve, and a 7010 sample injector valve, both equipped with pneumatic actuators (Model 70-01) and a 7120 manual loop injector which were obtained from Rheodyne. The 7010 and 7120 valves also equipped with 1-ml and 0.5-ml sample loops, respectively, were obtained from Rainin Instruments. A sample reservoir and buret were connected to a Swagelok T union which was obtained from Parker Hannifin. The fraction collection system

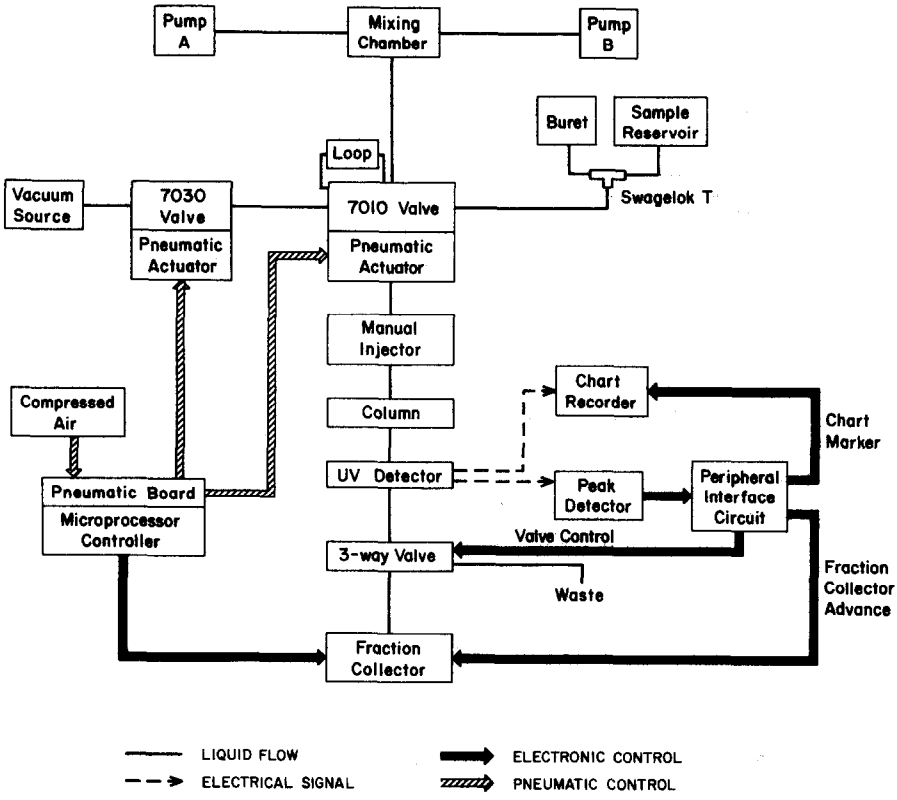

Fig. 2. Block diagram of the automated injection and fraction collection system.

consisted of an LKB 100-mV peak detector (Model 90002619), which was modified to accept input from a 10-mV UV detector, a three-way valve from General Valve Corporation (Model 1-17-900), an Altex UV 254-nm detector with a 20-μl flow cell (Model 153), a Kipp & Zonen chart recorder (Model BD41) and an LKB Multirac fraction collector (Model 2111). The peripheral interface circuitry shown in Fig. 3 was designed to convert a single electronic event, the peak detector's output voltage change, into three simultaneous control signals. These involved marking a chart recorder, stepping a fraction collector and actuating a three-way solenoid valve. The three-way solenoid valve was switched on by a Darlington transistor pair capable of sinking 500 mA of current without overheating. This occurred when the output from the peak detector went from low to high at the base of transistor 2N 3053.

The fraction collector advance function was initiated by the positive going edge of the peak detector's output which in turn caused a momentary relay closure (K_1) of 1 sec duration, and hence triggered a 556 timer circuit to send two consecutive pulses to a normally open relay connected to the fraction collector's remote input. The delay between pulses, which allowed for flush time, was adjustable from 7–37 sec by turning a 0.2-MΩ potentiometer through its range.

The chart mark circuit was operated by closing a single-pole double-throw relay (K_2), connected across the recorder's input, in response to both positive and negative

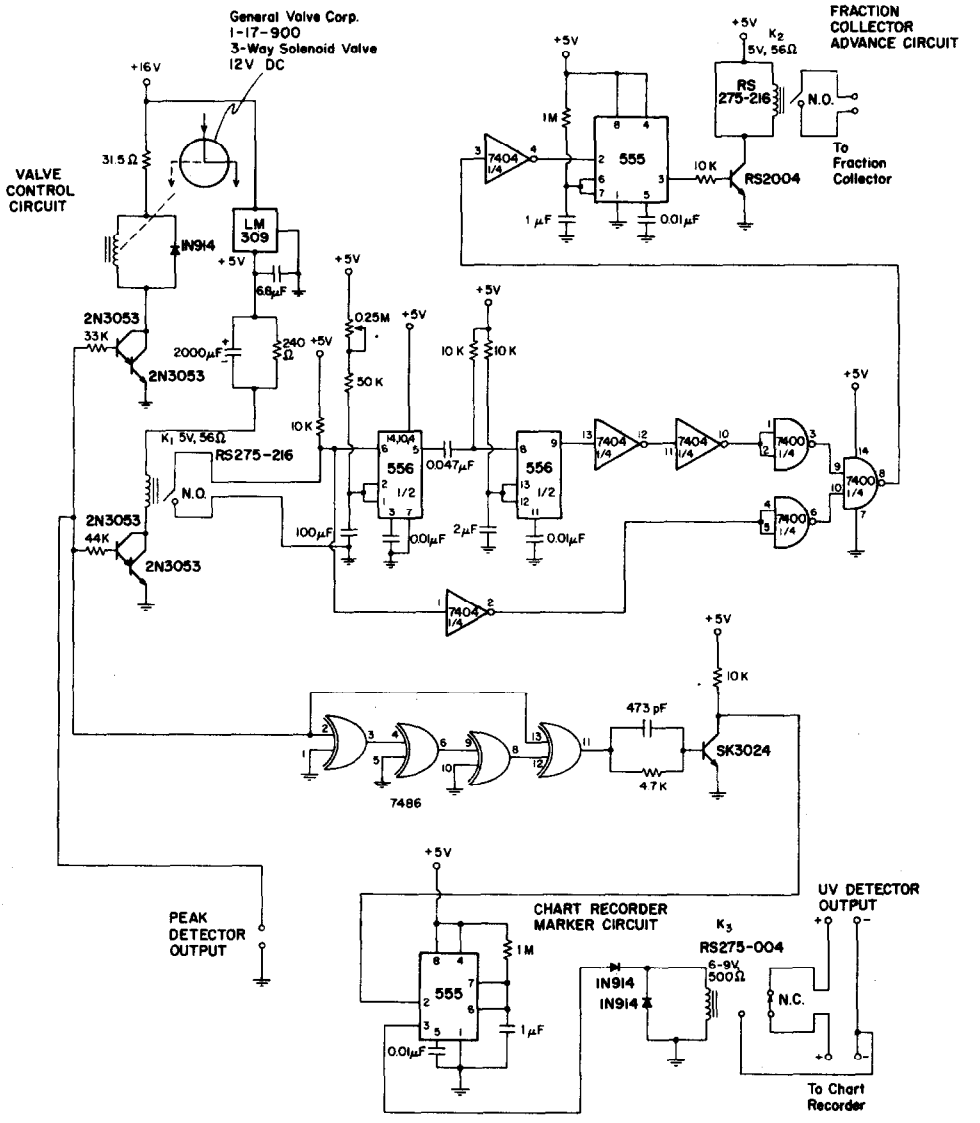

Fig. 3. Peripheral interface circuitry. K = kΩ; M = MΩ.

going edges from the peak detector's output. A single quad exclusive-OR gate package was used to sense the peak detector's output voltage change, which was further shaped by a 555 timer that drove the relay previously described.

RESULTS AND DISCUSSION

Automated semi-preparative HPLC

A block diagram of the components which constitute the automated injection

and fraction collection systems is shown in Fig. 2. This consisted of a gradient liquid chromatographic system employing two Altex pumps, a mixing chamber, a manual loop injector, a microprocessor controller, a chart recorder, a fraction collector, and ancillary injection and fraction collection components. The automatic fraction collecting function of this system was controlled in part by the peak detector, which was linked directly to the peripheral interface circuit. When the UV output exceeded that of the peak detector's threshold level, a voltage signal from the peak detector triggered the peripheral interface circuit to simultaneously operate a chart marker and the three-way valve, which either directed mobile phase flow to the head of the fraction collector or to a separate waste flask independent of the fraction collector. It was also responsible for initiating the head of the fraction collector to move along a series of collection tubes each time the threshold was exceeded. The fraction collector was linked directly to the microprocessor by two relay contact closure switches which positioned the head of the fraction collector in front of the first tube and returned it to waste.

Injection system operation

Sample injection was initiated by placing the 7010 and 7030 valves in their load and vent positions, respectively, and the 7120 manual injector in its load position. A 1.2-sec pulse of compressed air, at a minimum of 80 p.s.i., caused alignment of the 7030 valve with that of the 7010 valve and the vacuum source, thereby allowing sample to be drawn into the sample loop. The volume was predetermined with the sample reservoir's stopcock valve shut which allowed a time/buret volume displacement relationship to be established. Another 1.2-sec pulse of air then operated valve 7030, thereby isolating the vacuum source from the loop. This was followed by an additional 1.2-sec pulse of air to realign valve 7010 with the mobile phase and column. Finally, when the sample had been deposited on the column and the sample loop had been adequately flushed, the 7010 valve was returned to its load position.

Fraction collector operation

With the head of the fraction collector positioned in front of the first tube, sample collection was initiated by the peak detector. When the UV threshold of the peak detector was exceeded, the peripheral interface circuit simultaneously marked the chart, advanced the fraction collector to the first tube and switched the three-way valve to collect. When the UV output fell below the set threshold, the peak detector triggered the peripheral interface circuit to mark the chart and switch the three-way valve back to waste. The peripheral interface circuit initiated flushing of the tubing and three-way valve for an adjustable time period of 7 to 37 sec into a waste collection tube prior to advancing the head of the fraction collector over the sample collection tube; by this preliminary step, unwanted cross contamination of samples was avoided.

Programming for automated semi-preparative chromatography

An injection technique similar to that described by Murdock[12] has been employed which involves the regular injection of samples onto a column, such that the interval of time between the first peak of one injection and the last peak of a prior injection is at a minimum, thereby optimizing the number of samples that can be

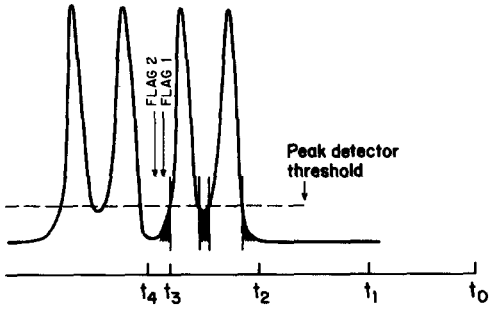

Fig. 4. An idealized chromatographic profile from two sequential injections of a two-component mixture.

injected in a given period. Although this technique was first described for use in gas chromatography[12], to the best of our knowledge this is its first application to preparative liquid chromatography; this methodology should not be confused with recycle chromatography[13,14].

Fig. 4 is an idealized chromatographic profile from two sequential injections of a two component mixture, which will be used as a model for describing the time course of events that occur during this type of chromatography, and the relationship of this process to the sequence of instructions used to program the microprocessor controller. The time points between t_0 and t_4 are defined as follows: t_0, start program; t_1 , end of the program's first cycle and simultaneous start of the second cycle; t_2, arbitrary time point before elution of first peak; t_3, termination of collection of final peak; t_4, arbitrary time point after final peak. The time period between $t_4 - t_2$, the elution interval, was input into the program, where it was equated with the time interval $t_1 - t_0$ so that in Fig. 4, $t_4 - t_2 = t_1 - t_0$.

The software for conducting the injection technique we have described involves a series of commands loaded into the memory of the microprocessor which operates the injection valves and fraction collector via two interface boards attached to the Model 420 controller. The board bearing flags 1–4 was a standard feature and controlled four independent relay contact closure switches, of which flags 1 and 2 were linked to the fraction collector through its remote input connector. Flags 3 and 4 were not employed in this system. The board bearing flags 5–8 was an ancillary solenoid pneumatic actuator component and controlled the 7010 and 7030 valves. Prior to starting the program, the 7010 and 7030 valves were set in their load and vent positions, respectively, and the 7120 manual injector set in its load position.

The first program command, flag 6 rotated valve 7030 from its vent to uptake position. This resulted in sample being drawn via valve 7010 and the vacuum source into the sample loop. Flag 7 was set to return valve 7030 to its vent position after the desired sample volume had entered the sample loop, whereupon flag 8 rotated valve 7010 into its inject configuration. Finally, after the sample had been flushed from the sample loop, flag 5 returned valve 7010 to its original load position, and the injection cycle was then repeated continuously throughout the duration of the separation. Flags 1 and 2 were involved with returning the head of the fraction collector to waste and positioning the head of the fraction collector in front of the first tube. Flags 6, 7, 8 and 5 were set to operate sequentially within a time frame equal to the elution interval

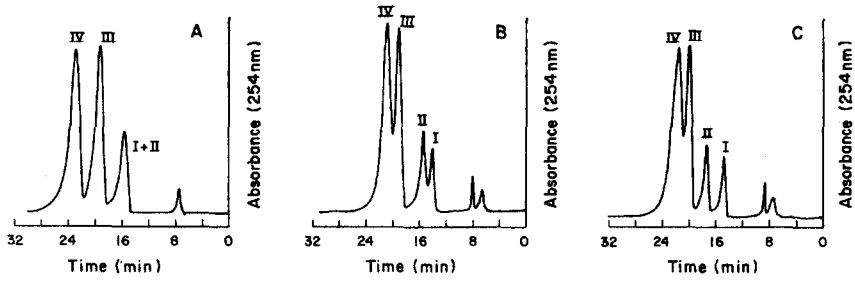

Fig. 5. Chromatographic profile of the four component benzyl D-glucoside mixture: 25-mg sample load at 50 mg/ml concentration; flow-rate, 2 ml/min; UV detection, 254 nm; mobile phase A, ethyl acetate–methanol (97:3); B, chloroform–ethyl acetate–methanol (27:9:4); C, chloroform–methanol (9:1).

($t_4 - t_2$) in question, and needed at least a 1.2-sec pulse of air at 80 p.s.i. Flag 7 was operated after a predetermined time interval which allowed the required sample volume to enter the sample loop (0.5 min = 1 ml in this system). Finally, flag 5 was set to operate after approximately two loop volumes of mobile phase had passed through the loop. Flags 1 and 2 were set to operate for 0.6 sec within the cycle $t_1 - t_0$ at a time point equivalent to the interval $t_4 - t_3$ in Fig 4, since the head of the fraction collector must be returned to waste (flag 1) at the end of one chromatographic cycle, prior to it being repositioned in front of the first tube (flag 2) for the start of the next cycle. Chart speed and mobile phase flow instructions were also input into memory, to initiate their function at time zero and terminate them at a time point after the flag 2 command, which allowed for the system to be automatically switched off at the end of the chromatographic period. After the program had been input into a selected file, the required number of repetitive injections needed to process a sample was then executed by addressing the start file command and selected file, with the microprocessor in its run mode, followed by a numerical duration parameter, which equalled the number of cycles. It should be mentioned that flags 1 and 2 had no operative significance in any cycle prior to collection of the first injection peaks. Although only two injections are shown in Fig. 4, in general the number of injections that occur between t_2 and t_0 is a function of the elution interval ($t_4 - t_2$) and is equal to $(t_2 - t_0)/(t_4 - t_2)$.

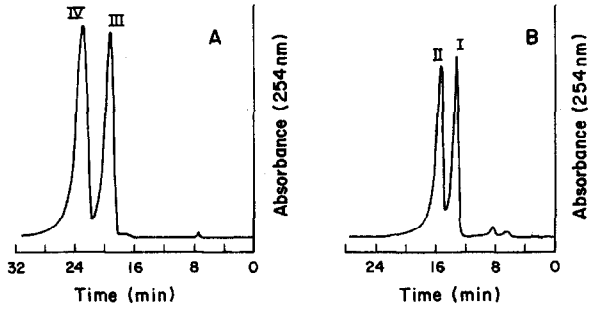

Fig. 6. Chromatographic profiles of compounds I + II (B) and III + IV (A) employing mobile phase systems chloroform–methanol (9:1) and ethyl acetate–methanol (97:3), respectively.

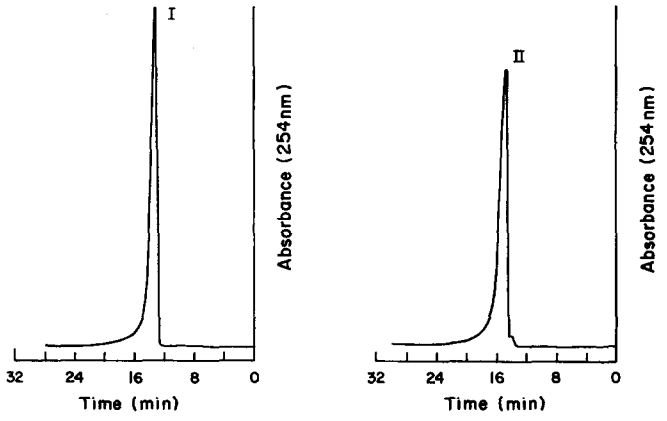

Fig. 7. Chromatographic profiles of purified compounds I and II employing the mobile phase system chloroform–methanol (9:1).

*Automated semi-preparative fractionation of a mixture of benzyl-*D*-glucosides*

The reaction between a monosaccharide and an alcohol under acidic conditions gives rise to a four-component complex mixture of products; such an example involving benzyl α- and β-D-glucofuranosides and glucopyranosides (Fig. 1) was used as a model for evaluating the efficiency of our semi-preparative fractionation system. Extensive silica gel TLC analysis of this mixture suggested that its components could be separated by HPLC using ethyl acetate and chloroform containing small quantities of methanol as mobile phase systems. Fig. 5 shows the elution profiles obtained when 25-mg sample loads were applied via the manual loop injector and eluted with ethyl acetate–methanol (97:3) (A), chloroform–ethyl acetate–methanol (27:9:4) (B) and chloroform–methanol (9:1) (C), at flow-rates of 2 ml/min. The peak numbers in Fig. 5 correspond to the appropriate numbers for structures shown in Fig. 1, and were assigned upon subsequent ^{1}H NMR spectral analysis of the pure components. Profiles A and C (Fig. 5) indicate that good resolution of compounds III and IV was achieved with the mobile phase used in A, while the corresponding compounds I and II were well separated with the mobile phase employed in C. Fractionation of this

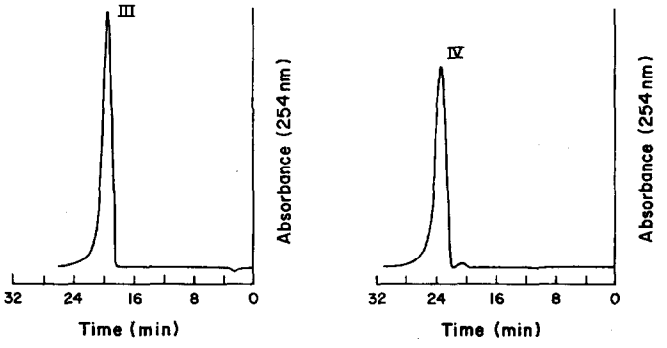

Fig. 8. Chromatographic profiles of purified compounds III and IV employing the mobile phase system ethyl acetate–methanol (97:3).

mixture, therefore, was based on the preliminary separation of peaks I and II from III and IV, which was accomplished with the mobile phase described in B, and involved the injection of 50-mg sample loads and elution at 3 ml/min with continuous repetitive injections being carried out by the technique previously described. Where ethyl acetate–methanol (97:3) was used as the mobile phase, samples were dissolved in ethyl acetate–methanol (9:1) to prevent solubility problems.

By this procedure, compounds III and IV were separated from compounds I and II, the profiles of which are shown in Fig. 6. A comparison of profile B in Fig. 5 with profiles A and B in Fig. 6 clearly indicates that a high degree of resolution was obtained by this initial separation. The final fractionation of compounds I from II and III from IV was conducted in an analogous manner employing the mobile phase systems described in B and A, respectively, as indicated in Fig. 6. The elution profiles of individual compounds I and II are shown in Fig. 7 and compounds III and IV in Fig. 8. As can be seen, the first peaks I and III of the mixtures I + II and III + IV, shown in Fig. 6, were obtained in high purity, while the subsequent peaks II and IV of each mixture contained a very small amount of impurity, which could be removed efficiently if necessary. Throughout the various chromatographic separations conducted, approximately 50% of materials injected were isolated from the peaks collected; the overall recovery of material, however, which included waste products was greater than 95%. At any time in the separation, waste materials could be reconstituted in the mobile phase and continually reapplied to the sample reservoir until essentially all of the material had been processed. Although the loading concentration was a limiting factor due to the relative insolubility of the glucosides, we were able to load 250 mg/h for the four component mixture and 200 mg/h for each of the two component mixtures by the injection technique described herein; this procedure resulted in the isolation of 2.5–3 g of fractionated material every 24 h. In one continuous run in which about 8 g of mixture was processed without recycling waste products, we isolated approximately 0.25 g and 1 g for each of peaks I + II and III + IV, respectively. Continuous recycling of waste products obviously would have resulted in a much higher yield of the purified individual components.

CONCLUSIONS

The automated system we have described allows users of an analytical high-performance liquid chromatograph to significantly increase the capability of their equipment by the purchase of a few commercially available components and the construction of the peripheral interface circuit that we have described. This system clearly has advantages over the method of manual repetitive application of samples, so widely employed to data in conducting semi-preparative chromatography on analytical equipment. For example, not only does this technique allow 24-h continuous sample processing, but when the readily programmable injection technique described is used, an overall improvement in sample throughput of approximately six-fold is obtained during a 24-h period as compared to manual injection of samples over an 8-h period. This system should be particularly useful for the purification of biologically active compounds having poor solubility characteristics, since its continuous operating capability can efficiently provide the required quantities of material. Furthermore, the means to continuously recycle waste products adds to the versatility of this tech-

nique. When operating at a flow-rate of 3 ml/min, approximately 2 l of waste eluent were collected every 24 h; this quantity of material could be readily evaporated and the material reapplied to the sample reservoir without difficulty.

It should be mentioned that column regeneration is required after a few days continuous use. This is achieved successfully by the method of Bredeweg *et al.*[11], although considerable flushing of the column with mobile phase is necessary to restore its efficiency. Finally, while the solubility characteristics and complexity of the mixture we have separated resulted in the isolation of approximately 2.5–3 g of processed material per day, much larger quantities could be obtained in the case of mixtures having good resolution and solubility properties.

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