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HIGH-RESOLUTION PREPARATIVE GAS CHROMATOGRAPHY

I. A MICROPROCESSOR-CONTROLLED SYSTEM FOR AUTOMATED FRACTION COLLECTION

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SUMMARY

A control system for automated high-resolution preparative gas chromatography is described. On the basis of recorded data from a reference chromatogram, the computer-controlled device automatically corrects for the drift in retention that may occur within a run or during extended operation, and synchronizes fraction collection. Failure to track the reference chromatogram activates an error status and protects the traps for contamination. A specified number of consecutive tracking failures discontinues the repetitive fractionation process. The system can be operated in a simulated mode to facilitate optimization and also includes a control routine to drive auxiliary devices such as an automatic on-column injector or flow switching valves.

Cyclic preparative high-resolution fractionation of an essential oil was carried out to evaluate the system. Retention shifts of several seconds were adequately corrected for by the real-time synchronization process, which allowed the precision trapping of closely spaced fractions. Trapping accuracy was also maintained during nearly 800 unattended high-resolution separations of *cis*- and *trans*-isopiperitenol. This resulted in the isolation of milligram amounts of the purified isomers and enabled ^{13}C NMR spectra of the components to be recorded.

INTRODUCTION

A considerable proportion of current research in organic analytical chemistry is focused on the characterisation of trace components, notably in fields such as aroma, pheromone and environmental chemistry, where many of the components that occur in very low concentrations are of key importance. In addition to various pattern recognition studies and quantitative determinations of known components, the structural elucidation of unknown ultra-trace constituents is a central activity in the disciplines mentioned. When dealing with complex natural products or samples of biological origin, such work is a demanding analytical task and requires extensive clean-up and fractionation of the starting material, usually followed by capillary gas

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chromatography–mass spectrometry (GC–MS) and other on-line techniques such as GC coupled with Fourier transform infrared spectrometry.

However, the information obtained in this way is seldom sufficient for the structural elucidation of unknown compounds. It is frequently necessary to obtain complementary chemical and/or spectroscopic (*e.g.*, ^{1}H NMR, ^{13}C NMR, UV) data, which necessitates the isolation of the individual components. Isolation is also imperative if the biological or toxicological properties of unknown compounds are to be evaluated. Isolation is usually accomplished by high-performance liquid chromatography (HPLC). However, the resolving power of this technique is not always sufficient when dealing with very complex samples. Moreover, recovery problems are encountered when the total amount of material is at the micro- or nanogram level. Particularly for volatiles the recovery becomes very poor, notably when reversed-phase systems are used.

Preparative GC would be a more suitable method in such instances, but the separation efficiency of the packed columns that are usually employed is not adequate. Adsorption of polar compounds on the packing material is another factor that limits the applicability of packed columns. Capillary columns provide an attractive alternative and have been used by a number of workers (*e.g.*, refs. 1–5). Most systems described involve manual collection techniques. However, the sample capacity of high-resolution capillary columns is very limited, and the collection of fractions for NMR studies, where micro- to milligram amounts of material are required, may involve more than 1000 repetitive runs. It is obvious that the use of an automated system is a more realistic approach for such work.

In a previous paper[6], we reported a system where the external event outputs of an integrator were used to control automated fraction collection from capillary columns. However, during practical work it was found that retention shifts can occur, particularly during long-term operation. Hence the accurate collection of narrow-spaced fractions was not possible unless frequent timing adjustments were made. Retention instability caused considerable disturbances when complex samples having a wide volatility range were fractionated.

The recovery of the small amounts of sample can be associated with difficulties[7]. Various trapping methods have been described (*e.g.*, refs. 8–10), and some workers have reported close to 100% trapping efficiency. However, the recovery studies were carried out under various conditions and are therefore not readily comparable. In particular, the handling and transfer of microgram or submicrogram amounts of (volatile) fractions is a critical step, which can lead to serious losses. Little systematic work has been devoted to this aspect.

In this series of papers, we shall explore the potential of preparative high-resolution GC and present improved methodology to allow a more versatile use of the technique. This paper describes a microprocessor-based control system, where a real-time correction of the trapping events allows unattended repetitive fractionation over extended periods. It is demonstrated that milligram amounts of pure compounds can be isolated from complex mixtures.

INSTRUMENTAL

Control system

In order to obtain a flexible system, computerized control was regarded as necessary. Precise timing of the trapping events is of critical importance when capillary columns are employed owing to the narrow width of the eluting peaks. A fraction collection period in an area with closely spaced or fused peaks may be as short as a few seconds, and a timing delay of a fraction of a second can significantly influence the composition of the trapped material. Therefore, a system for automatic time correction of the trapping events was constructed. Fig. 1 shows a schematic diagram of the hardware setup.

A home-made printed circuit board configured around a Motorola 6809 microprocessor was used. The program (mainly in assembler language) was stored in EPROM memory (32 kb), whereas the chromatographic data were stored in RAM memory (8 kb). Four VIAs (Mos-Tech 6522) were used in interfacing a printer (Epson RX-80), an ASCII keyboard (Cherry G-80) and a trapping unit (driving micro valves or rotary valves). A remote auxiliary keyboard was used to evoke the trapping events and to start or stop the recording process. A video display unit (NEC JB-902M) was interfaced to the processor bus via a video processor unit, configured around a Motorola M 6845. Apart from the control of the trapping valves, four inputs and twelve opto-coupled power outputs were installed to control auxiliary devices such as an automatic on-column injector.

The analogue detector signal from the chromatograph is transferred to the microprocessor via an instrumental amplifier and a V/f converter (INA 101 AN and VFC 320 BM, respectively; Burr-Brown). When a start pulse is obtained at the moment of sample injection (manually or from the auto-injector), the signal level is sampled every 20 ms and the measured values are compared over a specific time interval. Thus, the slope of the baseline (dV/dT) can be determined. With suitable criteria for dV and dT, the start, the end and the maximum of a peak can be distinguished from baseline drift and noise. This principle of peak detection is commonly employed in commercial integrators.

In order to allow a real-time correction of the trapping events, a reference chromatogram of the sample is first recorded. All retention data (0.1 s resolution), peak heights (in mV) and peak widths (0.1 s resolution) are stored in the RAM memory of the data system. Trapping events are evoked manually by pressing a function key, and the corresponding event times are thereby also stored in the computer memory.

A suitable number of recorded peaks are selected to serve as references, and identification windows are defined. During automatic fraction collection, the peak data are continuously compared and synchronized with the corresponding data of the stored reference chromatogram. The principle of this procedure is depicted in Fig. 2.

In principle, every peak in the reference chromatogram can be employed for the time correction procedure. However, this is usually neither necessary nor recommendable. Poorly separated or very small peaks are unsuitable for reference purpose.

Automatic fraction collection can be simulated in order to check the trapping

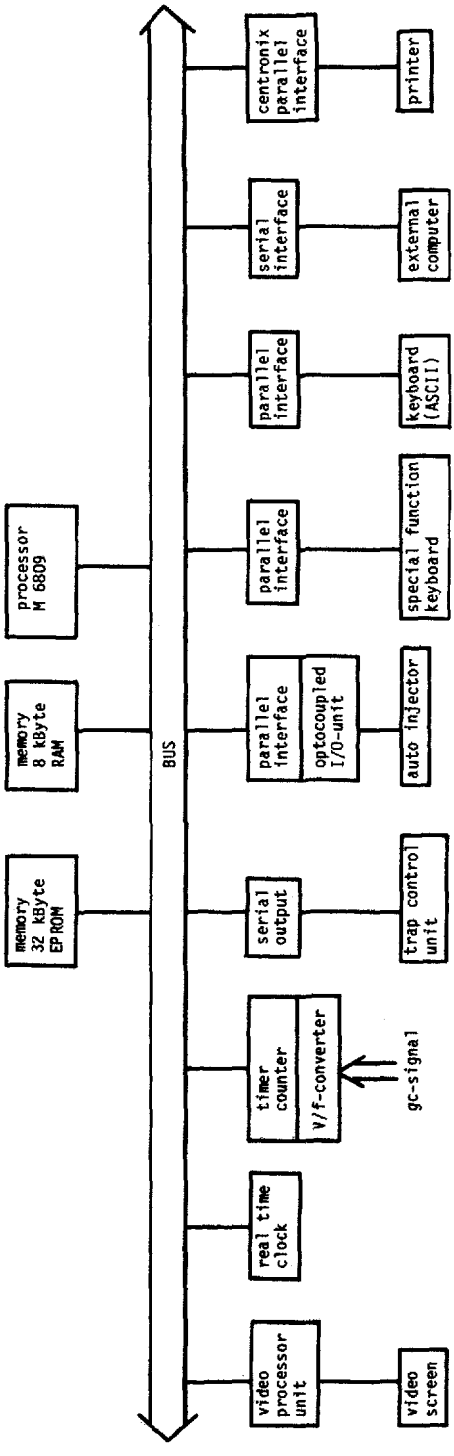

Fig. 1. Hardware configuration of the microprocessor-operated control unit.

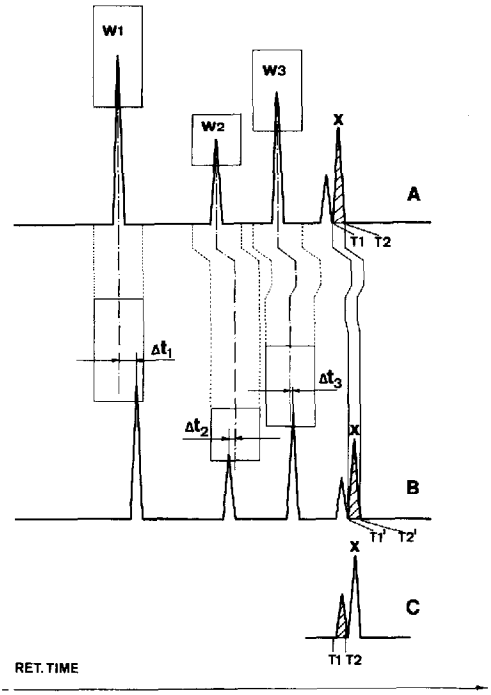

Fig. 2. Schematic principle of the fraction collection synchronization. A represents a model of a reference chromatogram, where the first three peaks are used as references. Time/height windows for each of those peaks are indicated by the areas W1, W2 and W3. The hatched area represents the region of fraction collection (compound X). B represents a chromatogram of a subsequent run, where sampling inaccuracy has resulted in reduced peak heights. However, the reference peaks still fall within the specified windows. A drift in retention is additively adjusted for, and a correct fraction collection is accomplished. C shows the last part of chromatogram B, and indicates how the fraction is collected when no retention adjustment is made.

precision and to verify that the chromatogram is properly tracked. In the simulated mode of operation, the eluting compounds are guided to a "waste" trap, to protect the individual collection tubes for contamination with unwanted material. After simulation (or any forced interruption of the process), the trapping and reference data can be edited and simulation can be repeated until satisfactory operating conditions have been created. Flexibility for optimization is obtained by using a menu-structured program. A flow sheet of the principal elements of this program is shown in Fig. 3.

Fraction collection

Trapping of the eluted fractions was accomplished according to the same principle as described previously[6]. However, some hardware modifications were made, as illustrated in Fig. 4. The effluent from the capillary column was guided to a three-way tee, where make-up gas (2 ml/min) was added, and further connected to a manifold (five or fifteen outlet ports). Part of the effluent (1/30) was guided to a flame ionization detector. Both the make-up tee and the manifold were made of fused-silica tubing, which was precision-fitted into polyimide bodies and glued with polyimide

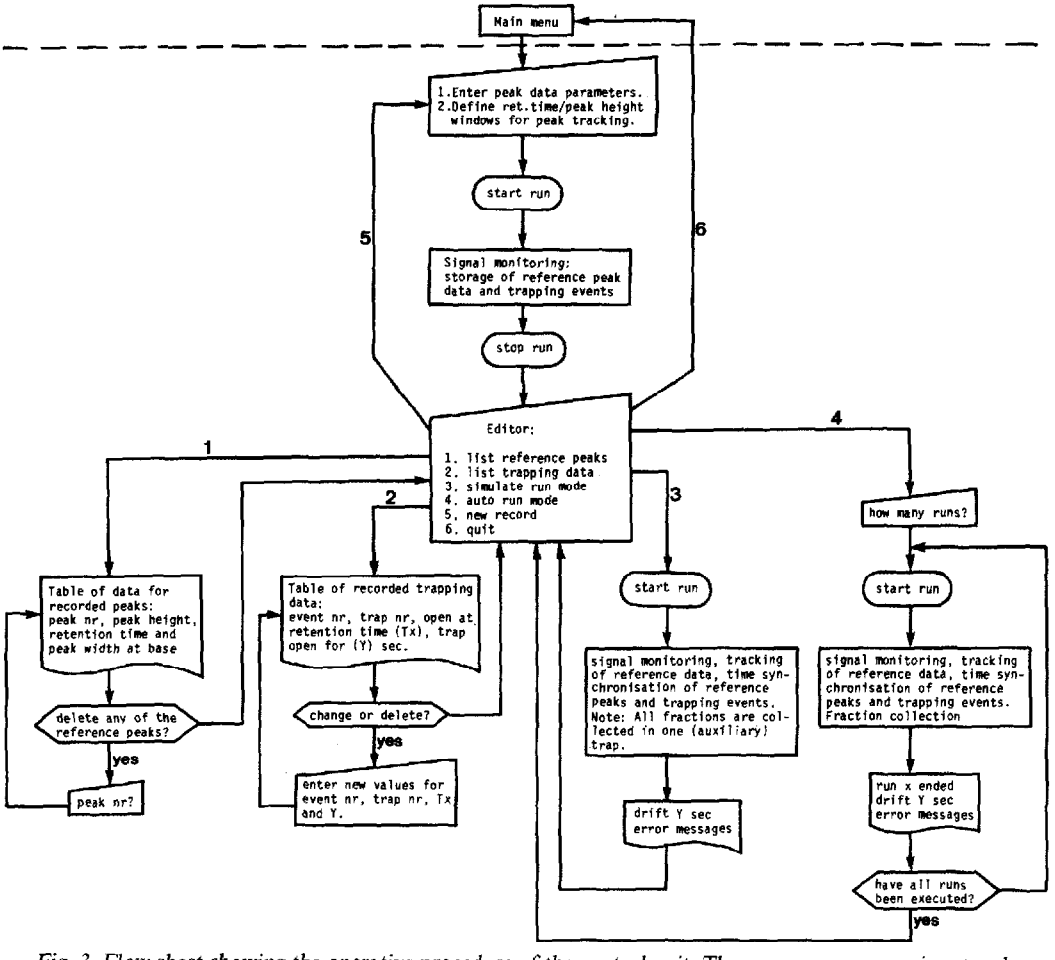

Fig. 3. Flow sheet showing the operative procedure of the control unit. The program sequence is entered via a "main menu", which comprises auxiliary entries for control of the individual functions of an auto-injector, flow switching, etc. Automatic injection is normally initiated at the "start run" command, but can also be delayed or repeated any time during the run. The cycle time for a run is automatically defined when the recording process is stopped. The recorded trapping events are numbered in consecutive order (default), but can be renumbered by using editor-2 (*e.g.*, to create mixtures of fractions). The tracking process (simulated or auto-run mode) can be interrupted at any instance. The program will then return to the editor.

prepolymer according to a procedure described by Sandra *et al.*[11]. Thus an inert flow distribution system was obtained.

Coiled glass capillary tubes (2 m × 0.6–0.7 mm I.D., coil diameter 130 mm) were used to trap the effluent. The tubes were straightened at both ends over a length of approximately 20 cm to facilitate connection. The inlet ends of the traps were drawn down to the same outer diameter as the tubes from the manifold. Connection was accomplished using shrinkable Teflon or miniature connectors[12]. The connections were positioned in a gradient-heated aluminium interface tube to reduce problems with condensation and fog formation. The outlet of the traps were connected

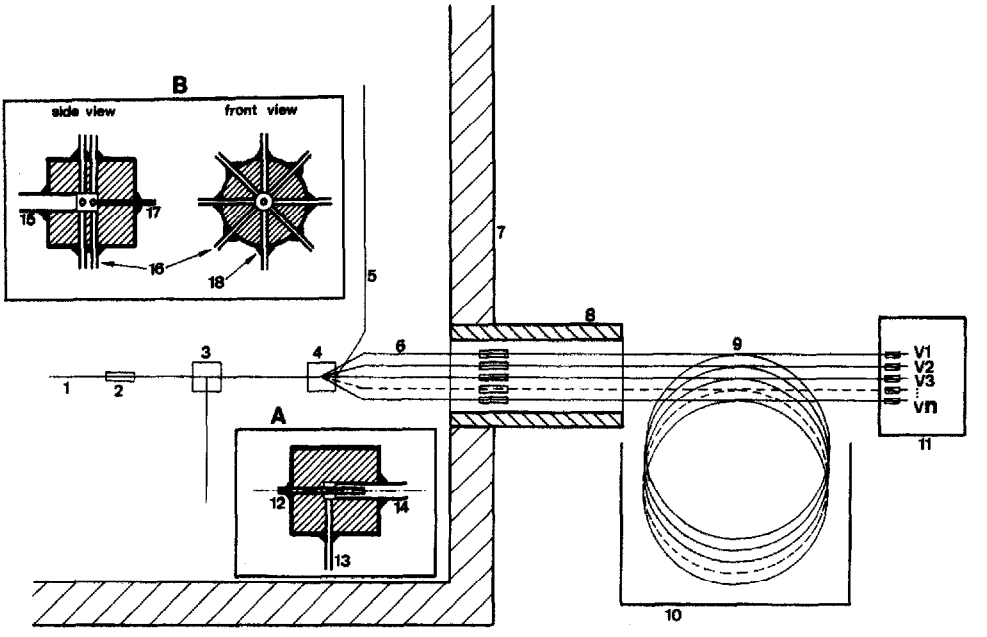

Fig. 4. Manifold and trapping arrangement. 1 = Capillary column; 2 = miniature connector; 3 = polyimide make-up tee; 4 = multi-channel manifold; 5 = outlet tube to detector; 6 = outlet tubes to the traps; 7 = GC oven wall; 8 = heated aluminium tube; 9 = glass capillary traps; 10 = Dewar (dry-ice–ethanol); 11 = trapping control unit with magnetic valves (V1, V2, V3, etc.). (A) Enlarged view of the polyimide make-up tee. 12 = Inlet tube connected to the capillary column (I.D. 0.32 mm); 13 = inlet for make-up gas (I.D. 0.32 mm); 14 = outlet tube to the manifold (I.D. 0.5 mm). (B) Enlarged view of the multi-channel manifold. 15 = Outlet from the make-up tee; 16 = outlets to the traps (I.D. 0.2 mm); 17 = outlet to the detector (I.D. 0.1 mm); 18 = polyimide glue.

to micro-magnetic valves (Brunswick Scientific, Model 407-C). A maximum of 32 valves could be driven from the processor unit via an opto-coupled serial input driver (MM5451; National Semiconductors). The setting of the valves was indicated by corresponding light-emitting diodes. The control unit can also be used to drive a rotary valve system instead of magnetic valves. However, the use of a manifold splitter and a common waste trap offers several advantages. First, there are no moving parts in the region of the sample passage. Second, the fractions can be guided to any of the traps in an optional sequence. This offers the attractive possibility of collecting mixtures of substances in their original proportions. Such a procedure is of particular value in flavour and pheromone chemistry when searching for key combination of compounds.

An important aspect in a system for unattended automated fraction collection is the elimination of trapping errors and associated risks of contamination of the collected material. A subroutine was included in the control program to protect the trapped material in events of failure. Under normal collection conditions, the common waste valve V1 (and corresponding trap) is kept open as long as no other valve is activated. During recording of the reference chromatogram and during test runs in the simulated mode, the fractions to be trapped are collected in a second waste trap (V2). Thus, flow conditions identical with those in the automated collection

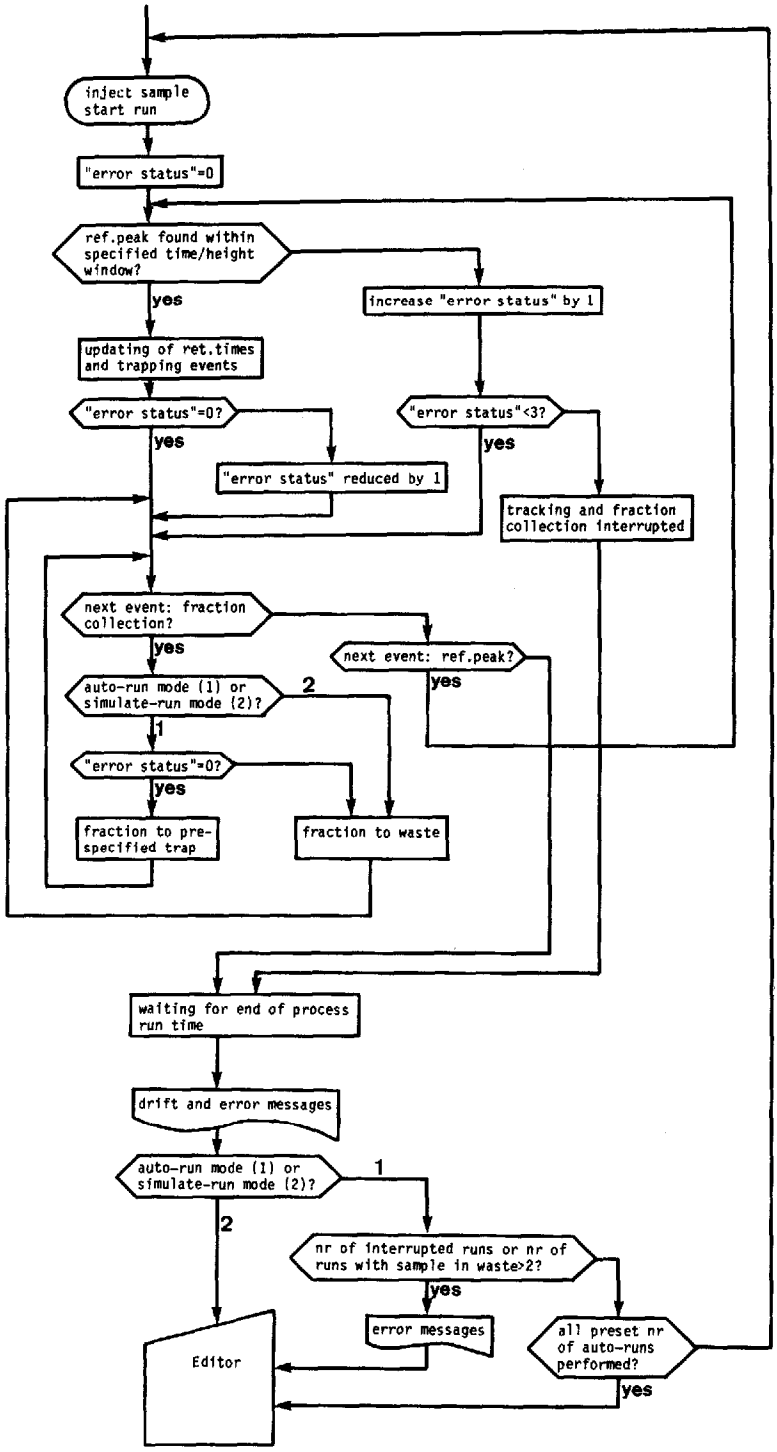

Fig. 5. Flow sheet of the automatic control and error handling procedure.

mode are established. This is particularly important when flow switching systems[6] are included in the splitter and manifold unit. Failure to track a reference peak activates an error status, and the remaining fractions to be collected during the ongoing run are switched to V2. The error status is reverted to normal when reference peaks are tracked again. The repetitive separation process will be halted if V2 has been activated in three consecutive runs. Fig. 5 shows a flow sheet where the error handling procedure is included.

Chromatographic equipment

A gas chromatograph (Varian Model 6000) equipped with a flame ionization detector and a split/splitless injector was used together with the described control and trapping unit. The electrically heated aluminium interface tube (length 175 mm, I.D. 14 mm) was inserted in the oven wall under the control box for the pneumatics. Automatic repetitive sample injection was accomplished using a modified autosampler (Varian Model 8000). The regular sample carousel was replaced by an automatic micro-dispenser (Hamilton Microlab-P), equipped with a 0.5-ml syringe. This syringe was coupled to the side inlet of the auto-injector syringe via a narrow-bore PTFE tube. The dispenser was actuated by a micro-switch when the plunger of the injection syringe reached the filling position. The micro-dispenser was set to fill the syringe of the auto-injector with sample portions of 5 μl. After 99 injections, the stock syringe was refilled with sample solution. For manual injections and fraction evaluation, an on-column injector (SGE-OCI-3) was used.

A computing integrator (Spectra-Physics SP-4270) was employed for area integration. A strip-chart recorder (Servogor 120), coupled in parallel, served as an additional monitor. The capillary columns used were prepared according to the procedure of Grob *et al.*[13]. The stationary phase (SE-54) was cross-linked with azoisobutyronitrile[14].

RESULTS AND DISCUSSION

Precision trapping

Several methods can be employed to ensure accurate fraction collection. In systems where packed columns are used, timer-based control devices without automatic retention adjustment are usually regarded as satisfactory[15,16], and a time resolution of the order of 1 s may be sufficient. A few systems for work with packed columns have employed a time correction procedure[17,18]. Hupe[19] employed a peak level detector, combined with a peak counting procedure. However, this technique is not suitable in cases where trace components are to be collected or in configurations where a flow switching splitter[6] is utilized. Additional advantages of time-based systems for fraction collection have been summarized by Roz *et al.*[20].

For high-resolution preparative work, stringent timing is necessary. Although a high degree of reproducibility is obtained with modern GC equipment, long-term deviations in retention can occur, *e.g.*, owing to accumulation of non-volatile material in the column, column deterioration, injection imperfections and sometimes also sample decomposition. Linear shifts in retention are frequently encountered when splitless or on-column injection in combination with the solvent effect is employed.

The present control system has the appropriate time resolution to be com-

patible with rapidly eluting compounds. Hence fused peaks can be "sliced" into optimized fractions. The real-time correction process ensures correct timing throughout long-term collection, and the error handling program allows safe unattended operation.

Proper selection of the reference peaks is important. The best trapping synchronization is obtained when reference peaks are chosen that elute just prior to the fractions to be trapped. Retention windows should be chosen large enough to allow for the maximum expected drift, even when the area embraces more than one peak. A mix-up of the peaks is not possible as long as the peaks can be discriminated in height. The signal obtained during collection can also be used for reference purpose. It is important to realise that the described retention correction system does not eliminate the need for precise control of the chromatographic variables (temperature, flow, etc.) Non-linear shifts are not fully corrected for, and it is hardly possible to provide a satisfactory algorithm that compensates for such errors. It would, of course, be possible (and simple) to include a level detector, which is activated in a defined retention window, but the advantages of the time-operated system are thereby sacrificed. However, as judged from the work carried out so far, no additional correction measures are necessary.

Optimization

In production-scale preparative GC, it is of vital importance to optimize the preparative efficiency[21], *i.e.*, to maximize the preparation of material of a defined purity per unit time. In this context, it is usually advantageous to use pre-fractionation procedures[22] or temperature programming. In principle, this approach should also be followed in preparative work using capillary columns. In fact, it is even more important here, considering the very limited sample capacity of capillary columns. Therefore, the strategy for the isolation of a compound from a complex mixture should include an extensive pre-fractionation on high-capacity systems (LC columns, packed GC columns).

However, in many situations, the total amount of available sample is limited to a few milligrams or less (*e.g.*, flavour concentrates, pheromone isolates, gland extracts). The collection of such material is usually a laborious procedure, and the preparation of substantially larger amounts involves an unreasonable amount of work or is simply not possible. Thus, the risk for losses due to sample work-up, adsorption, etc., must be reduced to a minimum, which largely prohibits the use of classical pre-fractionation methods. In such instances, preparative capillary GC has a unique potential.

Usually, it is desirable to isolate as many components as possible when dealing with limited amounts of important isolates, and a one-step preparative fractionation using an inert capillary column may be the preferred approach. If only a few target compounds are to be isolated, a preparative pre-separation on a capillary column with a different stationary phase may be advantageous. Column choice and optimization of microfractionation is a complex procedure, where many factors such as volatility, polarity and available amounts of starting material must be taken into consideration. In a forthcoming paper in this series, this subject will be considered in more detail.

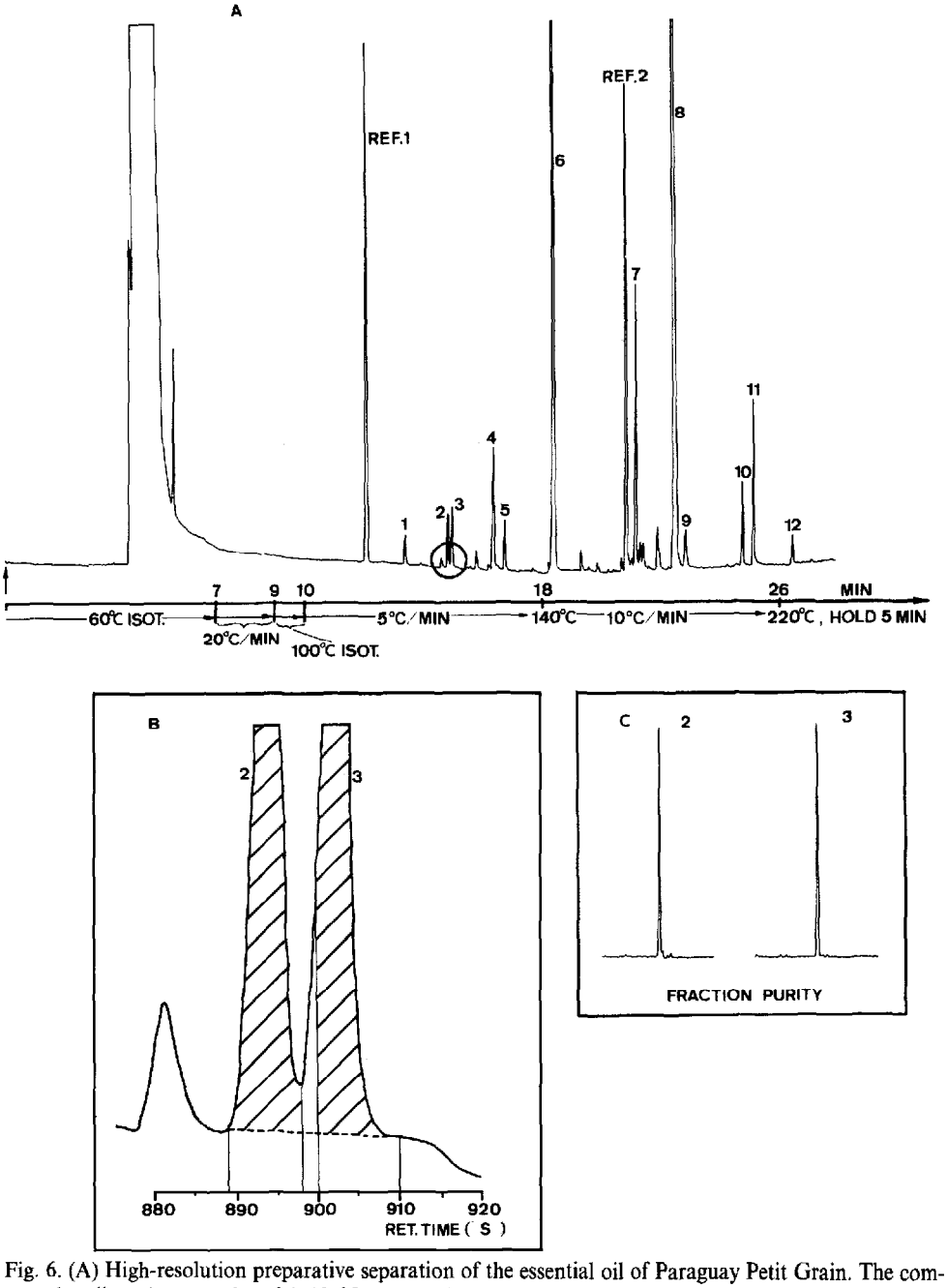

Fig. 6. (A) High-resolution preparative separation of the essential oil of Paraguay Petit Grain. The compounds collected are numbered 1–12. Nonane and dodecene (refs. 1 and 2) were added to the sample (125 μg/ml) and served as reference compounds for the retention synchronization. Chromatographic conditions: sample concentration, 1 mg/ml in hexane; injection, splitless, 1 μl; column, 50 m × 0.31 mm I.D., SE-54, 0.5 μm, cross-linked; carrier gas, nitrogen, head pressure, 100 kPa; time window for the reference peaks, 40 s; maximum allowed height variation of the reference peaks, 50%. (B) Enlargement of the encircled area around peaks 2 and 3. The hatched regions represent the areas of fraction collection, as defined in the stored reference chromatogram. (C) Chromatograms of compounds 2 and 3 after 76 repetitive collections.

System performance

In order to evaluate the performance of the control system, a direct fractionation of a complex mixture (the essential oil of Paraguay Petit-Grain) was carried out. Twelve components from this mixture were isolated from 76 repetitive automatic separations under temperature-programmed condition. A representative gas chromatogram and the corresponding operating conditions are shown in Fig. 6A.

Nonane and dodecene were used as reference compounds for the synchronization of the process. The setting of the identification windows employed allowed a maximum retention shift of 10 s in the forward or backward direction. The maximum drift observed was 4.5 s. The peak-height variations were not more than 30% from run to run. Hence faultless tracking of the reference peaks occurred. The retention correction was accomplished with a trapping precision of *ca.* 0.2 s, as could be observed from the event marks in the chromatograms. Fig. 6B shows an enlargement of the lower area around two closely spaced peaks (2 and 3). As can be seen, a drift of a few seconds would have caused appreciable cross-contamination. On comparing the chromatograms of the collected material (Fig. 6C) with the set points for the fractionation (Fig. 6B), an excellent correspondence in expected fraction purity is observed, which confirms the observed trapping accuracy.

It should be mentioned that the fractions collected were found to contain small amounts of impurities of a wide volatility range. The origin of this material is not known. It is possible that the fractions were contaminated during handling, transfer or re-injection, or that the impurities stem from the solvents employed. Another

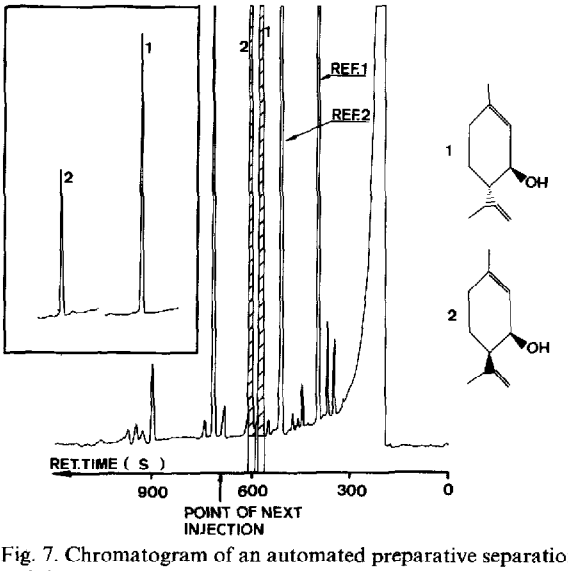

Fig. 7. Chromatogram of an automated preparative separation of *trans*- and *cis*-isopiperitenol. Undecane and dodecane (refs. 1 and 2) were used as reference compounds for the retention synchronization. Chromatographic conditions: column, glass capillary 25 m × 0.3 mm I.D.; film thickness 2 μm; stationary phase, SE-54, cross-linked; column temperature, 180°C, isothermal; carrier gas nitrogen; inlet pressure, 35 kPa; injection, 3 μl; split, 1:10 of a solution in hexane (this corresponds to a column load of 8 and 2 μg of components 1 and 2, respectively). The interspersed chromatograms depicted on the left-hand side show the purity of the fractions after 796 repeated collections.

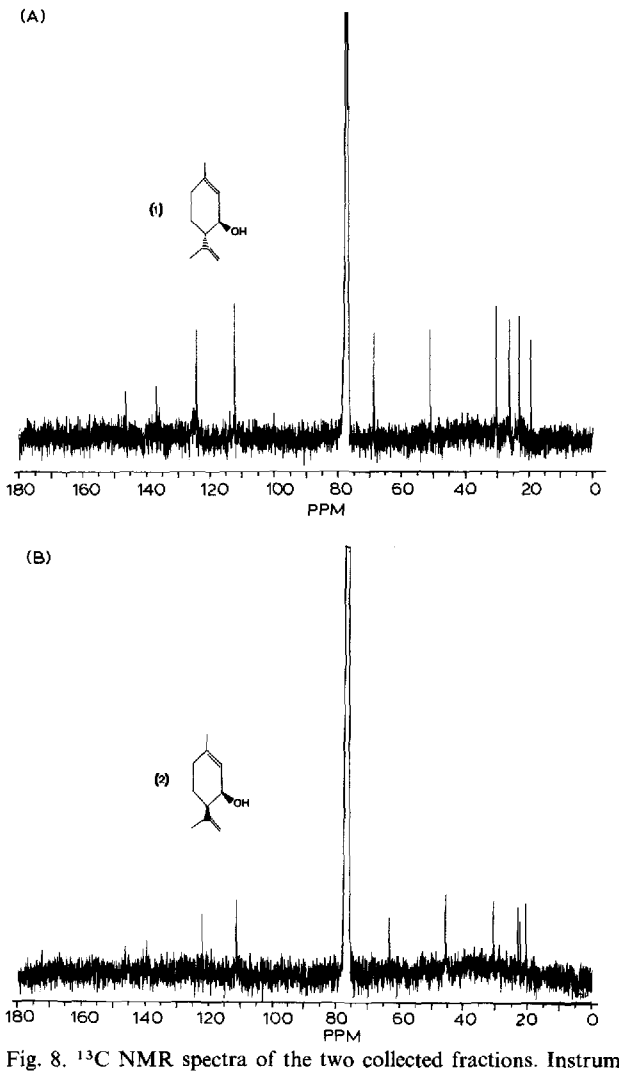

Fig. 8. ^{13}C NMR spectra of the two collected fractions. Instrument: Jeol GX-400; sample tube, 5 mm diameter; solvent, $CDCl_3$. (A) 6500 scans; (B) 25 000 scans.

problem concerns the sample recovery, which never exceeded 80%. This may be due to inefficient trapping, but could also be caused by losses in the injection system or during sample transfer. Both questions are at present being studied in detail.

Long-term collection

An additional experiment was undertaken to evaluate the long-term performance of the control system. Two isomers of isopiperitenol, obtained in a mixture from a synthesis, were subjected to automatic preparative fractionation. Two hydrocarbons (undecane and dodecane) were added to serve as references for the time synchronization. A chromatogram of the mixture and the corresponding chromatographic conditions are shown in Fig. 7. Optimization of the cycle time was accom-

plished by injecting the sample during the elution of the preceding injected material.

The average drift during the collection period was about 1 s. The maximum retention time deviation during a run from the nearly 800 unattended cycles proved to be 6 s, which demonstrates the need for the time correction system. Faultless tracking was observed throughout the experiment, and the collected material had the expected high purity. The extended preparative fractionation afforded several milligrams of the pure isomers, sufficient for ^{13}C NMR spectroscopy. Fig. 8 shows the spectra of both fractions. As can be seen, all the carbon atoms of the monoterpene structure can be assigned. The spectroscopic data obtained are at present being used in a structural study of the isomers.

CONCLUSIONS

The real-time retention correction system described provides an accurate and versatile means of control for automated preparative capillary GC. The automatic protection for collection errors allows reliable and unattended operation under extended periods. This opens up new opportunities for the straightforward isolation of trace components from complex mixtures, and makes it possible to obtain ^{1}H NMR or ^{13}C NMR spectral data for such components.

There should be several other applications of the described system, such as the control of multi-dimensional column switching or on-column trace enrichment and for preparative HPLC.

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