AN IMPROVED PRECOLUMN TECHNIQUE FOR HIGH-TEMPERATURE GAS CHROMATOGRAPHY OF BIOLOGICAL EXTRACTS

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INTRODUCTION

Isothermal gas chromatography instruments, which are available to date, can only accept a small volume of solvent vehicle with the samples, a consequence of the limited capacity of the systems to evacuate the vapor formed.

ABEL[1] has stressed some of the advantages of improving the solvent-handling capacity of these analyzers; and evaluated the use of programmed temperature precolumns as a means of selectively trapping the sample components, while the solvent vapor is vented to the atmosphere before it could reach the partition column. In ABEL's paper several pertinent literature references are also discussed.

In our experience of fecal sterol and bile acid analysis using an argon chromatograph, it was necessary before quantitative analysis could be achieved, to prevent the introduction of volatile sample contaminants, as the latter caused poor tracings and the sensitivity of the detector cell to vary across a chromatogram.

Of the different improved sampling methods tried, one was found safe for prolonged use with many types of biological extracts. The device, as described in the present paper, includes a conventional flash-heater injector; a vented programmed temperature precolumn the packing of which, unlike that in the ABEL system, is coated with the partitioning liquid phase; and an isothermal analytical column.

APPARATUS

This is shown schematically in Fig. 1. A Pye (Cambridge, England) argon chromatograph, Cat. No. 12,001, is used as the basic instrument. The analyzer has an isothermal oven for a 4 ft. straight, vertical glass column.

A 21 cm Pyrex glass tube (1) (8 mm O.D. × 4.5 mm I.D.) is packed over 16 cm in the same manner as the main column (*e.g.* for steroids, 1 % SE-30 on 100–120 mesh Gas Chrom Q). The top of this tube (precolumn) is equipped with a simple brass "tee" device (2) for injection through a septum and carrier gas inlet. Just below the tee the tube is jacketed for 70 mm, which includes 35 mm of packed area, with a bored aluminium cylinder (3) which can be electrically heated up to 300°. This arrangement affords a flash-heater chamber for sample vaporization, and a packed isothermal path for vapor insertion into the precolumn.

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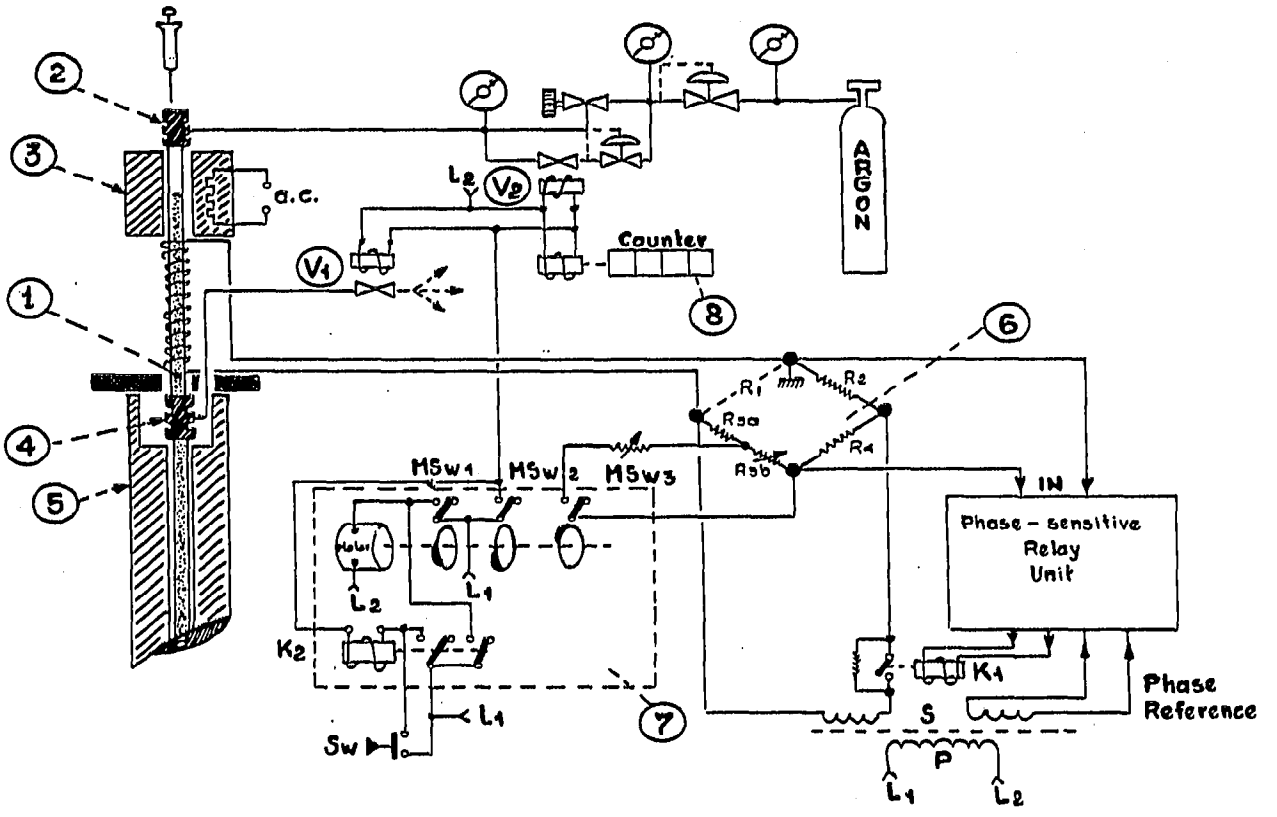

Fig. 1. Schematic diagram of the precolumn sampling system. L_1, L_2 = a.c. mains.

The remaining part of the tube (over 12 cm), between the flash-heater block and the junction (4) with the main column, has a continuous coil of bare 1 mm diameter No. 304 stainless alloy wire around it. A minimal distance between the coil turns is secured to avoid short circuits, and both coil ends are insulated from adjacent metal bodies by a layer of Teflon foil.

The precolumn tip extends into the isothermal column heating jacket (5) through a hole in the marinite insulating top plate, and is connected to the main column by means of a 5/16 in. to 5/16 in. swagelock union (4) having a 1/16 in. stainless steel tube outlet. The latter, externally to the oven, is connected to a one-way solenoid valve, V_1, opening to the atmosphere which is normally closed. Argon is supplied to the precolumn inlet from a constant flow (restrictor type) to constant pressure switchover device using a normally closed solenoid valve V_2.

The stainless steel coil around the precolumn serves as a programmed heater and as a screen against fluctuations of ambient air temperature. Therefore, the coil a.c. power circuit is wired so that it comprises one arm of a resistance-thermometer bridge (6), the coil serving as its own transducer, this by virtue of the temperature coefficient of resistivity of stainless steel. Two reproducible settings of the coil average temperature can be obtained by changing the value of the resistor R3 in the bridge, by means of the normally closed microswitch contact MSw_3 (see below). The bridge output is levelled by means of a diode limiter network, and is then amplified and rectified in a conventional phase-sensitive relay unit. The in-phase bridge output closes the mercury power relay K_1 for a fall of temperature at the coil, which increases by ten-fold the heat dissipation at the coil (for 5 Ω at 20°, 85 W under 24 V bridge

input). Because there is no thermal resistance between the heat source and the temperature detector, the advantages of the low heat capacity of the heater and operation without overshooting are combined. For adjustment purposes, the actual temperature at various levels inside the precolumn is measured by means of imbedded thermocouple junctions. An approximation of the temperature at any level of the coil is obtained from a low-inertia thermocouple welded on the wire. The profile of precolumn temperature changes when desired can be recorded on a 20 mV chart potentiometer.

TIMING OF THE PROCEDURE

Prior to operation of the apparatus the flash-heater is hot, and the coiled precolumn area is maintained isothermal at a temperature too low for migration of the sample components. Sample injection is preceded by opening the venting valve and switching on the constant-pressure diaphragm regulator, which increases the flow rate. The scavenging flow is maintained after injection, until carrier gas of high purity again flows through the system. Meanwhile the sample components regroup as a narrow band at the beginning of the coiled area, which acts as a trap. The subsequent steps consist in closing the venting valve, restoring the constant-flow supply through the precolumn, main column and detector system, and raising the precolumn temperature to achieve sample migration, which ultimately proceeds isothermally in the main column. Resetting the precolumn to the lower stand-by temperature as soon as the sample vapors have reached the main column junction, makes it possible to obtain rapid requilibration for a subsequent run.

Automatic timing of all these operations is effected by means of a three-cam synchronous motor-driven, two-minute repeat cycle timer (7). The cycle is push-button started (Sw), involving the locking relay K_2 and holding microswitch MSw_1. A few seconds later, microswitch MSw_2 energizes the parallel-wired constant-pressure supply and venting valves V_1 and V_2, and resets relay K_2. Microswitch MSw_3 finally controls precolumn warm-up by increasing the value of R_3.

PERFORMANCE AND DISCUSSION

The effect of the precolumn procedure upon peak shape and column efficiency, if any, was an improvement over conventional sampling.

The usual isothermal relative retention time data are no longer available with the new procedure. The peak recorded shortly after closing of the venting valve marks the emergence of a trace of solvent which infiltrates by diffusion to the top of the main column during venting and bears no direct relationship with the delayed and programmed insertion of the sample. The present discussion is thus restricted to the aspect of reproducibility of absolute retention times with a given instrument. A leak at the injection septum or at the column junction is a source of error shared with any instrument having a constant-flow supply. The effect of moderate temperature instability at the flash-heater was not detectable, because this affects the site of regrouping of the sample components prior to precolumn warm-up very little. Reliability of timing only depends upon the quality of the timer used. Reproducibility of precolumn temperature settings with the control system used is independent of line voltage or ambient air-condition changes. The profile of the precolumn tempera-

ture increase is potentially much more important; in contrast to the above, it is line voltage dependent and to some extent influenced by the room temperature, because the very fast temperature rise used (in the 150°/min range) is obtained on a heat exchange basis between the coil and the precolumn glass body. In practice, however, any influence upon the retention times due to small distortions of the profile of temperature increase was not observed, because elution of the short precolumn is very rapid. In conclusion, it may be said that the principal source of temperature disturbance lies inside the precolumn owing to the heats of warm-up and of vaporization of the solvent; the latter factor is discussed below.

Since the entire coil heater serves for temperature detection, the control operates on an average temperature basis. For any setting the proximity at the coil ends of the flash-heater and the column-oven, respectively, is a cause of superheating localized at these sites. In practice this is rather desirable and helps efficient mobilization of the sample during warm-up and correct vapor transfer through the precolumn–column junction.

During the venting period the gas flow is interrupted in the main column and detector. With an argon cell this is not objectionable, provided that the column-to-detector coupling is gas tight to avoid air diffusion. A compensating gas supply for flow sensitive detectors, such as the one described by DEANS[2] for a different application, could be used.

EFFECTS OF SOLVENT LOAD AND CONTAMINANTS

When a scavenging gas flow during injection of the order of 1 l/min S.T.P. (argon inlet pressure 1.5 kg/cm^2 and precolumn particle size 100–120 mesh) was used, identical chromatograms from microgram loads of usual test mixtures (*e.g.* cholestane–cholesterol) were obtained after varying the volume of solvent (*e.g.* petroleum ether or acetone) from 1 to 100 μl (for acetone, yielding respectively 0.3 and 30 ml of vapor S.T.P.). Such independence of the solvent load was confirmed with other solvents and solvent mixtures, different precolumn stationary phases, and over thousands of injections with various steroids and their derivatives (sterols, bile acid methyl esters, and their acetates, trifluoroacetates, or trimethylsilyl ethers). The following example demonstrates the expedient of mixed-solvent injection. Submilligram amounts of steroids are acetylated in test tubes (*e.g.* with acetic anhydride–pyridine); the mixture is then quenched with water, and diethyl ether is added. After mixing, 0.1 ml of the ethereal supernatant may be injected into the precolumn. The procedure thus does not require a concentration step.

The cooling effect of repetitive injection (*e.g.* at 8 min intervals) of a large amount of solvent clearly had no influence upon the retention times or upon the efficiency of sample insertion. The combined heat capacity (liquid and gas) and heat of vaporization of acetone for a 250° rise is approximately 0.2 cal/μl, which justifies the use of a large flashheater block, and a fast response programmer thermostat.

Under the scavenging condition used, abrupt discharge of a full 0.1 ml syringe onto the bare top of the precolumn packing does not disturb the support particles, which excludes vapor lock formation.

By appropriate selection of the precolumn lower isothermal temperature (*e.g.*

100° for steroids), it is possible to prevent contaminants more volatile than the compounds of interest from reaching the main column (*e.g.*, fatty acid methyl esters in the analysis of bile acid esters). Venting volatile contaminants of any nature generally results in a better return to base line after injection of samples insufficiently purified to be dealt with by conventional isothermal chromatography and might be a better method for analyzers used in conjunction with digital data computing devices.

Accumulation of non-volatile residues from crude samples at the top of the column, with conventional sampling, tends to interfere with correct insertion of sample vapors, and ultimately induces peak broadening and tailing. The maintenance of clean packing at the top with the precolumn system was found to be of minor importance, because chromatography starts at a clean precolumn level. Furthermore, supression of flash-back effects during injection eliminates spreading of contaminants on the walls of the injection port.

Impurities from biological samples which are often tested, can migrate slowly on an isothermal column. This difficulty can be solved by different methods. The precolumn may have a special program to vent slowly-migrating bands after insertion of the desired components into the main column. Alternatively, the main column can be back-flushed. Details of a back-flushing device using external valves are given in the paper by DEANS[2].

QUANTITATIVE ANALYSIS

Precolumn sampling in our experience with sterols and bile acids boosts the sensitivity of the argon detection system, making this detector reliable for delicate quantitation problems such as the measurement of several peaks differing widely in their retention times. Indeed a major difficulty with the argon cell lies in unpredictable quenching of response when certain sample contaminants forced through the entire column and detector line at high temperature happen to dehydrate.

MAINTENANCE

The performance of precolumns at various stages of aging was investigated with a short isothermal oven and argon detector assembly. It was found that neither repetitive solvent scavenging, abrupt changes of gas velocity, nor sharp temperature rises, had a long-term effect upon precolumn partitioning properties. Bulky top contamination from sample impurities apparently was the only source of loss of performance, and may lead to base line instability due to carrier gas contamination by pyrolysis products. The preparation of spare precolumns includes conventional pretreatment in a small oven under a flow of inert gas, *e.g.*, at 25° in excess of the maximum operating temperature.

A suitable precolumn replacement schedule for each analytical problem treated on the routine scale can be fixed by experiment, where each analyzer unit has a digital counter (8) (see Fig. 1) which records the number of injection cycles elapsed.

Attention should be paid to correct main column conditioning. The column at each opening of the venting valve is subject to a sudden pressure release to the atmosphere; it is thus essential to guard against the possibility of particle stirring and crushing from a loosely packed column. Therefore after conventional pretreatment

TABLE I

EXAMPLES OF ANALYSES WITH THE PRECOLUMN SAMPLING SYSTEM

Average common operating conditions. Basic instrument and sampling system, as in the text. Argon flow rate, 60 ml/min S.T.P. Scavenging constant-pressure inlet, 2 kg/cm^2. Precolumn venting period: 40 sec. Precolumn warm-up period: 70 sec. Time interval between valve closing and start of warm-up period: none. Sample loads, 1–10 μg range. Solvent loads (acetone): 50–80 μl.

Operating conditions	*Source of sample and compounds chromatographed*		
	Rat feces: crude unsaponifiable matter (sterols)	*Chicken feces: bile acids (acetylated methyl esters)*	*Rat feces: fatty acid methyl esters (C_{12}–C_{20})*
Analyzer temperature	220	250	160
Flash heater temperature	250	270	200
Precolumn stand-by temperature (average)	50	50	50
Precolumn upper temperature	230	250	200
Number of runs without maintenance	over 500	400–600	not determined
Precolumn packing	0.7% SE-30	0.7% SE-30	1% XE-60
Main column packing	0.7% SE-30	0.7% XE-60	12% diethyleneglycol succinate

the packing of each new column should be completed by ensuring that the top is efficiently secured against back pressure surges.

The absence of flash-back effects during injection permits the silicone rubber septum to be kept at an unusually low temperature (for steroids, 200° or less); while sample cross-contamination from the syringe needle is prevented by the use of dilute solutions. Using the Hamilton No. 710-N syringe, septum replacement after each series of 100 injections may be advisable.

APPLICATIONS

Some of the routine problems of quantitation which have been successfully treated in this laboratory over the past two years using the precolumn sampling procedure, are listed in Table I, which also details suitable operating conditions.

CONCLUSIONS

Among the most evident advantages of the present sampling procedure, the gas flow through the injection port can be considered best, as it is probably the only conceivable way of eliminating flash-back effects with large volumes of solvent.

A common feature with conventional programmed temperature chromatography is that the system ensures rearrangement of the sample at a clean packed level prior to insertion into the main column.

The present study may serve to emphasize the usefulness of better methods of discrimination between sample components and their contaminants at the instrument level. Efforts in this direction are complementary with those made by biomedical analysts to improve their methods of sample purification, thus enlarging the field of application of the gas chromatography technique.

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SUMMARY

The use of a vented and programmed temperature precolumn packed with a partitioning liquid phase is described as a means of improving high-temperature isothermal gas chromatography of contaminated biological extracts. The system eliminates flash-back effects at the injection port, improves the capacity of analyzers to deal with large amounts of volatile solvents, and retards long-term degradation of performance when crude samples are routinely analyzed.

REFERENCES

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