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THE LIQUID SPECTRORADIOCHROMATOGRAPH—AN AUTOMATED METHOD FOR THE SIMULTANEOUS SEPARATION, IDENTIFICATION, PURIFICATION, ESTIMATION AND RADIOASSAY OF THE COMPONENTS OF A MIXTURE OF UNSAPONIFIABLE LIPIDS

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SUMMARY

An automated system which we have termed the Liquid Spectroradiochromatograph, capable of monitoring the effluent from a liquid chromatography column for solute concentration, radioactivity and ultraviolet and visible light absorption, is described. Its usefulness in the analysis of mixtures of unsaponifiable lipids is discussed.

INTRODUCTION

Many studies on the biosynthesis of unsaponifiable lipids in plants, animals or bacteria involve feeding a radioactively labelled precursor (*e.g.*, [^{14}C]acetate or [^{14}C]-mevalonate) to the organism or enzyme system, and the subsequent extraction of the lipids and isolation of the unsaponifiable material. Separation of the unsaponifiable lipids is usually accomplished by column chromatography using stepwise elution and the arbitrary fractions, each of which may contain several components, are further resolved and analysed qualitatively to determine the nature of the components and quantitatively to determine the mass and radioactivity of these components[1,2]. As the chromatographic separations and subsequent analyses are time-consuming and often tedious, the construction of automated equipment capable of carrying out these procedures rapidly and reliably would be of considerable advantage to workers in this field.

This paper describes such a method; after the components of a lipid mixture have been separated by automated gradient elution chromatography, they are automatically assayed for UV or visible light absorption, radioactivity and mass before they are collected separately in an automatic fraction collector. The entire equipment is shown in Fig. 1.

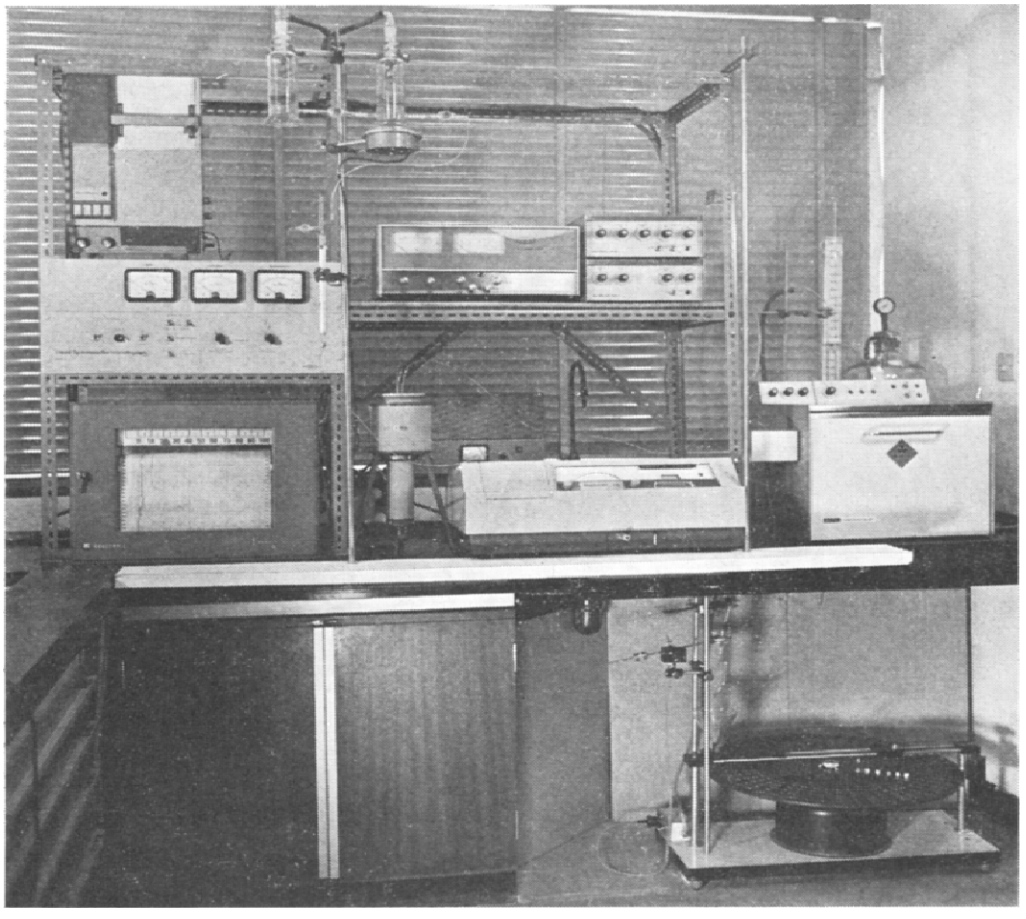

Fig. 1. Overall view of the instruments.

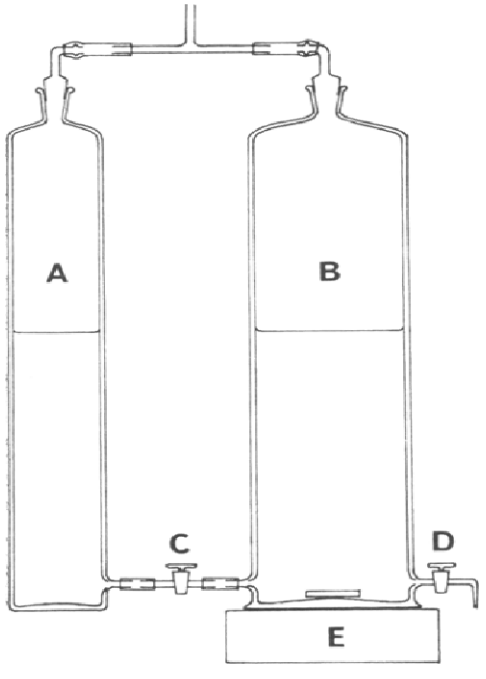

Fig. 2. Construction of the gradient elution apparatus. For key, see text.

MATERIALS AND METHODS

Gradient elution apparatus

The apparatus (Fig. 2) is based on those of PARR[3] and BOCK AND LING[4]. It consists of a reservoir (A) attached to a mixing chamber (B). The mixing chamber (B) consists of a cylindrical glass vessel (5 cm I.D.; 500 ml volume) fitted with a B24 Quickfit ground-glass socket and two side arms sited diametrically opposite to each other 1 cm from its base. Each side arm is fitted with a ground-glass tap, one of which (C) is connected by means of a short piece of nylon tubing [3.5 mm bore, Baird & Tatlock (London) Ltd.] to the side arm of the reservoir (A), the other (D) being connected through a length of nylon tubing (1.9 mm bore) to the sample injection device. The reservoir (A) is a cylindrical glass vessel fitted with a B24 Quickfit ground-glass socket and with a short glass side arm positioned 1 cm from its base. Four such vessels with I.D. of 5, 4, 3 and 2.5 cm, respectively, are available for use as the reservoir, enabling four different gradients to be obtained (see below).

During use, the reservoir (A) contains the polar solvent (*e.g.*, diethyl ether) and the mixing chamber (B) the non-polar solvent (*e.g.*, light petroleum, b.p. 40–60°) used in the filling of the chromatography column. At the beginning of the chromatographic analysis, the levels of the solvents in the two chambers are normally equal and the taps (C and D) are opened simultaneously. Should the particular separation require it, however, the level of the solvent in the mixing chamber could be higher than that in the reservoir. In this case, the chromatogram is started by opening the tap (D) and allowing the non-polar solvent to pass through the column until the level in the mixing chamber falls to that in the reservoir when the other tap (C) is opened and the gradient commenced.

Mixing of the two solvents is accomplished by a magnetic stirrer (E) positioned underneath the mixing vessel. Care is taken not to stir too vigorously so as to avoid vortex formation; this would raise the effective level of solvent in the mixing chamber and upset the shape of the gradient. The ground-glass sockets of both vessels are fitted with B24 adaptors connected by rubber tubing to a glass T-piece which is in turn similarly connected to a 20-l aspirator bottle. This bottle is fitted with a mercury manometer and the air pressure inside can be raised by means of a bicycle pump. This simple device enables a constant head of pressure to be maintained throughout the chromatography on the solvent passing through the column; the flow rate is therefore constant.

When the 5 cm I.D. reservoir (A) is used in conjunction with the mixing chamber (B), a linear gradient is obtained. The concentration (C_v) of the polar solvent in the solvent mixture leaving the mixing chamber after a volume (V) has flowed through the apparatus is given by the equation:

$$C_v = C_2 + (C_1 - C_2)\frac{V}{V_{\text{total}}}$$

where C_1 is the concentration of the polar solvent in the reservoir (A), C_2 is the concentration of the polar solvent initially present in the mixing chamber and V_{total} is the total volume of liquid in both vessels[5].

When the reservoir vessel (A) has an I.D. of 2.5, 3 or 4 cm, a concave expo-

nential gradient is obtained, the shape of which can be calculated by the equation:

$$C_v = C_1 - (C_1 - C_2)\left(1 - \frac{V}{V_{total}}\right)^P$$

where V, V_{total}, C_v, C_1 and C_2 have the above meanings and P is the ratio of the cross-sectional area of the reservoir (A) to that of the mixing chamber (B).

Sample injection device

The sample injection device (Fig. 3) consists of a piece of 0.2-in. bore glass tubing ending in a Bio Quickfit cone; the overall length is 3.5 in. Two side arms of the same gauge tubing are attached, diametrically opposite to one another, half an inch above the cone. One is fitted with a ground-glass tap (F) and is bent downwards at a right angle. The other side arm has an upward right-angle bend and is tapered to fit tightly into the 1.9-mm bore nylon tubing bringing solvent from the gradient elution apparatus. A rubber injection septum (G), such as is used in glass gas–liquid chromatography columns, is firmly plugged into the end of the tubing which is opposite to the cone. The ground-glass cone fits into the Bio Quickfit socket of the chromatography column. The injection device is held securely to the column by passing elastic bands over its side arms and attaching them to the hooks (H) on the side of the column.

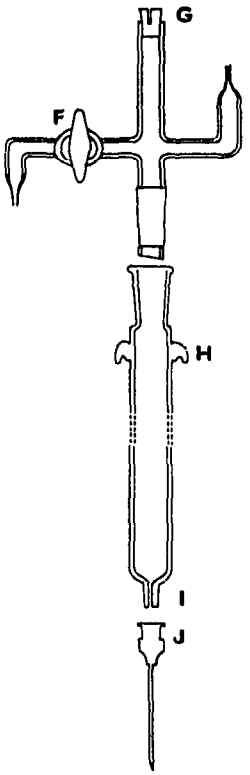

Fig. 3. Sample injection device and column assembly. For key, see text.

Chromatography column

Several glass chromatography columns have been made for use with this apparatus. Each has a Bio Quickfit ground-glass socket at one end to receive the sample

injection device and a cone (I) specially ground to connect with a Luer fitting hypodermic needle (J) at the other. In some cases, this ground cone is replaced by a 1-cm length of 3-mm bore nylon tubing fitted to the end of the column, which has been narrowed by pulling out in a flame; this size of nylon tubing fits perfectly into the Luer socket of the hypodermic needle. The columns differ from one another in respect of their I.D. (0.5 cm to 1.0 cm) and lengths (10 cm to 30 cm). The 1.5-in. hypodermic needles are of stainless steel (G19, Philip Harris and Co., Birmingham, England). They fit tightly into the 1-mm bore flexible nylon tubing [Baird & Tatlock (London) Ltd.] used to interconnect the column effluent monitoring devices. This tubing is sufficiently narrow to minimise the delay time between a solute arriving at the different monitoring stages (volume of tube = 0.785 ml/m).

Column filling and sample injection

The chromatography column is filled with adsorbent, irrespective of the column length, to within 2 cm of the bottom edge of the ground-glass socket. The remaining 2 cm of column space are filled with anhydrous sodium sulphate. Thus when the sample injection device is fitted, the only dead space filled with solvent is the volume of the Quickfit joint plus that of the 0.5 in. of tube up to the side arms of the injection device, a total volume of 2 ml. Developing solvent passes from the gradient elution apparatus through the 1.9-mm bore nylon tubing to the tapless side arm of the injection device. Should the level of solvent rise too high in the main tube of the injection device, the excess can be run off through the other side arm by opening the tap (F); this is necessary only during the initial setting-up of the apparatus.

The mixture to be chromatographed is injected on to the column once the flow rate of solvent from the gradient elution apparatus through the column is constant and the effluent monitoring devices are stabilised and set to register zero response. The sample is dissolved in the minimal volume of the non-polar solvent, present initially in the mixing chamber of the gradient elution apparatus. This solution is taken into a 2-ml graduated glass syringe fitted with a 6-in. stainless-steel hypodermic needle. The needle is pushed through the rubber septum (G) and into the layer of anhydrous sodium sulphate which diffuses the sample over the whole cross-sectional area of the column. The plunger of the syringe is slowly depressed to deliver a known aliquot of the sample (usually 1 ml) and the needle is then withdrawn.

Detection of light-absorbing solutes

On emerging from the column, the eluate passes in turn via 1-mm bore nylon tubing to a number of monitoring devices (Fig. 4). The first of these is a Beckman DB recording spectrophotometer (Beckman Instruments Ltd., Glenrothes, Fife, Scotland). This can be set at any wavelength within the range 205–770 nm and will continuously monitor the transmittance of the eluate at this wavelength throughout a chromatographic separation, recording the trace on the Honeywell multi-channel recorder. Alternatively, the spectrophotometer can be used to monitor the absorption spectra of eluted solutes, its output being fed, in this instance, to the Beckman potentiometric strip-chart recorder which is fitted with a Beckman scale expansion accessory, enabling the instrument to be used for solutions of low concentration.

The eluate from the chromatography column is fed by 1-mm bore nylon tubing into a flow cell mounted in the sample compartment of the spectrophotometer. The

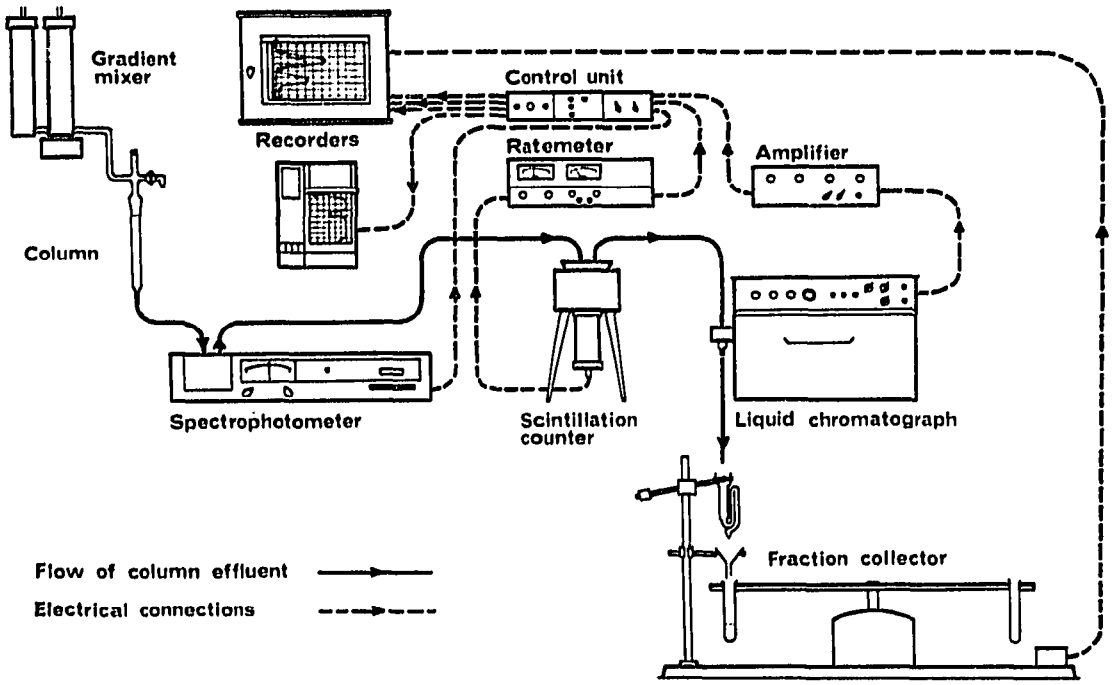

Fig. 4. Arrangement of the instrumentation, showing the flow of solvent and the electrical connections.

reference compartment contains a 0.5-cm blank cell containing the appropriate solvent (*e.g.*, light petroleum). The sample flow cell is constructed from two plates of spectroscopic grade silica held apart by a metal spacer machined to give a 0.5-cm light path through the sample. The total volume of the cell, defined by the size of the cavity through the spacer, is 0.25 ml. The inlet and outlet ports, at the bottom and top of the cell respectively, consist of lengths of stainless-steel tubing cut from G19 1.5 in. hypodermic needles brazed into drilled holes in the metal spacer. These connect with the 1-mm bore nylon tubing through which the eluate enters and leaves the spectrophotometer, passing through light-tight holes drilled in the hinged lid of the sample compartment. The silica–metal joints of the flow cell are solvent tight under the small positive pressures used, and are maintained with the epoxy resin 'Araldite' [CIBA (A.R.L.) Ltd., Duxford, Cambridge, England]. This flow cell has recently been replaced by a 0.5-cm path length Chandos Type 42 silica flow cell (Chandos Intercontinental Ltd., Stockport, Cheshire, England).

Radioassay of components in eluate

After leaving the spectrophotometer, the eluate from the column is monitored for radioactivity by passing it through an NE 808 flow cell (Nuclear Enterprises Ltd., Sighthill, Edinburgh 11, Scotland) positioned against the face of an I.D.L. scintillation counter [type 663, Isotope Developments Ltd., Beenham, Reading, Berks., England (now Nuclear Enterprises Ltd.)] by means of a special mounting assembly supplied by Nuclear Enterprises Ltd. Good contact between the face of the flow cell and that of the photomultiplier of the scintillation counter is maintained by a thin film of Nujol. The whole assembly is mounted in a lead shielding unit (type 722, Isotope Developments Ltd.). The output from the scintillation counter is fed into

a transistorised ratemeter and high-voltage unit (type 7070, Isotope Developments Ltd.); this unit also supplies the high voltage (1400 V) to the scintillation counter. The output (linear or logarithmic) from the ratemeter is fed into one of the channels of the Honeywell recorder.

The flow cell consists of a flat spiral glass tube containing europium-activated calcium fluoride crystals. It has a capacity of approximately 0.5 ml and can be used for the continuous monitoring of β-emitters either in organic liquids or vapours at temperatures of up to 150° if required. The makers quote figures of 1.55 % counting efficiency for ^{3}H with a background of 3.0 c.p.s. and 16.6 % efficiency for ^{14}C with a background of 1.34 c.p.s.; our results bear out these figures. The inlet and outlet ports are slightly modified in order that they can be connected to the standard 1-mm bore nylon tubing used to carry the column eluate. The pieces of glass tubing constituting these ports are reduced in length to approximately 1 cm and the Luer fitting of a G19 1.5 in. stainless-steel hypodermic needle is fitted over each and bonded with Araldite. The length of each needle is reduced to 1 cm prior to fitting so that the modified flow cell fits into the mounting assembly. Suitable lengths of 1-mm bore nylon tubing are firmly pushed over each stainless-steel tube and led out of the mounting assembly and surrounding lead shield through light-tight exit ports in order to carry the column eluate flow to and from the scintillation counter.

Detection of organic compounds in eluate

Organic compounds in the column eluate are detected by means of a Pye liquid chromatograph (Series 1, W.G. Pye and Co. Ltd., Cambridge, England), which also gives an idea of their relative quantities.

In this instrument, the column eluate is led into a very narrow stainless-steel tube whose end is bevelled. A high-precision stainless-steel wire moves at constant speed past the end of the tube, within the bevel, and is continuously coated with a very thin film (approximately 10 μl over 4.5 cm of wire) of the column eluate. The vast majority of the eluate passes on and is collected by the fraction collector. After being coated, the wire passes through an evaporator oven which is held at a temperature slightly higher than that of the boiling point of the chromatography solvent and which is swept by argon gas; the solvent is removed, leaving any solute adhering to the wire. The wire travels into a pyrolyser oven set, usually, at 700°. Here the organic solute on the wire is pyrolysed and the volatile pyrolysis products are swept by a stream of argon (30 ml/min) into an argon ionisation detector. The resulting signal is amplified and the output from the detector amplifier is fed to the multi-channel Honeywell recorder.

The performance of the system depends to a considerable extent upon the cleanliness of the moving wire. Cleaning the wire is effected by passing the wire through a cleaner oven also set at 700° and swept by a stream of argon (40 ml/min) before it is coated with chromatographic eluate.

Collection of fractions

After the eluate has passed through the liquid chromatograph, and a small sample has been removed by the moving wire, the remainder passes downwards through a metal funnel fitted with 1.9-mm bore flexible nylon tubing. An increase

in the tube diameter is made at this point because the pressure head established at the top of the chromatography column is lost at the wire-coating stage and bubbles of air tend to be introduced. The flow of the eluate is much improved by using the wider bore tube.

The fraction collector (Beaumaris Instrument Co., Beaumaris, North Wales) holds 150 15-ml tubes in three concentric rows, and the sample tube changer is controlled by the syphon balance arm method, the syphon having a capacity, and therefore the fraction a volume, of 3 ml. This method of control is preferred to that of drop-counting because the fraction volumes are constant irrespective of the nature of the eluting solvent. Every time the syphon empties and the fraction is changed, a microswitch is closed to complete the circuit of the event marker pen in the Honeywell recorder, thus giving a permanent record of the fraction changes.

Recorders and control assembly

Two recorders are used as part of the instrumentation, a Honeywell ElectroniK 15 multi-point strip chart recorder (4-channel; Honeywell Controls Ltd., Newhouse, Lanarkshire, Scotland) and a Beckman 100504 U linear potentiometric strip chart recorder fitted with a Beckman S151060 scale expansion accessory (both from Beckman Instruments Ltd., Glenrothes, Fife, Scotland).

The Honeywell recorder is a continuous balance potentiometer which measures and records the magnitude of a number of process variables. In this case, three process variables are fed into the four channels of the recorder, the spectrophotometer output into channel 4, the scintillation counter ratemeter output into channel 2 and the liquid chromatograph argon ionisation amplifier output into both channels 1 and 3. Each separate channel has a print-out cycle of 10 sec and each record is printed on the chart paper as a series of dots, a different colour being used for each channel. The chart speed used is 12 in./h. The operation pen (event marker) of the recorder is connected to the event marking output of the fraction collector and thus records the fraction changes.

The Beckman recorder, a continuous pen recording instrument, is included for use in following parameter changes which may be too fast for the multi-point recorder. It can, of course, be used for only one channel at a time, but the control unit has switches to connect it to any of the three instrument outputs. Its most useful function is for the rapid monitoring of the absorption spectrum of an eluted fraction. When the whole of the instrumentation is not in use, this recorder can still be used with the spectrophotometer for normal laboratory purposes. Indeed, any of the individual instruments may be used independently of the others as the liquid flow system is so simple and versatile.

In addition to switching the various instrument outputs to the appropriate recorder, the control unit also has an important function as a voltage attenuator. This facility is necessary because while the 0–10 mV output from the liquid chromatograph argon ionisation amplifier is matched to the −1 to +10 mV Honeywell recorder input, the outputs from the scintillation counter ratemeter and the spectrophotometer are both of the order of 0–100 mV. The attenuators used for these two channels, each consisting of a simple resistor network, nominally reduce each of the voltages to one tenth of their value, but are variable by turret switches and further resistors to give 2, 5 and 10 times effective "amplification" of the reduced signals.

This attenuation is not necessary for the Beckman recorder as it accepts an input voltage of 0–100 mV. It is necessary, however, to use the 10 × sensitivity setting on the scale expansion accessory when monitoring the output from the liquid chromatograph argon ionisation amplifier with this recorder. The control unit also has three meters (Fig. 1) which continuously display the responses of the spectrophotometer, scintillation counter and liquid chromatograph, respectively.

An additional function of the control unit is to reverse the polarity of the input from the spectrophotometer to the Honeywell recorder as the former measures transmittance rather than absorbance. This signal, now having a negative value, is brought back within the range of the recorder by a backing voltage supplied by a mercury battery fitted with a continuously variable attenuator. This variable resistor is used as a "set zero" control for the spectrophotometer record. The linear recorder now records the percentage of light absorbed by the eluate in the spectrophotometer flow cell, and its extinction and hence the concentration of a known solute may be calculated from this.

RESULTS AND DISCUSSION

While the equipment has been used with considerable success in studies on unsaponifiable extracts from several natural sources, its performance can best be assessed when using a standard mixture of defined components. Thus the apparatus has been used to separate and assay the isoprenoid compounds in a mixture containing per millilitre of light petroleum solution: [^{14}C]squalene (specific activity 1×10^4 d.p.m./mg), 1.0 mg; phytoene, 25 μg; β-carotene, 40 μg; lanosterol, 2.5 mg; [^{14}C]-cholesterol (specific activity 1×10^4 d.p.m./mg), 2.5 mg; and [^{14}C]ergosterol (specific activity 1×10^4 d.p.m./mg), 2.5 mg.

The column used for the separation had an I.D. of 1 cm and was packed to a depth of 20 cm with aluminium oxide (Woelm acid-washed, deactivated with water to Brockmann activity grade III) by allowing the adsorbent to settle out from a slurry in light petroleum (b.p. 40–60°). The sample (1 ml) was added to the column which was developed first with 30 ml of light petroleum, run in from the mixing chamber (B). This vessel had an I.D. of 5 cm and contained, initially, 250 ml of light petroleum. After 30 ml of light petroleum had passed down the column, gradient elution was commenced by opening the tap (C) and allowing the more polar solvent from the reservoir (A) to run into the mixing chamber. This reservoir had an I.D. of 3 cm and contained an initial charge of 80 ml of 40 % diethyl ether in light petroleum (v/v).

As there was only one component in the standard mixture that absorbs visible light (β-carotene, λ_{max} in light petroleum at 448 nm), the spectrophotometer was set throughout at a wavelength of 280 nm to monitor phytoene (λ_{max} at 285 nm) and ergosterol (λ_{max} at 282 nm, both in light petroleum) and was used at 450 nm only to follow the elution of the yellow β-carotene band. The passage of ^{14}C-labelled solutes through the scintillation counter was monitored with the photomultiplier operating at 1400 V. The count-rate range used was 0–400 c.p.s. and the time constant on the ratemeter was 2.5 sec. As solutes of known radioactivity were being eluted from the column, the linear output mode of the ratemeter was used; the logarithmic output is used when the range of radioactivity to be recorded is not known. The portion of the eluate stream that entered the liquid chromatograph was carried by the wire,

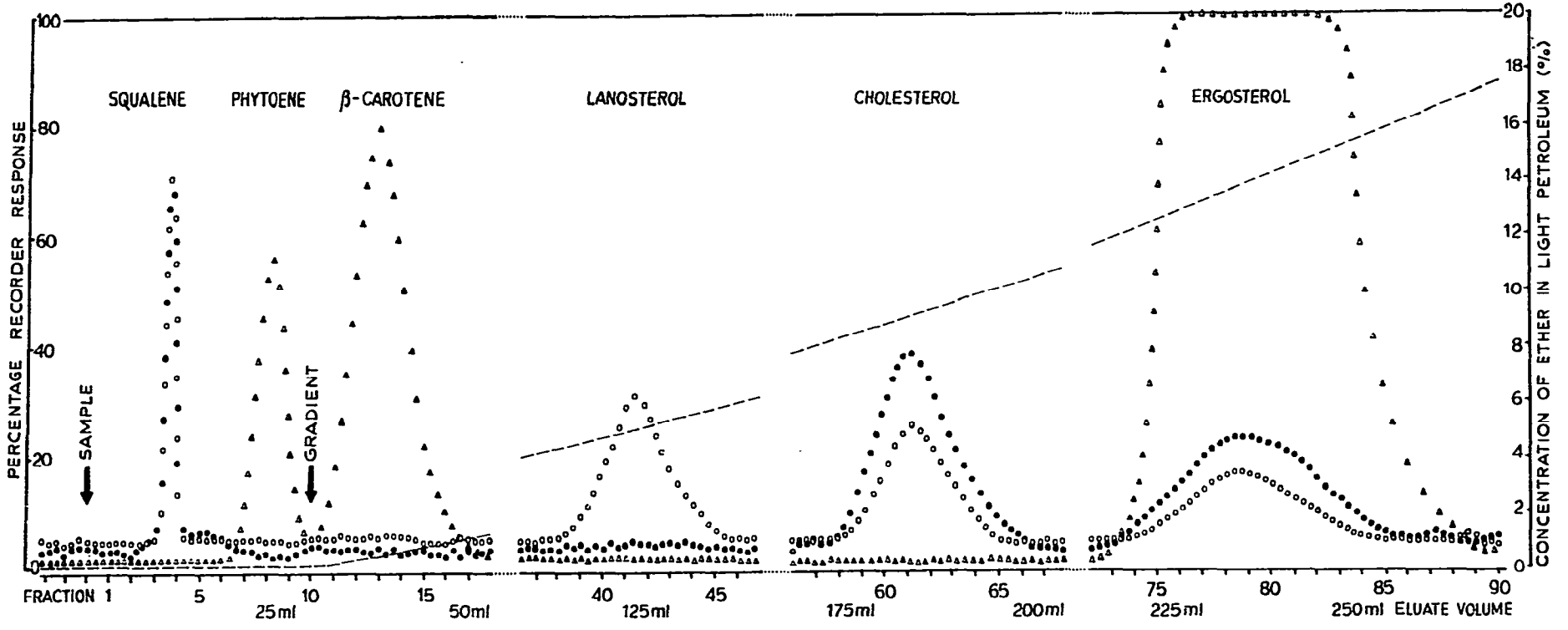

Fig. 5. Tracing of multi-point recorder chart after the analysis of the standard test mixture (see text for details). Key: △△△, absorption at 285 nm and ▲▲▲, absorption at 450 nm (from spectrophotometer); ●●●, radioactivity (from scintillation counter); OOO, concentration of solute (from liquid chromatograph); – – –, composition of solvent (percentage of ether in light petroleum).

moving at a speed of 8 in./sec, first into the evaporator oven, which was held at a temperature of 80° in order to remove the solvent, and then into the pyrolyser oven set at 700°. A detector voltage of 1250 V gave the best response and an amplifier attenuator setting of $\times 3$ ensured that the scale of the response was acceptable to the recorder.

The results of the analysis are shown in Fig. 5. Squalene is the first compound to leave the column, being eluted as a sharp band with light petroleum and appearing in fraction 4. The recorder peaks from the scintillation counter and the liquid chromatograph are coincident, indicating that the squalene is radioactive. The combination of these two monitoring devices thus results in a reliable method of detecting squalene and assessing its radioactivity and is more rapid than either detecting squalene on thin-layer chromatograms of random column fractions and scanning the thin layer for radioactivity[1,2] or reducing to the corresponding saturated hydrocarbon and identifying this compound and measuring its radioactivity on a gas radiochromatograph[6].

Phytoene leaves the column after 25 ml of light petroleum have passed through and appears in fractions 8 and 9. While an insufficient amount is present to be detected by the liquid chromatograph at the setting used, its high molar extinction coefficient ($\varepsilon = 50 \times 10^3$ at 285 nm) leads to a strong response (55 % full scale) by the spectrophotometer. Similarly, the strong absorption of visible light by β-carotene ($\varepsilon = 134 \times 10^3$ at 448 nm) leads to a large (80 %) response by the spectrophotometer although the liquid chromatograph does not record the presence of this solute at the sensitivity employed. The β-carotene is eluted with 0–2 % diethyl ether in light petroleum (v/v) and appears in fractions 12 to 15.

Lanosterol, on the other hand, has no conjugated double bonds in its molecule and cannot be detected by light absorption but only by the liquid chromatograph. It is eluted from the column with 4–6 % ether in light petroleum in fractions 40 to 45. Previous methods of lanosterol detection have depended on evaporating the solvent from the column fractions and carrying out a Liebermann–Burchard test on the residue; lanosterol yields a yellow colour (maximal absorbance at 458 nm), the intensity of which is at its maximum after 40 min (at 25°)[7]. The Liebermann–Burchard test has also been the basis of previous methods for the detection of cholesterol, which yields a blue-green colour in the test. The liquid chromatograph, however, is able to respond to cholesterol and the coincidence of this response and that from the scintillation counter shows that the cholesterol is radioactive. It is eluted from the alumina column with 8–10 % ether in light petroleum and appears in fractions 59 to 66. The ergosterol, which is eluted from the column with 12–16 % ether in light petroleum as fractions 75 to 88, is also radioactive. This sterol, however, absorbs UV light ($\varepsilon = 12.3 \times 10^3$ at 282 nm); the spectrophotometer response is very high compared with those for phytoene and β-carotene since a relatively high concentration of radioactive ergosterol is included in the standard sample mixture.

Throughout the analysis, the spectrophotometer gives an extremely stable response. In contrast to this, the outputs from the liquid chromatograph and the scintillation counter show random variations, but only to the extent of ± 1.5 % and ± 2.5 % of the full-scale deflections respectively at the sensitivities used. The sensitivity of these instruments can be increased to respond to smaller quantities of solute and to lower ^{14}C activities. This results, however, in a loss of signal-to-noise

ratio so that the interpretation of small peaks in the record becomes rather difficult. The nature of the chromatographic separation is such that the bands of the more strongly adsorbed solutes are broader than those of components eluted earlier from the column. There is not, however, any significant difference in the width of the peaks representing the responses of different instruments to a given solute, indicating that the volume and shape of the flow cells are satisfactory in that they do not allow diffusion of the solutes in the solvent stream. The coincidence of the responses from different instruments to the same component shows that the bore of the tubing used to connect the flow cells and the volumes of the flow cells themselves are small enough to eliminate any significant time-lag. The retention volumes of given components eluted from standard columns are sufficiently reproducible to be used for the identification of solutes by their adsorption characteristics[8].

The prime function of the instrument, as it is used in the authors' laboratory, is in the routine qualitative analysis of lipids, especially unsaponifiable lipids such as the terpenoid hydrocarbons, carotenoids and sterols, extracted from biological systems which have been incubated with radioactive substrates. The recorded responses from the spectrophotometer and the scintillation counter, while quantitative, are only sometimes given absolute quantitative significance as the light-absorbing and radioactively labelled components are often subjected to further purification prior to assay by standard methods. The liquid chromatograph record is only semiquantitative because of the variable nature of the pyrolysis products of different solutes; standardisation of the liquid chromatograph for individual components is required for its results to be absolutely quantitative.

The instrumentation described in this paper is capable of carrying out not only the separation and identification of the components of a lipid mixture but also the estimation of the quantity and radioactivity of these components. The analysis is complete in about 2.5 h and is thus much more rapid than was possible by previous methods in which the fractions were first collected and then analysed individually. While the equipment is completely automatic and will operate unattended, the analysis of a mixture of completely unknown quantitative proportions requires some attention on the part of the operator in order that the sensitivities of the various monitoring devices can be adjusted to give responses of acceptable magnitude.

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