Journal of Chromatography, 142 (1977) 459–467

CHROM. 10,430

HIGH-SENSITIVITY METHOD OF RADIO GAS CHROMATOGRAPHY FOR ^{3}H- AND ^{14}C-LABELLED COMPOUNDS

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SUMMARY

Water and carbon dioxide produced by the flame ionisation detector (FID) of a gas chromatograph can be trapped very efficiently into an absorbing fluid using the gas absorption cell described here. $^{3}H_2O$, $^{14}CO_2$, or both, if present in the FID effluent, can then be estimated using liquid scintillation counting. If desired, it is possible to differentiate ^{3}H and ^{14}C by chemical separation of the $^{3}H_2O$ and $^{14}CO_2$ or by dual label counting. The basic apparatus described can be constructed cheaply, and it is possible to automate it. The method is extremely sensitive and can be used to detect below 100 dpm injected per peak.

INTRODUCTION

The detection of radioactivity in the effluent from a gas–liquid chromatography (GLC) column is often an important aspect in experiments involving volatile radio-labelled compounds. The two basic approaches to radio gas chromatography (RGC) are continuous detection and collection of fractions for subsequent counting; in each case, methods have been used which involve detection with or without prior combustion. Continuous on-line detection has the advantage that the radioactive trace is produced simultaneously with the mass trace, but as the residence time of the radioactivity in the detector must be limited, the sensitivity suffers. For continuous assay, the column effluent has been trapped into a liquid scintillation fluid[1] or combusted and detected by a variety of methods, of which the gas proportional counter is perhaps the most sensitive.[2] A means of increasing the counting time without fractionation has been devised[3], by absorbing the combustion gases in a liquid, storing this in a long tube and allowing it then to flow slowly through the detector. Discontinuous assay methods have involved trapping the column effluent in fractions for subsequent liquid scintillation counting[4] or combustion using the flame ionisation detector (FID) of a GLC instrument followed by condensation of the water produced for $^{3}H_2O$ detection[5] or absorption of the carbon dioxide with ethanolamine for $^{14}CO_2$ detection[6]. The FID was found[6] to be an efficient combuster.

The ability to detect ^{3}H and ^{14}C simultaneously in column effluents has been

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approached by a continuous method[7], but it is more suited to collection of fractions. With this in mind, together with the need for high sensitivity, the apparatus described here has been developed. The method involves combustion of compounds using the FID and trapping the $^{14}CO_2$ or $^{3}H_2O$ in an absorption fluid, which after addition of a scintillation fluid, is collected in fractions and counted in a liquid scintillation counter.

EXPERIMENTAL

Materials

2-Methoxyethanol (2-ME) is used as the absorption fluid for H_2O. It has good properties for this role (it is high boiling and wets glass) and has been widely used in scintillation cocktails. To prevent collection of $^{14}CO_2$ when this is present with $^{3}H_2O$, 1% 1 *M* HCl is added to the 2-ME. For collection of $^{14}CO_2$, 10% redistilled 2-phenylethylamine (2-PE) is added to the 2-ME. These absorption fluids attack the flexible (Isoversinic) tubing used, but the addition of 5% H_2O to the 2-ME prevents this. The scintillation fluid used was 0.6% butyl PBD in toluene.

A mixture of N-TFA, [^{3}H]methyl ester derivatives of amino acids was produced by acylating the amino acid mixtures with trifluoroacetic anhydride, then methylating with ethereal CH_2N_2 in the presence of $^{3}H_2O$. The specific activity of the evaporated mixture was $4.16 \cdot 10^6$ dpm/mg.

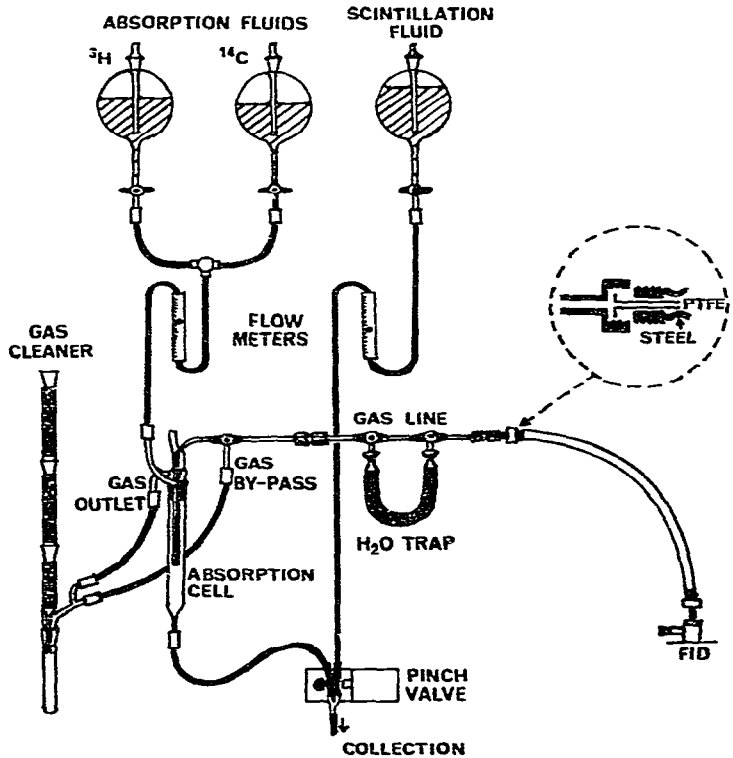

Fig. 1. Diagram of radio gas chromatography system

Methyl [^{14}C]palmitate and methyl [^{3}H]stearate were produced by methylating (CH_2N_2) [^{14}C]palmitic acid and [^{3}H]stearic acid (Radiochemical Centre, Amersham, Great Britain) which had been diluted with the corresponding unlabelled fatty acid. The specific activities were $4.04 \cdot 10^6$ dpm/mg and $3.12 \cdot 10^6$ dpm/mg, respectively.

GLC and liquid scintillation counting

A Pye 104 gas chromatograph was fitted with a silanised glass column (274 × 0.2 cm I.D.) packed with 5% OV-1 on Gas-Chrom Q. Flow-rate of nitrogen through the column was 30 ml/min, and the air and hydrogen flow-rates to the FID were 370 and 40 ml/min, respectively. The FID temperature was 300°. Samples were injected in 1 μl of acetone. The amplifier attenuation was × 5000.

Liquid scintillation counting was performed using polyethylene vials in a Nuclear Enterprises (Model 8310) counter. Background counting rates were 14 cpm and 19 cpm for ^{3}H and ^{14}C, respectively. Counting efficiency was estimated using external standard channels ratio and was typically in the region of 37% for ^{3}H and 82% for ^{14}C with the mixtures used.

Apparatus

This is shown in Figs. 1 and 2. The gas–liquid chromatograph used was a Pye 104, but the apparatus can be connected to any instrument which has an FID that can be sealed. The gases are led from the FID to the apparatus by a PTFE tube (4 mm I.D.) inside a flexible steel tube (United Flexible; see inset of Fig. 1 for coupling). Connections in the glass gas line are made with tube connectors (Swagelock) with

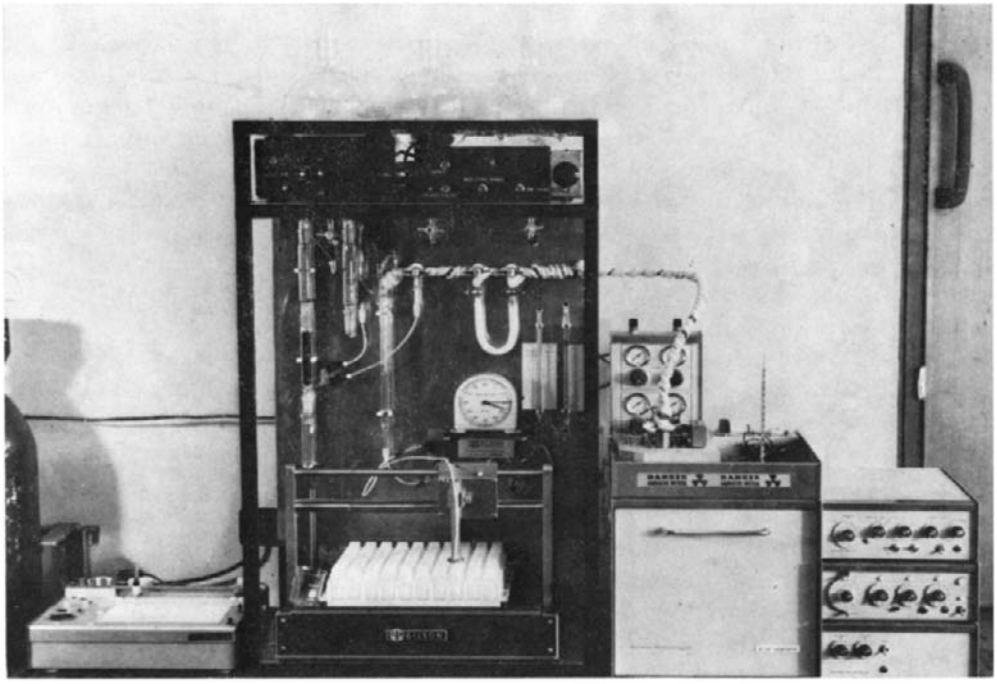

Fig. 2. Radio gas chromatography system.

PTFE ferrules. The H_2O-trap, which is packed with anhydrous magnesium perchlorate, can be by-passed using the 3-way PTFE taps (Springham), and is connected into the line with greaseless ball joints (Young, Acton, London, Great Britain). A further 3-way PTFE tap makes it possible to divert the gases to atmosphere without passing through the absorption cell. The gas line from the FID to the cell is wrapped with a 400-W heating tape (Electrothermal) and heated to *ca.* 120° to keep the H_2O gaseous. After passing through the cell, the gases are cleaned before venting to the atmosphere by passing them through columns of activated animal charcoal, soda asbestos (Carbosorb) and self-indicating silica gel to remove solvent, CO_2 and H_2O, respectively. Any condensation in the gas tubes falls into the trap below these columns.

The fluid reservoirs are modified 2-l round bottom flasks fitted with dip tubes to give a constant head. The fluid flow from the reservoirs is adjusted using all-PTFE fine control stopcocks (Springham) and measured by float flow meters (Glass Precision Engineering, Hemel Hempstead, Great Britain). Tubes from the ^3H-reservoir and the ^{14}C-reservoir join at a 3-way junction so that either one fluid or the other can be supplied to the cell. The glassware is linked by standard PTFE tubing (2 mm I.D.) and connectors (Altex). After passing through the absorption cell, the fluid is led to a fraction collector via a flexible tube (Isoversinic) in a pinch valve. The scintillation fluid joins the absorption fluid at a T-junction after also being controlled by the pinch valve. As an alternative the pinch valve may be replaced by two pneumatically actuated slide valves in tandem, one for each fluid, thereby preventing the problem of solvent attack on the flexible tube. It is essential to interrupt the flow between fractions to prevent spillage between the vials. In the system described here a relay is operated by the pulse from the fraction collector control unit when vials are changed. This relay, when activated, closes the pinch valve and moves the event marker pen on the recorder, giving a mark alongside the trace from the FID. In the case of the Gilson fraction collector (illustrated in Fig. 2) the electrical pulse given is too short to operate the pinch valve effectively, so the pulse is extended using a self-resetting synchronous motor timer. A switch that cuts off the power to the pinch valve is added to stop the flow of the fluids while the apparatus is on standby. With the system outlined it is possible to dispense with the fraction collector and operate manually by activating the relay with a hand or foot switch whilst changing the vials by hand.

Descending film absorption cell

The absorption cell, shown in Fig. 3, is constructed entirely from glass. It consists of four concentric tubes. The innermost tube (A) carries the gases from the FID into the cell. It has a jacket tube (B) and the space between the tubes (A) and (B) is heated by a resistance wire (Nichrom V, 23 SWG, 2Ω) to prevent condensation of H_2O inside tube (A). The outermost tube (D) acts as a condensation/absorption surface for H_2O and CO_2, and during operation the inner surface of this tube is constantly flushed by an even film of absorption fluid. This even film is produced by introducing the fluid into both sides of the cell between tube (D) and the short tube (C) (see Fig. 3b) between which there is only a narrow gap (1 mm). Capillary action ensures that the fluid is spread round evenly between these tubes. It is important for even distribution that the cell is vertical. Thus the absorption fluid is supplied by tube (F) from reservoirs via flow controllers and a flow meter. After descending through the cell inside tube (D), it leaves the bottom of the cell via tube (G). The

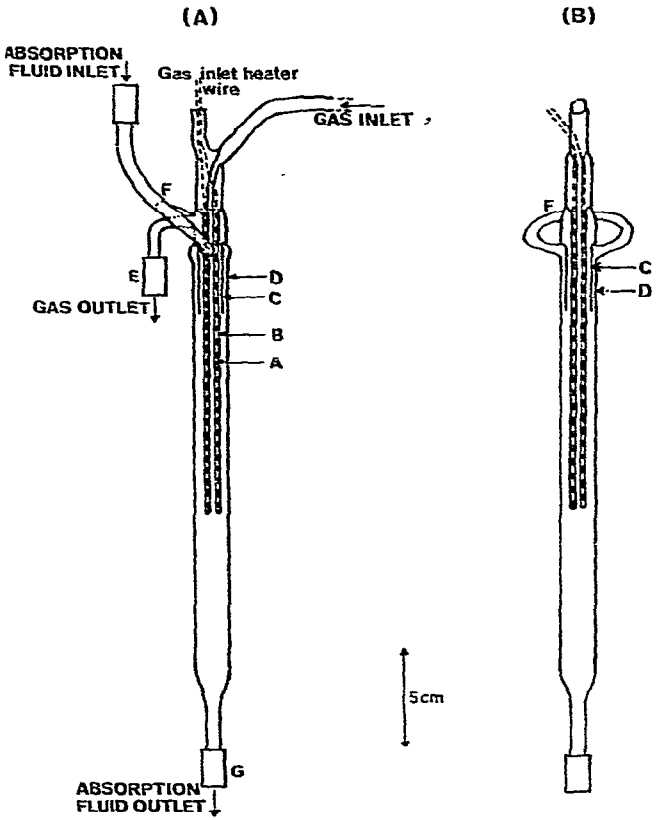

Fig. 3. Descending film absorption cell. (a) Front view; (b) view from right side. Dimensions: tube A, 4 mm O.D., 2.3 mm I.D.; tube B, 6.5 mm I.D.; tube C, 13 mm O.D.; tube D, 14 mm I.D.

combustion gases travel along a heated tube from the FID, and after leaving tube (A) in the cell, they travel in the opposite direction to the fluid and exit between tubes (B) and (C) and then through tube (E). Thus the gases have a free path through the cell. This is important to prevent back-pressure which can adversely affect the column and detector.

Operation

After allowing the gas line to heat up, the appropriate absorption fluid is run through the cell and adjusted to 8 ml/min. The gas inlet heater is switched on and the FID effluent is allowed to pass through the cell. When all condensation has disappeared from the gas inlet tube (A) the fraction collector is started and the scintillation fluid allowed to run and adjusted to 15 ml/min. The sample is injected into the GLC and fractions collected. When the run is complete the gases are diverted directly to the gas cleaning column. After switching off the gas inlet heater, the absorption cell is allowed to fill to the top with fluid to clean it. For dual label counting, it is possible either to use the CO_2-absorption fluid and collect both ^3H_2O and $^{14}CO_2$ and differentiate these by dual label counting, or to separate the two gases chemically as

follows. One chromatographic run is performed using the CO_2-absorption fluid with the gases diverted through the H_2O-trap which contains magnesium perchlorate. After flushing the cell with the H_2O-absorption fluid (containing 1% of 1 *M* HCl), the gases are diverted past the H_2O-trap and another chromatographic run is performed using the ^3H_2O absorption fluid. The vials from the second run are placed in a fume cupboard for 30 min at room temperature to allow any residual $^{14}CO_2$ to evaporate.

RESULTS AND DISCUSSION

A typical radio gas chromatogram is shown in Fig. 4, in which amino acids were separated as their N-TFA [^{3}H]methyl ester derivatives. The radioactivity detected

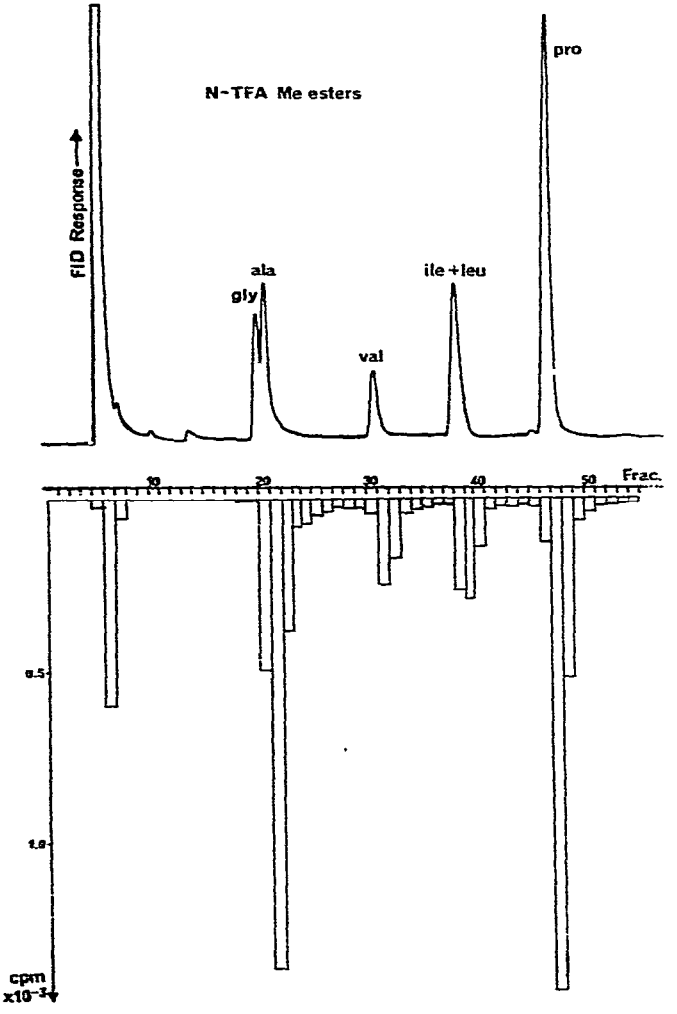

Fig. 4. RGC run of N-TFA amino acids, [^{3}H]methyl esters. Column temp., initially 75° rising to 130° at 6°/min; 5 μg (20,800 dpm) of mixture injected; 20-sec fractions; collection efficiency, 92%.

TABLE I

VARIATION OF $^{14}CO_2$ COLLECTION EFFICIENCY WITH 2-PE CONCENTRATION

For GLC conditions for methyl [^{14}C]palmitate, see legend to Fig. 5.

Concentration 2-PE in 2-ME (%)	*Collection efficiency (%)*
1	25
2	46
5	68
10	85
20	84

in each fraction is plotted as a histogram beneath the FID trace, the bars corresponding to the event marks. Firstly, it can be seen that there is an approximately 15 sec delay between the mass peak and radioactive peak, which indicates the time taken for the radioactivity to travel from the FID to the vial. Secondly, there is little loss of resolution apart from that involved in the taking of fractions. This latter point mainly accounts for the lack of resolution of the glycine and alanine peaks. The overall collection efficiency for this chromatographic run (*i.e.*, injection to collection) was 92%. It is suspected that the major loss of radioactivity is due to absorption on the GLC column, and as this varies from compound to compound and with variation in quantity injected, it is difficult to quantitate with a standard compound. The overall collection efficiency for methyl [^{3}H]stearate was 95% (see Fig. 5) and for other compounds this value has been close to 100%[8]. This means that quantitative combustion of compounds occurs in the FID (with conditions set for optimum sensitivity) and quantitative collection of ^3H_2O can be achieved. Any loss of activity due to non-absorption by the cell could be assessed by imitating the FID by use of a ^3H_2O or $^{14}CO_2$ generator[9]. As the absorption of CO_2 from the FID gases was felt to be more dependent on the operating conditions of the cell than the condensation of H_2O would be, several parameters were varied to find the optimum conditions for collection of $^{14}CO_2$ from methyl [^{14}C]palmitate. The collection efficiency of $^{14}CO_2$ increased with concentration of 2-PE in 2-ME up to 10%, and it was the same at 20% (see Table I). The total gas flow-rate through the cell (above 60 ml/min) had

TABLE II

VARIATION OF $^{14}CO_2$ COLLECTION EFFICIENCY WITH ABSORPTION FLUID TEMPERATURE

For GLC conditions for methyl [^{14}C]palmitate, see legend to Fig. 5.

Temperature of CO_2 absorption fluid (°C)		*Collection efficiency (%)*
Inlet (tube F)	*Outlet (tube G)*	
−10	19	43
5	23	67
14	28	84
16	29	86
19	30	85
23	33	84

very little effect on the efficiency of collection, as did the flow-rate of absorption fluid (above 5 ml/min). An interesting effect was found with the variation in temperature of the absorption fluid in that the collection efficiency decreased with lowering of the temperature (see Table II). Thus no cooling of the cell was necessary for optimum collection. No systematic study on the variation of collection efficiency with H_2O content of the absorption fluid was done, but addition of 5% H_2O did not affect the absorption of ^3H_2O or $^{14}CO_2$ into their respective fluids.

In experiments designed to test the sensitivity of the method, it was found that if the radioactivity from a single peak was collected in two or three fractions, then activity as low as 100 dpm in that peak could be detected (10 min counting time per fraction). This value applies to both ^3H and ^{14}C. This brings radio gas chromato-

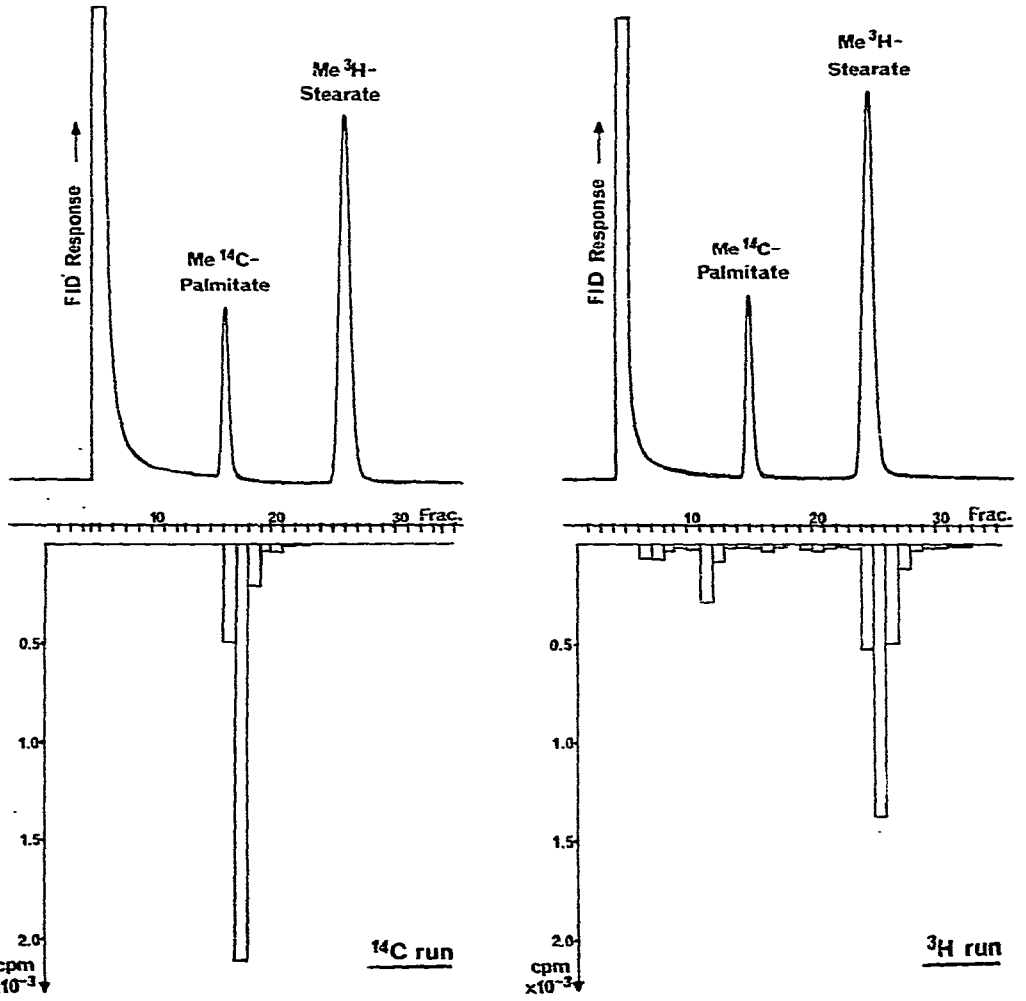

Fig. 5. Dual label RGC run of methyl [^{14}C]palmitate and methyl [^{3}H]stearate. Column temp., 220°; 1 μg (4040 dpm) of methyl [^{14}C]palmitate and 3 μg (9370 dpm) methyl [^{3}H]stearate injected; 20-sec fractions; collection efficiency: ^{14}C, 86%; ^{3}H, 95%.

graphy into the range required by many experiments, where the total activity to be analysed is small or where the specific activity is very low.

The determination of specific activities is an easy task with this system. It requires quantitation of the FID response for a given compound, and the determination of overall collection efficiency of the radioactivity.

The results of a dual label RGC run in which the $^{14}CO_2$ and $^{3}H_2O$ from methyl [^{14}C]palmitate and methyl [^{3}H]stearate were separated chemically are shown in Fig. 5. It can be seen that whereas the methyl [^{14}C]palmitate was radiochemically pure, the methyl [^{3}H]stearate sample gave rise to several impurity peaks. Chemical separation was found to be preferable to differentiation using the liquid scintillation counter because of the difficulties associated with dual label counting, especially if the activity of each label is very different. Less than 0.1% ^{14}C was detected in the ^{3}H run by chemical separation and *vice versa*, and this result would be impossible to achieve by dual label counting. The disadvantage of this method is that two GLC runs are required, but these can be compared directly by comparison of the FID trace. The ability to detect ^{14}C and ^{3}H simultaneously will not only extend the use of RGC to experiments of a dual label nature but also to those in which a second label can be used as an internal standard or in the formation of a radioactive derivative, to quantitate the other label used in the experiment.

CONCLUSION

The high sensitivity and the dual label counting ability of the apparatus described here will extend the range of experiments which can use RGC. In addition, the simplicity and cheapness of construction of the equipment will mean that any laboratory which possesses a GLC and a liquid scintillation counter can, for only a small outlay, carry out RGC.

ACKNOWLEDGEMENTS

I thank the Chemistry Department of Nottingham University for facilities to carry out this work, the Royal Society for a grant for the gas chromatograph and the Science Research Council for financial support. I am indebted to Mr. A. D. Buckland for his skilled glass blowing in the development of the glassware.

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