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HIGH-SENSITIVITY RADIOASSAY BY GAS CHROMATOGRAPHY

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SUMMARY

An approach to radioassay of gas-liquid chromatographic effluents is described in which the sensitivity is increased by expanding the time available for the radioassay. Radioactive compounds in the effluent of the column are burned over heated copper oxide to $^{14}\text{CO}_2$ and $^3\text{H}_2\text{O}$. The effluent of the combustion tube is then passed through a scrubber consisting of a small-bore, simulated gas chromatographic column that is simultaneously supplied with a small, continuous flow of aqueous sodium hydroxide. The liquid effluent of the scrubber is pumped, segmented with air bubbles, into a long length of tubing for storage. The contents of this tube are subsequently pumped through a flow-through scintillation detector at a rate determined by the quantity of radioactivity present.

INTRODUCTION

Just as gas-liquid chromatography (GLC) with conventional detectors provides information about the identity and quantity of individual compounds in a mixture, assaying a GLC effluent for radioactivity can provide similar information about the identity of radioactive compounds and the quantity of radioactivity in each.

Of the several approaches to measuring the radioactivity that have been described, one of the most straightforward is to pass the column effluent through a combustion tube and then directly through a radiation detector, such as a proportional counter, ionization chamber or gas-flow scintillation counter¹⁻³. The record of the radioassay is then available at the completion of the chromatographic separation. Unfortunately, this approach requires that a rather large minimum quantity of radioactivity be present. The reasons for this limited sensitivity are the same as those that make GLC attractive as a method of analysis, *viz.* the rapidity and the high resolution of the separation. The random nature of radioactive emission causes the precision of any radioassay to be a function of the number of radioactive events that are recorded. Therefore, if a compound remains in the sensitive volume of a radiation detector for a short time, few events will be recorded. In GLC, compounds are eluted from columns in rapid succession. In order to preserve the resolving power of the system, the time of passage of the effluent gas through the detector must be short so that the de-

tector can respond to the rapid changes in concentration that occur. In practical terms, this generally means that the average molecule must pass through the detector in no more than 15–30 sec. A radioactive compound decaying at a rate of 1000 dpm will thus give rise to from 250 to 500 recorded events or "counts", assuming 100% efficiency. Because of the random fluctuations in the rate of disintegration, not much less than this radioactivity in a single compound can be quantified by this approach. At least 25,000–30,000 cpm must therefore be present in the sample. When samples containing less radioactivity are to be assayed, the effluent may be fractionated and each fraction subsequently assayed for sufficient time to achieve the precision required. Unfortunately, fractionating the effluent is generally less convenient than employing an on-line detector, particularly if a sufficiently large number of fractions are collected to preserve the accuracy of identification of compounds by retention time.

In a previous study⁴ of the overall problem of detection in chromatographic systems, we suggested that it was not always advantageous to perform separation and detection simultaneously with on-line detectors. In many liquid chromatographic (LC) separations, for example, the time required for the separation is very long. Relatively expensive electronic detection systems, capable of rapid responses, are unnecessarily tied up in monitoring slow processes. We suggested an off-line approach consisting of storing the separation achieved in a slow chromatographic process in a "buffer storage" (computer terminology) for subsequent rapid detection as a means for making the two processes more temporally compatible. Although it was envisioned that separation by LC would take appreciably longer than the usual detection process, we also suggested that the use of a similar buffer storage in rapid GLC might be advantageous in order to slow the delivery of material to a detector that might respond more slowly, such as a mass spectrometer or a radiation detector. We report here the development of a model for using buffer storage to preserve the resolution of a mixture by GLC for subsequent radioassay. The time for radioassay and, therefore, its sensitivity can thus conveniently be varied according to the quantity of radioactivity in the sample.

METHODS AND MATERIALS

It has been demonstrated in the past by many investigators that labeled compounds in a column effluent can be quantitatively burned to $^{14}\text{CO}_2$ and $^3\text{H}_2\text{O}$ by passing the effluent through a narrow-bore quartz tube containing copper oxide wire that is maintained at 700° (refs. 1–3). On-line combustion simplifies and makes uniform the process of handling compounds that have widely different volatilities, and permits the radioassay method to be more generally applicable.

For the experiments described here, we assumed that quantitative combustion of ^{14}C - and ^3H -labeled compounds to $^{14}\text{CO}_2$ and $^3\text{H}_2\text{O}$ could be accomplished without difficulties from either incomplete combustion or "memory" effects by the methods we used previously.³ Our primary objective was therefore to develop a practical method for "scrubbing" these compounds from the carrier gas stream and collecting them in a buffer storage for subsequent radioassay. The effluent of the combustion tube was therefore imitated through the use of a $^{14}\text{CO}_2$ generator⁵. Simulated chromatographic peaks of $^{14}\text{CO}_2$ were produced by injecting 5- μl aliquots of a solution of sodium [^{14}C]carbonate into an acid (1 M sodium dihydrogen phosphate) solution in a 5-ml

stopped test tube fitted with gas inlet, outlet and injection port and supplied with a metered flow of nitrogen gas. This procedure permitted us to test the effects of changing various parameters on the fraction of the radioactivity collected. Injection of the same quantity of sodium [^{14}C]carbonate solution at different places in the train of components permitted isolation and testing of the effect of each component on the response characteristics of the system.

Scrubber

$^{14}\text{CO}_2$ was condensed from the nitrogen carrier gas stream into 0.1 *N* aqueous sodium hydroxide. In one series of experiments, scrubbing was accomplished by bubbling the gas through the solution contained in the segment of a circle of 3-mm-I.D. glass and flexible plastic tubing, also containing a "de-bubbler" or gas outlet. The flowing gas caused the solution to re-circulate through the circle. This device was modeled after that described by Popjak *et al.*⁶ for monitoring a GLC column effluent for radioactivity by continuous, cumulative scintillation counting. Glass T-connections were added to the circle of tubing to permit fresh sodium hydroxide solution to be added and withdrawn at approximately equal, constant rates.

The second scrubber, the "GC-LC" scrubber, consisted of a 7 cm \times 3 mm I.D. glass tube fitted with a silicone rubber stopper at each end. The tube was filled with either 80–100 mesh Chromosorb W or 30–60 mesh Chromosorb T polytetrafluoroethylene (Johns Manville, Denver, Colo., U.S.A.), held in place by glass wool. The tube was mounted vertically. Two 1.5-mm-O.D. stainless-steel hypodermic tubes pierced the inlet stopper. The first delivered the gas; the second delivered a metered flow of 0.1 *N* sodium hydroxide solution that percolated downward through the solid support.

De-bubbler

The frothy effluent of the scrubber was directed through another 1.5-mm-O.D. hypodermic tube against the inside wall of a small polyethylene cone open at the top—a "disposable" tip of an Eppendorf micropipette. The bubbles broke on contact with the wall, the gas escaped and the solution settled to the narrow apex of the tube where it tended to be held by capillary action. The solution was removed from the de-bubbler by pumping it into flexible polymeric tubing of different diameters either at the same rate as the solution was added to the scrubber, which produced a solid stream of solution, or, most often, at approximately twice that rate, which produced a stream of liquid fairly uniformly interrupted by air bubbles.

The pumping of the solution and the air were performed with a Technicon AutoAnalyzer I proportioning pump.

The radiation detector was a 3 cm \times 6 mm I.D. glass tube filled with anthracene crystals⁷ placed between the photomultiplier tubes of a Packard Tricarb[®] flow analyzer-scintillation counter. The counting rate, monitored by an analog counting rate meter, was recorded on a strip chart recorder.

The stream of solution from the de-bubbler was either pumped directly into and through the detector cell or into a buffer storage consisting of different lengths of 3-mm-I.D. polymeric tubing wound into a helix around a 15-cm-diameter cylinder the axis of which was vertical.

RESULTS

Efficiency of CO₂ trapping

Popjak-type re-circulating liquid scrubber. With a nitrogen flow-rate of 20 ml/min or more, tubing with at least 2.5 mm I.D. was required in order to maintain a uniform bubble pattern and orderly re-circulation of the sodium hydroxide solution. Quantitative collection of ¹⁴CO₂, as measured by liquid scintillation counting, was achieved in a 15-cm length of this tubing followed by 1 ml of a solution in a 10-mm-I.D. de-bubbler tube. The minimum volume of solution needed to accomplish this, including the volumes contained in the similar segment of tubing necessary for re-circulation and that in the T-connections, was 6 ml. Since it was desirable to replace the contents of the scrubber about four times per minute, the volume of the buffer storage needed to contain a chromatographic separation became cumbersome. This problem prompted the development of the GC-LC scrubber.

GC-LC scrubber. In the GC-LC scrubber, with sodium hydroxide solution flowing at a rate of 1–2 ml/min, ¹⁴CO₂ was quantitatively trapped from nitrogen streams flowing at a rate of 20–60 ml/min. When fractions of the effluent were taken every 15 sec, the pattern of distribution of the radioactivity reproduced the pattern of ¹⁴CO₂ evolution from the generator, as indicated by direct scintillation counting of the gas. There was no discernable peak distortion or broadening. The two kinds of solid support, the diatomaceous earth and the polytetrafluoroethylene, were equally effective.

The detector response following injection of sodium [¹⁴C]carbonate directly into the afferent detector line was used as a basis of comparison to determine possible peak broadening at each of the more distal components. For example, when flow-rates of 1.4 ml of sodium hydroxide solution were both added to the scrubber and withdrawn from the de-bubbler, moderate peak broadening was produced. When the flow to the scrubber was reduced to 1 ml/min and 1.4 ml/min was withdrawn from the de-bubbler, thus producing an air-segmented stream, the peak shape was restored to that produced by direct injection into the detector.

Changes in the ratio of air to solution delivered to the detector from 0 to 1.5 produced no detectable effect. The total number of counts recorded during the passage of the radioactive plug through the detector seemed to be determined almost entirely by the liquid flow, probably as a result of incomplete filling of the detector. The peaks were narrowed by introducing bubbles into the afferent line, because of the efficiency of the bubbles in preventing mixing in these lines. Substituting an air flow for part of the liquid flow increased the number of counts recorded in proportion to the decrease in liquid flow. These observations indicated that the detector acted as a mixing volume in which the liquid flow determined the turnover characteristics.

Bubbles produced no detectable increase or decrease in detector efficiency; there was no change in the maximum counting rate produced when the radioactivity injected was presumably all present in the detector at the same time.

Effect of buffer storage. Diverting the air-segmented effluent of the de-bubbler through a length of 3-mm-I.D. tubing sufficient to delay its arrival at the detector for 5 min resulted in no appreciable peak broadening. It was found helpful to pump air from the end of the helix or from the detector, when they were connected, at the same rate as fluid was pumped into it to maintain the flows constant. After the ef-

fluent was delivered to this tubing, stopping the flow and replacing the inlet tubing with tubing delivering fluid at 1/5th of the previous rate increased the number of counts recorded by the predicted five fold. There was no appreciable decrease in the maximum counting rate, indicating minimal mixing from one segment to another and no loss of resolution. The stability of the segmented stream was adequate to permit undisturbed standing for several hours although the relatively large bore of the tubing caused the segments to be susceptible to mechanical agitation.

DISCUSSION

In previous work with on-line, simultaneous GLC-radioassay, we often had the impression that the high speed of GLC, desirable in almost all other instances, often unnecessarily restricted the sensitivity of radioassays by allowing too little time for counting. The use of the buffer-storage approach permits the sensitivity to be expanded to meet the requirements of the many biochemical experiments in which only limited quantities of radioactivity can be taken for analysis.

In the past, the on-line flow analyzer-scintillation counter has often been preferred to other radioassay methods for assaying effluents of ion-exchange amino acid analyzers; the slowness of the separation permitted very long counting times and consequent high sensitivity. The objective of the present work was to make this kind of sensitivity available in GLC.

The sensitivity is largely determined by the time allowed for the radioassay. It is also a function of the resolution required. In the usual on-line, flow-through system, the resolution is a function of the volume of the radiation detector and the flow-rate of gas through it. In the buffer-storage approach the gas flow-rate term is replaced by the flow-rate of sodium hydroxide solution through the scrubber.

The most critical portion of the experimental apparatus described here is the scrubber. The use of a very-large-volume buffer storage is possible but it is experimentally cumbersome. The small liquid flows necessary to scrub $^{14}\text{CO}_2$ continuously from flowing gas in the GC-LC column made the use of this device attractive.

It is tempting to speculate that only minor modifications of this technique should be required to make continuous recovery of compounds separated by GLC possible. This approach might simplify the use of ancillary wet chemical and physical detection methods.

Combustion of the GLC effluent, which can conveniently be accomplished, simplifies all of the subsequent handling processes in radioassay by GLC. For example, it makes construction of a Popjak-type continuous, cumulative, on-line counter much simpler if the problems associated with preventing premature condensation and then accomplishing quantitative scrubbing can be avoided.

The counting efficiency of the anthracene crystal flow cell for ^{14}C in aqueous systems is somewhat less than that obtainable by liquid scintillation counting in toluene or xylene-based solutions stabilized as gels by detergents. The efficiency for ^3H is much higher by the liquid technique. The possibility of using continuous-flow liquid scintillation counting, particularly for ^3H , is consequently very attractive. Advantages of the use of a solid scintillator as described here, or a narrow-bore tube of scintillating plastic as a detector, are its re-usability and the consequent low reagent cost.

It should also be possible to precipitate the $^{14}\text{CO}_2$ continuously, for example, as barium carbonate on to a moving paper strip. The radioassay could then be performed subsequently by techniques used to assay radioactivity on paper chromatograms.

The efficiency of the GC-LC scrubber prompted us to consider the use of the same principle in reverse. $^{14}\text{CO}_2$ could presumably be released from the flowing liquid alkaline stream by mixing it with acid as it is delivered to a "de-scrubbing" column. The $^{14}\text{CO}_2$ could then be assayed by gas flow proportional counters, ionization chambers, or solid scintillation counters. Again, the time and sensitivity could be expanded to meet the needs of the experimental situation with minimal sacrifices of resolution.

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