## Radioassay of low activity fractions encountered in gas-liquid chromatography of long-chain fatty acids*

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» Methods have been developed for continuous radioassay of vapor emerging from a chromatographic column (1 to 5), or being collected in an accumulating trap (6, 7). These techniques are convenient and precise when used with high activity material; but when the activity is low, the time for assay generally is not adequate for dependable averaging of the counting rate. For satisfactory assay, fractions must be collected (7, 8) and counted for longer periods of time than are provided by the usual separation of peaks in the chromatographic outflow.

Low activity fractions (less than 1,000 $\mu\mu$c, or about 1,000 cpm with detectors of 50% efficiency) are fre-

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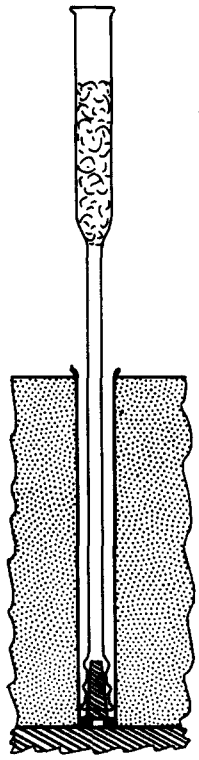

FIG. 1. Unit for collection of chromatographic fractions.

quently encountered in studies of fatty acid metabolism. Often the initial dose of radioactive label is limited by insolubility, low specific activity, toxicity, or by the need to minimize radiation dosage. The amount brought to analysis may be further reduced by low rates of synthesis and by dilution of the label. The present paper describes a simple and precise method for collection and assay of these low activity fractions.

The potential errors of this method, and of assay methods in general, fall into three categories: losses due to retention of material in the chromatographic column and mass detector, losses in collection and transfer of samples, and errors in counting due to instrumental or statistical limitations. These were evaluated in the present study.

*Procedure.* Effluent vapors are trapped in a glass collection unit (Fig. 1) firmly attached to the outlet of the mass detector. For leak-free attachment it is important that the glass joint of the unit and the outlet nipple of the detector be accurately machined to a standard taper. The insulating block surrounding the detector maintains a temperature gradient, holding the joint near detector temperature, while the distal half of the unit outside of the block is near room temperature. Most of the effluent material condenses in the stem of the unit just outside of the block. To trap the remainder, a loose cotton plug, damp with toluene, packs the proximal portion of the funnel. This packing must fill the space yet be loose enough to permit a free exit of gas.

The gas-liquid chromatography was performed as previously described (9), using Apiezon and ethylene glycol adipate polyester (EGA) columns operating at 197° and 185°, respectively.

During a chromatographic run, collection units are successively attached to the outlet, the number of fractions depending on the degree of resolution required. For many studies, it has proved sufficient to collect each peak in a single unit and to collect baseline outputs between peaks in one or two units. When greater resolution is needed (as in testing the homogeneity of material contained in a single peak for detection of radioactive material emerging between peaks, or for studying the distribution of activity in partially resolved peaks), the units can be changed more frequently. The radioactivity in each unit will, of course, be smaller and require a correspondingly longer period of assay.

After the chromatographic run is completed, the units are set up vertically and eluted into 20-ml counting vials with 15 ml of toluene in three portions of 5 ml. Scintillator (5 ml of a solution of 16 g of 2,5-diphenyloxazole and 400 mg of *p*-bis-2-(5-phenyloxazolyl)-benzene in 1 liter of toluene) is then added and the samples are assayed in a liquid scintillation counter. Since incomplete and variable washing can cause significant error with low activity material, the washing procedure should be checked in laboratory use and then carefully standardized.

To test the resolution of the method, a high activity sample (0.3 $\mu$c of chromatographically pure methyl palmitate-1-$C^{14}$) was loaded on an Apiezon column, and six collections were made during emergence of the palmitate peak. The radioactivity recovered during these intervals and the areas under the corresponding portions of the mass tracing varied over a hundredfold range (Table 1). The specific activity, however, as measured by the ratio of cpm to area, showed a coefficient of variation of only 7%. The constancy of this ratio indicates a consistent percentage recovery, and incidently shows that the trapping of an emergent component in the collection unit was not significantly delayed compared to its registration by the mass detector.

*Recovery of Radioactivity.* The percentage recovery of a measured load was determined in 13 experiments. An Apiezon column was loaded with 0.125 $\mu$l of radioactive methyl palmitate; an EGA column with 0.125 $\mu$l of a mixture of radioactive methyl palmitate and unlabeled methyl stearate (1:3). The eluant was col-

TABLE 1. SUBFRACTIONATION OF METHYL PALMITATE-1-$C^{14}$ PEAK

| Fraction | Area* | Counting Rate (cpm) | cpm/Area |
|---|---|---|---|
| 1 | 11.20 | 14,393 | 1,285 |
| 2 | 98.66 | 111,179 | 1,127 |
| 3 | 161.32 | 185,759 | 1,151 |
| 4 | 71.28 | 75,923 | 1,058 |
| 5 | 8.72 | 10,502 | 1,204 |
| 6 | 1.64 | 1,787 | 1,090 |
| | | | 1,154 ± 82† |

* The areas are expressed as the weights (mg) of the corresponding portions of the chart paper.
† Mean ± standard deviation.

TABLE 2. RECOVERY OF METHYL PALMITATE-1-$C^{14}$ LOAD

| | Apiezon Column | Apiezon Column | EGA Column |
|---|---|---|---|
| | No. of Experiments | | |
| | 5 | 5 | 3 |
| | *cpm* | *cpm* | *cpm* |
| Full pipette (0.125 μl) | 1,503 ± 83* | 6,411 ± 120 | 1,996 ± 36 |
| Left in pipette after loading | 23 ± 22 | 5 ± 2 | 43 ± 16 |
| Load | 1,480 | 6,405 | 1,953 |
| Recovered in palmitate peak | 1,332 ± 75 | 5,832 ± 124 | 1,731 ± 95 |
| Recovery | 90% | 91% | 88.5% |

* Mean ± standard deviation.

lected before, during, and after emergence of the palmitate peak. About 90% of the load was recovered with the peak (Table 2). Further experiments were done to determine how much of the remaining 10% was lost because of failure of collection and elution and because of delay in emergence of activity from the column and detector.

*Losses in Collection and Transfer.* The gas emerging from the collection unit was passed through a second unit to collect any vapor that might have escaped the first. In eight collections of material ranging in activity from 749 to 504,000 cpm, the activity recovered from the second trap averaged only 0.23% of that recovered from the first. In another experiment, gas from a collection unit was bubbled through toluene before discharge; no significant activity appeared in the second trap although the collection unit yielded 2,674 cpm. These experiments suggested that the incomplete recovery was not due to passage of radioactive vapor through the collection unit.

To test the completeness of washing, a group of collection units containing labeled palmitate was washed in the usual way (three washes with toluene), and then filled with a solution of unlabeled methyl plamitate in toluene (200 mg/liter) and left overnight. The units were emptied into counting vials and further washed with 10 ml of the palmitate solution. In five separate collections averaging 2,911 ± 292 cpm, the residual radioactivity averaged only 5 ± 3 cpm, or 0.17%. Another series of collection units was washed in the usual manner, following which the cotton packings were placed in counting vials containing scintillator solution. Nine units yielded an average of 5,822 ± 485 cpm in the original eluate, while the residual activity of the packings averaged only 2 ± 3 cpm. The presence of cotton in the vials did not prevent accurate detection of radioactivity, since cotton added to counting vials with standard palmitate caused no quenching. These results suggested that the incomplete recovery was not caused by losses occurring in the transfer procedure.

*Dispersion in the Column and Ionization Chamber.* Dispersion of radioactivity beyond the apparent limits of the mass peak would cause incomplete recovery with any method of assay since the fraction is identified by the response of the mass detector. That an appreciable dispersion occurs was demonstrated in the following experiment. A sample of palmitic acid-1-$C^{14}$ was methylated and loaded on an Apiezon column (0.125 μl of material assaying at 6,400 cpm). The outflow was collected in two 20-minute intervals before emergence of the peak, a 10-minute period that included all of the peak shown by the recording system, and three 20-minute intervals after the peak had passed and the tracing had returned to baseline. In five such experiments (Table 3), it was found that 0.44% of the activity emerged in the 40 minutes preceding the peak, and 1.09% in the hour after. In a parallel series of experiments performed on a fast EGA column, using 2.7-minute collection intervals instead of 20-minute intervals, a similar pattern of radioactive output was found: 0.12% of the activity emerged during the 5.4 minutes preceding the palmitate peak and 4.46% during the 10.8 minutes after.

The trailing could not be attributed to radioactive impurity, since rechromatographing of material collected during the central 10-minute period showed the same distribution of output as the original material (Table 3). The dispersion was independent of the presence of unlabeled compounds; chromatographically purified, radioactive methyl palmitate mixed with unlabeled methyl stearate showed the same pattern of radioactive output as the methyl palmitate alone.

TABLE 3. RADIOACTIVITY COLLECTED DURING RUNS WITH METHYL PALMITATE-1-$C^{14}$

| | | Commercial Palmitate-1-$C^{14}$ | | Palmitate-1-$C^{14}$ Purified by GLC* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Additions | | | | | | | |
| | | None | | None | | Methyl stearate | | Methyl palmitate† | | None† | |
| | | No. of experiments | | | | | | | | | |
| | Fractions Collected | 5 | | 4 | | 5 | | 3 | | 3 | |
| *No.* | *min* | *cpm* | % | *cpm* | % | *cpm* | % | *cpm* | % | *cpm* | % |
| 1. | 20 | 2 ± 2‡ | 0.03 | 0 ± 0 | 0 | 3 ± 4 | 0.11 | 5 ± 5 | 0.10 | 4 ± 2 | 0.10 |
| 2. | 20 | 24 ± 11 | 0.41 | 8 ± 4 | 0.17 | 9 ± 5 | 0.30 | 37 ± 16 | 0.85 | 43 ± 4 | 0.98 |
| 3. | 10(palmitate) | 5,832 ± 124 | 98.47 | 4,706 ± 387 | 98.74 | 3,255 ± 620 | 98.06 | 4,165 ± 155 | 96.77 | 4,251 ± 207 | 96.60 |
| 4. | 20 | 30 ± 12 | 0.50 | 29 ± 6 | 0.60 | 31 ± 5 | 0.97 | 49 ± 31 | 1.15 | 52 ± 51 | 1.19 |
| 5. | 20 | 23 ± 13 | 0.38 | 17 ± 6 | 0.35 | 10 ± 3 | 0.33 | 27 ± 34 | 0.62 | 24 ± 20 | 0.55 |
| 6. | 20(stearate) (palmitate) | 12 ± 7 | 0.21 | 7 ± 7 | 0.14 | 7 ± 8 | 0.24 | 15 ± 4 | 0.34 | 16 ± 14 | 0.35 |
| 7. | 20 | | | | | | | 7 ± 2 | 0.20 | 8 ± 5 | 0.19 |

* Material collected during the central 10-minute period of a previous run was rechromatographed in these experiments.

† Immediately following the third collection, the run was interrupted and a load of unlabeled palmitate or, as control, no load was put on the column.

‡ Mean ± standard deviation.

TABLE 4. FRACTIONATION OF STANDARD MIXTURE OF 1-$C^{14}$-LABELED METHYL ESTERS

| | Standard Mixture | | Collection No. | Recoveries from Apiezon Column | | | | Recoveries from EGA Column | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Radioactivity | Mass | | Time | Radioactivity | | Mass | Time | Radioactivity | | Mass |
| | % | % | | *min* | *cpm* | % | % | *min* | *cpm* | % | % |
| | | | 1 | 8.6 | 12 | 0.1 | 0 | 2.8 | 0 | 0 | 0 |
| Myristate | 39.8 | 11.5 | 2 | 4.5 | 4,118 | 37.8 | 11.4 | 1.3 | 1,416 | 37.5 | 9.3 |
| | | | 3 | 9.0 | 149 | 1.4 | 0 | 1.7 | 27 | 0.7 | 0 |
| Palmitate | 22.8 | 24.1 | 4 | 6.0 | 2,616 | 24.0 | 27.7 | 2.3 | 870 | 23.0 | 26.0 |
| | | | 5 | 24.8 | 87 | 0.8 | 0 | 2.5 | 15 | 0.4 | 0 |
| Stearate | 37.4 | 64.4 | 6 | 10.6 | 3,895 | 35.7 | 61.0 | 3.4 | 1,356 | 35.9 | 64.8 |
| | | | 7 | 9.0 | 29 | 0.3 | 0 | 3.4 | 64 | 1.7 | 0 |

A second load of unlabeled palmitate, applied to the column immediately following the emergence of the labeled palmitate peak, did not affect the pattern of radioactive output (Table 3). These results indicate that a pure substance emerges from a column over a wider time interval than is shown from the record of mass output. The distribution, moreover, is non-Gaussian; the 1.5% of activity falling outside the central period is dispersed more widely than would be expected from a normal distribution with mean and standard deviation fitting the central period. Even longer trailing was demonstrated by use of a new Apiezon column for analysis of a single, high activity (1 $\mu$c) load. The background count remained significantly elevated for days; indeed, before the equipment could be used again for analysis of low activity material the column had to be replaced and the ionization chamber cleaned with a solvent (hot chlorobenzene).

The total amount of activity dispersed beyond the limits of the mass peak could not be measured accurately because of the exceedingly slow rate of emergence, but it probably did not exceed 3% to 4% of the load. The remaining few per cent not recovered may have escaped as vapor during loading when the sample was applied to the hot packing of an open column; if so, the actual load was smaller than estimated and the true percentage recovery greater. In any case, losses in chromatography, rather than losses associated with collection and transfer, appeared to be the major cause of incomplete recovery. Comparable losses have been encountered with another method of collection and assay. Silicone-coated anthracene columns used for collection and radioassay of high activity material gave 87% to 95% recoveries (7), no greater than that obtained in the present study with low activity material.

*Fractionation of a Standard Mixture.* A load consisting of a known mixture of three chromatographically purified $C^{14}$-labeled methyl esters was applied to the column and the effluent was collected in seven fractions—three containing the material emerging with the mass peaks and four representing the baseline output (Table 4). The load applied to the Apiezon column (0.125 $\mu$l of the mixture) assayed at 12,211 cpm; 10,906 cpm, or 89% of this, was recovered. Approximately 0.042 $\mu$l (4,100 cpm) was loaded on an EGA column and 3,748 cpm, about 91%, was recovered. The three fractions collected during the emergence of the mass peaks contained more than 97% of all the activity recovered during the run, and showed a percentage distribution of radioactivity similar to the known distribution of activity between the three components of the mixture (Table 4). The results showed a consistent percentage recovery of three esters while the total amounts varied from about 3 $\mu$g to about 65 $\mu$g; they therefore suggest that relative specific activities, calculated as ratios of counts recovered and area of peak, would not be seriously in error.

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