Determination of the specific radioactivity of fatty acids separated as their methyl esters by gas–liquid chromatography

C. Bishop, R. F. Glascock, Elizabeth M. Newell, and V. A. Welch

National Institute for Research in Dairying, University of Reading, Shinfield, Reading, Berkshire, England

SUMMARY Free or combined ^{3}H-labeled fatty acids are converted to their methyl-^{14}C esters or, if labeled with ^{14}C, to their methyl-^{3}H esters. For a given specific radioactivity of the methyl group, the nuclide ratio in the esters separated by GLC is a direct measure of the specific radioactivity of the fatty acids, and quantitative collection is unnecessary. Methods of methylation with minimum quantities of labeled methanol, and of deriving nuclide ratios from channel ratios in a scintillation spectrometer, are given.

SUPPLEMENTARY KEY WORDS dual nuclide · recovery indicator

A number of techniques have been described for measuring the radioactivity of the methyl esters of fatty acids separated by GLC. These include conversion of the esters to carbon dioxide and hydrogen, whose radioactivity is continuously measured in a proportional counter (1, 2), and trapping them on various substances, followed by scintillation counting (2–7).

In order to obtain specific radioactivity it is necessary to know both the radioactivity and the mass of methyl ester collected. Mass may be determined either by chemical analysis (8) or by measurement of the area of the peak on the recorder tracing, the response of which has previously been calibrated by the use of standard compounds (7). A different approach to the problem which avoids the necessity of quantitative collection is to add an internal standard to a solution of the GLC-resolved ester and to analyze again by GLC (9).

The necessity for quantitative collection could also be avoided by the use of recovery indicators. In one method that has been tried in this laboratory, a portion of the methyl esters of the mixed ^{3}H-labeled fatty acids was separated on an analytical column and then, after addition of the ^{14}C-labeled ester of the fatty acid whose specific radioactivity was required, another portion was separated on a preparative column. From the ^{14}C and ^{3}H contents of the eluted ester and from the peak areas in both separations the ^{3}H specific radioactivity of the ester in question was calculated. The success of this method was, however, dependent upon the precision with which the area measurements were made. Because of the difficulties associated with the precise measurement of peak areas, another method has been devised in which a radioactive nuclide in the methyl group is used as a recovery indicator of methyl esters. Thus the methyl-^{14}C esters are prepared when the fatty acids are labeled with ^{3}H, and vice versa. In practice this means that when radioactive methanol of known specific radioactivity is used in the methylation procedure, the specific radioactivity, with respect to the other nuclide, of the esters separated from the GLC column is directly proportional to the nuclide ratio found in them.

Diazomethane-^{14}C has been used for the determination of NEFA as their methyl esters (10, 11), and since the present work was completed the method of Fischer and Kabara (12), in which methanol-^{3}H is used to determine the specific radioactivity of mixtures of ^{14}C-labeled fatty acids, has come to the authors' attention. A similar use of methanol-^{3}H to assay total fatty acids in tissue extracts was reported by Poledne and Mayes (13).

Preparation of Methyl Esters. What follows is the procedure for measuring the specific radioactivity of ^{3}H-labeled fatty acids by the use of methanol-^{14}C (98%, Radiochemical Centre, Amersham, Bucks, England). As already noted, however, the reverse arrangement of nuclides would be equally possible.

For the preparation of methyl-^{14}C esters, existing methods had to be modified according to the lipid to be treated so that the minimum amount of ^{14}C could be used. For glycerides, a method based on that of Morgan, Hanahan, and Ekholm (14) was used. The reagent consisted of equal volumes of a molar solution of sodium methoxide (prepared from metallic sodium and dry methanol) and of methanol-^{14}C of specific radioactivity preferably not more than the lowest specific radioactivity to be measured nor less than $^{1}/_{7}$ of the highest (see below). Samples of glyceride (up to 2 mg) were dissolved in 0.3 ml of chloroform (free from carbonyl chloride and alcohol), ether, benzene, or toluene, and 10 μl of the labeled sodium methoxide solution was added. After 10 min, 1 ml of hexane, 1 drop of 6 M HCl, and 2 ml of water were added, and the contents of the tube were well mixed. The hexane extract was washed with water and concentrated to about 50 μl before application to the GLC column.

For NEFA, cholesteryl esters, and phospholipids, a method based on that of Carroll (15) was used; in this procedure the lipid is heated in methanolic solution with acetyl chloride, which serves to generate HCl *in situ*. The reaction mixture consisted of benzene, acetyl

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; NEFA, nonesterified fatty acids.

chloride, and methanol-^{14}C in the proportions of 75:1:10. The samples were heated at 120°C in sealed ampoules with quantities of reaction mixture for periods of time which depended on the nature of the lipid. Thus, NEFA were treated with 100 μl of reaction mixture per mg for 2 hr, and cholesteryl esters and phospholipids with 500 μl per mg for 16 hr. The contents of the ampoules were extracted with hexane and transferred to stoppered tubes. The extract was washed with $NaHCO_3$ solution (5% w/v) and then with water and was finally concentrated to a small volume. The methyl esters were isolated by chromatography on a column of deactivated Florisil as described by Carroll (15). It was necessary to store the reagents containing methanol-^{14}C in vials sealed by a solvent-resistant serum cap and to withdraw the reagent by means of a syringe needle. If this precaution was not taken the reagent rapidly deteriorated and quantitative methylation was not then achieved.

Thin-layer Chromatography. TLC was performed as previously described (16). Since the phospholipids were eluted with methanolic HCl, care was taken to remove all traces of the methanol before the sample was methylated.

Preparative Gas–Liquid Chromatography. A stainless steel column 274 cm long and 6 mm in diameter was charged with diatomaceous earth (mesh 80–100; Phase Sep, W-AW-DMCS; Phase Separations Ltd., Queensferry, Flintshire, Wales) loaded with 10% (w/w) diethylene glycol succinate polyester (Applied Science Laboratories Inc., State College, Pa.). The carrier gas was N_2 at a flow rate of 66 ml/min and the column temperature was 175°C. The effluent stream was divided so that 15% went to the flame ionization detector and 85% went to the heated exit port and into 10 ml of scintillator solution by way of a stainless steel cannula. After each ester had been collected, the cannula was rinsed with 2 ml of scintillator solution. The trapping efficiency was approximately 85%.

Purification and Determination of the Specific Radioactivity of Standards. Carrier methyl palmitate (99%, British Drug Houses Ltd., Poole, Dorset, England) was added to palmitic acid-^{3}H (>96%, Radiochemical Centre) which was methylated and then purified by preparative GLC and TLC. The purified ester was saponified, the fatty acid was liberated, and its specific radioactivity was determined by weighing and titration followed by counting (16).

Carrier tripalmitin (99%, Sigma Chemical Co., St. Louis, Mo.) was added to tripalmitin-^{3}H (>98%, Radiochemical Centre) and the mixture was purified by chromatography on deactivated Florisil as described by Carroll (17). The purified tripalmitin was saponified and the specific radioactivity of the palmitic acid was determined as summarized above.

Measurement of Radioactivity. Radioactivity was measured at 12°C in a Packard Tri-Carb liquid scintillation spectrometer (model 3314). The scintillator solution was a mixture of 2-(4′ *t*-butylphenyl)-5-(4″ biphenylyl)-1,3,4-oxadiazole (butyl-PBD) and 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP) dissolved in toluene at concentrations of 6 and 0.3 g/liter, respectively. The instrument settings were similar to those described by Bush (18) and were adjusted so that the ratios of the counting rates in channels 1 and 2 were linearly related to the nuclide ratios in mixtures of toluene-^{3}H and toluene-^{14}C (19). In our equipment the linear relationship held good for ^{3}H/^{14}C ratios between 2 and 14. Efficiencies were determined by the use of toluene-^{3}H and toluene-^{14}C as internal standards.

Determination of Nuclide Ratios from Counting Rates. Samples of labeled esters obtained in biological experiments and separated by GLC normally amount to less than 1 mg each and would therefore be expected to be counted with the same efficiencies as the toluene standards. This was confirmed by measurements of efficiency after addition of standard toluene-^{3}H and toluene-^{14}C to separate vials of scintillator solution as well as to individual samples. The average efficiency of counting of the two nuclides, as measured by the first procedure, was found not to differ significantly from individual efficiencies. This is illustrated in Table 1, where nuclide ratios are calculated for a number of samples of doubly labeled esters by these two methods and also from channel ratios. The channel ratio technique was clearly the most convenient when the nuclide ratio fell within the range 2–14.

Calculation of the Specific Radioactivity of ^{3}H-labeled Fatty Acids. The methyl esters of the fatty acids of the experi-

TABLE 1 COMPARISON OF METHODS FOR THE DETERMINATION OF ^{3}H/^{14}C RATIO IN DOUBLY LABELED METHYL ESTERS

Methyl Ester Fraction	^{3}H/^{14}C Ratio as Calculated from: Individual Counting Efficiencies*	Average Counting Efficiencies†	Channel Ratio‡
Mixture	2.93	2.90	2.91
Palmitate	3.17	3.18	3.18
Mixture	3.89	3.95	4.00
Palmitate	4.69	4.68	4.78
Palmitoleate	4.81	4.76	4.88
Palmitoleate	6.61	6.59	6.75
Palmitate	13.47	13.44	13.58
Palmitate	14.29	14.31	14.42

* Efficiency of counting of ^{14}C and ^{3}H was determined by addition of known amounts of standard toluene-^{14}C and toluene-^{3}H successively to each sample.

† Average counting efficiency of nuclides in whole batch of samples was determined by addition of standards to separate vials (see text).

‡ Nuclide ratio determined from its linear relationship with channel ratios (see text).

mental samples and of a standard fatty acid were prepared by the use of the same batch of methanol-^{14}C. The specific radioactivity of the fatty acids of the experimental samples was then given by the expression:

$$S_x = \frac{R_x}{R_s} \cdot S_s$$

where S_s was the specific radioactivity with respect to ^{3}H of the standard fatty acid, S_x was that of the experimental sample, and R_s and R_x were the corresponding ^{3}H/^{14}C ratios. If the specific radioactivity of the standard ^{3}H-labeled fatty acid was expressed in radioactivity/μmole, then the same expression, where R_x represented the nuclide ratio in the mixed methyl esters, gave the specific radioactivity of the mixture in the same units.

Results and Discussion. The validity of the method was tested by measurement of the nuclide ratio in methyl-^{14}C palmitate-^{3}H prepared from standard tripalmitin-^{3}H. The SEM expressed as a percentage of the mean of a set of 12 samples was 0.78, and of another set of 18 samples was 0.29. This is an acceptable reproducibility for most biological work. Table 2 shows that the specific radioactivity of the methyl palmitate obtained by direct treatment of tripalmitin was in good agreement with that observed after methylation of the free palmitic acid obtained from the tripalmitin by hydrolysis. The table also shows that the values obtained for the specific radioactivity of the ester before and after GLC were in good agreement. This implies that no free methanol-^{14}C, methyl-^{14}C acetate, or unreacted ^{3}H-labeled lipid was present. Hence, if the method had been applied to a lipid containing a mixture of fatty acids, the nuclide ratio of the mixture before GLC would have been a reliable measure of the mean specific radioactivity of all the fatty acids present and hence of the lipid itself.

The applicability of the method to a biological problem is illustrated in Fig. 1, which shows some results obtained from a lactating cow previously dosed with palmitic acid-^{3}H. The lower curve gives the specific radioactivity of the total fatty acids of milk fat, and the upper curve gives that of the constituent palmitic acid. The maxima in the curves occur at the same time, and the relative areas under them are consistent with the facts, also ascertained in the experiment, that 90% of the radioactivity in the milk fat was in the palmitic acid and that the abundance of this acid was 32%.

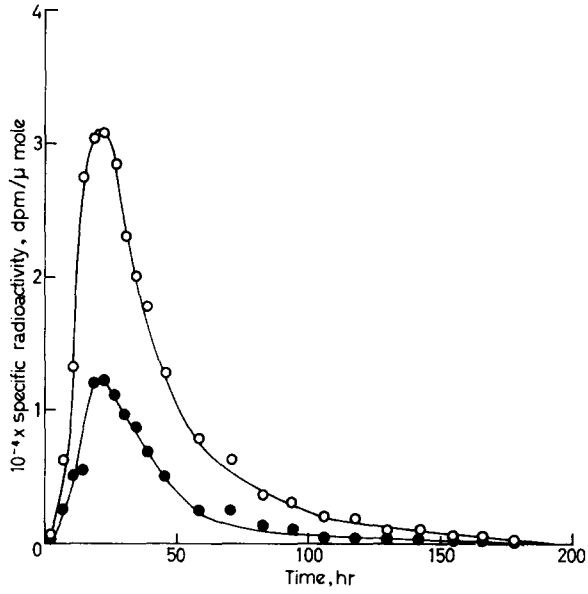

FIG. 1. Specific radioactivity of the fatty acids of milk fat obtained after intraruminal injection of a cow with palmitic acid-^{3}H. ○, palmitic acid; ●, total fatty acids.

TABLE 2 SPECIFIC RADIOACTIVITY OF PALMITIC ACID IN TRIPALMITIN

Sample*	Catalyst†	^{3}H/^{14}C of Sample (a)	^{3}H/^{14}C of Standard (b)	Specific Radioactivity of Standard Palmitate-^{3}H (c)	Specific Radioactivity of Sample‡ (ac/b)
				dpm/μmole	*dpm/μmole* ± SEM
Before GLC	base	2.897	9.038	6412	2055 ± 44
Palmitate	base	2.938	9.038	6412	2084 ± 39
Before GLC	acid	7.783	5.093	1260	1926 ± 42
Palmitate	acid	7.954	5.093	1260	1968 ± 59
Before GLC	acid	7.783	10.18	2657	2031 ± 46
Palmitate	acid	7.954	10.18	2657	2076 ± 63

* Nuclide ratios were measured both in the sample before separation on GLC and in the palmitate that was eluted.

† Methyl esters were produced by base-catalyzed methylation applied to tripalmitin in $CHCl_3$ or by acid-catalyzed methylation of palmitic acid obtained from tripalmitin after hydrolysis.

‡ Specific radioactivity of the sample of palmitic acid as determined gravimetrically was 1969 ± 10 dpm/μmole.

A possible disadvantage of the technique is one associated with all procedures involved in trapping of fractions rather than continuous recording of their radioactivity. As noted by Snyder (20), errors can arise from the trailing of compounds of high specific radioactivity and low abundance into the fraction being collected. Such errors could be revealed by the collection of small fractions as recommended by Thomas and Dutton (21). The specific radioactivity of the fraction (and hence the nuclide ratio) would then change as the substance responsible for the peak was collected. In that case a GLC technique giving better resolution would have to be sought.

The chief advantages of this method are that there is no need to achieve quantitative collection of the eluted esters and that once the reference samples of ^{3}H-labeled fatty acids and the standard dilutions of methanol-^{14}C have been prepared, specific radioactivities of fatty acids can be obtained directly from the counting rates, without further quantitative analysis.

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