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APPLICATION OF GAS–LIQUID CHROMATOGRAPHY TO ANALYSIS OF CARBON-14 BIOSYNTHETICALLY LABELLED COMPOUNDS

M. MATUCHA and V. SVOBODA
Institute for Research, Production and Application of Radioisotopes, Prague (Czechoslovakia)
and
E. SMOLKOVÁ
Department of Analytical Chemistry, Faculty of Sciences, Charles University, Prague (Czechoslovakia)

SUMMARY

Preparations of higher fatty acids and amino acids of 10–60 mCi/milliatom C specific activity, obtained from algae grown in a $^{14}CO_2$ atmosphere, were analysed by gas–liquid chromatography and radiochemical and chemical purities, content and specific activity of isolated compounds and mixtures were determined. Special attention was paid to the distribution of carbon-14 in the individual carbon positions in the fatty acids obtained by common labelling procedures. The results enable the choice of optimal growth conditions for the preparation of uniformly labelled compounds. In addition to mass detection, radioactivity was simultaneously detected by a gas-flow proportional counter, with digital and rate-meter outputs, after combustion on CuO of the split stream.

INTRODUCTION

In the preparation of ^{14}C-labelled compounds, as well as in their biochemical applications, gas–liquid chromatography (GLC) is generally used as an efficient analytical tool and has been described in the literature many times. Biosynthesis, still an important labelling method of various compounds of biochemical interest, provides substances the properties of which ought to be carefully checked with respect to the method of preparation and the applications intended. The following are possible GLC applications: identification of individual substances, control of radiochemical (and eventually also chemical) purity of isolated compounds, determination of content, and specific activity of preparations and compounds in mixtures.

In this paper are shown some applications of radio-gas chromatography for the analysis of biochemically ^{14}C-labelled compounds obtained from algae grown in a $^{14}CO_2$ atmosphere. These substances are mostly non-volatile and therefore use of a derivatization method is necessary. The problem of identification of higher G-^{14}C-labelled fatty acids, their radiochemical purity and analysis of ^{14}C-labelled pyrimidine bases have already been discussed elsewhere[1–3]. Now attention is directed to the estimation of the specific activity of G-^{14}C-labelled fatty acids, to the possibility of the

analysis of G-^{14}C-labelled amino acids, and to the possibility of the determination of isotopic abundance of carbon-14 in individual carbon positions in the molecule.

EXPERIMENTAL

For the purpose of analysis of algae lipids, the control of radiochemical purity of ^{14}C-labelled fatty acids (prepared from *Chlorella vulgaris* radioactive alga[4] of about 1 mCi/mg activity), and the determination of specific activity, we used a technique usually applied to the analysis of more complex mixtures of fatty acids, *viz.* ^{14}C radio-gas chromatography on two stationary phases, butane-1,4-diol succinate and Apiezon L. The uniformity of labelling was also controlled by chemical α-oxidation[5] and subsequent GLC of the degraded shorter-chain acids. G-^{14}C-labelled amino acids were derivatized to the commonly used trifluoroacetylated *n*-butyl esters[6] and then chromatographed on an OV-225 column.

Microorganism

A pure culture of the alga *Chlorella vulgaris* No. 82, from the collection of autotrophic organisms of the Czechoslovak Academy of Sciences, Prague, cultivated as reported by Baslerová and Dvořáková[7], was grown under conditions used for cultivation in an atmosphere of $^{14}CO_2$ (ref. 4).

Chemicals

Fatty acid methyl esters GLC 90 and GLC 110 were supplied by Supelco (Bellafonte, Pa., U.S.A.); K 108 and palmitic acid methyl ester by Applied Science Labs. (State College, Pa., U.S.A.); heptadecanoic acid methyl ester by Merck (Darmstadt, G.F.R.); boron trifluoride–ethyl ether complex by Serva (Heidelberg, G.F.R.); rectified methanol, p.a., by Lachema (Brno, Czechoslovakia); glycine, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, serine, threonine, aspartic and glutamic acids, lysine, arginine, proline and hystidine by Calbiochem (Los Angeles, Calif., U.S.A.); trifluoroacetic anhydride (20% solution in methylene chloride) by Serva; rectified *n*-butanol, p.a., by Lachema; rectified hexane, p.a., by Lachema; methylene chloride, p.a., by Merck; [G-^{14}C]palmitic acid (900 mCi/mmole), [1-^{14}C]oleic acid (59.7 mCi/mmole), and [1-^{14}C]linolenic acid (59.7 mCi/mmole) by Radiochemical Centre (Amersham, Great Britain); [G-^{14}C]palmitic acid (160 and 268 mCi/mmole), ^{14}C-labelled Chlorella lipid extract and G-^{14}C-labelled amino acid mixture by ÚVVVR (Prague, Czechoslovakia).

Preparation of fatty acid methyl esters

The extract of ^{14}C-labelled Chlorella lipids was dried and trans-esterified with methanolic hydrochloric acid. After the extraction of methyl esters by hexane, they were purified by vacuum sublimation. Free fatty acids were methylated by a methanolic solution of boron trifluoride.

Preparation of N-trifluoroacetylated amino acid n-butyl esters

An aqueous aliquot containing 5–50 μCi of protein hydrolysate or amino acid mixture was evaporated in a special 2-ml reaction vial on a vacuum rotary evaporator to dryness at room temperature, the residual water removed by evaporation with 0.2

ml methylene chloride and the amino acids then esterified with 0.2 ml 1.5 *N* methanolic hydrochloric acid for 30 min at room temperature and inter-esterified with 0.2 ml of 1.5 *N* butanolic hydrochloric acid for 150 min at 90° in a closed vial; the acylation was then accomplished by reacting dried esters with 0.2 ml of 20% trifluoroacetic anhydride in methylene chloride overnight at room temperature, again in a closed vial.

Degradation procedure for palmitic acid

To 100 μCi [G-^{14}C]palmitic acid in a thick-walled vial were added 50 mg $KMnO_4$ in 0.1 ml dry acetone and the closed vial was left to stand overnight at 56°. From the reaction mixture fatty acids were extracted and esterified by a boron trifluoride–methanol mixture.

Gas–liquid chromatography

GLC of fatty acid methyl esters was carried out on a 1 m × 4 mm I.D. column filled with 15% Apiezon L on Chromaton N-AW-HMDS, 0.16–0.20 mm, (Lachema) and on a 2 m × 4 mm I.D. column filled with 15% butane-1,4-diol succinate (BDS) on Chromaton N-AW-HMDS, 0.16–0.20 mm, in a Packard Model 7409 chromatograph (Packard, Downers Grove, Ill., U.S.A.).

Trifluoroacetylated amino acid butyl esters were analysed on a 2 m × 2 mm I.D. column filled with 1% silicone phase OV-225 on Chromosorb G (HP), AW-DMCS, 100–120 mesh, using a temperature programme with initial temperature 70°, programme-rate 5°/min, and final temperature 220° held for 30 min.

The simultaneous determination of radioactivity was accomplished after combustion over CuO of substances in the split carrier gas stream in a Packard Model 325 Tricarb furnace at 650°, with water absorption in a $MgClO_4$ tube and mixing with methane (1:1) by means of a 2-ml gas flow proportional counter. The counting unit consisted of a Berthold LB 2007 preamplifier, an LB 242K high-voltage supplier, an amplifier, a dual-channel rate-meter, an LB Scaler-timer 2520, a Kienzle D44 printer and a Goerz RE 520 dual-pen recorder.

RESULTS AND DISCUSSION

In *Chlorella vulgaris* lipids we have already identified the higher fatty acids from myristic to arachidic acid and their unsaturated homologues (investigated earlier by Mangold and Schlenk[8], Schlenk *et al.*[9] and Vereščagin and Kljačko-Gurvič[10]) and found peak overlapping in some cases. With the major constituents (they were all isolated) only oleic and palmitolinolenic acids overlapped; we expected also overlapping of minor acids, *e.g.* of palmitooleic and anteiso-heptadecanoic acids on a polar phase. We have tried to isolate anteiso-heptadecanoic acid by GLC on two stationary phases and have found that in this case there were also two components present, *viz.* palmitooleic and isoheptadecanoic acids on a polar phase, and heptadecanoic and isoheptadecanoic acids on a non-polar phase, thus simulating anteiso-heptadecenoic acid. A small amount (about 0.4%) of isoheptadecanoic acid can be seen in Fig. 1, demonstrating a radiochemical purity check of [G-^{14}C]palmitic acid. The absence of anteiso-derivatives was also confirmed by hydrogenation (hydrogen over an Adams catalyst). Further work is necessary in the field of identification of minor acids which may involve some difficult separations.

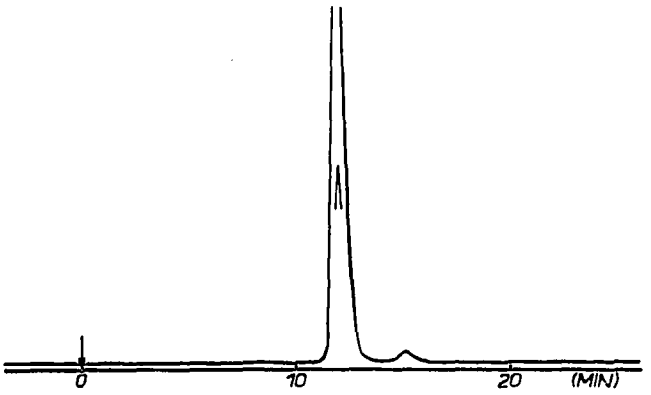

Fig. 1. Radio-gas chromatogram of 1 μCi [G-^{14}C]palmitic acid containing 0.4% of [G-^{14}C]isoheptadecanoic acid on an Apiezon L column at 230° and with an argon flow-rate of 60 ml/min for radiochemical purity control.

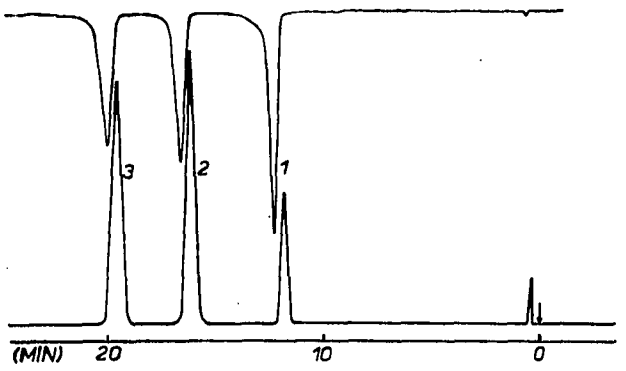

Fig. 2. Demonstration of specific activity determination of a mixture of [^{14}C]palmitic acid, 160 mCi/mmole (peak No. 1), [1-^{14}C]oleic and [1-^{14}C]linolenic acids, 59.7 mCi/mmole (peak Nos. 2 and 3, respectively), on a BDS column at 195° and with an argon carrier gas flow-rate of 40 ml/min. Upper curve: activity trace, 1000 imp./sec, full scale; lower curve: trace of mass detected by FID, sensitivity $1 \cdot 10^{-10}$ A.

The problem of specific activity determination was successfully solved by simultaneous detection of mass and activity. For the absolute specific activity determination it is appropriate to use an active internal standard (Fig. 2); the coefficient of variation of the determination obtained without use of electronic integrators was about $\pm 5\%$ and the method can be particularly recommended when using digital outputs. As can be expected from the biosynthetic viewpoint, the specific activities of Chlorella fatty acids originating from the same cultivation (at four- to fivefold growth of the alga) are not equal (Figs. 3 and 4). It is particularly obvious from Fig. 4 that the specific activity of unsaturated acids is substantially lower than that of palmitic acid, this being caused by fatty acid metabolism in the alga[11]. After hydrogenation the specific activities of the obtained palmitic and stearic acids are practically the same (Fig. 5). The problem of non-homogeneous labelling has been already described for "uniformly labelled" oleic and linoleic acid (with oxidative cleavage application)[12]

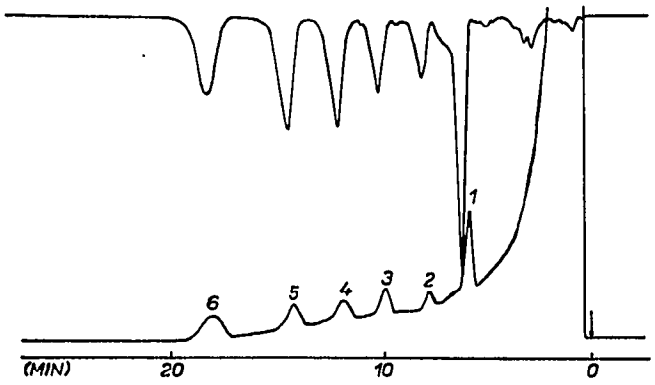

Fig. 3. Radio-gas chromatogram of the following ^{14}C-labelled Chlorella fatty acid methyl esters on a BDS column: 1 = 16:0, 2 = 16:2, 3 = 16:3, 4 = 18:1, 5 = 18:2, and 6 = 18:3.

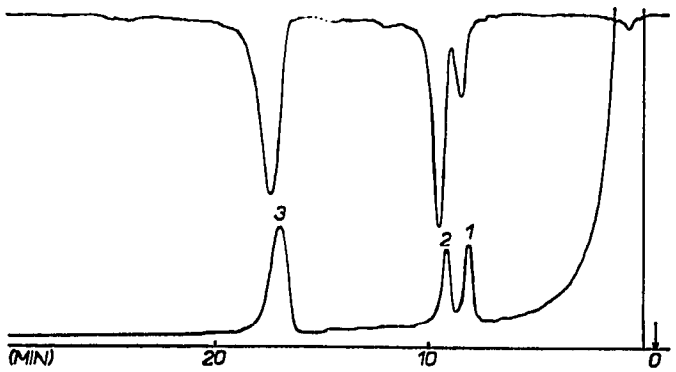

Fig. 4. Demonstration of differences in specific activity of the following ^{14}C-labelled Chlorella fatty acids on an Apiezon L column: 1 = 16:1, 16:2, and 16:3; 2 = 16:0 and 3 = 18:1, 18:2, and 18:3

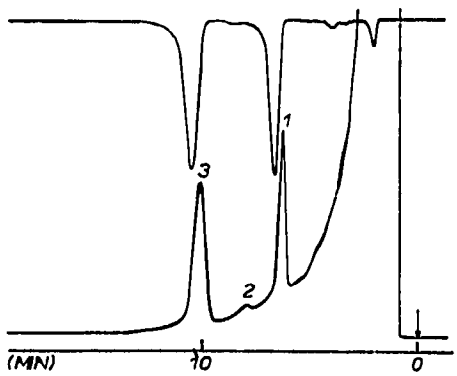

Fig. 5. Radio-gas chromatogram of the following hydrogenated ^{14}C-labelled Chlorella fatty acids on a BDS column: 1 = 16:0, 2 = 17:0, 3 = 18:0. Upper curve: activity trace; lower curve: mass trace.

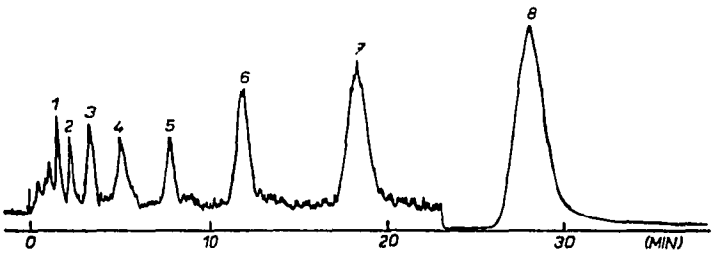

Fig. 6. Radio-gas chromatography of the following [G-^{14}C]palmitic acid degradation products on an Apiezon L column: 1 = 9:0, 2 = 10:0, 3 = 11:0, 4 = 12:0, 5 = 13:0, 6 = 14:0, 7 = 15:0, and 8 = 16:0 (ten times lower sensitivity).

and is actually known in studies of the carbon-14 pathway in growing algae (*e.g.* refs 13 and 14). Therefore we tried by means of the excellent degradation method used by James *et al.*[5] for [1-^{14}C]stearic acid to assay the distribution of carbon-14 in the palmitic acid molecule (Fig. 6). Our first results show that the specific activity of carbon atoms at the carboxyl end is higher than that of carbon atoms at the methyl end; this suggests the possibility of calculation of carbon-14 isotopic abundance in the ndividual carbon positions, *e.g.* for palmitic acid by the simple equations

$$C_1 = (C_{16})—(C_{15})$$
$$C_2 = (C_{15})—(C_{14}), \text{ etc.}$$

where C_1, C_2 are the isotopic abundances given by the specific activity of the carbon atoms in positions 1 and 2, respectively (mCi/milliatom) and (C_{16}), (C_{15}), (C_{14}) are the specific activities of palmitic, pentadecanoic and myristic acids obtained on degradation.

The differences in specific activity observed give evidence for the non-uniform labelling of fatty acids and suggest the need to apply longer cultivation times so that more generations of growing microorganisms are produced.

For amino acid analysis GLC has already been used many times in submicrogram quantities[15]. Del Favero *et al.*[16] also analysed ^{14}C-labelled amino acids but used an inactive carrier. The application of GLC in this field is complicated by the derivatization step and great differences in the structures of the molecules. The chosen trifluoroacetylated *n*-butyl esters seem to be generally applied. As stationary phase the silicone OV-225 recommended by Metz *et al.*[17] was used; although the separation was not satisfactory for all of the amino acids of the assayed mixture, the chromatogram in Fig. 7 well characterizes the composition of the mixture.

The amino acid derivatives used contain nitrogen and fluorine and can form electronegative compounds during combustion which influence gas-flow proportional counting by pseudoactivity and quenching[18]. With regard to this possibility we took a tenfold more amount of an amino acid than usual, but no influence on the counting of external radiation from a ^{60}Co-source at low counting rate was found (Fig. 8). Therefore we intend to use radio-gas chromatography also for ^{14}C-labelled amino acids of higher specific activities (10–60 mCi/milliatom C) as the method of radiochemical purity control.

Radio-gas chromatography as a method for assaying the quality of labelled compounds has several advantages over other chromatographic methods: higher

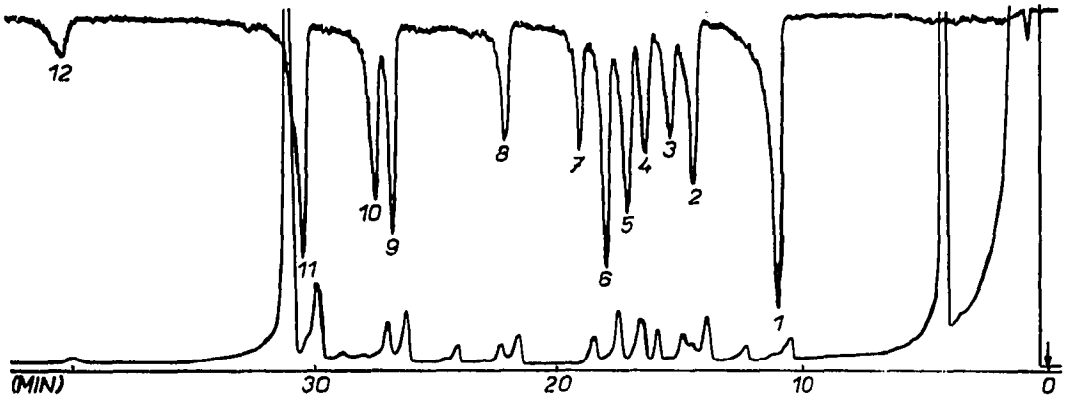

Fig. 7. Example of application of radio-gas chromatography to the analysis of the following ^{14}C-labelled amino acids: 1 = unknown, 2 = alanine, 3 = valine, 4 = isoleucine, 5 = glycine + threonine, 6 = leucine, 7 = serine, 8 = proline, 9 = aspartic acid, 10 = phenylalanine, 11 = glutamic acid + lysine, and 12 = arginine.

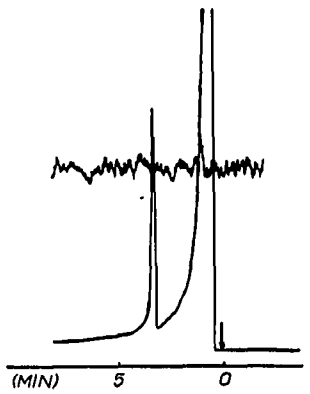

Fig. 8. Assay of the influence of amino acid derivatives on activity counting (external ^{60}Co radiation source and 3 μg of derivatized alanine were applied).

sensitivity for simultaneous mass and activity detections, possibility of easy determination of radiochemical impurities and specific activity, relatively short time of analysis and possibility of detecting some chemical (non-radioactive) impurities. There are naturally some limitations: the method cannot be used for every kind of labelled compound, and sometimes the derivatization is complicated, so that another chromatographic method is preferred.

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