RECENT DEVELOPMENTS IN THE CHROMATOGRAPHIC ANALYSIS AND PURIFICATION OF RADIOACTIVELY LABELLED LIPIDS.

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Received on September 27, 1973

SUMMARY

Methods for the analysis and purification of lipophilic substances labelled with ^{3}H or ^{14}C are outlined. The necessity of using various techniques based on complementary principles is emphasized. In addition, new developments in instrumentation for the quantitative analysis and preparative isolation of labelled substances are described.

INTRODUCTION

Chromatographic techniques applicable to the fractionation of lipophilic compounds became available long after methods for the separation of water-soluble substances had been developed. Both thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) were evolved primarily with an aim to analyze lipids.

Originally, thin-layer chromatography was used exclusively as an adsorption method, but soon other principles of chromatography were adapted to this technique and it became a highly expedient method also in laboratories engaged in work on water-soluble substances. Within a few years, the enormous resolving power of gas-liquid chromatography was recognized, and soon this technique was also rapidly adapted to the analysis of water-soluble substances.

Thin-layer chromatography and gas-liquid chromatography are the methods of choice for assessing both the chemical and the radiochemical purity of labelled aliphatic and alicyclic lipids.

Both thin-layer chromatography and gas-liquid chromatography are not only analytical methods, but their use as preparative tools is amply justified especially in work with radioactively labelled compounds where relatively small samples have to be purified by the most rigorous means.

The present review deals with new developments in technique and instrumentation for the chromatographic analysis and purification of labelled lipophilic substances. Some anxillary methods are discussed briefly. Only the literature from 1965 on is considered, as this topic has been treated comprehensively in monographs on ^{14}C- and ^{3}H- labelled compounds[1,2] as well as in chapters on thin-layer chromatography[3,4] and gas-liquid chromatography[5,6].

CHROMATOGRAPHY AS AN ANALYTICAL TOOL

Chromatographic methods are widely accepted for the analysis of labelled substances. It should, however, be realized that the complete resolution of a complex mixture can, as a rule, be accomplished only by the combination of several techniques based on complementary principles. It is advantageous to apply first a method that yields few but well defined fractions, and then to resolve each fraction by more discriminating techniques. For example, complex mixtures of lipids can be resolved completely by a stepwise fractionation, employing, first, adsorption chromatography, then, argentation chromatography and

finally reversed-phase partition chromatography or gas-liquid chromatography. Each of these steps effects a distinct pattern of fractionation, and the various principles of chromatography complement each other.

Thin-layer chromatography is popular owing to its simplicity, speed and versatility. The procedures used commonly in the evaluation of thin-layer chromatograms include elution analysis[3,4], strip scanning[7], autoradiography[3,4] including fluoregraphy[8] and zone analysis[9]. A spark chamber has also been devised for the detection of radioactive substances on chromatoplates[10].

Elution analysis can be quite reliable, but it is tedious. Strip scanning is suitable for qualitative work, however, this method is rather insensitive and often unsatisfactory for yuantitative analysis. Autoradiography, though sensitive, requires much time and quantitative results are difficult to achieve by this technique; at least when applied in conjunction with adsorption chromatography. Nevertheless, autoradiography of thin-layer chromatograms is considered indispensable as an adjunct to other methods because it permits visualizing all components separated, yields permanent records, and is inexpensive. -- Among the conventional methods, zonal profile scanning provides by far the highest sensitivity, best resolution and most precise quantitation in the assay of thin-layer chromatograms, however, this method is rather elaborate and costly.

During the past years, considerable progress has been made in quantitative adsorption thin-layer chromatography by the use of

vapour-phase detectors[11,12,13,14,15]. A scanner[16,17] described recently permits the detection and quantitative analysis of organic substances on thin-layer chromatograms.

Substances to be analyzed are chromatographed in glass or quartz tubes that are coated internally with a thin layer of silica gel or mixture of silica gel and cupric oxide. After removing the developing solvent, the tubular thin-layer chromatograms are assayed in the "TLC-Scanner"*. In this instrument the substances in the various chromatographic zones are vaporized consecutively, either by pyrolysis or by combustion, and the resulting products are monitored by a flame ionization detector or a thermal conductivity detector. Scanning is done by moving the tubular thin-layer chromatogram gradually through a ring-shaped furnace kept at 600-800°C. A carrier gas, nitrogen or helium, flowing through the tubular thin-layer chromatogram delivers the products of pyrolysis or combustion to the mass detector.

The flow diagram of a scanner equipped with a flame ionization detector, is shown in Fig. 1. The products of pyrolysis from the silica gel layer are monitored by the flame ionization detector, either directly, or after their conversion to methane. Similarly, the carbon dioxide formed by *in situ* combustion of the substances on the layer consisting of silica gel and cupric oxide is reduced to methane and monitored. If a thermal conductivity detector is used, the products of combustion are passed through a water trap and the carbon dioxide in the effluent is monitored.

*) Packard-Becker B.V., Delft, The Netherlands.

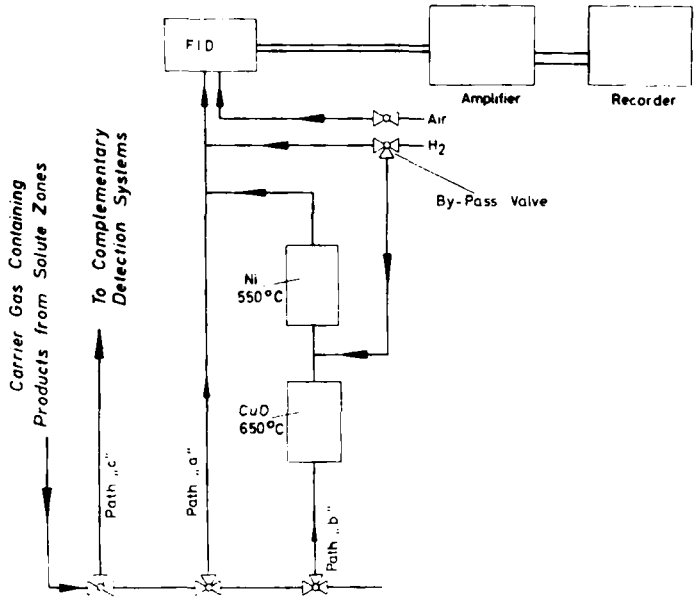

Fig. 1. Flow diagram of the "TLC-Scanner"[17]

This device can be used for the radio-assay of thin-layer chromatograms if a proportional flow counter is employed for monitoring radioactivity[15]. However, such arrangements are not very satisfactory, when low activities are to be measured.

The radioactivity in various zones of a tubular thin-layer chromatogram is determined accurately and conveniently when the TLC-Scanner is coupled with a collection device[18] as shown in Fig. 2.

The radioactively labelled compounds in tubular thin-layer chromatograms are quantitatively converted to $^{14}CO_2$ and/or $^{3}H_2O$, either by pyrolysis on layers of silica gel, and subsequent

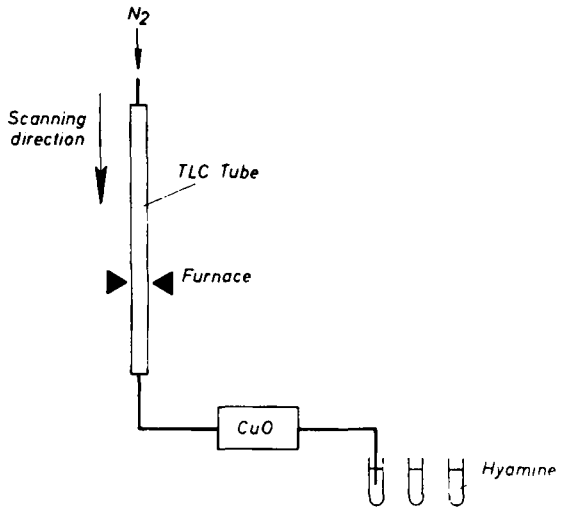

Fig. 2. Application of the "TLC-Scanner" in the quantitative analysis of labelled substances[18].

combustion over cupric oxide, or by combustion in situ on layers consisting of silica gel and cupic oxide. The products of combustion are collected serially in fraction tubes using a methanolic solution of Hyamine** as the trapping liquid. A three-way valve in the line of gaseous effluent emerging from the TLC-Scanner permits serial collection of fractions with negligible loss of radioactive material. The content of each fraction tube is transferred to a counting vial using a scintillator solution and the radioactivity is measured in a liquid scintillation spectrometer. The recovery of $^{14}CO_2$ and $^{3}H_2O$ is over 95 %.

**) Packard Instruments, Downers Grove, Ill., U. S. A.

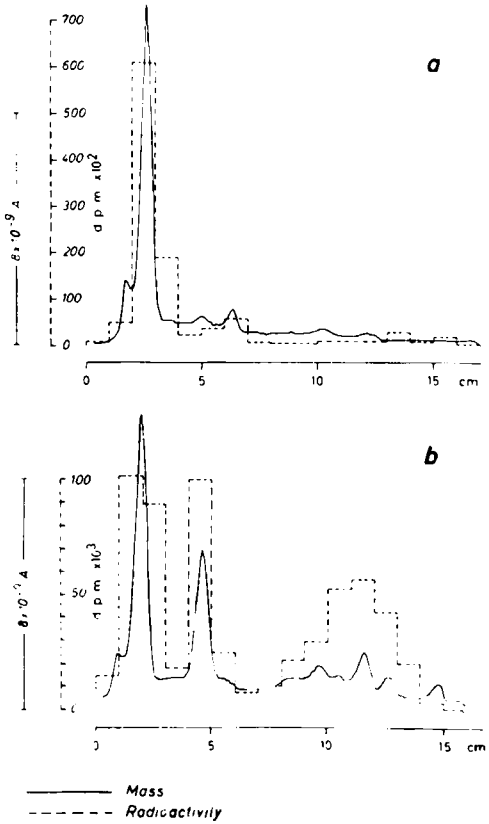

Fig. 3. Distribution of radioactivity and mass in the various zones of tubular thin-layer chromatograms of a, [1-^{14}C] dihexadecylglycerol, purified by a single crystallization, and b, other liquor from the above crystallization[18].

Fig. 3 shows typical applications of the TLC-Scanner in the analysis of labelled substances. A sample of α-[1-^{14}C]hexadecyl

glycerol, specific activity 7.2 uCi/mg, was purified by a single crystallization from ethanol. Both the crystalline product and the mother liquor were chromatographed in tubes coated with silica gel containing 15 % of cupric oxide; hexane:diethyl ether, 70:30, v/v, served as developing solvent. After removal of solvents, the tubes were scanned for both radioactivity and mass. The scans indicate that the product obtained by crystallization still contains about 30 % impurities.

This example shows that both radioactivity and mass in various zones of a thin-layer chromatogram can be conveniently monitored. Simultaneous recording of radioactivity and mass is accomplished by means of a stream splitter. Absolute determination of mass can be carried out by the use of an internal standard or by the injection of a known amount of carbon dioxide in the mass detection system[15]. Thus, both radiochemical and chemical purity of labelled compounds can be assessed and their specific activities determined. These are distinct advantages over the methods involving autoradiography of chromatoplates or liquid scintillation counting of scraped zones.

In gas-liquid chromatography two types of methods are generally used for detecting radioactively labelled substances. The effluent can be continuously monitored in the vapour phase using flow-through radiation detectors[19], such as ionization chambers, proportional counters and scintillation counters. Alternatively, the effluent can be collected serially or continuously in a trapping liquid and the radioactivity measured in a spectrometer using either counting vials or a flow cell. It is generally advantageous to either combust the radioactive substances to

$^{14}CO_2$ and $^{3}H_2O$ or to convert them into $^{14}CO_2$ and $^{3}H_2$ before the effluent carrier gas is delivered to the detector[20,21] or to the trapping liquid.

Some common drawbacks with most of the flow-through radiation detectors are their inability to monitor low activities, their poor resolution and their undesirable memory effects. In general, the systems involving collection of the radioactive effluent in a trapping liquid are superior for quantitative detection of substances, especially of those with very low specific activities.

Recently, several devices have been described which overcome some of the difficulties encountered in radio-gas chromatography.

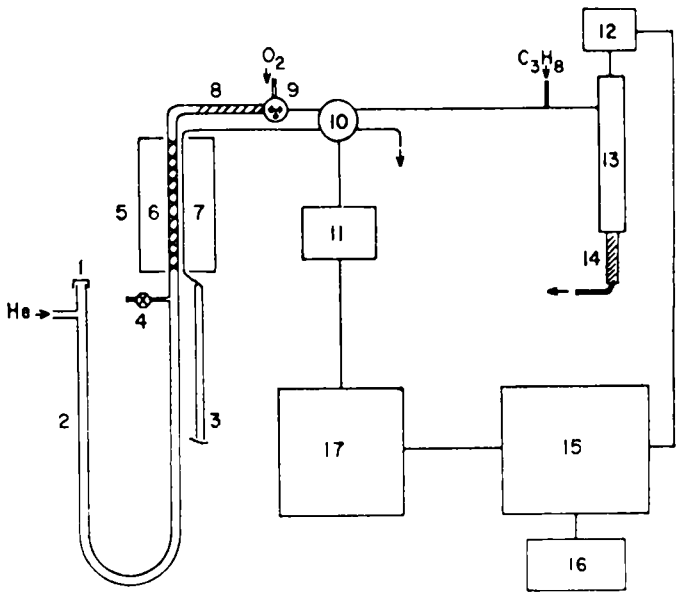

Fig. 4. A combustion-flow counting system for radio-gas chromatography[22].

1, Injection port; 2, Column; 3, Reference column; 4, Solvent venting valve; 5, Combustion furnace; 6, Copper shot; 7, Stainless steel tubing; 8, Packing of anhydrous magnesium perchlorate; 9, Three-way valve; 10, Microthermistor detector; 11, Detector control unit; 12, Amplifier; 13, Counter tube; 14, Trap for $^{14}CO_2$; 15, Digital integrator and power supply for rate meter; 16, Digital printer; 17, Dual pen recorder.

A continuous monitoring system[22] which uses a proportional flow counter is shown in Fig. 4. The total effluent from the gas chromatographic column is passed over cupric oxide at 800°C and the carbon dioxide formed is measured by a microthermistor. The radioactivity of the carbon dioxide, after mixing with propane, is measured in the proportional flow counter. Both mass detector and proportional counter operate without contamination, at ambient temperature, and 200–100000 dpm of ^{14}C can be detected in a given peak. Dynamic efficiency of the radioactivity detector at an effluent flow rate of 52 ml/min and a counter volume of 27 ml is about 40 %.

A specially designed flame ionization detector has been used to monitor the mass of radioactive components in the effluent of a gas chromatographic column[23]. From the detector, the combustion products, H_2O and CO_2, can be recovered quantitatively. The carbon dioxide is trapped in special absorbers which are connected with the detector. Subsequent measurement of ^{14}C activity in the absorbing liquid is carried out by heterogeneous

scintillation counting. Using suitable absorbers, other isotopes, such as ^{3}H and ^{35}S can be absorbed in the form of $^{3}H_2O$ and $^{35}SO_2$ and measured.

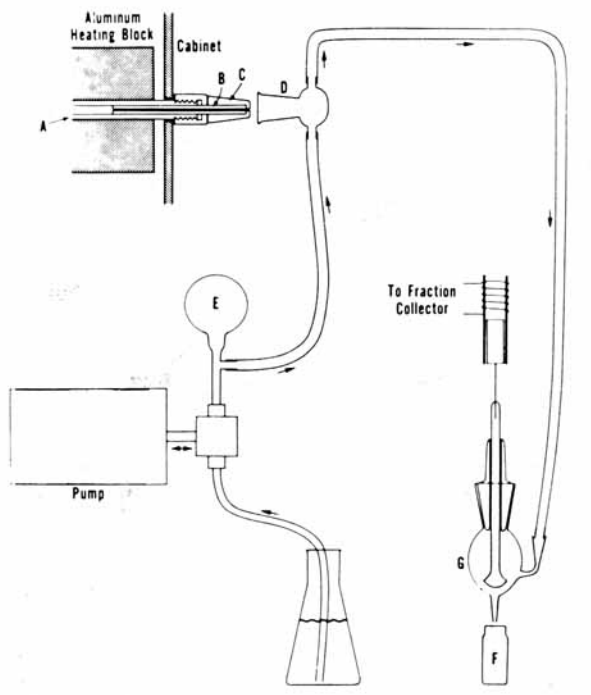

Fig. 5, An automated system for condensation of the effluent from gas chromatograph in a scintillation solvent[24]. A, Stainless steel collection tube; B, Aluminium tube; C, Teflon sleeve; D, Glass tee joint; E, Damping reservoir; F, Counting vials; G, Solenoid operated glass valve.

In a system shown in Fig. 5, ^{14}C and ^{3}H in compounds separated by gas chromatography can be detected by automated serial collection of fractions with subsequent scintillation counting[24].

A scintillation liquid is used for scrubbing the effluent. Samples having activities around 1-10 nCi/mg can be easily detected in this system. The counting efficiencies are of the same magnitude as in conventional liquid scintillation counting.

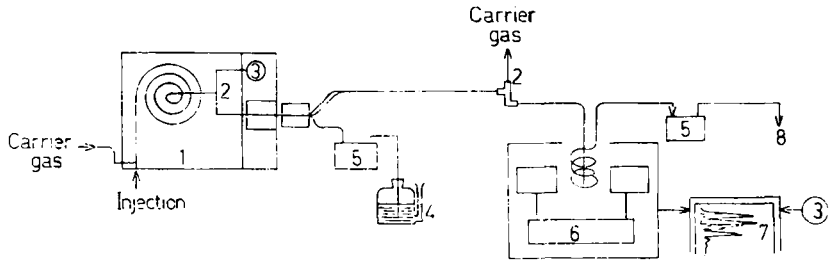

Fig. 6. A system for continuous collection and detection of radioactive effluents from a gas chromatograph[25].
1, Gas chromatograph; 2, Splitter; 3, Flame ionization detector; 4, Container with scintillation liquid; 5, Peristaltic pump; 6, Liquid scintillation spectrometer; 7, Two-pen recorder; 8, Fraction collector.

Another system[25] described recently for continuous collection and monitoring of radioactive effluent from a gas chromatograph is shown in Fig. 6. The effluent from the column is continuously dissolved in a stream of scintillation liquid. After most of the carrier gas has been separated, the liquid is passed through a helical flow cell which is inserted in a liquid scintillation counter. For accurate determination of radioactivity, fractions of the scintillation liquid are collected and measured in a liquid scintillation spectrometer. This system ensures good resolution and high sensitivity, and it is suitable for compounds

that are labelled with two different isotopes. The counting efficiency for ^{14}C is 85 % and for ^{3}H, 17 %.

Collection systems, in which the hot gaseous effluent is contacted with the cold trapping liquid, have an inherent disadvantage due to occasional formation of aerosols that are not trapped completely. Such a drawback is overcome, as mentioned earlier, if the effluent is combusted over cupric oxide and the resulting mixture of radioactive carbon dioxide and water is trapped serially in a suitable liquid, such as Hyamine hydroxide[26] or a solution of Hyamine hydroxide in a scintillation liquid[27]. The products of combustion are recovered quantitatively, and their radioactivity is measured.

Liquid chromatography in columns has received a good deal of attention during recent years despite of severe limitations in the detection systems and highly expensive instrumentation.

Several devices have been reported recently for monitoring in spite of radioactive effluents from liquid chromatographs. These are based on liquid scintillation counting in either heterogeneous [28,29,30] or homogeneous[30,31] systems.

A homogeneous system[30] is shown schematically in Fig. 7. The column effluent emerging from the UV-detector is mixed with the scintillation liquid and the mixture is passed through an empty helical flow cell located inside a liquid scintillation spectrometer. The counting efficiency is about 80 % for ^{14}C and 30 % for ^{3}H. In a similar setup, heterogeneous counting can be carried out by passing the effluent through an U-shaped flow cell filled with glass scintillation beads. However, the counting

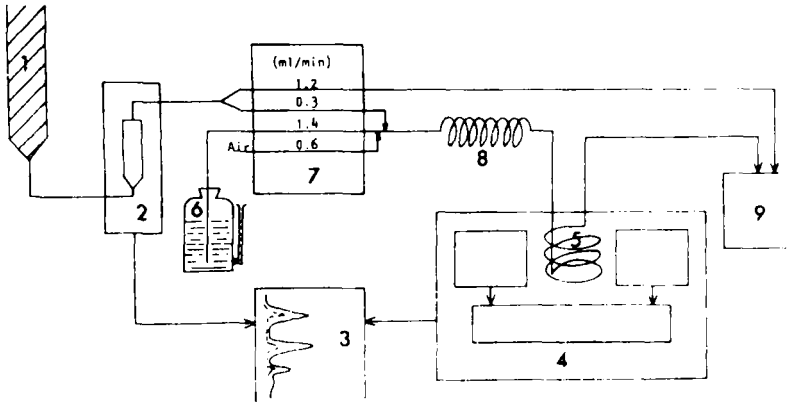

Fig. 7. A device for continuous monitoring of radioactive effluents from a liquid chromatograph[30].

1, Column; 2, UV-Detector; 3, Two-pen recorder; 4, Liquid scintillation spectrometer; 5, Helical flow cell; 6, Scintillator solution reservoir; 7, Proportionating pump; 8, Mixing spiral; 9, Fraction collector.

efficiencies are rather poor (17 % for ^{14}C and 0.2 % for ^{3}H).

In view of the high sensitivity and excellent accuracy of the new devices for monitoring radioactivity in the various chromatographic systems, one should consider analyzing "cold" mixtures by the isotopic derivative technique, that is, after labelling their constituents with a radioactive reagent[32].

CHROMATOGRAPHY AS A PREPARATIVE TOOL

Methods used in the analysis of radioactively labelled

substances are easily scaled up to permit the isolation of pure compounds from the products of a chemical synthesis or from a mixture of natural substances.

In synthetic preparations, the potential impurities can be surmized from the route of synthesis. As a rule, the starting material, the various intermediates, and the final product of a synthesis differ in regard to their functional groups and polarities. These substances can usually be resolved by adsorption chromatography, and the desired compound is isolated easily.

In preparations obtained by biosynthesis, impurities might comprise a wide range of substances, including homologous and vinylogous compounds as well as positional and geometrical isomers, which cannot be resolved completely by adsorption chromatography or by any other single process. Complementary principles of fractionation must therefore be exploited successively for isolating the desired compound. As in the analysis of such complex mixtures, adsorption chromatography should be followed by argentation chromatography and reversed-phase partition chromatography or gas-liquid chromatography. This sequence is not only logical, as it leads from the least discerning to the most discriminating principle of chromatography, but it is also practical in regard to the size of the sample that can be fractionated conveniently with the methods based on these principles.

Adsorption chromatography on layers of silica gel effects more satisfactory separations than column chromatography using the same adsorbent. Fractionation is most efficiently carried out

in two steps[33]: First, the major constituents are isolated by chromatography on adsorbent layers, 0.5 to 1 mm thick. Fractions that are not completely resolved on such rather "thick" layers contain the minor components in a ratio which is more favorable for an efficient fractionation at the subsequent stage. These fractions are resolved by chromatography on layers, 0.25 mm thick, and the minor components are thus isolated.

Substances that are not separated satisfactorily can sometimes be well resolved after converting some of them into derivatives.

The techniques described above are also applicable to argentation chromatography.

Reversed-phase partition chromatography is best carried out using columns [34] as the capacity of layers impregnated with a non-polar stationary phase is very low. The separations that can be accomplished by means of reversed-phase partition chromatography can be carried out much faster by gas-liquid chromatography and, therefore, reversed-phase partition chromatography in columns is seldom being used. In the authors opinion, this is not justified because the latter technique permits the fractionation of much larger samples. Moreover, there is no danger of decomposition due to heat.

Gas-liquid chromatography is especially useful for the isolation of the components of a homologous and vinylogous series[35]. Prior to its fractionation by gas chromatography, the samples should be purified by adsorption chromatography to ascertain that they are free of extraneous substances. Fractions isolated by gas chromatography can be freed of stationary phase ("bleed")

by adsorption chromatography. Especially with unsaturated substances it is worthwhile to check whether the material isolated has undergone chemical alteration such as isomerization and cyclization.

It is mandatory that both the chemical and radiochemical purity of substances isolated by chromatography be "cross-checked" by analyzing them with complementary techniques.

HOW PURE ARE "CHROMATOGRAPHICALLY PURE" SUBSTANCES?

The designation "chromatographically pure" has largely replaced other criteria of purity and is often considered to be the ultimate proof of purity. We feel that homogeneity in a particular system of chromatography is not in itself sufficient to verify the identity and purity of a compound.

The following example demonstrates that a substance that behaves as an entity in one or two chromatographic systems is not necessarily a pure compound. The methyl esters of uniformly labelled oleic, linoleic, and linolenic acids are analyzed by adsorption thin-layer chromatography, argentation thin-layer chromatography, reversed-phase partition thin-layer chromatography and by gas-liquid chromatography. Figure 8 shows autoradiographs of the chromatograms.

Adsorption chromatography (Fig. 8, a) verifies that each of the three esters is pure in regard to lipid class. However, argentation chromatography (Fig. 8, b) shows the presence of small amounts of configurational isomers in each sample of the supposedly all-<u>cis</u> compound. Reversed-phase partition

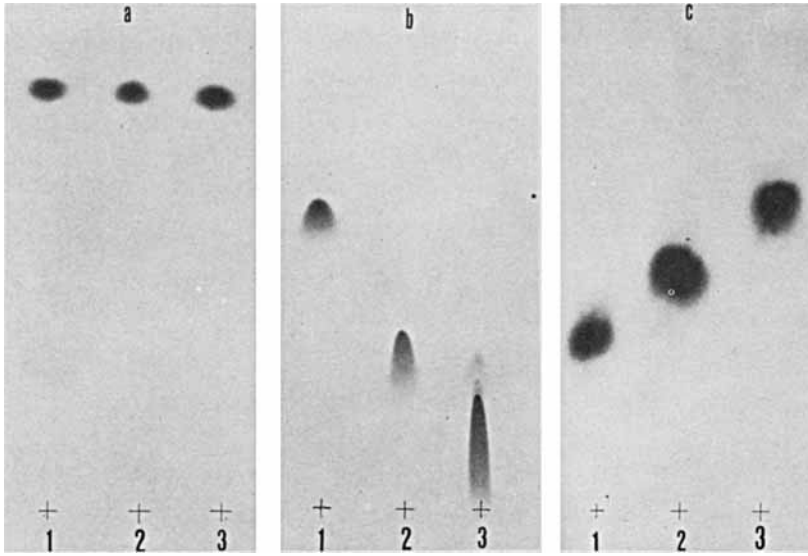

Fig. 8. Autoradiographs of chromatograms of 1, Methyl U-^{14}C oleate; 2, Methyl U-^{14}C linoleate, and 3, Methyl U-^{14}C linolenate. The esters were fractionated by a, Adsorption-TLC; b, Argentation-TLC, and c, Reversed-phase partition-PC[36].

chromatography (Fig. 8, c) indicates that the preparations of methyl esters are pure in regard to chain length and number of double bonds.

Figure 9 shows the autoradiograph of a chromatogram of methyl U-^{14}C linolenate on an adsorbent layer part of which was impregnated with silver nitrate[37]. Obviously, this methyl ester does not contain the corresponding fatty acid or other classes of compounds, but it is not uniform with regard to the configuration of double bonds.

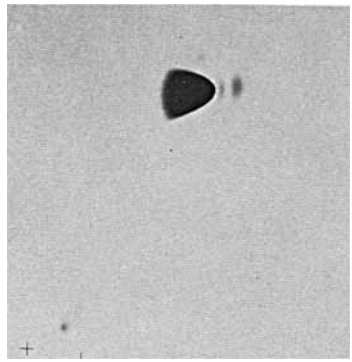

Fig. 9. Two-dimensional TLC of methyl U-^{14}C linolenate on an adsorbent layer, part of which is impregnated with silver nitrate.

Gas chromatographic analyses of methyl U-^{14}C oleate and methyl U-^{14}C linoleate indicates that these samples are pure compounds. However, a gas chromatogram of methyl U-^{14}C linolenate shows the presence of an impurity; the record is given in Fig. 10. This contaminant has been isolated by gas chromatography and found to be radioactive, but it could not be identified.

It is thus evident that for the complete analysis or purification of a sample it is essential that various separation methods be employed consecutively. The term "chromatographically pure" is meaningful only if various techniques based on complementary principles of chromatography do not effect any further fractionation of a substance. Nevertheless, chromatographically pure substances are not necessarily pure individual compounds, as can be demonstrated by classical methods of analysis. Physical constants and chemical reactions are still indispensable

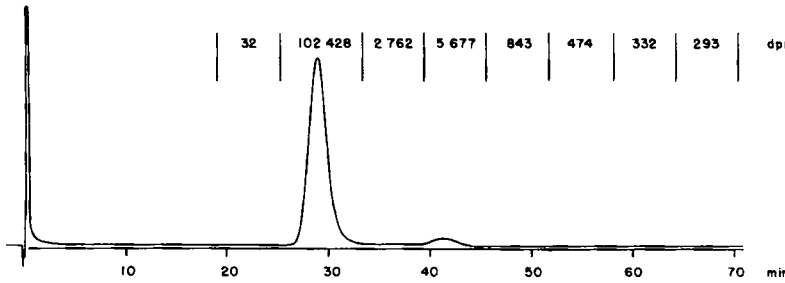

Fig. 10. Gas chromatogram of methyl U-^{14}C linolenate. The distribution of radioactivity in fractions of the eluate is indicated[36].

for the characterization and identification of substances isolated by chromatography.

STABILITY OF LABELLED COMPOUNDS

It is well known that radioactively labelled organic compounds, including lipids[38], suffer radiation-induced decomposition during storage[1,2,39,40]. This effect can be minimized if certain precautions are taken[39,40].

In our opinion, the extent of self-decomposition is frequently overestimated. The impurities found in preparations of labelled compounds are not necessarily products of radiation-induced decomposition. Quite often, such impurities are, in fact, intermediates and side-products in the synthesis of these compounds. In addition, samples of unsaturated substances may contain autoxidized and polymerized material; radiation certainly

enhances the formation of such products via free radical reactions, if oxygen is not rigorously excluded.

The methyl esters of oleic, linoleic and linolenic acids uniformly labelled with ^{14}C in the order of 100 uCi/mmole, a level sufficient for metabilic studies, are stable for years if stored under appropriate conditions[36]. Figure 8 shows chromatograms of methyl esters that had been kept under nitrogen in sealed ampoules at -30°C, for ten years.

Similarly, triglycerides labelled with either ^{125}I or ^{131}I barely undergo any self-decomposition during storage. The radioiodinated fats are stable for as long as five half-life periods, that is the time these substances can be of practical use in clinical diagnosis[41].

CONCLUSIONS

Users of radioactively labelled substances often complain about the unsatisfactory purity of commercially available products. It is generally accepted that the indiscriminate use of such preparations can lead to a waste of time and effort.

Although it is realized that impurities may arise by radiation-induced decomposition, many contaminants are, in fact, intermediates and side-products of syntheses.

Efficient methods for the analysis of radioactively labelled lipophilic compounds are now available, and techniques for the purification of such substances can be carried out in any laboratory. Therefore, investigators should be urged to analyze

labelled substances prior to their use and to purify them, if necessary.

The predicate "chromatographically pure" means little unless the purity of a substance has been established by various chromatographic procedures based on complementary principles. With the available methodology, specifications and criteria of pure labelled lipids and related compounds should now be established.

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