LIPID ANALYSIS

2nd Edition

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ISOLATION, SEPARATION, IDENTIFICATION AND STRUCTURAL ANALYSIS OF LIPIDS

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Preface to the Second Edition

IN THE Preface to the first edition of this book (1973), it was stated that "The development of chromatographic techniques, particularly gas-liquid chromatography and thin-layer chromatography, together with advances in spectroscopy, have led to an explosive growth of interest in lipids, and have revolutionised our knowledge of the role that they play in the structure and function of cell membranes, as essential dietary components and in numerous biological processes". In the intervening years, technical developments have continued apace, with the introduction of new materials and methods for lipid analysis. In particular, high performance liquid chromatography, which was virtually ignored in the first edition, is increasingly making a contribution. Nor has the need for improved methods lessened. It is increasingly being realised that not only the composition of lipids, but also their detailed structures and the physical form of their association with proteins in tissue, are of importance in understanding how lipids function and how they may potentially be involved in various human disease states. It has, therefore, been necessary to add a new chapter on the separation of lipoproteins to this edition. The basic plan of the book is the same as in the first edition but each of the original chapters has been extensively re-written to take account of new developments. I have resisted suggestions that the book should be expanded into an encyclopaedic compendium on the subject, in the hope that it will remain on the laboratory bench, not on the library shelf, guiding both the tyro and the expert through the complexities of the practice of lipid analysis.

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W. W. CHRISTIE

Summary

Introduction

THE approach of the research worker to the analysis of the lipids in a given sample will depend partly on the amount of material in the sample, partly on the equipment and instrumentation available, but principally on the amount of information required. For example, if large numbers of samples must be screened routinely, it may only be possible to perform the more basic compositional analyses on each one, while in other circumstances a detailed knowledge of the composition and structure of all the lipid components of a single sample may be necessary. Structural features of lipids of importance in analytical work are described in Chapter 1. Throughout the book, it is assumed that apparatus for gas-liquid chromatography (GLC) and thin-layer chromatography (TLC) will be available to the analyst. The principles of these and other procedures are described in Chapter 3.

In all analytical procedures, precautions must be taken to minimise the effects of autoxidation (see Chapter 3); in particular, lipids must be handled in an atmosphere of nitrogen wherever possible and anti-oxidants should be added to TLC sprays or to TLC solvent mixtures. Care should be taken to prevent the introduction of contaminants into samples. Also, many of the solvents and other chemicals used in lipid analysis are potentially hazardous to the operator and they should always be handled circumspectly.

Extraction of Lipids from Tissues

Detailed extraction procedures are described in Chapter 2. The method of choice depends partly on the nature of the sample, e.g. plant tissues are extracted first with *iso*-propanol to prevent enzymatic degradation of lipids, and also on the amount of the sample. For reasons of economy in time and materials, simplified extraction procedures may be preferred for very large samples or for numbers of small samples, although more exhaustive extraction procedures must be used when highly detailed analyses are intended. The analyst must also consider, when choosing a method of removing non-lipid contaminants from extracts, whether the gangliosides are required for analysis. The weight of the tissue and of the lipid obtained must be recorded and, in some circumstances, the amount of dry matter in the sample should be determined. This data can sometimes be obtained from a small representative aliquot of the sample.

Fatty Acid Compositions

Methods of determining the fatty acid compositions of lipid samples are discussed in Chapter 5. The methyl ester derivatives are first prepared as described in Chapter 4 (methods for preparing other lipid derivatives are also described here) and the method chosen will depend on the nature of the sample. If free fatty acids are present, an acidic reagent such as methanolic hydrogen chloride must be used although the milder and more rapid alkaline transesterification reagents are to be preferred for glyceride-bound fatty acids (special procedures may be required for short-chain, amide-bound or unusual fatty acids). Infrared and ultraviolet

spectroscopy may be of assistance in indicating the presence of some of the less common functional groups in fatty acid chains, and adsorption chromatography (especially TLC) is of value in indicating whether these are polar in nature. Gas-liquid chromatography is the chief method of determining the fatty acid composition of lipids and analyses should preferably be performed on more than one type of polyester liquid phase. Components can be identified provisionally by their retention times relative to authentic standards, by their equivalent chain length values, on the basis of possible biosynthetic relationships or by their behaviour in other ancillary chromatographic techniques, such as silver nitrate TLC or high performance liquid chromatography (HPLC). For unequivocal identification of a component, however, it is necessary to isolate it by some chromatographic procedure or combination of procedures, and establish its structure by definitive chemical and/or spectroscopic techniques. The GLC quantification method selected should be checked and calibrated regularly with standard mixtures of known composition similar in nature to the samples to be analysed. Results for each component fatty acid in a lipid class or mixture of lipids are generally expressed as "weight per cent" of the total but they must be converted to "mol per cent" for structural studies of lipids. In some circumstances, it is necessary to express results as "weight of each fatty acid per unit of tissue", but provided the weight per cent of lipid in the tissue is recorded, the methods of reporting data are interconvertible.

The Analysis of Simple Lipids

This topic is discussed in detail in Chapter 6. Thin-layer chromatography is usually the method chosen for the analysis of simple lipids and it may be performed on the microgram to 100 mg scale. Layers of silica gel G 0.5 mm thick are preferred for larger amounts and those 0.25 mm thick for micro-amounts; hexane-diethyl ether-formic acid (80:20:2 by vol.) is by far the most widely used developing solvent. A few simple lipids can be identified by specific spray reagents (cholesterol and derivatives, free fatty acids or ester bonds), but normally authentic standards are run alongside the samples under investigation. With very small amounts of material or with large numbers of similar samples, fluorometry or charring followed by photodensitometry are generally the methods of choice for lipid quantification; this can be performed while the samples are on the plate or after they have been eluted from the adsorbent. With large amounts of lipid (i.e. 2 mg or more), specific chemical techniques can be applied to quantify individual components, or gas chromatography of the fatty acid constituents of each lipid class in the presence of a known amount of a suitable internal standard may be used so that the fatty acid composition and the amount of each fraction are determined in the same analysis. When larger amounts of particular simple lipids are required for structural analyses say, they can be isolated by column chromatography on silicic acid or Florisil although, because of the difficulty of monitoring columns, it may often be simpler to fractionate samples by preparative TLC on several identical TLC plates with pooling of corresponding fractions.

Alkyldiacylglycerols and neutral plasmalogens are sometimes found with triacylglycerols in lipid samples and can be separated from each other with care by TLC. They can be determined by hydrogenolysis with lithium aluminium hydride and TLC separation of the products. Aldehydes, liberated from plasmalogens by acidic conditions, may be chromatographed as such or in the form of more stable derivatives on similar GLC columns as are used for the analysis of methyl esters of fatty acids.

Results obtained by the above quantification procedures are most often expressed for each component in terms of "weight per cent of the total lipid" although sometimes "weight per unit of tissue" is preferred. Provided that the total weight of lipid per unit of tissue is recorded, however, either method is suitable.

The Analysis of Complex Lipids

Procedures for the analysis of complex lipids are discussed in Chapter 7. Complex lipids can be separated from simple lipids for analysis by rapid column chromatographic procedures on virtually any scale and if

glycolipids are also major components of the sample, they can also be isolated at this stage. With small amounts of material, the complex lipids can be obtained by preparative TLC using the solvent system described in the previous section.

The more common phospholipids from animal tissues can be separated by TLC on silica gel H layers in a single dimension (on up to the 10 mg scale) with chloroform-methanol-acetic acid-water (25:15:4:2 by vol.) as developing solvent. Components are identified by their chromatographic behaviour relative to authentic standards or by means of specific spray reagents. Lipids can be determined by phosphorus analysis or charring-densitometry, or by GLC of their constituent fatty acids with a known amount of an internal standard so that the fatty acid composition and amount of each lipid class are determined in the same analysis. Other TLC systems in one direction are available for the analysis of the acidic lipids, which are generally minor components, but two dimensional TLC systems are a valuable alternative as they permit the separation of complex lipids into many more fractions on one TLC plate than is possible with one-dimensional systems. Related procedures are available for plant and bacterial lipids.

Glycosphingolipids can be separated from phosphatides by several chromatographic or chemical procedures. They can be fractionated into single components differing in the number and nature of the hexose units by one dimensional TLC and estimated by determining the amounts of the nitrogenous bases by chemical procedures. HPLC of benzoylated glycosphingolipids also provides excellent separations. Chromatographic methods are available for determining the fatty acid composition, the long-chain base composition and the hexose composition of individual glycosphingolipids.

When detailed analyses of all the minor components are required, more material must be used and it is advisable to convert all the components to the same salt form before commencing the separation process. Combinations of techniques such as DEAE or TEAE cellulose column chromatography in conjunction with preparative TLC or other column procedures must then be used to separate all the lipid components.

Alkyl- and alkenyl-forms of individual phosphatides cannot be separated from the diacyl- form of the phospholipid in the natural state but they can be determined by the hydrogenolysis procedure used for the analogous simple lipids. If the polar phosphorus group is removed or modified chemically, however, separation of the various forms of the phosphatide is sometimes possible.

Results of analyses are expressed in a variety of ways but generally reflect the method used for quantification. For example, "mol per cent phosphorus" or "weight of phospholipid per unit of tissue" are often used for each component when phosphorus analysis has been performed although "mol per cent phospholipid" can also be calculated from this data. "Mol per cent phospholipid" is normally the method of choice when GLC internal standard procedures are used for quantification. "Mol per cent of the total glycolipids" is the simplest method of expressing the results of glycosphingolipid analyses as these generally depend on determinations of the molar amounts of the nitrogenous bases. For the sake of uniformity, the author would prefer to see all results converted to "mol per cent of the total complex lipids."

Structural Analysis of Lipids

Methods of separating lipids into molecular species are described in Chapter 8, and for determining the positional distribution of fatty acids in lipids in Chapter 9. Lipids can be separated into simpler molecular species according to the combined properties of all the fatty acid constituents by silver nitrate and/or reverse phase TLC and often by high temperature GLC; HPLC in a reverse-phase mode is increasingly finding favour with lipid analysts. Combinations of two of these procedures should preferably be used wherever this is feasible. It is usually necessary to convert phospholipids to less polar derivatives prior to analysis in this manner, either by removing the phosphorus group entirely by means of phospholipase C hydrolysis, when the resulting diacylglycerols or a derivative thereof can be subjected to high temperature GLC in addition to the TLC separations, or by rendering the phosphate group less polar by enzymic and chemical means, when compounds labelled with ^{32}P can be studied. GLC of the component fatty acids with an added internal

standard or related procedures are generally the methods chosen for determining molecular species separated in this way.

Complicated stereospecific analysis procedures have been developed for determining the distribution of fatty acids in positions 1, 2 and 3 of L-glycerol in triacylglycerols but the composition of position 2 alone is obtained relatively easily by means of hydrolysis with pancreatic lipase. Phospholipase A_2 can be used to establish the distribution of fatty acids between positions 1 and 2 of glycerophosphatides.

In all lipid structural studies, results for fatty acid compositions and molecular species should always be expressed in terms of "mol per cent" of the total.

Other Analyses

Isotopically-labelled lipids can be assayed by essentially two approaches, i.e. either continuously or discontinuously. In the former, radioactivity and mass are monitored at the same time on chromatograms. In the latter, separate aliquots of lipids are isolated for mass determination or for liquid scintillation counting. The advantages and disadvantages of various methods are discussed in Chapter 10.

The techniques used for the separation of lipoprotein fractions are more the province of the protein than the lipid analyst. Refined procedures are available to the specialist, but some simplified procedures for the more important fractions are described in Chapter 11.

Contents

CHAPTER 1

The Structure, Chemistry and Occurrence of Lipids

A. INTRODUCTION

The term LIPID has traditionally been used to describe a wide variety of natural products including fatty acids and their derivatives, steroids terpenes, carotenoids and bile acids, which have in common a ready solubility in organic solvents such as diethyl ether, hexane, benzene, chloroform or methanol. A more specific definition is to be preferred, but has yet to be agreed internationally, and the term is nowadays frequently restricted to fatty acids and their naturally-occurring derivatives (esters or amides) and to compounds closely related biosynthetically to fatty acids. It is in this sense that the term is used in this book.

The principal lipid classes consist of fatty acid (long-chain aliphatic monocarboxylic acid) moieties linked by an ester bond to an alcohol, principally the trihydric alcohol, glycerol, or by amide bonds to long-chain bases ("sphingoids" or "sphingoid bases"). Also, they may contain phosphoric acid, organic bases, sugars and more complex components that can be liberated by various hydrolytic procedures. Lipids may be subdivided into two broad classes — "simple", which can be hydrolysed to give one or two different types of product per mol, and "complex", which contain three or more hydrolysis products per mol. The terms "neutral" and "polar" respectively are used more frequently to define these classes, but are less precise and may occasionally be ambiguous; for example, unesterified fatty acids are normally classed as neutral lipids despite the presence of the free carboxyl group.

A complete analysis of the lipids from a given source, therefore, involves separation of the lipid mixture into simpler types according to the number and nature of the various constituent parts, the identification and estimation of each of these and eventual determination of the absolute amount of each lipid type. Before progressing to this, however, a knowledge of the structure, chemistry and occurrence of the principal lipid classes and their constituents is necessary.

B. THE FATTY ACIDS

The common fatty acids of plant and animal origin contain even numbers of carbon atoms (4–24) in straight chains with a terminal carboxyl group and may be fully saturated or contain one, two or more (up to six) double bonds, which generally but not always have a *cis*-configuration. Fatty acids of animal origin are comparatively simple in structure and can be subdivided into well-defined families. Plant fatty acids, on the other hand, may be more complex and can contain a variety of other functional groups, including acetylenic bonds, epoxy-, hydroxy- or keto-groups and cyclopropene rings. Bacterial fatty acids usually consist of simpler saturated and monoenoic components, but may also contain odd-numbered, branched-chain and cyclopropane acids. Very complex high molecular weight acids, the mycolic acids, have been found in certain bacterial species.

1. Saturated fatty acids

The commonest saturated fatty acids are straight-chain even-numbered acids containing 14–20 carbon

atoms, although all the possible odd and even-numbered homologues with 2–30 or more carbon atoms have been found in nature. They are named systematically from the saturated hydrocarbon with the same number of carbon atoms, the final *-e* being changed to *-oic*. For example, the acid with sixteen carbon atoms and structural formula

$$CH_3.(CH_2)_{14}.COOH$$

is correctly termed hexadecanoic acid, although it also has a trivial name hallowed by common usage, i.e. *palmitic acid*. To simplify presentation and discussion of fatty acid compositions, shorthand nomenclatures also exist. In the simplest form, fatty acids are designated solely by the number of carbon atoms they possess, e.g. palmitic acid is a C_{16} acid. This compound can be defined more accurately, however, by listing both the number of carbon atoms in the acid and the number of double bonds, separating the two figures by a colon, i.e. taking the above example — 16:0. Table 1.1 contains a list of common saturated fatty acids together with their trivial and systematic names and shorthand designations.

TABLE 1.1. SATURATED ACIDS OF GENERAL FORMULA $CH_3.(CH_2)_n.COOH$

Systematic name	Trivial name	Shorthand designation
ethanoic	acetic	2:0
propanoic	propionic	3:0
butanoic	butyric	4:0
pentanoic	valeric	5:0
hexanoic	caproic	6:0
heptanoic	enanthic	7:0
octanoic	caprylic	8:0
nonanoic	pelargonic	9:0
decanoic	capric	10:0
hendecanoic	—	11:0
dodecanoic	lauric	12:0
tridecanoic	—	13:0
tetradecanoic	myristic	14:0
pentadecanoic	—	15:0
hexadecanoic	palmitic	16:0
heptadecanoic	margaric	17:0
octadecanoic	stearic	18:0
nonadecanoic	—	19:0
eicosanoic	arachidic	20:0
heneicosanoic	—	21:0
docosanoic	behenic	22:0
tetracosanoic	lignoceric	24:0

Acetic acid is only rarely found in association with higher molecular weight fatty acids in esterified form, although it has been found esterified to glycerol and to hydroxy-fatty acids in some seed oils. C_4 to C_{12} acids are found mainly in milk fats, although the C_{10} and C_{12} acids have also been found in quantity in certain seed oils. *Myristic acid* (14:0) is a minor component of most animal lipids, but is present in major amounts in seed oils of the family Myristicaceae. Palmitic acid is probably the commonest saturated fatty acid and is found in virtually all animal and plant fats and oils. *Stearic acid* (18:0) is also relatively common and may on occasion be more abundant than palmitic acid, especially in complex lipids. Longer chain saturated acids occur less frequently, but are often major components of waxes. C_{15} to C_{19} odd-chain acids can be found in trace amounts in most animal lipids, but can occur in larger quantities in certain species of fish or in bacterial lipids.

Decanoic and higher saturated fatty acids are solids at room temperature. Because of the lack of functional groups other than the carboxyl group, they are comparatively inert chemically, and natural or synthetic lipids containing only these acids can be subjected to more vigorous chemical conditions than those containing polyunsaturated fatty acids.

2. Monoenic fatty acids

Straight-chain even-numbered fatty acids of 10 to 30 carbon atoms containing one double bond of the *cis*-configuration have been characterised from natural sources. Monoenoic acids with double bonds in the *trans*-configuration are also known but are found comparatively rarely. Fatty acids of a given chain length may have the double bond in a number of different positions and a full description of any acid must specify the position and configuration of the double bond (in the recommended numbering system, the carboxyl carbon is C-1). For example, by far the commonest monoenoic acid is *cis*-9-octadecenoic acid, less accurately Δ^9-octadecenoic acid, which has the trivial name *oleic acid* and structure

$$CH_3.(CH_2)_7.CH = CH.(CH_2)_7.COOH$$

cis