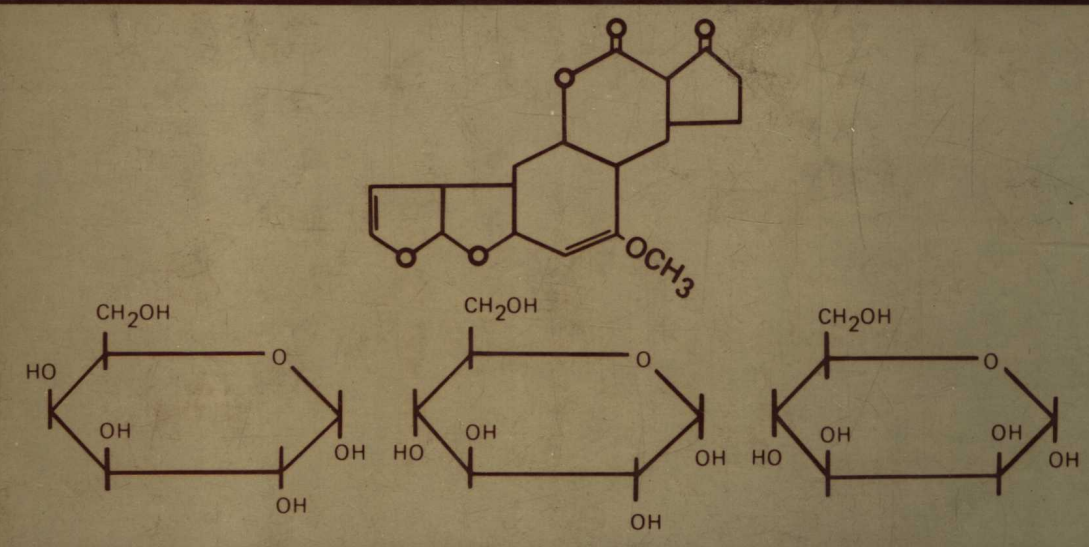
FOOD CONSTITUENTS AND FOOD RESIDUES

Their Chromatographic Determination



edited by JAMES F. LAWRENCE

Food Constituents and Food Residues

THEIR CHROMATOGRAPHIC DETERMINATION

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PREFACE

Over the past two decades, chromatography has played an important role in the determination of substances in foods. Gas chromatography and more recently liquid chromatography have been shown to be of invaluable assistance to food analysts, and numerous methods have appeared in the literature demonstrating this. The technique continues to be employed on a large scale and new approaches and applications are regularly being reported for solving problems related to chemical components of food, be they natural constituents or residues of agricultural chemicals, industrial contaminants, or additives. The purpose of this volume is to present to the reader up-to-date accounts of research in methodology development and recent applications of chromatography in the area of food analysis. Emphasis is placed on practical aspects rather than theoretical treatment or general fundamentals of chromatography, of which there are already an abundance of texts.

There are twelve chapters in the book that discuss chromatographic methods for natural food constituents as well as substances which may be present as residues resulting from their inadvertant or intentional addition. It is impossible in a volume such as this to include all natural or residual substances which may occur in foods. However, the material contained herein is considered to represent important areas of food analysis, and most of the approaches described may be extended to other substances of a similar nature. The chapters provide important information on recent developments in the analysis of compounds of a diverse nature from nonpolar to polar substances and at concentrations varying from trace levels up to percent composition.

The authors are all experts in their respective areas. Their individual chapters combine to create a volume which will be of interest to analysts in many fields who are involved in food analysis. I wish to express my sincere thanks to W. W. Christie, R. C. Noble, J. Pearson, E. Pfannkoch, F. E. Regnier, D. B. Parrish, J. F. Pirisino, G. A. Reineccius, S. Anandaraman, J. M. Hardin, C. A. Stutte, N. T. Crosby, T. Romer, T. Fazio, N. P. Sen, H. Roseboom, and V. Zitko for their time and efforts in providing material for this volume.

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CONTENTS

1

RECENT DEVELOPMENTS IN LIPID ANALYSIS

WILLIAM W. CHRISTIE and RAYMOND C. NOBLE

The Hannah Research Institute, Ayr, Scotland

I. INTRODUCTION

Any description of methodology must be prefaced by some definition of the basic materials under discussion. In the case of lipids, this is difficult, not only because of the range and variable nature of the substances encompassed by the term, but also because there is no generally accepted definition. Thus, the term "lipid" is usually used loosely to cover a wide range of substances that are insoluble in water and soluble in organic solvents, such as hexane, benzene, diethyl ether, chloroform, or methanol, and include long-chain hydrocarbons, fatty acids and their derivatives, alcohols, aldehydes, sterols, terpenes, carotenoids, and bile acids. Nowadays the term should preferably be applied in a more restricted way to fatty acids and their derivatives or metabolites, and it is used in this sense in this review. The principal lipid classes then consist of fatty acid (long-chain aliphatic monocarboxilic acid) moieties linked by an ester bond to an alcohol, in the main the trihydric alcohol glycerol, or by amide bonds to long-chain bases. They may also contain phosphoric acid, organic bases, sugars, and more complex components. Lipids can conveniently be divided into two major subgroups (1). These are the "simple" lipids, defined as those which contain one or two of the above hydrolysis products per molecule, and "complex" lipids which contain three or more types of hydrolysis products per molecule. The less precise and often ambiguous terms "neutral" and "polar" are frequently used to define these classes also; thus, in spite of the presence of a free

carboxyl group, unesterified fatty acids are normally considered to belong to the neutral lipids. A detailed description of lipid classifications is not within the scope of this review and a basic knowledge by the reader of the structure, chemistry, and occurrence of the principally known lipids and their constituents is assumed.

The role of lipids as essential cellular constituents involved in a range of biochemical and structural functions was known long before any meaningful fractionations and analyses were possible. However, the development of chromatographic procedures such as thin-layer and gas-liquid chromatography, commencing about 25 years ago, dramatically changed this picture and led to an explosive growth in our knowledge of the structure, composition, and function of lipids in natural biological materials. Although the pace of development in analytical methodology and instrumentation has been less dramatic over the past 6-8 years, nevertheless steady progress has been made and improvements to existing methods continue to appear. Many of these improvements have yet to be applied to foods but are discussed here because of their potential value in such analyses. These newer methods, together with some older but related procedures, form the subject of this review.

The development of new analytical procedures has been accompanied by progress in the automation of the manipulation of samples and in the handling of the acquired data. Such advances, useful and labor-saving as they are, must be treated with a certain amount of caution and respect. It is now possible for the time for the completion of an analysis to be considerably shortened and the operations involved to be reduced to a minimum, but the onus for ensuring the quality of the final results still rests with the operator's ability to apply various rules and checks. The capacity to remove needless drudgery from an analytical procedure is to be welcomed, but all too frequently it has been accompanied by a reduction in the validity of the final answers. Lipid analytical methodology has been the subject of several textbooks or review volumes (1-3).

II. LIPID EXTRACTION

Before any lipid analysis can be begun, quantitative isolation of the lipids, free from nonlipid contaminants, has to be achieved. Carelessness at this preliminary stage with the loss, partial or otherwise, of specific components will obviously put the validity of any further results at risk. Precautions are necessary to ensure that lipids are recovered unchanged and are representative of those in an unextracted material. For example, it is important that the risk of autoxidation of polyunsaturated fatty acids be minimized by handling tissues and extracts in an inert atmosphere of nitrogen and by the routine incorporation into solvents of a suitable antioxidant such as butylated hydroxy

toluene or 2,6-di-tert-butyl-p-cresol (BHT) (4). It is also important to minimize the effects of enzymic degradation during extraction as manifested mainly by hydrolysis reactions. When appreciable amounts of unesterified fatty acids, partial glycerides, or phosphatidic acid are found in an extract, for example, it can generally be assumed that there has been some carelessness in the extraction step. Fresh tissues, in particular, should be extracted with the minimum of delay. For example, rapid deacylation of phospholipids was found to occur in some bacterial extracts, even at -16°C (5). Lysophosphatidylcholine was long thought to be a biologically important lipid in certain adrenal organelles, but it is now considered to be largely an artifact of the extraction process (6). If immediate extraction is not possible, material should be frozen rapidly and stored in sealed glass containers at -20°C in an atmosphere of nitrogen, but some attention must then be paid to the possibility of enzymic action on the tissue lipids as a result of cell disruption. This may occur both during storage and on subsequent thawing, and it is generally advisable to extract without thawing.

The ideal solvent or solvent mixture for extracting lipids from tissues is one of sufficient polarity to remove all the lipids from their association with the cellular constituents while not reacting chemically with them, but at the same time it should effect solution of all the nonpolar lipids. Increasingly, attention has had to be given to the problems of possible toxicity of the chosen solvents to the operator. It is generally accepted that no single pure solvent is suitable as a general-purpose lipid extractant. For example, although chloroform and diethyl ether are excellent solvents for lipids, their ability to extract complex lipids from tissues is poor. Furthermore, their use may promote the action of phospholipase D during the extraction of plant tissues. As n- and isopropanol inhibit this reaction, it is generally recommended that they be used as preliminary extractants in this instance. Although limitations to its use are often voiced, it is still widely accepted that a mixture of chloroform and methanol in the ratio of 2:1 (v/v) will extract lipids more exhaustively from animal and plant tissues than any other simple solvent systems. Various procedural modifications in its use have been evolved over the years to improve the efficiency of lipid extractions for specific tissues and to minimize the possible effects from enzymatic degradation (7), but the well-established methods (8,9) can be recommended for most purposes. With particularly difficult samples such as cereals, butanol saturated with water has been recommended as extractant (10,1). Isopropanol-hexane (12) has been recommended as a low-toxicity mixture.

Partition of the chloroform/methanol extract with a suitable aqueous medium still remains the favored method of purification of lipid extracts, although more elegant, if time-consuming, procedures involving liquid-liquid partition chromatography on a column are available (13). It has recently been demonstrated that a pre-extraction of both animal

and plant tissues with 0.25% acetic acid will remove all potential non-lipid contaminants and will simultaneously deactivate the lipolytic enzymes (14,15).

If the extracts themselves must be stored for any length of time, then further checks for artifact production during the storage period are necessary. With the increasing capacity to detect ever-smaller amounts of materials, the potential problem of lipid contamination from solvents, sample handling, and laboratory apparatus has assumed greater importance. It is imperative that solvents should be distilled before use and that contact with any plastic container or apparatus should be avoided, as plasticizers are leached out surprisingly easily and may give rise to spurious peaks on chromatograms and affect ultraviolet (UV) spectra.

III. RECENT DEVELOPMENTS IN CHROMATOGRAPHIC TECHNIQUES AND INSTRUMENTATION

A. Principles

Lipid samples are inevitably complex mixtures of individual lipid classes, and each of these may contain a wide range of molecular species, so that a complete analysis of a given sample can be a difficult task. In common with all fields of research, the techniques and instrumentation available for lipid analyses have become increasingly sophisticated. Inevitably, this has been accompanied by increasing costs. However, although such techniques and instruments are valued weapons in the armory of the lipid analyst, they are not indispensable for good work; much excellent work can be done with comparatively simple apparatus.

The four basic principles that essentially govern the chromatographic separation of lipids, together with examples of the types of separation that can be achieved, are listed in Table 1. In adsorption chromatography, separation is effected through the degree to which lipid components are adsorbed onto a solid support (by hydrogen bonding, Van der Waals forces, and ionic bonding) relative to their solubility in an appropriate solvent or solvent mixture. As a result, the presence and nature of polar functional groups within the lipids determine, in the main, the ability to achieve separations. Developments over recent years in the quality of the absorbents available have led to marked improvements in the resolution obtained by adsorption-column or thin-layer methods. In partition chromatography, separation of the lipids is achieved according to differences in the partition coefficients between two immiscible phases, generally two liquid phases or a liquid and a gas phase. The first chromatographic procedures used for lipid separations, namely countercurrent and reversed-phase liquid-liquid chromatography, utilized this principle as does gas-liquid chromatography (GLC), which has revolutionized the analysis of lipids.

TABLE 1 Chromatographic Procedures Used in the Separation of Lipids

Procedure	Examples	Type of separation
Partition chromatography	Gas-liquid or liquid-liquid (including reverse-phase chromatography)	Homologous or isomeric series of lipids
Adsorption chromatography	Column, thin-layer (silicic acid, alumina, Florisil)	Number and nature of functional groups
Ion-exchange	Columns of DEAE-, CM, or TEAE-cellulose	Degree of acidity and (to some extent) polarity
Complexation chromatography	Silver nitrate or boric acid with silicic acid	Differing numbers or configurations or functional groups

Such procedures are particularly useful for subdividing lipid classes consisting of homologous or vinologous series of aliphatic residues and, in particular, for the isolation of fatty acids or their esters. High-performance (or high-pressure) liquid chromatography (HPLC) can be used to effect separations through either the principle of reversed-phase partition or adsorption. Essentially this latter technique is similar in principle to earlier methods but takes advantage of modern technological advances in the preparation of liquid phases in order to achieve the separations, and of automated instrumentation to record and quantify them. The separation processes involved in ion-exchange chromatography involve a combination of ion exchange of the ionic moieties of polar lipids, together with the adsorption of any highly polar nonionic parts of the lipid. Techniques involving complexation chromatography are used in conjunction with other methods of lipid analysis, but have proved particularly useful in the separation of molecular species and of configurational isomers of lipids.

In spite of the advances that have been made, often no single procedure will achieve the desired separations, and combinations of techniques must be used until the required lipid classes are obtained in a pure state. This is particularly true of separations of molecular species. For example, a natural triacylglycerol mixture with only five different fatty acid constituents may contain 75 different molecular species, not counting enantiomers. On the basis of the combined chain lengths of the fatty acid moieties, high-temperature gas chromatography is able

to separate four different fractions; using silver nitrate thin-layer chromatography, some eight distinct fractions can be separated on the basis of the total number of double bonds contained in each molecule of triacylglycerol (as shown in Fig. 1). By fractionating the sample first on silver nitrate thin-layer chromatography and then subjecting each separated component to gas chromatography, some 32 distinct molecular species can be discerned. Although this falls short of a complete analysis, it is a considerable enhancement on the original separative abilities of the techniques when used in isolation. Certain combinations have been found to be particularly useful in a variety of analytical situations. For example, gas chromatography or reversed-phase chromatography in conjunction with silver nitrate chromatography have been found to be useful combinations for identification and estimation of fatty acids or molecular species of lipids. Ion-exchange chromatography in combination with thin-layer chromatography has been found to be particularly useful for the separation of individual complex lipid classes.

B. Thin-Layer Chromatography

Many improvements in the techniques of thin-layer chromatographic (TLC) separation and quantification have been described in recent years. These range from very simple modifications to well-tried and accepted methods to the introduction of entirely new concepts, some involving sophisticated instrumentation. The workhorse of the average lipid analytical laboratory still remains the glass plate on which a thin layer of suitable adsorbent is held. The effects of different proprietary brands and batches of adsorbents on separations can be variable, but more vigorous control is now exercised by the manufacturers over the quality of their products. The use of commercially prepared precoated layers on plastic or aluminum backing is becoming more popular because of their convenience.

In recent years, high-performance thin-layer chromatography (HPTLC) procedures have been developed giving separation efficiencies of approximately 5000 or more theoretical plates, that is, approximately three times the normal efficiency (16). In one form, a special apparatus is used in which the solvent front is continuously evaporated and renewed, resulting in greatly improved separation efficiencies; however, only a few applications to lipid analyses appear to have been described. A simpler form of the technique consists of the use of commercially prepared plates precoated with adsorbent of a smaller and more uniform particle size than is generally employed. With such plates, both the resolution and speed of separations are improved especially with polar solvent mixtures, and there are indications that more accurate quantifications may be achieved. Disadvantages are that only very small amounts of sample can be applied to the plate without overloading,

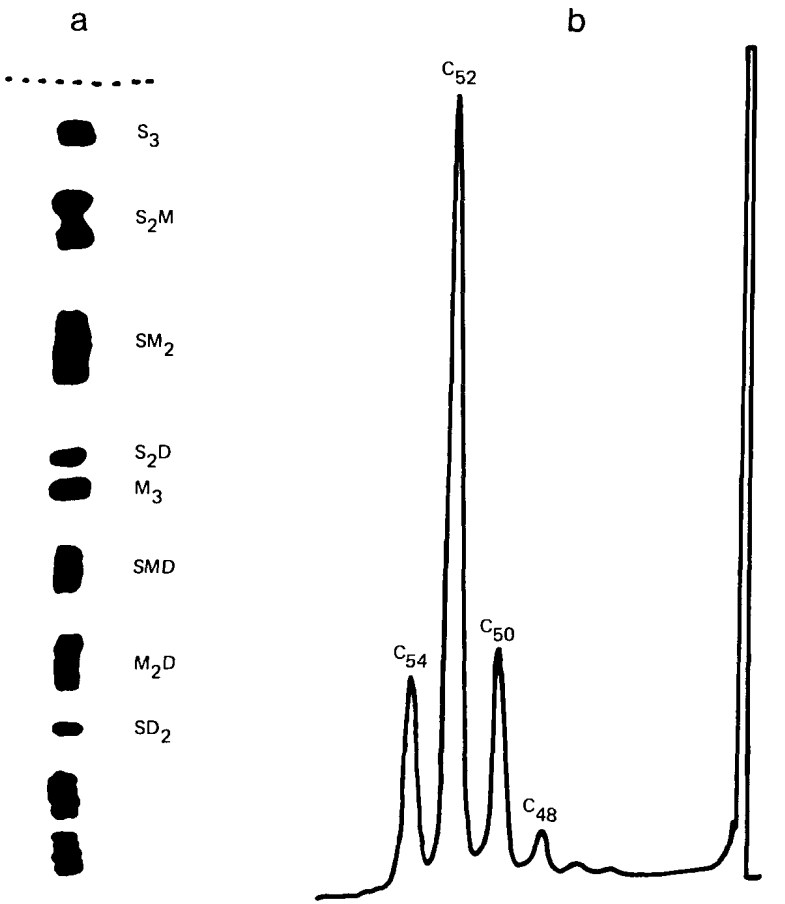

FIGURE 1 Separation of pig adipose tissue triglycerides. (a) On layers of silica gel G impregnated with 10% (w/w) silver nitrate with chloroform-methanol (99:1, v/v) as developing solvent. (b) By high-temperature GLC (50 × 0.4 cm (i.d.) glass column packed with 1% SE-30 in Chromosorb W. Carrier gas: Nitrogen at 50 ml/min. Temperature: Programmed from 280 to 330° at 2°/min).