Chromatography for the Analysis of Lipids

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To my wife Nuala and children
Noel, Caitrióna and Ciarán

Thanks for your patience

FOREWORD

I well remember turning up for my first day's work in June 1964. It was a hot month and the smell of chromatography solvents was quite strong, something that is not tolerated under today's safety legislation. Chromatography was a virtually unknown area to me then and I did not realize just how much its development and use were to transform and affect my life. I was in a very privileged position, for I was working in the Unilever Group set up and managed by Dr. A. T. James, which was in many ways at the cutting edge of chromatography and lipid biochemistry.

At my interview for this job, one of the questions I was asked by Dr. James was, "Have you ever constructed anything electronic or electrical?", and the next question was, "Did they work?". As it happened I had an interest in amateur radio and electronics and had indeed built both valve (am I that old?) and transistorized radios for my own use. So my answers were affirmative. I got the job, by sheer luck I thought. On my second day of employment I found out why these answers were so important; I was presented with the plans of a "radio-gas chromatograph", shown the tools and the bits, and told "build it" (the radio-gas chromatograph simultaneously measured organic mass and radioactivity). Such was the commercial situation in chromatography instrumentation that we had to build our own.

This single piece of instrumentation was to ensure that the work, which Dr. Lindsay Morris and myself were to do, would not only achieve results but more particularly would cover a wide range of topics in a relatively short time. Something we could only achieve because chromatography was so well developed and so much used in that laboratory. Then the excitement began for me and truly has never gone away.

Gas liquid chromatography (GLC) by then was already quite a sophisticated technique, yielding excellent powers for the separation, detection, and quantitation of complex mixtures of fatty acid components. The period of development up to this point is elegantly and humorously described by James (1986). However, you definitely needed to be a "jack of all trades" to ensure the best results. To this end, Dr. James set high standards and was quite intolerant of poor chromatographic results. His group was involved in a broad research brief under Unilever's umbrella and this umbrella enabled continued development of chromatographic techniques alongside the biochemical research. It was a time of intense but exceptionally interesting work which gained worldwide exposure for the scientists in the group.

The experience I gained during those years and built upon throughout my career has been put to good use. One thing I learned was that each different chromatographic technique has an optimum area for its best application while being complementary to the others. We therefore used GLC and thin layer chromatography (TLC) to their fullest extent. As column liquid chromatography developed into high performance liquid chromatography (HPLC) so we also applied this. Although the research we did on each technique explored

most areas of lipid chemistry, we never attempted to do everything with just one technique. Flexibility in approach to analysis is an important quality for persons in analytical chemistry. So it is my intention to represent GLC, TLC, and HPLC applications together in this book and hopefully in a coordinated manner. I shall also review the present situation regarding supercritical fluid chromatography (SFC). Thus I hope that the all too often separated techniques which are so complementary will be seen as such. After all, no self-respecting lipid laboratory restricts itself to a single chromatographic technique if it wishes to move forward apace. In this way, then, perhaps this book will be of help to both new and established lipid analysts.

In assembling and writing this treatise I make no apologies for having recorded things done "my way" (to coin a well-known song!). However, I do not claim to be infallible nor the only worldwide "expert". There are many who deserve that label better than I. For this reason I have included and reviewed where necessary as comprehensive, relevant, and up-to-date a bibliography as I have been able. To those who might feel left out or ignored, I apologize, it is not intentional. In many cases I have quoted and shown certain manufacturers' equipment or materials. This information is given in good faith as example of something which I know works well in my application. However, where I may appear to be critical of certain equipment I do not claim exclusion of other manufacturers' similar equipment or materials in these cases. My comments are a reflection of my own preferences and ways of working. Each analytical group should make their own choices based upon cost, effectiveness, and application.

Safety is an important feature of all work, especially that done in laboratories, so that I have included prompts where there may be particular safety risks. Please do not ignore these points on safety; many are common sense but some have been learned over time by experience.

ACKNOWLEDGMENTS

This book is dedicated to my family and I wish to acknowledge their tremendous support in its preparation. I also thank my employers United Biscuits (UK) Ltd., and Professor R. Marian Hicks for their permission and great encouragement, without which I certainly would not have succeeded. I thank also my many colleagues for their critical advice. Among those colleagues I single out are Professor F. D. Gunstone, Professor R. J. Hamilton, Professor K. Aitzetmüller, Professor C. H. S. Hitchcock, Professor A. T. James, and Professor R. Wood. I thank my staff for help in proofreading and particularly Ian Fare, my senior analyst, for running most of the chromatograms shown in Chapters 4, 5 and 6 of this book.

I thank in particular Professor A. T. James and Dr. L. J. Morris for providing the very stimulating research atmosphere in which I learned my craft; better tutors I could not have found. Last but not least I thank Unilever Research & Development, Colworth House, U.K. for providing the exciting and varied environment which supported my first 24 years work.

PREFACE

Is chromatography becoming too much of a "black box" technique? It is often true that those using the technique are relatively untrained in the theory and the art. Those workers in an academic environment are perhaps more privileged, but certainly the modern day approach in industrial concerns seems more and more to be one of use rather than understanding, with less time granted by employers to the learning of the science and art of chromatography. This type of approach is creating a reliance upon suppliers of chromatography equipment and materials, who set up their own technical centers purporting to solve your every problem with, of course, their equipment. These are controversial comments, no doubt, which will perhaps attract some interesting replies. It is not all bad, of course; sweeping generalizations are never accurate. There are indeed some very reputable commercial organizations who perform high quality development work, create reliable techniques, and produce excellent materials and equipment. I do feel, however, that to understand and be trained in the application of chromatography, particularly to the range of materials of interest to yourself, provides you with a great advantage. You make the decisions, you optimize the conditions, you understand the constraints and capabilities of the technique.

I hope this book will provide information which can be either used directly or built upon and perhaps aid both new and established lipid analysts in their endeavours. However, I do not presume to dictate technique but I do recommend, offer, or review information which will lead to data that can be interpreted to yield a detailed structure of lipid components. I hope that it may become obvious that the techniques of thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas liquid chromatography (GLC) are truly complementary and that a combination of techniques will often provide more or better information. Attempting to make one technique "universal" in the field of lipids will not work and ultimately the results required will dictate whether TLC, HPLC, GLC, or a combination of these is required. The emerging practice of supercritical fluid chromatography (SFC) is gaining in application as research unfolds the technique. However, it is my opinion that the technique is still at the prototype stage. It seems likely that in application, the attributes of SFC will lie somewhere between GLC and HPLC complementing both. However, it doubtless will play a major role in its link with mass spectrometry (SFC-MS). SFC demonstrates the ability to perform separations of temperature-sensitive compounds or compounds of very low vapor pressure, which are similar to those obtained on HPLC. However, the complications and problems caused by the organic solvents in HPLC are generally absent in SFC and this enables the use of gas chromatography detectors such as the flame ionization detector. Through the text of this book I shall cover TLC, GLC, and HPLC and also include information on sample preparation and some typical analytical data. I shall also provide a review of the present situation regarding

supercritical fluid chromatography of lipids. Throughout the text an attempt is made to coordinate the techniques so that their complementary nature will be seen. Background and introduction to each technique will be found in the relevant chapter.

TABLE OF CONTENTS

Chapter 1

INTRODUCTION

The term **lipid** describes or includes the broad range of natural materials which are soluble in organic solvents but are generally insoluble in water. Lipids which are solid at ambient temperatures tend to be referred to as "fats" while those which are liquid are called "oils". Kates (1986), in his treatise *Techniques of Lipidology*, includes in the definition "lipid" those substances which "contain long-chain hydrocarbon groups in their molecules and those present in or derived from living organisms." Kates also classifies two main groups of lipids which have significantly different natural functions and structures: the "neutral lipids" (including acylglycerides, fatty acids, alcohols, hydrocarbons, waxes) and "complex" or "polar" lipids (including phospholipids, glycolipids). The neutral lipids tend to form part of an energy store, for instance, in a seed or the adipose tissue of an animal. The polar lipids are functional in, for instance, the structure of membranes and cell organelles, or as complexes in the tertiary structure of some proteins and carbohydrate molecules. A very comprehensive treatment of the classification and structure of lipids is provided in Chapter 1 of Kates' book. His book is highly recommended as reading. A less broad coverage of lipid structure is provided below to aid the reader.

I. FATTY ACIDS*

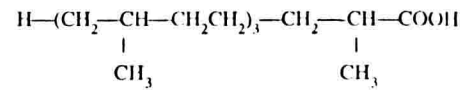
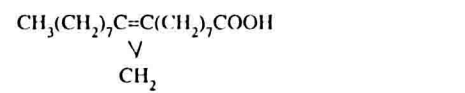
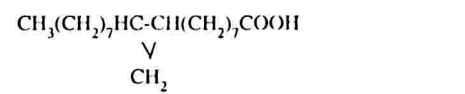
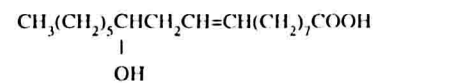
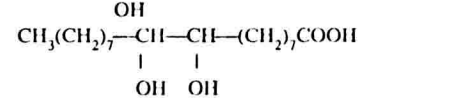
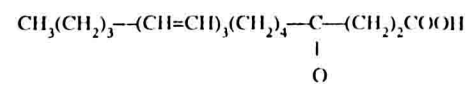
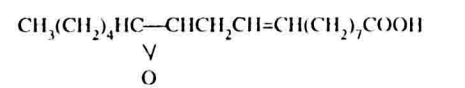
These form homologous series of "straight" chain carbon compounds terminating with a methyl group at one end and a carboxylic acid at the other. The chain length of natural fatty acids varies typically from C4 to C30, with the majority lying between C10 and C22. Fatty acid chains are generally composed of an even number of carbon atoms by virtue of their biochemical synthesis. This involves the cellular enzyme complex *fatty acid synthetase* which uses the 2 carbon fragment acetic acid as a building block. The acetic acid is in an "activated form" as a thiol ester of Coenzyme A (-CoA); this is carboxylated to malonyl-CoA and forms all but one of the two carbon units for fatty acid synthesis (Gurr and James, 1980). The product of this synthetase complex is palmitic acid (C16:0), and further chain elongation (including elongation of unsaturated fatty acids) requires a different complex of enzymes and takes place at the endoplasmic reticulum and under certain circumstances the mitochondrion. (For more general detail on the biosynthesis of these and the following fatty acids, see Gurr and James, 1980 and de Gruyter, 1988).

There are exceptions to the generality of straight chains and in many cases fatty acids substituted with hydroxy, keto, epoxy or having unusual unsaturation are found, sometimes in abundance (see review by Badami and Patil, 1981 and

* See Table 1.1 for examples of structures.

TABLE 1.1
Examples of Fatty Acid Structure

Common name	Systematic name	Formula	No. carbons and character
Lauric	*n*-Dodecanoic	$CH_3(CH_2)_{10}COOH$	12:0 – straight chain
Palmitic	*n*-Hexadecanoic	$CH_3(CH_2)_{14}COOH$	16:0 – straight chain
Palmitoleic	*cis*-9-Hexadecenoic	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$	16:1 – *cis* double bond
	cis-7-Hexadecenoic	$CH_3(CH_2)_7CH{=}CH(CH_2)_5COOH$	16:1 – *cis* double bond
Stearic	*n*-Octadecanoic	$CH_3(CH_2)_{16}COOH$	18:0 – straight chain
Oleic	*cis*-9-Octadecenoic	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	18:1 – *cis* double bond
Elaidic	*trans*-9-Octadecenoic	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	18:1 – *trans* double bond
Linoleic	*cis,cis*-9,12-Octadecadienoic	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	18:2 – meth. separated *cis* diene
Laballenic	(–)-5,6-Octadecadienoic	$CH_3(CH_2)_{10}{-}CH{=}C{=}CH{-}(CH_2)_4COOH$	18:2 – 5,6-allene
α-Linolenic	all-*cis*-9,12,15-Octadecatrienoic	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	18:3 – meth. separated *cis* triene
γ-Linolenic	all-*cis*-6,9,12-Octadecatrienoic	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_4COOH$	18:3 – meth. separated *cis* triene
α-Eleostearic	*cis*-9, *trans*-11, *trans*-13-Octadecatrienoic	$CH_3(CH_2)_3CH{=}CHCH{=}CHCH{=}CH(CH_2)_7COOH$	18:3 – *cis/trans* conjugated triene
Arachidonic	all-*cis*-5,8,11,14-Eicosatetraenoic	$CH_3(CH_2)_3(CH_2CH{=}CH)_4(CH_2)_4COOH$	20:4 – meth. separated *cis* tetraene
Clupanodonic	all-*cis*-7,10,13,16,19-Docosapentaenoic	$CH_3(CH_2CH{=}CH)_5(CH_2)_5COOH$	22:5 – meth. separated *cis* pentaene
	all-*cis*-4,7,10,13,16,19-Docosahexaenoic	$CH_3(CH_2CH{=}CH)_6(CH_2)_2COOH$	22:6 – meth. separated *cis* hexaene
Isostearic	16-Methylheptadecanoic	$CH_3{-}\underset{\displaystyle CH_3}{\underset{\vert}{CH}}{-}(CH_2)_{14}COOH$	18:0 – 16-methyl C17:0 (iso C18:0)
Anteisostearic	15-Methylheptadecanoic	$CH_3CH_2{-}\underset{\displaystyle CH_3}{\underset{\vert}{CH}}{-}(CH_2)_{13}COOH$	18:0 – 15-methyl C17:0 (anteiso C18:0)

Pristanic	2,6,10,14-Tetramethylpentadecanoic		19:0 – multiple methyl branched C15:0
Sterculic	*cis*-9,10-Methylene-9-octadecenoic		19:1 – *cis*-9,10-cyclopropene
Dihydrosterculic	*cis*-9,10-Methyleneoctadecanoic		19:0 – *cis*-9,10-cyclopropane
Ricinoleic	12-Hydroxy-*cis*-9-octadecenoic		18:1 – 12-hydroxyoleic
	erythro-9,10-dihydroxyoctadecanoic		18:0 – *erythro(cis)*-diOH stearic
α-Licanic	4-Keto-*cis*-9,*trans*-11,*trans*-13-octadecatrienoic		18:3 – 4-keto-α-eleostearic
Vernolic	*cis*-12,13-Epoxyoctadec-9-enoic		18:1 – *cis*-12,13-epoxyoleic

meth. = methylene (—CH_2—)

Gunstone, et al., 1986). Some plants (e.g., some members of the *Malvaceae* family) and bacteria have fatty acids containing cyclopropane and cyclopropene rings (Smith, 1970). Some plants, particularly the *Flacourtiaceae* family (Badami and Patil, 1981), exhibit fatty acids with a "methyl" terminal cyclopentene ring, with perhaps the most commonly known being *chaulmoogric acid* (13-[2-cyclopentenyl]-tridecanoic acid). These oils have been used extensively in the treatment of leprosy. A group of very important and biologically active unsaturated C20 fatty acids is the prostaglandins (de Gruyter 1988, p. 479). These contain a cyclopentenyl ring formed by cyclyzing the chain at carbons 8 and 12, for example, from arachidonic acid via the cyclooxygenase pathway. These unsaturated molecules are also substituted with hydroxy and keto groups. A further group of fatty acids contains a furanoid structure. Such an example was reported in *Exocarpus cupressiformis* seed oil (Morris et al., 1966), but they are found predominantly in fish (Glass et al., 1975).

Methyl branched fatty acids occur mainly in bacteria, although they are found in the fat of ruminant animals, where the origin of these is microbial metabolism in the rumen. Three main groups of branched chain fatty acids are identifiable: those with a single methyl branch at the carbon α to the methyl terminal — *iso* series, those with the branch ß to the methyl terminal — *anteiso* series, and those which exhibit multiple branching, for instance, the mycocerosic acids produced by the mycobacteria. A series of odd chain fatty acids also exists but again mainly in microbial systems.

Of the fatty acids with chain substitution groups, perhaps those with a hydroxy group are most prevalent. The most commonly known is that from castor bean oil *ricinoleic acid* (12-hydroxyoctadec-9-enoic acid), where it comprises up to 90% of the triglyceride fatty acids. The same acid can also account for up to 45% of the fatty acids in the lipid of the fungus ergot (*Claviceps purpurea*), which parasitizes certain cereal grain crops such as rye. Ergot fungal sclerotia also contain a series of medicinally important alkaloids and have been used as a base source of lysergic acid. Severe rye crop infections lead to contaminated flour and have been responsible in the past for the condition known as "ergotism" (sometimes called St. Anthony's fire). A variety of hydroxy and dihydroxy acids occur in the lipids of a number of plant families (Badami and Patil, 1981). The position of the hydroxy groups vary, typically (but not exclusively) being on carbons from 9 to 16, as measured from the carboxylic acid group. Where two hydroxy groups occur in the same fatty acid, these are invariably vicinal (on adjacent carbon atoms) but their geometry may be *threo (trans)* or *erythro (cis)*. Epoxy fatty acids (containing an oxirane ring) are also in abundance, particularly in some members of the *Compositae* and *Euphorbiaceae* families. Some of these epoxy acids have a commercial value and use, for instance, *vernolic acid* (12,13-epoxyoctadec-9-enoic acid) which comprises around 80% of the glyceride fatty acids in *Vernonia anthelmintica* seed oil. These epoxy groups tend to be positioned in the fatty acid chain where a double bond might otherwise exist, i.e., 9-10, 12-13, 15-16. Fatty acids with keto substitution are less common and less abundant, but

nevertheless are found at such chain positions as 4, 8, 9, 11, 13, 15, 17, and 19. For instance α-licanic acid (4-oxo-octadeca-9,11,13-trienoic acid) forms up to 60% of the fatty acids of *Licania rigida* seed oil. It might be noted that this acid also has a conjugated triene centre.

Normal straight chain fatty acids do not exhibit optical asymmetry. However, it is important to realise that when chain branching or substitution takes place, as in the *anteiso* branched series (but not the iso series) and the oxygenated acids described above, asymmetry exists about the carbons substituted. They can therefore exist in enantiomeric forms and optical rotation can be measured. Since these acids have a biochemical origin, their optical asymmetry is always quite specific, but may be different for the same structure isolated from different plant families.

Desaturation of normal straight chain fatty acids via regio-specific desaturase enzymes, yields an important family of fatty acids which contain one or more double bonds, of the *cis* isomeric form. This process in animals and plants is aerobic, but an anaerobic pathway does exist in some bacteria which produces only monoenoic (one double bond) fatty acids. Where there is more than one double bond each pair is normally separated by a single methylene unit (—CH_2—). The series of unsaturated fatty acids synthesized in plants is different from that synthesized in animals. This difference creates the requirement by animals for **essential dietary fatty acids** (EFA) produced in plants, although some carnivorous animals, such as the cat, require the EFA to be modified further by other animals. This fact, in a sense, makes these animals obligate carnivores, requiring arachidonic acid (C20:4) in their diet. Plants have the Δ9, Δ12 and Δ15 desaturase enzymes and thus create double bonds between the Δ9 double bond of oleic acid and the methyl end of the C18 chain, creating linoleic (*cis,cis* Δ9-12, octadecadenoic acid) and α-linolenic (all *cis* Δ9-12-15, octadecatrienoic acid), the primate essential fatty acids. Animals in general have the Δ9, Δ6, Δ5, and Δ4 desaturases and introduce double bonds between the carboxyl group and carbon 9 of a fatty acid. By combining desaturation and chain elongation, animals create two main series of polyunsaturated fatty acids, the ω6 and ω3 (measuring from the methyl end) with chain lengths usually up to C22. Some of the members of these series are precursors for the prostaglandins. These main pathways for desaturation and their products are common to plants and animals and produce the essential fatty acid requirements for all living tissues (see Gurr and James, 1980 and de Gruyter, 1988 for biosynthetic pathways).

Other unsaturated fatty acids occur which do not fit the pattern of the essential pathways, but these are mainly concerned with lipids used as energy stores in plant tissues, particularly the seeds. However, certain fungal mycelial growths can contain a wide range of unusual unsaturated fatty acids such as polyacetylenes. Animals also have fatty stores as energy reserves which may be in adipose tissue, the liver or even muscle, however, the fatty acids in these are typically in keeping with the pathways above. Where differences are found, these are inevitably due to their dietary lipids, for instance branched and odd chain fatty acids in ruminant depot fats. Plants can show a great variety of