# 5 Analytical procedures

## 5.1 Introduction

Quantitative analysis is an important part of lipid science, and developments in this field are often dependent on new and improved analytical procedures. Traditional procedures were essentially chemical in nature. They involved chemical reagents and solvents, were generally labour-intensive and many required gram quantities of material. Some of these still have a place, but increasingly they have been replaced by procedures based on physics rather than chemistry – in particular chromatography and spectroscopy. These are generally quicker, less labour-intensive, more accurate and require less material. Equipment is more sophisticated and more expensive: spectrometers and chromatography systems have largely replaced burettes and pipettes.

This chapter will outline the more commonly used analytical procedures. However, this is not a book devoted only to analysis – other texts concentrate on this topic and the fullest account at the present time is Christie's recent book (2003) and the website that he manages.

## 5.2 Classical analytical procedures

### *5.2.1 Introduction*

Analytical procedures for oils and fats are driven partly by the desire to identify and quantify materials being examined in the research laboratory and partly by commercial demands. Goods are bought and sold according to a specification and there is a need to check that the specification is being met. If goods are to be traded internationally, procedures of analysis must be robust and widely recognised.

Organisations such as those listed below provide similar but not identical directions. In addition there are other national oil/fat organisations that develop analytical procedures. Full details of these tests will not be found in this book but their nature and purpose will be described.

AOAC The Association of Official Analytical Chemists
AOCS The American Oil Chemists' Society

BSI The British Standards Institution
ISO The International Organisation for Standardisation
IUPAC The International Union of Pure and Applied Chemists

Before any test is carried out it is necessary to obtain a representative sample of material and perhaps to transport and store this before any measurement is made. There are standard procedures for all these stages. Attention must be given to the storage temperature, the nature of the container, the inhibition of enzyme activity and the possible addition of antioxidants. Unless care is taken in all these matters, even the most careful analysis will be valueless.

### *5.2.2 Extraction*

Different ways of quantitatively extracting lipid from a sample are available and depend on the nature of the matrix in which the lipid exists. For oilseeds, the oil is generally extracted from crushed seed by the Soxhlet procedure using hexane or a suitable hydrocarbon fraction such as that boiling between 40 and 60ºC. This method provides a sample of oil which can also be used for further tests. Non-destructive methods suitable for routine assessment of many samples are based on NMR (section 5.3.5) or NIR (section 5.3.8). Extraction of oils and fats on an industrial scale is described in section 2.1.

More complex methods are required for biological sources such as a liver or blood, often associated with a high proportion of water. In foodstuffs, lipid is accompanied by protein and/or carbohydrate and these sources may also require special procedures (McLean & Drake, 2002).

Biological samples are extracted with chloroform-methanol according to the well-established methods of Folch *et al*. (1957) or Bligh & Dyer (1959). In the Folch extraction, ground or homogenised tissue is shaken with a 2:1 mixture of chloroform and methanol, and the organic extract is subsequently partitioned with aqueous potassium chloride solution. The combined layers should have a volume ratio of 8:4:3 (chloroform/methanol/water). Extracted lipid is in the (lower) chloroform layer. The Bligh and Dyer method was developed for fish muscle and other wet tissue assumed to contain about 88 g of water in every 100 g of tissue. The tissue (100 g) is homogenised with chloroform (100 ml) and methanol (200 ml) and, after filtering, residual tissue is homogenised a second time with chloroform (100 ml). The two organic extracts are combined and shaken with aqueous potassium chloride (0.88%, 100 ml). After settling the lipid partitions into the lower layer.

Fat in food has been defined in Europe as total lipids including phospholipids and in the United States as fatty acids from monoacylglycerols, diacylglycerols, triacylglycerols, free acids, phospholipids and sterol esters. These assessments have generally been made by extraction with an appropriate

solvent assuming that all lipid is extracted and that the extract is only lipid. There may be problems when lipid is associated with protein or with carbohydrate, therefore modified methods are needed. An alternative method is to hydrolyse the total sample with acid or alkali after adding tri-undecanoin (glycerol ester of 11:0) as internal standard. The resulting fatty acids are extracted, converted to methyl esters, and examined by gas chromatography. The results are converted to triacylglycerol equivalents and expressed as fat.

### *5.2.3 Melting behaviour*

Fats are not pure organic compounds with sharp melting points but mixtures of many individual triacylglycerols, each of which may be solid or liquid. Many fats are plastic solids that deform under pressure as they are mixtures of solid and liquid components. The proportion of these two phases changes with temperature and it is necessary to know the solid/liquid ratio at appropriate temperatures. This is important in assessing the quality of spreading fats and confectionary fats (sections 10.2 and 10.6). The temperature at which solid first appears on cooling is also important in frying oils and in salad oils (sections 10.4 and 10.5).

The 'titre' denotes the solidification point (ºC) of the fatty acids derived from a fat while the slip melting point is the temperature at which a column of fat (10±2 mm), contained in an open capillary tube and immersed in water to a depth of 30 mm, starts to rise.

Of greater value is the measurement of solid fat content by low-resolution $^{1}$H NMR spectroscopy (section 5.3.5). Measurements made at a range of temperatures give a plot of solid content against temperature. The slope of this line and the temperature at which there is no solid phase provide useful information about the melting and rheological behaviour of the sample under investigation.

### *5.2.4 Unsaturation*

Oils and fats contain saturated and unsaturated acids, and many of their properties depend on the ratio of these two acid types. Traditionally, average unsaturation has been measured as the iodine value based on reaction with iodine monochloride (Wijs' reagent) or other mixed halogen under controlled conditions. It is still cited in most specifications relating to oils and fats. However, it has a number of disadvantages and limitations. The measurement is time-consuming, labour-intensive and uses undesirable reagents and solvents. For this reason it is now often calculated from the fatty acid composition determined by gas chromatography using the theoretical iodine values of individual components. The theoretical iodine values of methyl stearate, oleate, linoleate and linolenate are 0, 85.6, 173.2 and 260.3, respectively, based on the function: 25380 $\times$ (number of double bonds) $\div$

$$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$$

**Scheme 5.1** Linoleic acid with two allylic groups and one bis-allylic group.

molecular weight. However the agreement between the observed and the calculated value is not good. Reasons for this include the fact that no allowance is made for unsaponifiable material, which generally contains olefinic compounds and that the GC trace may contain several minor peaks which are ignored. The iodine values of polyunsaturated fatty acids may also be low through incomplete halogenation. An important limitation is that the iodine value does not distinguish between *cis* and *trans* isomers and this information is important when following partial catalytic hydrogenation.

Knothe (2002) has drawn attention to the fact that average unsaturation distinguishes between saturated and unsaturated acids, but does not reflect the important difference in reactivity between monounsaturated and polyunsaturated acids. He has suggested new indices measuring the allylic position equivalent (APE) related to mono and polyunsaturated acids and the bis-allylic position equivalent (BAPE) related to polyunsaturated acids only (Scheme 5.1). These can be determined by gas chromatography or from $^1H$ and $^{13}C$ NMR signals characteristic of each of these acid types (sections 6.2.4 and 6.2.5).

### *5.2.5 Acidity, saponification and unsaponifiable material*

The level of free acid is an item in most specifications for crude and refined oils. It is measured by titration with standard sodium hydroxide solution. The amount of alkali required to hydrolyse (saponify) a fat is a measure of the average chain length of the acyl chains, though this value is affected by unsaponifiable material also present in the oil. This parameter may be reported as 'saponification value' (SV) or 'saponification equivalent' (SE). These are inversely related by the expression SE = 56100/SV. With increasing chain-length, saponification equivalent rises, but saponification value falls. Typical saponification values for some common oils include coconut 248–265, palmkernel oil 230–254, cocoa butter 192–200, palm oil 190–209, cottonseed 189–198, soybean 189–195, sunflower 188–194, corn 187–195, groundnut 187–196, olive 184–196 and rape 182–193.

When a natural fat or oil is hydrolysed, it gives fatty acids (soluble in aqueous alkali), glycerol (soluble in water) and other material (insoluble in aqueous alkali). The latter can be extracted with an appropriate organic solvent and is described as unsaponifiable or non-saponifiable material. It includes sterols, tocopherols, hydrocarbons, long-chain alcohols, etc. There is a growing interest in these compounds and methods of analysing this fraction in more detail are available (section 5.4.4). Unsaponifiable material is

normally less than two per cent of the total oil though sometimes it will be higher. Wax esters, for example, are hydrolysed to long-chain acids and alcohols with the latter being part of the unsaponifiable fraction. Spreads with added phytosterol esters (section 9.8.2) will also have elevated levels of unsaponifiable material.

### *5.2.6 Measurement of oxidative deterioration and of oxidative stability*

In common with other olefinic compounds, oils and fats react with oxygen. The process is complex (section 7.2) and usually undesirable. Two major questions are asked of the analyst in this connection: how far has this sample already been oxidised and how long will this (food) sample last before it is unacceptable, i.e. what is its shelf-life? The first requires a measurement of present status, while the second requires a predictive measurement. The most common oxidative process (autoxidation) occurs with an induction period, during which deterioration is not severe and it is useful for food producers to know the length of this period. Several stages of oxidation can be recognised and tests are available for each stage:

- Primary products of oxidation are allylic hydroperoxides and are measured as peroxide value or as conjugated dienes.
- Secondary products are mainly aldehydes and are measured by the anisidine value.
- Tertiary oxidation products include short-chain acids measured by the Rancimat or oil stability index (OSI) and malondialdehyde measured by the TBA test.

Although oxidative deterioration is most important for goods stored at ambient or refrigerator temperatures, the changes can be accelerated at elevated temperatures. Unfortunately reaction at higher temperature is not always a good predictor of reactions occurring at lower temperatures. The most common method of assessing oxidative status is by measurement of hydroperoxides. These molecules react with acidified potassium iodide to liberate iodine that can be determined volumetrically by reaction with sodium thiosulfate. The value represents mmol of oxygen per 2 kg of fat and this means that in an oil with a peroxide value of 2, around 0.1% of the olefininic molecules have been oxidised. Freshly refined material should have a peroxide value below 1. A fat is considered to be rancid at a peroxide value of 10. Refining destroys hydroperoxides, but it does not regenerate the fat in its original form. Hydroperoxides are converted to aldehydes during refining. Volatile aldehydes are removed during subsequent refining but aldehydes attached to the glycerol moiety remain and can be detected by the anisidine value. Refining an oil that has already been oxidised will therefore reduce the peroxide value but increase the anisidine value. These two measurements may

be combined in a totox value representing the sum of twice the peroxide value plus the anisidine value. The anisidine value is based on the measurement of the intensity of the chromophore at 350 nm produced by reaction of anisidine (4-methoxyaniline, $ArNH_2$) with carbonyl compounds which are mainly 2-enals ($R'CH{=}CHCHO$). This value varies depending on the enals actually present and is therefore only strictly comparable across results for a single oil. An anisidine value of 1 corresponds with around 0.1% of oxidised material.

$$ArNH_2 + OCHCH{=}CHR' \rightarrow ArN{=}CHCH{=}CHR'$$

Early stages of autoxidation can also be detected by measurement of uv absorption at 234 nm resulting from conjugated dienes formed during oxidation of polyunsaturated fatty acids. The method is not suitable for heated fat, for fat that already contains conjugated dienes, and for fats with a high content of oleic acid and consequent low levels of linoleic acid.

The thiobarbituric acid test (TBA) depends on the reaction of this compound with malondialdehde ($OHCCH_2CHO$) formed during oxidative breakdown of polyunsaturated acids.

In the rancimat and omnium oxidative stability measurements, a stream of air is drawn through heated oil (100–140ºC) into a vessel containing deionized water. Short-chain acids – mainly formic – increase the conductivity of the water and the induction period is indicated by the time that elapses before there is a rapid increase in conductivity. These measurements may be of limited value for predicting the stability of a range of oils, but for repeated samples of the same oil they give useful comparative values. They have largely replaced older active oxygen methods (AOM).

In the older accelerated tests (Schaal, active oxygen) the oil or fat is held at a temperature up to 100ºC and the time taken to reach an arbitrary peroxide value is measured. This is taken as an indication of the induction period and hence shelf life under normal storage conditions.

In biological experiments, the presence of short-chain hydrocarbons in breath may be measured. Ethane comes from n-3 acids and pentane from n-6 acids.

Headspace analysis may be carried out in various ways using gas chromatography to separate and identify short chain compounds – mainly aldehydes – formed by decomposition of hydroperoxides. Compounds such as 4-heptanal, and the 2,6- and 3,6-nonadienals are considered to be the most significant flavour notes, but many of the volatile materials have little sensory effect.

## 5.3 Present-day analytical techniques

### *5.3.1 Chromatography and spectroscopy*

Most lipid analytical systems are now based on the powerful separating ability of a chromatographic procedure. Those of greatest interest are thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC). These will be described in the following sections and their use in specific lipid analyses will be reported in section 5.4.

Chromatography was first developed as a means of separating coloured compounds – hence its name – but with more sophisticated methods of following the separation, this technique can now be applied to the separation of a wide range of mixtures. Christie (2003) identifies several modes of chromatography in which a solute mixture is separated into its components by partition between a stationary and a mobile phase.

- Adsorption using mainly silica as stationary phase and a mobile phase consisting of either a single solvent (isocratic elution) or a mixture of two solvents of differing polarity (gradient elution).
- Normal-phase liquid-partition.
- Liquid-liquid or gas-liquid partition. In such systems the more polar phase is stationary (usually a liquid held on to a solid or a surface) and the less polar phase (liquid or gas) is mobile. In reversed phase chromatography, the less polar phase is stationary.
- Complexation chromatography with silver ions. Silver salts are incorporated into the stationary phase and silver ions react reversibly with pi electrons in unsaturated solutes. The strengths of these interactions vary with several structural features in the solute molecules and even small and subtle differences are sufficient to achieve useful separations. In some cases, the silver ions are bound to silica-based ion-exchange materials.
- Gel-permeation or size exclusion chromatography. Separation is based mainly on the size and shape of the solute molecules. Larger molecules elute before smaller ones, which are more easily trapped in the pores of the adsorbent.
- Chiral-phase chromatography. By incorporating a chiral molecule into the stationary phase it is possible to separate enantiomeric compounds. Alternatively, the enantiomers can be converted to diastereisomers with sufficiently different physical properties to be separated by more conventional chromatographic techniques.

All these separations result from differing molecular interactions between the several components of the solute and the mobile and stationary phases. With lipids, these interactions generally arise from (weak) polar forces.

Important spectroscopic procedures include ultraviolet and infrared spectroscopy, mass spectrometry (see Christie website), and nuclear magnetic

resonance spectroscopy (see Christie website). They are described further in sections 5.3.5, 5.3.6 and 5.3.8 and their use is illustrated in section 5.4.

### *5.3.2 Thin-layer chromatography (TLC) and related chromatographic systems*

In thin-layer chromatography a layer of adsorbent – usually silica – is held on a flat glass, metal or plastic surface. The mixture to be separated is placed as a spot or a streak, 1.5–2.0 cm from one edge of the plate. The plate is then placed upright in a closed glass jar or tank containing the developing solvent up to a level below the spot. The solvent rises up the plate through capillary attraction and carries the various components of the mixture to different heights thus effecting a separation. The solvent is often hexane alone or hexane with increasing proportions of diethyl ether, but other solvents and solvent mixtures can be used. If the spot is placed near a corner of the plate it is possible to develop it in one direction and then turn the plate through 90º and develop again in a second solvent (2D-TLC). When separation is complete the plate can be treated with material that will render the lipids visible. Typical examples are of such reagents are iodine vapour and 2′7′-dichlorofluorescein. Separation on a silica layer will depend on the polarity of the solutes. For example, 1- and 2-monoacylglycerols, 1,2- and 1,3-diacylglycerols and triacylglycerols can all be separated as can the various classes of phospholipids. Also, esters of the common fatty acids can be separated from those with an oxygenated function. These separations can be carried out on an analytical or semi-preparative scale.

Other separations are possible if an appropriate chemical is incorporated into the silica layer. Older examples include borates and arsonates to separate polyhydroxy compounds according to the number of hydroxyl groups, their position in the acyl chain and their stereochemistry. Better known in this connection is the use of silver nitrate. Argentation or silver ion chromatography is widely used to separate *cis* and *trans* isomers and esters according to the number of double bonds. Examples are given in sections 5.4.1 and 5.4.2.

Solid phase extraction (SPE) frequently involves the use of small commercial chromatography columns made of impermeable plastic material, which is generally pre-packed with about 0.5 g of a variety of adsorbents. Silica columns are most widely used by lipid analysts though those chemically bonded with octadecylsilyl groups (ODS) have many non-lipid applications. Columns packed with silica are used for lipid class separation as an alternative to TLC. They are also useful in the study of clinical samples to separate cholesterol (that might interfere with gas chromatography) from methyl ester. Columns with bonded phenylsulfonic groups can be converted to their silver ion forms (section 5.3.2).

Christie (2003) has described a useful procedure in which short ion-exchange solid-phase extaction columns are charged with $AgNO_3$-$CH_3CN$-$H_2O$. Esters (0.25 mg) with 0–6 double bonds are eluted with solvents ranging from dichloromethane to acetone-acetonitrile (60:40). The quantities are small but sufficient for further examination by GC and/or MS.

### *5.3.3 High-performance liquid chromatography (HPLC)*

HPLC involves chromatography with solvent mixtures as mobile phase and microparticles (3–10 micron) of silica- or alkyl- (most often $C_{18}$) bonded silica as stationary phase. In normal (straight) phase HPLC the stationary phase is polar (silica) and the mobile phase is non-polar. Reversed-phase HPLC refers to (non-polar) columns with alkyl-bonded silica and polar solvent mixtures. HPLC can also be used in the silver ion mode where the silver ions are bonded to the stationary phase. The most widely-used columns are 250 mm long with a 4–5 mm internal diameter and can be used in an analytical or semi-preparative mode. With careful use they have quite a long life.

Appropriate injection systems and high-pressure pumps are also required. Detection systems depend on UV absorption, differential refractometry, fluorescence, and evaporative light scattering. Each system has its limitations in respect of solute, solvent, sensitivity, linearity of response and must be selected carefully for each type of analysis. HPLC can be used for separations that can be achieved by TLC and *vice versa.* Examples of the use of HPLC are found in sections 5.4.1, 5.4.2 and 5.4.3.

Size exclusion chromatography (SEC) or gel permeation chromatography (GPC) effect separation by molecular weight and are useful for the separation of lipid polymers which are eluted before triacylglycerols and before free acids.

### *5.3.4 Gas chromatography (GC)*

Gas chromatography is the analytical procedure most widely used by lipid analysts. It is employed mainly to separate and quantify component acids in the form of their methyl esters. This efficient separation procedure is based on partition chromatography where the stationary phase is usually coated on the inner wall of a fused silica capillary tube 10–100 metres in length and is liquid, at least at the temperature at which it operates. This phase may be non-polar, weakly polar, or highly polar. The gas phase is usually nitrogen, helium or hydrogen in order of increasing resolving power. The column is heated to a range of temperatures, limited only by the thermal stability of the stationary phase and of the analyte. Elution is slower at lower temperatures, but separation is improved and it may be necessary to make a compromise between time of elution and efficiency of separation. Separation is monitored with a flame ionisation detector that is remarkable for its robust nature and for

its linear response over a wide range of concentration. If the analyte contains volatile components that co-elute with the solvent or non-volatile components that are not eluted, then it is desirable to add an internal standard so that absolute rather than relative values can be measured. The separation is carried out at constant temperature (isothermal) or according to a prearranged programme during which the temperature is gradually raised. Lipids can be examined in many forms and the column can be connected to a mass spectrometer (section 5.3.6).

The column allows partitioning of the separate constituents in the analyte between the stationary phase as a thin film on the inner surface of the capillary column and the mobile phase (gas). The components of the analyte travel down the column and are eluted after different times (retention time) depending on the proportion of time spent in the stationary and mobile phases. The efficiency of a chromatographic system depends on the nature and flow rate of the carrier gas, column dimensions, liquid phase thickness and column temperature. These parameters should be optimised within practical constraints such as the time that can be given to each analysis.

### *5.3.5 NMR spectroscopy*

Low-resolution $^{1}$H NMR spectroscopy is used to determine the ratio of solid and liquid phases in a plastic fat. This is important in chocolate manufacture and in the understanding of melting behaviour in spreads (sections 10.2 and 10.6). Low resolution NMR has almost completely replaced the older method of dilatometry to measure solid fat content. The percentage of solids is given by the expression 100 (hydrogen nuclei in the solid phase) ÷ (all the hydrogen nuclei in the sample). These two types of hydrogen environment can be distinguished by observation of the relaxation signal. The signal for hydrogen atoms in solids decays quickly – less than one per cent remains after 70 $\mu$sec – while that from liquids decays very slowly requiring about 10 000 sec. There are practical reasons why measurements cannot be made at the instant of the pulse and are usually made after 10 $\mu$sec (SS + SL) and after 70 $\mu$sec (SL only). Because some of the SS signal will have already decayed after 10 $\mu$sec the observed value has to be corrected by a factor determined by calibration of the system using samples of solid plastic (35–70%) in liquid paraffin.

These measurements require only about six seconds and are used routinely for the study of spreads and confectionery fats. However, fats needing polymorphic stabilisation such as cocoa butter have to be equilibrated before measurements are made and a tempering routine requiring up to about 40 hours has been described.

By further adaptation the NMR system can be modified to distinguish between oil and moisture and it is possible to measure the oil and moisture content of around 1000 samples of seeds per day.

The use of high resolution NMR spectroscopy is described in sections 5.4.1–5.4.3. $^{1}H$, $^{13}C$ and $^{31}P$ all find application and the technique is also discussed in sections 6.2.4 and 6.2.5.

### *5.3.6 Near-infrared and Fourier transform infrared spectroscopy*

The near-infrared region of the spectrum, composed of overtones and combinations of the fundamental bands, was considered unimportant until developments in computing made it possible to exploit this information. Near-infrared reflectance spectroscopy (NIRS), based on commercial instruments, is now used extensively in agriculture and beyond. It is used, for example, to determine the content of moisture, protein and fat in a batch of seeds. Its use has been extended to the determination of fatty-acid composition and this may be carried out on a single seed. This is of great benefit in breeding programmes. The procedure is rapid, non-destructive and involves neither sample-grinding nor chemical modification. Calibration equations based on a large number of samples are required, but instruments from different laboratories can be integrated in a network with calibration equations developed on a master instrument and then used in all the satellite instruments in the network.

Fourier transform infrared (FTIR) spectroscopy has advanced dramatically in recent years and is now being used as an alternative way of measuring several important properties for lipid analysts. An FTIR spectrometer can record the entire infrared spectrum in one second and this can be added to many other scans through a fast Fourier transform algorithm to produce a conventional infrared absorption spectrum. Such spectra based on interferometry have several advantages over spectra from more conventional dispersive instruments. There is a marked improvement in signal to noise ratio, higher energy throughput, superior resolution and greater wavelength accuracy through the use of an internal laser. Undiluted edible oils are particularly suited to FTIR analysis as they are liquid, easy to handle and have relatively simple spectra. Preliminary calibration is necessary to convert spectral information into useful data and once this is available the system may be used to measure parameters such as *cis-trans* ratios, iodine value, saponification number, free acid content, peroxide value, and anisidine value. Details are available on the website <www.agrenv.mcgill.ca.foodsci.irg.irhomepg.htm>

### *5.3.7 Mass spectrometry*

When a molecule (M) is bombarded with electrons of sufficient energy in the vapour phase at low pressure, the molecule becomes ionised to give a molecular ion ($M^{+}$). At greater electron energies, the ionised molecules fragment, usually in several ways, to give one uncharged particle (A) and one

positively charged particle ($B^+$). The mass spectrometer separates the charged particles ($M^+$ and $B^+$) according to their mass/charge ratio (*m/z* where *z* is usually 1). With high-resolution instruments, this value can be measured with such accuracy as to indicate the molecular formula of each ion.

$$M \underset{-2e}{\overset{+e}{\rightarrow}} M^+ \rightarrow A + B^+$$

Modifications of the spectroscopic system either inhibit fragmentation (soft techniques) so that that the molecular ion dominates or promote fragmentation. In the former case, the results can be used quantitatively to determine the relative amounts of all the molecular ions produced from the mixture. Enhanced fragmentation assists in structural identification. Sometimes it is desirable to bleed an inorganic ion into the system to promote ionisation.

The traditional form of mass spectrometry operates through electron impact (EI). Modifications include:

- APCI (atmospheric pressure chemical ionisation) which is a mild ionisation technique.
- MS-MS (tandem mass spectrometry). Molecular ions are separated and then fragmented to form daughter ions. These are separated and monitored in another part of the instrument.
- FAB (fast atom bombardment). Complex molecules not considered sufficiently volatile for study by the normal procedures are bombarded in an inert matrix with heavy atoms such as argon. Secondary ions are released from the matrix for MS examination.
- MALDI-TOF (matrix-assisted laser desorption/ionisation linked to a time-of-flight mass spectrometer). This involves direct injection of the lipid sample in an appropriate matrix.
- Electrospray ionisation is a mild but sensitive ionisation process used with compounds of high molecular weight. It gives information on molecular weight primarily, but in combination with tandem mass spectrometry a full structural determination may be possible. There are many applications to phospholipids and glycolipids. This technique is the basis of the new studies of lipidomics which require a large number of analyses without any chromatography.

The identifying ability of the mass spectrometer has been coupled with the separating ability of GC and HPLC to provide powerful techniques to analyse complex lipid mixtures both quantitatively and qualitatively. This requires the selection of derivatives that give good separation in the GC system and good fragmentation in the MS system (section 5.4.2). However, increasingly samples are being analysed without the chromatographic step using computers to analyse the complex spectra obtained.

### *5.3.8 Enzymatic procedures*

One challenge to lipid analysts is to distinguish between the fatty acids in the *sn*-1, 2 and 3 positions in triacylglycerols or between the *sn*-1 and 2 positions in phospholipids. This has been achieved through the use of lipases or phospholipases which effect deacylation stereospecifically. Because these reactions are frequently accompanied by acyl migration, the experimental instructions must be followed meticulously if meaningful results are to be obtained. Some examples are detailed in section 5.4.2.

## 5.4 Lipid analysis

Lipid samples isolated from a plant or animal system and available in mg to kg quantities can be examined at various levels of complexity:

- General properties of the sample such as iodine value, level of free acid, state of oxidative deterioration, solid/liquid ratio, etc. (section 5.2).
- Recognition of the proportion of different lipid classes such as acylglycerols, phospholipids and sphingolipids.
- Component acid analysis of the total lipid or of individual lipid classes.
- Further information on triacylglycerols such as separating fatty acids in the *sn*-1 and 3 positions from those in the *sn*-2 positions, separating triacylglycerols with different double bond numbers or measuring levels of molecular species.
- This last can be examined at various degrees of refinement. For example, there are six isomeric triacylglycerols, each containing one mol of palmitic, oleic and linoleic acid. The analyst has to decide how far these are to be distinguished.

It is necessary to appreciate the potential complexity of the problem. If a wax ester contains five acids and five alcohols, then these can be combined to give 25 possible esters. The situation with individual phospholipid classes is similar. Phosphatidylcholines with five component acids can exist in 25 different forms, so that a full analysis would involve the recognition of all these in both qualitative and quantitative terms. With triacylglycerols, the situation is even more complex. With five different acids there could be 125 ($5^3$) triacylglycerols, though some could be present at zero or very low levels. These figures become even larger: 10 acids could exist in 1000 triacylglycerols. Many of the triacylglycerols are isomeric and the researcher and analyst have to consider what level of information is required. There may also be a conceptual problem. If all 125 compounds were identified quantitatively would it be possible to grasp the significance of the information? The analyst would probably group the components or focus only on the major individuals.

The chromatographic and spectroscopic procedures used to conduct these analyses have been outlined (section 5.3) and the following sections provide a brief account of several types of lipid analysis.

### *5.4.1 Fatty acids*

Component acid analysis is the most widely practised analytical technique in lipid science. In its most common form, mixed triacylglycerols are converted to methyl esters and these are separated on an appropriate GC column. Modern chromatographic systems will provide a trace, retention times and an area percentage based on total eluted material (see Fig. 5.1). Careful attention to a number of parameters is necessary to get accurate results. Unless specially calibrated, the system will not identify the esters present in each peak. This has to be done by the analyst. For the most frequently-occurring esters this can be achieved by comparison with standards derived from oils of known composition or with mixtures available from lipid suppliers in qualitative or quantitative mixtures.

When closely related methyl esters are not separated (sometimes called critical pairs) then the GC conditions should be modified. The column should be replaced by one of differing polarity or the chromatogram can be run at a lower temperature. In the latter case, separation is improved but elution will

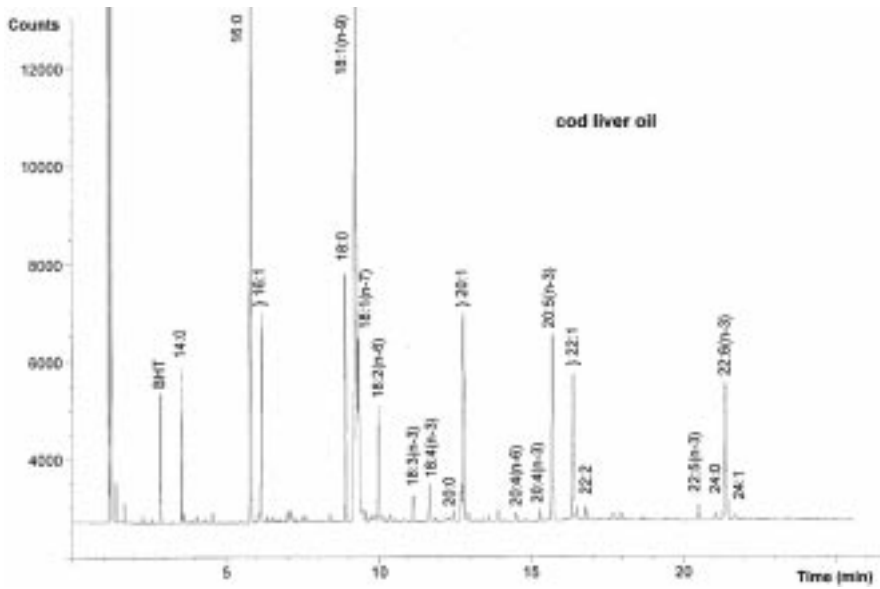



**Figure 5.1** GC separation of the methyl esters derived from cod liver oil by gas chromatography using a CP-Wax 52CB with hydrogen as mobile phase. The vertical axis is Counts and the horizontal axis is Time (min). Copied with permission of the author and the publisher from Christie (2003).

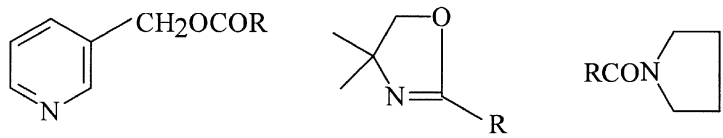



**Figure 5.2** Picolinate, dimethyloxazoline and pyrrolidide from the carboxylic acid RCOOH.

take longer. Sometimes replacing methyl esters with isopropyl esters will give better resolution. Ethyl, butyl and 2-methoxyethyl esters have also been used. For short-chain acids, the methyl esters may be too volatile, and butyl or decyl esters can be used in their place.

For GC-MS, the derivatives must be chosen to give good separations on the GC column and to give mass spectroscope information. The most favoured derivatives are N-containing compounds such as:

- picolinyl esters made by acylation of picolinyl alcohol either with fatty acid and 1,1′-carbonyldiimidazole or with acid chloride (Figs 5.2 and 5.3),
- pyrrolidides made by interaction of pyrrolidine and free acid or methyl ester,
- 4.4-dimethyloxazolines (DMOX) formed by reaction of 2-amino-2-methyl-1-propanol with free acid, ester or acid chloride (Figs 5.2 and 5.3).

Fatty acids are identified by comparison of GC retention time with standard materials, but GC-MS is the surest method of structural identification (section 5.3.6). Useful separations can also be made using silver ion chromatography in TLC or HPLC systems. By these means, *cis* and *trans* isomers are easily separated. The Δ6c, Δ9c and Δ11c-18:1 isomers can also be separated by silver ion chromatography.

By a combination of silver ion TLC and GC it is possible to separate *cis* and *trans* isomers, first as groups and then as individual isomers. Because there is incomplete separation of *cis* and *trans* isomers by GC alone, results obtained without the preliminary silver ion separation underestimate the content of *trans* isomers by an average margin of 35 per cent. Detailed analyses of this type show that while dairy fats and partially hydrogenated vegetable fats both contain a range of *trans* 18:1 acids, they vary markedly in the relative proportions of the different isomers.

More sophisticated methods of fatty-acid analysis can also be carried out by regiospecific and stereospecific analysis which provide information about the distribution of acyl chains at the various glycerol hydroxyl groups (section 5.3.2).

Interesting developments in GC are apparent in its application to biotechnology. Plant breeders may need to analyse hundreds or thousands of samples for fatty-acid composition and this can only be achieved by careful management and by automation of standard chromatographic procedure.

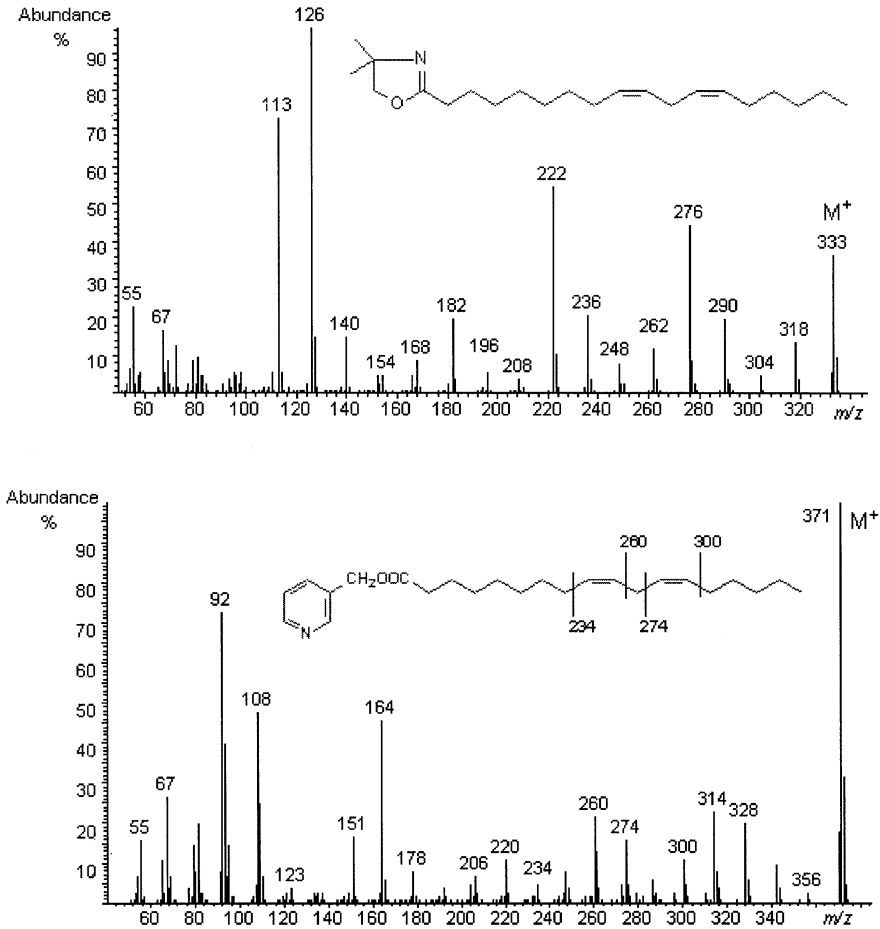



**Figure 5.3** Mass spectra of linoleic acid as DMOX derivative and as picolinate. Downloaded with permission from <www.lipid.co.uk>.

Individual seeds or small samples must be extracted, converted to methyl esters, chromatographed and the results monitored. In one project, all samples are bar-coded and are typically presented in 25-well breeder trays adapted so that seeds cannot mix. The seeds are crushed with a 25-pin pestle tool and a robotic system then carries out the passage from crushed seed to production of an ester solution placed in an autosampler vial. Each GC oven has two injectors, two columns, and two detectors.

Using high-resolution $^{1}$H NMR spectroscopy it is possible to determine the level of DHA in an oil sample and to measure total n-3 acids. In both cases ethylene glycol dimethyl ether can be used as an internal standard. DHA and

other acids with Δ4 unsaturation have characteristic signals at 2.38 ppm for the four hydrogen atoms on $C_2$ and $C_3$ while acids without Δ4 unsaturation have a signal at 2.28 ppm for the two $C_2$ hydrogen atoms. The proportion of n-3 acids can be determined from the $CH_3$ signal. This is at 0.81–0.89 ppm for all n-3 acids and at 0.90–0.98 ppm for all other acids/esters.

### *5.4.2 Acylglycerols*

The following description applies mainly to triacylglycerols, but the methods can usually be adapted to examine monoacylglycerols, diacylglycerols and phospholipids. In several of the procedures to be described, the original sample is separated into a number of fractions for independent examination. In producing a final composition it will be necessary to know the proportion of each fraction. When these are too small to be weighed appropriate information can be obtained by adding an internal standard to each fraction. Subsequent GC analysis of the methyl esters will then allow the relative weight of each fraction to be determined.

The first step in analysing triacylglycerol mixtures is to determine the fatty acid composition by GC as described in the previous section. If for no other reason, this information may be used at the end of a triacylglycerol analysis to check that the data are reliable. If, for example, a mixture has been separated into several fractions, then the fatty-acid composition of these, suitably weighted, should add up to that of the original mixture. If they do not then the analysis is faulty.

Procedures for distinguishing the acyl groups in the two $\alpha$ positions from those in the $\beta$ position provide a regiospecific analysis. Those that distinguish all three positions provide a stereospecific analysis. Both give insight into the nature of the mixed triacylglycerols beyond that provided by GC of the total esters, but neither gives complete information on molecular species. Some authors have used their data to calculate levels of individual molecular species based on assumptions concerning the association of acids in these differing positions. But these assumptions are not always justified.

In regiospecific analysis, GC gives information on the distribution of fatty acids between the primary OH groups of glycerol (*sn*-1 and 3) and the secondary OH group at the *sn*-2 position. This limited distinction can be made with a 1,3-specific enzyme such as pancreatic lipase. The mixed triacylglycerols in a tris buffer are shaken with calcium chloride, bile salt and pancreatic lipase for 2–4 minutes at 40ºC. The reaction is stopped by addition of ethanol and 6M HCl, and total organic products are extracted with diethyl ether. The product is separated by TLC and the recovered 2-monoacylglycerols are converted to methyl esters for examination by GC. This gives the composition of the fatty acids in the *sn*-2 position. These values can be subtracted from the composition of the original triacylglycerols to give the

$$GlOCOR + EtMgBr \longrightarrow GlOH + RC(Et)_2OH$$

$$TAG \longrightarrow DAG\ (1,2\text{-},\ 2,3\text{-}\ \text{and}\ 1,3) \longrightarrow MAG\ (1\text{-},\ 2\text{-},\ \text{and}\ 3\text{-})$$

**Scheme 5.2** Reaction of triacylglycerol with EtMgBr to give tertiary alcohols and deacylated triacylglycerols as detailed in the second line.

combined composition of the 1 and 3 positions. These may be the same but it must not be assumed that they will be.

Alternative procedures for conducting regiospecific analysis of triacylglycerols are based on non-specific partial deacylation of glycerol esters with the Grignard reagent EtMgBr in a reaction of less than one minute. This is combined with chromatographic methods for separating the partially deacylated products (monoacylglycerols and diacylglycerols) from each other and from the tertiary alcohol also formed. The experimental conditions must be selected to keep acyl-migration to a minimum. The separated glycerol esters are subject to further detailed chromatographic analysis in various ways (Scheme 5.2).

In one procedure, two monoacylglycerol fractions (*sn*-1/3 and *sn*-2) are isolated and each is converted to methyl esters for GC examination. The results compare favourably with those obtained by pancreatic lipase.

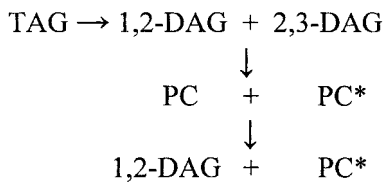
The acyl (C-1) signals in a $^{13}C$ NMR spectrum are also useful in that their chemical shifts depend on the position of the double bond closest to the carboxyl group and on whether the acyl chain is in the *sn*-1/3 positions or the *sn*-2 position. This makes it possible, for example, to compare the distribution of $\Delta 4$ acids (virtually only docosahexaenoic acid) and $\Delta 5$ acids (eicosapentaenoic acid and/or arachidonic acid) between the 1/3 and 2 positions The appropriate chemical shifts are detailed in section 6.2.5.

In stereospecific analysis, the reaction with EtMgBr has been extended to determine the fatty acid composition at all three of the glycerol positions. This has been achieved in two ways based on the conversion of a mono- or diacylglycerol fraction to a urethane by reaction with an isocyanate.

$$\text{Mono or diacylglycerol (ROH)} + R'NCO \rightarrow \text{urethane (ROCONHR}')$$

Takagi and his colleagues (Christie, 2003) react the mixed $\alpha$-monoacylglycerols with 3,5-dinitrophenylisocyanate, producing two enantiomeric sets of bisurethanes which are then separated on a chiral column. Each set is finally converted to methyl esters for GC analysis.

Christie (2003) avoids the need for a chiral column by reacting an enantiomeric isocyanate [S-(+)-1-(1-naphthyl)ethyl isocyanate] with the mixed 1,2- and 2,3-diacylglycerols. In this way the enantiomeric diacylglycerols are converted to two groups of diastereoisomeric urethanes which can be separated by silca-HPLC. Each group can then be converted to methyl esters for normal GC analysis.




**Scheme 5.3** Stereospecific analysis of triacylglycerols.
TAG = triacylglycerols, DAG = diacylglycerols, PC = natural phosphatidylcholines based on 1,2-diacylglycerols, PC* = unnatural phosphatidylcholines based on 2,3-diacylglycerols.

Older methods of stereospecific analysis exploit the selectivity of an appropriate phospholipase. As an example, one method involves reaction with EtMgBr for 25 seconds, isolation of the mixed 1,2- and 2,3-diacylglycerols, and reaction of this mixture with choline chloride to produce phosphatidylcholines. Those based on 1,2-diacylglycerol are identical to natural phosphatidylcholines and are hydrolysed to the diacylglycerols in the presence of phospholipase C in two minutes. In contrast, 2,3-diacylglycerols give a set of enantiomeric phosphatidylcholines which are not natural substrates for phospholipase and react much more slowly, requiring a reaction time of four hours. After a short reaction, it is possible to separate diacylglycerols (from the natural phosphatidylcholines) from unreacted unnatural phosphatidylcholines. It is also necessary to carry out a lipolysis of the original triacylglycerols with pancreatic lipase. Appropriate fractions are converted to methyl esters for GC examination and the composition of the fatty acids attached to each of the glycerol hydroxyl groups can be calculated by suitable arithmetical manipulation (Scheme 5.3).

As an alternative, the mixture of natural and unnatural phosphatidylcholines described above can be deacylated in the presence of phospholipase A2. This will give a lyso-phosphatidylcholine and fatty acid from the 2-position from the natural phosphatidylcholine only.

Because of the reliability of the flame ionisation detector it would be good to analyse triacylglycerol mixtures by gas chromatography, but this technique is not widely used for this purpose. Apart from the milk fats and the lauric oils with their short- and medium-chain acids the low volatility of the triacylglycerols require that the columns be run at high temperatures, usually close to their experimental limits. GC is therefore not widely used to analyse triacylglycerols except in a few limited cases. It has been used to analyse palm oil and cocoa butter and its alternatives in terms of their carbon numbers. Palm oil for example, can be characterised by the content of its glycerol esters having 48, 50, 52, and 54 carbon atoms. These numbers are the sum of the carbon atoms in the three acyl chains and do not include the three glycerol carbon atoms. The $C_{48}$ fraction contains three $C_{16}$ chains, the $C_{50}$ fraction contains two $C_{16}$ and one $C_{18}$ chain and so on. The $C_{18}$ chain will be mainly

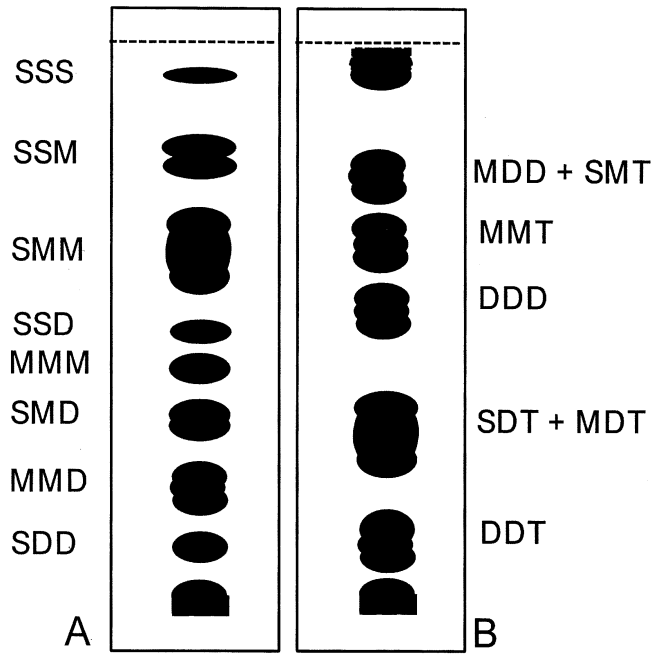



**Figure 5.4** Separation of soybean triacylglycerols by silver ion chromatography using chloroform-methanol at a 99:1 ratio for plate A and 99:6 for plate B. S, M, D and T relate to saturated, monoenoic, dienoic and trienoic acyl chains, respectively. The order does nor reflect positional distribution. Copied with permission of the author and the publisher from Christie (2003).

oleate but may be stearate or linoleate so these fractions refer to groups of triacylglycerols and not to individual compounds. These techniques were developed with packed columns. When short capillary columns are used, the groups are still apparent but each is further divided so that the $C_{52}$ peak, for example, becomes four peaks corresponding to POSt, POO/ PLSt, PLO and PLL, where P = palmitic, St = stearic, O = oleic and L= linoleic. Each of these three letter symbols includes all the possible isomers with the three acyl chains indicated.

Silver ion chromatography with thin layer chromatography or HPLC can be used effectively to separate triacylglycerols according to their degree of unsaturation. With improved procedures bands or peaks, containing for example, three double bonds, are further separated into triacylglycerols, such as MMM, DMS and TSS where S, M, D and T represent saturated, monoene, diene, and triene acyl chains. Since interaction between silver ions and double bonds is not linearly related to the number of unsaturated centres, it is not always easy to interpret a silver ion chromatogram and it may be necessary to investigate the fractions further (Figs 5.4 and 5.5).

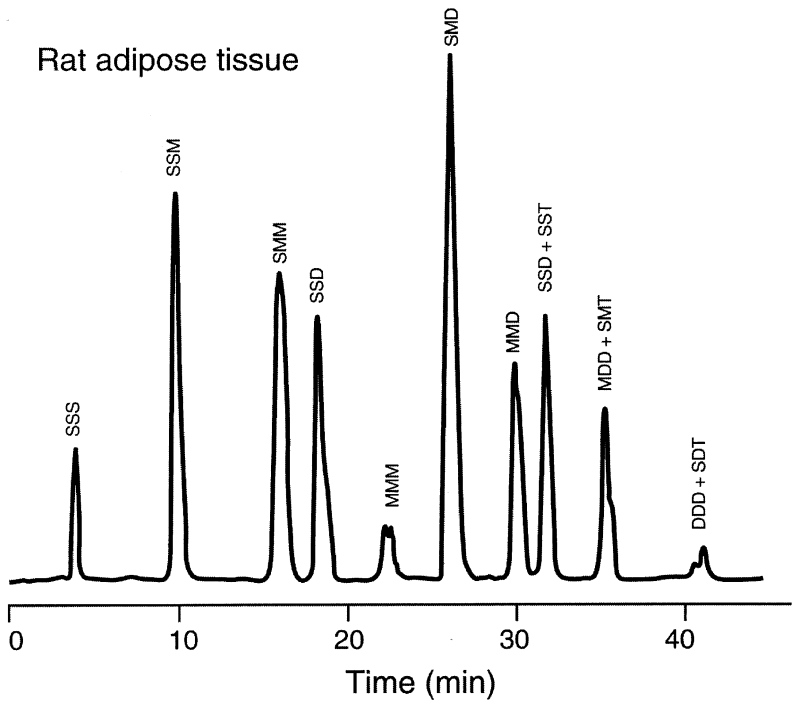



**Figure 5.5** Separation of triacylglycerols from rat adipose tissue by silver ion HPLC. S, M, D and T relate to saturated, monoenoic, dienoic and trienoic acyl chains respectively. The order does not reflect positional distribution. Copied with permission of the author and the publisher from Christie (2003).

RP HPLC provides one of the best methods of analysing triacylglycerol mixtures, but here again, recognition of peaks requires experience and/or further examination of fractions. This is because separation depends on the partition number (PN), related to both chain length, carbon number (CN) and the number of double bonds (db). The simple expression PN = CN – zdb (z = 2) is only of limited value because the claim that z = 2 is only approximate. For example, based on the acids 12:0 to 18:0 and 16:1, 18:1, 18:2, and 18:3 there are over 20 triacylglycerols with a partition number of 44 which can be separated from each other. The following examples, taken from the complete group, are eluted in the order given: OLL (54:5), OOLn (54:5), LaOO (48:2), PPLn (50:3) MStLn (50:3) MMP (44:0), where L = linoleic, La = lauric, Ln = linolenic, M = myristic, O = oleic and P = palmitic. An example of RP HPLC is given in Fig. 5.6.

Mass spectrometry can be used in conjunction with GC or HPLC to identify the triacylglycerol component present in a separated peak, but it is also possible to obtain valuable analytical information from mass spectrometry alone using tandem mass spectrometry (MS-MS). This provides information

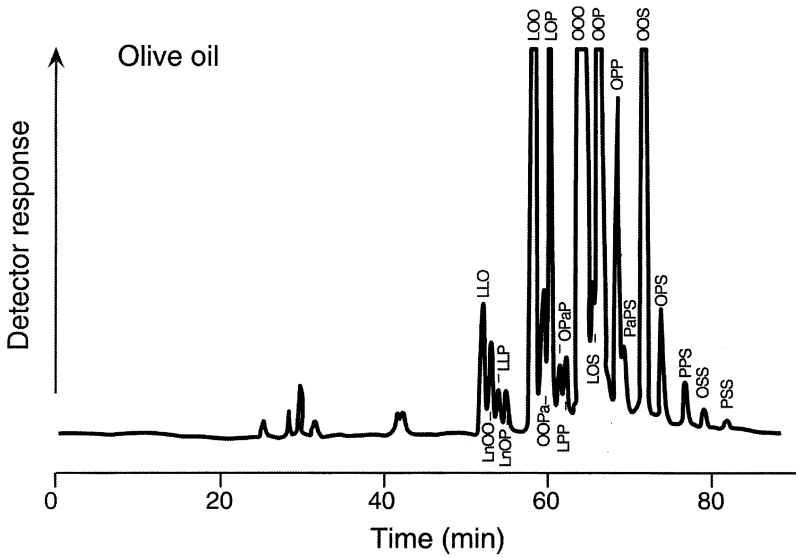



**Figure 5.6** Separation of olive oil triacylglycerols by reversed phase HPLC, L, Ln, O, P, Pa and S stand for the acyl chains of linoleic, linolenic, oleic, palmitic, palmitoleic and stearic acid respectively. Copied with permission of the author and the publisher from Christie (2003).

on the size of individual molecular species, the nature of the attached acyl groups, and distinguishes acyl groups at the *sn*-2 position from those in the *sn*-1/3 positions. One example is concerned with the triacylglycerols in human milk fat (Currie & Kallio, 1993). Ammonia negative ion chemical ionisation MS gave 29 molecular ions $[M\text{-}H]^-$ of molecular weight 666.7–886.7, with 38–54 carbon atoms, and 0–4 double bonds. In addition, 28 fatty acid molecular weights were recognised. The composition of each $[M\text{-}H]^-$ cluster was determined from a study of the $[RCOO]^-$ daughter ions and the distribution of acyl groups between the *sn* 1/3 and *sn*-2 positions from a study of the $[M\text{-}H\text{-}RCOOH\text{-}100]^-$ fragments. The most abundant glycerol ester is the symmetrical 1,3-dioleate 2-palmitate, making up about 10 per cent of the total.

Increasingly, triacylglycerols are being studied through RP HPLC followed by atmospheric pressure chemical ionisation (APCI). The spectrum from each separated glycerol ester contains four significant peaks: the molecular ion peak $[M+H]^+$ and three diacylglycerol peaks corresponding to the loss of one acyl group. For example, peaks at 879, 622 [M-256] 600 [M-278] and 574 [M-304] indicate a triacylglycerol containing 16:0, 18:3 and 20:4. Identification is not too difficult but procedures for good quantitation need to be improved.

In the MALDI-TOF spectrum each triacylglycerol appears as a sodiated molecular ion $(M + 23)^+$ which can be identified from the fatty acids present in the oil. A distinction is not made between isomers, and response factors

have to be measured to make the results quantitative. Fatty acids can also be identified in this way but there are simpler ways of doing this.

### *5.4.3 Phospholipids*

In studying the phospholipids present in an oil or biological extract, interest may lie in total phospholipid, individual phospholipid classes, or the molecular species present in the sample. At one time total phospholipids were determined by measuring the level of phosphorous by classical methods and multiplying that by a factor to express phospholipid level. This has been replaced by separation of lipid classes by TLC or HPLC. Phospholipid class analysis is achieved by normal HPLC using a silica column and appropriate mixed polar solvents. For molecular species separation a reversed-phase HPLC system is used with mixed polar solvents. Phospholipids can also be determined by $^{31}$P NMR since the phosphorus atom in each phospholipid class gives a distinct signal. For quantitative examination triphenyl phosphate is used as a standard.

### *5.4.4 Minor components (sterols and tocopherols)*

Sterols in free and/or esterified form are present in vegetable and animal fats at levels of 0–3 per cent but in products with added phytosterols this may rise to 13 per cent. Lipids of animal origin contain mainly the zoosterol cholesterol, but lipids from plant sources contain phytosterols (mainly sitosterol, campesterol, stigmasterol and avenasterol, section 1.6.3). Sterols can be studied at three levels: total content of sterols after hydrolysis, proportion of free and esterified sterols, analysis of individual sterols in free sterols, esterified sterols or total sterols.

Individual sterols are determined by gas chromatography of the unsaponifiable fraction using the free sterols or their trimethylsilyl ethers. To assist in quantification an internal standard such as betulin or $\beta$-cholestanol may be added to the analyte. If sterol esters and free sterols are to be distinguished these must be separated by silica chromatography before hydrolysis, eluting sterol esters first with a less polar solvent and then free sterols with a more polar solvent.

The sample may be hydrolysed or subjected to methanolysis and total sterols subsequently separated from other components by TLC. Individual sterols are then determined by gas chromatography as such (ROH) or as trimethylsilyl ethers ($ROSiMe_3$). Fast methods of GC have been devised for routine analysis. It is usually necessary to add appropriate internal standards to obtain quantitative information.

Gel permeation chromatography (GPC) can be used without saponification to separate sterols, sterol esters, squalene and tocopherols and each class of

compounds can be examined further by gas chromatography. Appropriate internal standards are added for each class.

Up to four tocopherols and four tocotrienols (section 1.6.4) may be present in a lipid sample. For analysis, the sample may be saponified when the tocopherols concentrate along with sterols in the unsaponifiable fraction, but saponification is not always necessary. Individual tocopherols can be separated by gas chromatography when the order of elution is $\alpha$-tocopherol, $\alpha$-tocotrienol, $\beta$-tocopherol, $\beta$- tocotrienol, $\gamma$-tocopherol, $\gamma$-tocotrienol, $\delta$-tocopherol and $\delta$-tocotrienol (though there is some doubt about $\delta$-tocotrienol). Tocopherols can also be separated from one another by HPLC and are best measured with a fluorescence detector or with a coulometric electrochemical array detector. In this method saponification is not necessary because of the high sensitivity of the fluorescence detector.

## Bibliography and references


Adlof, R. (2003) *Advances in Lipid Methodology – Five*, The Oily Press, Bridgwater.

Bligh, E.G. & Dyer, W.J. (1959) A rapid method for total lipide extraction and purification, *Canad. J. Biochem. Physiol.* **37**, 911–917.

Christie, W.W. (2003) *Lipid Analysis – Isolation, Separation, Identification and Structural Analysis of Lipids*, The Oily Press, Bridgwater.

Christie, W.W. (1992) *Advances in Lipid Methodology One*, The Oily Press, Dundee.

Christie, W.W. (1993) *Advances in Lipid Methodology Two*, The Oily Press, Dundee.

Christie, W.W. (1996) *Advances in Lipid Methodology Three*, The Oily Press, Dundee.

Christie, W.W. (1997) *Advances in Lipid Methodology Four*, The Oily Press, Dundee.

Christie, W.W. & (in part) Gunstone, F.D. <www.lipid.co.uk>

Currie, G. J. & Kallio, H. (1993) Triacylglycerols of human milk: rapid analysis by ammonia negative ion tandem mass spectrometry, *Lipids,* **28**, 217–222.

Folch, J., Lees, M. & Sloane, S.G.H. (1957) A simple method for the isolation and purification of total lipids for animal sources, *J. Biol. Chem.*, **226**, 497–509.

Knothe, G. (2002) Structure indices in fatty acid chemistry. How relevant is the iodine value? *J. Amer. Oil Chem. Soc*., 79, 847–854.

Knothe, G. (2003) Quantitative analysis of fatty compounds by $^{1}$H-NMR, *Lipid Technology*, **15**, 111–114.

McLean, B. & Drake, P. (2002) Review of methods for the determination of fat and oil in foodstuffs, Campden and Chorleywood FRA Group, Review No 37, 1–52.

FTIR spectroscopy <www.agrenv.mcgill.ca.foodsci.irg.irhomepg.htm>.