Analytical Parameters

4.1 Introduction

Those working in the food industry need to have some knowledge of lipid analysis. Some properties will be detailed in a specification, others relating to starting materials or food products may have to be measured in-house or in an external laboratory. In whatever form the information comes and from whatever source it comes the food scientist needs to know what it signifies and whether the information provided is acceptable or not. Standardised and widely-accepted analytical procedures are preferred to methods that are not generally practised in other laboratories. If goods are to be traded internationally analytical procedures must be robust and widely recognised. What follows is not a detailed account of these procedures but rather an outline of methods and the value of results so obtained. Other texts concentrate on this topic and the fullest account at the present time is Christie's recent book (2003) and his website *The Lipid Library*.

Traditional procedures of analysis were essentially chemical in nature. They involved chemical reagents and solvents, they were generally labour-intensive, and often 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 Some of these still have a place but increasingly they have been replaced by procedures based on physics rather than chemistry, particularly chromatography and spectroscopy. These latter are generally quicker, less labour-intensive, more accurate, and require less material. However, equipment is more sophisticated and more expensive. Spectrometers and chromatography systems have largely replaced burettes and the older type of glass pipettes.

Organisations such as those listed below provide similar but not identical directions.

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AOAC The Association of Official Analytical Chemists
AOCS The American Oil Chemists' Society
BSI The British Standards Institution
ISO The International Organization for Standardization
IUPAC The International Union of Pure and Applied Chemists

European Pharmacopoeia, 5th edition, Council of Europe, Strasbourg, 2004

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 temperature of storage, 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.

4.2 Oil content

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 other suitable hydrocarbon fraction such as that boiling between 40°C and 60°C. This method provides a sample of oil that can also be used for further tests. Non-destructive methods suitable for routine assessment of many samples are based on near infrared (NIR, Section 4.8) or nuclear magnetic resonance (NMR, Section 4.9). Extraction of oils and fats on an industrial scale is described in Section 3.1.

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

Biological samples are extracted with chloroform–methanol according to the well-established methods of Folch *et al*. (1957) or of Bligh and Dyer (1959) as described by Christie (2003). 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 and modified methods are needed. An alternative method is to hydrolyse the total sample with acid or alkali after adding triundecanoin (glycerol ester of undecanoic acid – 11:0) as internal standard. The resulting fatty acids are extracted, converted to methyl esters, and examined by gas chromatography (GC). The results are converted to triacylglycerol equivalents and expressed as fat (McLean and Drake, 2002).

4.3 Unsaturation – iodine value

Oils and fats contain saturated and unsaturated acids and many of their properties depend on the ratio of these two types of acids. Traditionally, average unsaturation has been measured as the iodine value based on chemical reaction with iodine monochloride (Wijs' reagent) or other mixed halogen compound under controlled conditions. The value is still cited in many 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 iodine value is now often calculated from the fatty acid composition determined by GC using the theoretical iodine values of individual components.

The calculated iodine values of methyl stearate, oleate, linoleate, and linolenate are 0, 85.6, 173.2, and 260.3 based on the function:

$$25380 \times (\text{number of double bonds}) \div \text{molecular weight}$$

However, the agreement between observed and calculated values is not good because (a) no allowance is made for unsaponifiable material which generally contains olefinic compounds and (b) the GC trace may contain minor peaks which are unidentified and ignored. Also, measured iodine values of polyunsaturated fatty acids may 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) from monounsaturated and polyunsaturated acids and the bis-allylic position equivalent (BAPE) from polyunsaturated acids only. These can be determined by GC or from ^{1}H and ^{13}C NMR signals characteristic of each of these acid types (Sections 4.9 and 4.10). This suggestion has not been widely applied but the principle remains valid (see the concept of oxidisability in Section 6.2).

4.4 Saponification – free acids, sap value

The level of free acid is listed in most specifications for crude and refined oils. It is measured by titration with standard sodium hydroxide solution and may be expressed as acid value (mg KOH required to neutralise 1 g of fat) or as percentage of free fatty acid. For most oils free fatty acid is expressed as oleic acid and is equal to the acid value divided by 1.99 (usually rounded to 2.0). Free acids present in crude oils are largely removed during the refining processes and the acid value should be below 0.1%.

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 numbers are inversely related by the expression SE = 56,100/SV. The SE is the average molecular weight of all the acyl chains. With increasing chain length, SE rises but SV falls. Typical SVs for some common oils include coconut 248–265, palm kernel 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. High values are associated with the two lauric oils and the lower values with oils rich in C_{16} and C_{18} acids.

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). This last can be extracted with an appropriate organic solvent (hexane or diethyl ether) 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 chromatographic and/or spectroscopic methods of analysing this fraction in more detail are available. Unsaponifiable material is normally less than 2% of the total oil though sometimes it will be higher. Wax esters are hydrolysed to long-chain acids and alcohols and the latter will be part of the unsaponifiable fraction. Spreads with added phytosterol esters (Sections 7.7 and 8.3) will also have elevated levels of unsaponifiable material.

4.5 Melting behaviour, solid fat content, low-temperature properties

Fats are not pure organic compounds with sharp melting points but mixtures of many individual triacylglycerols each of which may be solid or liquid at ambient temperature. Most spreads are plastic solids that deform under pressure as during the spreading operation because they are mixtures of solid and liquid components. The proportion of these two phases varies with temperature and it is frequently necessary to know the solid/liquid ratio at a range of selected temperatures. This is important in assessing the quality of spreading fats and confectionary fats (Sections 8.3 and 8.7). The temperature at which solid first appears on cooling is also important in frying oils and in salad oils (Sections 8.5 and 8.6). The

'titre' denotes the solidification point (°C) 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. This is a useful low-temperature property (see also Sections 5.1–5.3).

Of greater value is the measurement of solid fat content by low-resolution (pulse) ^{1}H NMR spectroscopy (Section 4.9). The percentage of solid determined by pulse-NMR is based on the ratio of the response from the hydrogen nuclei in the solid phase and that from all the hydrogen nuclei in the sample. 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.

4.6 Oxidation – peroxide value, anisidine value, stability, shelf life, stability trials, taste panels

In common with other olefinic compounds oils and fats react with oxygen. The process is complex (Section 6.2) and usually undesirable. Two major questions are asked of the analyst in this connection: how far has the sample already been oxidised and how long will the (food) sample last before it is unacceptable? (*i.e.* What is its shelf life?) The first requires a measurement of present status while the latter requires a predictive measurement. The most common oxidative process is autoxidation. This occurs with an induction period during which deterioration is not severe and it is useful for food producers to know the length of this. Several stages of oxidation are 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 formed during oxidation of PUFA.
- Secondary products are mainly unsaturated aldehydes and are measured by the anisidine value.
- Tertiary oxidation products include short-chain acids measured by the Rancimat or oil stability index (OSI) or malondialdehyde measured by the TBA test.

Though knowledge of 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 measured volumetrically by reaction with sodium thiosulphate. The value represents mmol of oxygen per 2 kg of fat and this means that ~0.1% of the olefinic molecules in an oil have been oxidised when the peroxide value is 2. Freshly refined material should have a peroxide value below 1. A fat is considered to be rancid at a peroxide value exceeding 10. Refining destroys hydroperoxides but it does not regenerate the fat in its original form. Hydroperoxides are cleaved to aldehydes during refining. While volatile aldehydes are removed during subsequent refining short-chain aldehydes attached to the glycerol moiety remain (sometimes called core aldehydes) and can be detected by the anisidine value. Refining an oil that has already been oxidised will therefore reduce the peroxide value but the anisidine value will not be reduced to zero. 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 arising from molecules of the type $ArN{=}CHCH{=}CHR'$ produced by reaction of anisidine (4-$MeOC_6H_4NH_2$ represented as $ArNH_2$) 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 type of oil. An anisidine value of 1 corresponds with ~0.1% of oxidised material.

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

Early stages of autoxidation can also be detected by measurement of ultraviolet absorption at 234 nm resulting from conjugated dienes formed during oxidation of polyunsaturated fatty acids. This method is not suitable for heated fat, for fat that already contains conjugated dienoic acids, nor for fats with a high content of oleic acid and consequent low levels of linoleic acid (Table 6.2).

$$CH_3(CH_2)_3\mathit{CH_2}CH = CH\mathbf{CH_2}CH = CH\mathit{CH_2}(CH_2)_6COOH$$

Figure 4.1 Linoleic acid with two allylic groups (italic) and one bis-allylic group (bold).

In the Rancimat and Omnium Oxidative Stability measurements a stream of air is drawn through oil heated at 100–140°C into a vessel containing de-ionised water. Short-chain acids – mainly formic (HCOOH) – 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 was held at a temperature up to 100°C and the time taken to reach an arbitrary peroxide value was measured. This was 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 (C_2H_6) comes from omega-3 acids and pentane (C_5H_{12}) from omega-6 acids.

Headspace analysis may be carried out in various ways using GC to separate and identify short-chain compounds – mainly aldehydes – formed by decomposition of hydroperoxides. Compounds such as 4-heptenal, 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.

The ultimate assessment of food flavour and texture is achieved by taste panels. These are discussed by Malcolmson in Shahidi (2005).

4.7 Gas chromatography

Lipid analysis is now dominated by chromatographic and spectroscopic procedures based on physics rather than chemistry. Procedures are increasingly automated with results available in electronic form. Some companies outsource their analyses of oils

and fats to specialist laboratories that have dedicated instruments and wide expertise.

Some chromatographic procedures (thin layer chromatography TLC, high-performance liquid chromatography HPLC, silver ion chromatography) and the mass spectrometric (MS) techniques that may be associated with them are more likely to be found in the research laboratory than in the quality control laboratory.

The most widely used property of any fat is its fatty acid composition. This indicates what fatty acids are present and at what level and is universally determined by GC of the methyl esters derived from the triacylglycerols. Minor components (sterols, sterol esters, and tocopherols) can also be determined by appropriate GC procedures. In considering fatty acid composition attention has to be given to the procedures for preparing the methyl esters, to the GC conditions, and to the ways in which the results are presented.

Triacylglycerols are easily converted to methyl esters by base-catalysed transesterification. This involves reaction with excess of methanol containing sodium methoxide and is complete in a few minutes at 50°C. It may be necessary to use a co-solvent such as toluene and antioxidant is generally added to protect the unsaturated esters throughout the analysis. This method works well with refined oils of low acidity but free acid is not esterified under alkaline conditions and when present, as in crude extracted oils, methyl esters are more generally made by acid-catalysed esterification and transesterification using methanol and hydrogen chloride, sulfuric acid, or boron trifluoride along with co-solvent and antioxidant. These may be combined in a process involving alkaline hydrolysis followed by acid-catalysed esterification. Special methods may be necessary when the oil contains fatty acids with acid-labile functional groups such as epoxide or cyclopropene.

GC is employed to separate and quantify component acids in the form of their methyl esters. This efficient separation procedure is based on partition chromatography in which the stationary phase is usually coated on the inner wall of a fused silica capillary tube 10–100 m in length and is liquid at the temperature of analysis. This phase may be non-polar, weakly polar, or highly polar. The gas phase is usually nitrogen or 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. Rapid procedures for GC separation have been described and are required when very large numbers of samples have to be examined. 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. The separation may be carried out at constant temperature (isothermal) or according to a prearranged programme during which the temperature is gradually raised. With automatic injection equipment can be organised to operate overnight without manual intervention.

The column allows partitioning of the separate constituents of the analyte between the stationary phase as a thin film on the inner surface of the capillary column and the mobile phase (gas). The individual 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 are optimised within practical constraints such as the time that can be given to each analysis.

The peaks in the chromatogram are identified on the basis of their elution time in comparison with data obtained from standard mixtures of esters under identical chromatographic conditions. If there is any doubt about the identification it may be necessary to repeat the GC with a column of differing polarity or to combine the GC separation with MS for structural identification.

The simplest way of reporting the results is to normalise all peaks (*i.e.* to express the area of each peak as a percentage of the total area under all peaks). Results should then total 100.0 even if some of the smaller peaks have not been identified with certainty. The GC may fail to detect volatile impurities (present in the uncounted solvent peak) and components which are not eluted during the time given to the analysis. These include oxidised and polymerised impurities and some minor components in the oil. Increasingly therefore results are expressed as milligram/gram. These can be determined with the help of an internal standard – usually the triacylglycerol of an odd-chain acid with 11, 17, 19, or 23 carbon atoms – which has to be added in a weighed amount to the fat being analysed. The internal standard is then subject to the same chemical reactions and extraction procedure as the sample being examined. The results should approach a total of 1000 but are more likely to be around 900.

4.8 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 much used 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 used as an alternative way of measuring several properties important 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. 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 and in the paper by Tseng and Wang (2007).

With developments in MS procedures the mass spectrometer itself is used as a quantitative instrument. The charge for individual peaks is compared with total ion current (Section 4.10). The MS

procedure has the added advantage of providing structural information about the material in the peak. This is in contrast to FID measurement where structure can only be inferred (though with a large measure of certainty in routine analyses).

4.9 ^{1}H NMR spectroscopy

^{1}H NMR spectroscopy is used in two ways in the study of lipids. With wide-line (low resolution or pulsed) instruments it is possible to determine the proportion of solid and liquid in a fat and the content of oil in a seed. High-resolution spectrometers, on the other hand, are used to examine solutions and give information about the solute, which may be an individual compound or a mixture, such as a natural oil or fat. Solids can also be examined when the spectrometer is used in 'magic angle' mode.

Low-resolution ^{1}H NMR or time-domain NMR is much used in quality control laboratories for the measurement of solid fat content, simultaneous determination of oil and moisture content, the study of oil and water droplet size distribution, and measurements that can be made through packaging. The technique has been reviewed by Meeussen in Hamilton and Cast (1999), Todt *et al*. in Dobson (2001), and Knothe (2003).

The ratio of solid and liquid phases in a fat is important in chocolate manufacture and in the understanding of melting behaviour in spreads (Sections 8.3 and 8.7). 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:

$$100\ (\text{hydrogen nuclei in the solid phase}) \div (\text{all the hydrogen nuclei in the sample})$$

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 1% remains after 70 μs – while that from liquids decays very slowly requiring about 10,000 s. There are practical reasons why measurements cannot be made at the instant of the pulse and are usually made after 10 μs (S_S+S_L)

and after 70 μs (S_L only). Because some of the S_S signal will have already decayed after 10 μs 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 6 s 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 h 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.

High-resolution spectroscopy, on the other hand, is used to examine solutions and gives information about the solute, which may be an individual compound or a mixture, such as a natural oil or fat.

A typical ^{1}H spectrum is shown in Figure 4.2. It contains signals that can be distinguished by chemical shift, coupling constant, splitting pattern, and area. The last of these provides quantitative information that can be presented as mol % in contrast to GC data given in wt %. The remaining parameters give structural information (Diehl in Dobson, 2001; Knothe, 2003; and *The Lipid Library*).

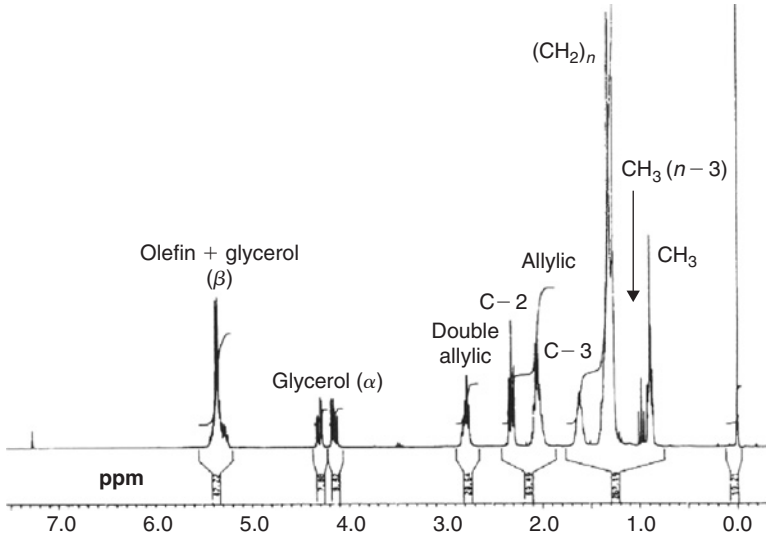

Figure 4.2 Typical ^{1}H NMR spectrum of a vegetable oil.

A saturated long-chain methyl ester has five signals with the following chemical shifts (ppm), number of hydrogen atoms, and splitting pattern:

•	CH_3	0.90	3	triplet
•	$(CH_2)_n$	1.31	$2n$	broad (many overlapping signals)
•	$-C\underline{H}_2CH_2COOCH_3$	1.58	2	quintet
•	$-CH_2C\underline{H}_2COOCH_3$	2.30	2	triplet
•	$-CH_2CH_2COOC\underline{H}_3$	3.65	3	singlet

Such a spectrum indicates the presence of a straight-chain saturated methyl ester but does not distinguish between homologues in a mixture. In olefinic esters there are additional signals corresponding to the:

- olefinic hydrogen atoms ($-C\underline{H}=C\underline{H}-$ 5.35 ppm, 2H for oleate, 4H for linoleate, 6H for linolenate)
- allylic hydrogen atoms ($-C\underline{H}_2CH=CHC\underline{H}_2-$ 2.05 ppm, 4H)
- doubly allylic hydrogen atoms ($=CHC\underline{H}_2CH=$ 2.77 ppm, 2H for linoleate, and 4H for linolenate)
- hydrogen atoms of the end methyl group of omega-3 esters produce a triplet at 0.98 ppm which is distinguished from the usual triplet at 0.90 ppm.

Glycerol esters have five hydrogen atoms associated with the glycerol unit. There is a one-proton signal at 5.25 ppm ($C\underline{H}OCOR$) overlapping with olefinic signals and a four-proton signal split between 4.12 and 4.28 ppm ($C\underline{H}_2OCOR$).

For vegetable oils containing the usual mixture of saturated acids and C_{18} unsaturated acids useful information can be obtained by ^1H NMR procedures that are non-destructive and require no chemical reactions. The signal at 2.30 ppm (α-methylene function) provides a measure of all the acyl groups. The signals at 0.90 and 0.98 ppm distinguish linolenate (omega-3) from all other esters. Signals at 2.77 ppm are a combined measure of triene (linolenate) and diene (linoleate) and those at 2.05 ppm relate to all of linolenate, linoleate, and oleate. The intensity of these signals can be used to calculate the composition (mol %) in terms of oleic, linoleic, linolenic, and total saturated acids but the results are less

accurate than those obtained by GC. The accuracy of this procedure is limited because the values are not determined independently but are dependent on each other. For example, an error in measuring omega-3 trienes will introduce errors in the subsequent assessment of diene and monoene esters.

4.10 ^{13}C NMR and ^{31}P NMR spectroscopy

^{13}C NMR spectra are based on natural ^{13}C atoms present at a level of 1.1% in organic compounds. The spectra provide two kinds of information: the chemical shift of each signal (up to 50 signals in a natural mixture of triacylglycerols) and their relative intensities. The former is of qualitative value and permits identification of important structural features. The latter, with appropriate safeguards, provides quantitative information of analytical value. Chemical shifts may vary slightly with concentration of the solution under study and (rather more) with the solvent employed. Most measurements are made with solutions of about 1M concentration and $CDCl_3$ is the solvent most commonly used. Other solvents include $(CD_3)_2SO$, C_6D_6, and mixtures of CD_3OD and $CDCl_3$. Figure 4.3 shows a typical spectrum for a vegetable oil.

In using ^{13}C NMR data (chemical shifts and intensities) the first step is to assign as many of the chemical shifts as possible. If the substance under study is a mixture, many individual signals

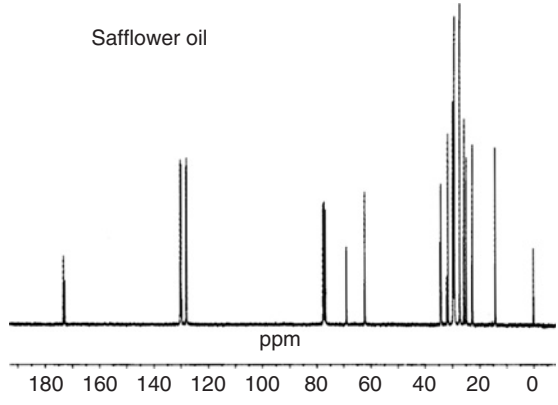

Figure 4.3 ^{13}C NMR spectrum of safflower oil.

will appear as clusters. This makes interpretation more difficult but eventually provides additional information. It is wise to ignore signals in the methylene envelope (29.4–29.9 ppm) resulting from mid-chain carbon atoms that are not greatly influenced by nearby functional groups. Instead, examine the easily recognised shifts (in ppm) associated with the following carbon atoms ω1 (around 14.1), ω2 (22.8), ω3 (32.1), C-1 (174.1), C-2 (34.2), C-3 (25.1), glycerol (68.9 and 62.1), olefinic (127–132), and allylic (27.3 and 25.6).

The chemical shift of a carbon atom depends on its total environment to a distance of six or more atomic centres. For example, in glycerol trioleate the signals for the olefinic carbon atoms (C-9 and C-10) differ from one another and also, to a small extent, on whether the oleate is an α or β chain (attached to primary or secondary glycerol hydroxyl groups). The C-1 signal is also slightly different for saturated and Δ9 unsaturated chains. In these examples the difference is produced by structural changes up to 11 atomic centres away. This makes the spectrum more complex but also more informative when all the chemical shifts have been assigned.

In another example, the methyl groups at the end of the acyl chains in glycerol tripalmitate give one signal at about 14.1 ppm well separated from other signals and hence easily recognised. The difference between α and β chains for this signal in this molecule is too small to be observed but in a vegetable oil, containing saturated and unsaturated chains the peak at 14.1 ppm appears as a cluster of two or more signals. Each is indicative of a different environment for the methyl group and may result from omega-3, omega-6, or omega-9 acyl chains where the closest double bond affects the chemical shift of the methyl signal.

The signals for the acyl carbon atoms (C-1) in triacylglycerol mixtures appear as a complex cluster. One group of signals around 173.1 ppm and a second group around 172.7 ppm correspond to acyl carbon atoms in the (two) α-position and the carbon atom in the β-position, respectively, with peak areas in the ratio 2:1. These shifts do not differ greatly between saturated esters and those having unsaturation starting at the 9 position as in oleate and linoleate. However, different carbon shifts are observed when the double bond gets close to the acyl function as in Δ4 acids (DHA), Δ5 (EPA and AA), and Δ6 (petroselinic, γ-linolenic, and stearidonic). This makes it possible to determine the proportion of each of these

acids in the α and β positions and this is now used in studies on fish oils which characteristically contain some of these acids (*European Pharmacopoeia*, 2005, 2006; Curtis in Breivik, 2007).

To obtain quantitative data attention has to be given to the protocol for obtaining the spectrum. In particular, the problem of relaxation has to be overcome either by adding a relaxation agent such as $Cr(acac)_3$ (chromium acetonylacetonoate) and/or by including a delay time between successive scans of the spectrum. This will add to the time required to collect the spectrum. Spectrometers now available operate at a frequency for ^{13}C of 68 MHz or more and spectra are generally obtained using an NOE-suppressed (nuclear Overhauser effect), inverse-gated, proton-decoupled technique. Exciting pulses have a 45–90° pulse angle and acquisition times (including delay times) are generally 1–20 s per scan. The number of scans is usually 1000 or more. The sample size for a routine ^{13}C NMR spectrum is normally 50–100 mg and the spectrum is obtained in 20–30 min. With smaller samples high-quality spectra can be obtained with as little as 1 mg but with a correspondingly longer acquisition time.

Assignments of chemical shift are often made on the basis of available knowledge. Existing information has been built up over the past 30 years assisted by the study of ^{2}H-containing compounds and the use of chemical shift reagents. Where the necessary information is not available more advanced spectroscopic procedures will assist assignment. This can also be made on the basis of line-width and relaxation measurements. Easily recognised carbon atoms present in most triacylglycerols have been cited above. This provides enough information to make a preliminary assignment to the signals in a spectrum such as that of safflower oil (Gunstone, *The Lipid Library*).

From the peak areas of appropriate signals the average number of double bonds per triacylglycerol molecule and the average molecular weight can be calculated and hence the iodine value (excluding unsaponifiable material). These are based on signals at 24.85 (C-3, a measure of total acyl chains), 25.62 (L11, a measure of linoleic acid), 27.15 (O8, O11, L8, L14, monoenes, and dienes), and the multiplet at 29.45 ppm (mid-chain methylenes).

^{31}P NMR spectroscopy is used in the analysis of phospholipids. The phosphorus atom in each phospholipid class (PC, PE, PS, PI, etc.) gives a distinct signal and it is possible to determine the proportion of each phospholipid type using triphenyl phosphate as a standard when quantitative results are needed (Diehl, 2002).

4.11 Mass spectrometry

MS is a procedure used to determine the structure of individual molecules. Originally these had to be isolated by standard methods but it is now more usual to combine the mass spectrometer with GC or HPLC so that individual components of a mixture are separated by chromatography and identified by MS. If the compound is already known then its mass spectrum can be compared with that already reported and contained in a data bank (Christie, *The Lipid Library*). If the compound is novel it should be possible to identify it by application of the basic principles of MS.

When a chromatographic separation precedes MS then it is also desirable to quantify the data so that the proportion of each molecular species is also known. This is usually achieved by measurement of the total ion current but accurate quantification requires calibration with standards or the use of isotopic internal standards. In the combined GC–MS procedures it is also necessary to select derivatives that combine ease and completeness of preparation with good chromatographic properties (satisfactory separation under simple GC or HPLC conditions) and good spectroscopic properties (molecular and/or fragment ions that lead to easy recognition of the molecule). This last may require a selection of the appropriate spectroscopic procedure.

When a molecule is ionised (electronically or chemically) it forms a molecular ion (M^+). This may fragment to give one ionised (A^+) and one unionised (B) particle and a mass spectrometer is a device for producing and examining the charged particles. These are separated according to their mass to charge ratio (*m/z,* where *z* is usually one). With high-resolution instruments this value can be measured with such accuracy as to indicate the molecular formula of each ion. The intensity of each peak is related to that of the base peak (largest) which is given a value of 100.

$$M \rightarrow M^+ \rightarrow A^+ + B$$

Electron ionisation (EI) has been the most widely used ionisation technique in the past. This occurs through an exchange of energy between electrons emitted by a glowing filament (usually at 70 eV) and vaporised sample molecules. Under these conditions the molecular ion usually fragments in one or more ways which can

be interpreted in terms of the stability of the various atom-to-atom bonds in the ion.

Chemical ionisation (CI) results from gas phase reactions between a small amount of sample and a large amount of reactant gas (such as methane, ammonia, or isobutene) itself ionised by EI producing reactant gas ions (CH_5^+, NH_4^+, $C_4H_9^+$). CI is usually 'softer' than EI with the consequence that more of the molecular ion is available for detection and fragmentation is less extensive. This generally makes interpretation simpler. The following CI techniques are used by lipid analysts:

- Atmospheric pressure chemical ionisation (APCI)
- Fast atom bombardment (FAB)
- Collision-induced dissociation (tandem mass spectrometry, MS/MS).

For the structural identification of fatty acids, MS procedures linked to GC or HPLC have replaced the older classical methods. MS was first carried out on methyl esters but this is not very satisfactory because under EI the double bonds migrate along the chain and cannot be located unequivocally. Several methods of 'fixing' the double bond were devised but only one of these, applied mainly to monoene esters, remains in use. For both mono- and polyunsaturated acids the methyl esters have been replaced by other acid derivatives that give useful structural information. Appropriate fatty acid derivatives are now generally examined by EI and triacylglycerols by one of the CI methods.

Olefinic esters react with dimethyldisulphide (MeSSMe) and iodine to give a bis(methylthio) derivative the mass spectrum of which shows a molecular ion and two or three large fragment ions that together clearly indicate the position of the SMe groups and hence of the double bond.

$$RCH{=}CHR' \rightarrow RCH(SMe)CH(SMe)R'$$

For example, methyl oleate gives a molecular ion at 390 ($C_{21}H_{42}O_2S_2$) and three large fragment ions at 173 ($C_9H_{18}SMe$), 217 ($C_{10}H_{18}O_2SMe$), and 185 (217–32 through loss of methanol). These clearly show that methyl oleate is Δ9–18:1 but do not indicate the configuration of the double bond. However, *cis* and *trans* monoenes form *threo* and *erythro* adducts, respectively and although these have similar mass spectra they are separated by

GC. The procedure is less satisfactory with polyunsaturated acids which are better examined in other ways.

Polyunsaturated acids are now usually examined as picolinyl esters or as 2-alkyl-4,4-dimethyloxazoles (DMOX). These compounds have the structures indicated in Figures 4.4 and 4.5. When these molecules are ionised the charge is carried on the nitrogen atom and double-bond ionisation and isomerisation are minimised. Radical-induced cleavage occurs evenly along the chain and gives a series of relatively abundant ions of high mass resulting from the cleavage of each C−C bond. When a double bond or other functional group is reached then diagnostic ions appear.

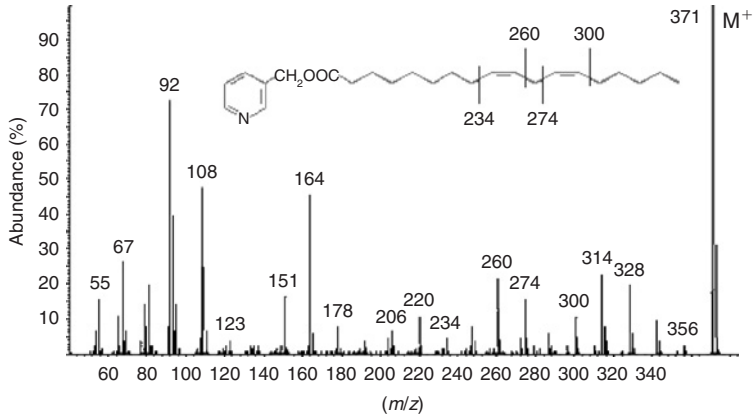

Figure 4.4 Mass spectrum of linoleic acid as the picolinate (*Source*: Downloaded with permission from MS files, www.lipid.co.uk).

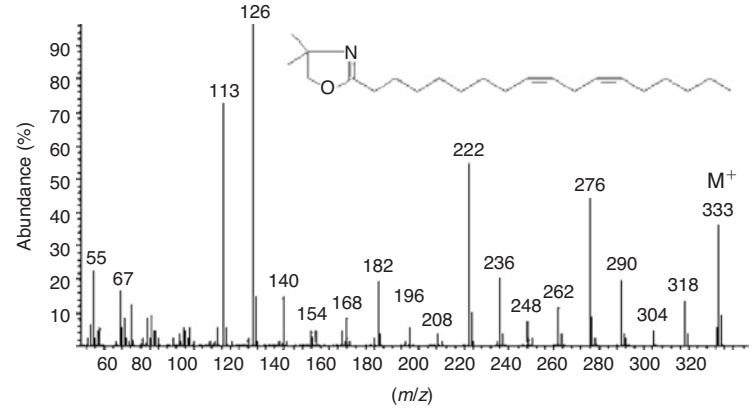

Figure 4.5 Mass spectrum of linoleic acid as the DMOX derivative (*Source*: Downloaded with permission from MS files, www.lipid.co.uk).

The picolinyl esters are made from the free acids and picolinyl alcohol either via the acid chloride (formed by reaction with oxalyl chloride) or through interaction with 1,1′-carbonyldiimidazole in dichloromethane in the presence of 4-pyrrolidinopyridine as catalyst. Another method involves interesterification of triacylglycerols or phospholipids with potassium *tert*-butoxide and 3-hydroxymethylpyridine for 2 min at room temperature.

The spectrum shows some fragments of low mass characteristic of picolinates resulting from $ArCH_2^+$ (93), $ArCH_2O^+$ (108), $ArCH_2OC(OH)=CH_2^+$ (151), and $ArCH_2OC(O)=CH_2^+$ (164) where Ar is C_5H_4N or C_5H_5N. In addition there is a molecular ion peak and a series of other high mass fragments which, correctly interpreted, will indicate a structure for the picolinate. For example the ester from linoleic acid (Figure 4.4) has peaks at 371 (M^+, the 18:2 picolinate which is a C_{24} compound), 356 (M^+-15), and a number of fragments with lower *m/e* values. Most of these ions differ by 14 mass units from their neighbour representing loss of CH_2 but something different happens between 300 and 274 and between 260 and 234 where there is a loss of 26 mass units (C_2H_2 representing a $-CH=CH-$ unit). These fragments indicate the presence of double bonds at Δ9 and Δ12.

DMOX derivatives are made by heating the lipid with 2-amino-2-methyl-1-propanol in a nitrogen atmosphere at 180°C (2 h for free acids, 18 h for methyl or glycerol esters). Their spectra show peaks at 113 and 126 common to all DMOX derivatives along with a molecular ion and a series of fragments differing by 14 mass units except that some pairs differ by only 12 mass units. The latter are indicative of olefinic centres and are interpreted according to the statement: 'if there is an interval of 12 mass units between the most intense peaks of clusters of ions containing *n* and $n-1$ carbon atoms then there is a double bond between carbon $n+1$ and *n* in the acyl chain'. Spectra of DMOX derivatives of many acids are available on Christie's website.

MS procedures, combined with a chromatographic separation system, also give valuable insight into the structure and composition of triacylglycerol mixtures such as milk fats, vegetable oils, and fish oils. In general, identification depends on molecular ions that define the number of both carbon atoms and double bonds in each triacylglycerol molecule. In addition, fragment ions indicate the nature of each acyl group in terms of its number of carbon atoms and unsaturated centres and in some cases will define the distribution

of fatty acyl residues between the primary (*sn*-1/3) and secondary (*sn*-2) glycerol positions. Quantitative determination of mixtures is still a problem because the MS responses of triacylglycerols vary with the molecular structure. This topic is intensively reviewed by Laakso and Manninen in Hamilton and Cast (1999) and by Laakso in Dobson (2002).

Reverse phase HPLC followed by APCI MS gives a molecular ion $(M+H)^+$ and fragment ions corresponding to M–RCOOH. For example, the StLO fraction gives peaks at 885.6 $(M+H)^+$, 605.4 $(StO)^+$, 603.5 $(StL)^+$, and 601.4 $(OL)^+$. Among the diacylglycerols, the 1,3 isomer is less intense than the 1,2 and 2,3 isomers and this makes it possible to identify the fatty acid at the 2 position.

Mass spectrometry of lipids has also been reviewed by Christie (1998 and *The Lipid Library*), by Laakso and Manninen and by Roach *et al*. in Hamilton and Cast (1999), and by Dobson and Christie, Laakso, and Korachi *et al*. in Dobson (2002).