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Plant Lipids: Generalities

1. GENERALITIES

Lipids are natural substances, esters of fatty acids and alcohols or polyols. They are constituents of cell structures such as membrane phospho- and glycolipids, coating elements such as waxes or cutins, and also reserve substances and sources of energy for the cell.

These lipids—also called «fats»—are hydrophobic, and sometimes amphophilic, substances, soluble in apolar or barely polar organic solvents, and non volatile: they are referred to as «fixed» oils, as opposed to «essential» oils.

The following are commonly distinguished:

- simple lipids, esters of a fatty acid and of an alcohol that may be:
  - glycerol, constituent of *triacylglycerols* or *triglycerides*;
  - a high molecular-weight aliphatic alcohol, a constituent of *waxy esters*;
- complex lipids: phospholipids, glycolipids. They play a fundamental role in

they have no pharmaceutical or industrial applications to date, therefore we shall not cover them here.

Along the same lines, and as we did for carbohydrates, we shall not dwell on the biosynthetic origin of lipids, their catabolism, their metabolic inter-conversions, nor on the fundamental properties that characterize them and which belong strictly in biochemistry.

Considering their uses in pharmaceutical technology and their various applications (dietetics, cosmetic formulations, food technology, and more) we shall study plant oils—*huiles grasses* or «fat» oils (!) according to the French Pharmacopoeia—and we shall do so after we recall some general concepts on the triacylglycerols that constitute them.

2. TRIACYLGLYCEROLS (TRIGLYCERIDES)

A. Natural Occurrence

Triacylglycerols are practically nonexistent in vegetative organs (leaves). They are stored as oily inclusions called oleosomes, which arise from the endoplasmic reticulum, and at times gather in large piles in the cells of reserve tissues; this is particularly true in seeds in which they may represent over 50% of the dry weight. The triacylglycerol content of seeds increases during the maturation process whereas in parallel, the phospholipids and glycolipids of young seminal tissues disappear. Exceptionally, seeds may accumulate not triacylglycerols, but esters of fatty acids and of long-chain aliphatic alcohols (see jojoba). Although this is less common, some fruits concentrate triacylglycerols in their pericarp: olive, avocado, bay berry.

B. Structure of Triacylglycerols

They are triesters of a triol, glycerol, and of fatty acids, in other words aliphatic carboxylic acids of variable length which normally have an even number of carbon atoms.

Nature of the Fatty Acids. The vast majority of vegetable fatty acids falls into two groups: saturated fatty acids and their unsaturated homologs. In both groups, 18 or 16 carbon atoms are most common.

Saturated Fatty Acids. Fatty acids with less than 12 carbon atoms are rare in plants: they occur, especially those in C_8 and C_{10}, in the triacylglycerols of palm seeds, mainly composed of lauric acid and myristic acid. Up to C_{14}, fatty acids are rarely present in substantial quantity: examples are bay butter (C_{12}) and nutmeg butter. Fatty acids with 20 or more carbon atoms are not common either: except in peanut oil, each normally represents less than 0.5% of the constituent fatty acids of

Examples:

$C_{6:0}$:	hexanoic acid	=	caproic acid
$C_{8:0}$:	octanoic acid	=	caprylic acid
$C_{10:0}$:	decanoic acid	=	capric acid
$C_{12:0}$:	dodecanoic acid	=	lauric acid
$C_{14:0}$:	tetradecanoic acid	=	myristic acid
$C_{16:0}$:	hexadecanoic acid	=	palmitic acid
$C_{18:0}$:	octadecanoic acid	=	stearic acid
$C_{20:0}$:	eicosanoic acid	=	arachidic acid
$C_{22:0}$:	docosanoic acid	=	behenic acid
$C_{24:0}$:	tetracosanoic acid	=	lignoceric acid
$C_{26:0}$:	hexacosanoic acid	=	cerotic acid
$C_{28:0}$:	octacosanoic acid	=	montanic acid
$C_{30:0}$:	triacontanoic acid	=	melissic acid

Unsaturated Fatty Acids. The most important ones are in the C_{18} series, the configuration of the unsaturation(s) is *Z* * as a general rule, and in polyunsaturated molecules, the double bonds occur in a 1,4-diene pattern **.

* Trans fatty acids occur in milk, butter, and animal fats—they are formed during ruminal hydrogenation—and arise, in vegetable fats, from isomerisation during hydrogenation (margarines). The French diet is thought to contain 8 to 10 g/day and questions have yet to be answered about their harmlessness.
See: Are Trans Fatty Acids a Serious Risk for Disease? Discussion (1997). *Am. J. Clin. Nutr.*, **66**, suppl., 1018S-1019S and the articles by A. Ascherio, A. and W.C. Willett: Health Effects of *trans* Fatty Acids (p. 1006S-1010S), and by S. Shapiro: Do Trans Fatty Acids Increase the Risk of Coronary Artery Disease? A Critique of the Epidemiologic Evidence (p. 1011S-1017S).

** Comments on nomenclature. Fatty acids do not escape the common rule: the carboxylic carbon is numbered 1, and unsaturations and substituents are named according to the classic rules. However, lipid experts (especially physiologists and nutritionists) frequently use a nomenclature of the "*n*-x" type where *n* is the number of carbon atoms of the fatty acid and x is the number of carbon atoms between the distal double bond and the methyl group at the end of the chain. Thus linoleic acid is a C_{18}, *n*-6, acid, and α- and γ-linolenic acids are *n*-3 and *n*-6, respectively. There are also ω-6 and ω-3 fatty acids, or fatty acids of the ω-6 and ω-3 family, with ω designating the carbon atom of the terminal methyl group (in relation to C-2 which is α); for example, linoleic acid, γ-linolenic acid, and arachidonic acid are of the ω-6 type. In practice the designation of fatty acids is often abbreviated by merely characterizing the number of carbons and the number of unsaturations, and separating the two numbers by a colon (e.g. $C_{18:1}$); in this case the position of the unsaturation must also be specified (e.g. $C_{18:2}\Delta^{9,12}$ or $C_{18:2(9,12)}$).

Examples (C18 series):

$C_{18:1}$:	9-octadecenoic acid	=	oleic acid
$C_{18:2}$:	9,12-octadecadienoic acid	=	linoleic acid
$C_{18:3}$:	9,12,15-octadecatrienoic acid	=	α-linolenic acid

Unsaturated acids with short chains ($\leq C_{16}$) or with 20 or more carbons are less common:

$C_{14:1}$:	9-tetradecenoic acid	=	myristoleic acid
$C_{16:1}$:	9-hexadecenoic acid	=	palmitoleic acid
$C_{20:1}$:	9-eicosenoic acid	=	gadoleic acid
$C_{22:1}$:	13-docosenoic acid	=	erucic acid

Positional isomers of these are also rare:

$C_{18:1}$:	6-octadecenoic acid	=	petroselinic acid
$C_{18:1}$:	11-octadecenoic acid	=	*cis*-vaccenic acid
$C_{18:3}$:	6,9,12-octadecatrienoic acid	=	γ-linolenic acid

Some are exceptional:

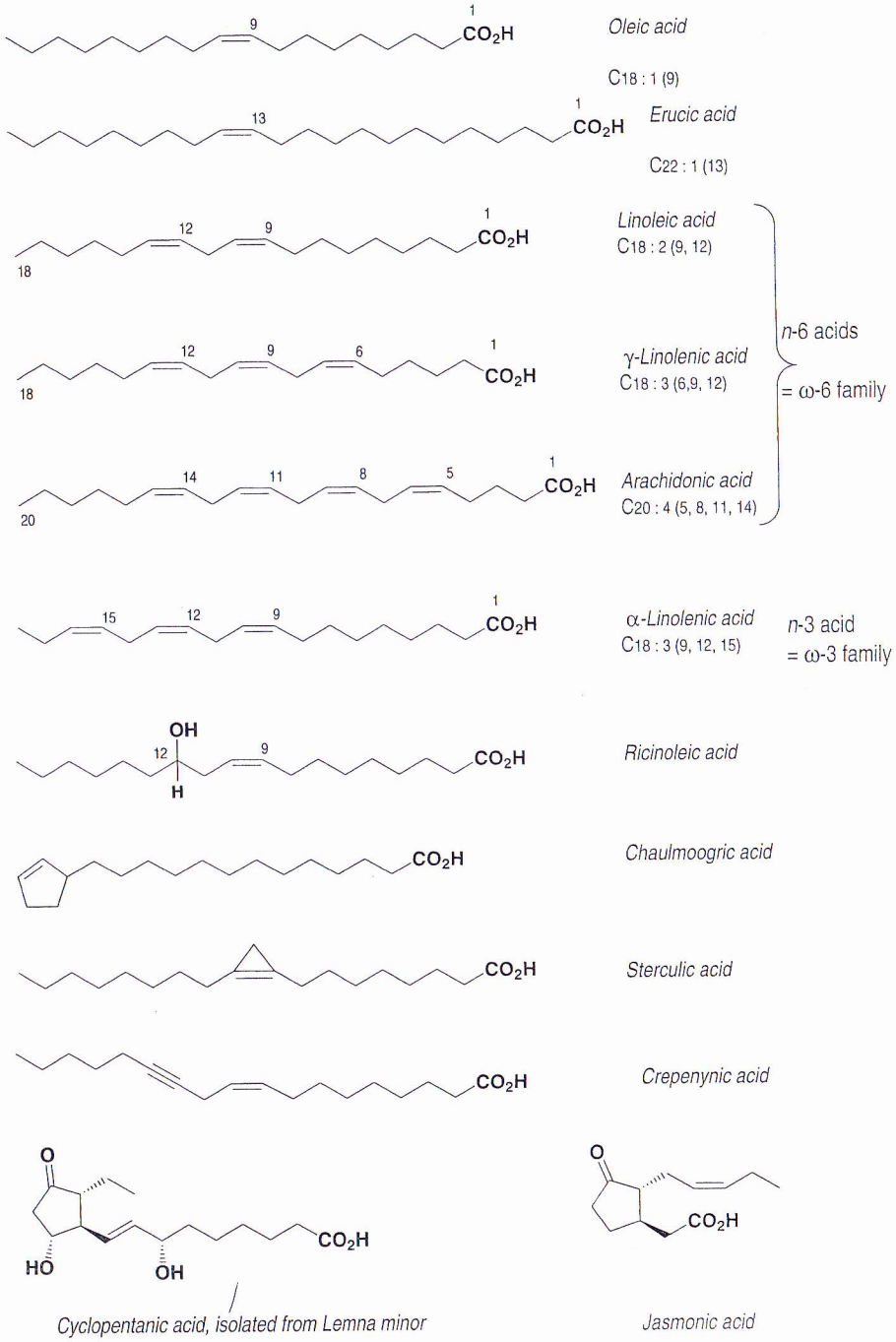
$C_{20:4}$:	5,8,11,14-octadecatetraenoic acid	=	arachidonic acid

Other unsaturated fatty acids. Next to these «classic» fatty acids, many rare structures are known which are generally limited in distribution to a genus, a family, or a group of families. For example, unsaturations that are commonly *Z* may be *E* (e.g., eleostearic acid, $C_{18:3\ (9Z,11E,13E)}$); one of the unsaturations may be a triple bond (crepenynic acid); there may be up to six unsaturations and they may be conjugated (allene fatty acids, e.g., parinaric acid, $C_{18:4\ (9E,11E,13E,15E)}$).

Oxidized fatty acids. The fatty acid may be oxidized:

- ketonic fatty acids such as licanic acid of the oils of Chrysobalanaceae (*Licania*, *Couepia*), particularly the oil of Brazil oiticica (4-oxo-9*E*,11*Z*,13*Z*-octadecatrienoic acid);
- hydroxylated fatty acids (e.g., ricinoleic acid = 12-hydroxy-9*Z*-octadecenoic, lesquerolic acid = 14-hydroxy-11*Z*-eicosenoic);
- epoxy fatty acid (e.g., vernolic acid, $C_{18:1(9),\ (epoxy\text{-}12,13)}$)

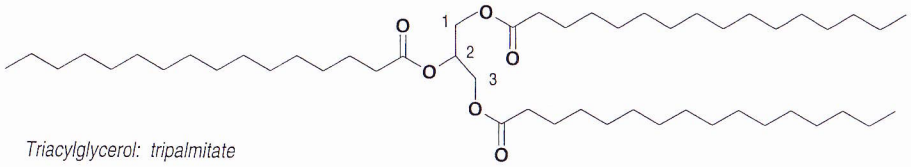
Cyclized fatty acids. In some cases the carbon chain is partially cyclized: cyclopropanic acid and cyclopropenic fatty acids



sterculic acids), or of the oil from the seeds of litchi (*Litchi sinensis* Sonn., Sapindaceae), cyclopentenic fatty acids of the Flacourtiaceae *.

Hydroxylated cyclopentane structures are also known which are reminiscent of animal prostaglandins. Some of these molecules—such as (–)-jasmonic acid and its derivatives—are plant growth regulators with hormonal properties.

Structures of Glycerol Esters. A triacylglycerol (triglyceride) may be homogeneous or heterogeneous depending on whether the fatty acid moieties that esterify the three alcohol functions of glycerol are identical or different. In general, triacylglycerols are heterogeneous, and a vegetable oil is a complex mixture of triesters. Note, however, that saturated fatty acids esterify preferentially the primary alcohol functions (α and α' positions) of glycerol and that unsaturated fatty acids esterify mainly its secondary alcohol function (β position).



Triacylglycerol: tripalmitate

In the official nomenclature of triacylglycerols, the classic α, α', and β are replaced by 1, 2, and 3, the number of the carbon atom of D-glycerol in the Fischer projection, with the secondary alcohol on the left, C-1 at the top, and C-3 at the bottom. The substituents are named using, for convenience, the common names (e.g., 1-palmityl-2-oleyl-3-sterylglycerol, 1,3-dipalmityl-2-linoleylglycerol). Abbreviated symbols are generally accepted: the above examples become POS and PLP.

* These acids (chaumoolgric [= 13-*cyclo*-pent-2-enyl-*n*-tridecanoic], hydnocarpic acid, gorlic acid) are constituents of the triglycerides of the chaumoolgra oils that were used for the treatment of leprosy before sulfones were discovered. They were prepared from the seeds of Flacourtiaceae native to India and to the Indochinese peninsula (*Hydnocarpus kurzii* [King] Warb., *H. anhelminthica* Pierre ex Lanessan and other spp.). These oils were also found in the seeds of African species (*Caloncoba echinata* [Oliver] Gilg.) and of South American species (*Carpotroche brasiliensis* Endl.). See Norton, S.A. (1994). Useful Plants of Dermatology. I. *Hydnocarpus* and Chaumoolgra, *J. Am. Acad. Dermatol.*, **31**, 683-686.

Except for *Aphloia madagascariensis* Clos., which is said to be diuretic and which still appears in a small number of proprietary drugs, Flacourtiaceae are not used in France. The leaves of this *Aphloia* provide a tetrahydroxanthone, tannins, and saponins that are esters in C-28 of hydroxylated urs-12-en-28-oic acids. See Dijoux, M.-G., Lavaud, C., Massiot, G., Le Men-Olivier, L. and Sheeley, D.M. (1993). A Saponin from Leaves of *Aphloia madagascariensis*,

C. Properties of Glycerides and Fatty Acids

Triacylglycerols are soluble in organic solvents, including acetone, and this differentiates them from phospholipids. On treatment by an alkaline hydroxide, they release one molecule of glycerol and three molecules of fatty acid: the saponification value determined by this method provides information on the average chain length (see below). Unsaturated fatty acid-containing triacylglycerols become rancid: when exposed to air they develop foul smells more or less rapidly. This phenomenon is linked to peroxidation of unsaturated fatty acids: the resulting peroxides may polymerize—the desired goal for paints based on flaxseed oil or other drying oils. They may also be cleaved and yield aldehydes, ketones, and acids of unpleasant odor.

At ordinary temperature, fatty acids are liquid if their carbon chain is shorter than 10 carbon atoms; otherwise they are solid. They are all insoluble in water and soluble in organic solvents. If unsaturated, they absorb UV light, and this may be applied to their quantitation. As acids they form salts: this is the basis of the soap and detergent industry (alkaline salts, organic base salts). As acids they may be esterified: the volatility of their methyl esters is greater than that of the acids and makes them amenable to GC analysis.

3. OIL PRODUCTION

From the description of olive oil preparation by Pliny or that, more ancient, of the Assyrian sesame oil press to the modern screw press, the principle of oil production has not changed: expression of the starting material yields the oil directly. Current procedures also use organic solvents, and in both cases the crude oil undergoes various refining steps. Before proceeding with the recovery of the oil contained in the vegetable organs to be processed, strict quality control of the starting material (e.g., absence of foreign matter and of deterioration) is in order and preliminary procedures are often required, whether they are general (cleaning, drying) or specific (washing the olives; delintering cotton; shelling peanuts, soybeans, or sunflower seeds).

- ***Extraction by Expression***. Generally screw presses are used because they afford a better yield than the older hydraulic presses: they operate at higher pressures and continuously, not in batches, which is an added advantage. Prior to expression, oilseeds rich in proteins undergo cooking at around 90°C, which frees the oil by bursting cell structures, but also coagulates the proteins. Most often a fast drying step follows.

- ***Extraction by Solvents***. This type of extraction is applicable to intact seeds as well as to seeds partially extracted by expression. The solvent, generally hexane (bp 65°C), is added to the cleaned, hulled, and rough-milled seeds. An organic

also a solvent-soaked defatted meal. Industrial setups commonly have a counter-current design. Oil recovery ranges from 95 to 99%.

• ***Refining the Crude Oil.*** Crude oils obtained by distillation of the miscella may contain water, free fatty acids, lecithins, resins, pigments (carotenes, chlorophyll), sterols, waxes, substances with odors and tastes, and external contaminants (pesticides). Refining includes the following sequence:

- *Degumming* (mucilage removal). Its role is to eliminate lecithins, proteins, and other constituents present in the oil in colloidal suspension. To accomplish this, the hot oil is hydrated, whereupon the colloids form a dense gel which separates from the lighter oil. The gel is discarded and the oil dried under vacuum. In most cases, this treatment is replaced by an injection of phosphoric acid into the hot oil: the phospholipids then precipitate upon neutralization by sodium hydroxide;

- *Neutralization*. The free fatty acids, always present in the crude oil, are neutralized by dilute sodium hydroxide. The soap formed (soap stock) adsorbs part of the impurities: coloring matter, phenols, sterols, wax esters, traces of metals, and miscellaneous oxidation products. The excess soap and sodium hydroxide are removed by washing with hot water;

- *Bleaching*. This is done by passing the oil through diatomaceous earths or activated charcoal. The bleaching agent is removed by filtration.

- *Wax removal*. The crude oils rich in waxes (sunflower, corn, cotton) are freed from those by cooling (winterization): the crystallized waxes are removed by filtration;

- *Deodorizing*. The aldehydes and ketones responsible for the unpleasant odor of crude oil are eliminated by injecting steam into the very hot oil (>200 °C) under high vacuum.

Subsequent treatments of the oils: they take place mainly in the food industry and include hydrogenation and interesterification in the margarine industry (see specialized texts). In all cases the cattle cake is recovered, treated (solvents removed), and if needed detoxified. Except for specific uses it is directed toward animal feeding.

4. QUALITY CONTROL FOR LIPID-CONTAINING DRUGS: TESTS FOR FIXED OILS

A. Generalities

Quality control for lipid-containing drugs does not differ from that of other

determination of the fixed oil content, are the main part of the assay, and are no major problem*. In the case of the oil itself, the control is more complex: the purity check is required to include sophisticated analytical techniques to determine the fatty acid composition, the glyceride structure, and the composition of the unsaponifiable fraction. The principle behind the methodology is detailed and reviewed in specialized texts, the methods themselves are standardized (by agencies such as the *Association Française de NORmalisation* [= AFNOR], the *American Oil Chemists Society* [= AOCS], or the *International Standardization Organization* [= ISO]), and they are applied mainly in the food industry. We shall cover here only a few basic concepts.

• Determining the fatty acid composition is easy; it is carried out on methyl esters obtained by methylation** subsequent to saponification or, more directly, by alkaline methanolysis. This method is by far the most often used for fat analysis. In isothermal chromatography, fatty acid esters are identified by their «equivalent chain length», i.e., the length of the saturated fatty chain that would have, in the same operating conditions, the same volume of retention as the fatty acid under study. This value is deduced from the relation between the logarithm of the reduced retention volume and the number of carbon atoms of the fatty acid (see French Pharmacopoeia, V.3.3.6.). The knowledge of the fatty acid composition is not always sufficient to confirm the purity of the oil: additional tests are required, especially the study of certain constituents of the unsaponifiable fraction which act as «tracers».

• The main constituents of the unsaponifiable matter are saturated and unsaturated, aliphatic or tetraterpenoid hydrocarbons (carotenes), sterols, triterpenoid alcohols, fatty alcohols, vitamins (tocopherols, tocotrienols). The sterols are, from the analytical point of view, the most interesting constituents of the unsaponifiable matter. They are generally represented by two to five major sterols that are commonly Δ^5-sterols (sitosterol, campesterol, stigmasterol). The composition of the sterols and their ratios are good identity markers: some sterols are specific (Brassicaceae brassicasterol) or their content is significant (Δ^7-stigmasterol in sunflower or safflower oil [Asteraceae]). In most cases, sterol analysis allows the detection of illicit adulterations. Sterols are analyzed generally and tocopherols occasionally. In both cases, preliminary extraction of the unsaponifiable matter is necessary (with diethyl ether or hexane), and so is separation, which is straightforward by preparative TLC. The recovered sterol fraction is analyzed directly by GC.

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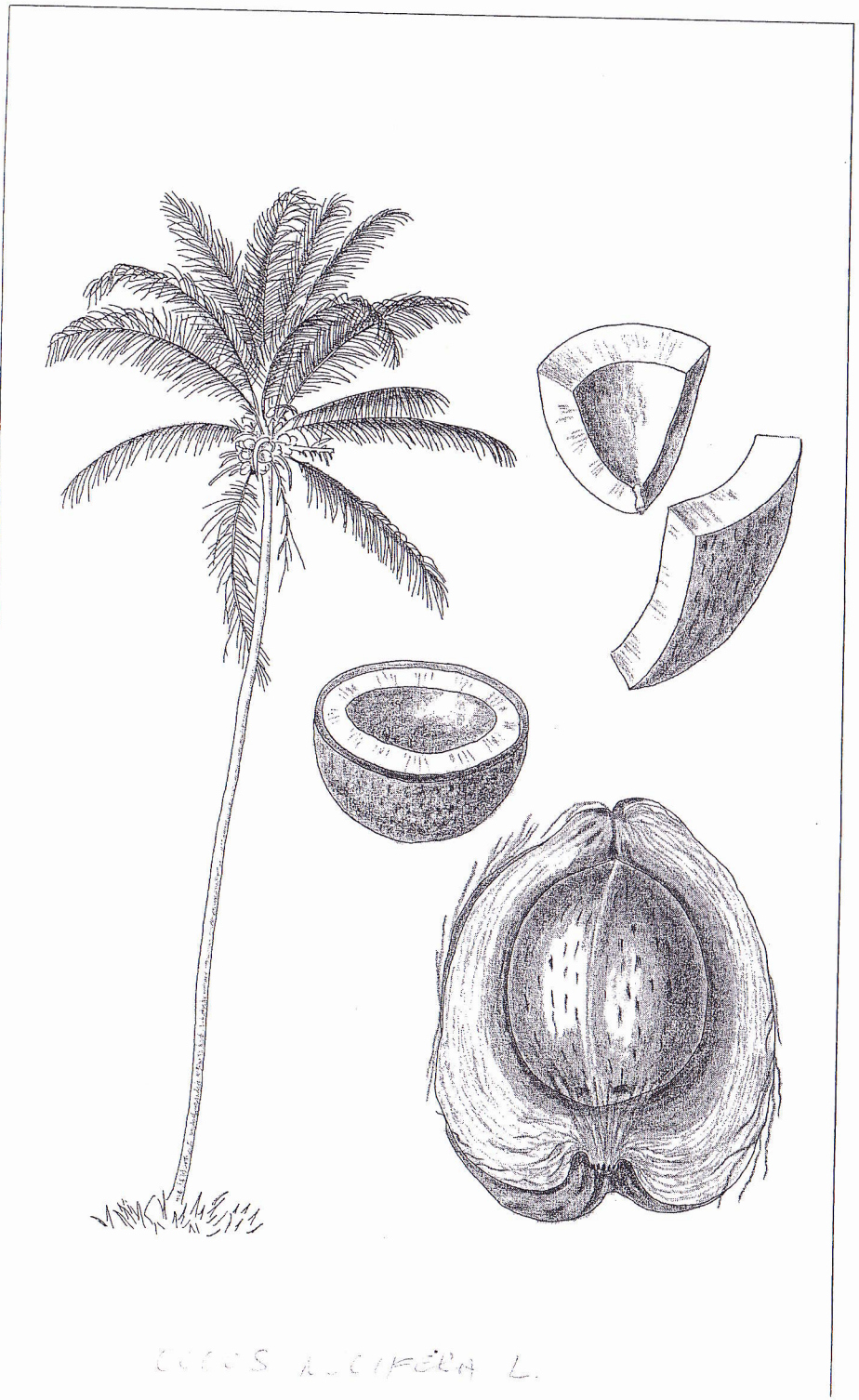
* In the oil crop industry, either the extraction method (determination on the hexane extract [NF ISO 659 standard]) or the low resolution, continuous wave (CW) NMR method (NF ISO 5511) are used.

** If the oil contains short-chain fatty acids, the methylation procedure must be adapted to



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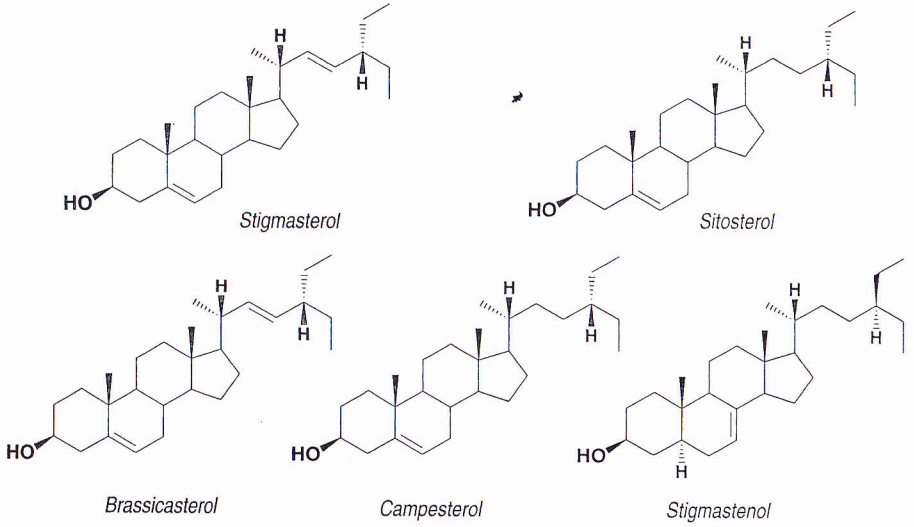
• The study of the glyceride structure requires a compromise between the complexity of the problem (the number of possible combinations of glycerol and fatty acids) and the need to do the work routinely. This is accomplished by determining a simplified structure by classic chromatography:
- distribution of triacylglycerol molecular weights (GC);
- separation and identification of the main triacylglycerols by reverse phase HPLC; separating triacylglycerols into groups as a function of their global unsaturation allows the detection of adulteration by other oils;
- selective hydrolysis and analysis of 2-monoglycerides.

• The determination of various indices or values is widely practiced by pharmacopoeias, sometimes interesting in terms of quality evaluation, but not always significant: for example a peroxidized oil heated above 120 °C will have a very low, misleading peroxide value. In this particular case, measuring the absorption, which detects peroxide cleavage products, is more significant (see below).



B. European Pharmacopoeia and Fixed Oils

Quality control of fixed oils listed in the European Pharmacopoeia includes common assays and, when relevant, tests specific to the oil under consideration: we shall mention those in the corresponding monographs. The common assays are the following:

1. Specific gravity (2.2.5 *).

* The numbers are the paragraph numbers in the 3rd edition of the European

2. Acid value (2.5.1). This is the number of milligrams of potassium hydroxide needed to neutralize the free acids present in one gram of substance. It is a measure of the degree of alteration of the oil or, if applicable, of the quality of the refining.

3. Peroxide value (2.5.5). This number expresses, in milli-equivalents of active oxygen, the amount of peroxide contained in 1000 g of substance (determined by the method described in the French Pharmacopoeia).

4. Unsaponifiable matter (2.5.7). This comprises the «substances that are not volatile at 100-105 °C and are obtained by extraction with an organic solvent of a solution of the substance of interest after saponification». In practice, the saponification medium is diluted with water then extracted with diethyl ether; following washes and solvent elimination, the residue is weighed. The absence of significant amounts of fatty acids in this residue must be verified by acidimetry;

5. Foreign oils in fixed oils. This test may be done by TLC (2.4.21), but the monographs that require it (all but that of castor oil) call for GC (2.4.22).

TLC plates first developed in a petroleum ether solution of paraffin * are used. The solution undergoing the test consists of the mixture of fatty acids from saponification, and the reference standard solution consists of the mixture of fatty acids from saponification of a 19:1 mixture of corn oil and rapeseed oil. After developing the chromatogram, the spots corresponding to the fatty acids are visualized by iodine vapor.

GC analysis is not applied to the oil, but to the methyl esters of its constituent fatty acids (methylation by refluxing in anhydrous methanol under nitrogen, then pentane extraction of the fatty acid esters). The analysis is carried out in parallel on a reference standard solution of methyl esters, and the chromatogram undergoes a double evaluation: qualitative (equivalent chain length) and quantitative (integration, standard curve). In each monograph, the French Pharmacopoeia specifies the minimum and/or maximum levels of fatty acids normally contained in the fixed oil of interest. A linear GC temperature program can also be used.

6. Most monographs require, in addition, the determination of the following:

- alkaline impurities (2.4.19): neutralization of an acetone solution of the oil in the presence of bromothymol blue;
- the refractive index (2.2.6);
- the saponification value (2.5.6): this is the number of milligrams of potassium hydroxide needed to neutralize the free acids and saponify the esters present in 1 g of substance **. The shorter the fatty acid chains within the triacylglycerols, the higher the saponification value.

* Very often TLC plates are coated with silica gel impregnated with an aqueous solution of silver nitrate: the interaction between the Ag^+ ions and the double bonds improves the resolution of unsaturated fatty acids (mono-, di- and trienes, *Z* and *E*).

- water (2.5.12), when the oils are to be used in pharmaceutical formulations for parenteral administration. Water is determined by microquantitation and must be «not more than» a limit set for each monograph (0.05, 0.1, or 0.3%). In addition, the limits for the acid and peroxide values are lower in the case of oils for parenteral administration.

- sterols (2.4.23). After isolation of the unsaponifiable matter (2.5.7) and separation of the sterol fraction by preparative TLC (2.2.27), this fraction is silylated for GC analysis (2.2.28). The chromatographic peaks are identified by comparison with standards (unsaponifiable matter of rapeseed and sunflower oils); the use of an internal standard (betulin = lup-20(29)-ene-3β,28-diol) allows the quantitation of the various constituents.

7. In some cases the French Pharmacopoeia also requires measuring the absorbance (2.2.25): conjugated dienes and enones resulting from peroxide decomposition absorb at 232 and 270 nm, respectively: the A_{232}/A_{270} ratio is determined (for olive oil), or else simply the absorption at about 270 nm (for persic oil, refined olive oil for parenteral preparations, castor oil) *.

The determination of other classic values (iodine value, hydroxyl value **) and of optical rotation (2.2.7) is required only for castor oil because of its particular composition.

The determination of the triglyceride composition by HPLC (2.2.29) is currently required only for sesame oil.

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* A232/A270 must be *greater than* a threshold value (the greater the decomposition, the higher the level of secondary oxidation products). In contrast the unique measurement at 270 nm must be *less than* the published limit. The oxidation of oils upon exposure to the air yields aldehydes: if necessary, it is possible to assess the degree of oxidation by determining the anisidine value (formation of colored derivatives by reaction of the aldehydes with *p*-anisidine).

** Iodine value (2.5.4): this is the number of grams of halogen, calculated as iodine, able to react in precise conditions with 100 g of substance. It is a measure of the overall unsaturation of the fat. Hydroxyl value: this is the number of milligrams of potassium hydroxide needed to