CHAPTER II

Methods
CHAPTER-II: METHODS

EXTRACTION

The aim is to separate cellular lipids from other constituents (Proteins, Polysaccharides) and to preserve the lipids for analysis. Removing the non-lipids without losing the lipids is a complex task. The high sensitivity of analytical methods needed for low amounts of extracted lipids requires the use of very pure solvents and clean glassware.

In order to extract lipids from tissues, it is necessary to find solvents which will not only dissolve the lipids readily, but will overcome the interactions between the lipids and the tissue matrix.

Lipids occur in tissues in a variety of physical forms. The simple lipids are often part of large aggregates in storage tissue from which they are relatively easily extractable whereas complex lipids are not extracted so readily. Various solvents or solvent combinations have been suggested for extracting lipids.\textsuperscript{1} Common methods include extraction with chloroform-methanol\textsuperscript{2} or hexane: isopropanol\textsuperscript{3} followed by addition of salt solution to result in partition between an aqueous and an organic phase. These may be regarded as general methods for the extraction of both neutral and polar lipids, but there are a variety of more specialized extraction methods. Steam distillation is used to produce the volatile essential oil, in which mono and sesquiterpenes dominate while continuous extraction with hot solvents is accomplished by Soxhlet extraction. A very simple extraction method is the short-
duration dip in organic solvents for epicuticular lipids. In some case, separation of lipid classes can be accomplished by partition during an extraction procedure e.g separation of neutral and polar lipids from very polar lipids.\textsuperscript{4} Sometimes extraction can involve a chemical treatment e.g. the dipolymerization of cutin and suberin (complex polyesters of hydroxy fatty acids) is required before the monomers are soluble in organic solvents.\textsuperscript{5} There are several points worth considering regarding extractions. These include problems of incomplete extraction, the release of lypolytic enzymes, and oxidative compounds. An extraction method should be standardized for exhaustive extraction of the lipids and, where possible, with regarding of tissues. Losses in partition methods may include highly polar lipids which partition into the aqueous phase e.g. sphingolipids\textsuperscript{6} and lyso phospholipids\textsuperscript{7} require direct extraction with water saturated n-butanol. Homogenization of plant tissues releases lypolytic enzymes.

Phospholipase is active even in organic solvents. A short heat treatment is usually done to inactive the lypolytic enzymes prior to homogenization.\textsuperscript{8,9} Moreau\textsuperscript{10,11} has demonstrated the variability of phospholipase activity in a range of plant tissues. Another problem in extraction is that of chemical stability. Oxidation is a problem, with polysaturated fatty acids.\textsuperscript{12} Precautions to minimize oxidation include the use of peroxide free solvents, the addition of antioxidants such as ethoxyquin or butyalated hydroxy toluene, shielding from strong light in order to prevent
photooxidation and photoisomerization and the use of nitrogen to evaporate solvents.

Exhaustive procedures for extraction of different plant tissues have been described by Zhukov et al.\textsuperscript{13}

Many a times, coloured contaminants hide lipid spots at the time of detection.\textsuperscript{14} For oil processing – refining is needed to remove cloudiness, carried by degumming.\textsuperscript{15} Isohexane is used presently, in place of n-hexane, as extraction solvent due to environmental considerations.\textsuperscript{16} After long storage at freezing temperature, lipid parameters (sterol glycosides) may alter, hence extraction should be done at the earliest possible time.\textsuperscript{17} To prevent labile lipids from oxygen attack, oxygen absorbed is placed inside the sample package. This allows transportation of sample without pretreatment at low temperature.\textsuperscript{18} Recent advances in extraction includes solid-phase extraction for fatty acid analysis.\textsuperscript{19}

For analysis of extractives chromatographic and spectroscopic methods were used.

**CHROMATOGRAPHIC METHODS**

According to IUPAC definition of chromatography: it is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in definite direction. The following component of the system of mobile phase is either a
liquid or a gas. It is of much value in separation science with techniques like column chromatography, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC).

A description of chromatographic methods has been given by Willard et al.\textsuperscript{20} and Poole and Schuette\textsuperscript{21}, Braithwaite and Smith\textsuperscript{22} and Weston and Brown.\textsuperscript{23} Application in the field of lipids has been comprehensively described by Kates,\textsuperscript{24} Christie\textsuperscript{25} and Mangold.\textsuperscript{26}

**Column Chromatography:**

It is convenient to use small pre packed cartridges of silica gel for small scale separations.\textsuperscript{27-32} Aminopropyl bonded phase cartridges have also been used for the isolation of sample and complex lipid fractions.\textsuperscript{33} By selection of an adsorbent of the correct grade and activity, isolation of animal\textsuperscript{34,35} plant\textsuperscript{36} and bacteria\textsuperscript{37} lipid fractions have been achieved. Alumina can also be used for the purpose.\textsuperscript{13} Complex lipids can be fractionated on silica gel.\textsuperscript{25}

**Thin Layer Chromatography:**

The technique of TLC has been described by Stahl,\textsuperscript{38} Skipski and Barclay\textsuperscript{39} with recent advances by Sherma and Fried.\textsuperscript{40} A wide range of functional groups – specific reagents are available.\textsuperscript{24,26,38} Another method is the use of TLC-flame ionization detection (TLC-FID). It is used for quantitating total and
specific lipids in small amounts.\textsuperscript{41} The Iatroscan is a system in which TLC is carried out on a silica gel fused to a quartz support rod and then the rod slowly passed through a flame ionization detector.\textsuperscript{42} Separation and quantitation of phospholipids in animal tissues has been done by Iatroscan TLC-FID.\textsuperscript{43} Salts of Ca, Co, Sn and other divalent metals have been applied to chromorods SII for use in separation of phosphorylated acylglycerols in TLC-FID.\textsuperscript{44} Crane \textit{et al.}\textsuperscript{45} pointed out, with complex relationship between detector response and sample loading lipid class and flame ionization detector scan rate, it is not possible to use the technique for accurate mass quantitation without extensive standardization. Radioactivity can be monitored by radio TLC scanners, while operate using an open counting chamber. For maximum resolution autoradiography is done.

There are various solvent systems for the separation of lipid classes\textsuperscript{46} by TLC. Some solvent systems for plant lipids using silica gel TLC have been given by Wilson and Rinne.\textsuperscript{47} One dimensional TLC can also utilize multiple developments with several solvent systems.\textsuperscript{48} Two dimensional TLC increases the potential resolution of a complex lipid mixture.\textsuperscript{25,40} Determination of phospholipids on two dimensional thin layer chromatographic plates by imaging densitometry has been tried.\textsuperscript{49} A three way system has recently been proposed.\textsuperscript{50}

A modern development in TLC is high performance TLC, where a well defined particle size is used rather than the normal
average. Improved resolution is obtained\textsuperscript{51-53} with preparative TLC, routinely used for small scale preparations. Recent modifications of preparative TLC include the use of wedge-shaped coatings\textsuperscript{54} and centrifugal TLC.\textsuperscript{55} Inclusion of silver nitrate in silica gel layers have been described by Morris.\textsuperscript{56} Due to double bond complexation with silver ions, a separation based on the degree and the stereochemistry of unsaturation is possible. This has been used for fatty acid ester separation and for intact lipids.\textsuperscript{57,58} The use of chemically bonded cross linked and capped reversed phase plates,\textsuperscript{59} which are commercially available, has made reversed phase TLC much easier and reproducible. Octadecyl, octyl, ethyl, phenyl and cyanopropyl phases are available. Phosphomolybdic acid and iodine represent the best destructive and non-destructive methods respectively.\textsuperscript{60,61} Affinity chromatography\textsuperscript{62,63} has been used for analysis of biochemical molecules.

For fatty acid analysis, the use of phenacyl ester is helpful.\textsuperscript{64} Stumpf \textit{et al.},\textsuperscript{65} have used phenacyl esters on C\textsubscript{18} coated plates impregnated with zinc. Jee and Svetashov\textsuperscript{66,67} describe the use of multiple-zone TLC plates, with C\textsubscript{18} phase in one dimension and silver nitrate-silica gel in the other. Rogers \textit{et al.},\textsuperscript{68} have used an octadecyl cartridge to concentrate organics from aquatic plants, while Juaneda and Rocquelin\textsuperscript{29} used a silica gel cartridge for a rapid separation of neutral and polar lipids. TLC technique has been used for the separation of four steryl derivatives from sitosterol β-D glucoside.\textsuperscript{69}
Weerheim\textsuperscript{70} has suggested a method for HPTLC. For analysis of isomeric phosphoinositides, 1.8\% boric acid impregnation has been suggested.\textsuperscript{71} A review of Phospholipid separation by TLC has been recently given.\textsuperscript{72}

**High Performance Liquid Chromatography:**

HPLC is used not only in the analysis of thermally labile, non-volatile ionic compounds but for all types of molecules from the smallest ions to macromolecules. Most important, HPLC has opened horizon in separations and has helped to make possible the development of biotechnology, where there is a great need for ultra pure products.

Johnson and Stevenson\textsuperscript{73} described the basic principles. The mobile liquid phase is forced under controlled high pressure through a relatively narrow bore column containing a stationary phase, which can be solid surface or a liquid phase, the later bonded chemically to an insert support. The detection system in continuous. Early work on the HPLC of lipids used refractive index detectors but these are not very sensitive and require calibration. UV detection is the dominant form of detection in HPLC, but for most lipids the chromophore will be either the ester carbonyl or an isolated double bond, so adsorption will occur at very short wavelengths (200-210 nm) and the extinction coefficient will be low. These limitations for most acyl lipids have hindered the development of HPLC analysis for plant lipids, but despite these disadvantages, the methods can be used with low
UV cut off solvents (190 nm). To obtain the possible separation the efficiency of the chromatographic system must be optimized in order to minimize band broadening.

Chemical derivatization of an analyte is performed to improve the selectivity and sensitivity of that analyte. It is a technique that is most commonly performed prior to UV adsorption or fluorescence detection. It has been used to aid in the detection of compounds such as thiols, steriods, fatty acids, and several inorganic species. Recent developments in HPLC detectors along with other development such as multididimensional liquid chromatography and microbore columns have been described. The development of flame ionization detectors for lipid analysis by HPLC has been mentioned by Phillips et al. HPLC has no limitations of thermal stability and is suitable for non-polar and highly polar lipids alike. Most reports concern non plant lipids. Separation of plant phospho-galactolipids and glycolipids have also been done. Use of HPLC in separations of molecular species of seed oil triacylglycerols as such using silver nitrate impregnated silica and by reverse phase or both have been given. Kesselmeier et al. have reported C₆ reverse phase HPLC separation of chloroplast lipids, point out lipids class separation is needed first and show that for UV detection at 200 nm for chloroplast lipids, the detector response is a function of the number of double bonds. The use of P-anisoyl derivation of 1,2 diacylglycerols derived from these lipids by phospholipase treatment for
galactolipids, phospholipids or a periodic acid oxidation, 1,1-dimethylhydrazine treatment for gatactolipids with UV monitoring at 250 nm, has been mentioned. Demandre et al. have used silica absorption HPLC followed by C\textsubscript{18} reverse phase HPLC to fractionate galacto and phospholipids.

A study of phosphatidylcholine by reverse phase HPLC has been made using UV detection at 206 nm to monitor total phosphatidylcholine species and at 234 nm to measure oxidized species by conjugated diene chromophore. HPLC separation for acyl-CoA has been achieved by C\textsubscript{18} stationary phase with counter ion in the mobile phase.

Apart from the use of silica gel and octadecyl reverse phase florisil and anion exchangers (di and tri ethylamino ethyl celluloses) have been used. Alumina, being basic in nature when used with organic eluants will absorb, aromatic unsaturated hydrocarbons, steroids, alkaloids and natural products. Kieselguhr (celite) due to its high porosity and large surface area, is used as a support for the stationary phase in partition chromatography. Gel permeation chromatography has been used free fatty acids in biological assays. Counter chromatography has been used for separation of polar lipids. Separation of wax esters from olive oils using HPLC has been tried. Excellent reviews of high speed chromatography separations and advances in the field have recently been appeared. Double piston pumps are the latest in HPLC system. Recently, separation of liposomes, free
sterols,\textsuperscript{107} assessment of lipid classes\textsuperscript{108} and measurement of labelled fatty acids\textsuperscript{109} have been carried.

**Gas Liquid Chromatography:**

The basic principles have been described by Mc Nair and Bonelli.\textsuperscript{110} In gas liquid chromatography, the mobile phase is an insert gas while the stationary phase is usually a liquid coating, either a capillary wall or an insert column packing. For detection and quantitation of the components, thermal conductivity, electron capture and flame ionization detectors are generally used. The flame ionization detector is used for lipids analysis, since it is a non-specific detector (total ion current monitoring) with the commercial availability of fused silica capillary columns, there has been a rise in the use of capillary gas chromatography. A large number of stationary phase are available. The general polarity of these is defined by Mc Reynolds constant.\textsuperscript{111} Apiezon greases and polyesters are replaced by polysiloxane and carborane phases, which are capable of use at higher temperatures. Retention behavior of fatty acid ester is described by equivalent chain length (ECL) values.

Ackman\textsuperscript{112} and Jamieson\textsuperscript{113} reviewed their use and the early literature on the gas chromatography behavior of fatty acid esters. Correction to the basic assumption of linear proportionality between carbon number and logarithm of retention time can be required.\textsuperscript{114} Positional isomers elute at different retention times facilitating their identification.\textsuperscript{115} Geometric isomers (cis and
trans unsaturated fatty acid esters) can be separated directly, with packed columns, using high polarity stationary phases.\textsuperscript{116}

Triglycerols can be determined on 60 cm (short packed) OV-I (a polar) column.\textsuperscript{117} Triglycerols have also been separated on polar columns.\textsuperscript{118} Mono and digalactosyl, diacylglycerols can be analyzed intact after derivatization.\textsuperscript{119,120} Capillary gas chromatography of high molecular weight neutral lipids e.g tocopherol and sterols have been reported.\textsuperscript{121} Analysis of free fatty acid, mono, di and tri acylglycerol content of processed vegetable oil using a shortened capillary column has been attempted.\textsuperscript{122} Derivatization methods, particularly those that reduce polarity, are important.\textsuperscript{24,25,123,126} The use of quaternary ammonium salts for transmethylation has been described.\textsuperscript{24,25,125,126} A rapid NaOMe procedure has been described by Christie.\textsuperscript{127}

For transesterification of lipophilic molecules, specialized conditions have been employed.\textsuperscript{128} Short chain ester may be lost due to their volatility especially when solutions have to be concentrated, while very short chain fatty acids can partition appreciably into the aqueous phase in extractions. To increase the size of the alkyl group – is one of the possibilities.\textsuperscript{129} Other possibilities include – gas chromatography of the reaction\textsuperscript{130,131} gas chromatography of free fatty acids or to concentrate acids\textsuperscript{56} as their phenyl esters. Halogenated alcohols or phenol derivatives e.g. pentafluorobenzyl esters\textsuperscript{132} give enhanced sensitivity of detection with an electron capture detector. A reductive
technique for gas chromatographic determination of the acyl portion (acyl thioesters) have been described but was shown to have insufficient specificity.\textsuperscript{134}

Gas liquid chromatography could be adapted to become a preparative method by addition of a radioactive detector.\textsuperscript{135} A system for simultaneous flame ionization and \textsuperscript{14}C detection for capillary columns have been described.\textsuperscript{136} A recent development, Super Critical Fluid Chromatography (SEC). The greater selectivity of adsorption chromatography for compounds of different chemical types may be extended using Super Critical Fluid (SFC) as the mobile phase.\textsuperscript{137}

A review\textsuperscript{138} of recent development in GLC includes head space analysis, multi dimensional gas chromatography, back flushing GC-FT-IR and other detection system, use of multiple detectors and theoretical and instrumental aspects. Head space gas – chromatography analysis employes a specialized sample and sample introduction technique, making use of the equilibrium established between the volatile components of a liquid or solid phase and the gaseous vapour in a sealed sample container. Reviews on packed\textsuperscript{139} and capillary column gas chromatography\textsuperscript{140} of lipids have been written recently.

GLC has been described as a total system for high accuracy in the analysis of the fatty acid composition of fats and oils.\textsuperscript{141} In recent studies capillary gas liquid chromatography has been used for the separation of phospholipids\textsuperscript{142} and phytosterols.\textsuperscript{143} The
occurrence of cyclopropenoid fatty acids has been studied using gas chromatography.\textsuperscript{144}

Recent development regarding film thickness is given by phase ratio. This is the ratio of the volume of the gas phase divided by the volume of the stationary phase. The internal coating of a capillary column is very thin, compared with the diameter of the column thus the phase ratio is in the same order as the average molecular weight of the compounds being analyzed.\textsuperscript{148}

Automated analysis of FAME\textsuperscript{146} and an effective method for the same shift in ECL on capillary column\textsuperscript{147} have been suggested. Application of different temperature and pressure programs, on a single capillary column has been proposed.\textsuperscript{148} Losses of PUFA (DHA) from high temperature of column during GLC of methyl esters of long-chain Omega-3 fatty acids has been reported.\textsuperscript{149} Analysis of isomeric diacylglycerol classes to evaluate the quality of olive oil in relation to storage, has been carried out.\textsuperscript{150}

\textbf{SPECTROSCOPIC METHODS}

Spectroscopy is concerned with the production measurement and interpretation of electromagnetic spectra arising from either emission or absorption of radiant energy by various substances. Organic spectroscopy has been covered in many texts.\textsuperscript{151-153} The subject of fatty acid spectroscopy has reviewed by Gunstone.\textsuperscript{154,155}
Mass Spectroscopy:

A comprehensive text on the technique has been given by Waller\textsuperscript{156} and Waller and Derner.\textsuperscript{157} The technique in essence involves the analysis of the mass/charge ratios of ions produced from the sample molecules. A detailed reviews on the resent development have been given by Burlingame \textit{et al.}\textsuperscript{158}

The early work on the analysis of acyl lipids is dominated by fatty acid analysis.\textsuperscript{159,160} Dommes \textit{et al.}\textsuperscript{161} suggested that O- trimethyl silylether derivatives are the best for the analysis of polyunsaturated fatty acids. A recent method,\textsuperscript{162} using derivatives seems suitable for the analysis of conjugated and non conjugated polyunsaturated fatty acids. Acyl pyrroldides appear to be useful for functional group localization.\textsuperscript{163} Mass spectroscopy has been extensively used in the structure determination of oxygenated fatty acids from cutin polymerization.\textsuperscript{164} Electron impact mass spectroscopy, though limited by its gas phase ionization, has been applied to neutral lipids quite successfully.\textsuperscript{165} A description of chemical ionization mass spectroscopy is given by Harrison.\textsuperscript{166} The technique has been applied to the analysis of fatty acids.\textsuperscript{167-170} A recent suggestion is the direct analysis of the TLC spots by fast atom bombardment mass spectroscopy.\textsuperscript{171} Examples of the use of desorption ionization methods on the lipids include the ammonia desorption – chemical ionization mass spectroscopy of triclyglycerols,\textsuperscript{172} of sucrose esters\textsuperscript{173} and of phosphatidyl cholines.\textsuperscript{174} Fujino \textit{et al.}\textsuperscript{175} have reported the field desorption
mass spectroscopy of mono, di and triglycosyl ceramides from wheat grain. Gas chromatography-mass spectroscopy has been used extensively in the analysis of plant lipids and fatty acids. Acyl composition of lipid can be quantitated by the technique.\textsuperscript{176} A similar analysis has been applied to diacyl-glycerol species derived from chloroplast lipids.\textsuperscript{177} There are reports\textsuperscript{178-181} of fragmentation patterns for stereo specific isomers of acyl lipids.

A variation of gas chromatographic mass spectroscopy is selective ion monitoring, sometimes known as mass chromatography or mass fragetography. The technique has been used in analysis of molecular species of plant cerebrosides and ceramides.\textsuperscript{182,183} Liquid chromatography interfaces are a more recent development.\textsuperscript{184-187} A potentially useful spectroscopy development for polar lipid analysis is liquid chromatography mass spectroscopy fast atom bombardment.\textsuperscript{188-189} Tandem mass spectroscopy (mass spectroscopy-mass spectroscopy) is another recent developments that may have applications on the analysis of lipid mixtures.\textsuperscript{190} In the technique, an ion is selected by primary mass analyzer, representing the molecule of interest withing the mixture.\textsuperscript{191,192} The applications in identification of Jojoba wax, Soyabean and phosphatidylcholine\textsuperscript{193} have been described. Morris\textsuperscript{194} reviewed the investigations on the stereochemistry of fatty acid desaturation, using deuterium labelling. Use of labelled malonyl CoA to assay fatty acid synthesis, with the measurement of the individual fatty acid has been attempted.\textsuperscript{195} Direct determination of phospholipid
structures in microorganisms have been reported by fast atom bombardment triple quadruple mass spectroscopy.\textsuperscript{196} Mass spectroscopy is useful in providing the structure of branched long chain hydrocarbons, fatty acids, alcohols, ketohydroxy, methoxy and unsaturated fatty acids.\textsuperscript{197}

Recent work in the field includes – mass spectroscopy of fatty acid derivatives,\textsuperscript{198} analysis of conjugated linoleic acid derivatives,\textsuperscript{199} oxazoline derivative of highly unsaturated fatty acids with determination of double bond positions,\textsuperscript{200} elucidation of chlorine location in dichloro fatty acids with DMOX derivatization,\textsuperscript{201} characterization of fatty acids distribution,\textsuperscript{202} software tools for analysis of Mass spectroscopy data,\textsuperscript{203} rapid characterization of fatty acyl composition of complex lipids\textsuperscript{204} and application of capillary electrophoresis (CE). Mass spectroscopy (MS) to characterization of bacterial lipopolysaccharides.\textsuperscript{205}

**Nuclear Magnetic Resonance Spectroscopy:**

The technique and spectral interpretations have been described in the standard texts.\textsuperscript{206-208} The phenomenon arises when a nucleus is placed in an external magnetic field. The nuclear-spin energy become non-degenerate. Transitions between these states occur with absorption or emission of radiation.

For lipids, the most commonly studied nuclei are $^1\text{H}$\textsuperscript{209} and $^{13}\text{C}$-NMR\textsuperscript{210} is more useful in biological studies. The use of high resolution NMR in the study of the chemistry and biochemistry of
lipids has been reviewed by Pollard. Studies on biological molecules particularly membrane have been carried.

Use of NMR in measurement of oil and water content in oil seeds have been made. ¹H-NMR is used in the assay of the cyclopropenoid content of vegetable oil upto 1% level following the ring methylene signal versus the terminal methyl resonance. Unsaturated fatty acids have been extensively examined. The shift reagents are used to pull apart overlapping resonance. The structure and stereochemistry of the glycosides of fatty alcohol and sterols have been determined by ¹H-NMR. Examination of hydroxy fatty acids and positional analysis of triacylglycerols have been done by NMR. Chiral shift reagents have been done applied to the stereospecific analysis of model triacylglycerols. The technique has found use in non-destructive quantitation of the lipids of oil seeds. It has been used for study of lipid-protein interactions, Ashworth et al. have described ¹³C acetate level experiments on Corn suspension. Soyabean plants were exposed to ¹³C carbondioxide and the carbohydrate and lipid pools examined by ¹³C-NMR. Gated decoupling and pulse decays can be obtained under normal conditions, if resonance have similar spin-spin relaxation times. Norton and Azoury have reported relaxation times for α and β crystal forms of tristearin and tripalmitin. Each have examined partly solidified margarine oil by high resolution FT-NMR. Recently application of two dimensional NMR in
structure determination have been reported. Sub micro inverse detector gradient NMR is an advanced technique for determining structures of lipid, other uses of sub micro NMR techniques are in $^{1}$H-$^{15}$N heteronuclear shift correlated at natural abundance in low level wide range.

**Other Spectroscopic Methods:**

The texts of Silverstein, Dykel and Gordon, contain general information, while Gunstone, has data more pertinent to acyl lipids.

Relatively few applications of ultraviolet spectro-photometry at 200-210 nm in the separation of simple lipids have been described, possibly because this form of detection is of limited value for quantitative purpose. Although UV detection at 205 nm has been used as a great deal for the detection of lipid classes separated by HPLC, it is of much less value for analysis of molecular species. The response of the detector is mainly to isolate double bonds in this region of the spectrum, and molecular species must differ in degree of unsaturation. It was pointed out that a UV detector operated at 215 nm, where ester bonds exhibit a weak absorbance, has a good sensitivity. Shukla et al. studied the operation of UV detection has been used in the fractionation of some leaf waxes and in lipid per-oxidation. The spectral range extents from 120 nm to 767 nm in recently developed state--of--the--art--instrument.
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Infrared detection at 5.72 to 5.75 μm has been used in the analysis of lipid classes for shortening and partial hydrolysis of seed oils.\textsuperscript{244} IR detection was used in the separation of wax constituents.\textsuperscript{245} The technique has not been used for quantitative purpose.\textsuperscript{246-248} A review on application of FT-IR spectroscopy has appeared.\textsuperscript{249}

DEGRADATIVE METHODS

These constitute analysis of constituent moieties of complex lipids, degradation of acyl chain\textsuperscript{250} and stereospecific analysis.\textsuperscript{251-253}

Lot of work has been done with analysis on methodology in our Laboratory e.g. effect of oxalic acid impregnation on chromarod on the separation of phospholipids for determination by TLC/FID,\textsuperscript{254} enhanced response of unsaturated lipids on TLC/FID after exposure of rods to iodine vapour,\textsuperscript{255} determination of lipolysis on plate. TLC\textsuperscript{256} determination of unsaturated by ozonolysis,\textsuperscript{257} esterification using trimethylsilyl alcohol,\textsuperscript{258} comparison between Iatroscan and TLC\textsuperscript{259} and methodology by GC/MS.\textsuperscript{260} The analysis by Iatroscan TLC/FID in general\textsuperscript{261} and recently with reference to lipids,\textsuperscript{262} have been included.

Recent trends in the methodology includes development of fat/oil determinator\textsuperscript{263} specifically designed to eliminate the need for hazardous chemicals in Soxhlet and other solvent extraction methods. Using compressed CO$_2$ as solvent the analyte is extracted from sample and transferred to ultra-efficient solid
phase collection traps. Liquids, solids and semisolids matrices can be analyzed easily with minimal sample preparation. Seeds, dairy products and snack foods are just a few of the proven applications. The sample is simply prepared, loaded into the thimble and dropped into the instrument. Once the automated extraction process is complete the vials are removed from the instrument and transferred to the balance. Weights are electronically transferred into the data management software and the results are automatically collected.

Depending on the degree of saturation and the natural or added antioxidants present, the storage period (hence stability) may be increased to a greater or lesser extent. For determination of the oxidation stability of oil and fats, the Rancimat method could be the method of choice where the samples are prematurely aged by thermal decomposition. The resulting products are blown out by air stream and transferred to measuring cell filled with distilled water, which contains a conductivity increasing cell. As soon as the volatile decomposition products reach the cell, the conductivity increases sharply the time which, elapses before such decomposition products appear is known as the induction time. Evaluation is carried out automatically. The method is the subject of AOCS official methods.

Recently TLC on zinc ferrocyanide-silica gel G plates have been attempted. New technique in fatty oil refining, lipid processing and preparation of oleochemicals were discussed in a
recent seminar.\textsuperscript{266} Applications of Chromarod-Iatroscan TLC-FID in lipid analysis have recently been presented.\textsuperscript{267} Latest is the parametric study of polysulphone membranes for their separation performance.\textsuperscript{268} Separation of alkenyl glycerol ether lipid content have been attempted\textsuperscript{269} and a method for analysis of phospholipid and its products have recently been proposed.\textsuperscript{270} Recently from this Laboratory Lariya\textsuperscript{271} has completed studies on lipids from some nonconventional sources, Soni\textsuperscript{272} has presented work on linoleic acid isomers, Thakur\textsuperscript{273} on microorganism mediated formation of hydroxy fatty acid and Vaidya\textsuperscript{274} on analytical technique for analysis of monoglycerides. Latest work is on quantification of diacylglycerols\textsuperscript{275,276} and acyl lipid composition.
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