Screening analysis of unknown seed oils

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In the present paper, modern tools for the screening analysis of unknown seed oils have been reviewed. The known plant fatty acid structures have been divided into three groups (usual, unusual non-oxygenated, unusual oxygenated fatty acids) and examples for each class are presented. Moreover, the importance of chemotaxonomy for the screening analysis of some lipid components is discussed. The main part of the work is focussed on various aspects of preliminary seed oil analyses for the detection of unusual fatty acids and lipid classes and the analysis of fatty acid derivatives by gas chromatography and gas chromatography/mass spectrometry. Furthermore, some useful methods to obtain rapid information on the configuration of double bonds are cited. The paper concludes with a scheme for the detection of unusual fatty acids and lipid classes in seed oils.

Screening-Analyse unbekannter Samenöle. Im vorliegenden Übersichtartikel werden moderne Methoden für die Screening-Analyse von unbekannten Samenölen vorgestellt. Die bekannten Pflanzenfettsäurestrukturen wurden in drei Gruppen unterteilt (gewöhnliche, ungewöhnliche nicht-oxidierte und ungewöhnliche oxidierte Fettsäuren), und entsprechende Beispiele für jede Gruppe werden präsentiert. Außerdem wird die Bedeutung der Chemotaxonomie für die Screening-Analyse diskutiert. Der Hauptteil des Artikels konzentriert sich auf die Methoden zur schnellen Detektion von ungewöhnlichen Fettsäuren und Lipidklassen in Samenölen und auf die gaschromatographische und gaschromatographisch-massenspektrometrische Analyse verschiedener Fettsäurederivate. Es werden weiterhin einige Methoden zur schnellen Bestimmung der Doppelbindungskonfiguration zitiert. Die Arbeit schließt mit einem Analysenschema zur Detektion von ungewöhnlichen Fettsäuren und Lipidklassen in Samenölen ab.

1 Introduction

Most of the seed oils consist predominantly of triacylglycerols, which are esters of fatty acids with glycerol. The main part of the fatty acids in the plant kingdom are saturated or olefinic unsaturated straight-chain C_{16} or C_{18} compounds with a terminal carboxyl group. In the diene and triene derivatives, respectively, the double bonds are interrupted by a methylene group. Typical members of these structure types are palmitic, stearic, palmitoleic, oleic, linoleic, and linolenic acid. *Smith* [1] classified these ubiquitous compounds as *usual* fatty acids. Consequently, all fatty acids with different structure features are considered as *unusual*. Due to the progress in fatty acid research in recent years, the classification of *Smith* [1] from 1971 is certainly somewhat outdated and discussible. Nevertheless, the author classified the known seed oil fatty acid structures mainly based on the approach of *Smith* [1]. In Tab. 1, a classification in three main groups (usual, unusual non-oxygenated, unusual oxygenated) and, depending on more detailed structure features, in further sub-groups is presented.

For the chemist, the isolation and elucidation of rare fatty acid structures has always been a challenge, and nowadays the number of known fatty acids exceeds thousand [28]. Recently, the interest in fatty acids with unusual structure features and in oils, that can provide a high concentration of a single fatty acid, has increased as they can be of high value for the chemical and pharmaceutical industry [28–33]. Global screening programs for the search of new plant oils as renewable sources have been initiated. This is also a preventive research as it is estimated that there is approximately only 50–100 years supply left in readily accessible fossil oil resources, which are actually the most important basis of the petrochemical industry [34]. About 14% of the world production of vegetable oils (> 60 million metric tons/annum) are already used as raw material for the production of e.g. biodiesel, lubricants, plastifiers, polishes, varnishes, detergents, paints, inks, nylon, cosmetics, pharmaceuticals, and many other products, and its utilization will probably be extended in the next years [28]. In contrast to fossil oil resources, the vegetable oils are renewable and have the ecological advantage to be CO_2-neutral [34].

When a wild plant with interesting seed oil properties is detected, one of the next steps to develop an industrial crop is its domestication. This is a difficult task and depends on a series of factors, e.g. climatic and soil conditions. However, the recent progress in genetic engineering, as transgenic rapeseeds, points to the future possibility to transfer the genes of interest from wild plants into established oil plants [35]. Thus, the production of special seed oils with a desired fatty acid composition in industrial scale may be possible in future without having the domestication problem. There are already attempts underway to transfer the gene for a hydroxy fatty acid (ricinoleic acid) from castor seeds to rape seeds [36].

The importance of the oil plants for the future demands that unknown plant species are chemically analyzed and evaluated. This task is a race with time as, up to now, only 10% of the plant kingdom has been chemically examined and many of the unknown species, especially in tropical and subtropical regions, are threatened to become extinct.

The present work may serve as a guide for a fast and aimed screening analysis of novel seed oils with modern analytical methods. Outgoing from a consideration of chemotaxonomical facts, a strategy for the detection/identification of various fatty acid and lipid structures is presented. It is limited to analytical techniques that in the author's opinion are available for most analysts in a chemical re-

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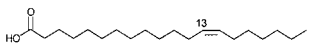
search environment. Methods for enrichment and isolation of minor, individual or chiral compounds are not included. Due to the great number of publications in the field of seed oil analysis, it was also not possible to consider all references and the selection was rather subjective without the intention to slight anyone not cited herein.

Tab. 1. The structures of seed oil fatty acids.

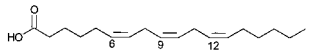
I Usual fatty acids

II Unusual non-oxygenated fatty acids (Example of occurrence[a])

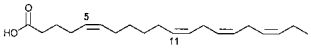
1 Mono-olefinic fatty acids (paullinic acid; *Paullinia elegans* [2])



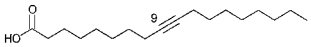
2 Non-conjugated poly-olefinic fatty acids, methylene-interrupted (γ-linolenic acid; *Borago officinalis* [4])



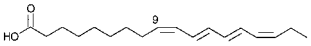
3 Non-conjugated polyolefinic fatty acids, non-methylene-interrupted (all *cis* 5, 11, 14, 17-eicosatetraenoic acid; *Biota orientalis* [6])



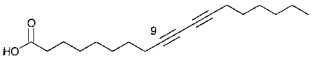
4 Non-conjugated acetylenic fatty acids (sterolic acid; *Iodina rhombifolia* [3])



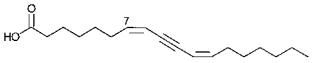
5 Conjugated olefinic fatty acids (α-parinaric acid; *Sebastiana brasiliensis* [8])



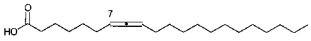
6 Conjugated acetylenic fatty acids (isanic acid, *Curupira tefeensis* [10])



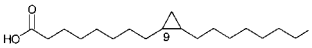
7 Conjugated olefinic-acetylenic fatty acids (heisteric acid, *Heisteria silvanii* [11])



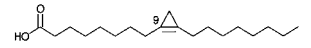
8 Allenic fatty acids (phlomic acid; *Phlomis tuberosa* [13])



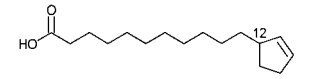
9 Cyclopropane fatty acids (dihydrosterculic acid; *Pachira aquatica* [14])



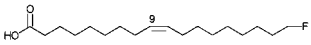
10 Cyclopropene fatty acids (sterculic acid; *Pachira aquatica* [14])



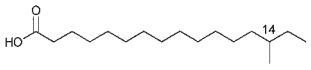
11 Cyclopentene fatty acids (hydnocarpic acid; *Hydnocarpus anthelmintica* [16])



12 Fluoro fatty acids (ω-fluorooleic acid; *Dichapetalum toxicarum* [18])

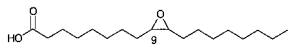


13 Methyl-branched fatty acids (14-methylpalmitic acid; *Ginkgo biloba* [26])

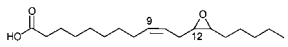


III Unusual oxygenated fatty acids (Example of occurrence[a])

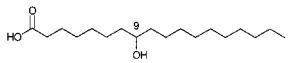
1 Saturated epoxy fatty acids (9,10-epoxystearic acid; *Iodina rhombifolia* [3])



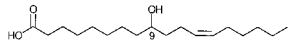
2 Unsaturated epoxy fatty acids (vernolic acid; *Bernardia pulchella* [5])



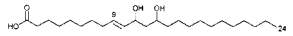
3 Saturated hydroxy fatty acids (9-hydroxystearic acid; *Iodina rhombifolia* [3])



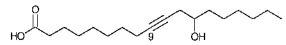
4 Non-conjugated ethylenic monohydroxy fatty acids (isoricinoleic acid; *Semecarpus kurzii* [7])



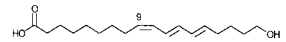
5 Non-conjugated ethylenic polyhydroxy fatty acids (axillarenic acid; *Baliospermum axillare* [9])



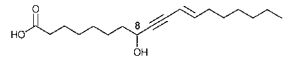
6 Non-conjugated acetylenic monohydroxy fatty acids (12-hydroxy-octadec-9-ynoic acid; *Agonandra brasiliensis*)



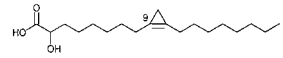
7 Conjugated ethylenic hydroxy fatty acids (kamlolenic acid; *Trewia nudiflora* [12])



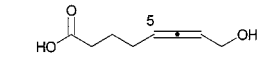
8 Conjugated acetylenic hydroxy fatty acids (8-hydroxyximenynic acid; *Iodina rhombifolia* [3])



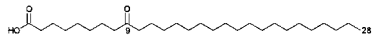
9 Hydroxy cyclic fatty acids (2-hydroxy sterculic acid *Pachira aquatica* [14])



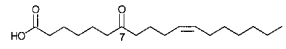
10 Hydroxy allenic fatty acids (8-hydroxy-5,6-octadienoic acid; *Sebastiana commersoniana* [15])



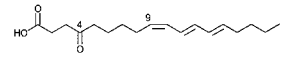
11 Saturated keto fatty acids (9-oxooctacosanoic acid; *Argemone mexicana* [17])



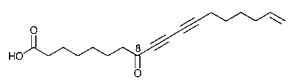
12 Non-conjugated ethylenic keto fatty acids (7-Ketooctade-*cis*-11-enoic acid; *Gardenia lucida* [19])



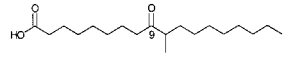
13 Conjugated ethylenic keto fatty acids (α-licanic acid, *Couepia longipendula* [21])



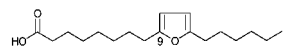
14 Conjugated acetylenic keto fatty acids (8-oxo-octadeca-9,11-diyn-17-enoic acid; *Ongokea gore* [22])



15 Methyl-branched keto fatty acids (9-oxo-10-methyl-stearic acid; *Pseudobombax elliptica* [23])



16 Furanoid fatty acids (8-(5-hexyl-furyl-2)-octanoic acid; *Exocarpus cupressiformis* [24])



[a] Further examples for each fatty acid class are found in ref. [1, 35–37].

2 Fatty Acids and Lipids vs. Chemotaxonomy

Depending on the objective of the research project, one can make:

- a screening for distinct fatty acids,
- a screening within particular plant families or genera or
- a random screening of novel oil seeds.

In all cases, a careful consideration of the available chemotaxonomical data is highly recommended as seed oils from plants, that belong to the same family, show frequently similarities in their fatty acid composition. Especially the presence of certain unusual fatty acids correlates sometimes very well with the botanical classification. A presence or absence can even indicate closer or less close relationships between the species concerned within a family [37]. Typical examples of fatty acids with chemotaxonomical importance are the conjugated acetylenic fatty acids of the Santalaceae and Olacaceae, the cyclopropenoid fatty acids of the Malvaceae and Sterculiaceae and the cyclopentene fatty acids of the Flacourtiaceae (see Tab. 1).

On the other hand there exist some plant families, such as the Euphorbiaceae, that demonstrate a great diversity of unusual lipid structures and no common pattern can be concluded. In this case for instance, a random screening may yield interesting results. There are also plant families that do not have a direct botanical relationship, but show a very similar fatty acid pattern. As an example, the families Lauraceae and Palmae can be mentioned, as both of them accumulate lauric acid. Finally, it must also be brought up that there is a large number of plant families, in which no unusual fatty acids occur [38] and their patterns of usual fatty acids do not have chemotaxonomical significance [37].

In conclusion, the usefulness of fatty acids as an unequivocal guide to plant classification is somewhat limited and often restricted to particular families. Nevertheless, the importance of the chemotaxonomical data should not be overlooked. Before analyzing novel seed oils, it is always recommendable to know the lipid components of known species of the same family. If special fatty acid structures are required, the evaluation of the chemotaxonomical data can save time as some plant families can be excluded and the work can be focussed firstly on distinct plant families. In the author's opinion, a careful consideration of all known chemical data on the seed oils of the respective plant family is an essential part of the screening analysis and will help to sharpen and direct the analytical eye. An excellent source of plant chemistry data is the series "*Chemotaxonomy of Plants*" from *Hegnauer* [39]. Some special compilations of the occurrence of rare fatty acids are also available. They are classified due to plant families [25], fatty acid structures [1, 40, 41], and plant species [42], respectively. The most recent general reference of fatty acid structures is "The Lipid Handbook" [43]. A further useful data source can be found in the internet. Researchers from the NCAUR, ARS/USDA (National Center for Agricultural Utilization Research) published the analytical data of about plant species, and the data can be searched without any charges in the World Wide Web at http://www.ncaur.usda.gov/nc/ncdb/search.html-ssi [43].

It must also be mentioned here that in some special cases the main part of the fatty acids can be bound in other molecules than triglycerides. Some examples of these unusual lipid structures are summarized in Tab. 2. Their occurrence can be restricted to only one or a few families. For instance, toxic cyanolipids and wax esters were reported only for Sapindaceae [48] and Jojoba seed oils [47], respectively. Acetoglycerides were found in the seed oils of species from the Celastraceae, Lardizabalaceae, Ranunculaceae, Rosaceae [49, 50], and Balsaminaceae [51], and tetra- and pentaacylglycerides appeared in Euphorbiaceae, Cruciferae, Polygalaceae and Compositae [45].

Tab. 2. Examples of unusual lipid classes in seed oils.

Lipid class	Occurrence
α-Acetotriglycerides	*Maytenus ssp.*, Celastraceae [44]
Cyanolipids (Type I–IV)	*Paullinia elegans*, Sapindaceae [2]
Tetraacylglycerides	*Trewia nudiflora*, Euphorbiaceae [45]
Tetraacylallenestolides	*Sebastiana commersoniana*, Euphorbiaceae [15]
Pentaacylglycerides	*Chamaepeuce afra*, Compositae [46]
Waxester	*Simmondsia californica* (Jojoba), Buxaceae [47]

Although the amount of available data has increased dramatically in the last years, a lot of plant families are still under-exploited, their chemical data are incomplete or became obsolete because the analytical results were obtained with antiquated methods. Thus, every analysis of novel seed oils is a valuable contribution to our knowledge on lipid chemistry and its expansion may help to make even more use of it.

3 General Remarks on Screening Analysis

To prevent a misidentification of the plant material, it is recommended to collect and identify the seed samples only with a botanist, who knows the respective plant families. Every plant collection should be moreover documented by a reference specimen. If it is possible, the collection at different places should be considered to recognize eventual genetic variations [29].

The seed sample amount for a screening analysis varies with the absolute oil content and the type of the fatty acids present. If the seed oil only consists in usual or more frequently occurring unusual fatty acids, only a few mg of oil will be needed, owing to the high chromatographic separation capacity and sensible detection methods. The presence of rare unusual or novel fatty acids demands higher oil quantities as further separation/isolation steps and spectroscopic measurements may be necessary. Generally, it can always be recommended to store a part of untouched seed sampled for eventually necessary further experiments.

The extraction and storage of the oil should be done with all precautions to avoid excessive autoxidation of highly unsaturated compounds. The natural tissue antioxidants, such as the tocopherols, give some protection to the oil extracts, but it is generally advisable to add about 10–100 mg/l of a synthetic antioxidant, such as butylated hydroxytoluene (BHT), to the extraction solvent [52]. Before the complete extraction of the seed oil, it may be also desirable to make a preliminary extraction by crushing a very small amount of seeds with few ml of petroleum ether and sodium sulfate in a mortar with a pestle. The resulting supernatant extract can be applied on a silica thin-layer plate and examined under ultraviolet light (254 nm). If conjugated unsaturated compounds are detectable, it is advisable to do

the further extraction and sample handling under a nitrogen atmosphere.

Further details on lipid extraction methods have been reviewed recently [52–54].

4 Analyses of the Seed Oil

4.1 Preliminary analyses

In most of the cases, the main target of screening analyses of novel seed oils is the identification of the fatty acids. However, to make a first evaluation of a novel oil, it is advisable to get a general knowledge of its total composition. As mentioned before, it is possible that a seed oil consists of other molecular species than only usual triglycerides. The presence of such compounds (Tab. 2) may have a significant influence on the technological, toxicological, and nutritional properties of the seeds and its oil, respectively. An analysis of the fatty acids only can totally prevent the detection of these compounds or some of their esterified fatty acids [55] and may even lead to a misidentification of the fatty acids present. It is also important to check the oil for the presence of unusual unsaponifiable matter as observed, e.g. in *Curupira tefeensis* [56] or *Peschiera australis* [57].

4.1.1 Thin-layer analysis of the seed oil

An easy way to get preliminary information on the oil composition is thin-layer chromatography (TLC) on silica gel layers with a solvent system as e.g. hexane/diethyl ether/acetic acid = 80/20/2 (v/v/v) [52]. Detection of each lipid class can be made first under UV light (254 nm) and then after spraying with H_2SO_4/MeOH (50%) and subsequent heating. Generally, about seven spot sections can be observed:

1. alkanes/alkenes/squalene/sterolester/waxester (Rf ≈ 98-94);
2. triglycerides (Rf ≈ 60; dominant);
3. free fatty acids (Rf ≈ 39);
4. sterols (Rf ≈ 19);
5. diglycerides (Rf ≈ 15-21);
6. monoglycerides (Rf ≈ 2);
7. phospholipids (Rf ≈ 0).

The TLC analysis enables a preliminary detection of:

- the presence of compounds with conjugated double/triple bonds (detection under UV light),
- the presence of free fatty acids (Rf value comparison),
- the presence of oxygenated fatty acids, unusual lipid classes or other unusual compounds.

There exist also some special spraying reagents that permit the selective detection of e.g. keto, epoxy, hydroxy, and cyclopropene groups [58]. However, the author prefers the use of instrumental analytical methods (see 4.1.2–4.2), as these techniques provide more complete information and are not hazardous to the analyst.

If significant unusual TLC spots are detectable, the oil can be hydrolyzed, and the resulting non-saponifiable matter and free fatty acid fractions can be separated and further analyzed. To prevent the destruction of acid-labile fatty acids, as those with an epoxy and cyclopropene ring, the time with acidic conditions must be as short as possible [59].

For more details on TLC analysis of lipids see e.g. ref. [60–62].

4.1.2 Ultraviolet spectroscopy of the seed oil

Ultraviolet spectroscopy (UV) is useful for the detection of conjugated unsaturated systems and is discussed in section 6.3.

4.1.3 Infrared spectroscopy of the seed oil

Most of the usual seed oils show very similar infrared (IR) spectra between 4000 and 660 cm^{-1}. Therefore, the presence of additional IR absorptions can be attributed to less common functional groups. This technique has been employed in a number of works, and in Tab. 3, some IR absorption bands, that are useful for the detection of unusual lipid components, are summarized.

It is of advantage to record the IR spectra with a *Fourier* transform IR spectrometer, as good spectra can be obtained with small quantities of oil samples in a short time. More details on IR spectrometry and double bond configuration are discussed in 6.2.

4.2 Nuclear magnetic resonance spectroscopy of seed oils

In the past, NMR spectroscopy was mainly a tool for the analysis of pure compounds. Due to the now widely available high-resolution spectrometers, the immense improvement of computer power, and the introduction of new pulse sequences, the technique was recently also applied with great success to mixtures, such as seed oils.

Tab. 3. Some infrared absorption bands of uncommon functional groups in seed oils.

Absorption [cm^{-1}]	Functional group	Absorption [cm^{-1}]	Functional group
3550/1265/1219/1110	α-hydroxy [63]	1002	*trans*-double bond α-β unsaturated carboxyl [64]
3450	chain hydroxy [28]	987	conjugated *trans*-*trans*-*trans*-double bonds [65]
3077/1020	cyclopropane [66]	985	conjugated *trans*-*trans*-double bonds [65]
2250	cyanolipids with cyanogenic cyano group [67]	968	non-conjugated *trans*-double bond [28]
2220 (weak)	conjugated diacetylene [65]	958/987	conjugated *cis*-*trans*-*trans* double bonds [65]
1960	allene [15]	955	conjugated acetylenic-*trans* system [3]
1852/1010	cyclopropene [28]	952/985	conjugated *cis*-*trans*-double bonds [65]
1720	α-β unsaturated carboxyl [64]	910	terminal vinyl [3]
1705	chain keto [68]	848/826	epoxy [5]
1235/1370/1050	α-acetoacetates [44]	820	*cis*-double bond in α-β unsaturated carboxyl [64]

4.2.1 ^{1}H Nuclear magnetic resonance spectroscopy

With about 1 mg of seed oil, a ^{1}H NMR spectrum can be recorded in a few minutes. In Fig. 1 the spectrum of an Euphorbiaceae seed oil is shown as an example. The signals with the numbers 1, 2, 4, 5, 6, and 8–12 (Fig. 1) can be observed in all triglyceride seed oils that contain usual fatty acids, including linolenic acid. As only a relatively small number of signals (> 9) can be observed, the information impact of ^{1}H NMR spectra is limited in comparison with that of ^{13}C NMR spectra (see 4.2.2). Nevertheless, the detection of a number of functional groups in seed oils, such as allenes [15, 69], conjugated double bonds [8, 15, 70, 71], conjugated double-triple bonds [3, 10, 11, 72], cyclopropane [73] and cyclopropene rings [74], cyclopentene [75], epoxy [5], hydroxy [14, 76], and terminal vinyl groups [3] is possible by their characteristic ^{1}H NMR signals. The methylene groups adjacent to a keto group show similar shift values as those adjacent to the carboxyl group when located remote from the carboxyl or other functional groups. However, in some special cases the presence of a keto group can be indicated by its ^{1}H NMR spectrum as shown, e.g. for 4-keto-α-eleostearic acid [77].

By a careful interpretation of the coupling patterns and consideration of the integral values of the signals the configuration of the double bonds in conjugated systems can be elucidated even in mixtures [3, 8, 15, 78]. Furthermore, it is possible to distinguish fatty acids that contain a double bond at the *n*-3 terminal [8, 79, 80] from other unsaturated fatty acid isomers by means of their terminal methyl signals. In the case of an Euphorbiaceae seed oil, that contained two *n*-3 acids (linolenic and α-parinaric acid (18:4(9*c*, 11*t*, 13*t*, 15*c*)), even the separation of their terminal methyl and allylic methylene ^{1}H NMR signals (Fig. 1) could be observed in the oil mixture [8].

By integration of distinct ^{1}H NMR signals, a quantification of unusual fatty acids in lipid mixtures can be effected in an easy and non-destructive way, as was shown for cyclopropene [74], *n*-3 fatty acids [79], and epoxy [5] fatty acids.

The ^{1}H NMR data can also permit the detection/quantification of unusual lipid classes, e.g. acetolipids [44], cyanolipids [2, 67], and allene estolides [15].

4.2.2 ^{13}C Nuclear magnetic resonance spectroscopy

Because of the naturally low content of the ^{13}C isotope (^{12}C/^{13}C NMR = 98.9/1.1), ^{13}C NMR spectroscopy is an inherently insensitive method and for routine analysis about 50 mg of oil are necessary to obtain a spectrum. However, by measuring overnight, good spectra can be made with 1 mg of oil. The detection limit for each fatty acid is 2–5%, depending on its structure and measuring parameters. It may be important to mention, that the NMR operator must consider the possible presence of quaternary carbon atoms, such as from acetylenic or keto groups. When a spectrum is recorded routinely in the broad band decoupled mode, the signals of the latter functional groups are weak in comparison to the methyl, methylene, and methine signals (nuclear *Overhauser* effect) and may be overlooked.

As an alternative for the routine measurement of the oil in deuteriochloroform solution, in some cases the direct analysis of whole seeds is possible by using "Magic Angle Sample Spinning NMR" [81, 82].

The chemical shift and the intensity of the ^{13}C NMR signals provide structural information on the individual fatty acids and lipid classes, and in some cases, even the elucidation of the distribution of the different acyl groups on the glycerol backbone and the position of the unsaturated centers and functional groups in the acyl group is possible [83]. The analyst decides in which depth he wants to explore the data, and for screening analysis, the identification of the fatty acids and unusual lipid classes will be the main goal in most cases. Depending on the seed oil examined and the available resolution, more than 50 signals can generally be observed, and by using techniques as "Attached Proton Test" (APT) or "Distortionless Enhancement by Polarization Transfer" (DEPT), immediate information on the multiplicity of the individual carbons can be obtained additionally [76].

The chemical shift of a carbon atom depends on its environment to a distance of six and more atomic centers. Beside the shift value of the considered carbon atom with a functional group, the shift values for the carbon atoms in their immediate neighborhood are of great importance. Some typical chemical shift ranges of ^{13}C NMR signals from fatty acids are summarized in Tab. 4 and a spectrum from an Olacaceae seed oil is presented as an example in Fig. 2A. Six characteristic signal clusters due to the terminal methyl, the methylene, the glycerol, the acetylenic, the olefinic, and the carbonyl carbon atoms can be observed, and in Fig. 2 B–D more spectral details are shown. By comparison with literature data, a first look at the spectrum permits already a series of conclusions, as for example:

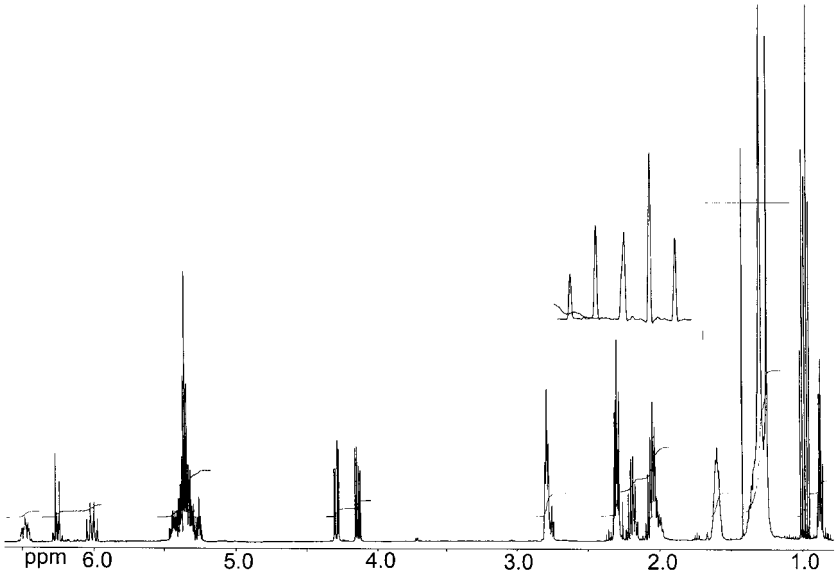


Fig. 1. ^{1}H NMR spectrum of an Euphorbiaceae seed oil (500 MHz). Signal groups (ppm) at δ 0.88 (CH_3, all fatty acids); 0.968 (CH_3, linolenic acid); 1.0 (CH_3, parinaric acid), 1.28 (bulk CH_2), 1.58 ($C\underline{H}_2$-CH_2-COOR, all FA); 2.05 (allylic CH_2), 2.2 (allylic CH_2, parinaric acid), 2.3 (-$C\underline{H}_2$-COOR); 2.8 (double allylic CH_2); 4.1–4.3 (glycerol CH_2); 5.25 (glycerol CH); 5.35 (olefinic CH); 5.43–6.5 (conjugated olefinic CH, parinaric acid). For more details see ref. [8].

Tab. 4. Chemical ^{13}C NMR shift ranges (in ppm) of some carbon atoms from seed oils[a].

Signal range	Carbon atoms of	Signal range	Carbon atoms of
> 210	–*C*=O	62	–CH_2–O– (Glycerol)
205	–CH=*C*=CH–	60–75	–*C*HOH
173–172	–*C*OOR	58.5	*C*H– in *trans* epoxy
165	–CH=CH–*C*OOR	56.5	*C*H– in *cis* epoxy
156/105	*C*H– in furanoid fatty acid	33	–CH_2–CH=CH– (*trans*)–
150–100	–*C*H=*C*H– (isolated, conjugated)	27	–CH_2–CH=CH– (*cis*)–
109	*C*H– in cyclopropenoic fatty acid	19.5	–CH_2–C≡C–
80–90	–*C*H=C=*C*H–	16	*C*H cyclopropane ring
68	–*C*HO– (Glycerol)	11	*C*H_2 cyclopropane ring
65–92	–C≡C–	7.5	*C*H_2 cyclopropene ring

[a] Most of the shift values were adapted from ref. [83].

- Fig. 2B: Presence of oleic acid as main unsaturated fatty acid and a low concentration of linoleic and linolenic acid proved by the small satellite peaks that surround the intense oleic acid signals. Presence of olefinic carbons (X, Y, Z) in an unsymmetrical environment (such as conjugated double/triple bonds).
- Fig. 2C: Presence of at least six acetylenic carbons. The distribution of their intensities suggests that they may originate from three different fatty acids (X, Y, Z). As the signals for X,Y are very close a similar structure is probable.
- Fig. 2D: Presence of at least two methylene groups in allylic position to a *trans*-double bond. Presence of methylene groups in allylic position to a *cis*-double bond. Presence of methylene groups in propargyllic position to a triple bond. The signals between 29.4 and 30 ppm derive from the methylene carbons ("methylene envelope") that do not have functional groups in their direct neighborhood. They are generally excluded from spectra interpretation.

The quantitative integration of the ^{13}C signals is only possible with approved safeguards concerning the relaxation problem of the individual ^{13}C NMR nuclei [83].

A number of papers provided a wide range of detailed ^{13}C NMR data of fatty acids and lipids, e.g. for acetylenes [84–87], allenes [15, 69], conjugated acetylenes [10], conjugated dienes [5, 85, 88], trienes [89–92], tetraenes [8, 91, 93], conjugated olefin-acetylenes [3, 11, 72], cyclopropenes [14, 83], epoxy [94], hydroxy [14, 95, 96], keto [95, 97], and olefins (monoenes [84, 85, 87, 98]; polyenes, methylene-interrupted [99–102]; non-methylene-interrupted [98, 103, 104]).

The detection of acetolipids [83], allene estolides [15], cyanolipids I–IV [2, 105, 106], and free fatty acids [83] is also possible by ^{13}C NMR spectroscopy. In an Apocynaceae seed oil, even the detection and identification of two apolar alkaloids was possible through analyzing the ^{13}C NMR data of the lipid mixture [57].

Gunstone has reviewed and tabulated most of the results in ^{13}C NMR spectroscopy of lipids of the last years in a comprehensive manner [83, 107–109], and these data collections are certainly of great value for every lipid analyst.

The ^{13}C NMR data of seed oils lack direct information on the chain length of the individual fatty acids. Therefore, a combined interpretation of the ^{13}C NMR and GC (or

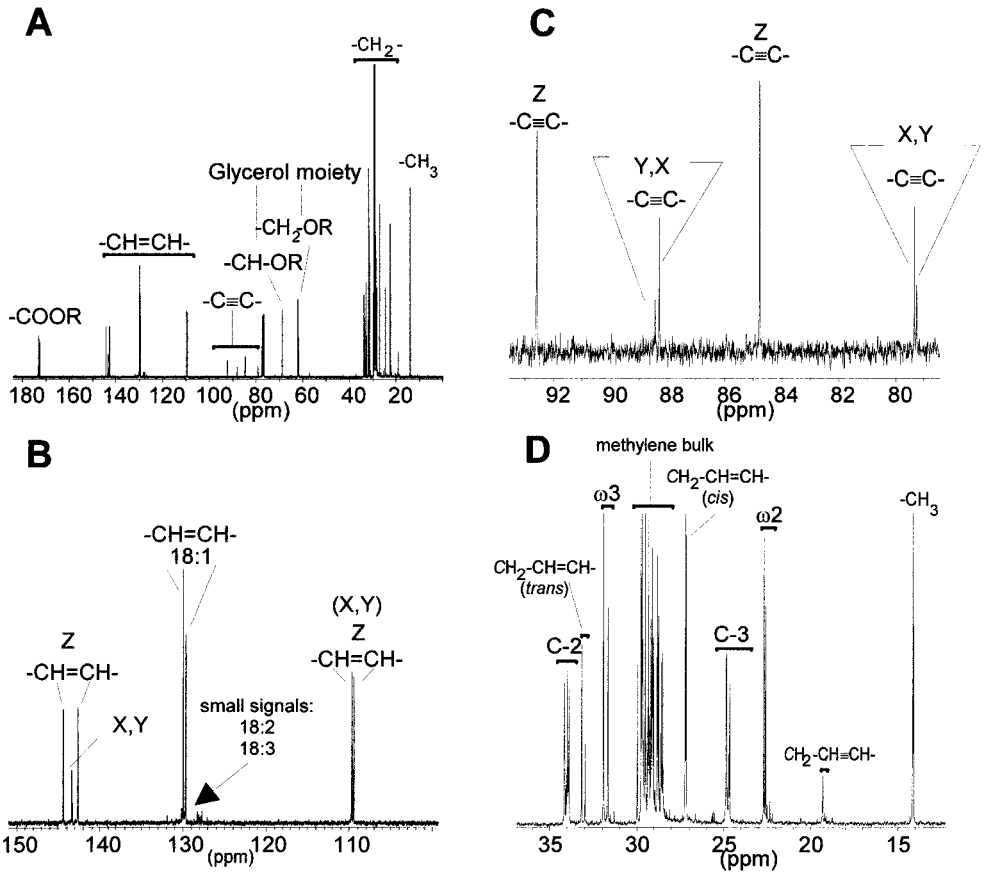


Fig. 2. The ^{13}C NMR spectrum (recorded at 100 MHz) of an Olacaceae seed oil. The characteristic features are explained within the figure.

GC/MS) data is recommended when a novel seed oil has to be evaluated.

4.2.3 Two-dimensional nuclear magnetic resonance spectroscopy of seed oils

The measurement of two-dimensional NMR spectra of the whole seed oil can provide useful structure information. It was shown that the ^{1}H-^{1}H homonuclear (COSY) and ^{1}H-^{13}C NMR heteronuclear correlation spectroscopy (HETCOR) enabled the identification of the configuration of the conjugated triene system of α-cleostostearic acid in cherry oil [78] and the configuration and localization in the fatty acid chain of the conjugated tetraene system of α-parinaric acid in an Euphorbiaceae seed oil [8].

An interesting high-field ^{1}H NMR (600 MHz) study of olive oil showed that the simultaneous detection of minor compounds, such as aldehydes, diacylglycerols, linolenic acid, water, acetic acid, phenols, and sterols is possible by using two-dimensional totally correlated spectroscopy (TOCSY) and nuclear *Overhauser* effect spectroscopy (NOESY) [110].

Up to now, the two-dimensional NMR spectroscopy was used only in few works for the analysis of seed oils and further investigations in this area are desirable.

5 The Analysis of Fatty Acids

5.1 Gas chromatography

5.1.1 Preparation of fatty acid methyl esters

Gas chromatography (GC) is still the most valuable method for separation and quantification of fatty acid mixtures. To increase the volatility, the fatty acids are commonly converted to their methyl esters before analysis. This analytical step must be critically evaluated as unwanted structure modifications can occur depending on the type of fatty acids and the chosen reaction conditions.

For unusual seed oils, it is generally advisable to use a mild and fast reacting basic transesterification reagent, such as sodium methylate. If large amounts of free acids are present, these can be converted to their methyl esters with diazomethane [59]. However, diazomethane is extremely toxic and is known to form artifacts in some circumstances by reacting with double bonds or carbonyl groups [111]. The acid-catalyzed transmethylation procedure, e.g. using sulfuric acid (1–2%) in methanol, have the advantage to transform esterified and free fatty acids in one step. However, long reaction times are required and, when fatty acids with cyclopropene rings, epoxy groups or conjugated double bonds are present, some drastic structure changes, such as ring disruption and isomerization, can occur [112]. The newer quaternary ammonium reagents, such as tetramethylguanidine hydroxide, are a mild and fast alternative for transmethylation [112].

Excellent surveys with more details on fatty acid derivatization techniques were published recently [59, 111, 113].

5.1.2 Sample injection, separation, and detection of the fatty acid methyl esters

The most frequent used injector is certainly the split injector. It is universal, flexible and the injection can easily be automated. Albeit this injector type has also some drawbacks, such as sample discrimination for compounds with a broad range of molecular weights, it can be recommended for screening analysis. On-column injectors might be the most appropriate system for analyzing oil samples [114], but it is less frequently installed in GC systems and its handling is more difficult. The importance of the injection technique in lipid analysis has been discussed in detail [115, 116].

The use of WCOT (Wall Coated Open Tubular) fused-silica capillary columns for the separation of fatty acid methyl esters has been widely accepted in recent years and a variety of stationary phases with different polarities are commercially available. On non-polar phases, the fatty acid methyl esters are separated due to their boiling point, and the unsaturated compounds elute in front of the corresponding saturated acid with the same chain length. Unfortunately, partial overlapping of biologically important unsaturated fatty acids, such as linolenic, linoleic, and oleic acid is observed [111, 114, 117]. In contrast, columns with polar phases permit the separation of essentially all chain lengths and unsaturated bond number homologues, including a number of monoene and polyunsaturated isomers [114, 118, 119].

Certainly none of the commercially available columns is able to completely separate all theoretically possible fatty acid mixtures, but very good results can be obtained by analyzing with a capillary column coated with polar stationary phases. In Tab. 5 some typical examples are summarized.

5.1.3 Identification of the fatty acid methyl esters

The identification of the usual fatty acids can be carried out by comparison of the relative retention times or ECL

Tab. 5. Some examples of capillary columns used for the separation of unusual fatty acids.

Capillary column	Used for the separation of:
BPX-70™	Δ5-unsaturated polymethylene-interrupted fatty acids in Ranunculaceae [120]; γ-linolenic, α-linolenic, and stearidonic acid in Boraginaceae [121]; acetylenic fatty acids in Olacaceae [11]
Carbowax 20M™/DB Wax™	Conjugated triene fatty acids in Cucurbitaceae [90], positional monoene isomers in Sapindaceae [2, 122], Δ5-unsaturated polymethylene-interrupted fatty acids in Cupressaceae, Pinaceae, and Taxaceae [123]
CP-SIL-88™	Unusual unsaturated double bond and configuration isomers in Ranunculaceae [124] and Pinaceae [125]
CP-WAX 52CB™	Methyl-branched and Δ5-unsaturated polymethylene-interrupted fatty acids in *Ginkgo biloba* [20]
DB-23™	Cyclopropanoic, cyclopropenoic, and hydroxy fatty acids in Bombacaceae [14], positional monoene isomers in Sapindaceae [2, 122], conjugated triene, tetraene, and keto fatty acids in Chrysobalanaceae [21, 77, 126] and Euphorbiaceae [6], acetylenic acids in Olacaceae [3, 128] and Santalaceae [3, 128]
SILAR 5 CP™	Δ5-unsaturated polymethylene-interrupted fatty acids in Ranunculaceae [129], allenic fatty acids [13]
SP2330™	Polyunsaturated fatty acids in various species [130]

Detection of the individual compounds can be made with a flame-ionization detector (FID).

(equivalent chain-length) values with standards or with fatty acid methyl ester mixtures from well-studied oils. A comprehensive retention data collection for a series of other unsaturated fatty acids is available [117, 131, 132], and the general GC characteristics of some unusual fatty acid types have been reviewed by *Christie* [118]. All these data permit with certain restrictions a tentatively identification of unknown peaks.

The increment in ECL value of a given fatty acid methyl ester over that of the saturated ester with the same chain length, called "fractional chain length" (FCL), is dependent on the structure of the compound, and thus influenced by the number of double/triple bonds and/or functional groups and their relative position(s) in the fatty acid chain [118]. It was shown that the ECL values of fatty acids can be predicted by using suitable reference compounds [118]. For instance, the ECL values obtained by arithmetic calculation using appropriate standards on CP-SIL 88™ and DB-Wax™ capillary columns were, e.g. employed successfully for the identification of fatty acids from conifer seed oils [123, 125].

The analyst should know that retention data, such as ECL values and relative retention time, may vary a little with certain column and separation conditions and are also influenced by compounds that elute adjacent to the fatty acid in consideration [118]. For instance, *Wolff* [133] demonstrated that the elution order of 20:1(n-9) and 18:3(n-3) varied with temperature using a CP-SIL 88™ column. It must also be mentioned that the separation characteristics of two columns of the same type can demonstrate slight variations from charge to charge and even different elution orders, e.g. for Silar 5CP columns were reported [124]. A recent paper from *Stransky* et al. [134] demonstrated that the accuracy of the ECL values depends also on the method of calculation and the same authors proposed in a following work [132] that the linear dependence of the ECL values on the number of carbons should be substituted by a higher order polynomial to obtain more accurate results.

Several authors recommended the employment of at least two columns with different selectivity [111] and it was stated that reliable retention data on three different phases have even higher information value for the identification of organic compounds than their mass spectra [132]. A three-dimensional *Kovats* retention index system using four different columns for identification of seed oil fatty acids was also proposed [135].

It was also suggested to analyze a given sample first on a non-polar capillary column. In this way, peak groups indicating the fatty acids with the same chain length are obtained and must be accounted for the following subsequent analysis on a polar capillary column [136]. As an alternative, a total reduction of the unsaturated systems with hydrogen under PtO_2-catalysis [59] and subsequent analysis on a polar column, can be carried out and yield the same results.

In the author's opinion, the use of various columns with different polarities is certainly advantageous but not always essentially necessary, when the results of the GC analysis are completed with other chemical/spectroscopic methods and "hyphenated techniques", e.g. GC/MS analysis of various fatty acid derivatives. The utilization of further identification methods seems almost indispensable to evaluate new seed oils. Otherwise it is possible that compounds, having accidentally the same retention time as an usual compound, are misidentified or can be overlooked. This is for instance possible in the case of the allenic C_{18} fatty acid, labellenic acid (18:2(5,6)), which is difficult to separate from linoleic acid, even by capillary GC [137].

5.1.4 Pre-separation of complex samples

In the case of complex fatty acid mixtures or peak overlapping, it may be necessary to simplify the sample by an additional separation step before GC analysis.

Fatty acid methyl esters with polar functional groups, such as hydroxy, epoxy, and keto, can easily be separated from non-polar compounds by preparative adsorption TLC on silica gel [138].

A method to simplify a mixture, that contains fatty acids with double bonds, is the formation of acetoxymercurimethoxy derivatives. The subsequent separation of these derivatives, e.g. by TLC, depends only on the degree of unsaturation, and not on the stereochemical configuration or position of the double bond [139]. By a treatment with hydrochloric acid the original fatty acids can be recovered quantitatively without stereochemical changes. Unfortunately, the method cannot be applied to conjugated fatty acids as the recovery is uncomplete as decomposition occurs [140]. The reaction is also irreversible for acetylenic compounds. Oxymercurated acetylenic fatty acids are converted into oxo and hydroxy compounds depending on the experimental conditions [141]. More details on this technique can be found in the review from *Sebedio* [139].

Reversed-phase high performance liquid chromatography (RP-HPLC) can also be used as a micropreparative technique for isolating individual fatty acids or simpler fractions from complex samples [142, 143].

The method of choice to obtain fractions separated according to their degree of unsaturation is the silver nitrate ion chromatography and the chromatographic pattern obtained gives also very helpful hints for the identification of the compounds in question, as positional and stereoisomers can be separated from each other [118, 144–147]. The technique is predominantly used in conjunction with TLC or column chromatography, including HPLC. An alternative elegant, clean and fast method was proposed by *Christie*, using silver nitrate impregnated Bond Elut™ columns [148]. Silver ion chromatography has been reviewed in great detail recently [149].

All of these procedures provide simpler fatty acid fractions and the choice of one of these techniques for the screening analysis of seed oils depends on the particular case. Applications for the separation of various seed oil fatty acids have been published, e.g. ref. [3, 11,13, 20, 129, 145].

5.2 Gas chromatography/mass spectrometry of fatty acids

For the complete structure elucidation of fatty acids, information about:

1. chain length,
2. chain type (straight/branched/cyclic parts),
3. number, position, and configuration of the double bond(s),
4. number and position of the triple bond(s),
5. type, number, position, and configuration (sometimes) of further functional groups are needed.

A part of these information can be acquired by GC/MS analysis in the electron impact mode by analyzing various fatty acid derivatives.

Generally, the first derivatives to be analyzed are the methyl esters. It is advisable to examine the sample under the same conditions as used in the GC analysis. Doing this, it is possible to check the homogeneity of the individual peaks by mass spectrometry. Peak overlapping, that cannot always be recognized by GC analysis only, is so detectable and the analyst can take the necessary steps to achieve a better GC separation or to make a pre-separation prior to GC analysis.

Many methyl esters show a molecular ion, that permits the determination of the carbon number, of the double bond equivalents, and, in some cases, the presence and nature of functional groups can be elucidated. If no molecular ion can be measured, it can be helpful to reduce the ionization potential from 70 eV to 25 eV or to use another mild ionization technique, such as chemical ionization [150, 151]. The lack of molecular ions can be observed frequently with hydroxy fatty acids [152]. In this case, a derivatization to the corresponding trimethylsilyl ether [153] or the use of chemical ionization [154] is useful to overcome the problem.

Some methyl esters yield also characteristic fragmentation patterns, permitting the complete structure assignment. This is the case (with restrictions), e.g. in simple acetylenic [155, 156], allenic [157], saturated epoxy- [153], saturated hydroxy- [152], saturated keto- [158], saturated cyclopentene [159], and saturated furane fatty acids [24]. In contrast, the spectra of simple cyclopropane [160] and cyclopropene fatty acids [161] are not very helpful for structure elucidation of unknown derivatives.

The presence of unsaturated chain carbons has a significant influence on the fragmentation pattern. During the ionization process, the double and triple bonds isomerize along the hydrocarbon chain [162]. Consequently, most of the biologically important positional and stereoisomers of olefinic fatty acids show almost identical mass spectra and neither the double bond position nor its configuration (exception 2-unsatured fatty acids) can be assigned [160]. Further details on the mass spectroscopy of fatty methyl esters have been reviewed elsewhere (e.g. ref. [151, 163, 164]).

To not disclaim the "high-resolution isolation" of the fatty acids in the gas phase and the high sensibility of the GC/MS method, there are two possibilities, that can manage the problem of bond migration and of unstable functional groups:

1. on-site derivatization,
2. remote-site derivatization

(Methods, such as tandem MS and chemical ionization are not discussed in this chapter).

5.2.1 On-site derivatization

For the on-site derivatization of olefinic/acetylenic fatty acids, the unsaturated bonds are fixed by conversion to special derivatives (Tab. 6). The mass spectra of these compounds give intense α-fragmentation ions, permitting the elucidation of the original structure [160, 163, 175]. However, with an increasing number of unsaturated bonds, the spectra become more complex and the volatility decreases significantly, which can prevent the passage through the GC column.

The objective of the chemical modification of other functional groups than double/triple bonds is the formation of more stable compounds with improved mass spectrometric properties. The trimethylsilyl ethers of the hydroxy fatty acids provide also favorable gas chromatographic behavior [151]. In Tab. 6 typical examples are presented. The spectra of these derivatives are readily interpretable in terms of the original position of the modified functional group by significant ion peaks produced by α-fragmentation.

If only a few unusual fatty acids are present in the sample and the analyst has already some structural information from other spectroscopic measurements or from GC analysis, the on-site derivatization methods can be very useful for structure elucidation. However, it must be taken in account that only the fatty acids containing the reactive functional group are transformed and further steps for the elucidation of other compounds may still be necessary. Due to the change of the GC pattern in comparison with the unmodified methyl ester mixture, peak overlapping of the altered fatty acids with the other compounds is also possible.

5.2.2 Remote-site derivatization

The second alternative to obtain structure specific mass spectra, the remote-site derivatization, has some important advantages in comparison to the methods described in 5.2.1. In the remote-site derivatives, the carboxyl groups of all of the fatty acids are allowed to react with a nitrogen-containing compound. Typical examples of remote-site derivatives are summarized in Fig. 3.

As the ionization in the ion source occurs predominantly at the nitrogen of the ring system, the fragmentation in the fatty acid chain is suppressed and structure specific fragmentation patterns can be observed [172]. In brief, the regular 14 mu series for the cleavage at each fatty acid methylene group is interrupted in a characteristic manner, when an unsaturated bond or a functional group is present [172–175]. This is generally valid for all remote-site derivatives.

The mass spectrum and the fragmentation pattern of α-eleostearic acid as 4,4-dimethyloxazoline derivative is illustrated in Fig. 4 as an example. As explained before, the

Tab. 6. Some chemical modifications of double/triple bonds and functional groups in fatty acids for GC/MS analysis.

Structure element	Reaction	Derivative (References with seed oil application, except [160, 163])
Double bond(s)	Addition of dimethyldisulphide (I_2-catalysis)	Dimethyldisulphide adducts, adduct formation is stereospecific, geometric isomers can be separated by GC [120, 127, 166]
	Oxidation with $KMnO_4$ or OsO_4, followed by silanization	Silylated vicinol diols, reaction is stereo specific, geometrical isomers can be separated by GC [163]
	Methoxymercuration/Demercuration	Two positional methoxy isomers for each double bond [156]
Triple bond(s)	Oxymercuration/reduction with $NaBH_4$ and silanization	Two positional silylated monohydroxy isomers for each triple bond [156]
Cyclopropane	With boron trifluoride-methanol	Two positional methoxmethyl isomers for each cyclopropane ring [160]
Cyclopropene	Silver nitrate/methanol	Two positional methoxy and keto isomers for each cyclopropene ring, methoxy derivative predominantly [14, 167, 168]
Hydroxy	Silanization	Silylated hydroxy group(s) [3, 15, 153, 169]
Epoxy	Boron trifluoride-methanol/Silanization	Two positional methoxy/silylated hydroxy isomers [5, 153, 170, 171]

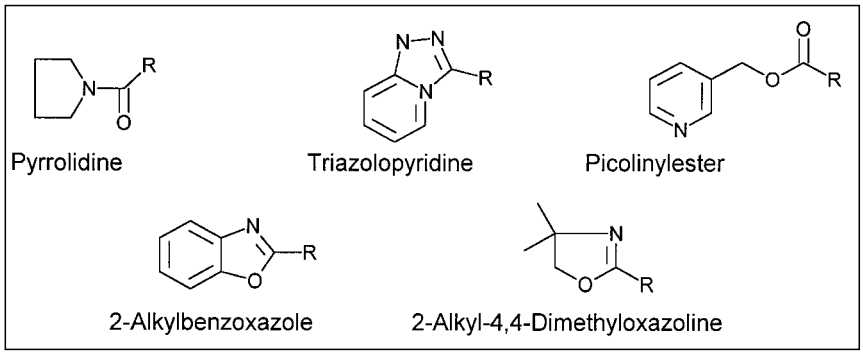


Fig. 3. Some typical remote-site derivatives from fatty acids (R = fatty acid chain).

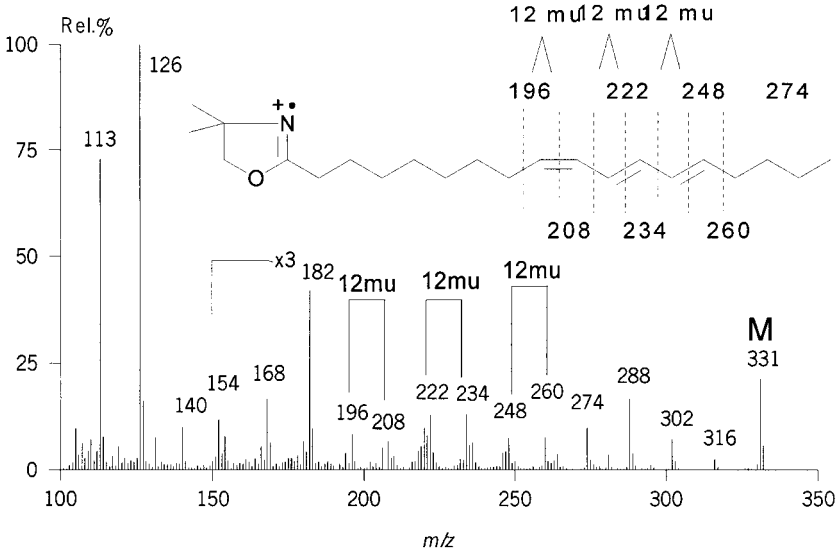


Fig. 4. Mass spectrum and schematic fragmentation pattern of the dimethyloxazoline derivative of α-eleostearic acid.

14 mu gaps (*m/z* 140/154/168/182 …) are interrupted by 12 mu gaps (*m/z* 198/208; 222/234; 248/260), indicating clearly the position of all the conjugated double bonds [21].

While the fragmentation mechanism is principally identical in all remote-site derivatives, significant differences in the intensity of the diagnostic ions and in the GC properties are noticeable. In recent years, the 4,4-dimethyloxazoline derivatives, introduced by *Zhang* et al. [176], and the picolinyl ester derivatives, introduced by *Harvey* [177], have been preferred by most of the researchers (see Fig. 3). A critical comparison of both of these derivatives demonstrated, that the 4,4-dimethyloxazoline derivatives are more convenient for polyunsaturated and conjugated fatty acids and that the identification of cyclopropane and branched fatty acids is more convenient by using the picolinyl ester derivatives [174]. The mass spectroscopical properties of the DMOX [143, 174, 175, 178] and picolinyl ester derivatives [143, 172–174] have been reviewed recently in detail. In Tab. 7, the fatty acid structure types, that have been investigated by both derivatization methods and that might be of importance for the seed oil analysis, are summarized.

A significant advantage of the 4,4-dimethyloxazoline derivatives is that their elution temperatures are only about 10 °C higher than those of the analogous methyl esters [176]. The quality of the GC separation is generally as good as that from the methyl esters and allows an effective resolution of complex mixtures using polar capillary columns [180]. However, the derivatization reaction to the 4,4-dimethyloxazoline derivatives requires relatively high temperatures of about 175 °C [176, 180]. This can be a problem for the derivatization of some fatty acid structures, such as conjugated tetraenes (*Spitzer* 1997, unpublished) and epoxy derivatives [5].

The picolinyl esters elute about 50 °C above the corresponding methyl esters [172]. Thus, the analyst has to apply higher GC temperatures, what can be a problem for the analysis of higher molecular weight fatty acids using polar columns. However, owe to the now available cross-linked, low-bleed, high-temperature polar capillary columns, the major part of the picolinyl ester derivatives is now also suitable for a high-resolution separation [174]. As an advantage for the picolinyl ester derivatives, a mild derivatization procedure is available [200].

5.2.3 Recommendations for the seed oil fatty acid analysis by GC/MS

The derivatives here presented for GC/MS analysis of fatty acids provide a wide range of information about their individual structure elements. For the analysis of novel seed oils it can be recommended to try both 4,4-dimethyloxazoline and picolinyl ester derivatives before to use an on-site derivatization method. In many cases, the information on the position of the functional groups will be obtained (Tab. 7). For instance, the 4,4-dimethyloxazoline derivatives of more than 80 unsaturated fatty acid isomers provided easily interpretable and structure specific mass spectra.

Both remote-site derivatization techniques have been applied successfully for the structure elucidation of various unusual seed oil fatty acids, e.g. ref. [2, 3, 10, 14, 16, 21, 23, 77, 120, 122, 126, 129, 180, 187, 206] for 4,4-dimethyloxazoline derivatives and ref. [20, 185, 186, 196, 207] for picolinyl ester derivatives.

For some unsaturated fatty acids it can be advantageous to analyze also the deuterated derivatives [174]. A series of deuterated polyunsaturated [208] and acetylenic fatty acids [209] has been analyzed as picolinyl esters with great suc-

Tab. 7. Information on the fatty acid structure by their dimethyloxazoline and picolinylester derivatives.

Fatty acid type	Dimethyloxazolines	Picolinylesters
Non-Oxygenated fatty acids		
Saturated fatty acids	M^+, full structure specific [176]	M^+, full structure specific [179]
Non-conjugated polyolefinic fatty acids	M^+, easy location of up to six double bonds [176, 180]	M^+, location of up to six double bonds with restrictions for fatty acids with more than three double bonds [179, 181–186]
Non-conjugated acetylenic fatty acids	M^+, location of triple bond [3, 187]	M^+, location of triple bond(s) with some restrictions [188, 189]
Conjugated olefinic fatty acids	M^+, location of up to three conjugated double bond(s) [21, 190]	M^+, location of up to three conjugated double bond(s) [172, 191]
Conjugated acetylenic fatty acids	M^+, location of conjugated ene-yne and yne-yne (with restrictions) systems, additional terminal vinyl group recognizable [3, 10, 128, 187]	M^+, conjugation/location of conjugated yne-yne systems [10, 192]
Non-oxygenated allene fatty acids	Not published	M^+, location of allenic system [157]
Cyclopropane fatty acids	M^+, location of cyclopropane ring [193]	M^+, location of cyclopropane ring [172, 194]
Cyclopropene fatty acids	M^+, location of cyclopropene ring [14]	M^+, location of cyclopropene ring [195]
Cyclopentene fatty acids	M^+, recognition of cyclopentenyl ring and location of chain double bond, recognition of cyclopentanyl ring [16]	M^+, recognition of cyclopentenyl ring [196]
Methyl-branched fatty acids	M^+, location of branched methyl groups [197]	M^+, location of branched methyl groups [20, 179, 198]
Oxygenated fatty acids		
Saturated epoxy fatty acids	M^+, location of epoxide ring [199]	Not published
Olefinic epoxy fatty acids	M^+, location of epoxide ring and double bond(s) with restrictions [199], vernolic acid derivative not detectable [5]	M^+, location of epoxide ring and double bond(s) [200]
Saturated hydroxy fatty acids	M^+, location of OH-position [201]	M^+ (as TMS derivative), location of OH-position [181]
Non-conjugated monohydroxy olefinic fatty acids	M^+, location of OH-position and double bond [14, 202]	M^+, location of OH-position and double bond [198]
Conjugated monohydroxy olefinic fatty acids	M^+, location of OH-position and conjugated system [201]	Not published
Conjugated monohydroxy acetylenic fatty acids	M^+, location of OH-position, conjugated system unclear [203]	Not published
Hydroxy cyclopropene fatty acids	Location of OH- and cyclopropene ring position [14]	Not published
Saturated keto fatty acids	M^+, location of keto position with restrictions [204]	M^+, location of keto position [205]
Olefinic keto fatty acids	M^+, location of keto position, conjugated system unclear [21]	Not published
Methyl-branched keto fatty acids	M^+, location of keto position and branched methyl group [23]	Not published

cess. If the results with these remote-site methods are not indicative for the structure, the analyst may try to use an adequate on-site derivatization method [3, 5].

6 Information on the Configuration of Double Bonds and Functional Groups

Due to the measurement principle of mass spectrometry, information on the stereochemistry of the fatty acids cannot be obtained directly from the fragmentation pattern (with very few exceptions). Some useful methods for this approach are summarized in this section.

6.1 The importance of the retention data

As described in 5.1.2, modern polar GC capillary columns enable the separation of many positional and stereoisomeric fatty acids. Thus, the complete stereochemical identity of an unknown fatty acid can be proven, when the retention data *and* the mass spectra of the respective derivatives of a known standard and an unknown compound are in full agreement. When used with care, literature retention data or calculated ECL values can also be employed as reference (see 5.1.3). As stated in 5.1.4, in some cases the results can be more apparent when a pre-separation is done before GC/MS analysis.

Careful consideration of both, GC and MS data, enabled for instance the identification of olefinic-acetylenic fatty acids in a Santalaceae seed oil [3] and led to the unambiguous structure confirmation of a series of non-methylene-interrupted olefinic fatty acids in Ranunculaceae [120, 129] and Conifer [206] seed oils.

6.2 Infrared spectroscopy and double bond configuration

IR spectroscopy can be a further simple tool to get stereochemical information. It can be applied to the whole seed oil,

to the fatty acid methyl ester mixture and to the fatty acid methyl ester fractions obtained by pre-separation steps (see 5.1.4), respectively. The utilization of IR spectroscopy for the identification of stereoisomers is based on the fact that the presence of *trans* double bonds can easily be proven by prominent absorption band(s) between 940 and 999 cm^{-1}.

If *trans* absorptions are detectable, the frequency value gives a first hint about the nature of the *trans* double bond(s) [65]. For non-conjugated *trans* derivatives the IR band appears at 968 cm^{-1} and this frequency does not change for additional non-conjugated double bonds [28]. If one or more *trans* double bonds are part of a conjugated system, the IR absorption band is shifted about ± 30 cm^{-1} and up to two absorptions in this range can be observed. The IR absorptions of a series of conjugated olefinic and olefinic-acetylenic fatty acids were tabulated by *Hopkins* [65] and some values are presented in Tab. 3. IR spectroscopy was employed to prove preliminarily the presence of conjugated olefinic [8, 21, 77, 210] and olefinic-acetylenic [3, 10, 11] systems in novel seed oils.

Most of the natural occurring unsaturated fatty acids have *cis* double bonds, that do not give rise to prominent IR absorptions (1620, 715 cm^{-1}). Consequently, the absence of *trans* double bands in the IR spectrum of a seed oil or its fatty acid methyl esters proves that only *cis* double bonds are present (within the detection limit). In this way, IR was used to verify the lack of *trans* double bonds in e.g. Boraginaceae [121], *Ginkgo biloba* [20], and Sapindaceae [2, 122] seed oils. The use of IR spectroscopy in oil analysis has been reviewed in detail [211].

A more sophisticated technique is the coupling of GC with *Fourier* transform (FTIR) spectroscopy, that permits to obtain IR spectra from each GC separated fatty acid. However, in polyenoic fatty acids it is not possible to determine, which of the double bonds has *trans* configuration. Once again, the consideration of GC retention data can be helpful in this circumstance. The topic has been reviewed recently [212, 213].

6.3 Ultraviolet spectroscopy and double bond configuration

Ultraviolet spectroscopy (UV) (220–400 nm) is a sensitive method, that permits the detection of conjugated systems, such as conjugated double, double-triple and triple-triple bonds by characteristic absorption bands. In some cases, it is possible to distinguish geometrical isomers. As IR spectroscopy, it can be applied to the whole seed oil and to the fatty acid methyl ester fractions, respectively.

If only one or few conjugated fatty acids are present, it may be possible to exclude the already identified fatty acid GC peaks and to link the unknown GC peak(s) with the respective UV absorption. This was for instance shown for a conjugated triene fatty acid in cherry seed oil [78] and for conjugated tetraene fatty acids in Rubiaceae [210] and Euphorbiaceae seed oils [8], respectively.

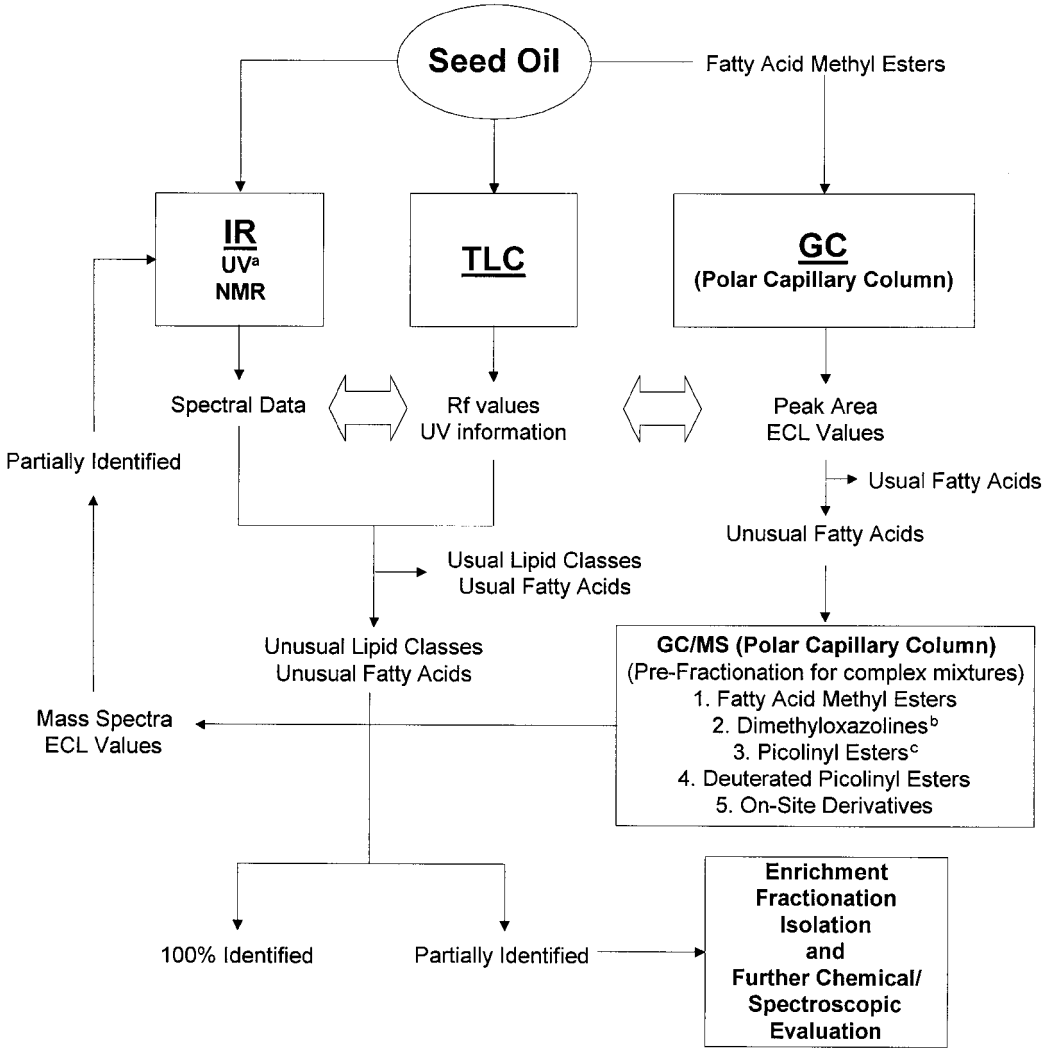


Fig. 5. Scheme for the detection of unusual fatty acid and lipid classes ([a] if indicated by TLC; [b] can be made from free fatty acid or their methyl esters; [c] must be made from free fatty acids). Abbreviations: TLC = thin-layer chromatography; GC = gas chromatography; IR = infrared spectroscopy; UV = ultraviolet spectroscopy; NMR = ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

Although, the UV data may be helpful for the identification of conjugated systems, their positions or sequences in the fatty acid chain can generally not be recognized. The use of UV spectroscopy for the analysis of fatty acids has been reviewed [211] and a valuable collection of UV data for fatty acids has been published [65].

6.3.1 ^{13}C Nuclear magnetic resonance spectroscopy and double bond configuration (see also 4.2.2)

A change from *cis* to *trans* configuration in non-conjugated and conjugated fatty acids has a significant effect on the shift values of the olefinic and allylic carbon atoms [83]. In many cases, a careful interpretation of the ^{13}C NMR data in the double bond and allylic methylene group range (Tab. 4) will permit the correct assignment of the configuration of the olefinic systems, even in seed oils with fatty acid mixtures.

For more details see 4.2.2 and ref. [83, 107–109].

6.3.2 Silver ion chromatography

As already mentioned in 5.1.4, silver ion chromatography is a suitable method for the fractionation of fatty acid methyl esters according to their degree of unsaturation, type of unsaturation (olefinic or acetylenic), and the position and stereochemical configuration of double bond(s). The elution pattern can thus be helpful to obtain structure information in relation to unsaturated systems. Some aspects of the silver ion chromatography of unusual fatty acids have been reviewed by *Christie* [52].

7 Schema for the Detection of Unusual Fatty Acids and Lipid Classes in Seed Oils

The combination of the analytical methods described here, enables the development of a strategy for the analysis of novel seed oils. The analytical sequence shown in Fig. 5 permits the detection of a number of unusual lipid components in little time and few chemical manipulations are needed. It is certainly not possible to consider or predict all possibilities that can occur in seed lipids and in some cases it will be necessary to isolate the unknown compound(s) (especially true for new compounds) and to use chemical and spectroscopic methods for the complete elucidation not presented herein.

As illustrated in Fig. 5, a combined interpretation of the analytical methods is important and the analyst should always compare the analytical data from various sources. For the analysis of new seed oils, it is essential to check the oil sample first by TLC, IR spectroscopy and GC. In this way, the detection of the presence of unusual fatty acid *and* lipid classes is possible in most cases. If TLC spots are detectable under UV light (260 nm), a UV spectrum will provide further important information. Depending on these preliminary results, the analyst has to decide if further steps, such as GC/MS of various derivatives and NMR analyses are necessary and in which depth they have to be applied to get more complete results.

8 Outlook

For the future it is desirable to complete and to update the chromatographic and spectroscopic data from fatty acids and other lipids, including those from rare compounds. As the major part of the analyses of seed oils was made in times where high resolution chromatography and spectroscopy were not available, it would be interesting to carry out a reinvestigation in some cases.

Particularly valuable for screening analysis are the GC retention data from polar capillary columns, the mass spectroscopic data from remote-site derivatives and the ^{13}C NMR spectral data, as their information impact is high and permits a fast seed oil analysis. In many cases, the combined interpretation of these analytical data is a very powerful tool to get fast and unambiguously structure information on fatty acids and lipids in mixtures, without the need to isolate individual compounds. The development of computer software, that allows to link the various analytical data to a logical structure conclusion, would also be interesting.

At the end of this century, lipid analysis has reached a high level that enables the characterization of novel seed oils in a short time with only a small amount of sample. The rapid development in analytical science, data processing and communication possibilities will certainly yield further progress in the next century.

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[Received: August 4, 1998; accepted: October 20, 1998].