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Analytical Techniques for Hydroxy Acids in Fats and Oils

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Abstract

Recent work on separation techniques such as column, thin-layer, gas-liquid, and liquid-liquid partition chromatography; chemical and physical characterization techniques, e.g., derivatization, X-ray, infrared, nuclear magnetic resonance and mass spectrometry; and the possibilities for qualitative and quantitative analysis using these and other methods are discussed.

Introduction

LONG-CHAIN HYDROXY acids occur widely in nature. Downing (1) reviewed the knowledge up to 1961 regarding the long-chain, aliphatic, hydroxy acids found in animals, plants, and microorganisms and some of the methodology for their detection, isolation, and structure determination. Fontell (2) and co-workers reviewed some new separation and analytical techniques for fatty acids and other lipids in 1960 and chapters in Markley's recent treatise (3) give general information on hydroxy acids. This report will consider developments in the fields of separation and analysis since the above reviews. It will discuss qualitative methods, but give particular attention to quantitative techniques.

Column Chromatography

The most significant advances in separation techniques have occurred in all types of chromatography. Column chromatography on benzene-methanol-wetted silicic acid with benzene-methanol eluants, as described by Frankel and his co-workers (4), is most useful for the separation of non-hydroxy acids from mono- and di-hydroxy acids and their esters. Binder and co-workers have used the method for preliminary separations in the analysis of the fatty acid composition of castor (5) and dimorphotheca (6) oils. The acid classes were titrated or weighed, and the results of replicate analyses agreed well. The compositions of the non-hydroxy fractions were determined by quantitative gas-liquid chromatography (GLC) and other standard methods. This column chromatographic method is invaluable for such preliminary separation. It has good capacity (about 20 mg/g), yields good separations, and is reproducible. Similar systems have been used, for example (7), in fractionating less polar phosphate esters from more polar alcohols before applying other complementary methods.

Gunstone and Sykes (8) have described celite-paraffin oil or celite-acetylated castor oil columns useful in separating non-hydroxy, hydroxy, and acetylated hydroxy acids with aqueous-acetone solvents, but the capacities are low. Chobanov and co-workers (9,10) separated ricinoleic and dihydroxystearic acids from non-hydroxy carboxylic acids on silica gel columns with loadings of about 5 mg/g and combinations of methanol-hexane-acetone or acetic acid-hexane-acetone. Their titration results on castor oil fatty acid compositions (10) are in good accord with literature values (cf. 5).

Other column separations employ magnesium silicate (11), alumina (12,13) or silica gel (14,15,16) adsorbents with benzene-ether or hexane-ether solvent systems. Hydroxy acids from peat (12), apple, or carnauba waxes (11), from brain and other tissue lipids (14), from bacteria (15), from metabolic breakdown of ricinoleic acid (16), and from oxidation of petroselinic acid (13) have been isolated by adsorption chromatography. Generally, clean separations were achieved allowing further characterization.

Additional notable new column chromatographic methods involve complexing agents. de Vries (17) described the separation of *cis* unsaturated fatty acids from their *trans* isomers using silica gel impregnated with silver nitrate and eluants of benzene-hexane or ether-hexane. He also separated the alcohol, cholesterol, from its standard analog, cholestanol. Unfortunately, this system has a limited capacity for unsaturated compounds. Two research groups (18, 19) have also described ion exchange resins as supports for silver ion in columns useful for these separa-

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tions. We have found modifications of the system described by Dutton and co-workers (19) particularly useful in work with unsaturated hydroxy acids.

Liquid-Liquid Partition Chromatography

Several recent studies described the partition of castor esters (20,21) and wool wax acid esters (22, 23) between polar and nonpolar solvents. Smith and co-workers (24) employed hexane—85% aqueous methanol in the countercurrent distribution of a mixture of hydroxy acids from *Lesquerella densipila* seed oil. The methyl esters were resolved into a C_{16} hydroxymonoene, a C_{18} hydroxymonoene (presumed to be ricinoleic acid), and the C_{18} hydroxydiene, densipolic acid (12-hydroxy-*cis*-9, *cis*-15-octadecadienoic acid) after 400 transfers. The related dimorphecolic (9-hydroxy-*trans*-10,*trans*-12-octadecadienoic) and lesquerolic (14-hydroxy-*cis*-11-eicosenoic) acids also have been isolated for characterization and structure proof by this group (25) using countercurrent distribution. Liquid-liquid partition chromatography is an excellent separation method, but it requires an expensive and somewhat temperamental piece of equipment and considerable experience.

Thin-Layer Chromatography

A phenomenal separation method recently adopted in lipid chemistry is thin-layer chromatography (TLC) . Mangold wrote a comprehensive review of its applications to lipids in 1961 (26). Other areas are covered in reviews by Wollish (27) and there are probably a dozen or more other reviews and monographs available. We routinely use the original Kirchner chromatostrip technique (28) in much of our work on the separations of mixtures of the methyl esters of hydroxy-, keto-, and non-hydroxy acids (29). TLC is also very useful in examining other complex reaction mixtures; illustrations are given in most current publications dealing with hydroxy acids. Adsorption chromatography on silica gel layers with hexane-ether solvents is favored (5,6,29,30,31,32) for hydroxy acid mixtures, but both adsorption chromatography with $CHCl_3$: methanol:acetic acid (90:10:2) (33) and "reversed phase" chromatography with Nujol on celite developed in aqueous acetic acid (32) reportedly yield excellent separations.

Quantitative TLC systems dealing with hydroxy acids have been suggested by Mangold and co-workers (35) who used diazomethane–C^{14} or acetic anhydride –C^{14} to label castor fatty acids. After fractionation by TLC, the methyl–C^{14} ester spots were scraped off the plates, eluted, and their radioactivity counted. The non-hydroxy spot was further analyzed by paper chromatographic fractionation and radioactivity counting. Viogue and Holman (36a) separated diazomethane-derived castor methyl esters by TLC, eluted the spots, and estimated esters by the ferric hydroxamate test. Similar separation and measurement methods were employed by these authors for derivatives obtained by lipoxidase-catalyzed oxidation of ethyl linoleate (36b). Non-hydroxy acids of castor oil were quantitatively determined by GLC analysis of the material eluted from the proper TLC spot. These results (35,36) suggest that TLC readily adapts to quantitative measurement of hydroxy acid derivatives. Etherification of alcohol functions (5,36) is a drawback in the use of diazomethane. This and other known side reactions involving diazomethane may lead to quantitation errors. Yamada and Stumpf (37) recently used TLC and radioactivity count to assess the incorporation of C^{14}–labeled acetate or oleate into ricinoleic acid in a synthesis system catalyzed by castor seed extracts.

Some interesting new approaches involve use of complexing agents during TLC of fatty acids. Three different groups (38,39,40) suggested impregnation of TLC adsorbents with silver nitrate to aid resolution of *cis* and *trans* isomers and higher unsaturates. Morris (39,41) also proposed the separation of mono-, di-, and poly-hydroxy acid esters through the use of adsorbents impregnated with boric acid and other recognized glycol-complexing agents. Recently TLC with silver nitrate was used in examining the fatty acid composition of isano oils (42,43). We have found the silver nitrate system of particular value, and anticipate that glycol-complexing agents will prove worthwhile for specific analytical problems.

Although TLC is rapid, has good capacity, shows good resolution, and provides clues to structure, certain problems do occur in its use and interpretation. Many of these problems and their solutions are discussed in the review articles and monographs and attention to them is recommended.

Paper Chromatography

Good reviews including discussion of hydroxy acids appeared in 1961 by Rouser and co-workers (44) and in 1962 by Kaufmann and Ko (45). Paper chromatography suffers generally from low capacity and slow development but the separations are often better than those obtained in other systems. Recent articles describe separations of α-hydroxy (46) and ω-hydroxy (47) fatty acids.

Gas-Liquid Chromatography

GLC has been universally adopted and almost every fatty acid paper routinely refers to GLC analyses. Publications covering fatty acids and referring to hydroxy acids include those of Miwa and co-workers (48,49), Litchfield and co-workers (50), and Kaufmann et al. (51). O'Brien and Rouser recently published an extensive study on the GLC analysis of the hydroxy acids (52) stressing the need for using proper response factors. Morris et al. (53) studied the alteration of vicinally-unsaturated hydroxy fatty acid esters during GLC and determined that changes occurred in the heated injection block. The results of collaborative studies on GLC analyses including castor and dehydrated castor esters were recently reported by the AOCS Instrumental Techniques Committee (54). The long retention times of ricinoleate esters caused some problems, but the method appears acceptable if proper precautions are observed.

GLC techniques have been used in the analysis of many mixtures containing hydroxy acid esters, for example: castor acids (5,21,36,54), *Dimorphotheca* acids (6,25,55), *Lesquerella densipila* acids (24), *Lesquerella lasiocarpa* acids (25,56), *Vernonia anthelmintica* acids (32,57), *Tragopogon porrifolius L.* acids (58), cork acids (59), isano acids (42,43), apple and carnauba wax acids (11), oxidized petroselinic acid (13), brain and tissue acids (14), *Serratia marcescens* acids (60), and *Azobacter agilis* acids (15). These techniques have been of special value in studies on the metabolism of hydroxy acids in animals fed oxidized corn oil (61) or ricinoleic acid (16,62), or in man after ingestion of castor oil (63,64) as well as in studies on the biosynthesis of ricinoleic acid (65).

Many workers use nonpolar silicone rubber or Apiezon stationary phases with hydroxy acid esters

to avoid long retention times and interesterification losses with polyester substrates. Further, the non-polar columns have better temp stability. Now, with dual column systems, stabilized polymers, and temp programming one can use polyesters to achieve better separation of non-hydroxy acids as well as to reduce retention times for hydroxy acids. We find "on-column" injection helps to avoid the flash-heater reactions reported (53).

Quantitative GLC requires attention to another problem. Response factors must be determined and used in the separation and quantitation of hydroxy acids by GLC. As mentioned above, O'Brien and Rouser (52) recently clearly pointed out the extent of this problem.

Chemical modification of hydroxy acids and subsequent GLC analysis is also useful. A number of groups (15,24,25,42,43,58,60) have used oxidative degradation coupled with GLC to determine the exact location of hydroxyl groups in fatty acid chains. Others have acetylated the hydroxy acids before GLC analysis (11,14,59) or carried out Beckmann rearrangements on ketones derived from hydroxy acids (42). These techniques appear satisfactory and lead to lower GLC retention times, but they do add steps to the analyses.

Chemical Modification

All of these separation techniques can be used in conjunction with chemical modifications, but chemical modifications also serve as direct tools. Critchfield recently reviewed analytical techniques for hydroxy compounds (66). In the official AOCS method pyridine catalyst and excess acetic anhydride are employed for acetylation and the acetic acid liberated is back-titrated. Schenk and his co-workers (67) advocated the use of perchloric acid as a catalyst for acetylations in ethyl acetate or pyridine solvents. They claim very rapid and complete acetylations. Stetzler and Smullin (68) used p-toluenesulfonic acid catalyst in ethyl acetate because they observed hydroxyl values 20–30% higher than theory predicts with perchloric acid catalyst. They suggested that extra alcohol functions are liberated during perchloric acid catalyzed hydrolysis. In our laboratories we observed high hydroxyl values when perchloric acid catalyst was used in ethyl acetate, but this catalyst gives satisfactory results in pyridine solution. Sully (69) suggested the use of excess stearic anhydride in place of acetic anhydride with back titration of the stearic acid liberated. The advantages claimed do not appear great enough to justify the more involved procedures.

Kaufmann and Schmuelling (70) recently re-examined the use of acetyl chloride in pyridine. They concluded that satisfactory results could only be obtained with free hydroxy fatty acids if 150–200% excess acetyl chloride was used in toluene-pyridine solution. Robinson and co-workers (71) suggested the use of excess 3,5-dinitrobenzoyl chloride in pyridine with back titration of the acid liberated. Johnson and Critchfield (72) also used 3,5–dinitrobenzoyl chloride but they dissolved the derivatives in hexane and observed (at 525 mμ) the color that developed after adding dimethylformamide and propylenediamine. Kyriacon (73) suggested metallic zinc as a catalyst for use with acetyl chloride alone, claiming increased acetylation rates.

Reed et al. (74) described the reaction with excess isocyanate. Following urethane formation, the excess isocyanate is decomposed with excess dibutylamine and the amine is titrated with standard acid. This technique has value in special applications, but known isocyanate side reactions may prove troublesome.

Two research groups (75,76) recently suggested acetylation of hydroxyl functions, treatment of the ester with hydroxylamine and ferric ion and measurement of the "ferric hydroxamate" complex at 520 mμ. This approach appears satisfactory, but corrections must be made for other reactive functions by colorimetric determinations before and after acetylation.

Critchfield and Hutchinson (77) suggest oxidation of secondary alcohols to ketones with acid dichromate. The ketones are then converted to 2,4–dinitrophenylhydrazones which are measured at 480 mμ. Primary alcohols are oxidized to carboxylic acids and do not interfere; however, the method seemed to require considerable care for good results.

Kortha (78) suggested a simple gravimetric scheme which involved direct determination of hydroxyl value from the wt gain on acetylation. He claimed a maximum deviation of 0.5 unit in hydroxyl value.

Kumar (79) suggested observation of the turbidity in oil samples containing castor oil following treatment with molybdic acid as a qualitative test. Another qualitative scheme advanced by Malins and co-workers (80) involved nitration of hydroxy compounds with acetyl nitrate, separation of the derivatives by TLC, and examination of the derivatives' IR spectra which change uniquely.

Infrared Spectrometry

IR spectroscopy is widely used in lipid chemistry. O'Connor (81) and Chouteau (82) recently reviewed the field. Studies dealing primarily with the effects of inter- and intramolecular interactions on the IR spectra of hydroxy acids and related compounds by Kummerow and co-workers (83) and by Eddy and colleagues (84) appeared recently.

A number of groups have suggested the use of the hydroxyl absorption around 2.8μ (32,85,87,88) or at the overtone near 1.4μ (89) for quantitative analytical work. O'Connor and Chipault (90) presented results from a collaborative study on the determination of hydroxyl by IR measurements near 1.4 or 2.8μ. They concluded that the method was limited to the determination of primary hydroxyl groups in samples containing no secondary hydroxyl functions. This limitation is inherent, for the different absorption maxima observed are a direct result of the structural differences between primary and secondary alcohols (cf 84). For analysis of samples containing only secondary alcohol derivatives we routinely use the fundamental absorbance near 2.76μ. Concns of unknown are obtained by reference to a calibration curve. Hydrogen bonding may introduce error into such measurements. Dilute solutions (below 0.02 M) and solvents incapable of entering into hydrogen bonding should be used. Condensed phase measurements should also be assessed carefully, and instrumental limitations in the near IR region should be recognized. A recent paper (88) proposed a method of measuring hydroxyl value which was based on calibration with high concentrations of a known alcohol in benzene. An ordinary IR spectrophotometer was used. The IR absorption band shown at about 2.9μ seems very broad. It would appear that effects of hydrogen bonding and instrumental limitations may prove troublesome in such systems.

Within the normally used IR region of 2.5 to 15μ the hydroxy acids and their derivatives have some

readily recognizable features, i.e., the bands found in the 2.7–3μ region (hydroxyl), the 5.7–6μ region (carboxyl), and the 8μ region (carbon-oxygen stretching). These and related spectral features are discussed generally in the review articles (81,82) and more specific examples of their use are given in articles devoted to derivatives of castor acids (5,16, 80), *Dimorphotheca* acids (6,53,55), apple and carnauba wax acids (11), *Azobacter agilis* acids (15), *Lesquerella* acids (25,56), olive oil acids (31), hydroxy acids derived from naturally occurring epoxy acids (32), hydroxy acids derived from oxidized linoleic acid (36b,53), hydroxy acids from isano oil (42, 43, 53), hydroxy acids obtained from *Vernonia* oil (57), hydroxy acids of *Tragopogon porrifolius L.* oil (58), cork acids (59), *Serratia marcescens* acids (60), and nitrate esters of hydroxy acid derivatives (80).

Ultraviolet Spectroscopy

UV spectroscopy is not directly applicable to analysis of most hydroxy acids. It has been used to detect the presence and the dehydration of vicinally unsaturated acids such as dimorphecolic acid (6,7,25, 37,53,55). The unsaturation adjacent to the hydroxyl group confers extreme sensitivity to acids or elevated temps. Such compounds are routinely examined between 200 and 300 mμ for the absorption by the conjugated diene of dimorphecolic acid at 231 mμ and by the conjugated triene of its dehydration product around 280 mμ. As dehydration proceeds the triene peak near 280 mμ increases while the diene peak near 230 mμ decreases. Similar studies have been carried out on trace components from olive oil (31), products from lipoxidase treatment of linoleate (36b), the acetylenic hydroxy acids from isano oil (42,43), and the hydroxy conjugated dienes from *Tragopogon porrifolius L.* (58).

X-Ray Analysis

A review of X-ray analysis by O'Connor appears in Markley's recent book (3). The classic work on long-chain hydroxy and keto acids is that of Stenhagen and his co-workers (91). They observed the diffraction patterns for all of the hydroxystearic and ketostearic acid isomers. Positional isomers of ketostearic acids can be determined with this method but hydroxystearic isomers cannot be distinguished when the hydroxyl groups are between C-6 and C-13. In our laboratories we were unable, however, to duplicate the literature results for either methyl 12-ketostearate or methyl 9-ketostearate (92). We have exchanged samples with Professor Stenhagen and are currently cooperating in re-evaluating the earlier work. The results should prove of value for future use of the method.

Nuclear Magnetic Resonance

Hopkins and Bernstein (93) and Hopkins (94) published reviews on the use and interpretation of nuclear magnetic resonance (NMR) with fatty acid derivatives and included brief references to hydroxy acids. NMR is an ideal nondestructive method. It is rapid, uses small samples, gives good structure clues, and can be reasonably quantitative. For example, when methyl ricinoleate is acetylated to methyl 12-acetoxyoleate both the appearance of the acetate methyl peak and shift of the C–12 proton peak in the NMR spectra immediately give excellent structure clues. Such results are quantitative because all protons have the same absorption value in NMR, and an integral of the area under a peak or group of peaks immediately indicates the number of protons of that type. Such results usually have an error of 5% or less and are exceedingly valuable for determination or proof of structure. As in IR spectroscopy, hydrogen bonding may shift the peak of the hydroxyl proton, depending on the concentration, solvent, and temp. Such shifts are well-recognized, however, and should not cause trouble. Recent examples citing NMR applications were concerned with dimorphotheca oil analysis (6), metabolism of ricinoleic acid (16), and structure proof of densipolic acid (24).

Mass Spectrometry

Dutton recently reviewed some applications of mass spectrometry to fatty acid research (95). Classical studies on hydroxy acids and related compounds have been carried out by Ryhage and Stenhagen. Their original work in 1960 (96) has been followed by two more recent reviews (97,98). In studying hydroxy acid esters Ryhage and Stenhagen (96) particularly noted the value of the characteristic peaks from the cleavage alongside the hydroxyl groups and the peak at M-50.

Mass spectrographs also have been used as supersensitive GLC detectors (cf. 95), essentially allowing simultaneous separation and structure proof of components of mixtures. The instruments are very expensive and the results require considerable interpretation, but the method has a valuable place in fatty acid research particularly for structure proof and mol wt determinations.

Optical Rotation

Over ten years ago Bolley (99) suggested the use of optical rotation for assessing castor oil composition. Recently, Priester (100) attempted to use the same technique to quantitatively follow the dehydration of castor oil. He failed because the estolides formed during dehydration have rotations much higher than the starting hydroxy glycerides. He was able, however, to assess the ability of the dehydration catalysts to prevent undesired estolide reactions.

A more recent use of optical rotation is in optical rotatory dispersion (ORD). By continuously varying the wavelength of the light source and measuring the optical rotation, an optical rotatory dispersion spectrum is obtained which shows the change of rotation with wavelength. The curves provide clues to absolute configuration and conformation of molecules. Additional information on the theory and practice may be found in Djerassi's monograph (101) or other reviews on the topic.

With respect to hydroxy acids considerable work has been done by Sjöberg and his co-workers (102). These groups have published ORD curves for α- and β-hydroxy acid derivatives and have assigned absolute configurations based on the results. Gunstone and Baker recently synthesized 9-D-hydroxystearic acid and compared it with a 9-hydroxystearic acid derived from *Strophanthus* oil (103). They could not detect any measurable rotations at the D-line and they indicated that the ORD curves were not significant. Schroepfer and Bloch (104) recently reexamined the rotation of Gunstone's 9-hydroxystearic acids and found them to have slight negative specific rotations. They also found that 10-hydroxystearic produced by the action of *Pseudomonas* species on oleic acid had a negative specific rotation. They did not provide ORD data in this preliminary communi-

cation, but they did note that the rotations increased with decreasing wavelength. Schroepfer and Bloch (104) thus have established the absolute configurations of two naturally occurring hydroxy acids and are continuing to elucidate related enzymatic conversions. In our laboratories we are interested in similar approaches to absolute configurations and we have such ORD studies in progress.

This is the current status of analytical techniques for hydroxy acids. Many new techniques allow rapid separation, characterization, and measurement of the components of natural and synthetic mixtures. All of the methods are complementary and some of them can answer the most subtle questions concerning these products.

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REFERENCES

1. Downing, D. T., Rev. Pure Appl. Chem. *11*, 196–211 (1961).
2. Fontell, K., R. T. Holman and G. Lambertsen, J. Lipid Res. *1*, 391–404 (1960).
3. Markley, K. S., "Fatty Acids," 2nd ed., Vol. 1, Interscience Publishers, Inc., New York, 1960.
4. Frankel, E. N., C. D. Evans, H. A. Moser, D. G. McConnell and J. C. Cowan, JAOCS *38*, 130–134 (1961); Frankel, E. N., C. D. Evans, D. G. McConnell and E. P. Jones, *Ibid. 38*, 134–137 (1961); Frankel, E. N., D. G. McConnell and C. D. Evans, *Ibid. 39*, 297–301 (1962).
5. Binder, R. G., T. H. Applewhite, G. O. Kohler and L. A. Goldblatt, *Ibid. 39*, 513–517 (1962).
6. Binder, R. G., T. H. Applewhite, M. J. Diamond and L. A. Goldblatt, *Ibid. 41*, 108–111 (1964).
7. Diamond, M. J., T. H. Applewhite, R. E. Knowles and L. A. Goldblatt, *Ibid. 41*, 9–13 (1964).
8. Gunstone, F. D., and P. J. Sykes, J. Chem. Soc. 5050–5057 (1960); J. Sci. Food Agr. *12*, 115–123 (1961).
9. Chobanov, D., A. Popov and I. Mitzev, Compt. Rend. Acad. Bulgare Sci. *14*, 463–466 (1961).
10. Popov, A., I. Mitzev, D. Rankov and D. Chobanov, Rev. Franc. Corp Gras *10*, 595–601 (1963).
11. Mazliak, P., Phytochemistry *2*, 253–261 (1963).
12. Rauhala, V. T., JAOCS *38*, 233–235 (1961).
13. Fore, S. P., T. L. Ward and F. G. Dollear, *Ibid. 40*, 30–33 (1963).
14. Kishimoto, Y., and N. S. Radin, J. Lipid Res. *4*, 130–138 (1963); *loc. cit.* 139–143 (1963).
15. Kaneshiro, T., and A. G. Marr, Biochem. Biophys. Acta *70*, 271–277 (1963).
16. Uchiyama, M., R. Sato, and M. Mizugaki, *Ibid. 70*, 344–346 (1963); Okui, S., M. Uchiyama, M. Mizugaki and A. Sugawara, *Ibid. 70*, 364–348 (1963); Okui, S., M. Uchiyama and M. Mizugaki, J. Biochem. (Japan) *53*, 265–270 (1963); *54*, 536–540 (1963); Okui, S., M. Uchiyama, R. Sato and M. Mizugaki, *Ibid. 55*, 81–86 (1964).
17. de Vries, B., Chem. and Ind. (London) 1049–1050 (1962); JAOCS *40*, 184–186 (1963).
18. Wurster, C. F., Jr., J. H. Copenhaver, Jr., and P. R. Shafer, *Ibid. 40*, 513–514 (1963).
19. Emken, E. A., C. R. Scholfield and H. J. Dutton, *Ibid. 41*, 388–390 (1964).
20. Lakshmanan, C. M., and G. S. Laddha, *Ibid. 37*, 466–468 (1960).
21. Philip, K. J., P. Venkatarao and K. T. Achaya, Ind. J. Technol. *1*, 427–431 (1963).
22. Noble, W. R., A. Eisner and J. T. Scanlan, JAOCS *37*, 14–16 (1960).
23. Downing, D. T., Aust. J. Appl. Sci. *14*, 50–56 (1963).
24. Smith, C. R., Jr., T. L. Wilson, R. B. Bates and C. R. Scholfield, J. Org. Chem. *27*, 3112–3117 (1962).
25. Smith, C. R., Jr., T. L. Wilson, E. H. Melvin and I. A. Wolff, J. Am. Chem. Soc. *82*, 1417–1421 (1960); Smith, C. R., Jr., T. L. Wilson, T. K. Miwa, H. Zobel, R. L. Lohmar and I. A. Wolff, J. Org. Chem. *26*, 2903–2905 (1961).
26. Mangold, H. K., JAOCS *38*, 708–727 (1961).
27. Wollish, E. G., M. Schmall and M. Hawrylyshyn, Anal. Chem. *33*, 1138–1142 (1961); Wollish, E. G., Microchem. J. Symp. II, 687–696 (1962).
28. Kirchner, J. G., J. M. Miller and G. J. Keller, Anal. Chem. *23*, 420–425 (1951).
29. Applewhite, T. H., M. J. Diamond and L. A. Goldblatt, JAOCS *38*, 609–614 (1961).
30. Subbarao, R., M. W. Roomi, M. R. Subbaram and K. T. Achaya, J. Chromatog. *9*, 295–300 (1962).
31. Vioque, E., L. J. Morris and R. T. Holman, JAOCS *38*, 489–492 (1961).
32. Morris, L. J., R. T. Holman and K. Fontell, J. Lipid Res. *2*, 68–76 (1961); Morris, L. J., and R. T. Holman, *Ibid. 2*, 77–82 (1961); Chalvardjian, A., L. J. Morris, and R. T. Holman, J. Nutrition *76*, 52–58 (1962).
33. Sgoutas, D., and F. A. Kummerow, JAOCS *40*, 138–141 (1963).
34. Kaufmann, H. P., and Y. S. Ko, Fette Seifen Anstrichmittel *63*, 828–830 (1961).
35. Mangold, H. K., R. Kammereck and D. C. Malins, Microchem. J. Symp. II, 697–714 (1962).
36. (a) Vioque, E., and R. T. Holman, JAOCS *39*, 63–66 (1962); (b) Arch. Biochem. Biophys. *99*, 522–528 (1962).
37. Yamada, M., and P. K. Stumpf, Biochem. Biophys. Res. Commun. *14*, 165–171 (1964).
38. Barrett, C. B., M. S. J. Dallas and F. B. Padley, Chem. and Ind. (London) *1962*, 1050–1051.
39. Morris, L. J., *Ibid. 1962*, 1238–1240.
40. de Vries, B., and G. Jurriens, Fette Seifen Anstrichmittel *65*, 725–727 (1963).
41. Morris, L. J., J. Chrom. *12*, 321–328 (1963).
42. Gunstone, F. D., and A. J. Sealy, J. Chem. Soc. *1963*, 5772–5778.
43. Morris, L. J., *Ibid. 1963*, 5779–5781.
44. Rouser, G., A. J. Bauman, N. Nicolaides and D. Heller, JAOCS *38*, 565–581 (1961).
45. Kaufmann, H. P., and Y. S. Ko, Fette Seifen Anstrichmittel *64*, 434–438 (1962).
46. Skipski, V. P., S. M. Arfin and M. M. Rapport, Arch. Biochem. Biophys. *87*, 259–265 (1960).
47. Wakabayashi, K., and N. Shimazono, Biochim. Biophys. Acta *70*, 132–142 (1963).
48. Miwa, T. K., K. L. Mikolajczak, F. R. Earle and I. A. Wolff, Anal. Chem. *32*, 1739–1742 (1960).
49. Miwa, T. K., JAOCS *40*, 309–313 (1963).
50. Litchfield, C., R. Reiser and A. F. Isbell, *Ibid. 40*, 302–309 (1963).
51. Kaufmann, H. P., G. Mankel and K. Lehmann, Fette Seifen Anstrichmittel *63*, 1109–1119 (1961).
52. O'Brien, J. S., and G. Rouser, Anal. Biochem. *7*, 288–296 (1964).
53. Morris, L. J., R. T. Holman and K. Fontell, J. Lipid Res. *1*, 412–420 (1960).
54. Sallee, E. M., R. R. Allen, W. T. Coleman, J. R. Chipault and R. T. O'Connor, JAOCS *41*, 158–160 (1964).
55. Earle, F. R., I. A. Wolff, C. A. Glass and Q. Jones, *Ibid. 39*, 381–383 (1962).
56. Mikolajczak, K. L., F. R. Earle and I. A. Wolff, *Ibid. 39*, 78–80 (1962).
57. Miwa, T. W., F. R. Earle, G. C. Miwa and I. A. Wolff, *Ibid. 40*, 225–229 (1963).
58. Chisholm, M. J., and C. Y. Hopkins, Can. J. Chem. *38*, 2500–2507 (1960).
59. Duhamel, L., Ann. Chim. *13*, 315–346 (1963).
60. Bishop, D. G., and J. L. Still, J. Lipid Res. *4*, 81–86 (1963); *4*, 87–90 (1963); Biochem. Biophys. Res. Commun. *7*, 337–341 (1962).
61. Perkins, E. G., J. G. Endres and F. A. Kummerow, Proc. Soc. Exptl. Biol. Med. *106*, 370–372 (1961).
62. Perkins, E. G., J. G. Endres and F. A. Kummerow, J. Nutrition *73*, 291–298 (1961).
63. Watson, W. C., and R. S. Gordon, Jr., Biochem, Pharmacol. *11*, 229–236 (1962).
64. Watson, W. C., R. S. Gordon, Jr., A. Karmen and A. Jover, J. Pharm. Pharmacol. *15*, 183–188 (1963).
65. Canvin, D. T., Can. J. Biochem. and Physiol. *41*, 1879–1885 (1963).
66. Critchfield, F. E. "Organic Functional Group Analysis," MacMillan Co., New York, 1963, pp. 81–106.
67. Fritz, J. S., and G. H. Schenk, Anal. Chem. *31*, 1808–1812 (1959); Schenk, G. H., and M. Santiago, Microchem. J. *6*, 77–82 (1962).
68. Stetzler, R. S., and C. F. Smullin, Anal. Chem. *34*, 194–195 (1962).
69. Sully, B. D., Analyst *87*, 940–943 (1962).
70. Kaufmann, H. P. and E. Schmülling, Fette Seifen Anstrichmittel *64*, 319–321 (1962).
71. Robinson, W. T., Jr., R. H. Cundiff and P. C. Markunas, Anal. Chem. *33*, 1030–1034 (1961).
72. Johnson, D. P., and F. E. Critchfield, *Ibid. 32*, 865–867 (1960).
73. Kyriacon, D., *Ibid. 32*, 291–292 (1960).
74. Reed, D. H., Critchfield, F. E. and D. K. Elder, *Ibid. 35*, 571–573 (1963).
75. Vioque, E., and M. P. Maza, Grasas y Aceites *31*, 207–210 (1962); Anal. Abs. *10*, 3286 (1963).
76. Gutnikov, G., and G. H. Schenk, Anal. Chem. *34*, 1316–1319 (1962).
77. Critchfield, F. E., and J. A. Hutchinson, *Ibid. 32*, 862–865 (1960).
78. Kartha, A.R.S., and J. M. S. Mathur, Ind. J. Chem. *1*, 494–495 (1963).
79. Kumar, R., JAOCS *40*, 80 (1963).
80. Malins, D. C., J. C. Wekell and C. R. Houle, Anal. Chem. *36*, 658–661 (1964).
81. O'Connor, R. T., JAOCS *38*, 648–659 (1961); *Ibid. 38*, 641–648 (1961); in "Fatty Acids," edited by Markley, K. S., 2nd ed., Vol. 1, Interscience Publishers, Inc., New York, 1960, pp. 422–474.
82. Chouteau, J., Rev. Franc. Corps Gras *8*, 267–284 (1961).
83. Kitagawa, I., M. Sugai, and F. A. Kummerow, JAOCS *39*, 217–222 (1962).
84. Eddy, C. R., J. S. Showell, and T. E. Zell, *Ibid. 40*, 92–96 (1963).
85. Bayzer, H., E. Schauenstein and K. Winsauer, Monatsh. *89*, 15–22 (1958).
86. Morris, L. J., and R. T. Holman, J. Lipid Res. *2*, 77–82 (1961).
87. Subbarao, R., and K. T. Achaya, J. Sci. Ind. Res. *21D*, 446–448 (1962).
88. Brunelle, T. E., and S. B. Crecelius, Soap Chem. Specialities, *39*, (10) October, 1963, 63–65.
89. Crisler, R. O., and A. M. Burrill, Anal. Chem. *31*, 2055–2057 (1959).
90. O'Connor, R. T., and J. R. Chipault, JAOCS *40*, #3, *14*, 32–34 (1963).
91. Bergström, S., G. Aulin Erdtman, B. Rolander, E. Stenhagen and S. Ostling, Acta Chem. Scand. *6*, 1157–1174 (1952).
92. Palmer, K. T., and D. R. Black, unpublished work.
93. Hopkins, C. Y., and H. J. Bernstein, Can. J. Chem. *37*, 775–782 (1959).
94. Hopkins, C. Y., JAOCS *38*, 664–668 (1961).
95. Dutton, H. J., *Ibid. 38*, 660–664 (1961).
96. Ryhage, R., and E. Stenhagen, Arkiv Kemi *15*, 545-574 (1960).
97. Ryhage, R., and E. Stenhagen, J. Lipid Res. *1*, 361–390 (1960).
98. Ryhage, R., and E. Stenhagen, in "Mass Spectrometry of Organic Ions," ed. F. W. McLafferty, Academic Press, New York, 1963, pp. 399–452.
99. Bolley, D. S., JAOCS *30*, 396–398 (1953).
100. Priester, R., Fette Seifen Anstrichmittel *63*, 361–364 (1964).
101. Djerassi, C., "Optical Rotary Dispersion, Applications to Organic Chemistry," McGraw-Hill, New York, 1960, 293 pp.
102. Sjöberg, B., A. Fregda and C. Djerassi, J. Am. Chem. Soc. *81*, 5002–5003 (1959); Sjöberg, B., Acta Chem. Scand. *14*, 273–295 (1960); Sjöberg, B., B. Hansson and R. Dahlbom, *Ibid. 16*, 1057–1059 (1962); Sjöberg, B., D. J. Cram, L. Wolf and C. Djerassi, *Ibid. 16*, 1079–1096 (1962).
103. Baker, C. D., and F. D. Gunstone, J. Chem. Soc. *1963*, 759–760.
104. Schroepfer, G. J., Jr., and K. Bloch, J. Am. Chem. Soc. *85*, 3310–3311 (1963).

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