Hydrocarbons and polychlorinated biphenyls from the unsaponifiable fraction of anhydrous milk fat

Vincent P. Flanagan and Aldo Ferretti

Dairy Products Laboratory, Agricultural Research Service,
U.S. Department of Agriculture, Washington, D.C. 20250

Abstract Using a combination of gas–liquid chromatography and mass spectrometry, the presence of 39 aliphatic hydrocarbons was firmly established in the unsaponifiable fraction of anhydrous milk fat. The hydrocarbons were the C_{14} to C_{27} and the C_{29} to C_{31} straight-chain paraffins, their monoolefin analogs, and the C_{25} to C_{29} branched alkanes. Phytene (3,7,11,15-tetramethyl-*n*-hexadec-2-ene), identified for the first time in milk fat, was isolated and identified by high-resolution mass spectrometry and infrared analysis. The total hydrocarbon content amounted to 30 ppm of the milk fat. Polychlorinated biphenyls also were detected in trace amounts in the area of the chromatogram between the C_{18} and the C_{23} hydrocarbons.

Supplementary key words straight-chain alkanes · alkenes · branched alkanes · phytene · gas–liquid chromatography · mass spectrometry

Several investigators have reported the presence of hydrocarbons in milk fat, or butterfat, and anhydrous butter oil (1–6). They include the normal C_1 to C_{48} alkanes, many of their 2-methyl and 1-cyclohexyl derivatives, mono- and diolefins, branched-chain alkanes, alkynes, alkyl-substituted benzenes, and squalene. In many instances, prior to isolation of the hydrocarbons the milk fat had undergone some sort of treatment, including heating to 210°C for 2 hr (2), gamma irradiation (4, 5), or copper treatment (6).

Identification of hydrocarbons containing less than 17 carbon atoms was based on gas–liquid chromatographic retention times and mass spectrometry (2, 4–6). The higher hydrocarbons, however, were identified only on the basis of chromatographic evidence (1, 3).

The present work was undertaken with the purpose of unequivocally establishing the presence of hydrocarbons in the unsaponifiable fraction of anhydrous milk fat. The isolated hydrocarbons were analyzed by GLC, and the column effluent was fast-scanned with a mass spectrometer. Identifications were always based on comparison of GLC retention times and mass spectral data of the unknowns with those of authentic materials.

Along with the hydrocarbons, certain polychlorinated biphenyls also were detected. Their mass spectra were compared with those reported in the literature (7). Reference retention times were measured from a PCB chromatogram whose peak compositions had previously been determined by GLC–MS under identical conditions in our laboratory.

EXPERIMENTAL PROCEDURES[1]

Isolation of the hydrocarbon fraction from the unsaponifiable matter

100 g of anhydrous milk fat (prepared from mixed-herd milk, Beltsville, Md.) was saponified without a solvent according to a previously published procedure (8). After cooling to room temperature, the saponified material was ground in a mortar to a fine powder. The powder was then divided into two equal parts, which were placed in two 250-ml centrifuge bottles, and extracted seven times with a total of 1000 ml of redistilled *n*-hexane (Phillips Petroleum, Bartlesville, Okla.). The supernatant fractions from each extraction were decanted after centrifugation. The combined extracts were dried over Na_2SO_4 and filtered through Whatman no. 1 filter paper. The solvent was removed by heating on a

---

Abbreviations: GLC, gas–liquid chromatography; MS, mass spectrometry; AMF, anhydrous milk fat; PCB, polychlorinated biphenyl(s); DMCS, dimethylchlorosilane.

[1] Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

steam bath under a gentle stream of air. The residue (HC-1), consisting primarily of cholesterol, weighed 0.25 g.

Column chromatography

HC-1 was dissolved in several milliliters of *n*-hexane and chromatographed over a column of 50 g of acid alumina, Brockmann activity grade I (J. T. Baker Co., Phillipsburg, N.J.), previously hydrated to a 6% water content and washed with 75 ml of *n*-hexane. The column was irrigated with hexane, and 200 ml of eluate was collected. This was evaporated to dryness as described above. The residue (HC-2) weighed 9.4 mg. HC-2 was dissolved in 2 ml of *n*-hexane and chromatographed over 35 g of Florisil (Fisher Scientific Co., Silver Spring, Md.), previously activated by heating at 200°C for 16 hr and washed with 75 ml of hexane. The eluant was *n*-hexane, and 200 ml of eluate was collected, which, after solvent evaporation, yielded 3.0 mg of a waxy residue (HC-3). This represents 30 ppm of the milk fat. The procedure described above, for the isolation and purification of the hydrocarbon fraction from the unsaponifiable matter, was repeated with four more 100-g batches of AMF. The five HC-3 residues were combined, and ca. 0.2 mg of the mixture was analyzed for presence of carbonyl (9) and hydroxyl (10) compounds. The test was negative for both classes of compounds.

For GLC solvent blank, 1000 ml of *n*-hexane was evaporated to near dryness. The chromatogram showed only the solvent peak.

GLC-MS

Prior to further processing, the various residues, as well as the final fraction HC-3, were chromatographed on an 8 ft × 0.125 inch (O.D.) stainless steel column treated with DMCS and packed with 3% JXR silicone on 80-100 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.). The instrument was a Hewlett-Packard 5750A coupled to an LKB-9000 mass spectrometer (11). Three sets of temperature programming conditions were employed, depending upon the area to be scanned. Condition A: 70 to 200°C at 2°/min; this was used through the elution, and scanning, of the C_{20} hydrocarbon. Thereafter, the column was heated rapidly to 210°C, and this temperature was maintained through the end of the chromatogram. Condition B: 70 to 210°C at 4°/min, and isothermal thereafter; this condition was used to scan the C_{21}–C_{29} area. Condition C: 100 to 210°C at 4°/min, and isothermal thereafter; this condition was used to scan the area beyond the C_{29} hydrocarbon. The base line after the elution of the C_{31} hydrocarbon was essentially a straight line for 1.5 hr. In all cases the carrier gas (He) was supplied at a head pressure of 40 psi. All peaks were continuously scanned at an ionizing energy of 70 eV. Valleys between major peaks were not scanned continuously because preliminary observation indicated that the relative spectra consisted essentially of fragments from the adjacent major compounds.

Because of its relative abundance and interesting low-resolution mass spectrum (see under Results), the material that was eluted between the C_{18} and the C_{19} hydrocarbons was trapped and subjected to high-resolution mass spectrometry and infrared analysis. The material was isolated from residue HC-3 by GLC (programming condition A) by trapping the effluent in a capillary glass tube cooled with dry ice. The instrument used was a Hewlett-Packard 5750B gas chromatograph equipped with a flame ionization detector. The column effluent was split 10:1 with one part going to the detector. The high-resolution mass spectrum was determined with a CEC 21-110B double-focusing mass spectrometer operating at 70 eV. The spectrum was recorded on a photoplate, and accurate mass measurements were made by means of a comparator, with perfluorokerosene as internal standard.

Infrared analysis

The infrared spectra were determined with a Beckman IR-5A instrument, employing an ultramicro cell (0.1 mm thickness) or NaCl plates. Carbon tetrachloride was the solvent used with the ultramicro cell.

Reference hydrocarbons

The reference straight-chain hydrocarbons were purchased from Chemical Samples Co., Columbus, Ohio. *trans*-Phytene was prepared from phytol (Mann Research Laboratories, New York) by hydrogenolysis with sodium in liquid ammonia (12). All reference compounds were purified by GLC before determination of their mass spectra.

RESULTS

Hydrocarbons

Based on GLC analysis, cholesterol was by far the largest constituent of the residue HC-1. After passing HC-1 over the alumina column, cholesterol was removed entirely. The gas-liquid chromatogram of HC-2 showed that three large peaks, which did not fit the pattern for a homologous series of hydrocarbons, still remained. These peaks were located in the C_{18}–C_{19}, C_{23}–C_{24}, and C_{28} areas. Further purification over Florisil removed two of these peaks, probably associated with oxygenated compounds. The remaining peak was located between the C_{18} and the C_{19} hydrocarbons. The purified hydrocarbon fraction HC-3, whose chromatogram is shown in Fig. 1, was used for the GLC-MS analysis. The chromatogram

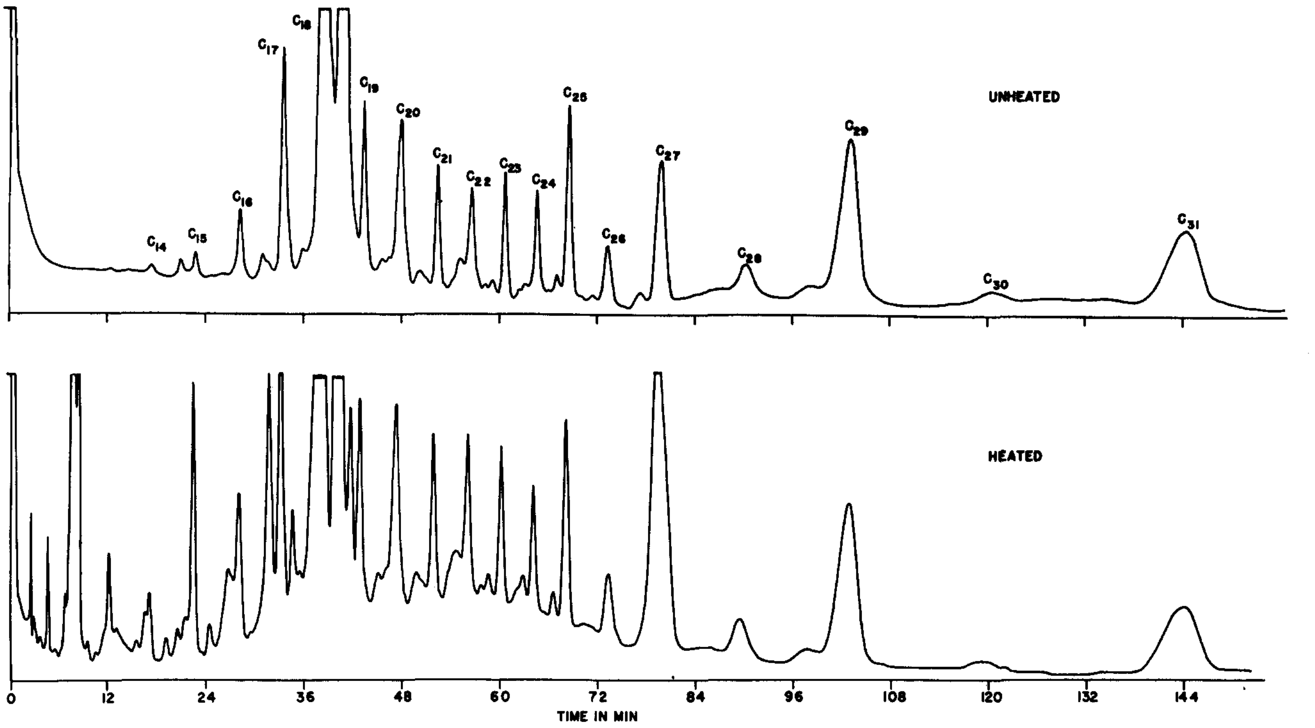

FIG. 1. Gas–liquid chromatogram (flame ionization detector) of the hydrocarbon fraction from the unsaponifiable matter of unheated (residue HC-3) and heated anhydrous milk fat. Programming conditions: 70 to 210°C at 2°/min and isothermal thereafter.

is similar to that published by McCarthy, Kuksis, and Beveridge (1).

Continuous mass scanning of the chromatographic peaks indicated that most of them represented mixtures. The following hydrocarbons were identified positively: the C_{14} to C_{27} and C_{29} to C_{31} straight-chain alkanes, the corresponding monoalkenes, and the C_{25} to C_{29} branched alkanes.

The branched alkanes and the alkenes were uniformly eluted a few scans earlier than the straight-chain alkanes having the same number of carbon atoms. This afforded detection of all three classes of compounds. However, the position of the double bond and the site of branching remained undetermined. Beginning with the C_{21} alkane, the odd-number hydrocarbons are more abundant than the even-number ones and become increasingly so as the chain length increases (Fig. 1). The observed intensity of the molecular ion of the alkenes was invariably weaker than that of the corresponding alkane. On the basis of the general rule that the molecular ion of monoolefins is more pronounced than in saturated analogs (13), we concluded that the alkanes are the predominant components of the hydrocarbon mixture.

Phytene

The unknown compound associated with the large peak between the C_{18} and the C_{19} hydrocarbons (see Fig. 1) was provisionally designated as 280-II because of its apparent molecular weight and the presence of another compound (designated as 280-I), present in a much smaller amount, whose retention time was very close to that of the C_{18} alkane. High-resolution mass spectral measurements indicated that the exact molecular weight was 280.3153, consistent with the elemental composition $C_{20}H_{40}$. Therefore, the molecule must have one site of unsaturation. A monocyclic structure was ruled out primarily on the basis of the relatively low intensity of the molecular ion (see Fig. 2). The great number of rearrangement ions confirms the presence of double bond unsaturation. Furthermore, the elution of 280-II several minutes ahead of the C_{20} alkene strongly indicates that the molecule contains a number of branching sites. The absence of infrared absorption near 990 cm^{-1}, 910 cm^{-1}, and 890 cm^{-1} ruled out a terminal vinyl and a terminal methylene moiety (14). Keeping this in mind, along with the observation that the base peak was at *m/e* 70, structures such as those shown in Fig. 3 are very probable, if not the only ones possible. The double bond apparently is in the 2 or the 3 position. Then the formation of a strong $[C_5H_{10}]^{+\cdot}$ ion could be rationalized on the basis of a McLafferty rearrangement (15). The partial structure II of Fig. 3 resembles the unsaturated tail of phytene. The *trans*-phytene synthesized in our laboratory exhibited GLC retention time and a mass spectrum

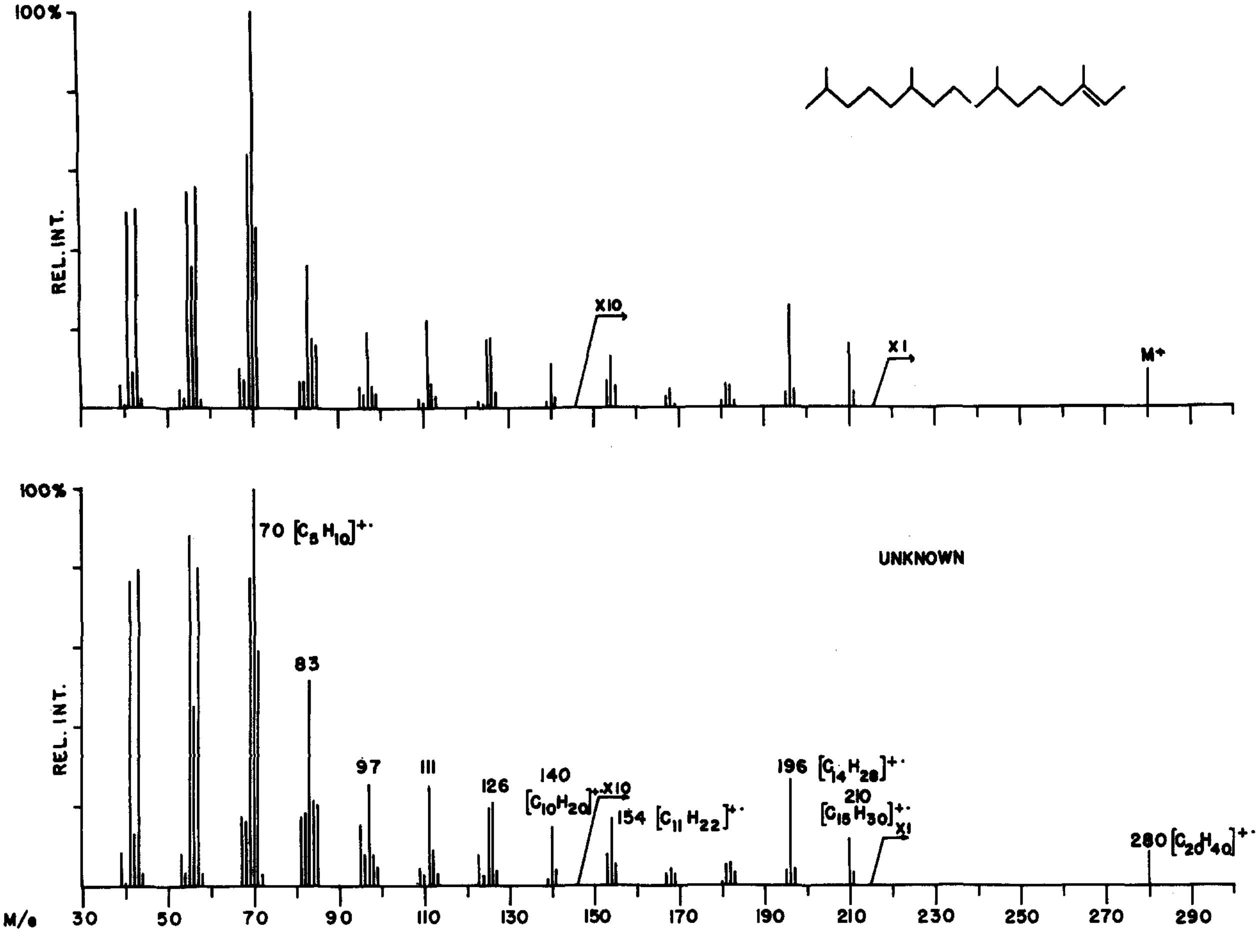

FIG 2. Mass spectrum of the unknown compound 280-II and of synthetic *trans*-phytene.

(Fig. 2) identical with those of compound 280-II, including metastable peaks at the following *m** values: 61.5, 63.0, 76.3, 88.0, 89.6, 101.5, 103.0, and 157.5. Therefore, the identification of compound 280-II as a phytene rests on a firm basis. However, given the expected qualitative similarity between the mass spectra of *cis-trans* isomeric hydrocarbons, and the unavailability of the *cis*-phytene for chromatographic and spectroscopic comparison, the geometry of the double bond in 280-II remains uncertain. The scarcity of compound 280-I, whose mass spectrum is very similar to that of 280-II, precluded further analytical work to confirm the speculation that it could be the *cis* isomer.

Polychlorinated biphenyls

The PCB, although present only in traces, were clearly indicated by their relatively intense molecular ion followed by the M + 2, M + 4, and M + 6 ions from the isotopic contribution of the chlorine atoms and by the typical fragmentation pattern resulting from the successive losses of chlorine. Polychlorinated biphenyls having the empirical formulas $C_{12}H_7Cl_3$, $C_{12}H_6Cl_4$, $C_{12}H_5Cl_5$, and $C_{12}H_4Cl_6$ were detected only in the area of the chromatogram between the C_{18} and the C_{23} hydrocarbons.

DISCUSSION

Phytene has been identified in milk fat for the first time in the present investigation. Its origin is uncertain but, owing to its structural relationship with phytol, a degradation product of chlorophyll, the introduction of phytene or of its precursor via feed is probable. Another possible precursor of phytene is vitamin K_1.

Only some of the hydrocarbons reported herein have been identified previously, with a reasonable degree of certainty, in milk fat and related materials. The C_{14} to C_{17} alkanes and 1-alkenes were found by Khatri, Libbey, and Day (4) in the steam distillate of gamma-irradiated milk fat. The C_{14} to C_{17} *n*-alkanes, the C_{14}, C_{16}, and C_{17} 1-alkenes, along with many lower homologs, were found by Siek and Lindsay (2) in milk fat vacuum

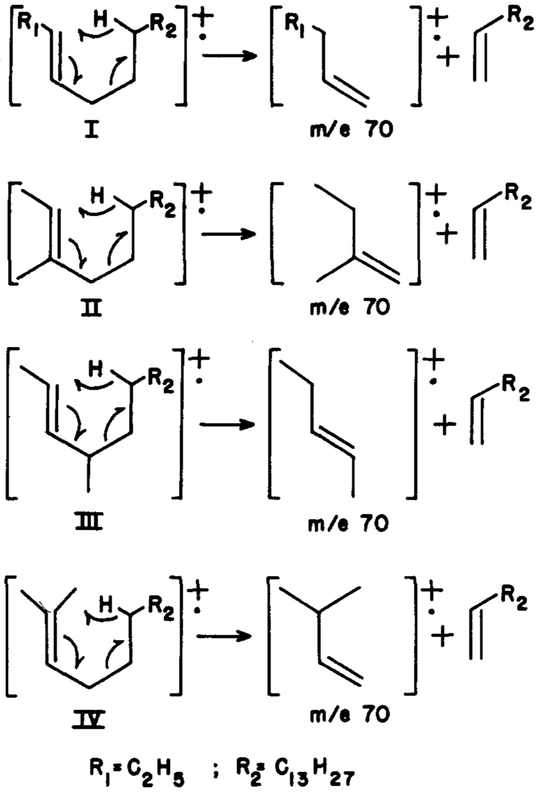

FIG. 3. Formation of the base peak (m/e 70) from three possible molecular ion structures in the mass spectrum of the unknown compound 280-II.

steam distillates, but predominantly in samples heated at 210°C for 2 hr. Hydrocarbons with less than 14 carbons were not detected during our investigation. This observation prompted us to conduct an experiment comparable to that described by Siek and Lindsay (2) to determine whether the lower homologs could have been lost during our isolation process. The experiment consisted of heating 100 g of anhydrous milk fat to 210°C for 2 hr and isolating the hydrocarbons using the procedure described above to obtain the fraction HC-3. The chromatogram of the residue thus obtained (Fig. 1) clearly proves not only that the more volatile compounds are not lost during processing but also that at least some of them may be heat-induced. A similar conclusion was reached by Siek and Lindsay (2).

In a study of seasonal variation in hydrocarbon content in milk fat, Ristow and Werner (3) identified the C_{14} to C_{35} *n*-alkanes along with several branched-chain monoolefins. However, identifications were made solely on the basis of GLC retention times, and the results from such experiments must be interpreted with caution. Analogous considerations apply also to the results by McCarthy et al. (1). They reported finding the C_{17} to C_{48} alkanes of the normal, iso, and cyclohexyl series, and an unspecified number of alkenes. However, in the words of the authors, "On the basis of gas chromatographic behavior alone, . . . only squalene could be identified with any degree of certainty" (1). Conclusive evidence has been offered for the presence of 11-cyclohexylundecanoic acid in butter (16). On the other hand, convincing proof for the occurrence of cyclohexyl alkanes has not yet been provided. Cyclohexyl alkanes have mass spectra distinctly different from those of straight-chain paraffins and olefins. Not only the intensity of their molecular ion is relatively high, but a pair of intense peaks normally are present at m/e 82 and m/e 83, the latter often being the base peak (17). In the present investigation we did not find any spectrometric evidence for the presence of cyclohexyl alkanes in fraction HC-3.

The origin of hydrocarbons in milk fat is obscure. Although some experimental evidence suggests that several of them are heat-produced (2), the mechanism of their formation is unknown. Moreover, the presence of certain hydrocarbons in unheated milk fat seems certain (2). No flavor significance is attached to the hydrocarbons because of their generally low concentrations and high flavor thresholds.

The finding of PCB during the present investigation was unexpected. Although present only in traces, their detection can only serve to emphasize the widespread contamination of our environment. Their origin and significance is beyond the scope of this paper, and the reader is referred to the review article by Edwards (18).

The authors are indebted to Dr. John M. Ruth, Agricultural Environmental Quality Institute, ARS, U.S. Department of Agriculture, Beltsville, Md., for the high-resolution mass spectral measurements.

Manuscript received 16 October 1972; accepted 10 January 1973.

REFERENCES

1. McCarthy, M. J., A. Kuksis, and J. M. R. Beveridge. 1964. Composition of molecular distillates of butter oil: isolation and identification of components other than glycerides. *J. Lipid Res.* **5:** 609–615.
2. Siek, T. J., and R. C. Lindsay. 1968. Volatile components of milk fat steam distillates identified by gas chromatography and mass spectrometry. *J. Dairy Sci.* **51:** 1887–1896.
3. Ristow, R., and H. Werner. 1968. Jahreszeitliche Veränderung des Gehaltes an Kohlenwasserstoffen im Milchfett. *Fette Seifen Anstrichm.* **70:** 273–280.
4. Khatri, L. L., L. M. Libbey, and E. A. Day. 1966. Gas chromatographic and mass spectral identification of some volatile components of gamma-irradiated milk fat. *J. Agr. Food Chem.* **14:** 465–469.
5. Merritt, C., Jr., D. A. Forss, P. Angelini, and M. L. Bazinet. 1967. Volatile compounds produced by irradiation of butterfat. *J. Amer. Oil Chem. Soc.* **44:** 144–146.
6. Forss, D. A., P. Angelini, M. L. Bazinet, and C. Merritt,

Jr. 1967. Volatile compounds produced by copper-catalyzed oxidation of butterfat. *J. Amer. Oil. Chem. Soc.* **44:** 141–143.

7. Bagley, G. E., W. L. Reichel, and E. Cromartie. 1970. Identification of polychlorinated biphenyls in two bald eagles by combined gas-liquid chromatography-mass spectrometry. *J. Ass. Offic. Anal. Chem.* **53:** 251–261.
8. Schwartz, D. P., L. H. Burgwald, and C. R. Brewington. 1966. A simple quantitative procedure for obtaining the unsaponifiable matter from butteroil. *J. Amer. Oil Chem. Soc.* **43:** 472–473.
9. Schwartz, D. P., H. S. Haller, and M. Keeney. 1963. Direct quantitative isolation of monocarbonyl compounds from fats and oils. *Anal. Chem.* **35:** 2191–2194.
10. Schwartz, D. P. 1970. Quantitative micro determination of alcohols as esters of pyruvic acid 2,6-dinitrophenylhydrazone. *Anal. Biochem.* **38:** 148–157.
11. Ferretti, A., and V. P. Flanagan. 1971. Volatile constituents of whey powder subjected to accelerated browning. *J. Dairy Sci.* **54:** 1764–1768.
12. Bhati, A. 1966. Epoxides of phytol and related compounds. *Perfum. Essent. Oil Rec.* **57:** 563–566.
13. Budzikiewicz, H., C. Djerassi, and D. H. Williams. 1967. Mass Spectrometry of Organic Compounds. Holden-Day, San Francisco, Calif. 49–72.
14. Nakanishi, K. 1962. Infrared Absorption Spectroscopy, Practical. Holden-Day, San Francisco, Calif. 24–25.
15. McLafferty, F. W. 1959. Mass spectrometric analysis. Rearrangements in vinyl derivatives. *Anal. Chem.* **31:** 2072–2075.
16. Schogt, J. C. M., and P. H. Begemann. 1965. Isolation of 11-cyclohexylundecanoic acid from butter. *J. Lipid Res.* **6:** 466–470.
17. Levy, E. J., R. R. Doyle, R. A. Brown, and F. W. Melpolder. 1961. Identification of components in paraffin wax by high temperature gas chromatography and mass spectrometry. *Anal. Chem.* **33:** 698–704.
18. Edwards, R. 1971. The polychlorobiphenyls, their occurrence and significance: a review. *Chem. Ind.* (London). 1340–1348.