# Characterization and Identification of Lipids by Their Critical Solution Temperatures

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**Abstract**

The mutual solubility of two liquids which are not miscible in all proportions is essentially a function of the temp. Rising temp usually increases the solubility, possibly reaching a point, i.e., the upper critical solution temp (CST), at which the two components become miscible in all proportions. The CST is characteristic for the two liquids involved.

A microtechnique developed by Fischer simplifies rapid and accurate determinations of the upper critical solution temp. This technique is described and its application to the characterization and identification of pure lipids and lipid mixtures is demonstrated. The significance of this simple laboratory procedure and its relation to complementary analytical methods is discussed.

IT IS WELL KNOWN that the solubility properties of organic compounds can be utilized for the separation of mixtures and for the characterization of their constituents. The solubility of a compound in a liquid is dependent upon the temp. Two liquids which are not completely miscible at room temp may dissolve in each other to a higher degree at either increasing or decreasing temp. The temps at which complete solubility is achieved are, therefore, the maximum or minimum temps at which this pair of liquids can exist in two liquid phases. These upper and lower critical solution temps are characteristic for each of the two liquids. Occasionally, both the upper and lower critical solution temps of a pair of liquids can be realized. In the majority of cases, a rise in temp increases the mutual solubility. Hence, determinations of the upper critical solution temp (CST) offer a means for characterizing a system of two liquids. With a suitable test liquid, many compounds can be characterized by their CST in the same sense as by their melting points and boiling points.

Initial studies of liquid-liquid systems were reported by Alexejew (1) and Rothmund (42). Various aspects of the solubility phenomena of "immiscible" liquids have since been extensively investigated (30, 35). The standard monograph by Hildebrand and Scott (24), and a volume of the Discussions of the Faraday Society (14) summarize the problems of phase equilibria and solubilities. Data on solubility characteristics of organic compounds have been compiled by Seidell (48), and physicochemical constants of binary systems have been collected by Timmermans (49). Francis (21) recently published a volume dealing exclusively with critical solution temps.

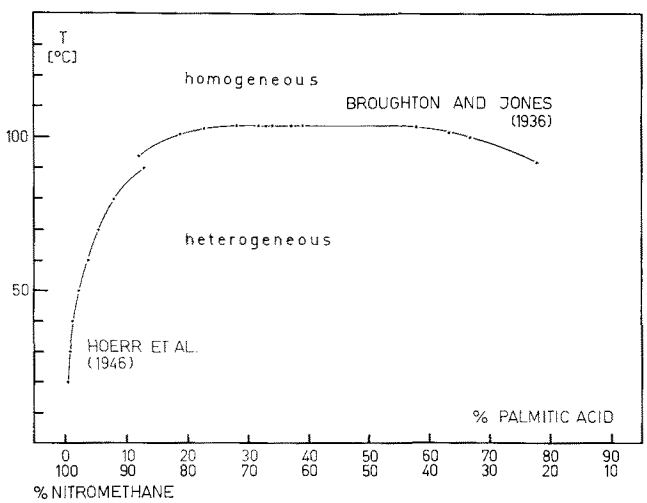


FIG. 1. Solubility diagram of palmitic acid and nitromethane (6,28).

Generally, the total miscibility of two liquids at the upper critical solution temp is achieved instantly (Fig. 1), i.e., little temp change results in a significant change of the mutual solubility. This effect facilitates CST observations with great accuracy (6).

The percentage of palmitic acid dissolved in nitromethane at various temps is depicted in Figure 1, according to data reported by Broughton and Jones (6) and Hoerr et. al. (28). The two solubility curves do not exactly merge. It can be assumed that the substances used were of different qualities.

Determinations of upper and lower critical solution temps are used mainly in petroleum chemistry for: characterizing hydrocarbons, studying the properties of selective solvents, estimating impurities, and analyzing multiple mixtures. The method usually employed is to place at least 1 ml of each of the two liquids together in a test tube, and to observe the temp at which the two liquids just mix on heating or just cloud on cooling (21). The temp readings can be rendered difficult due to color, the formation of precipitations or the occurrence of isooptical mixtures (the refractive indices of both liquids become identical) (22). Refinements in observation of CST-values or methods of expediting the procedure have been described by numerous authors (3,7,21). In contrast to melting point determinations, super cooling does not occur. Therefore, the temp readings of the CST are the same during the heating and the cooling processes, both of which may be repeated several times.

In the lipid field several procedures have been proposed for assessing the quality of fatty raw material by determining their solubility characteristics. For example, the Crismer number represents the temp at which a homogeneous mixture of fat and solvent starts to separate into two phases (9–11). A modification of this procedure has been adapted as an AOCS Official Method (2). Related techniques have been proposed by Valenta (50,51), Fachini and Dorta (15), and many others (32). As all of these procedures require rather large amts of sample, they are hardly applicable for the characterization of highly purified compounds.

R. W. Fischer devised a method for the determination of upper critical solution temps, which permits the rapid and accurate analysis of as little as 0.5–2 $\mu$l sample sealed into a glass capillary together with a suitable test substance (16–20,23). This capillary is heated on a hot stage, and the interface between the two liquids is observed under a microscope. Fischer and co-workers applied this microtechnique to characterize petroleum products (20), fats and oils (17,19,31), and other naturally occurring substances.

The small amts of sample required for a CST de-

termination can be isolated easily by chromatographic techniques.

Critical solution temps determined with the micromethod agree in many cases with data reported by authors who had obtained these values through solubility studies (6,25–28,47). Deviations can be due to differences in the purity of the preparations analyzed and/or the purity of the test substances used.

We have applied the micromethod of Fischer to characterize a large number of chromatographically pure aliphatic compounds. CST-values of homologous and vinylogous fatty acids and methyl esters (44), triglycerides (45), alcohols, dialkyl ethers and wax esters (46), α-glyceryl ethers (4) and hydrocarbons (5) have been reported.

The purpose of the present communication is to describe the micromethod of Fischer as used in our laboratory, to review and discuss present knowledge about critical solution temps of lipids, and to present the results of some new studies.

## The Micromethod of Fischer

The apparatus, the amt of sample used, the speed and accuracy of CST determinations are similar to the microdetermination of melting points as described by Kofler (34,40). Following will be a description of the equipment and procedure used, and the test substances employed will be specified.

### Equipment

1) Microscope. The Reichert "RCH" (Reichert Optische Werke A. G., Wien 17, Austria) is especially designed for use in connection with a heating stage. It is equipped with an objective 10X and an Huyghenian eyepiece 10X, thus yielding a total magnification of 100X. However, any upright transmitted light microscope can be used provided that the stage top contains two plug holes to accept clips for mounting a micro heating stage. The stage top of the microscope should be all metal and nonrotary in order to resist higher temps.

When using the Reichert-Kofler micro heating stage, the objective used in the microscope must have at least a free working distance of 6 mm (e.g., the Reichert Dry Achromatic Objective with a magnification of 10X, a numerical aperture of 0.25 and a working distance of 7.1 mm).

2) Micro heating stage. Suitable micro heating stages are available from Reichert Optische Werke, No. 4060 and 4065, and Arthur H. Thomas Co. (Philadelphia) No. 6886-A and 6886-B. These heating stages are equipped with exchangeable mercury in glass thermometers. The Reichert thermometers range from 20 to 230C and 120 to 350C, whereas the Thomas thermometers have ranges from 30 to 230C and 60 to 350C.

3) Slide and shifting device. All determinations of CST-values listed in this communication have been carried out using the adapted microscope slide illustrated in Figure 2.

Figure 2 shows a sealed capillary containing sample and a test substance (*a*), an adapted microscope slide (*b*) and the shifting device supplied with the Reichert micro heating stage (*c*).

A 38 × 26 × 1 mm microscope slide was adapted by gluing two glass strips (38 × 4 × 1 mm) onto it, leaving a channel of 0.5 mm in width. Recent experiments with brass slides and aluminum slides proved their suitability. A shifting device (Cat. No. 6887 M) with an aluminum slide (Cat. No. 6888 Q) for determination of CST-values by the micromethod is available from A. H. Thomas Company.[3]

4) Capillaries. Glass capillaries of 0.2–0.3 inner diam and about 15–20 cm length may be drawn from glass tubing (7 mm diam). They are also available commercially from A. H. Thomas Company.

### Test Substances

The use of CST determinations as an analytical procedure requires standard test substances, in order to obtain reproducible results. Valuable is their CST determination with pure standard liquids such as benzene or hexadecane (see Table I).

The following requirements have to be considered when selecting a suitable test substance.

*General Standards.* The substance should be available in high purity and storable without the use of special precaution (e.g., against autoxidation, etc.). It must be chemically uniform at different temps, or at least its solubility characteristics must be reproducible at any specific temp used. Furthermore, the test substance should not show a tendency to react or decompose and preferably be a liquid at room temp.

*Special Requirements.* The test substance should yield a CST with the sample in a temp range from 20 to 230C.

At the CST the refractive indices of sample and test substance must be sufficiently different to allow one to see the interface between them.

Approaching the CST total miscibility should occur instantly making possible exact observations of the disappearing interface.

The following five liquids (see Table I) have been selected as test substances for characterizing various lipid classes. They proved to meet most of the requirements specified above. Only the 1,3-butanediol (Matheson, Coleman and Bell, Norwood, Ohio) was purified by vacuum distillation. All other liquids were used as obtained in order to facilitate their application.

By selecting a suitable test substance the CST-values of a series of compounds may be determined at various temp ranges.

### Procedure (16,44)

The test substance and the liquid sample are intro-

TABLE I

Test Substances

| Substance | Supplied by | $n_D^{20}$ | $d_n^{20}$ | CST (°C) |
|---|---|---|---|---|
| 1,2-Ethanediol (ethylene glycol) | Fisher[a] (certified No. E–178, Baker (reagent, No.9300), Fluka[c] (puriss., p.a., No. A 50297) | 1.4316 | 1.112 | 188 with Benzene (Baker,[b] thiophene free, reagent, No. 9154) |
| 1,3-Butanediol.... | MC&B[d] (No. BX 1640) (dist.) Fluka (puriss., No. A 51581 | 1.4402 | 1.006 | 78 with Benzene |
| Nitromethane..... | Fisher (certified No. N–98), MC&B (spectroquality No. NX 613) | 1.3820 | 1.134 | 151.5 with Hexadecane (Lachat,[e] No. A 705 > 99.5% by GLC) |
| Acetonitrile ..... | Baker (reagent, No. 9011), MC&B (chromato-quality No. AX 148), Fluka (No. A 50057) | 1.3445 | 0.780 | 144 with Hexadecane |
| Acetic anhydride | Baker (reagent, No. 0018), Fluka (No. A 53856) | 1.3910 | 1.076 | 125 with Hexadecane |

[a] Fairlawn, N.J.; [b] Phillipsburg, N.J.; [c] Switzerland; [d] Norwood, Ohio; [e] Chicago, Ill.

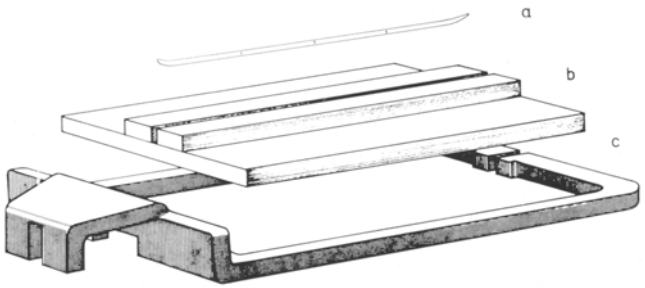


FIG. 2. a) Sealed capillary, b) adapted microscope slide, c) shifting device.

duced into the capillary by dipping it consecutively into both liquids. Each liquid may be drawn up to a height of about 7–10 mm. The ratio of the volumes is not important because only a small volume on either side of the interface is involved in the mixing process (16). The capillary is then sealed with a microburner at both ends, the total length of the sealed capillary being about 30–35 mm. It should contain no more than about half its volume of liquids to allow room for expansion. Care has to be taken in sealing the capillary to avoid the formation of glass clots at either end. The sealed ends should be slightly bent to the same side, as shown in Figure 2, in order to secure proper contact between slide and capillary when the latter is inserted into the channel. Samples which are solid at room temp can be melted on a glass slide over a microburner.

Figure 3 shows the step-by-step procedure of sampling a substance which is solid at room temp. Its melt is drawn up into the capillary which already contains the liquid test substance (*a*). Because the sample usually solidifies immediately after it has ascended, thus preventing proper sealing, it is liquified again in the capillary through contact with a hot glass strip (*b*). The capillary is then sealed at the other end by drawing off its remaining part over the microburner in such a way that the end is slightly bent upward (*c*). If sample and test substance in the capillary are separated by air, they are brought into contact by centrifugation. Before centrifuging, solids may be liquified again by inserting the sealed capillary into a centrifuge glass which contains water and which is then heated just above the melting point of the sample. Centrifuging is also employed to separate two liquids which form several segments in the capillary. This is necessary because a number of interfaces migrating during the heating process could prevent exact observation of the CST. When working with substances that may react with each other, the sealed capillary is inserted in such a way that the more dense liquid is toward the bottom of the centrifuge glass to minimize contact while centrifuging.

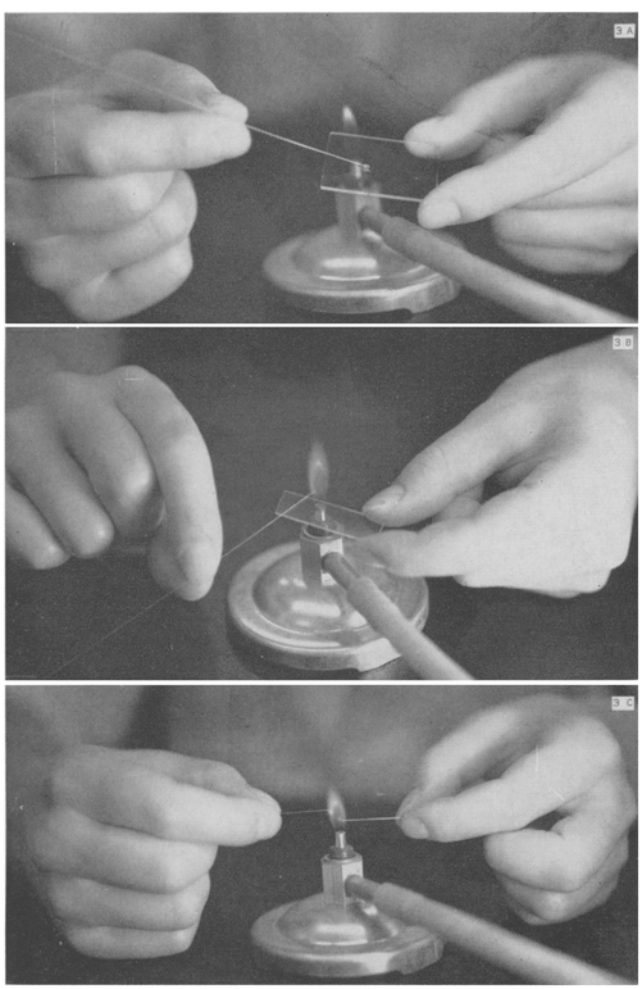

FIG. 3. Sampling of solid compounds: *a*, melting the sample and drawing it up into the capillary; *b*, sealing the capillary at one end; *c*, sealing the capillary at the other end.

The capillary tubing is inserted into the channel of the slide and heated on the micro heating stage. The interface between the two liquid phases is observed under the microscope. A slow movement of the interface may be encountered during the heating process as the two components begin to dissolve in each other. The meniscus in most cases remains clearly visible and it is kept in the field of view by using the shifting device.

The three microphotographs of Figure 4 illustrate the disappearance of the interface as the CST is reached. The clearly visible meniscus (*a*) disappears at the CST with a characteristic movement. In many cases it separates into two parts moving into either one of the two liquids (*b*). Above the CST one single liquid phase is formed (*c*).

Upon cooling the interface reappears at the same temp and at the same place in the capillary. Generally, the temp readings obtained were reproducible within ± 0.5C, i.e., the deviations did not exceed the experimental error of the equipment used. Some difficulty in observing the meniscus may occur when the refractive indices of both compounds become identical, making the interface invisible. This is observed, for example, with the pair methyl arachidate/1,3-butanediol at temps below the CST. No characteristic movement of the meniscus is observed when it disappears over a certain temp range, but the interface becomes visible again upon further heating. If identity of refractive indices occurs near the CST, exact temp readings are difficult. Adding a small amt of dye to the test substance may permit visualizing the disappearing of the interface but may also influence the CST. No change in the temp readings with methyl esters was observed if 0.5% w/v of methylene blue was added to the 1,3-butanediol used as the test substance.

Repeating the heating process with the same sample should yield identical temp readings at the CST; if not, decomposition or reaction between the compounds may be assumed. The amts of "impurities" formed by reactions between sample and test substance, e.g., fatty acid-ethylene glycol, can be retarded if the capillary is placed on the preheated stage at a temp just below the expected CST in order to shorten the heating process. A first test gives, in this case, the

approximate CST. After the determination the sample may be recovered from the capillary for further use and analysis.

Measurements of CST-values below room temp are possible if cooling equipment is employed. In this case the two liquids must be first separated into two phases by centrifuging the capillary at a temp below their CST. Determinations of CST-values above 200C are less useful and less easily measured, because of possible decomposition of the sample or the test substance.

#### Determination of Refractive Indices

We have found that compounds exhibiting identical critical solution temps can often be distinguished and characterized by determining the temps at which their refractive indices equal the refractive indices of standard glass powders (45).

The method described by Kofler (34,40) is suitable for determining refractive indices of less than 1 mg of a liquid or a melt under the microscope. A set of glass powders and minerals of known refractive indices is available. Sufficiently uniform and monochromatic illumination is provided by a red glass filter and a 100-watt lamp bulb. (Materials available from Reichert and Thomas.)

A few particles of the selected standard powder are added to a microscopic preparation of the sample on a glass slide. The refractive index of the glass particles must be slightly higher than that of the melt of the sample. The slide is then placed upon the heating stage and the temp raised above the melting point of the sample. When the refractive index of the particles differs from that of the melt, the particles are clearly visible, sharply outlined against the surrounding liquid by a black line. Upon raising the microscope's drawtube, the Becke line, i.e., a bright line moving from the substance of lower refractive index to the substance of higher refractive index, appears.

At rising temps the refractive indices of all liquids decrease according to their temp coefficients, whereas the refractive index of the standard glass particles is not appreciably affected (34). The temp range, at which neither glass particle nor Becke line is visible, is recorded. Upon further heating the Becke line can be seen again, moving in the opposite direction, as the refractive index of the glass particles becomes higher than that of the sample.

#### Analysis and Preparation of Lipids

Most substances were obtained from The Hormel Institute, Austin, Minnesota; Lachat Chemical Co., Chicago 43, Ill.; and Applied Science Laboratories, State College, Pennsylvania. Some diglycerides were gifts from Dr. B. J. F. Hudson, Unilever Research Laboratory, The Frythe, Welwyn, England. Di- and triacid triglycerides were obtained from Dr. F. H. Mattson, The Proctor and Gamble Co., Cincinnati, Ohio. Cholesteryl esters (36) were supplied by Dr. V. Mahadevan, The Hormel Institute, Austin, Minnesota. α-Glyceryl ethers were prepared as described recently (4).

Each preparation was checked for purity by adsorption–TLC on Silica Gel G (39). In special cases, reversed-phase partition chromatography on siliconized Whatman No. 1 paper (38), and gas-liquid chromatography (8,29) in a Beckman GC–2 instrument, were employed.

All samples contaminated with other classes of compounds were purified by adsorption chromatography on layers of Silica Gel G, 0.25, 0.5, or 1.0 mm thick. As a rule, 10–20 mg of a preparation was fractionated on a plate, 20 × 20 cm. In preparative thin-layer chromatography (TLC), the desired major fraction was generally visible without the use of an indicator. It was scraped off the glass plate with a razor blade, eluted from the adsorbent by repeated slurrying with anhydrous peroxide-free diethyl ether, and filtered through a sintered glass funnel. After evaporation of the solvent *in vacuo*, the purity of the recovered material in regard to other classes of compounds was verified by adsorption–TLC. Some lipids contaminated with compounds of the same homologous series were purified by preparative gas-liquid chromatography (GLC) in a Beckman GC–2 instrument using columns of acylated cyclodextrins as stationary phases (43). It was found useful to flash distill in vacuo, wherever feasible, the material recovered after TLC and/or GLC.

TABLE II

Suitable Test Substances for Characterizing Compounds of Various Lipid Classes by Their Critical Solution Temperatures

| Homologous series | Test substances | | |
|---|---|---|---|
| | Nitromethane | Acetonitrile | 1,3-Butanediol |
| Hydrocarbons | +[a] | + | Immiscible |
| Bromides | + | + | Immiscible |
| Prim. alcohols | + | + | Miscible |
| Sec. alcohols | + | + | Miscible |
| Methyl ketones | + (1)[b] | + (1) | + |
| Fatty acids | + | + (1) | Miscible |
| Esters | | | |
| (Me-, Eth-, But-) | + (1) | + (1) | + |
| Cetyl esters | + | + | Immiscible |
| Cholesteryl esters | + | Immiscible | Immiscible |
| α-Glyceryl ethers | + | + | Miscible |
| α-Monoglycerides | + (1) | Miscible | Miscible |
| α,α′-Diglycerides | + (1) | + (1) | + |
| Triglycerides | + | + | Immiscible |

[a] CST-values can be expected within the experimental temp range.
[b] Long-chain compounds only may exhibit CST-values.

## Applications

#### Critical Solution Temperatures of Pure Lipids

The compounds of the different lipid classes investigated exhibit widely different solubility characteristics. Therefore, a variety of test substances is required for the CST determinations of those compounds. Table II facilitates selecting a suitable test substance for testing compounds of chain lengths from $C_8$ to $C_{22}$.

Ethylene glycol was not included in the table, since it was suitable as a test substance for fatty acids only (44). With alcohols, using this test substance, no accurate temp readings were obtained, monoglycerides were miscible. CST–values with all other lipid classes indicated in Table II were above the experimental temp range.

Acetic anhydride is a suitable test substance for triglycerides and hydrocarbons; however, it reacts

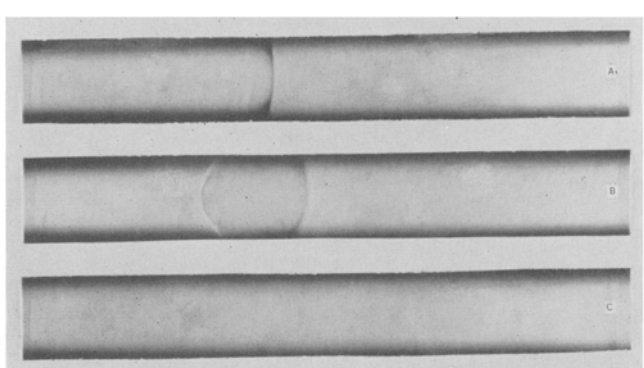

FIG. 4. Microphotographs of the capillary containing sample and test substance: *a*, interface below CST; *b*, interface at the CST; *c*, liquid system above CST.

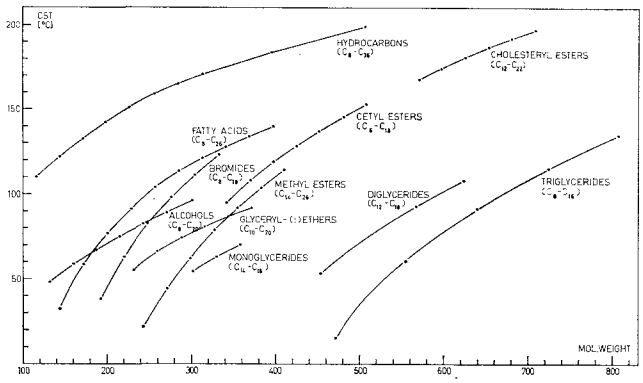


FIG. 5. Critical solution temps of various homologous series of saturated lipids plotted against their mol wts. Carbon numbers indicate the lengths of straight aliphatic chains. (See Tables III, IV and VII).

with hydroxyl groups and is miscible with acids and esters. Of the five liquids employed as test substances, nitromethane has the widest application. Acetonitrile can be used similarly for various lipid classes.

**Homologous Series**

*Saturated Compounds.* A given compound will exhibit widely different CST-values, depending on the identity and nature of the test substance. Thus, for example, methyl caprylate has CST-values of 60.5C with 1,2-propanediol, 69C with 1,3-butanediol, 116.5C with 1,5-pentanediol, 132C with 1,4-butanediol, and 197C with ethanediol. For a relatively wide range of lipid materials and closely related compounds that we have examined, nitromethane proved to be a particularly useful test substance. CST-values of the members of several homologous series were determined with nitromethane. These CST-values are plotted against the mol wts of these compounds in Fig. 5.

TABLE III
Critical Solution Temperatures of some Saturated Lipids with Nitromethane and Acetonitrile (see Tables IV and VII also)

| | CST (°C) | | |
|---|---|---|---|
| | Nitromethane | Acetonitrile | Difference |
| *Hydrocarbons* | | | |
| n-Octane | 110.5 | 92.5 | 18 |
| n-Decane | 122.5 | 107.5 | 15 |
| n-Dodecane | 132.5 | 121.5 | 11 |
| n-Tetradecane | 142 | 133.5 | 8.5 |
| n-Hexadecane | 151 | 144 | 7 |
| n-Octadecane | 159 | 155 | 5 |
| *Bromides* | | | |
| n-Octyl bromide | 38 | 17.5 | 20.5 |
| n-Decyl bromide | 63 | 45.5 | 17.5 |
| n-Dodecyl bromide | 82.5 | 69 | 13.5 |
| n-Tetradecyl bromide | 98 | 87.5 | 10.5 |
| n-Hexadecyl bromide | 111 | 104.5 | 6.5 |
| n-Octadecyl bromide | 123.5 | 120 | 3.5 |
| *Alcohols* (46) | | | |
| n-Octanol | 46.5 | —[b] | |
| n-Decanol-1 | 57 | 23 | 34 |
| n-Dodecanol-1 | 66 | 34.5 | 31.5 |
| n-Tetradecanol-1 | 74 | 445 | 29.5 |
| n-Hexadecanol-1 | 81.5 | 54.5 | 27 |
| n-Octadecanol-1 | 89 | 64 | 25 |
| *Fatty Acids* (44) | | | |
| Myristic acid | 91.5 | 53.5 | 38 |
| Palmitic acid | 104 | 68.5 | 35.5 |
| Stearic acid | 113.5 | 81.5 | 32 |
| Arachidic acid | 121 | 91.5 | 29.5 |
| Behenic acid | 128 | 101 | 27 |
| Lignoceric acid | 134 | 109 | 25 |
| *Methyl esters* (44) | | | |
| Methyl palmitate | 44 | 30 | 14 |
| Methyl stearate | 62 | 51 | 11 |
| Methyl arachidate | 78.5 | 71 | 7.5 |
| Methyl behenate | 91.5 | 88 | 3.5 |
| *Cetyl esters* (see Table IV) | | | |
| *Cholesteryl esters* | | | |
| Cholesteryl laurate | 168 | 201 | 33 |
| Cholesteryl myristate | 175 | 212 | 37 |
| Cholesteryl palmitate | 181.5 | —[a] | |
| Cholesteryl stearate | 187 | — | |
| Cholesteryl arachidate | 192 | — | |
| Cholesteryl behenate | 197.5 | — | |
| *α-Glyceryl ethers* (4) | | | |
| n-Decyl | 55 | 25.5 | 29.5 |
| n-Dodecyl | 66.5 | 38 | 28.5 |
| n-Tetradecyl | 74 | 48 | 26 |
| n-Hexadecyl | 81 | 57 | 24 |
| n-Octadecyl | 87.5 | 65 | 22.5 |
| n-Eicosyl | 93 | 72 | 21 |
| *α-Monoglycerides* | | | |
| Monolaurin | 42 | —[b] | |
| Monomyristin | 54 | — | |
| Monopalmitin | 63 | — | |
| Monostearin | 70 | — | |
| *α,α'-Diglycerides* | | | |
| Dilaurin | 53.5 | —[b] | |
| Dimyristin | 73 | 60 | 13 |
| Dipalmitin | 92 | 82 | 10 |
| Distearin | 108 | 103 | 5 |
| *Triglycerides* (see Table VII) | | | |

[a] Above experimental temp range.
[b] Below experimental temp range or below m.p.

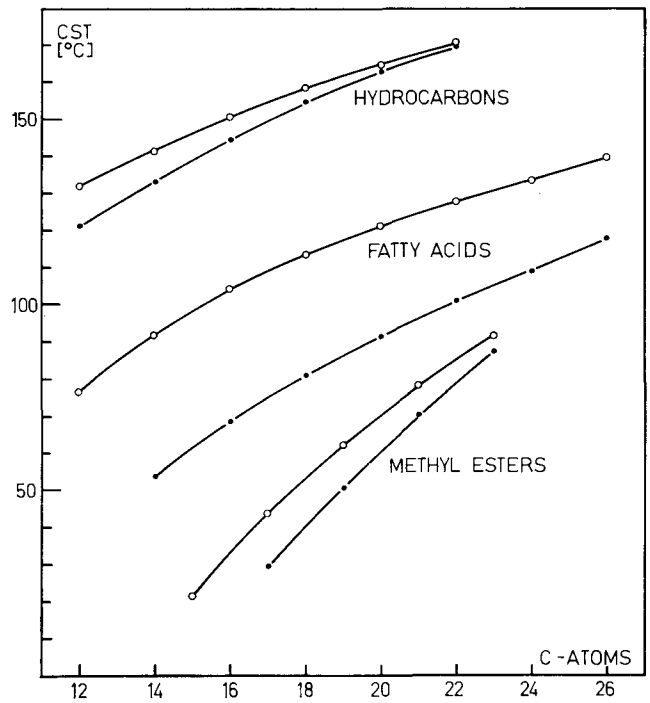


FIG. 6. Critical solution temps of hydrocarbons, fatty acids and methyl esters with nitromethane (o) and acetonitrile (.) (see Table III).

It is evident from Figure 5 that the type of functional group (s) per molecule has a significant influence on the CST. The position of a functional group is also a contributing factor. This is demonstrated by the differences in the CST-values of methyl esters and cetyl esters of identical mol wts. The influence of the position of a functional group was studied in more detail with a series of isomeric alkyl esters and dialkyl ethers (46). In all homologous series the CST-values increase with increasing mol wts of the compounds. The shape of a curve, which is an expression of the differences between the CST-values within a certain homologous series, is characteristic for each class of compounds. The series of alcohols, for example, yields CST-values whose curve is distinctly different from all other series of compounds having similar mol wts. This is probably due to the pronounced association of the free hydroxyl groups in the alcohols.

It is to be observed that the CST values of compounds with an odd number of carbon atoms, for example, fatty acids with 11,13,15,17 and 19 carbon atoms, fall on the same curve as their even-numbered homologues (44). This is quite different from what is observed in melting point curves and makes it possible to predict CST-values for all members in a given homologous series, whether the carbon numbers be odd or even. This observation was found to be true for all homologous series and for all test substances examined.

The comparative use of different test substances

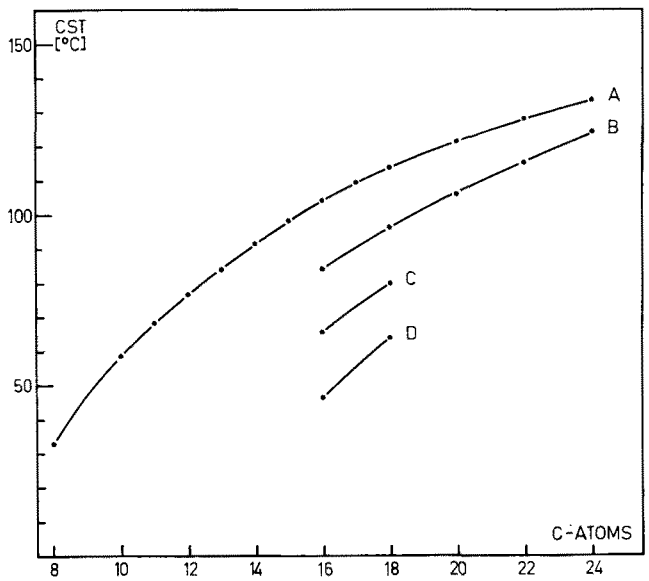


FIG. 7. Critical solution temps of saturated (*A*), mono- (*B*), di- (*C*) and tri- (*D*) unsaturated fatty acids with nitromethane (44).

yields further information about the identity of a compound. Complete miscibility or immiscibility over the whole experimental temp range, or even reaction with a certain test substance, characterize to some extent the type of compound being analyzed. The application of two test substances of similar solubility characteristics, for example, nitromethane and acetonitrile can provide additional information. This is demonstrated by Table III and by Figure 6.

The CST-values of hydrocarbons, fatty acids and methyl esters, each with nitromethane and with acetonitrile, are listed in Table III, and are plotted against the total number of carbon atoms in Figure 6.

For the compounds of the three homologous series in Figure 6, as in others, the CST-values with acetonitrile are lower than the corresponding values with nitromethane. The values for both of these two test substances rise with increasing chain lengths of the compounds, but at a different rate. It was observed that the differences between the CST-values with acetonitrile and nitromethane decrease in either of the three homologous series with increasing chain length, until the values with acetonitrile become higher than those with nitromethane. This is demonstrated in Table IV, with the example of cetyl esters.

Besides the chain length (mol wt), the difference between the CST of a compound with nitromethane and acetonitrile depends mainly upon its functional group. Thus, the differences between the CST-values with nitromethane and acetonitrile are different for $C_{16}$-hydrocarbon, $C_{16}$-acid, and $C_{16}$-methyl ester (see Table III and Figure 6). Similar relations were recognized in the homologous series of alcohols and bromides.

*Unsaturated Compounds.* Lipids containing one or more double bonds can be characterized with the same accuracy as saturated compounds. With a given test substance, the CST-values of a series of monounsaturated compounds are lower than those of the corresponding saturated substances. Increasing degree of unsaturation per molecule effects a further decrease of the CST. This rule is demonstrated in Figure 7 for saturated and *cis*-unsaturated fatty acids with nitromethane as test substance; it applies to vinylogous series of all chain lengths studied (44).

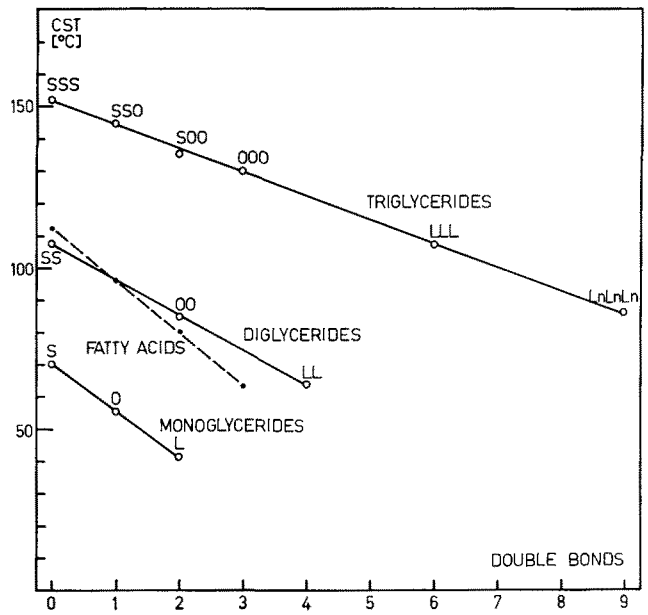


FIG. 8. Critical solution temperatures of mono-, di- and triglycerides and free fatty acids (44) with nitromethane. Constituent fatty acids are: S = stearic acid, O = oleic acid, L = linoleic acid, Ln = linolenic acid.

Similar relations exist in other lipid classes such as mono-, di- and triglycerides (see Figs. 8 and 9). In many cases, the effects of double bonds on the CST of a compound are additive. For example, the CST-values of stearic, oleic, linoleic and linolenic acids with nitromethane are 113.5C, 96.5C, 80C and 64C (44) (see also Fig. 8). The effect of double bonds on the CST generally decreases with increasing mol wts of the compounds. In Figure 8, the CST-values of saturated and unsaturated $C_{18}$-acids, their mono-, di- and triglycerides are plotted against the total number of double bonds per molecule.

Positional isomers of *cis*-unsaturated lipids, such as the methyl esters of octadecenoic acids with the double bonds in 6,9,11 or 12 position, cannot be distinguished by their CST-values with the test substances used in the present study. *Trans*-unsaturated compounds exhibit higher CST-values, with a given test substance, than their *cis*-unsaturated isomers (44,45). Methyl oleate and methyl elaidate, for example, have CST-values with nitromethane, of 41C and 46C, respectively (44).

**Isomeric Alkyl Esters and Isomeric Dialkyl Ethers**

The position of the functional groups in long-chain compounds exerts a marked effect upon the critical solution temp. This can be demonstrated with a series of isomeric alkyl esters (46). As an example, the CST-values with nitromethane were determined for isomeric alkyl esters having a total carbon number of 18 (mol wt: 284.5). Several isomeric dialkyl ethers

TABLE IV

Critical Solution Temperatures of Cetyl Esters with Nitromethane and Acetonitrile

| C-Atoms | Cetyl ester | CST (°C) | | |
|---|---|---|---|---|
| | | Nitromethane | Acetonitrile | Difference |
| 18 | acetate | 47.5 | 36 | 12.5 |
| 20 | butyrate | 79 | 68 | 11 |
| 22 | caproate | 95 | 89 | 6 |
| 24 | caprylate | 108 | 104 | 4 |
| 26 | caprate | 119 | 118 | 1 |
| 28 | laurate | 128 | 130 | 2 |
| 30 | myristate | 136.5 | 140.5 | 4 |
| 32 | palmitate | 145 | 150.5 | 5.5 |
| 34 | stearate | 152.5 | 159.5 | 7 |

TABLE V

Critical Solution Temperatures of Isomeric Esters (Mol wt–284.5) and Ethers (Mol wt–270.7) with Nitromethane (46)

| C-Atoms | Ester | CST (°C) | Ether | CST (°C) |
|---|---|---|---|---|
| $C_1$–$C_{17}$ | Methylmargarate | 53.5 | Methyl-heptadecyl | 105 |
| $C_2$–$C_{16}$ | Ethylpalmitate | 59.5 | Ethyl-hexadecyl | 112.5 |
| $C_4$–$C_{14}$ | Butylmyristate | 65 | Butyl-tetradecyl | 119 |
| $C_6$–$C_{12}$ | Hexyllaurate | 67.5 | Hexyl-dodecyl | 120.5 |
| $C_8$–$C_{10}$ | Octylcaprate | 68 | Octyl-decyl | 120.5 |
| $C_{10}$–$C_8$ | Decylcaprylate | 68 | | |
| $C_{12}$–$C_6$ | Dodecylcaproate | 66.5 | | |
| $C_{14}$–$C_4$ | Tetradecylbutyrate | 63.5 | | |
| $C_{16}$–$C_2$ | Hexadecylacetate | 47.5 | | |

with a total number of 18 carbon atoms (mol wt: 270.5) were also tested with nitromethane. Table V shows that similar influences on the critical solution temps are exerted by different positions of the ether bonds.

### Triglycerides

Determinations of CST-values are especially valuable for characterizing triglycerides and other compounds which occur in different polymorphic forms. The practical significance of this may be demonstrated by Table VI. Their CST-values with nitromethane distinctly characterize the triglycerides listed in Table VI, whereas the melting points of the polymorphic forms are hardly suitable for distinguishing between these substances.

The CST-values with nitromethane of various saturated and unsaturated triglycerides are plotted against the mol wts of these compounds in Figure 9.

As in other homologous series the CST-values of triglycerides increase with increasing mol wts and decrease with increasing number of double bonds per molecule. The triglycerides of *cis* and *trans*-isomeric fatty acids can be distinguished by their CST-values (Table VII). Isomeric diacid and triacid triglycerides, such as 1-stearo-2,3-diolein and 2-stearo-1,3-diolein, or 1-palmito-3-stearo-2-olein and 1-palmito-2-stearo-3-olein have identical CST-values.

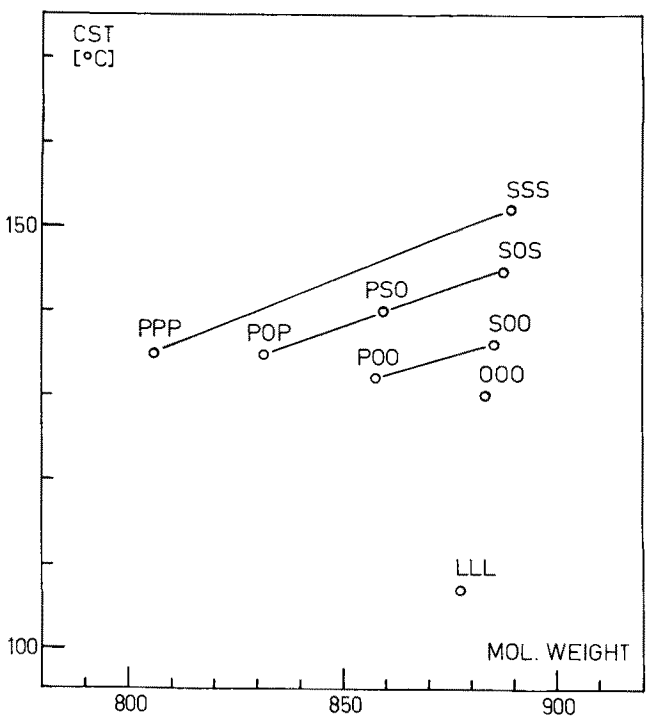


FIG. 9. Critical solution temperatures of mono-, di- and triacid triglycerides with nitromethane (45). Constituent fatty acids are: P = palmitic acid, S = stearic acid, O = oleic acid, L = linoleic acid (see Table VII).

TABLE VI

Melting Points (37) and Critical Solution Temperatures (45) of Triglycerides

| Triglyceride | Vitreous | α | β′ | β | CST Nitromethane |
|---|---|---|---|---|---|
| Trilaurin | 15.0 | 35.0 | — | 46.4 | 91.5 |
| Trimyristin | 33.0 | 46.5 | 54.5 | 57.0 | 115.5 |
| Tripalmitin | 45.0 | 56.0 | 63.5 | 65.5 | 135.0 |
| Tristearin | 54.5 | 65.0 | 70.0 | 72.0 | 152.0 |

It is evident from Figure 9 and Table VII that many saturated and unsaturated triglycerides yield identical or almost identical CST-values with a certain test substance. The identification of triglycerides having identical CST-values is often possible by a determination of their refractive indices. The temp ranges at which refractive indices of the melted triglycerides equal those of standard glass powders were determined according to the procedure described above. An increasing number of double bonds per molecule increases the refractive index of a triglyceride. The following example may demonstrate the advantage of combining determinations of CST-values with determinations of temps of which certain refractive indices are attained: tripalmitin and 1-stearo-2,3-diolein yield identical CST-values with nitromethane (135C). The melt of tripalmitin reaches a refractive index of 1.4339 at 91–93C, whereas 1-stearo-2,3-diolein reaches the same refractive index at 110–113C. This pronounced difference permits a means of distinguishing between the two triglycerides (see Table VII).

### Critical Solution Temperatures of Mixtures

Complex mixtures of compounds can yield accurate and reproducible critical solution temps with a suitable test substance, providing that none of the components of the mixture reacts with the test substance or decomposes during heating. This fact has been utilized by various authors to characterize mixtures or to determine quantitatively one of their major components (16,21). In the lipid field, besides the initially mentioned techniques, studies with aniline as the test substance have been reported (33). Relations between the "aniline points" of various fats and oils and their other characteristics such as iodine number, saponification number, percentage of free fatty acids or peroxides and their contents of hydroxyl groups, have been stated by these authors.

In many cases the micromethod described here can

TABLE VII

Critical Solution Temperatures of Triglycerides and Temperatures at which Arbitrarily Selected Refractive Indices are Attained (45)

| Triglyceride | CST (°C) | | | T (°C) | |
|---|---|---|---|---|---|
| | Nitro-methane | Aceto-nitrile | Acetic anh. | 1.4584 | 1.4339 |
| Tricaprylin | (15) | — | — | — | 55–57 |
| Tricaprin | 60.0 | 53 | 34 | — | 69–71 |
| Trilaurin | 91.5 | 91.5 | 68 | — | 78–81 |
| Trimyristin | 115.5 | 120 | 93.5 | — | 86–88 |
| Tripalmitin | 135 | 145 | 116 | — | 91–93 |
| Tristearin | 152 | 170 | 135 | — | 95–98 |
| Triarachidin | 167.5 | 189 | 150.5 | — | 99–101 |
| Tripalmitolein | 110.5 | 114.5 | 88.5 | 44–45 | 116–118 |
| Triolein | 130 | 140.5 | 110 | 47–48 | 120–122 |
| Tripetroselinin | 132 | 142 | 111.5 | 46–48 | 120–122 |
| Trielaidin | 133.5 | 145 | 113.5 | 45–46 | 117–119 |
| Tri-ll-eicosenein | 145 | 160.5 | 126 | 48–50 | 122–123 |
| Trierucin | 160 | 182 | 141.5 | 49–50 | 123–125 |
| Trilinolein | 107 | 113.5 | 85.5 | 73–75 | 149–151 |
| Trilinolenin | 86 | 88 | 63.5 | 101–102 | — |
| 1,3-Dipalmito-2-olein | 135 | 145.5 | 115 | — | 102–103 |
| 1-Palmito-2,3-diolein | 132 | 142 | 111.5 | 38–39 | 110–112 |
| 2-Palmito-1,3-diolein | 132 | 142 | 111.5 | 38–39 | 110–112 |
| 1,3-Distearo-2-olein | 144.5 | 158 | 124 | — | 106–108 |
| 1-Stearo-2,3-diolein | 135 | 147.5 | 115 | 39–40 | 110–113 |
| 2-Stearo-1,3-diolein | 135.5 | 147.5 | 116 | 39–40 | 110–113 |
| 1-Palmito-3-stearo-2-olein | 140 | 152 | 120 | — | 102–104 |
| 1-Palmito-2-stearo-3-olein | 140 | 152 | 120 | — | 102–104 |

TABLE VIII

Critical Solution Temperatures of Mixtures of Two Homologous Compounds

| % Methyl palmitate in Methyl myristate | CST (°C) Nitromethane | CST (°C) 1,3-Butanediol |
|---|---|---|
| 0.0 | 21.5 | 131.5 |
| 0.1 | 21.5 | 131.5 |
| 0.5 | 21.5 | 131.5 |
| 1.0 | 22 | 131.5 |
| 5.0 | 22.5 | 132.5 |
| 10.0 | 23.5 | 133.5 |
| 50.0 | 32.5 | 140 |

be applied successfully (17,19,31). However, widely different solubility properties of the components of a mixture to be tested can make CST determinations by the micromethod impossible. In these cases the interface between the two liquids collapses, then reappears immediately, and upon further heating sereval different menisci may be observed in the capillary. Each of these interfaces may disappear at a different temp, thus indicating that the original mixture of the sample tested disintegrates during the heating process into various fractions of different compositions. This was observed, for instance, with a sample containing equal amts of mono-, di- and tripalmitin tested with nitromethane.

*Synthetic Mixtures of Pure Compounds.* The effect of amt and nature of impurities on the CST of a compound is demonstrated by the following experiments. Various amts of methyl palmitate were added to methyl myristate and the CST-values of the mixtures were determined using nitromethane and 1,3-butanediol as the test substances.

Table VIII shows that the limit of detection of methyl palmitate in methyl myristate is approximately 3–5%.

Smaller amts of myristic acid can be recognized in methyl myristate. Using nitromethane as the test substance, we could detect about 2% free fatty acid in the ester, whereas with 1,3-butanediol the limit of detection was 1% (Table IX).

Mixtures of four or five methyl esters (GLC reference standards of The Hormel Institute) containing equal amts of each compound, by weight, were characterized by their CST with nitromethane and with 1,3-butanediol. CST-values measured and those calculated from the CST-values of the components (the arithmetic mean) are listed in Table X. The values found for the mixtures of esters agreed closely with those calculated from the CST-values of the components. It is evident from Table X that with methyl esters of fatty acids the CST-value of the mixture resembles the arithmetic mean of the CST-values of the components, if each is within the experimental temp range, or may be estimated within a certain homologous or vinylogous series (44). This fact can be used to predict CST-values of mixtures and to estimate the influence of contaminants upon the CST of a compound. Furthermore, the CST of a mixture can be used to substantiate the results of quantitative analyses.

TABLE IX

Critical Solution Temperatures of Mixtures of Two Compounds of Different Lipid Classes

| % Myristic acid in methyl myristate | CST (°C) Nitromethane | CST (°C) 1,3-Butanediol |
|---|---|---|
| 0.0 | 21.5 | 131.5 |
| 0.1 | 21.5 | 131.5 |
| 0.5 | 21.5 | 131 |
| 1.0 | 22 | 130 |
| 5.0 | 25 | 123.5 |
| 10.0 | 29 | 118 |
| 50.0 | 57–61[a] | —[b] |

[a] Not reproducible.
[b] Below m.p. of mixture.

TABLE X

Critical Solution Temperatures of Synthetic Mixtures of Methyl Esters

| Synthetic Mixture | CST (°C) Nitromethane | | CST (°C) 1,3-Butanediol | |
|---|---|---|---|---|
| | Found | Calc. | Found | Calc. |
| Ref. Mixt. No. 1 | 34 | (33.2) | 147.5 | 147.1 |
| Methyl palmitate | 44 | | 148.5 | |
| Methyl stearate | 62 | | 163 | |
| Methyl oleate | 41 | | 150 | |
| Methyl linoleate | 19 | | 141.5 | |
| Methyl linolenate | (0)[a] | | 132.5 | |
| Ref. Mixt. No. 5 | 70 | 69.0 | 168 | 168.5 |
| Methyl palmitate | 44 | | 148.5 | |
| Methyl stearate | 62 | | 163 | |
| Methyl arachidate | 78.5 | | 176 | |
| Methyl behenate | 91.5 | | 187 | |
| Ref. Mixt. No. 8 | 32.5 | (32.4) | 139 | 139.6 |
| Methyl tridecanoate | (9)[a] | | 122 | |
| Methyl myristate | 21.5 | | 131.5 | |
| Methyl pentadecanoate | 34 | | 140 | |
| Methyl palmitate | 44 | | 148.5 | |
| Methyl margarate | 53.5 | | 156 | |

[a] CST below experimental temp range, value estimated.

*Naturally Occurring Lipid Mixtures.* The CST-values of some fats and oils have been determined by conventional method (21,32) and also by the micromethod described here (17,19,31). In Table XI, some CST-values of several typical naturally occurring lipid mixtures with nitromethane and acetonitrile are listed, as determined by the method of Fischer. Another suitable test substance for characterizing complex lipid mixtures is ethylene glycol monoethyl ether (31). Some waxes, such as bees wax, which contain large proportions of mainly saturated long-chain alkyl esters of fatty acids and cholesteryl esters, did not exhibit reproducible CST-values with nitromethane and acetonitrile. The samples of lanolin, carnauba wax and some mineral oils disintegrated at high temps. Vegetable oils, which are rich in unsaturated triglycerides, show comparatively low CST-values with nitromethane and acetonitrile. The CST-values of hydrogenated fish oils are, of course, much higher than those of the genuine oils (cf Fig. 5 and Fig. 8). It is well known that the CST-values of triglyceride mixtures decrease with increasing iodine values of these mixtures (19,33), and it has been stated that CST-values of fats and oils can be calculated from the CST-values of their constituents (32). However, experimental proof of this postulate has become possible only recently, after efficient methods for the fractionation of lipid mixtures had been developed.

**Integration of the Technique with Other Methods**

Determinations of CST-values aid in assessing the composition of fractions isolated from natural mixtures or synthetic preparations by crystallization (4,

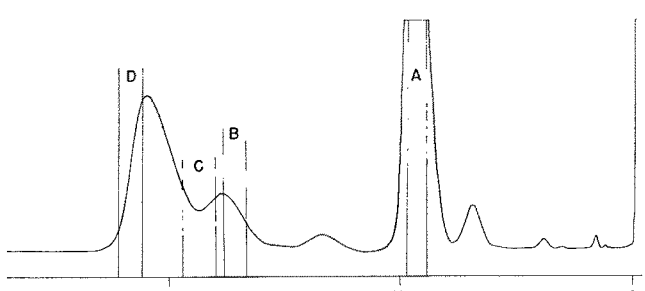


FIG. 10. Separation of methyl esters of fatty acids from Maratti (*Hydnocarpus Wightiana*) seed oil. A = methyl hydnocarpate, B = methyl gorlate, C = methyl gorlate + methyl chaulmoograte, D = methyl chaulmoograte. Beckman GC-2, 4' column of 15% Apiezon M on Gas Chrom P. (mesh size 80/100), 225C, Helium at 35 psi.

TABLE XI

Critical Solution Temperatures of Naturally Occurring Lipid Mixtures

| Lipid material | CST (°C) Nitromethane | CST (°C) Acetonitrile |
|---|---|---|
| Animal waxes | | |
| Bees wax | 142–145[a] | ~180[b] |
| Spermaceti | 141 | 146 |
| Vegetable oils | | |
| Olive oil | 128 | 140 |
| Safflower oil | 114 | 124 |
| Fish oils | | |
| Herring oil | 121 | 128 |
| Capelin oil | 135 | 148 |
| Hydrogenated fats | | |
| Hydrog. herring oil | 135 | 149.5 |
| Hydrog. capelin oil | 140 | 162 |

[a] Not reproducible.
[b] Reaction.

5), distillation (12), liquid-liquid countercurrent distribution and solid-liquid countercurrent distribution (12), and by chromatographic techniques (13,44).

The complexity of mixtures of substances migrating as one spot in adsorption–TLC, can be demonstrated easily by determining the CST-values of material contained in the leading part and in the tail end of the spot. Subfractions of mixtures yield different CST-values, whereas various parts of a spot containing an individual compound give identical CST-values. This is illustrated by the following examples: A mixture of methyl myristate and methyl stearate (A) containing equal amts of each compound was spotted on a 20 × 20 cm thin layer plate. The chromatogram was developed with a mixture of petroleum hydrocarbon-diethyl ether, 90:10. The "upper" and "lower" parts of the spots were scraped off separately, eluted from the adsorbent with anhydrous diethyl ether and, after evaporation of the solvent in vacuo, the CST-values with nitromethane and 1,3-butanediol were determined. The same procedure was carried out with the GLC reference mixtures No. 5 (B) and No. 8 (C). (Their composition is listed in Table X). Table XII indicates the CST-values of the subfractions with nitromethane and 1,3-butanediol.

Also, mixtures of triglycerides were fractionated by adsorption–TLC on Silica Gel G containing silver nitrate (41), the major triglyceride fractions were eluted from the adsorbent and after evaporation of the solvent in vacuo the various fractions were identified by detemining their CST-values and their refractive indices. Gas-chromatographic analyses of the constituent fatty acids in the form of their methyl esters confirmed the results of the thermo-analytical studies (13).

Compounds eluted from gas-chromatographic columns can be collected and their identities can be substantiated by determining their CST-values. Figure 10 shows a typical chromatogram of the methyl esters of the fatty acid mixture isolated from Maratti (*Hydnocarpus Wightiana*) oil.

Fractions A–D were eluted from the column. Their CST-values were determined with 1,3-butanediol and are listed in Table XIII.

Overlapping of compounds in GLC also can be demonstrated by CST determinations, as indicated by fraction C, containing about equal amts of methyl gorlate and methyl chaulmoograte. When using an

TABLE XII

Critical Solution Temperatures of TLC–Subfractions

| Mixture[a] | CST (°C) Nitromethane | | CST (°C) 1,3-Butanediol | |
|---|---|---|---|---|
| | Front end | Tail end | Front end | Tail end |
| A | 55 | 43 | 158 | 145.5 |
| B | 76 | 64 | 173 | 164 |
| C | 39 | 31 | 147 | 140 |

[a] See text.

TABLE XIII

Critical Solution Temperatures of GLC–Fractions

| Fraction[a] | Methyl ester of | CST (°C) 1,3-Butanediol |
|---|---|---|
| A | Hydnocarpic acid | 128 |
| B | Gorlic acid | 136 |
| C | Gorlic and chaulmoogric acids | 141.5 |
| D | Chaulmoogric acid | 145 |

[a] See Figure 10.

ethylene glycol succinate column (52), the methyl esters of oleic acid and hydnocarpic acids emerge together. Subfractions of the "oleate-hydnocarpate" peak indicated that the end of the peak was enriched in methyl oleate (CST with 1,3-butanediol, 130.5C).

## Summary and Discussion

The adaptation of a micromethod for the determination of upper critical solution temps has been described in detail. It has been demonstrated that closely related substances can be distinguished by their critical solution temps. As only milligram amts of sample are required, the technique can be generally applied to the characterization of organic substances which are either liquid at room temp or melt without decomposition.

Microdeterminations of critical solution temps proved to be valuable for substances which cannot be adequately characterized otherwise because they have a very low melting point or melt over a wide range. Compounds occurring in several polymorphic modification can be characterized by this method. For polymorphic compounds the melting points are uncertain and unreliable for characterizations. In contrast, each of these compounds yields one reproducible CST-value with any suitable test substance.

The use of two or more test substances in the CST determination facilitates the identification of a compound.

To distinguish between compounds which yield identical CST-values, the determination of the refractive indices as described by Kofler has been applied. For example, it has been found that saturated and unsaturated lipids having identical CST-values can be characterized by the refractive indices of their melts.

Saturated and unsaturated lipids of different homologous series have been characterized by their CST-values. It has been found that with straight chain aliphatic compounds, these values depend on mol wt, the type and position of the functional group(s) and the number and configuration of double bonds. In all homologous series studied, the CST-values with a given test substance increase with increasing mol wts of the compounds in a continuous manner. Unlike the melting points, no alternating behavior between the even and odd members of homologous series is observed. Increasing numbers of double bonds in the compounds tested reduce the CST-values in many cases proportionally, but their influence upon the CST decreases with increasing mol wts of the substances. Isomeric straight-chain and branched-chain lipids usually can be distinguished, and straight-chain compounds and their alicyclic isomers also exhibit different CST-values with a given test substance.

Critical solution temps may be used in combination with various separation methods to analyze fractions. As a rule, identity and purity of a substance are established if complementary fractionation procedures, such as thin-layer adsorption chromatography and gas-liquid partition chromatography have not yielded fractionation and if it exhibits an accurate CST.

The rapid and inexpensive laboratory procedure described is well established as an adjunct to other physical and chemical techniques for the characterization and identification of organic compounds. Systematic studies on homologous series, as summarized in the present report, may further the knowledge of the relationship between the chemical structures and the CST-values of organic substances. The CST-values determined so far may serve as a basis to predict with sufficient accuracy the critical solution temps of many more pure compounds of various lipid classes. This procedure may be helpful in confirming the structures of synthetic preparations and of materials isolated from the complex natural fats, oils and waxes. Moreover, CST-values of pure compounds may be used for estimating the CST-values of their naturally occurring mixtures. This would be a simple procedure for checking and substantiating the results of quantitative analyses.

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REFERENCES

1. Alexejew, W., Ann. physik. Chem. *28*, 305 (1886).
2. AOCS Official Methods Cb 4–35, American Oil Chemists' Society, 35 E. Wacker Drive, Chicago 1, Ill.
3. ASTM Method D611 and D1012, American Society for Testing Materials, 1916 Race Street, Philadelphia 3, Pa.
4. Baumann, W. J., and H. K. Mangold, J. Org. Chem. *29*, 3055 (1964).
5. Baumann, W. J., and H. K. Mangold, J. Org. Chem., in press.
6. Broughton, G., and D. C. Jones, Trans. Faraday Soc. *32*, 685 (1936).
7. Brown, C. W., Ind. Eng. Chem., Anal. Ed., *18*, 739 (1946).
8. Craig, B. M., and N. L. Murty, Can. J. Chem. *36*, 1297 (1958).
9. Crismer, L., Bull. Assoc. Belge de Chim. *9*, 71, 143 (1895).
10. Crismer, L., *Ibid.* *9*, 359 (1896).
11. Crismer, L., *Ibid.* *10*, 312 (1897).
12. Cubero, J. M., and H. K. Mangold, unpublished.
13. Cubero, J. M., and H. H. O. Schmid, unpublished.
14. Discussions Faraday Soc. *15*, 1953. The Equilibrium Properties of Solutions of Non-Electrolytes.
15. Fachini, S., and G. Dorta, Z. dtsch. öl–u. Fettind. *45*, 498 (1925).
16. Fischer, R. W., in Microchemical Techniques (N. D. Cheronis, ed.) Microchem. J. Symp. *2*, 977 (1962).
17. Fischer, R., and J. Horner, Mikrochim. Acta 1953, 386.
18. Fischer, R., and G. Karasek, Mikrochemie ver. Mikrochim. Acta *33*, 316 (1947).
19. Fischer, R., and T. Kartnig, Fette, Seifen, Anstrichmittel *60*, 904 (1958).
20. Fischer, R., and H. Moser, Erdöl Kohle *9*, 377 (1956).
21. Francis, A. W., "Critical Solution Temperatures," No. 31, Advances in Chemistry Series. American Chemical Society, Washington, D.C., 1961.
22. Francis, A. W., Ind. Eng. Chem. *45*, 2789 (1953).
23. Gölles, F., Microchim. Acta 1963, 376 and previous publications.
24. Hildebrand, J. H., and R. L. Scott, "The Solubility of Nonelectrolytes," American Chemical Society Monograph Series, 3rd ed., Reinhold Pub. Corp., New York, 1950.
25. Hoerr, C. W., and H. J. Harwood, J. Phys. Chem. *56*, 1068 (1952).
26. Hoerr, C. W., H. J. Harwood and A. W. Ralston, J. Org. Chem. *9*, 267 (1944).
27. Hoerr, C. W., and A. W. Ralston, *Ibid.* *9*, 329 (1944).
28. Hoerr, C. W., R. S. Sedgwick and A. W. Ralston, *Ibid.* *11*, 603 (1946).
29. Horning, E. C., E. A. Moscatelli and C. C. Sweeley, Chem. Ind. 1959, 751.
30. International Critical Tables, McGraw-Hill Book Co., New York, 1928.
31. Kartnig, T., Sci. Pharm. *29*, 187 (1961).
32. Kaufmann, H. P., "Analyse der Fette und Fettprodukte," Band I, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1958.
33. Kaufmann, H. P., and J. G. Thieme, Fette, Seifen, Anstrichmittel *58*, 585 (1956).
34. Kofler, L., and A. Kofler, "Thermo-Mikro-Methoden zur Kennzeichnung Organischer Stoffe und Stoffgemische," Universitäts-Verlag Wagner, Innsbruck, 1954.
35. Landolt-Börnstein, Physikalisch-Chemische Tabellen; Zahlenwerte und Fünktionen, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1923–1960.
36. Mahadevan, V., and W. O. Lundberg, J. Lipid Res. *3*, 106 (1962).
37. Malkin, T., The *Polymorphism* of Glycerides, in "Progress in the Chemistry of Fats and Other Lipids," R. T. Holman, W. O. Lundberg, and T. Malkin, eds., Vol. 2, Pergamon Press, New York, 1954, pp. 1–50.
38. Mangold, H. K., B. G. Lamp and H. Schlenk, J. Am. Chem. Soc. *77*, 6070 (1955).
39. Mangold, H. K., H. H. O. Schmid and E. Stahl, Thin-Layer Chromatography, in "Methods of Biochemical Analysis," Vol. 12, D. Glick, ed., Interscience, Publ., a division of John Wiley & Sons, Inc., New York, London, Sydney, 1964.
40. McCrone, W. C., "Fusion Methods in Chemical Microscopy," Interscience, Publ., Inc., New York, 1957.
41. Morris, L. J., J. Chromatog. *12*, 321 (1963).
42. Rothmund, V., Z. physik. Chem. *26*, 433 (1898).
43. Schlenk, H., J. L. Gellerman and D. M. Sand, Anal. Chem. *34*, 1529 (1962).
44. Schmid, H. H. O., H. K. Mangold and W. O. Lundberg, Microchem. J. *7*, 287 (1963).
45. Schmid, H. H. O., H. K. Mangold and W. O. Lundberg, *Ibid.* *7*, 297 (1963).
46. Schmid, H. H. O., H. K. Mangold and W. O. Lundberg, *Ibid.*, in press (1965).
47. Sedgwick, R. S., C. W. Hoerr and H. J. Harwood, J. Org. Chem. *17*, 327 (1952).
48. Seidell, A., "Solubilities of Organic Compounds," Vol. II, D. Van Nostrand Co., Inc., New York, 1941; Seidell, A., and W. F. Linke, "Solubilities of Inorganic and Organic Compounds, Supplementary Volume." D. Van Nostrand Co., Inc., New York, 1951.
49. Timmermans, J., "The Physico-Chemical Constants of Binary Systems in Concentrated Solutions," Vols. I–IV, Interscience, Publ., Inc., New York, 1959–1960.
50. Valenta, E., Dinglers Polytechn. J. *252*, 296 (1884).
51. Valenta, E., *Ibid.* *253*, 418 (1884).
52. Zeman, I., and J. Pokorny, J. Chromatog. *10*, 15 (1963).

# Lipid Analysis by Quantitative Thin-Layer Chromatography

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## Abstract

Techniques for the quantitative analysis of lipids using thin-layer chromatography (TLC) are reviewed. The general procedures are divided into two groups on the basis of whether or not the methods involve the recovery of substances from chromatoplates.

Recovery methods are elaborated under detection of spots, recovery of substances and quantification. Methods are described for the recovery of labile compounds from chromatoplates and for the determination of the structures of triglycerides and lecithins.

Methods for the direct quantitative analysis of spots on chromatoplates are reviewed. These include measurements of spot size, reflectance, absorbance of transmitted light, and fluorescence. Details of the photodensitometric method, particularly, spot visualization and instrumentation are described. The analysis of lipid classes using a combination of DEAE cellulose chromatography and TLC by the densitomery of charred spots is illustrated.

## Introduction

THE SPEED AND VERSATILITY of thin-layer chromatography (TLC) and its ability to resolve compounds with very minor differences in chemical structure, make it especially valuable as an analytical technique for the quantitative analysis of lipids. In addition to providing a method of analysis for many lipids which cannot be analyzed otherwise, it is also used to facilitate the analysis of many lipid compounds whose quantitative determination pose special problems. Methods for quantitative analysis by TLC may be divided into two broad groups, those which involve recovery of the separated compounds from the chromatoplate followed by an analysis using well-established analytical procedures, and methods based on a direct analysis of the spots on the chromatoplate. The more common techniques employed in quantitative