[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF CALIFORNIA]

ADSORPTION AND CHROMATOGRAPHY OF FATTY ACIDS ON CHARCOAL[1]

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The separation and analysis of mixtures of saturated and unsaturated fatty acids by displacement chromatography on charcoal has been studied rather carefully (1) and shown to have significant utility; however, the application of displacement development to mixtures of unknown compounds is relatively difficult. In particular, the separation of zones of desired components by use of carriers can be accomplished effectively only when the adsorbabilities of the desired components are known. Since development by elution has certain advantages for separation of mixtures of unknown identity, this method has been investigated by us for application to separation of the fatty acids from the tubercle bacillus. One such application of the method has been reported in an earlier paper (2). The present report is concerned with certain features of the scope and limitations of the method.

The separation of normal fatty acids in the C_{12} to C_{18} range by chromatography on charcoal has been studied by Cassidy[2] (3), who used elution development with petroleum ether. Difficulties with the method evolved at that time were the large volumes of solvent and very long periods of time required for development. The report of Hagdahl and Holman (4) that separation may be effectively accomplished in displacement chromatography with alcohol as solvent prompted investigation of this solvent for the present work. Among the advantages of alcohol are the possibility for direct titration of eluent for determination of acid content, and improvement of separation (4) by adding water to reduce solubility. The adsorbent used was Darco G-60, which was employed by the previous workers (3, 4).

In order to secure information as to the types of acids which may be separated by chromatography on charcoal with alcohol as solvent, static adsorption isotherms were determined on appropriately selected normal, branched-chain, and unsaturated acids. These isotherms are assembled in Fig. 1. Cassidy (5) has expressed some doubt as to the usefulness of adsorption isotherms on pure substances as a criterion of the separability of these substances from a mixture. He found the predictions from isotherms to be applicable to behavior in chromatography of mixtures when one charcoal was used, but inapplicable when two other charcoals were used. It may be of significance that the adsorption characteristics of the two latter charcoals indicated considerable polar character (pres-

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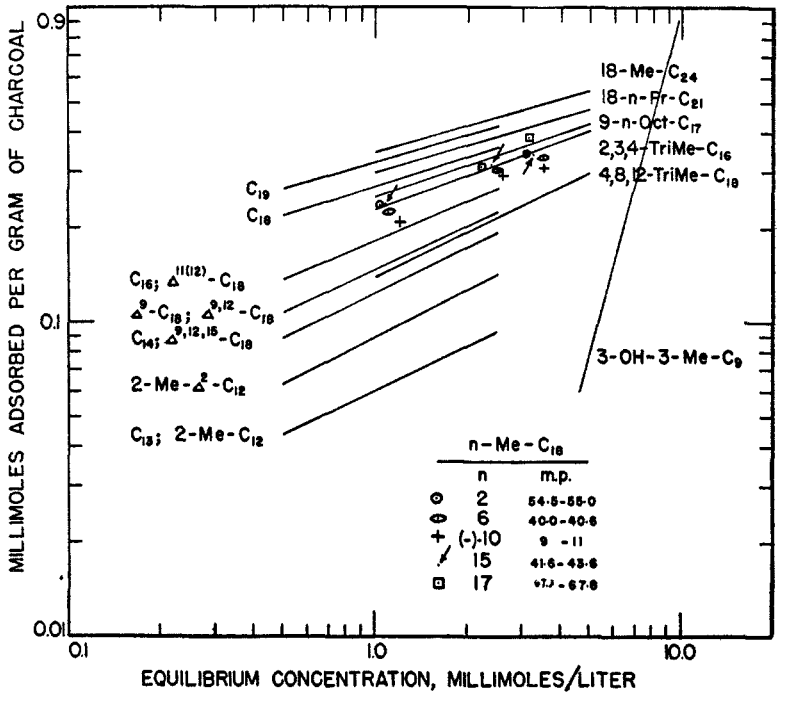

FIG. 1. STATIC ADSORPTION ISOTHERMS OF FATTY ACIDS ON DARCO G-60, WITH 95% ETHANOL AS SOLVENT.

ence of metallic oxides). This suggests an inhomogeneous adsorbing surface, which may have been responsible for the difference in adsorption of pure substances and mixtures of substances. All our experiments, thus far, with Darco G-60 indicate that the isotherms are rather good criteria of potential separability in chromatography. Some of these experiments are mentioned below. Also, several of the substances included in Fig. 1, or substances of comparable structures, have been subjected to separation by displacement chromatography by Holman and Williams (6), and their results are generally consistent with predictions to be made from the static adsorption isotherms shown in Fig. 1. Among these comparisons, the following are noteworthy:

(a) Non-conjugated unsaturation significantly lowers intensity of adsorption in relation to the saturated acid, but α,β-unsaturation increases adsorption.

(b) Oleic acid falls between myristic and palmitic acids.

(c) Oleic and linoleic acids have very similar adsorbabilities.

The last-mentioned comparison is subject to some quantitative uncertainty, in that Holman and Williams reported separation of oleic and linoleic acids to be difficult, but possible, whereas our isotherms are identical. The analysis used by these authors was based on selective attack by the enzyme, lipoxidase, on linoleic acid. They also stated, however, that no separate zones could be detected by a difference in refractive index in the interferometer of the Tiselius-Claesson ap-

paratus. In connection with separation of oleic acid or linoleic acid (in separate experiments) from myristic and palmitic acids, they stated that the two unsaturated acids did show zones of different refractive index, also that each of them lay between myristic and palmitic acids. These observations seem difficult to reconcile with the reported separation based on enzymatic analysis. In our experiments, using elution development, fourteen different attempts to separate the oleic and linoleic acids from olive oil, using various techniques and solvents, failed to accomplish the separation. There was a slight concentration of linoleic acid in the earlier fractions when elution was with 75% alcohol. As noted by Hagdahl and Holman (4), dilution of the alcohol and consequent reduction of solubility increases the separability of fatty acids.

It may be observed in Fig. 1 that branching of the chain lowers adsorbability, and a larger branch or several methyl branches lower adsorbability more than a single methyl branch. The position of a single branching methyl has only a small effect. The adsorption is slightly stronger when methyl is near the end of the chain, but the magnitude of this difference is probably too small to allow separation. Adsorbability of the monomethyloctadecanoic acids gives a certain correlation with melting point, but it may be noted that the 15-methyl isomer is out of line. When the difference caused by position of branching is magnified by presence of several branches it becomes relatively large; 2,3,4-trimethylhexadecanoic acid is adsorbed considerably more strongly than the higher molecular weight 4,8,12-trimethyloctadecanoic acid. The isotherm for 3-hydroxy-3-methylnonanoic acid is unusual in that the slope of the log log plot is greater than unity. This acid was eluted from a column much more rapidly than *n*-tridecanoic acid. The synthetic 11(12)-octadecenoic acid was adsorbed more strongly than oleic acid. This is presumed to be due not to the different position of the double bond but to the fact that oleic acid is *cis* and the synthetic acid would be expected to be mostly *trans*, since it was prepared by dehydrohalogenation (7). Behavior of *cis* and *trans* isomers of unsaturated acids in elution chromatography has not yet been examined. Holman (1) has reported the adsorbabilities too close together to permit separation by displacement elution.

The deductions to be reached from the data in Fig. 1 suggest that many types of mixtures may be separated especially effectively by a combination of fractional distillation and chromatographic adsorption. For example, distillation readily separates stearic and oleic acids from palmitic, and the former pair is readily separated by chromatography. Also, acids with large branches or several branching methyls may be chromatographically separated from the monomethyl acids or normal acids of the same molecular weight. Neither distillation nor chromatography of the acids appears able to separate isomeric monomethyl-substituted acids, or a mixture of oleic and linoleic acids. The latter pair has been separated by chromatography of the esters on silica gel (8).

Both the 11(12)-octadecenoic acid and palmitic acid could be separated readily from stearic acid in a column. Since analysis of mixtures of palmitic and stearic acids is so often desired in biological work, separation of this pair was examined rather carefully. This pair was also used to test the validity of static adsorption

TABLE I

CHROMATOGRAPHY OF PALMITIC AND STEARIC ACIDS WITH DIFFERENT QUANTITIES OF CHARCOAL

Per cent of theoretical[a] amount of charcoal.....	70	76	105
Per cent of palmitic acid eluted in pure condition[b].....	65	70	84

[a] For calculation of theoretical amount of charcoal from isotherms, refer to Experimental. [b] A mixture of equal weights of the two acids was chromatographed in a manner similar to that described for the run shown in Fig. 2 and Table II.

isotherms for estimation of the amount of adsorbent to be used for a given separation (9).

In Table I is shown the relative effectiveness of separation when various amounts of charcoal were used. These data give excellent support to the utility of isotherms for estimating the amount of charcoal required to accomplish a separation. Highly satisfactory separation was secured when there was used 5% more charcoal than that calculated from the isotherms. This procedure is recommended as very convenient and effective for separation of milligram quantities of these two acids. In Fig. 2 are plotted the data secured when elution was with

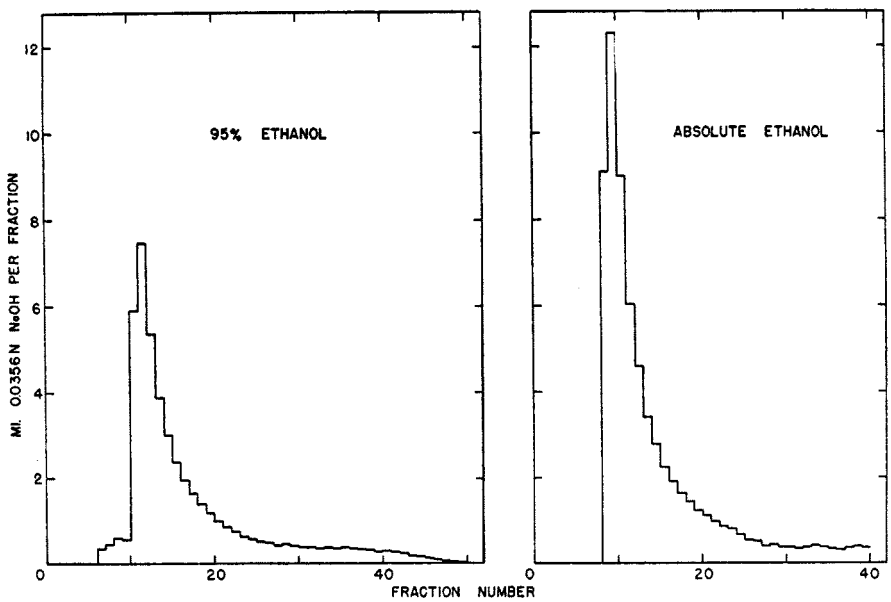

FIG. 2. CHROMATOGRAPHIC SEPARATION OF PALMITIC AND STEARIC ACIDS. The volume of each fraction is 30 ml. The titration of each fraction is corrected for charcoal acid by subtraction of 0.26 ml. of base for 95% ethanol and 0.10 ml. for absolute ethanol (*cf.* discussion of charcoal acid in Experimental, under description of the adsorbent). There was used 6.8 g. of Darco G-60 (105% of the amount calculated from the 95% ethanol isotherm) and 13.6 g. of Celite 521. Height to diameter ratio of the column of adsorbent was 2.6. There was applied to the column a solution of 400 mg. of each acid in 200 ml. of solvent.

TABLE II

SELECTED DATA ON FRACTIONS FROM CHROMATOGRAM SHOWN IN FIG. 2.

95% Ethanol			Absolute Ethanol		
Frac. No.	M.P., °C.[a]	Fatty acid eluted, % of total C_{18}	Frac. No.	M.P., °C.[a]	Fatty acid eluted, % of total C_{18}
6–9		"charcoal acid"[b]	8	59.4–61.7	
10–13	61.0–62.2		10	59.9–62.1	70
21	61.0–62.2	84	11	54.0–55.2	84
22–24	60.1–61.8	88	12	55–57	93
25–27	54–58		13		100
37		100	21, 22	61.6–66.3	
42–50	63.7–66.5		38	66.8–68.8	
53	65.0–67.2				
60–63	66.0–68.3				
68[c]	67.4–69.1				
69	67.5–69.2				

[a] For isolation of the fatty acid, the titrated eluent was concentrated, diluted with four volumes of water, acidified with sulfuric acid, and extracted with hexane. Acid left after removal of hexane was dried (not recrystallized), and the corrected m.p. was determined. [b] For a description of charcoal acid, refer to Experimental. [c] At Frac. 67, the eluent was changed to absolute ethanol.

95% alcohol or with absolute alcohol, and additional data are included in Table II. With 95% alcohol, 84% of the palmitic acid was eluted (Fracs. 10–21) in pure condition with a total of 630 ml. of eluent, and about 88% of the palmitic acid was eluted (to Frac. 24) before significant quantities of stearic acid appeared in the eluent. Pure stearic acid appeared after about two liters of 95% alcohol had been used as eluent. The stearic acid may be rapidly eluted with absolute alcohol followed by benzene. Shift to absolute alcohol at Frac. 25 would result in much faster elimination of palmitic acid with only a small additional loss in intermediate fractions. The data show the much poorer results obtained when initial elution is with absolute alcohol, a behavior consistent with that reported in displacement chromatography (4). These results may be due, in part at least, to the need for more charcoal when absolute ethanol is used. The specific factors involved in this different behavior have not yet been investigated further.

The basic investigation reported in this paper is being extended to separation of specific mixtures encountered in synthetic work and in isolation of fatty acids from natural sources.

EXPERIMENTAL

The adsorbent, Darco G-60. A satisfactory rate of flow was obtained in chromatography when Darco G-60 was mechanically mixed with two parts by weight of Celite 521. The Celite was found to give no observable adsorption of the fatty acids.

In latter stages of elution of the charcoal, when benzene was used as eluent, traces of the neutral "petroleum-like" material mentioned by Cassidy (3) were eluted. This could be separated from the acids by taking advantage of its neutral nature. Elution with alcohol, including absolute alcohol, did not displace this neutral material, but alcohol displaces an

acidic substance termed *"charcoal acid"* in this work. This solid material appears to be inorganic since ignition to red heat only darkens and partly volatilizes it. Its removal from fatty acids is simple since it is quite soluble in water and insoluble in hexane or benzene. It appears to have no effect on adsorption isotherms, and its removal from the charcoal is so difficult that removal from the fatty acids was chosen as the more convenient procedure.

Removal of charcoal acid from the charcoal with alkali is rapid, but removal of alkali from the charcoal is tedious. In chromatography of fatty acids with alcohol as eluent, a significant amount of charcoal acid is usually displaced ahead of the first fatty acid (refer to Fig. 2, 95% ethanol), then it is eluted at a fairly constant but slowly decreasing rate. This rate is equivalent to about 0.85 ml. of 0.0356 *N* base per 100 ml. of 95% ethanol eluent from 5–10 g. of charcoal. The charcoal acid is eluted more rapidly with 50% ethanol, less rapidly (0.35 ml. of 0.0356 *N* base per 100 ml.) with absolute ethanol. These values were used as correction factors in determining rate of elution of fatty acids by titration (*Cf.* Fig. 2).

The 100-g. sample of washed charcoal prepared as a check on adsorption characteristics was eluted with 12 l. of 1:1 ethanol, water. One gram of this material, on equilibration with 50 ml. of 95% ethanol, yielded charcoal acid equivalent to 0.06 ml. of 0.045 *N* base (unwashed charcoal, 0.26 ml. per g.). The adsorption isotherms and behavior in chromatography were indistinguishable from those of unwashed charcoal. It was concluded that complete removal of charcoal acid is impractical and that presence of charcoal acid is no disadvantage in chromatography. Previous workers (4, 6, 8) appear to have not recognized the presence of this substance on Darco G-60.

Static adsorption isotherms. Except for 17-methyloctadecanoic acid, which was in very short supply, three determinations were made for each isotherm. For stearic acid, numerous determinations were made in order to make sure that temperature variations ($20 \pm 3°$) and other factors were not introducing variations significant to the present work. In a typical procedure, each determination was with 1.000 g. of charcoal, and the three measurements were made respectively with 0.1000 g., 0.2000 g., and 0.2000 g. of acid with 100 ml., 100 ml., and 150 ml. of 95% ethanol (5% water by volume). The charcoal was added to the solution of acid, and the mixture was shaken by hand in a glass-stoppered flask for about two minutes, then intermittently for one hour. After the flask had stood undisturbed for 12–24 hours, the supernatant liquid was withdrawn through a medium sintered-glass disc with precaution against evaporation. Titrations were carried out on 25-ml. aliquots. In some instances, duplicate sets of determinations were made.

Calculation of the theoretical amount of charcoal for chromatography. An illustrative calculation is for the run shown in Fig. 2, involving 400 mg. each of palmitic and stearic acid applied to the column in 200 ml. of 95% ethanol. This solution contains 1.40 mmoles of stearic acid and 1.56 mmoles of palmitic acid, hence is 7.0 *mmolar* in stearic and 7.8 *mmolar* in palmitic acid. From the adsorption isotherms (Fig. 1), at this concentration Darco G-60 will retain about 0.5 mmoles of stearic acid per g. and 0.43 mmoles of palmitic acid per g. Thus, there is required to retain the stearic acid about 2.8 g. of charcoal and for the palmitic acid about 3.6 g., a total of 6.4 g. For a discussion of the theory pertaining to the fact that this quantity of charcoal retains the applied acid essentially quantitatively, refer to Ref. 9.

Apparatus for chromatography. The complete assembly used for chromatography, except for the fraction collector, is shown in Fig. 3. Fractions were received in vessels contained in an automatic fraction changer, actuated at a selected time interval. Elution was slightly more rapid at higher temperature, but the volume per fraction remained sufficiently constant. Various features of this apparatus were adopted after trial and error, and several simple devices were found more satisfactory than more expensive items. For example, the indicated rubber stoppers, secured with clamps, were more satisfactory than glass joints.

It became apparent early in this work that a constant pressure atop the column of adsorbent is highly critical. Pressure fluctuation of more than about 5 mm. of mercury, especially interrupting the pressure during a run, nearly always leads to formation of air bubbles and channeling of the adsorbent. The use of the mercury head in tube C for regulation of pressure proved more simple and reliable than an electronically controlled barostat

or a mechanical needle valve. The needle valve S_3 was used to approximately control nitrogen input to a value about 25% above rate of flow of eluent from the column. This avoids wasting nitrogen. The by-pass stopcock S_4 was used when temporarily increased flow was needed during change of solvent or addition of solvent *via* the funnel F. By closing the stopcock U and the screw clamp at W, the pressure in the system is maintained while the funnel is opened for addition or removal of solvent. When the funnel is open to the air, careful opening of stopcock U allows solvent already placed in B to flow into the funnel where it may be removed. Solvent is never stored in the funnel F where it can dissolve stopcock

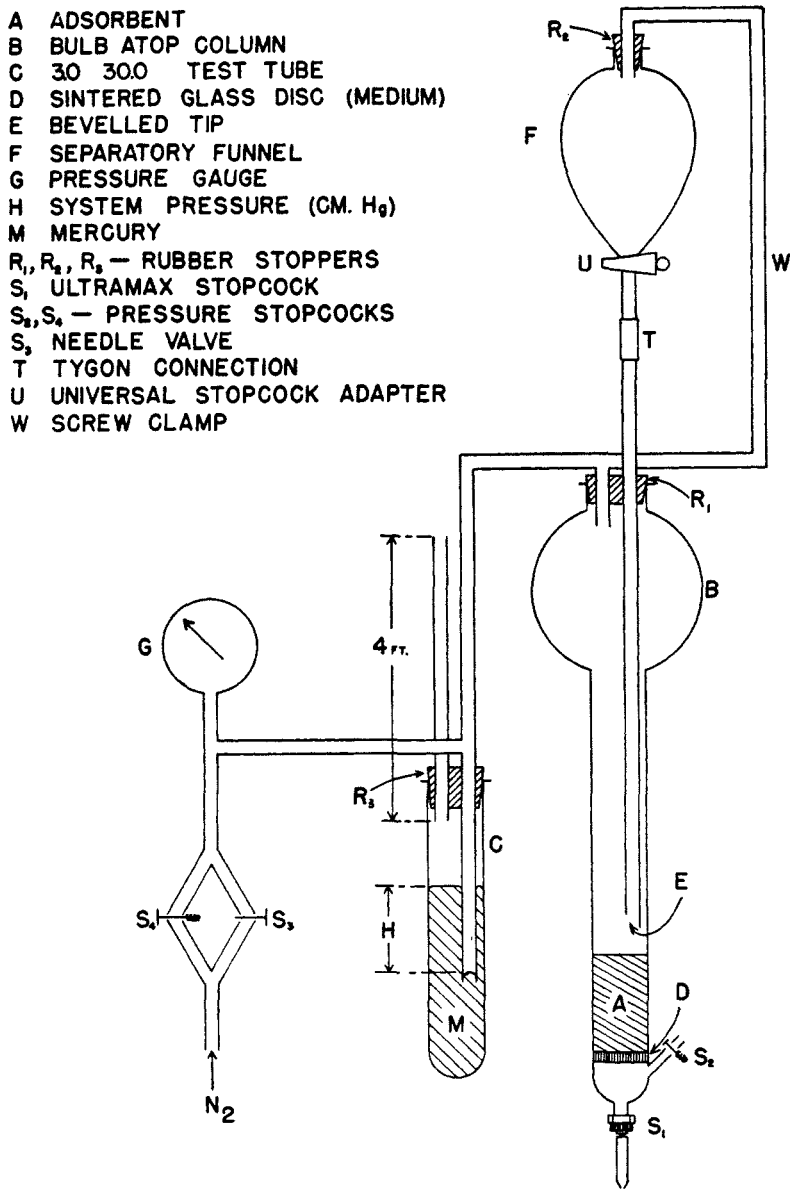

FIG. 3. APPARATUS FOR CHROMATOGRAPHY BY ELUTION DEVELOPMENT.

grease (even silicone grease dissolves slowly), but is stored in bulb B. Funnel F is used for transfer operations.

It is often convenient, if not mandatory, to interrupt a chromatogram, and this is accomplished by closing the Ultramax stopcock S_1 and leaving the system under pressure. During a period of standing, liquid flows into the space below the sintered glass disc. When operation is resumed, stopcock S_2 is carefully opened and the liquid is allowed to flow out by opening S_1. If continuous operation can be accomplished, a simple drip tip below the sintered glass disc is sufficient. This method must be used if pressures in the column are to exceed about 25 cm. of mercury, for the Ultramax stopcock leaks above this pressure. Use of a Teflon plug in a glass stopcock barrel was unsatisfactory, for pressure on the plug is necessary to prevent leakage and long contact of the Teflon with solvent always resulted in enough swelling to crack the barrel of the stopcock.

The pressure ordinarily used was about 20 cm. of mercury. At this pressure, rate of flow through a 2 x 25 cm. column of Darco and Celite (1:2) is 10–12 ml. per hour with alcohol and 13–15 ml. per hour with benzene. Pressures as high as 40 cm. of mercury give a proportional increase in rate of flow. In much of the work described in this paper (as shown in Fig. 2), a much higher rate of flow was secured because a height to diameter ratio of about three was used.

Of the various methods which have been described for packing a column (*Cf.* ref. 9), the use of a slurry was found greatly superior for the adsorbent used in this work. After the adsorbent mixture had been slurried with solvent in a beaker it was poured into the column and allowed to settle for at least 6 hours. After about an hour, tapping the column with a rubber mallet helped to eliminate air bubbles. One gram of the adsorbent mixture, wet with alcohol, packed under 20 cm. of mercury pressure, occupies a volume of about 2.5 ml.

Application of the solution of adsorptive was made directly with a pipette into the column as the last of the solvent was passing into the adsorbent, then the system was set up as in Fig. 3 and the pressure was maintained constant until the run was completed.

Acid in each fraction was determined by titration to a phenolphthalein end-point. For isolation of acids from the eluent, refer to Table II, footnote *a*. No difficulty with esterification during chromatography has been experienced; however, as a precaution, excess alkali may be added before the titrated solutions are concentrated.

Fatty acids. Normal fatty acids, C_{14}, C_{16}, and C_{18}, were commercial products, purified by fractional distillation of the methyl esters and recrystallization of the acids from acetone. The C_{13} and C_{19} acids were prepared *via* the cyanide from the next lower even-carbon bromides. Melting points were: C_{13}, 41.4–42.4°; C_{14}, 52.6–53.4°; C_{16}, 61.2–62.0°; C_{18}, 67.8–68.8°; C_{19}, 67.3–67.8°.

Oleic, linoleic, and linolenic acids were commercial products (Hormel). 11(12)-Octadecenoic acid was prepared from 12-hydroxyoctadecanoic acid obtained by saponification of hydrogenated castor oil. The sequence of steps was: fractional distillation of methyl ester (to remove about 15% of stearic acid), bromination with phosphorus tribromide, dehydrohalogenation with potassium hydroxide in ethanol, esterification and fractional distillation, and saponification. The acid melted at 29–36°, and its infrared spectrum indicated about 66% *trans* isomer and 34% *cis* isomer.

The branched-chain acids were the best samples prepared in this laboratory and reported in the series of papers entitled, *Branched-Chain Fatty Acids.*

SUMMARY

Static adsorption isotherms on Darco G-60 charcoal, with 95% ethanol as solvent, have been determined for a series of saturated, unsaturated, and branched-chain acids. There is discussed potential use of these isotherms for estimation of separability by chromatography, and certain applications are illustrated.

A simple apparatus is described for elution chromatography on charcoal.

A convenient chromatographic separation of palmitic and stearic acids is described.

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