[CONTRIBUTION FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

Adsorption Analysis. IV. Separation of Mixtures of Higher Saturated Fatty Acids

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This paper describes a method for separating small amounts of mixtures of higher, straight-chain, saturated fatty acids into their components.

The separation procedure in question involves the use of Tswett's "chromatographic" adsorption method.[1] The Tswett-column adsorption method has been applied also by others to the separation of fatty acids or their derivatives. Thus Kondo[2] obtained incomplete separations of oleic from palmitic or stearic acids by adsorption on alumina. Manunta[3] found that frankonite could be used to obtain separations of mixtures of palmitic and stearic acids, but, as judged from the criteria given, these separations were not very complete. Better separations were obtained by Kaufmann[4] in an extensive series of experiments in which he used alumina or silica gel as adsorbents. He studied a variety of solvents and methods of application of the mixtures to the columns, and he obtained good separations of mixtures of fatty acids. (He has also studied unsaturated as well as saturated members of the fatty acid series, as well as glycerides.)

The method described in this paper employs a commercial carbon as the adsorbent. The fatty acids are colorless and their other characteristics make it additionally difficult to locate by the usual methods[5] zones which might be formed on the columns.[6] For these reasons it was not necessary to limit the study to white adsorbents. A commercial carbon was found which had excellent characteristics for the separation of fatty acids and the technique of washing the zones through the column, the so-called "liquid chromatogram," used elsewhere with a carbon column[7] was employed.

Low-boiling petroleum ether was used for carrying the mixed acids onto the adsorption column and for development. Elution was accomplished with low-boiling petroleum ether, ligroin, or benzene, to which had been added 2 to 4% by volume of acetone or methanol. Methanol was found to be a more powerful eluting adjuvant than acetone in an eluant mixture, but it was abandoned in later experiments in order to obviate possible ester formation.

The method has the disadvantage that a quantitative separation of a complex mixture may require an experiment of long duration. The advantages of the method are that the separation procedures require no unusual or expensive apparatus, that they may be made semi-automatic so that they require a minimum of attention and that quite small amounts of mixtures may be handled and separated without great loss of material. The duration of the experiments may be shortened if only qualitative information is desired.

The chromatographic method has the further advantage, as Kaufmann[8] has shown, that it constitutes a most powerful tool for proving the purity or homogeneity of a sample of fatty acid.

Materials.—All solvents were distilled before use in order to remove non-volatile material. *Petroleum ether* (a low-boiling petroleum fraction with a boiling range of *ca.* 30–70°), *ligroin* (boiling range *ca.* 90–105°) and *benzene* were usually not specially dried. *Fatty acids* were the same as those described elsewhere.[9a] The *adsorbent* used was

(1) L. Zechmeister and L. Cholnoky, "Principles and Practice of Chromatography," translated from the 2nd German ed. by A. L. Bacharach and F. A. Robinson, Chapman and Hall, Ltd., London, 1941; L. F. Fieser, "Experiments in Organic Chemistry," 2nd ed., D. C. Heath and Co., Boston, 1941, section on chromatographic technique; H. G. Cassidy, *J. Chem. Education*, **16**, 88 (1939).

(2) H. Kondo, *J. Pharm. Soc. Japan Trans.*, **57**, 218 (1937).

(3) C. Manunta, *Helv. Chim. Acta*, **22**, 1156 (1939).

(4) H. P. Kaufmann, *Fette und Seifen*, **46**, 268 (1939).

(5) In some (unpublished) preliminary work the colored *p*-aminoazobenzene derivatives of several higher fatty acids were prepared by reaction of the acid chlorides with the amino compound in pyridine. It was not found possible to separate these colored derivatives by any of the chromatographic means tried. Such commonly used white adsorbents as calcium carbonate, magnesium carbonate, magnesium oxide, zinc oxide and cane sugar showed no adsorption. Aluminas and fuller's earth showed adsorption but no separation. The substances were insoluble in petroleum ether, so carbon disulfide had to be used as the solvent. It is probable that the relatively large aromatic radical when attached to the fatty acids lessens the differences in their adsorption behaviors. Thanks are due to Dr. M. L. Crossley of the Calco Chemical Division, American Cyanamid Co., for supplying the *p*-aminoazobenzene and other dyestuffs used in this work.

(6) An ingenious new approach to the problem of finding zones on adsorption columns has been made by W. Trappe, *Biochem. Z.*, **306**, 316 (1940). He observed that silica gel columns became transparent or translucent when wetted by certain solvents, and under these conditions it was possible to observe zones formed by colorless substances. The substances changed the light transmission of the column at the region of zone-formation. This approach might be applicable to the separation of fatty acids since these are adsorbed by silica gel.[9]

(7) H. N. Holmes, H. Cassidy, R. S. Manly and E. R. Hartzler, THIS JOURNAL, **57**, 1990 (1935).

(8) H. P. Kaufmann, *Angew. Chem.*, **53**, 98 (1940).

(9) H. G. Cassidy, (a) THIS JOURNAL, **62**, 3073 (1940); (b) **62**, 3076 (1940).

the same kind as a commercial carbon which may be obtained from the Darco Corporation, New York City, under the name G-60.

Experimental.—The data can be given concisely in a few figures and summaries since the experiments all followed the same pattern. (a) A mixture of lauric and stearic acids (acids with a six-carbon difference between them) is readily separated into its components. Such an experiment has already been given in another connection.[9b] A mixture of palmitic and stearic acids (2-carbon difference) is more difficult to separate. An experiment of long duration, (b), and one of short duration, (c), are given. The separation of mixtures of lauric, myristic, palmitic and stearic acids is discussed in two experiments, one, (e), a short-duration experiment which gives qualitative information, and, (d), a more complete separation which is considered in sufficient detail to show the general method used in all this work.

(a) A mixture of equal weights of lauric and stearic acids was separated almost quantitatively on a carbon column.[9b] The course of the experiment is shown in Fig. 1, which is self-explanatory. Of 0.3044 g. of mixed acids, 93% was recovered and of this a small middle fraction, about 8%, consisted of mixed acids. One gram of carbon was used. The developer was petroleum ether.

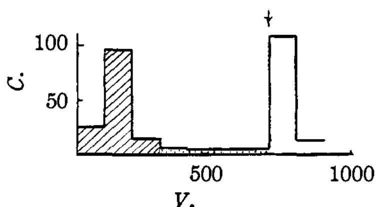

Fig. 1.—Separation of lauric and stearic acids: *V*, volume of percolate in cc., collected in units as shown; *C*, concentration of percolate, in mg. per 100 cc.; ■, lauric acid; ⁝, mixed acids; □, stearic acid; ↓, eluant solution added at this point.

(b) Separation of an equimolecular mixture of palmitic and stearic acids is shown in Fig. 2. Here carbon was mixed with a filter-aid[10] and packed to give a long column. The separation required about a hundred days but through the use of the apparatus shown in Fig. 3 very little attention was needed during this period. The column of adsorbent was formed in tube A. The developer was allowed to siphon from the 2-liter flask B, and a low pressure of nitrogen gas (about 11 or 12 cm.) was applied through the tubes C. The flask was shielded and all the rubber connections were wired. The pressures used in this apparatus were always low, so that the complications needed for working with high pressures[11] were avoided. A well-made round-bottomed flask can stand considerable internal

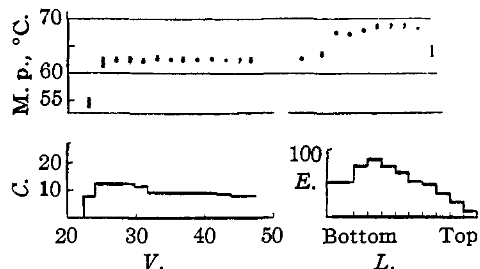

Fig. 2.—Separation of palmitic and stearic acids: *V*, volume of percolate in liters; *C*, concentration of solids in mg. per liter for each fraction of percolate collected; *L*, relative length of sections of column taken for elution; *E*, milligrams eluted per 4-cm. section of column. M. p., melting range in °C. (cor.) for each fraction of material obtained from percolates and from sections of column.

pressure[12] far in excess of that used at any time here. The height of the liquid in the shielded reservoir could be read from its level in the adsorption tube. An additional inlet was provided for the reservoir at D so that it could readily be refilled. The apparatus needed attention only every third or fourth day when the reservoir was refilled and the percolate fraction removed.

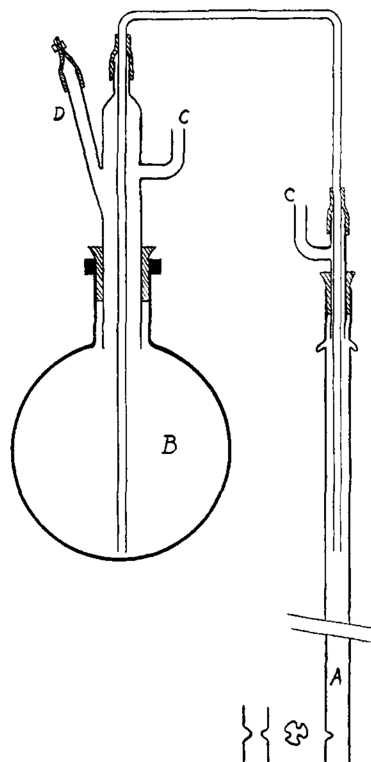

Fig. 3.—Apparatus for separations of long duration; the hatched portions are rubber connections.

The separation of palmitic and stearic acids is shown in Fig. 2 and is summarized below: palmitic acid 0.3872 g. and stearic acid 0.4281 g. (1.5 millimols of each) were dis-

(10) Although these filter-aids do not appear to adsorb fatty acids[9b] yet they should not be considered to be inert in all cases. For example Gallagher, Koch and Dorfman, *Proc. Soc. Exptl. Biol. and Med.*, **33**, 440 (1935), found that male sex hormone was adsorbed from urine by a diatomaceous earth. The cotton used for the pads upon which the columns of adsorbent are packed has already been shown not to adsorb from petroleum ether fatty acids such as those used here.[9b]

(11) A. M. Potts and F. C. Koch, *Proc. Soc. Exptl. Biol. and Med.*, **37**, 300 (1937).

(12) See, for example, F. B. Dutton, *J. Chem. Education*, **18**, 15 (1941).

solved in 100 cc. of petroleum ether and applied to an adsorption column consisting of 10 g. carbon mixed with 20 g. filter-aid. The column measured 43 cm. long and 1.5 cm. in diameter. Acid began to appear in the percolate after 22 liters of petroleum ether had passed through the column of adsorbent. This 22 liters of percolate had been collected in 2-liter fractions, each being distilled and the recovered solvent returned to the column. From the distillation of this 22 liters there was obtained a total residue of 6.1 mg. of substance consisting of lint and some organic material which was non-acidic and appeared to be petroleum-like in nature. Material of this kind is always obtained from this adsorbent and it is therefore advisable to wash the adsorbent thoroughly before use.

Development was accomplished with 25 liters of petroleum ether, and the column of adsorbent was then cut into ten sections. Each section was eluted with benzene containing a little acetone. Elution was efficiently carried out by packing the section of adsorbent into a small tube and passing the eluant down through it. Fractions of acid were identified by m. p., and in many cases also by equivalent weight.

Over-all recovery of acids:	99%
Recovery of palmitic acid:	54%
Recovery of stearic acid:	52%

An additional 40% of the total acid was recovered as the mixed fraction from sections 1 to 4 of the column, and since this came on the column between the palmitic and stearic acids, its nature was known, that is, it consisted of palmitic and stearic acids only, and their amounts could have been calculated, within the error of the titrations, from the equivalent weight of the mixture. There was also some 0.56% of unidentified material comprising the first two fractions of the percolate and the material eluted from the top section of the column of adsorbent.

(c) A more rapid separation of palmitic and stearic acids was carried out in the following way: palmitic acid 0.1919 g. and stearic acid 0.2131 g. (0.75 millimole of each) were dissolved in 100 cc. of petroleum ether and added to an adsorption column containing 4 g. carbon mixed with 4 g. filter-aid. The column measured 6.1 by 2.3 cm. About 3 liters of petroleum ether passed through this column without bringing out any acid, and ligroin was then added to hasten development. This developer (1.6 l.) produced several fractions of pure palmitic acid, totaling about 57% of the added palmitic acid. Petroleum ether containing about 2% acetone was then added as a mild eluant, 1.95 liters being used, and this was followed by 0.98 liters of petroleum ether containing 2% methanol. In the last of the petroleum ether–methanol fractions about one-third of the stearic acid appeared in a pure (by m. p.) form. The time required for this experiment was about seven days.

(d) The separation of a mixture of lauric, myristic, palmitic and stearic acids was carried out in a similar manner. One very careful separation is summarized in Fig. 4.

For this separation 10 g. of carbon mixed with 20 g. of filter-aid was used. The column of adsorbent was given a preliminary washing with 2.3 liters of petroleum ether. Lauric acid, 0.2040 g., 0.2322 g. myristic, 0.2603 g. palmitic and 0.2877 g. stearic acids (1.0 millimole of each acid) were dissolved in 100 cc. of petroleum ether and applied to the column. The melting points of the individual fractions are shown in the figure. The rate of flow of percolate was about 500 cc. in twenty-four hours and the duration of the experiment was about one hundred days.

Total recovery of acids added:	*ca.* 93.5%
Recovery of pure lauric acid:	78% of that added
Recovery of myristic acid:	85%, substantially pure
Recovery of palmitic acid:	77%
Recovery of stearic acid:	60%, nearly pure

Discussion of this Experiment.—The curves in Fig. 4 show the course of this separation very well. As each fraction of percolate was collected, the solvent was distilled off for re-use and the residue was kept *in vacuo* overnight and then weighed. The melting points given are for these fractions without further treatment, and could probably have been improved by recrystallization. The weights and melting points were plotted to give the curve shown, and the course of the separation was judged from the developing shape of the curve. The first fraction which came through the column was impure. It melted over a range of several degrees. The equivalent weight of this fraction (no. 4) was 214.5 ± 2, which is high for lauric acid, and it is probable that this fraction contained some of the non-acidic petroleum-like material mentioned above. This material is always found in this carbon, and although most of it is removed in the preliminary washing of the column, yet there is some which seems to be held back and which only appears under the "displacing" influence of the more strongly adsorbed substances being separated. This material is not unsaturated, as judged by testing it with bromine in carbon tetrachloride. (If any ester had been present it might have been expected to appear at this point also.)

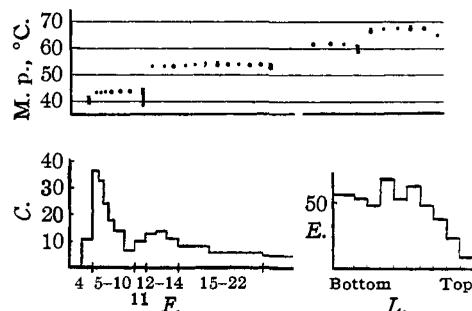

Fig. 4.—Separation of lauric, myristic, palmitic and stearic acids: *F*, numbers of percolate fractions and combinations of fractions referred to in text. These fractions are plotted on the basis of their relative volumes; *C*, *L*, *E*, and m. p. are the same as in Fig. 2.

Following this impure fraction there was seen to appear a "zone" of material. This was recognized to be a zone by the peak followed by decreasing amounts of material coming through the column. It was clear, from the sharp melting points, that here was a zone of lauric acid, and the equivalent weights, 201.7, 203.3 ± 2, confirmed this conclusion (fractions 5 to 10).

The next fraction (11) comprised the mixture which was to be expected where the tail of the lauric acid zone overlapped the head of the myristic acid zone. Equivalent

weight confirmed this and, moreover, since this fraction appeared between the lauric acid and the myristic acid zones it might reasonably be concluded to contain only these two acids, and their proportions could then be estimated from the equivalent weight of the mixture.

Myristic acid appeared next. The sharp melting points and the equivalent weight determination (225.4 ± 2) indicated that this was substantially pure myristic acid.

It is quite probable that continued long washing would have brought out the palmitic acid next, and, even more slowly, the stearic acid. However, it was decided instead to cut the column into sections and examine these,

When the sections were examined, it was found that pure palmitic acid was at the bottom of the column of adsorbent (it is less well adsorbed than stearic acid from the mixture by this carbon). Then there was a portion of the column which contained an overlapping of the palmitic and stearic acid zones. Then in the upper part of the column there was very nearly pure stearic acid, and finally, at the top of the column, very tightly adsorbed, there was a small amount of colored impurity which was present in the original acids which were added to the column.

The melting points and the equivalent weights were in accord. The melting points rose stepwise with increasing molecular weights of the fractions.

(e) A more rapid analysis of a mixture of these four acids was carried out as shown below. An adsorption column consisting of 4 g. of carbon mixed with 4 g. of filter-aid and measuring 9.2 by 1.9 cm. was used to analyze a mixture of 0.75 millimole each of lauric (0.1503 g.), myristic (0.1725 g.), palmitic (0.1921 g.), and stearic (0.2137 g.) acids, which were applied to the column in 100 cc. of petroleum ether. The column was developed with 2.1 liters of petroleum ether, and it was then extruded and cut into nine sections each 1 cm. long. Each was extracted with ligroin. The over-all recovery of the mixed acids was 91%, and most of the rest could have been recovered by continued extraction. Pure lauric and nearly pure stearic acids were obtained, and some estimate of the other acids could be derived from comparisons of the melting points and equivalent weights of the different fractions, together with a consideration of their positions on the adsorption column. The time required for this partial analysis was nine hours.

General Discussion.—The data given above indicate that even complex mixtures of fatty acids may be separated by Tswett adsorption analysis. If only a partial separation is desired the analysis may be quite rapid. More complete, and even quantitative, analyses may be obtained, but here experiments of longer duration are usually needed.

The separations depend upon many factors. From a practical standpoint not every carbon is suitable for separating these fatty acids.[9b] It is conceivable that any carbon might be used if enough of it were employed, but in some cases this would require a great deal of adsorbent. Also, carbons which have been treated with alkali, and which retain alkaline material, might react with the acids and prevent separation from occurring. The carbon used here was the most suitable among ten which have so far been examined.

In these separations it is important that enough carbon be used to hold back, almost completely, all of the more strongly adsorbed materials, yet allow the less strongly adsorbed ones to pass successively into the percolate. With a six-carbon difference between a pair of homologous acids in this molecular weight range a ratio of 4 parts by weight of carbon to 1 part by weight of the mixture of acids allows a safe margin provided that the column is of sufficient height. The separation of two such widely different acids is relatively easy by any method, and the use of this adsorption technique instead of crystallization or distillation of the esters is a matter of choice.

The amount of carbon must be larger when the two acids are more closely related. A two-carbon difference between a pair of acids requires for the separation of the mixture about 10 parts of carbon for each part of mixture. With mixtures of three or four acids, the ratio should be 10 or 15 to 1. In the latter cases, if there is enough carbon present and the column is long enough to separate the two most closely related of the highest molecular weight acids present, then the lower molecular weight acids may be expected to be well separated.

The types and amounts of developer used are of importance. In the cases of the fatty acids and adsorbent under investigation here the developer solvent of choice seems to be petroleum ether. The higher molecular weight acids (C_{16}, C_{18}), in distinction to those of lower molecular weight (C_{12}, C_{14}), are well adsorbed from this solvent, and their zones migrate down the column slowly. Ligroin is too good a solvent for all of these acids, and it brings about in most cases too rapid migration of the zones. One could alter the rate of development, if the labor involved were justified, by appropriate mixing of solvents such as petroleum ether and ligroin. This type of adjustment of the rate of development has been suggested by others, and may be useful in certain cases.

It should be stated that adsorption methods of this kind, in which the substances dealt with are difficult to localize on the column, are to be considered as "last resort" methods until they have been worked out, and that they should not ordinarily be used if the conventional chemical methods will work as well. There are, however, cir-

cumstances in which Tswett column separation is the method of choice, and it is hoped shortly to report further on some of these.

Since small amounts of pure acids can readily be obtained from mixtures by this method it may be expected that the Tswett column separation technique would find application in conjunction with an isotope dilution method such as that of Rittenberg, *et al.*,[13] if it were to be shown that fatty acids with abnormal isotope contents are inseparable from their normal analogs in these columns.

(13) D. Rittenberg and G. L. Foster, *J. Biol. Chem.*, **133**, 737 (1940).

I am indebted to Professor R. J. Anderson who provided some of the materials used in this work and who gave advice and criticism during its progress, and to the National Tuberculosis Association which furnished funds in aid of this work.

Summary

A method has been described and illustrated for separating mixtures of higher fatty acids by Tswett adsorption analysis on a column of adsorbent carbon.

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[CONTRIBUTION FROM THE DERMATOLOGICAL RESEARCH LABORATORIES DIVISION OF ABBOTT LABORATORIES]

N[1]-Heterocyclic Sulfanilamide Derivatives*

BY G. W. RAIZISS, L. W. CLEMENCE AND M. FREIFELDER

The replacement of an amide hydrogen in sulfanilamide by a benzene nucleus did not produce compounds showing therapeutic activity in pneumococcus infections. However, replacement by a heterocyclic nucleus containing nitrogen such as in sulfapyridine,[1] or containing nitrogen and sulfur as in sulfathiazole,[2] resulted in high therapeutic activity.[3] A nucleus containing two nitrogens, such as pyrimidine, also was found to promote activity.[4]

It appeared of interest to us to prepare derivatives with nuclei containing two or three nitrogens; five-membered rings containing two nitrogens such as pyrazole and hydantoin; six-membered rings containing nitrogen and sulfur in comparison with thiazole and thiazoline, which are both five-membered rings (the latter described in this paper); and also compounds with both benzene and heterocyclic rings containing nitrogen and sulfur. The compounds were prepared in the usual way by the condensation of *p*-acetaminobenzene sulfonyl chloride with the heterocyclic amine. The acetylsulfanilyl derivatives thus formed were hydrolyzed to give the sulfanilylamino heterocycle. The various intermediate amines were prepared by methods described in the literature.

The therapeutic activity of these compounds as determined in preliminary experiments in lower animals infected with pneumococcus type II proved to be generally low except sulfapyrazine (XIV),[5] sulfahydantoin (XV) and sulfathiazoline (XVI).[6] The last-named product proved to be particularly interesting both on account of low toxocity and high therapeutic effect. For these experiments mice were infected intraperitoneally with 2 to 20 minimum lethal doses of type II pneumococcus of which the average minimum lethal dose was 0.5 cc. of 1:10,000,000 dilution of broth culture. The drugs were given by mouth in a dose of 10 mg. Mice were treated immediately after infection, 3 times daily for five days, twice on the sixth day, and once on the seventh—a maximum of 18 treatments.[7]

Experimental

The N[1]-substituted sulfanilamides were prepared in the usual manner by the condensation of *p*-acetylaminobenzenesulfonyl chloride and the corresponding heterocyclic amine in pyridine; however, in some cases an additional solvent such as acetone was added to promote solution (I, X, XI, XII, XIII, XVI). In several cases, also, the pyridine was replaced by using sodium bicarbonate in acetone-aqueous solution (II, III, VI, VIII). The crude acetylsulfanilyl derivatives were precipitated by the addition

* This paper was presented before the Division of Medicinal Chemistry of the American Chemical Society in St. Louis, April, 1941.

(1) Whitby, *Lancet*, **1**, 1210 (1938).

(2) Fosbinder and Walter, THIS JOURNAL, **61**, 2032 (1939).

(3) Reinhold, Flippin and Schwartz, *Am. Jour. Med. Sci.*, **199**, 393 (1940).

(4) Roblin, Williams, Winnek and English, THIS JOURNAL, **62**, 2002 (1940).

(5) After this paper was submitted for publication, Joiner and Spoerri, THIS JOURNAL, **63**, 1929 (1941), described the preparation of 3-sulfanilamino-2,5-dimethylpyrazine.

(6) During the completion of this paper, Sprague and Kissinger, *ibid.*, **63**, 578 (1941), described the preparation of this compound, 2-sulfanilylaminothiazoline.

(7) We are indebted to Dr. M. Severac and Mr. J. Moetsch for their coöperation in conducting the animal experiments.