Chromatography

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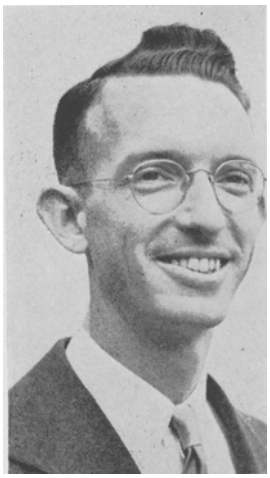
OF THE various techniques that have been devised for the resolution of mixtures, chromatography is one of the most effective. First described by Tswett in 1906, there have been found an ever increasing range of applications both for separations and for quantitative analysis of a wide variety of substances.



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The principle of chromatographic separations on columns depends on repeated partition of the solute between an adsorbent or stationary phase and a solvent or moving phase. A single solute molecule migrates only when it is in solution in the moving phase. When it is held by the adsorbent or stationary phase, it remains stationary as the solvent flows past. Thus the longer the solute is held in the stationary phase, the more slowly it will migrate down the column. If two solute molecules are held stationary for differing periods of time, they will migrate through the column at different rates and thus are separated. There are a number of choices for both the stationary and the mobile phases which are the basis for a classification.

Chromatography is usually classified as either partition, adsorption, or ion exchange, depending upon the type of stationary phase employed. Three methods of operating a chromatographic column are employed, elution, frontal analysis, and displacement. Thus there are nine main classes, all of which have not been investigated up to the present time. Since there are many materials available for both the stationary and the mobile phases, the possible experimental systems are almost infinite. Although application of the technique at the present time is largely empirical, there have been a very wide range of procedures devised both for separation and quantitative analysis.

In the lipid field chromatography has been applied to many problems of separation and analysis. In this presentation only a few of the many examples will be given to illustrate this extremely useful technique in the field of fats and oils.

The earliest and most widely known method of chromatography is the adsorption technique. The apparatus consists of a column of finely divided adsorbent wetted by one liquid moving phase. The material to be separated is dissolved in a suitable solvent and applied to the top of the column of adsorbent. The amount of material loaded on the column is kept small in relation to the total capacity of the adsorbent column. A solvent is allowed to percolate through the column, which distributes the components in zones that migrate down the column with the least adsorbed component migrating at the greatest speed. The zones can be washed through the column (eluted) and the effluent solution (eluate) collected in fractions; or the solid adsorbent can be extruded from the column, the zones separated, and the components washed from the adsorbent.

The use of adsorption chromatography has certain disadvantages, especially for quantitative analysis. The adsorption may be only slowly or incompletely reversible, which results in poor separation and poor recovery of solutes. However the separations are usually not specific with regard to adsorbent or solvent so that the success of a given separation depends upon trial of different combinations of adsorbent and solvent until a suitable pair is found.

Adsorption chromatography has been used to effect the separation of quite a wide variety of lipid materials. Trappe (11) found that the degree of adsorption of the various lipid materials on silica or alumina were in the decreasing order, phosphatides, fatty acids, cholesterol, triglycerides, cholesterol esters, and hydrocarbons. Thus in accord with this series, fatty acids may be removed from esters by percolation through a silica or alumina column. Triglycerides may be partially separated on alumina. For example, Walker (12) found, when linseed oil in hexane was passed through a column of alumina, that the more unsaturated glycerides migrate down the column behind the less unsaturated. Also soybean oil was separated by the adsorption technique into fractions having a difference in iodine value of 69 units (9). The separation of saturated fatty acids on alumina has not met with much success. However other methods of chromatography permit this separation and will be discussed later.

The separation of unsaturated fatty acids has been accomplished by the use of derivatives to increase the difference in adsorbability between the acids. Simmons and Quackenbush (10) prepared the 2,4-dinitrobenzenesulfenyl chloride derivatives of oleic, linoleic, and linolenic and found that they could be separated from each other and from the saturated acids on a magnesium sulfate column by the use of a benzene-ethyl ether mixture (95:5) as developing solvent. The recovery was above 95%. A method of analysis was developed which compared favorably with the spectrophotometric method.

Separation of the bromine derivatives of unsaturated fatty acids was accomplished by Howton (6) on a column of alumina-celite employing ether in n-pentane as a developing agent. The di- and tetrabromides were separated, but the recoveries were not quantitative. Therefore no quantitative method could be developed. However this could be used as an aid in preparative work.

Since absorption or elution chromatography does not usually give quantitative results, the method is most useful in separations which need not be quantitative. For example, the separation and subsequent identification (8) of the 2,4-dinitrophenyl hydrazones of carbonyl compounds resulting from the oxidation of unsaturated fatty acids have yielded much information on the cause of soybean oil flavor reversion.

To overcome the disadvantage of irreversible ad-

sorption, the technique known as displacement analysis was developed. This technique is carried out in the same manner as adsorption chromatography except that a "displacer" or "developing solute" is added to the solvent. The displacer is a material that is more strongly adsorbed on the column than any of the components in the sample. This acts to displace the adsorbed solute from the adsorbent and drive the material down the column. The adsorbed solutes also displace each other as they migrate down the column with the most strongly adsorbed component of the sample being displaced by the developing solute. The effluent from the column is collected in a series of fractions, and when the developing solute comes through, all of the components of the sample have been displaced. This method of operating a column has been used with a number of adsorbents including the ion exchange resins. However there may be some overlap between one band and the next so that the method does not always achieve quantitative separations. Holman has done considerable work with this method of chromatography, using an apparatus which is quite elaborate. The column consists of a series of "coupled filters," which is actually a set of chromatographic columns in series. They have the effect of sharpening the fronts of the zones because the effluent of one filter is collected, mixed, and presented to fresh adsorbent in a smaller filter. By repetition of this procedure, very sharp fronts can be obtained. The effluent from the column is directed through an interferometer to detect the fronts. Using this apparatus, it was shown that fatty acids from formic through eicosanoic (5) could be separated, using charcoal columns and fatty acid solutions in aqueous alcohol as displacer. Separations of a wide variety of materials have been made, including various glycerides (3). This separation was carried out on a charcoal column, and tristearin in benzene was used as a displacer. Beef fat was analyzed by this procedure and was separated into 14 fractions, the melting points of which ranged from 60° to liquid at room temperature. The value of this type of chromatography as a preparative tool is illustrated by the separation of highly unsaturated fatty acids from cod liver oil (1). By the use of a charcoal column and 1% methyl behenate in isopropyl alcohol as the displacer, methyl eicosapentaenoate and ethyl dodecapentaenoate and hexaenoate were purified. It would appear that displacement chromatography is very useful in the field of lipid separations because substantial quantities of each component of a sample may be obtained in a relatively pure state. However the choice of the displacer is not always an easy task, and although special equipment is not necessary, it is desirable because of the increase in utility of the apparatus.

For quantitative work the method known as partition chromatography has been most successful. This modification utilizes the difference in concentration of solutes in two liquid phases or a liquid and a gas phase. One liquid is held stationary by a solid carrier while the other liquid or a gas flows past. The basic difference between partition and adsorption chromatography is that in the former the stationary phase is a liquid and separations depend on the difference of the partition coefficients of solutes in the two liquid phases. The most common arrangement is one in which an aqueous or polar phase is held stationary while the non-polar phase flows past. However, reversed phase, the technique in which the non-polar liquid is held stationary and the polar solvent flows, is sometimes employed.

Paper chromatography is a type of partition chromatography which utilizes a paper support for the immobile liquid phase. The value of paper chromatography lies in the extremely small sample size that may be used. The method has found its greatest use in the field of amino acid analysis. However a number of applications have been devised in the lipid field. The apparatus consists of a strip or sheet of filter paper surrounded by the vapor of the stationary phase held in a closed container. The mobile phase is held in a trough at the top of the paper to obtain a descending solvent front or at the bottom which results in an ascending front. The sample is introduced as a spot on the paper near the solvent trough. If the solutes are colorless, it is necessary to spray the paper with a suitable reagent to cause the separated components to become visible. Materials are usually characterized by the R_f value, which is defined as the distance the material moved from the original position expressed as a fraction of the distance moved by the solvent front. The process may be one-dimensional by the use of a paper strip or two-dimensional, in which the components are separated along one line and then introduced into a second solvent which travels at right angles to the direction of the first solvent. The method may be illustrated by the separation of the unsaturated fatty acids in the form of their mercuric acetate derivatives (13). Tetralin was used as the stationary phase on the paper, and the developing solvent was a mixture of methanol-acetic acid-petroleum hydrocarbon. After elution the paper was sprayed with diphenylcarbazone in alcohol to reveal the separated components. Samples of 10–300 μg. of the mixed mercuric acetate derivatives were used. Although quantitative measure of the spots was not made, the absorption spectra of solutions of the mercuric acetate derivatives could be used for quantitative work.

Partition chromatography on a column requires a solid for the support of the non-mobile phase. For example, the use of silicic acid as a support for aqueous buffered solutions has resulted in excellent quantitative methods for the separation and determination of fatty acids from formic through pelargonic and dicarboxylic acids of 3- to 16-carbon chain lengths. For analysis of a mixture of monobasic acids as described by Corcoran (2), silicic acid is mixed with an aqueous solution of glycine which has been adjusted to the desired pH with sodium hydroxide. The pH of the aqueous phase determines which acids will be resolved. To separate the 9-, 8-, 7-, and 6-carbon acids a 2M solution of glycine at pH 10 is used while a solution that has a pH of 8.4 resolves the acids of 3 to 6 carbons, and the longer chain acids come through the column together. The aqueous buffer-silicic acid is packed into a column, a sample of 10–50 mg. of acids introduced and the column eluted with mixtures of butanol in chloroform which are increased with respect to butanol content as elution proceeds. Small fractions of the effluent are collected and titrated. If the titration is plotted against the fraction number, a series of peaks result, and the area under the peak is a measure of the quantity of acid. Recoveries of 100% are obtained by this method. Dibasic acids may also be determined in a similar system which employs citric acid solutions as the aqueous phase (4).

The control of the pH of this aqueous solution also determines which dibasic acids will be resolved. We have made use of these methods in our laboratory for the study of positional isomerization of unsaturated fatty acids and have found them to be very excellent methods.

Another good example of partition chromatography was described in a recent publication (14). This chromatographic system employed a methyl cellosolve-water ammonia system on a silicic acid support as the stationary phase and petroleum ether-butyl ether mixtures as the mobile phase. Monocarboxylic acids from 14- to 2-carbon chain lengths are resolved by the column, using petroleum ether as the solvent. Dibasic acids from 22 carbons down are eluted with butyl ether-petroleum ether. Samples of only a few milligrams of the mixed acids are used, and the mono- and dicarboxylic acids can be resolved in one column. This chromatographic method should find increasing use in the analysis of fatty acids.

Fatty acids may also be separated by the technique known as gas-liquid chromatography. This method makes use of the partitioning of the fatty acids between a mobile gas phase and a liquid stationary phase. As described by James and Martin (7), the apparatus consists of a long column packed with a Celite-silicone oil mixture. The column is surrounded by a heating jacket so that the vapor of a boiling liquid surrounds the column and controls the temperature of the apparatus. The gas phase, a stream of nitrogen, carries the vaporized acids through the column into a titration cell, where they are absorbed in water and titrated. The method gives a very good resolution of fatty acids from formic to lauric and will also separate some isomeric acids such as isovaleric, methyl ethyl acetic, and n-valeric acids. The temperature of the column, which is maintained by a boiling liquid, depends on the vapor pressure of the acids to be separated. This should be between 10 and 1,000 mm. of mercury. For example, cellosolve, which boils at 137°, is suitable for the separation of acids from valeric to lauric. This method permits automatic titration of the acids as there is no large volume of liquid-eluting solvent to collect.

This presentation has only touched on a very few of the procedures that have been described for chromatographic separations in the field of lipids. In view of the infinite experimental procedures that are possible using this technique, it is indeed a very fertile field of research both for preparative work and analysis.

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Distillation

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DISTILLATION is a separation process—one of four included in the 1955 Short Course. The others are chromatography, crystallization, and solvent extraction. All four have the common characteristic that distribution occurs between two phases that are mechanically separable:

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distillation
vapor-liquid
chromatography
fluid-stationary
crystallization
liquid-solid
extraction
liquid-liquid

Separation is possible only when one component is enriched in one phase and depleted in the other relative to another component. In practice these separation processes are not sharply differentiated. Distillation may be combined with extraction to yield the familiar extractive distillation process. Gas chromatography combines some of the characteristics of distillation and extraction as well as the chromatographic technique.

For present purposes the subject of "Distillation" is limited in scope to "analytical techniques." Techniques of vacuum analytical distillation applicable to fatty oils and their derivatives include equilibrium distillation and molecular distillation. We will consider the mixture, the apparatus, and the procedure and will briefly outline distillation theory.

Mixtures of greatest interest to members of the American Oil Chemists' Society are animal and vegetable fats and waxes. The fats are glycerol esters of fatty acids, and the waxes consist mainly of higher alcohol esters of fatty acids. Derived from these by saponification are mixtures of alcohols or acids that, in turn, are the raw materials of a growing chemical industry. Some of the mixtures, such as lard, tallow, cottonseed, soybean, or marine oils, have great economic value. Others, like human hair oil or the bacterial waxes, have little commercial value but may be interesting scientifically.

Apparatus suitable for distilling such a range of mixtures includes equilibrium stills and molecular stills. The choice for a particular analysis will depend primarily on the vapor pressure and thermal stability of the sample. Multistage equilibrium stills are widely used for fractionation of free fatty acids, esters of fatty acids with low boiling alcohols, ni-