GAS CHROMATOGRAPHIC ANALYSIS OF PROTEINS, LIPOPROTEINS AND LIPIDS IN ANIMAL TISSUES

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The science of chromatography is a strikingly original and simple invention which has projected the light of discovery into the mazes of chemical substances that constitute all things -- both organic and inorganic. This invention was conceived in 1906 in Warsaw at the hands of M. Tswett. Tswett was a botanist and he worked with plant pigments, performing a simple experiment that was to form the basis for the chromatographic adsorption method. He was successful in separating chlorophylls from xanthophylls in green leaves using what we now call liquid-solid column chromatography. Tswett termed his method "chromatography" which literally means "color writing" and although the term is a misnomer for many modern chromatographic methods, the principles are still the same.

In 1941, Martin and Synge published a paper entitled "A New Form of Chromatogram Employing Two Liquid Phases". This was the beginning of liquid-liquid partition chromatography and, no doubt the beginning of gas chromatography since the authors state that "The mobile phase need not be a liquid but may be a vapor. Very refined separations of volatile substances should be possible in a column in which permanent gas is made to flow over gel impregnated with a non-volatile solvent".

Although the implication above was direct, a period of eleven years passed before the classic work of James and Martin (1952) describing the separation and microestimation of C_{4} to C_{12} (formic to lauric) fatty acids by gas-liquid partition chromatography.

During the past several years gas chromatography has become established as a rapid, efficient and relatively simple technique for the separation and analysis of mixtures of volatile substances.

A large number of gas chromatographic instruments in varying degrees of sophistication are currently commercially available. All have certain basic components and requirements which are essential for proper operation. These are: (1) An injector block (2) A packed column containing a non-volatile stationary liquid phase (the solvent) coated on an inert phase of fairly large surface area such as glass beads, ground firebrick or diatomaceous earth. Another form of column of very small diameter called a capillary column is also in use. In this case, the liquid phase is coated on the inside of the column wall and no inert phase is necessary. (3) A device called a detector which senses the components of the volatile mixture as they emerge from the column. (4) An electrometer which amplifies the sensings of the detector and (5) a strip chart recorder which records the amplified sensings of the detector on a strip chart. (6) A cylinder of compressed gas or a gas generator to supply the carrier gas (mobile phase).
We shall briefly consider the operation and principle of certain of the instrument components.

(1) The injector block is equipped with a septum made of heat resisting silicone rubber so as to allow sample injection using a micro-syringe. The injector block should preferably be heated to a higher temperature than that of the column. This is necessary to insure that the carrier gas enters the column at column temperature and that the sample components be made to vaporize very rapidly on injection in order to avoid artifacts of efficiency or resolution.

(2) The column may be looked upon as that part of the chromatographic equipment for which all others are made. All chromatographic separations involve the transport of a sample of a mixture through a column (or a physical equivalent of a column). The mixture may be a liquid or a gas. The transport is effected by moving gas (the mobile phase).

Owing to the selective retardation exerted by the stationary phase, the components of the mixture move through the column at different effective rates. Thus, they tend to segregate into separate bands or zones. The rate at which a sample component is carried through the column is governed by its partition coefficient between the gas phase and the stationary liquid phase. Each compound has a characteristic partition coefficient (K) which is given by the equation:

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K = \frac{\text{Weight of solute / ml stationary phase}}{\text{Weight of solute / ml mobile phase}}
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Therefore, if the partition coefficient of compound A is small, the amount of compound dissolved in the stationary liquid phase will be small compared to that in the gas phase. Consequently, the compound passes through the column rapidly, since it is not retarded by the stationary liquid. If the partition coefficient of compound B is large, the greater proportion of it resides in the solvent; therefore, passage through the column is slow and peak B will emerge subsequent to peak A. The time required for a component to emerge from the column is known as its retention time and is characteristic of the compound under a specified set of conditions. It should be remembered that the solute present in the gas phase is at all times in dynamic equilibrium with the same solute in the liquid phase. Martin and Synge (1941) visualized the chromatographic column as divided into a number of regions, called theoretical plates, with a perfect equilibrium between the solute in the mobile and stationary phases existing in each. Each plate corresponds to one funnel in the extraction train. The height equivalent to one theoretical plate (HETP) is taken as the height of a
layer at right angles to the column such that the mean concentration of the solute in the stationary phase in this layer is in equilibrium with the solute in the mobile phase leaving this layer. The number of theoretical plates in a column is a measure of the number of extractions which may be thought to have occurred that is, the efficiency of the column.

Some capillary columns are reputed to have 750,000 theoretical plates. (N-butane on a 1000 foot nylon capillary).

As was mentioned above, sample components move at different rates due to selective retardation in the liquid phase. Components are retarded by the forces of interaction between solutes and solvent. These forces can be divided into four types (Keulemans, 1959).

(a) Dispersion, London or non-polar forces arising from synchronized variation in the instantaneous dipoles of two interacting species. These are present in all cases and are the only sources of attraction energy between two non-polar substances.

This attraction comes about because the electrons in a molecule are in constant motion and a chance occurrence may lead to a momentarily unsymmetrical charge distribution. This in turn may effect the neighboring molecule inducing a simultaneous and opposite electrical dipole. Thus, two molecules such as two hydrogen molecules, which have on an average over a period of time complete symmetry of negative charge about the nuclei, may experience mutual attractive forces through synchronization of electronic motions. These interactions are responsible for the condensation of gases when the temperature becomes sufficiently low.

(b) Induced dipole, induction forces or Debye forces resulting from the interaction between a permanent dipole in one molecule and the induced dipole in a neighboring molecule. These forces are usually relatively small.

The induced dipole may be produced on approach of another molecule which has a permanent dipole. The direction of the induced dipole is always that which causes attraction to the inducing dipole. Consider a non-polar particle the helium atom and as the approaching polar molecule a water molecule. If the negative end of the water molecule approaches the helium atom, it repels electrons from the portion of the atom nearest to itself, producing in the region of the helium atom a positive charge which will attract the negative end of the water molecule. If on the contrary, the positive end of the water molecule happens to be nearest the helium atom, electrons in the latter will be
drawn toward the water molecule, placing a negative charge on the part of the helium atom closest to the water molecule; the opposite charges will again attract one another.

(c) **Orientation or Keesom forces** resulting from the interaction between two permanent dipoles, the association energy depending upon both sizes and positions of the dipoles. The "hydrogen bond", that is, the relatively strong interaction between negatively charged atoms and positively charged hydrogen in groups such as OH, NH, and SH is a particularly important type of orientation force and is of considerable practical use in gas-liquid partition chromatography.

(d) **Specific interaction forces** and chemical bonding, e.g., complex formation between solutes and metal ions, or loose adduct formation between solutes and solvents. These forces of interaction between solutes and solvents govern the relative volatilities or the "escaping tendency" caused by thermal agitation of the solute molecules. In gas chromatographic separations one deals with ideally dilute solutions and thus the attractive forces occur between solute and solvent and not between solute molecules as in the case of pure solute in the liquid state.

With a non-polar solvent, non-polar solutes will tend to be separated approximately in the order of their boiling points since the forces of interaction (dispersion forces) between solute and solvent will be similar to the forces between the solute molecules themselves. Polar solutes will be eluted from a non-polar solvent more rapidly than non-polar solutes of a similar boiling point as they will have lost the strong dipole-dipole association energy which is present in their own liquid. As the polarity of the solvent is increased, the polar solutes will be retarded to a greater extent.

According to Keulemans (1959), there must be a certain compatibility between the stationary phase and the sample components. A useful practical rule is that, for an efficient normal separation, the components of the mixture and the stationary liquid should show some resemblance, that is, polar with polar and non-polar with non-polar. There are many exceptions to this "rule of similarity" especially if one desires to separate components of about equal boiling points but of different chemical nature. Choice of a liquid phase is still partly a matter of trial and error.

(3) There are several types of detectors in use on chromatographic instruments. The more common ones will be considered briefly.
(a) Thermal conductivity cells. Heated elements are cooled by the carrier gas and assume definite resistance. When the eluted component passes over the element, the rate of heat loss is changed and the resistance of the element changes due to its temperature change. There are both reference and sensing elements incorporated into a Wheatstone bridge and the out of balance signal is applied to a recorder.

(b) Hydrogen Flame and Thermocouple. Hydrogen or hydrogen and nitrogen is used as the carrier gas and the effluent from the column is burned in a fine jet placed below the thermocouple. The appearance of an organic compound in the effluent results in a lengthening and rise in temperature of the flame and an increase in voltage of the thermocouple. The normal output of the later is balanced by a potentiometric network, differences in voltage being fed to a recorder.

(c) Ionization Detectors. Ionization detectors are inherently more sensitive than thermal ones, responding to as little as $10^{-10}$ to $10^{-15}$ mole of solute in the carrier gas. The principle of operation of the several types of ionization detectors is basically the same. An organic compound is ionized, and the ions (or electrons) formed are used to carry an electric current. The magnitude of the signal is then recorded with or without intermediate amplification. A number of ionization detectors have been described in the literature but only three have found widespread use as yet and are available commercially. These are the argon detector (Lovelock, 1958) the hydrogen flame ionization detector and the radio frequency detector.

In general, ionization detectors are considerably less sensitive to temperature changes and fluctuations in the flow rate of the carrier gas than are thermal detectors. Furthermore, the signal-to-noise ratio is so high that reference cells are not needed.

Analysis of Animal Tissues

1. Lipids

One of the requirements for gas chromatographic analysis of a substance is that it be volatile. The lipids, proteins and lipoproteins of animal tissue either are generally notoriously high boilers or decompose on exposure to high temperatures. Thus, some means is necessary for rendering the compound more volatile either by the formation of derivatives or hydrolysis or pyrolysis. The usual procedure for the higher fatty acids, the most common lipid analyzed by gas chromatography,
is to convert the acid to its corresponding methyl ester. Stoffel et al. (1959) list the following essentials for an optimal micromethod for methyl ester preparation: quantitative yield, absence of change in double bond structure of highly unsaturated acids and technical convenience. These workers used interesterification to form methyl esters stating that this method has proved to be technically simpler, milder and more quantitative than diazomethanolsis. The latter method may produce poor yields because of the formation of addition products of diazomethane at ethylene bonds.

Metcalfe and Schmitz (1961) outlined a rapid method for the preparation of methyl esters of fatty acids using boron trifluoride and methanol. It is claimed that esters are formed in about two minutes and are comparable to those obtained by other procedures. It should be pointed out that free fatty acids must be used. Thus, the fatty acid must be liberated from its parent compound before methylation.

Vorbeck et al. (1961) made a quantitative comparison of four methylation techniques including diazomethane, methanol-hydrochloric acid with sublimation, methanol-hydrochloric acid on ion exchange resin and methanol-boron trifluoride. Their data indicate that the choice of methylation procedure depends on the nature and composition of the sample. The greatest variation among methods occurred in the mixture of lower molecular weight acids and the authors state that these acids are best methylated with diazomethane. The methods are comparable when only the higher fatty acids are present. No significant loss of polyunsaturated fatty acids was found as a result of treatment with diazomethane.

At North Carolina State College we have used the transesterification method of Tove (1961) for fatty acid methylation. This method consists of dissolving glycerides in a mixture of benzene, dry methanol containing 5 percent hydrogen chloride and 2-2 dimethoxypropane. The lipid solution is kept at room temperature overnight, and the methyl esters extracted with hexane. The hexane extract is washed with water and simultaneously dried and neutralized by treatment with a mixture of anhydrous sodium sulfate and sodium carbonate.

A recent approach to the analysis of free fatty acids from C₄ to C₂₂ has been reported (Metcalfe, 1960) whereby a polyester treated with phosphoric acid is the liquid phase. The addition of acid to the polyester is necessary because of concentration-dependent molecular association of the solutes in the partition liquid. If no acid is added, the fatty acids tend to dimerize rather than associate with the column packing.

The literature with respect to lipid analyses is accumulating in great volume. Dal Nogare (1962) states that more than 200 papers and addresses appeared in 1961 alone on the determination of fatty acids in biologically important materials and foodstuffs. The invention of gas chromatography has made it possible to discover that fats from a number of different natural sources, including ruminant body and milk fat, contain small amounts of branched chain and normal odd-numbered fatty acids. Hawke et al. (1959) presented retention volume data on the gas chromatographic separation of acids of the above type using three different liquid phases: Silicone grease, Apiezon M and polydiethylene glycol succinate.
Hornstein et al. (1961) studied the fatty acid composition of meat tissue phospholipids and neutral lipids for their possible contribution to meat flavor. The methyl esters were chromatographed on an 8 foot copper column packed with polyvinylacetate. Beare (1962) also studied the fatty acid composition of meat fats using columns packed with butanediol succinate and with silicone. The results obtained with both columns generally agreed.

The effects of added dietary fat on fatty acid composition of steer depot fat was studied by Edwards et al. (1961). The fatty acids were methylated by diazomethane and separated on a Celite column coated with Reoplex 400. It is of interest to note that the addition of animal fat to the diet of fattening dry lot steers resulted in a significant increase in the stearic acid content of subcutaneous fat.

At North Carolina State College we have used gas chromatography to study the fatty acid composition of beef depot fats as influenced by ration and the variation in composition with respect to location of fat deposits. The relationship between flavor and individual fatty acids has also been studied. Fat from fresh hams and cured hams in various stages of aging has been analyzed so as to note any change in fatty acid composition during the aging process.

Volatile fatty acids in aged country style hams were analyzed by Ockerman (1961) who converted them to ethyl esters by heating with potassium ethyl sulfate. The esters were chromatographed on a diisodecyl phthalate column using a thermal conductivity detector.

Oette and Ahrens (1961) describe a simple procedure for quantitative formation, recovery and gas chromatographic analysis of 2-chloroethanol esters of short chain fatty acids. The column used was ethylene glycol adipate, ethylene glycol succinate or Apiezon M on Celite. Gehrke and Linskoff (1961) used a silicone 550-stearic acid column at 137°C for the separation of unesterified steam-volatile fatty acids. The preparation technique resulted in nearly quantitative recovery. It should be added that stearic acid can be used as a part of the liquid phase when operating temperatures are no higher than these. Higher temperatures cause bleeding of the acid from the column and subsequent detector response.

Emery and Koerner (1961) published a paper on the gas chromatographic determination of trace amounts of fatty acids in water. The column used was 20 percent Tween 80, 2 percent phosphoric acid (85%) on Chromasorb W. The method has since been applied to the analysis of volatile fatty acids in blood and rumen juice. Very little sample preparation is required. It is necessary, however, to use a detector which is insensitive to water such as flame ionization. It is also preferable to use a gold plated detector housing so as to avoid corrosion problems which might result from the phosphoric acid.

O'Neill and Gershbein (1961) advanced procedures for the determination of cholesterol and squalene in various biological mixtures. The column used was a stainless steel U-shaped column 24 inches long packed with Apiezon L on 30 - 60 mesh inert phase. Column temperature was 350°C. Under these conditions, these two compounds emerged from the column in 10 minutes. Column life at this temperature is only about one week.
Long chain fatty aldehydes present in plasmalogens were studied by Gray (1960). Plasmalogens are a class of phospholipids. These aldehydes are rather unstable and tend to polymerize over a period of time, thus they are unsuitable for gas-liquid chromatographic analysis. Gray converted them to their corresponding dimethyl acetics which were satisfactorily separated on Apiezon L and Reoplex 400.

There is evidence that surface area of the inert phase effects separation in gasliquid chromatography. Ettre (1960) investigated firebrick, Chromosorb, Chromosorb W, Celite 545 and Teflon and drew the following conclusions regarding data obtained. Chromosorb, Celite and Chromasorb W have similar results with regard to separation effect; tailing of polar compounds is considerable with Chromosorb and firebrick; the specific surface area of the support material does not influence particularly the separation effect of the column if the surface area is over a certain limit; the use of Teflon is preferable where the extremely high polarity of the sample makes it necessary. Hornestein and Crowe (1961) also found that Chromosorb R and Celite 545 gave similar results. Their study included the use of glass beads as an inert phase. This material was reported to be poor for use as a column support for separation of fatty acid methyl esters.

Recently considerable attention has been given to the adsorption of a solute onto the inert phase. Such adsorption results in peaks with wide bases and unsymmetrical peaks. Perrett and Purnell (1962) used hexamethyldisilazane to react with five different solid supports. Their results show that adsorption by the support is reduced very significantly after treatment. The reduction is brought about predominantly by a change in the available surface area. This change is thought to result from loss of active sites and not from blocking of the pores with the consequent loss of internal area. The least adsorptive support found was silazane treated Celite (or Chromosorb W).

There is some evidence that the use of a polyester stationary phase results in a contamination of the esters owing to transesterification process. This was reported by Pascaud (1963). James (1960) has compiled data from various sources showing that temperature and residence time in the column have a marked effect on transesterification in polyester columns. Even at 200°C there can be an appreciable loss of the slower components resulting in an apparent increase in the faster. At 240°C, the errors could amount to as much as 40 percent in extreme cases. Optimum resolution with a minimum of artifacts can probably be obtained with small liquid-to-solid support ratios coupled with low temperatures, high flow rates, small samples and sensitive detection systems (Burchfield and Stores 1962).

One can also obtain misleading results by overloading of the hypersensitive detectors, giving rise to an extreme non-linear condition. Blunted or inverted peaks usually result. Gerson (1961) reported that medium overloading results in a marked reduction in the amount of C12 and C14 fatty acids present in a sample. Very high overloading can result in a recovery of only 6 percent of C12. Novak and Janak (1960) report that linearity of the flame ionization detector is roughly valid up to a concentration of 0.5 volume percent of the component in the carrier gas.
They also state that the flow rate, as well as the position of the lower electrode, has an influence on the linearity of the detector signal response and the inversion of the chromatographic curves.

One of the advantages of gas chromatographic analysis is that one can obtain quantitative data, provided that collection, separation and derivative preparation are quantitative and that composition is not altered by the liquid phase and the response of the detector is linear. If these conditions are met, it is only necessary, especially with a homologous series, to determine the relation of each peak on the strip chart to the total of all peaks. This is done in a number of ways. At North Carolina State we use a planimeter to measure peak areas. Should the pen not return to the base line before starting another peak, a perpendicular is dropped from the low point so as to separate the peaks. In severe cases, the two adjacent peaks are reconstructed before their areas are determined. This method has estimated satisfactorily the fatty acid content of standard mixtures.

2. Proteins

Proteins per se cannot be analyzed by gas chromatography. They must be analyzed as their component parts. Even amino acids are nonvolatile (alanine with a molecular weight of only 89 melts at 297° C.) and must have their vapor pressure increased and polarities reduced prior to analysis by gas chromatography through removing or masking functional groups by oxidation, acylation, alklylation, or through other means. Comparatively little work has been done on the separation and determination of amino acids despite the obvious advantages of such a procedure.

Youngs (1959) converted glycine, alanine, valine, leucine, and isoleucine into their n-acetylbutyl esters using acidified butanol and acetic anhydride. He used a 6 foot x 0.6 inch copper column containing 20 grams of 20-40 mesh firebrick coated with safflower oil, hydrogenated to an iodine value of less than one. He claimed a quantitative determination.

Ulehla (1960) analyzed 19 common amino acids by a combination of pyrolysis on a heated platinum wire and gas chromatographic separation of the products. Several amino acids gave specific pyrolytic spectra.

Zlalkis et. al. (1960) exposed amino acids to oxidation by ninhydrin resulting in the formation of aldehydes and CO₂. The aldehydes were chromatographed on a firebrick column coated with an equal mixture of ethylene and propylene carbonates. After separation, aldehydes were cracked and reduced to methane and water. The water is removed and only methane is detected. Seven acids were analyzed.

Johnson et. al. (1961) chromatographed to n-acetylamino acid esters of isobutyl, n-butyl and n-amyl alcohols on Carbowax columns using 0.5 to 5.0 percent liquid phase on Chromosorb. Thirty five acids were investigated and detection limits as low as 10⁻¹⁰ moles of acid were obtained. The authors include a standard procedure for preparation of the derivatives.
Saroff et al. (1962) used a six foot long column of polyethylene glycol adipate on Chromosorb W with nitrogen and anhydrous ammonia as the carrier gas. Amino acids were analyzed as their ester hydrochloride salt dissolved in alcohol.

By treating $\alpha$-monoamino-monocarboxylic acids with a mixture of concentrated HCl and HNO$_3$ Melamed and Renard (1960) obtained the corresponding chloro-acids in a yield of almost 100 percent. The chloroacids are converted to their methyl esters by means of diazomethane and analyzed on a $\frac{4}{5}$ meter column consisting of 2 meters of polyethylene glycol followed by 2 meters of silicone oil - stearic acid. The eight amino acids analyzed could not be properly separated using only one column packing.

The quantitative analysis of amino acids by gas chromatography is difficult for several reasons, one of which is the involved procedures necessary to prepare the acids for analysis. In addition, none of the methods currently available is satisfactory for all the amino acids. This is also true for fatty acid analysis now that more acids have been found in biological materials. One development in instrumentation which will aid in the analysis of a wider spectrum of both amino and fatty acids is temperature programming. This procedure consists of a gradual increase in temperature of a column as the mixture of components passes through. The increase can either be linear or non linear. Compounds are chromatographed at the temperature best suited for the separation of its components. Programming the temperature spreads out peaks which appear early in isothermal runs at high temperatures and sharpens up peaks which have long retention times at low temperatures; thereby resulting in more uniform presentation of the data.

3. Lipoprotein

No references were found concerning the analysis of lipoprotein, per se. This material, consisting of protein and lipid must be fractionated into its component parts for analysis. One paper (Suffis et al., 1963) presented a method for the analysis of peanut lipoprotein in comminuted meats. The procedure is based on the difference in behenic acid (C$_{22}$, saturated) composition between peanut lipoprotein and comminuted meats as measured by gas chromatography using a six foot x $\frac{1}{8}$ inch column of Carbowax 20M on Chromosorb W.

In gas chromatography we have a powerful analytical tool which in spite of many advances is still in its infancy. Many research areas have been uncovered or made easier by its use. Application of the method can be as universal as the ability and imagination of the investigation will allow.
REFERENCES CITED


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MR. PEARSON: Thank you, Dr. Craig.

Our next paper this morning is going to follow along the same lines and is going to be on the subject of Glass-Paper and Thin Layer Chromatography. This paper will be presented by L. D. Williams who is working in our laboratory at Michigan State University. He obtained his M.S. and B.S. Degrees at the University of Arkansas and is presently doing graduate work with us. He has been using this technique to a considerable extent in our laboratory, so we will now hear from L. D. Williams.

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