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THE HISTORY OF GAS–LIQUID CHROMATOGRAPHY

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CONTENTS

1. INTRODUCTION

> I am of your Opinion, said Carrasco; but 'tis one thing to write like a Poet, and another thing to write like an Historian. 'Tis sufficient for the first to deliver Matters as they ought to have been, whereas the last must relate 'em as they were really transacted, without adding or omitting any thing, upon any Pretence whatever.
>
> Miguel De Cervantes, *Don Quixote*, Part II, Book III, Ch. iii.

The earliest known gas chromatographic (GC) separations are more exactly described as examples of gas–solid chromatography, since each depended upon the adsorption of a vapor on to some solid support. Although these demonstrations antedated gas–liquid chromatography (GLC) by nearly three decades, it is vital to recognize that they did not contribute to the subsequent development of the more versatile gas–liquid techniques. Rather, GLC evolved as a rational extension of liquid–liquid partition studies.

Gas–solid analyses operate according to the principles of adsorption chromatography, whereas GLC, founded upon totally different precepts, involves the partition of a volatile component between a liquid phase and a gaseous phase. In fact, great efforts are expended to avoid gas–solid adsorption by use of chemically deactivated supports.

2. THE FORMATIVE YEARS (1941–1960)

> No great thing is created suddenly, any more than a bunch of grapes or a fig. If you tell me that you desire a fig, I answer you that there must be time. Let it first blossom, then bear fruit, then ripen.
>
> Epictetus, *Discourses*, Ch. XV.

Thirty years ago Martin and Synge[1], experimenting in the Wool Industries Research Laboratories at Leeds, co-authored their first paper on liquid–liquid partition chromatography. It was here that they first introduced the concept of GLC.

> The mobile phase need not be a liquid but may be a vapour... Very refined separations of volatile substances should therefore be possible in a column in which permanent gas is made to flow over gel impregnated with a non-volatile solvent in which the substances to be separated approximately obey Raoult's law[1].

This farsighted idea lay dormant for another decade until Martin, now pursuing his studies with James at the National Institute for Medical Research at Mill Hill, published the first report devoted to the subject of GLC[2].

Employing their novel technique Martin and James were able to separate and quantitate a mixture of eleven volatile acids (acetic to hendecanoic) on a 4 ft. × 4 mm column of Kieselguhr coated with DC-550 silicone oil containing 10% stearic acid. Nitrogen was used for the carrier gas, and detection was achieved by titration with sodium hydroxide using a phenol red indicator. The minimum amount of acid detectable by this method was 0.02 mg (for acetic acid).

The great success which accompanied their use of GLC for the microanalysis of these acids led Martin *et al.*[3] to apply the new method to the examination of volatile bases.

Acid-washed Kieselguhr, pretreated with methanolic sodium hydroxide to prevent adsorption, was used for the support, and the liquid phase was hendecanol–15% liquid paraffin. They separated ammonia, mono-, di- and trimethylamine, utilizing automatic titrations with sulfuric acid for the detection system. The lowest limit of amine detectable was reported to be 2 μg (for ammonia).

These workers soon perceived that a wider application of GLC methods was dependent upon improved modes of detection.

> The lower limit of quantity of material used is determined only by the efficiency of detection... The method of detection described here is acid–base titration, but many methods of detecting changes in the composition of a gas stream could be used...[2]

Thermal conductivity detectors (katharometers) were available, and actually had seen some use in gas–solid analyses. But they were relatively insensitive and failed to give a uniform response for all solutes. In later years, improvements in design and construction circumvented these disadvantages, making these detection systems standard items for nearly all the early commercial gas chromatographs.

Aware of its many drawbacks, Martin did not use a katharometer. Instead, he concentrated his efforts on developing a superior detector, and the results were impressive.

Once again an advance of consequence was provided by Martin and James[4] through their invention of the gas density meter. This detector exhibited high sensitivity and gave responses independent of the chemical structure of the substances being separated. Using nitrogen for the carrier gas Martin and James reported the detection of 1 molecule of pentanol in 10000 molecules of nitrogen. Although this detection system is no longer in use, its development was of great significance because it made possible the use of GLC for the separation of complex mixtures of organic compounds.

Following this lead, the next few years witnessed rapid advances in detector technology, with the best methods exploiting the physical properties of ionized gases for their operation.

Only two years after the introduction of the gas density meter, McWilliam and Dewar[5] at Ascot Vale, Australia, reported on a new detector which they designated as the flame ionization detector.

> It has been found that the electrical conductivity of a flame burning a mixture of hydrogen and nitrogen (in air) is very sensitively affected by the vapours of organic substances, and this effect can be used for detection purposes in GC[5].

This device could detect 4×10^{-10} g of diethyl ether; that is, one part (organic molecule) in 400 million (carrier gas). Its simplicity of design, stability of performance, linearity of response and uninfluenceability by such common contaminants as water vapor were attractions which soon made this detector the most intensively studied of all ionization methods.

About this time Lovelock[6], at the National Institute for Medical Research at Mill Hill, introduced the argon ionization detector, an apparatus which permitted the detection of as little as 2×10^{-12} moles of most organic substances. This detector, which was easy to construct, had several desirable properties: it was highly sensitive to vapors, yet insensitive to changes in temperature, pressure and gas flow-rate. Also, decomposition of organic molecules within the detector was negligible. 10 mCi of ^{90}Sr bonded in silver foil was used as the source of ionizating radiation, and argon was the choice for carrier gas.

> In practice only two rare gases are available in cylinder form and at prices which would permit the economic use of such an apparatus, namely helium and argon. By a fortunate coincidence the cheaper of these gases, argon, functions well in the device when used directly from commercial cylinders. Carefully purified helium would do even better but the commercial gas contains sufficient impurity, probably neon and argon, to discharge the metastable helium atoms shortly after their formation[6].

Lovelock, now working with Lipsky at Yale University, provided the next improvement in ionization technology[7]. Their new development, labelled as the electron capture detector, was a device for the qualitative analysis of a gaseous mixture based upon the ability of certain organic substances to capture free electrons.

> With many organic compounds the electron affinity is determined principally by the predominant functional group present in the molecule particularly when this includes some atom other than carbon or hydrogen. The size and configuration of the hydrocarbon moiety has relatively little effect. The electron affinities of such classes of compounds as esters, ethers, ketones and alcohols are all different and it is possible to assign an unknown organic compound to a class following the measurement of its electron affinity[7].

The achievability of the electron capture device is best illustrated in an experiment where, using a 100 ft.$\times$0.01 in. capillary column coated internally with a thin film of squalene, Lovelock and Lipsky chromatographed a 22-component hydrocarbon mixture containing 5% of a mixture of two ketones.

> The identification of the two ketones amidst the forest of hydrocarbon spikes would have been a formidable task with the conventional quantitative method (argon ionization detector). Using the qualitative method (electron capture detector), however, the presence of the two ketones is clearly distinguished[7].

One other aspect of GLC technology, which was received with great interest at this time, was the introduction of high resolution capillary columns by Golay of the Perkin-Elmer Co. in Norwark, Conn. At the Gas Chromatography Discussion Group held in Amsterdam in May 1958, Golay[8] presented a rigorous mathematical examination of the theory of capillary columns. Making use of an electrical analogy of resistances and condensers, Golay reasoned that the gas resistance in a routinely packed column of solid particles must be 10000 times greater than an ideal capillary column having the same HETP*. This finding led Golay to experiment with copper and steel capillary columns coated internally with a thin film of stationary liquid. Utilizing a 150 ft.×0.01 in. column coated with 1% didecyl phthalate, he separated *p*- and *m*-xylene within 1 h.

Researchers at Unilever in The Netherlands followed Golay's presentation with the first practical demonstration of the use of coated capillary columns[9]. These workers separated a mixture of fatty acid methyl esters using a 120-m copper capillary column coated internally with silicone grease.

3. THE EXPANSIVE YEARS (1960–1969)

> By their fruits ye shall know them.
> Matthew VII, 20.

The petroleum and polymer industries found immediate use for GLC, but its application to biological and medical problems was nearly non-existent. In 1960, however, GLC entered a new era. High-molecular-weight, structurally complex substances of great biological and medical importance were shown to be amenable to GLC. VandenHeuvel *et al.*[10], collaborating at the National Heart Institute in Maryland, published the first practical demonstration of the use of GLC for the separation of steroids. Although other workers[11,12] in Scotland and The Netherlands had shown that some steroids could be analyzed by GLC, thick coatings of liquid phase, high temperatures and protracted retention times obviated their general use. VandenHeuvel *et al.*, operating with a column of 2–3% SE-30 methyl silicone gum coated on to Chromosorb W, eluted cholesterol within 35 min at a temperature of 222° which precluded thermal decomposition.

> When a column containing 2-3/100 SE-30 silicone... was used... all of the compounds... were eluted as single components with no sign of decomposition; these included hydrocarbons, ketones, alcohols, ethers and acetyl esters. The short retention times observed at this relatively low temperature for comparatively high-molecular-weight compounds may be related to the effects observed when very thin liquid films are employed...[10]

A group of alkaloid chemists, closely associated with Horning in Maryland, were quick to apply this powerful new tool. Previously, the separation of alkaloids from crude alkaloid mixtures was a difficult endeavor, and was achieved only by laborious fractional crystallization, precipitation and countercurrent extraction**. The Maryland chemists, using the same 2–3% SE-30 column, reported the separation of forty-five alkaloids with retention times varying from 1.5 to 90 min (ref. 13).

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* HETP = Height equivalent to a theoretical plate. For a discussion of the plate concept refer to A. I. M. Keulemans, *Gas Chromatography*, Reinhold, New York, 1959, p. 108.

** *Note by Editor*: Paper and thin-layer chromatography were then already in extensive use and have not been superseded by GLC.

The effectiveness of GLC was well illustrated in the separation of codeine, morphine, thebaine, laudanosine, papaverine and gnoscopine from a mixture of *Papaveraceae* alkaloids.

In March 1961 VandenHeuvel and Horning, now joined by Haahti, a visiting scientist from the University of Turku in Finland, introduced a new liquid phase for steroid separations[14]. In their earlier work the Horning group used SE-30, a non-selective liquid phase which effected separation on the basis of molecular weight and molecule shape. Their new phase was Dow Corning's QF-1, a fluorinated alkyl silicone polymer. This was shown to be a highly selective phase which was particularly effective for the separation of closely related steroid alcohols, ketones and esters.

> QF-1 showed a selective behavior. An increase in retention times was observed for compounds with oxygen-containing functional groups in the order ether < hydroxyl < ester < keto. Further, the retention times for hydroxy and keto steroids varied with structural variations to a far greater extent than has been observed for other phases[14].

Dramatic separations of cholesterol and cholestanol were achieved.

> Another example is the separation of 5α-pregnane-3β,20α-diol and 5α-pregnane-20β-ol-3-one; this pair has not been amenable to separation with other phases[14].

Continuing in their pursuit of improved steroid separations, Horning *et al.*[15] examined five different polyester liquid phases: neopentyl glycol adipate, neopentyl glycol succinate, ethylene glycol adipate, ethylene glycol succinate and ethylene glycol isophthalate. Neopentyl glycol succinate became the polyester phase which found the most extensive use in GLC. This phase has a high degree of thermal stability, and is selective for ketones, esters and carbon–carbon unsaturated bonds.

Among other types of liquid phases investigated the methyl phenyl siloxane polymers were found to possess the desirable property of selectivity based upon the amount of carbon–carbon unsaturation present[16]. The separation of desmosterol from cholesterol, for example, could be achieved most effectively with low-efficiency columns. Unfortunately, the inability of this phase to separate stereoisomers precludes its application to steroid identification work.

Up to this point in the evolution of GLC there existed only two ways to effect an acceptable separation of closely related hydroxy- and keto-substituted steroids. One could use either the capillary columns of Golay or one of the newer selective phases, such as QF-1. Soon, however, reports began to appear in the literature delineating additional methods, each of which involved the formation of derivatives.

The Maryland group published the results of their experience with trifluoroacetylation[17]. This method of derivatization, coupled with the use of selective phases, resulted in sharp separations of a closely related series of high-molecular-weight bile acid methyl esters (mol. wt. >700).

Subsequent to the Maryland report, Wotiz and Martin[18] of the Boston University School of Medicine published a paper in which they recommended the use of acetic acid esters for the separation of estrogens. Acetates had a major drawback: high temperatures were required to obtain reasonable retention times, even with short columns.

Shortly after their studies with the trifluoroacetyl derivatives the Horning

group, which now included Luukkainen, a scientist from the University of Helsinki, released their work on trimethylsilyl ether derivatization[19].

> With the non-selective phase SE-30 the retention times for the ethers were somewhat greater than those for the parent compound. This is not unexpected, since separations with an SE-30 phase follow approximately the order of molecular weight. With selective phases, the retention times of the ethers are significantly less than those of the parent compound, and the effect is particularly great for polyhydroxy compounds[19].

They went on to describe the several useful properties observed for these derivatives.

> Epimer separation factors may be materially increased; for example, the cholesterol/epicholestanol separation factor is 1.03 on a phenylsilicone phase, but the value for the trimethylsilyl ethers under the same conditions is 1.42... The relatively rapid elution and excellent resolution... as trimethylsilyl ethers suggest that these derivatives may be particularly useful in work with estrogens[19].

This fundamental research was soon followed by a practical demonstration of the useful properties of trimethylsilyl ethers of estrone, estradiol-17β and estriol[20]. This study is noteworthy from one other aspect. Temperature-programming was applied to a strictly biological problem: evaluation of the steroid profile of first trimester pregnancy urines.

As a result of a suggestion by Lovelock, Wotiz, now collaborating with Clark, prepared heptafluorobutyrate derivatives of hydroxyl-containing steroids[21]. Using a commercial gas chromatograph with a Lovelock electron capture detector they could detect subnanogram amounts of estrogens.

This method of derivatization has been used to great advantage in recent years. For example, heptafluorobutyrate derivatives have often replaced other more cumbersome and tedious methods (double-isotope-derivative techniques) for measuring plasma levels of androgens.

Some five years after Wotiz reported on the heptafluorobutyrates, Kirschner and Coffman[22] at the National Institute of Health in Maryland isolated and quantified subnanogram amounts of testosterone and Δ^4-androstenedione from human plasma samples.

Improvements were made at Oxford for measuring testosterone in peripheral plasma[23]. Analyses could now be accomplished with as little as 2.5 ml of plasma, whereas the American group needed ten times as much[22]. Exley[23] reported that one could detect 40 pg of testosterone in male peripheral plasma using such derivatives.

> The lack of development of highly sensitive techniques with these esters appears to have been due to differences in the quality of the commercially available esterification reagent, and to confusion because testosterone 17-monoheptafluorobutyrate and testosterone diheptafluorobutyrate (produced by esterification of the 3-enol) could be produced by different esterification conditions[23].

Exley overcame these difficulties by preparing pure heptafluorobutyric anhydride reagent, thereby producing nearly quantitative yields of testosterone diheptafluorobutyrate.

> The diheptafluorobutyrate is five times as electron-absorptive as testosterone 17-monoheptafluorobutyrate, and is possibly one of the most sensitive of all the electron-capturing derivatives yet devised for steroids[23].

Horning, now Director of the Institute for Lipid Research at Baylor College of Medicine in Houston, Texas, became interested in these studies by Wotiz and Exley. Horning's previous investigations of testosterone enol ethers had revealed that two isomeric enol ethers were always formed under silylating conditions. He reasoned therefrom that acylation with heptafluorobutyric anhydride might also lead to two isomeric enol esters. Indeed, such was found to be the case[24].

> Our results indicate that two products are always formed when this reaction is carried out for testosterone, and by using GC–mass spectrometry to study the structures it was found that both compounds were diacyl derivatives corresponding to enol esters[24].

These compounds were shown to be the diheptafluorobutyric esters of 2,4-androstadiene-3,17β-diol and 3,5-androstadiene-3,17β-diol.

Because the products are unstable in the standard reaction mixture (a benzene solution) and their ratio of formation varies with acylation conditions, Horning emphasized that, contrary to Exley's recommendations, this procedure is less than suitable for the most exact work. Acylation in a pyridine solution, on the other hand, appears applicable to quantitation.

Perhaps the most significant development in the last few years has been the development of a combined gas chromatograph–mass spectrometer. The key to the practical operation of such a system lay in the molecular separator designed by Ryhage[25] at the Mass Spectrometry Laboratories of the Karolinska Institutet, Stockholm.

Ryhage analyzed mixtures of fatty acid methyl esters. He used an instrument employing a column packed with 1% SE-30 on silanized Gas-Chrom P connected directly to an Atlas CH 4 mass spectrometer, equipped with a fast recording system for scanning the mass range *m/e* 12 to 500 in 1–2 sec. The column effluent was directed into two molecular separators connected in series where 99% of the carrier gas (helium) was removed by two pumping systems, and the sample presented to the ion chamber in a relatively concentrated state.

The direct coupling of the gas chromatograph to the mass spectrometer was a great improvement over earlier attempts to collect samples from a gas chromatograph in a cold trap, and subsequently analyze them in a mass spectrometer.

Ryhage was aware, however, that direct connections also created problems.

> The bleeding from... columns is much more critical when a mass spectrometer is used as a detector, as it will give a high background for the mass spectra[25].

To illustrate the usefulness of the combined instruments Ryhage separated a naturally occurring complex mixture of fatty acids from butterfat.

> From the mass spectra it was possible to identify a homologous series of *n*-saturated, *n*-unsaturated, and branched-chain fatty acid methyl esters present in this mixture[25].

These instruments are not confined to fatty acid studies, however.

> Several other classes of organic compounds such as diterpenes, ketones, alcohols, ketonic steroids, and sterols have been analyzed with good results and these experiences have shown that all compounds which can be subjected to GC may also be analyzed with the compound instrument[25].

Ryhage and his coworkers[26] soon exhibited the versatility of this novel combined instrumentation in a clinical study in which they measured the neutral fecal steroids from patients confined to a carefully standardized diet. It is interesting to note that the serum cholesterol level fell in all subjects when corn oil replaced butter in the diet. Correlative data disclosed that cholesterol excretion was elevated during the corn oil period.

4. ENVOI

GLC is a young discipline —all of its major contributors are still alive and practicing their art. Yet, it has had an immense impact on science. Many difficult analytical problems, once approached with trepidation, are now considered commonplace. Truly, GLC stands high as a classic example of the vital role technological advances play in the evolution of basic research.

> In the experimental sciences all progress is measured by improvement in the means of investigation... Each time that a new and reliable means of experimental analysis makes its appearance, we invariably see science make progress in the questions to which this means of analysis can be applied... In a word, the greatest scientific truths are rooted in details of experimental investigation which forms, as it were, the soil in which these truths develop.
>
> Claude Bernard, *Introduction à l'étude de la médecine expérimentale*, 1865, Ch. I.

Editor's comment. The author of this history quotes his favourite authors frequently and thus prompted me to quote one of mine:

> It is reported that one day a Jesuit, who was a friend of Pope Pius XII, said to His Holiness: "Holy Father, I have been speaking at some length to a most learned man. He told me that only half of history was true. Does Your Holiness consider that correct?
> Pope Pius XII, who was a learned man himself, thought for a while before he answered. Then he said:
> "Less than half."
>
> Aubrey Menen, *Rome revealed*, Thames and Hudson, 1960, p. 9.

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