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# The Application of the Silica Gel Partition Chromatogram to the Estimation of Volatile Fatty Acids

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The lower fatty acids, with the exception of formic acid, are so similar in their chemical properties that their quantitative separation and estimation by purely chemical methods have not proved possible. On the other hand, marked changes in physical properties occur as the series is ascended; for example, the volatility in steam increases and the partition coefficient between water and immiscible organic solvents decreases. It is not surprising, therefore, that the methods available for the estimation of these compounds in mixtures should be physical rather than chemical.

The distillation procedure of Duclaux (1874) and its numerous refinements make use of the observation that the volatile fatty acids distil in steam at different rates, and that in a mixture each distils at its own characteristic speed regardless of the presence of others. The partition method introduced by Behrens (1926), subsequently improved by Werkman (1930), depends on the fact that each fatty acid partitions itself between water and an immiscible organic solvent irrespective of the other acids present. Although both these methods, in the absence of more satisfactory ones, have found favour with the analyst, it should be emphasized that both have their limitations. They are inadequate for work with more than two acids, they are of little use by themselves for the identification of the constituents of a mixture, and neither achieves a complete resolution of even a binary mixture into its components. In consequence, the amount of each acid present is obtained by calculation rather than by direct assay. Indeed, until recently, no method has been available for the separation, identification and estimation of the lower fatty acids in a mixture Schicktanz, Steele & Blaisdell (1940) introduced a procedure based on the fractional distillation, with an efficient column, of the azeotropes formed be tween the lower fatty acids and aryl hydrocarbons. In this manner they were able to separate a mixture containing formic, acetic, propionic, *n*-butyric and *iso*-butyric acids into its components. This represented a notable advance in technique, but the method is not quantitative and is scarcely suitable for routine analysis.

Lester Smith (1942) demonstrated that the silica-gel partition chromatogram, developed by Martin & Synge (1941), would separate a mixture of formic, acetic, propionic, *n*-butyric and *n*-valeric acids in chloroform into its component acids. Only compounds with differing partition coefficients between water and the organic developing solvent can be separated by this method. Martin & Synge (1941) incorporated in the gel an indicator of suitable pK for the location of colourless organic acids on the column, and for the separation of acetylated amino-acids they initially recommended methyl orange; this indicator was subsequently supplemented by anthocyanins (Gordon, Martin & Synge, 1943, 1944) which are not leached so readily as methyl orange. More recently Liddell & Rydon (1944) have introduced an azo dye, 3:6-disulpho-$\beta$-naphthalene-azo-*N*-phenyl-$\alpha$-naphthylamine, with similar properties and the added advantage that it is readily prepared in quantity in the laboratory. Lester Smith (1942) found that methyl orange was of little use when the partition chromatogram was used for the separation of lower fatty acids, owing to lack of sensitivity, the

volatile fatty acids having a significantly higher pK than the acetylated amino-acids, and he recommended bromocresol green (pK 4·66) as a suitable alternative. This has been confirmed, and in addition it has been found that both anthocyanins (pelargonin) and the Liddell & Rydon indicator are insensitive as compared with bromocresol green.

Lester Smith published no investigation of the quantitative aspects of the method, but the procedure was of value for the identification of lower fatty acids present in a chloroform solution; furthermore the results appeared so promising that a further investigation has been made with the object of developing a routine method for the separation, identification and quantitative estimation of volatile fatty acids in biological materials, and particularly in fermentation fluids.

## EXPERIMENTAL

### *Preparation of the chromatogram*

Silica gels prepared by the method of Gordon *et al.* (1943) gave the most suitable chromatograms when 3 g. of gel were mixed with 1·8 ml. of water; such a preparation, however, was acid to bromocresol green, and remained so after washing with large volumes of distilled water. It was found expedient therefore to add a quantity of 0·1 N-NaOH to the bromocresol green solution such that 1·8 ml. of the indicator mixed with 3 g. of gel gave a light blue powder. A solution of bromocresol green (0·2%), made according to Cole (1933), and containing in addition 17·4 ml. of 0·1 N-NaOH/100 ml., was employed. This quantity of NaOH has been found adequate for all preparations of gel so far made.

The developing solvents used were a mixture of chloroform and butanol. Technical chloroform was purified by washing thoroughly with tap water to remove ethanol, drying over $CaCl_2$, and distilling over a packed column, the fraction boiling at 61–62° being collected. Used chloroform was recovered in the same way after a preliminary extraction with NaOH solution to remove acid substances. Two chloroform-butanol mixtures were employed containing 1 and 5% (v/v) of butanol respectively ($CB_1$ and $CB_5$). They were stored dry in brown glass bottles; prior to use on the chromatogram they were saturated with distilled water.

The chromatogram tubes were about 40 cm. in length and constricted at one end. A perforated silver disk, bearing a circle of filter paper, was lodged on the constriction and this served to support the column. The gel (3 g.) was weighed, transferred to a clean mortar, and ground to a fine powder. The indicator solution (1·8 ml.) was added and the mixture ground with the aid of a pestle and a stainless steel spatula until a homogeneous, finely divided, light blue powder was obtained. A small amount of wet $CB_1$ was added and the resulting green paste ground for a further period; more wet $CB_1$ was added, to a total volume of some 30 ml., and the slurry poured into the chromatogram tube.

The tube was immediately rotated smartly between the hands to remove any air bubbles trapped between the particles of gel. The column was then allowed to pack down. During this stage it is important that the surface be kept covered with the developing solvent, for if allowed to dry, even for a short period, the column is liable to crack; should this occur the column is extruded, mixed with $CB_1$ and remade. The developing solvent must be added carefully without disturbing the surface of the column, as this leads to unevenness of the bands. When the column ceased to contract, 1 ml. of 0·03 N-*n*-valeric acid (Kahlbaum) in $CB_1$ was run on to the column and washed through with the same solvent. This served two purposes, (*a*) of testing the column for evenness of packing, and (*b*) of 'neutralizing' any local concentration of alkali, which occasionally appears as a result of uneven mixing of the alkaline indicator solution with the gel.

### *The behaviour of fatty acids on the chromatogram*

Preliminary experiments with columns prepared as described above, and using $CB_1$ as the developing solvent, confirmed the observation of Lester Smith that the partition chromatogram would resolve a chloroform solution containing formic, acetic, propionic, *n*-butyric and *n*-valeric into its component acids. Formic acid remained fixed at the top of the chromatogram and the remainder passed down the column as discrete yellow bands, moving at different rates; *n*-valeric acid moved fastest and acetic acid the slowest. The leading edge of each band was sharp and well defined, whereas the rear edge tended to be diffuse and its termination not always easy to detect. Propionic, *n*-butyric and *n*-valeric acids were washed through the column with $CB_1$; but to remove acetic acid it was necessary to use $CB_5$ and this resulted in the leaching out of some of the indicator. Formic acid could only be washed out by raising the butanol concentration to 20% (v/v).

Martin & Synge (1941) demonstrated that, in the case of the partition chromatogram, $R$, the ratio between the rate of movement of a compound down the column and the rate of movement of the developing solvent in the tube above the chromatogram is related to the partition coefficient of the compound between water and the non-polar phase. The value $R$ is thus characteristic of the compound. In the case of the volatile fatty acids it has been established that the partition coefficient varies with concentration (see Smith & White, 1929), and it is to be expected therefore that the $R$ values of these compounds will vary in a similar fashion. In consequence, if the $R$ value is to be used for the identification of the volatile fatty acids, the degree of variation must be determined over the range of concentrations likely to be met in practice.

Stock solutions of acetic, propionic, *n*-butyric and *n*-valeric acids (Kahlbaum) were made up in pure chloroform, and from these a suitable range of concentrations of each acid prepared. Samples of 1 ml. were used for the determination of the $R$ value. The

sample was delivered on to the surface of a column with a pipette calibrated to deliver between two marks. When the sample had percolated into the gel the chromatogram was developed with $CB_1$; at intervals during the development the distances travelled both by the meniscus of the developing solvent and the leading edge of the band were measured, and the $R$ values calculated. The values so obtained for different concentrations were plotted against concentration and the resulting curves, given in Fig. 1, show that the $R$ values vary with

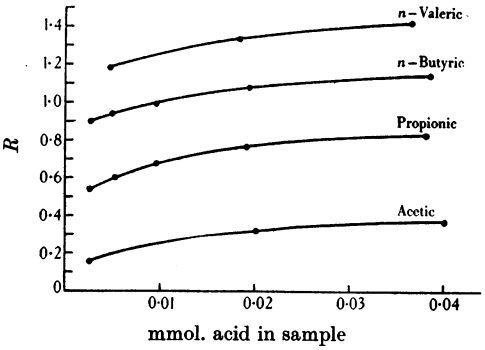


Fig. 1. The effect of concentration on the $R$ value of fatty acids. ($R$ = mm. moved by band/mm. moved by developing solvent in the tube above the chromatogram.)

concentration. To identify an unknown fatty acid therefore, it is necessary to determine both the $R$ value and the concentration. In all probability, however, the method will not distinguish between *n*-butyric and *iso*-butyric acids on account of the similarity of their partition coefficients; nor, for the same reason, would it be possible to separate the four isomers of valeric acid.

### *The estimation of volatile fatty acids*

Two preliminary steps are necessary before the chromatographic procedure can be applied to the analysis of volatile fatty acids in biological materials: (1) the volatile fatty acids must be separated from all other acidic substances, (2) the volatile fatty acids must be transferred quantitatively from aqueous solution to as small a volume of chloroform as possible. For stage (1) steam distillation is best, and of the numerous procedures available, the double distillation method of Friedemann (1938) has proved the most reliable. In the second distillation formic acid is oxidized so that the final distillate contains all the other volatile fatty acids. The exclusion of formic acid is desirable because it is difficult to remove from the column. Chemical methods are available for its estimation (Pickett, Ley & Zygmuntowicz, 1944; Dakin, Janney & Wakeman, 1913).

The fatty acids present in the distillate were quantitatively transferred to chloroform in the following manner. A drop of phenol red was added, the aqueous solution made alkaline with a slight excess of 0·1 N-NaOH, and evaporated to about 0·1–0·2 ml., the last stages being carried out in a long-necked 100 ml. round-bottomed flask heated on a boiling water-bath. Evaporation was facilitated by a blast of compressed air. When the fluid had been reduced to the desired volume, the flask was set aside to cool and drain. Finely powdered A.R. anhydrous $KHSO_4$ (3–4 g.) was added until a fairly dry, pink powder was obtained. The product was then loosened by tapping the flask and gently swirled to make certain that small amounts of liquid were not retained on the walls. The solid was now extracted with six 4 ml. portions of dry $CB_5$ and the combined extracts diluted to 25 ml. When pure chloroform or $CB_1$ was used the extraction was not quantitative.

The total volatile acids in this extract were determined as follows. Using a pipette calibrated to deliver between two marks, a suitable volume was transferred to a 6 × 1¼ in. boiling tube and *c.* 20 ml. distilled water added, followed by 1 drop of 0·1 % phenol red as indicator. A brisk stream of $CO_2$-free air was bubbled through the aqueous phase for 3 min. (a sintered glass gas distributor was used to provide a stream of very fine bubbles). The acids were titrated with 0·01 N-NaOH from a 5 ml. burette graduated in 0·01 ml. with the current of $CO_2$-free air continued throughout the titration. Towards the end of the titration it was essential to shake the tube vigorously to complete the extraction of the acids from the solvent mixture; with these precautions a very sharp end-point was obtained. When large volumes of chloroform were used, emulsions sometimes formed which resulted in the addition of excess standard alkali, and it was found convenient to back titrate this excess with 0·01 N-acid. The efficiency of the extraction procedure can be judged by reference to column 1 of Table 1.

A suitable volume of the chloroform extract (see below) was pipetted on to a chromatogram, prepared as described in the previous section, and the chromatogram developed with $CB_1$. Each fraction except acetic acid was collected separately in a 6 × 1¼ in. boiling tube and titrated with 0·01 N-NaOH with the precautions outlined above. To ensure the complete recovery of each fraction it was necessary to collect the developing solvent from the time the leading edge of the first was about to leave the column until the leading edge of the succeeding band was about to leave. The determination of acetic acid by difference was necessary owing to the fact that $CB_5$, used to 'elute' acetic acid, leached considerable quantities of indicator, which obscured the end-point of the titration. This procedure is not

so satisfactory as a direct determination, but must continue to be employed until an indicator is found with the sensitivity of bromocresol green, but which, at the same time, is not leached by the developing solvent.

To assess the accuracy and precision of the method, 30 ml. of an aqueous solution containing known amounts of acetic, propionic and *n*-butyric acids were evaporated down and put through the extraction and chromatographic procedures outlined above. The results of fifteen such tests are given in Table 1. It will be seen that the recovery of each acid is adequate for most purposes.

Table 1. *Precision and accuracy of the determination of acetic, propionic and butyric acids in a mixture*

(Fifteen determinations were carried out on a standard mixture of the three acids and the results are expressed as the mean recovery and standard deviation in terms of 0·01 N-reagent. Volume of chloroform extract, 1 ml.)

| | Total acid (ml.) | Butyric acid (ml.) | Propionic acid (ml.) | Acetic acid (ml.) |
|---|---|---|---|---|
| Theoretical | 28·93 | 9·31 | 9·58 | 10·04 |
| Observed | 28·86±0·35 | 9·4±0·18 | 9·52±0·26 | 9·93±0·31 |

At the end of an estimation the chromatogram was freed from acetic acid by a thorough wash through with $CB_5$, followed by $CB_1$, and it was then ready for further use. In this way columns have been used as many as six times without any loss of performance, and in spite of the leaching of considerable quantities of indicator. A chromatogram, once made, can be preserved for a number of days by immersing the column in a tube of $CB_1$.

This chromatographic procedure has been applied over the past two years to the analysis of the volatile fatty acids in the rumen of the sheep, and has been found satisfactory.

*Additional precautions.* If good results are to be obtained with this technique, the following additional points should be borne in mind.

(1) If too large a volume of chloroform extract is taken for analysis on the partition chromatogram, the bands become broad and diffuse with a strong tendency to overlap. Table 2 records two experiments where 5 ml. samples were used; there was an apparent loss of some 30% of the *n*-butyric acid despite the fact that the recovery of total acid was good. This apparent loss was due to the fact that the butyric band was unnecessarily broadened and during the development the rear edge of the band was entirely engulfed by the leading edge of the propionic acid band. The overlapping was not due to the fact that the propionic acid was in excess of the *n*-butyric acid. The volume of chloroform extract taken for analysis should not exceed 3 ml. and should preferably be less.

Table 2. *Effect of volume of sample on the recovery of acetic, propionic and butyric acids*

(Volume of chloroform extract, 5 ml. Results in terms of 0·01 N-reagent.)

| | Total acid (ml.) | Butyric acid (ml.) | Propionic acid (ml.) | Acetic acid (ml.) |
|---|---|---|---|---|
| Theoretical | 86·5 | 9·5 | 47·2 | 29·9 |
| Observed: (1) | 86·3 | 7·22 (76%) | 48·4 (102·5%) | 30·7 (102·5%) |
| (2) | 85·1 | 6·97 (73·5%) | 48·9 (103·5%) | 29·2 (97·5%) |

(2) The 3 g. column should not be overloaded with acid, and the sample taken for analysis should not contain more than a total of 10 ml. of 0·01 N-acid.

(3) Each batch of gel should be thoroughly tested before being put to routine use. Recent batches of gel have necessitated the use of $CB_5$ throughout, and were observed to bind the indicator much more strongly than previous preparations; at the same time there was no evidence of loss of performance. The reason for this change in properties is as yet unknown.

## SUMMARY

1. The silica-gel partition chromatogram, with bromocresol green as indicator, is found to resolve a chloroform solution of the acids formic, acetic, propionic, *n*-butyric and *n*-valeric.

2. The *R* values of the fatty acids vary with the concentration.

3. The procedure has been adapted for the identification and quantitative estimation of acetic, propionic and butyric acids in a mixture containing all three acids.

4. Formic acid cannot be estimated by this method and must be removed from the solution taken for analysis.

5. Elution of acetic acid is accompanied by the leaching out of the indicator in amounts sufficient to obscure the end-point in the final titration. Acetic acid therefore is determined by difference.

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# The Action of Arsine on Blood

## OBSERVATIONS ON THE NATURE OF THE FIXED ARSENIC

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A striking feature of poisoning by the inhalation of arsine is the haemolysis which occurs after a short latent period. It is known that arsine is 'fixed' in some non-volatile form by blood in the presence of oxygen and that this 'fixation' may be accompanied by changes in the colour and spectroscopic characteristics of the blood pigment, but little is known with certainty regarding the chemical changes involved in these phenomena and no satisfactory explanation of the mechanism of the haemolysis has been advanced.

That oxygen plays an essential part in the chemical changes was first indicated by Naunyn (1868), who showed that in its absence haemolysis did not occur in blood treated *in vitro* with arsine. This observation was repeatedly confirmed, e.g. by Meissner (1913), who recognized that arsine is fixed by blood in a non-volatile form in the presence of oxygen. He ascribed this fixation to oxidation to arsenious or arsenic acid, or to the formation of a complex compound, and discovered the important fact that haemoglobin is the only constituent of blood which will react vigorously with arsine. That the fixed arsenic is largely confined to the erythrocytes up to the time of commencement of haemolysis was made clear by the experiments of Thauer (1934), and this has been confirmed in experiments preliminary to the present work.

The chemical nature of the fixed arsenic formed in blood was considered as early as 1865 by Eulenberg, who examined the blood of an arsine-poisoned cat, and on the basis of precipitation experiments with magnesia mixture reported that arsenious acid but not arsenic acid was present. The use of such methods for the detection of small amounts of arsenious and arsenic acids in animal experiments was criticized by Joachimoglu (1916). Stricker (1882) also reported that arsine is oxidized in blood to arsenious acid, but we have been unable to consult the original work.

Labes (1928, 1937) suggested that in the presence of oxygen arsine becomes oxidized within the erythrocyte to colloidal elementary arsenic, which he supposed to be the active haemolytic agent. He observed that colloidal solutions of elementary arsenic haemolyze erythrocyte suspensions, but presented no direct evidence that intracellular colloidal arsenic is formed when blood is treated with arsine.

Extensive studies of arsine fixation by haemoglobin and of the nature of the haemolytic agent have been made by Heubner and his co-workers, who allowed whole blood, erythrocyte suspensions, or haemoglobin solutions to react with arsine in the presence of oxygen. From the arsenic content of the reaction products and from the amount of oxygen utilized surmises as to the probable nature of the fixed arsenic were made. Heubner & Wolff (1936) considered that arsenic dihydride $(AsH_2)_2$, was first formed and that this compound was the haemolytic agent. Their figures further indicated that the final product of oxidation of arsine in the presence of haemoglobin was arsenious acid. Wolff (1936) agreed that this was the final product, but suggested that the intermediate compound was hydroxyarsine ($AsH_2$-OH). Gebert (1937) suggested that arsenic dihydride was formed as an intermediate in the oxidation of arsine to elementary arsenic, the latter being eventually further oxidized to 'arsenik'.

These conclusions of Labes and of the Heubner school seem to be coloured by the assumption that an oxidation product of arsine must necessarily be the direct haemolytic agent.

In the present work an attempt has been made to gain a further insight into the chemical mechanisms involved in the arsine-blood reaction, by a direct examination of the properties and behaviour of the fixed arsenic formed in the blood of arsine-poisoned rabbits, and in normal rabbit blood and haemoglobin solutions treated *in vitro* with arsine.