recently been shown (Peters & Wakelin, 1948; Grob, 1946) that trypsin and chymotrypsin are inactivated by thiol compounds, whereas the skin proteinase is not, the conclusion stands firm at present that it is a proteinase of different type.

## SUMMARY

1. The proteinase present in acetone-dried extracts and in Fruton-type extracts of fresh skin from the rat have been compared and further characterized in the light of Fruton's results.

2. The acetone-dried extracts have high proteinase activity and low dermo- and amino-peptidase activity with only slight activation effects of manganese, whereas the fresh skin extracts have a high peptidase and low proteinase activity.

3. The skin proteinase differs from previously recognized enzymes.

4. Manganese activates the leucine aminopeptidase optimally at 0·002M; cobalt and magnesium also activate.

We are indebted to Prof. K. Linderstrøm-Lang for his hospitality and kindness in showing his technique; also to the Nuffield Committee and to the Osler Trustees (Oxford) for grants in aid of this work.

## REFERENCES

Beloff, A. & Peters, R. A. (1945). *J. Physiol.* **103**, 461.
Berger, J. & Johnson, M. J. (1939). *J. biol. Chem.* **130**, 641.
Fruton, J. S. (1946). *J. biol. Chem.* **166**, 721.
Grob, D. (1946). *J. gen. Physiol.* **29**, 219.
Linderstrøm-Lang, K. (1927). *C.R. Lab. Carlsberg*, **17**, no. 4.
Maschmann, E. (1940). *Naturwissenschaften*, **28**, 780.
Maschmann, E. (1942). *Biochem. Z.* **311**, 29, 252, 374.
Peters, R. A. & Wakelin, R. W. (1948). *Biochem. J.* **43**, 45.

# Separation and Estimation of Saturated $C_2$–$C_8$ Fatty Acids by Buffered Partition Columns*

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The lack of adequate small-scale methods for the analysis of mixtures of fatty acids has long been felt. Following up an observation of Lester Smith (1942), Elsden (1946*a*) has recently introduced an important new technique, based on partition chromatography, for the separation and estimation of the lower members of the saturated series of fatty acids. A wet silica gel was impregnated with bromocresol green, a small sample of the mixture of acids, dissolved in chloroform, was introduced at the top of the column and, after its penetration into the gel had taken place, a suitable mixture of butanol and chloroform was allowed to percolate through the column. By using the colour change of the indicator as a pointer to the positions occupied by the acids, Elsden was able to estimate propionic and butyric acids. Under certain conditions valeric acid also could be estimated, but the precise behaviour of the gel is conditional upon its properties. Acetic acid could not be estimated except by difference. The method has been used by Elsden (1946*b*) and by Elsden, Hitchcock, Marshall & Phillipson (1946) with excellent results in the analysis of rumen contents, but it has certain disadvantages. Different batches of silica prepared in the same manner often differ considerably in their properties, and of several batches only one may be suitable for use. The range of the method is very limited: acetic acid can only be estimated by difference and the method cannot ordinarily be applied to acids higher than butyric; such higher acids fail to undergo separation and can only be determined collectively. Finally, no separation is possible between the isomers of any given acid.

We have now devised a procedure which obviates most of the disadvantages of Elsden's method and are able to identify, separate and estimate most of the naturally occurring, steam-volatile members of the saturated fatty acid series with a high order of accuracy and recovery. The use of indicators on the column is dispensed with, acetic acid can be determined directly, small differences in the properties of the silica are of no consequence, and almost complete resolution of certain isomeric valeric acids has been achieved.

* A preliminary account of this work was communicated by one of us (R. S.) to the Biochemical Society on 31 October 1947 (Scarisbrick, Baldwin & Moyle, 1948).

In order to extend the range of the method it was necessary to find conditions which would increase the relative solubility of the higher acids in the stationary, aqueous phase. We tried the use of alkaline columns with the idea of altering the effective partition coefficient in favour of the aqueous phase, and even in our earliest experiments we were able to achieve virtually complete separations of caproic and valeric acids (Fig. 1). It was

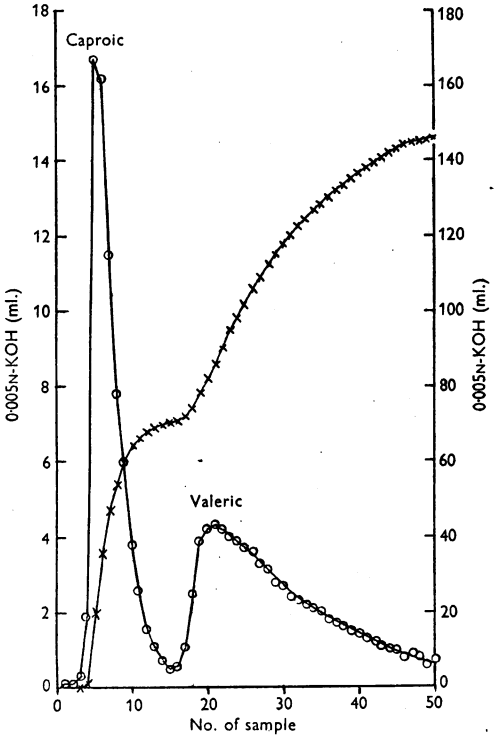


Fig. 1. Separation of caproic and valeric acids. Column: 5 g. silica, 0·5 ml. litmus, 2·5 ml. 2·5M-$K_2HPO_4$, 0·34 ml. 10N-KOH. Solvent: chloroform. Ordinates: titres of successive 5 ml. samples of chloroform elutriates, o individual (scale on left), × aggregate (scale on right).

hoped that by means of even more alkaline columns still higher acids would prove to be separable but, unfortunately, as the length of the fatty chain is increased, the solubility of the soaps decreases rapidly and sets an eventual upper limit to the range of the method; lauric ($C_{12}$) and higher acids cause blockage of the columns. It is necessary, therefore, to effect a preliminary separation of the lower acids from lauric acid and higher members of the series; this can be done satisfactorily by steam distillation by the method of Friedemann (1938) whereby formic acid, if present, is destroyed. The free, steam-volatile acids are then recovered in solution in a suitable mixture of chloroform and butanol by a procedure similar to that of Elsden (1946*a*, *b*).

Throughout our experiments we have used columns heavily buffered with phosphate. Neither this nor the employment of alkaline gels is entirely novel (see Synge, 1946, for further references) but, so far as we are aware, columns of the type used here have not been previously used for the same purpose, although Sato, Bary & Craig (1947) have used buffers in a method based on 'counter-current distribution' which allows the separation of fatty acids as far as valeric. Goodall & Levi (1946) have also used buffered chromatograms in a micromethod for the separation of the various types of penicillins on filter paper.

## EXPERIMENTAL

### *Reagents*

*Solvents.* Chloroform, previously washed with water and dried over $CaCl_2$, and *n*-butanol are redistilled in an all-glass apparatus. Normally we use mixtures containing 1, 10 and 30% (v/v) of *n*-butanol in chloroform (referred to in the text as '1% butanol', etc.), all of which are equilibrated by shaking with 2M-$K_2HPO_4$. It is advisable to filter the equilibrated solvents through dry paper before use.

*Silica.* Silica may be prepared according to the directions given by Gordon, Martin & Synge (1943) or Isherwood (1946). Several different preparations were used in the course of these experiments but, as we shall show, there is little if anything to choose between different samples.

*Buffers.* Stock 2M solutions of $KH_2PO_4$, $K_2HPO_4$ and $K_3PO_4$ are required and should be made up from the purest specimens available. We found that a commercial sample of $K_3PO_4$ gave very poor recoveries compared with a specimen made by adding the theoretical amount of A.R. KOH to A.R. $KH_2PO_4$. From these stocks the following standard buffers are prepared: I, 2 vol. $K_2HPO_4$ + 1 vol. $KH_2PO_4$; II, 2·5 vol. $K_2HPO_4$ + 3·5 vol. $K_3PO_4$; III, $K_3PO_4$ alone; IV, $KH_2PO_4$ alone. These are referred to as 'buffers I, II', etc.

### *General procedure*

*Columns.* Five g. silica are intimately mixed with 3 ml. of the appropriate buffer by grinding in a small mortar. The mass is suspended in about 40 ml. 1% butanol and poured through a funnel into a tube of the usual design (a drawing of the apparatus actually employed is reproduced in Fig. 2), and the tube refilled with 1% butanol by means of a separating funnel with a bent outlet (Fig. 2*A*). The solvent is then allowed to percolate through the column for 20–30 min. to permit proper packing of the gel and the formation of a firm surface.

*Introduction of acids.* The acids to be analyzed are introduced in the form of a solution, usually in 1% butanol. Samples as large as 50 ml. may be taken if necessary, but for most purposes 1–5 ml. is convenient. When small samples are to be introduced the device illustrated in Fig. 2*B* is useful: the delivery tube is placed in position with its tip a few millimetres above the surface of the gel. As soon as the last of the solvent has drained into the gel the sample is cautiously run in through the delivery tube. At the same moment the tap (*T*) is closed and the automatic siphon (*S*) placed in position with a sampling tube

below. The tap (*T*) is then reopened. As soon as the sample has drained into the gel the delivery tube is washed down twice with about 1 ml. 1% butanol and then withdrawn. The separating funnel, already charged with 1% butanol, is placed in position. As soon as the washings have entered the gel the tap of the funnel is carefully opened and the solvent admitted to the column.

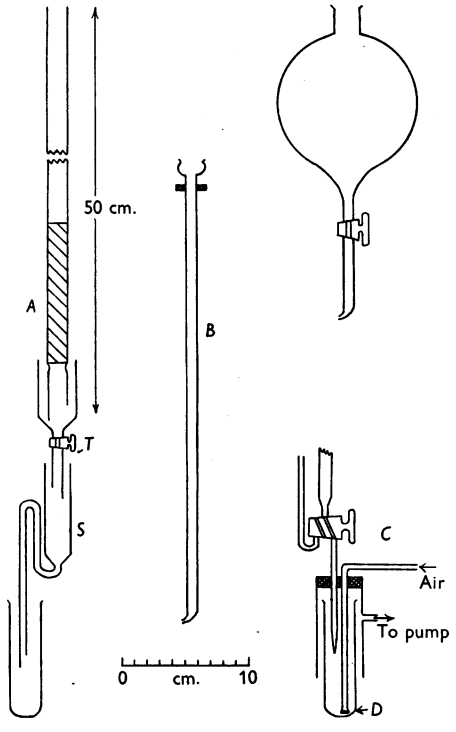


Fig. 2. Apparatus (see text).

*Sampling.* Successive, approximately equal samples of about 5 ml. (the exact volume is immaterial) are collected in dry tubes (4 in. × 1 in.) by means of the automatic siphon (*S*). Each sample is titrated in turn and the titres, after correction for blanks, are plotted against the number of samples. It is usually convenient to plot the titres both individually and cumulatively.

*Titrations.* To avoid the inconvenience of titrating in a heterogeneous medium 0·005N-KOH is used in $CO_2$-free methanolic solution. The solution is stored in a bottle guarded against $CO_2$ and delivered by gravity to a guarded, self-filling 10 ml. micro-burette, the stopcock of which is lubricated with a silicone preparation.

A stream of dry, $CO_2$-free air is passed through the sample throughout the titration, using a small sintered glass distributor (*D*) to obtain a stream of fine bubbles. The air should be passed for at least 30 sec. before beginning to titrate; shorter periods of preliminary gassing fail to remove $CO_2$ while long periods may lead to loss of acids by volatilization. The butanol, chloroform and methanol vapours are sucked away by the pump (see Fig. 2*C*).

*Indicator and end point.* In our early experiments we used phenolphthalein as indicator, as is usually advised, but we later abandoned this in favour of cresol red. A 0·04% solution in methanol is used, two drops being taken for each 5 ml. solution undergoing titration. The colour changes from yellow through a brownish red and finally, very sharply indeed, to a bluish red: even when 0·005N-alkali is being used, 0·01 ml. suffices to produce the final change of colour, which is more easily seen by daylight than by artificial illumination.

Cresol red has certain disadvantages which, however, can easily be overcome, and are compensated for by the sharpness of the end point. It is preferentially soluble in water so that the samples for titration must be collected in dry tubes, and the air stream must be dried. There is a slight lag in the establishment of the final end point, especially in 30% butanol, but this can be overcome by waiting for 30 sec. after the end point has apparently been reached in order to see whether the brownish red colour returns. There is a further and more subtle difficulty. If, when the total titre in 1% butanol is less than about 0·5 ml., a dropwise titration is carried out, a false end point may be reached with the addition of a few drops of the alkali. On the addition of a further 1–2 drops the indicator again turns yellow, and only then begins to approach the true end point. This curious phenomenon, which is not observable in 10 or 30% butanol, can be avoided by the addition of a few drops of methanol before starting to titrate samples in which a small titre is expected.

*Blanks.* Blank values are determined by running samples of the solvents through virgin columns. They vary slightly from one sample of silica to another, but the errors introduced by these variations into the final estimates are of the order of not more than 1% when total titres of 10 ml. or more are being handled. In our own experiments the following average blanks were found:

| | Blank (ml. 0·005N-KOH/5 ml. sample) | | |
|---|---|---|---|
| Buffer | 1% | 10% | 30% |
| I | 0·023 | 0·034 | 0·036 |
| II | 0·024 | 0·032 | 0·038 |
| III | 0·022 | 0·034 | 0·036 |

*Change of solvent.* When one acid has been eluted from the column the elution of the next can be hastened by replacing the solvent by another containing more butanol. Generally speaking, the exchange is made when the titre has dropped to the blank level or as soon as an increase of at least 0·1 ml. in titre is observed.

*General operation.* It is usually convenient to collect samples every 4–5 min. The rate of flow diminishes with increasing concentrations of butanol and with increasing pH, but depends mainly upon the porosity of the particular sample of silica in use. It can be controlled by modifying the proportions of silica and buffer in the mixture, but it is better to rely upon alterations in the hydrostatic pressure of solvent above the column. It is also possible to mix samples of different porosities together in order to obtain suitable rates of flow.

An experimental run can be interrupted, if necessary, by fitting to the top of the tube a rubber bung pierced by a small stopcock. The tube is filled very nearly to the top with solvent, the bung placed securely in position and the tap closed; in this way it is possible safely to discontinue operations for several hours.

Table 1. *Behaviour of aliphatic acids* ($C_2$ *and upwards*) *on columns I, II and III*

| Column | pH* | % butanol | Acids: Eluted | By-passed† | Retained |
|---|---|---|---|---|---|
| I | 6·6 | 1 | $C_4$ | $C_5$ and higher | — |
| | | 10 | $C_3$ | | |
| | | 30 | $C_2$ | | |
| II | 8·4 | 1 | $C_6$ | $C_7$ and higher | $C_3$, $C_2$ |
| | | 10 | $C_5$ | | |
| | | 30 | $C_4$ | | |
| III | 9·5 | 1 | $C_7$ and higher | — | $C_4$ and lower |
| | | 10 | $C_6$ | | |
| | | 30 | $C_5$ | | |

* Determined by glass electrode (16°) on mixtures of 5 g. silica with 3 ml. buffer suspended in 10 ml. water: these figures are more useful as a check on the properties of the silica than as indications of the operative pH of the columns.
† In the first five to six samples.

*Operational columns.* It is not possible to carry out complete separations of more than three acids upon any one column under the conditions we employ, and we have therefore developed three types of columns buffered, respectively, with buffers I, II and III. These columns, referred to as 'columns I, II and III', cover the whole useful range of the method: their performances are summarized in Table 1 (see also Figs. 3–5).

## RESULTS

### *Separation and recovery of acids*

Recovery experiments were first carried out on carefully redistilled specimens of single acids, the total load taken being of the order of 20 ml. 0·005N in each case. The recoveries are listed in Table 2. Feeling satisfied that virtually quantitative recoveries of single acids can be achieved, we prepared a series of mixtures of known acids and put them through the usual procedure. The results (Table 3, Figs. 3–5) showed that virtually complete separations of naturally occurring acids are possible up to $C_8$, and that quantitative recoveries of the separated components can be achieved. Oenanthic acid ($C_7$) is separable from caproic ($C_6$) but not from caprylic acid ($C_8$); pelargonic acid ($C_9$) is inseparable from caprylic ($C_8$) and higher acids.

*Behaviour of isomers.* *n*- and *iso*Butyric acids behave so similarly on the columns that they are indistinguishable and the same is true of *n*- and *iso*caproic acids. We made a special study of the valeric acids, viz. *n*- and *iso*valeric, trimethylacetic and DL-methylethylacetic acids.

Since sharper separations are normally found between acids that come late through the columns (see Figs. 3–5) we studied the behaviour of the isomers on columns of type III, from which they are eluted only by 30% butanol. Each experiment was performed on a separate column and all the columns were prepared in the same way and under identical conditions. It was found that by increasing the relative proportion of buffer to silica better separations were obtained: these columns were therefore made from 4 g. of silica and 3 ml. of buffer III. The results showed that *n*-valeric and trimethylacetic acids behave almost identically, while *iso*valeric and DL-methylethylacetic acids come through together but considerably later than the other two. We prepared an equimolecular mixture of the *n*- and *iso*-acids and examined this on one of these columns. The resulting curve (Fig. 6A) showed two well-separated peaks, indicating that a partial separation of the two isomers had been accomplished. Similar mixtures of trimethylacetic and DL-methylethylacetic (Fig. 6B), *n*-valeric and trimethylacetic (Fig. 6C) and *iso*valeric and trimethylacetic acids (Fig. 6D) were similarly treated and again gave partial separations.

Although we have not succeeded in accomplishing total separations even in these favourable cases, it is, nevertheless, established that *n*-valeric and trimethylacetic acids can easily be distinguished from *iso*valeric and DL-methylethylacetic acids.

### *Influence of some experimental conditions*

*Properties of the silica.* Several different samples of silica were used in the course of our work. These included one too acid and another too alkaline for use in Elsden's (1946*a*) procedure, two more that gave good results by his method, and a sample of Isherwood's (1946) non-adsorbent silica. Virtually identical results were obtained with all samples.

Fig. 7 presents the results obtained by analyzing samples of the same mixture of acids (*n*-caproic, *iso*valeric and *n*-butyric acids) on four different silicas. In examining these curves the following facts must be borne in mind. The precise position taken up by a given acid is influenced to some extent by the rate of flow of the column and therefore by the porosity of the gel. This is shown by slight differences in the positions of the first bands in Fig. 7. More important, however, are the differences due to the fact that the butanol content of the

Table 2. *Recovery of single acids*

| No. of C atoms | Acid | Column | Amount taken (ml. 0·005 N) | Amount recovered (ml. 0·005 N) | Recovered (%) |
|---|---|---|---|---|---|
| 2 | Acetic | I | 20·6 | 20·6 | 100 |
| 3 | Propionic | I | 18·8 | 18·2 | 97 |
| | Propionic | I | 18·8 | 18·4 | 98 |
| 4 | *n*-Butyric | I | 19·6 | 19·4 | 99 |
| | *n*-Butyric | II | 19·3 | 18·8 | 98 |
| | *iso*Butyric | I | 21·5 | 21·4 | 99 |
| | *iso*Butyric | II | 21·5 | 20·7 | 96 |
| 5 | *n*-Valeric | II | 18·5 | 18·3 | 99 |
| | *n*-Valeric | II | 18·5 | 17·7 | 96 |
| | *iso*Valeric | II | 19·6 | 20·0 | 102 |
| | *iso*Valeric | III | 19·6 | 19·2 | 98 |
| | Trimethylacetic | II | 19·9 | 20·6 | 103 |
| | Trimethylacetic | III | 19·9 | 19·7 | 99 |
| 6 | *n*-Caproic | II | 17·3 | 17·3 | 100 |
| | *n*-Caproic | III | 19·3 | 19·1 | 99 |
| | *iso*Caproic | III | 19·2 | 19·3 | 101 |
| 7 | *n*-Oenanthic | III | 18·8 | 18·3 | 98 |
| 8 | *n*-Caprylic | III | 19·3 | 19·2 | 99 |
| 9 | *n*-Pelargonic | III | 19·2 | 19·2 | 100 |
| 10 | *n*-Capric | III | 18·6 | 18·5 | 99 |

Table 3. *Recovery of acids from mixtures*

| Column | Acids taken | Amount taken (ml. 0·005 N) | Amount found (ml. 0·005 N) | Recovered (%) | Total recovered (%) |
|---|---|---|---|---|---|
| I | *n*-Butyric | 19·6 | 20·0 | 102 | 101 |
| | Propionic | 18·8 | 17·9 | 96 | |
| | Acetic | 20·6 | 21·4 | 104 | |
| II | *n*-Caproic | 17·8 | 17·8 | 100 | 99 |
| | *n*-Valeric | 18·5 | 18·6 | 100 | |
| | *n*-Butyric | 19·3 | 18·6 | 97 | |
| II | *n*-Caproic | 17·8 | 18·0 | 101 | 98 |
| | *n*-Valeric | 18·5 | 18·6 | 100 | |
| | *n*-Butyric | 19·3 | 18·9 | 98 | |
| II | *n*-Caproic | 17·8 | 18·6 | 104 | 101 |
| | *n*-Valeric | 18·5 | 18·9 | 102 | |
| | *n*-Butyric | 19·3 | 18·8 | 98 | |
| III | *n*-Capric | 18·6 | 37·7 | 100 | 99 |
| | *n*-Caprylic | 19·3 | | | |
| | *n*-Caproic | 19·3 | 19·0 | 99 | |
| III | *n*-Capric | 18·6 | 37·8 | 100 | 99 |
| | *n*-Caprylic | 19·3 | | | |
| | *n*-Caproic | 19·3 | 18·8 | 98 | |
| III | *n*-Capric | 18·6 | 37·5 | 99 | 100 |
| | *n*-Caprylic | 19·3 | | | |
| | *n*-Caproic | 19·3 | 19·4 | 100 | |

developing solvent was not increased at the same time in all cases. Such an increase results in a sharp rise in titre as the next acid comes through, and any delay in changing the solvent is naturally followed by a later rise in the curve. When allowances are made for these factors, the differences between the four curves become insignificant, indicating that it is unnecessary to take elaborate precautions in preparing the silica and, incidentally, that different samples of silica can safely be mixed together in order to obtain columns running at convenient speeds.

*Effect of concentration of buffer.* We adopted the use of 2 M buffers as standard procedure. Fig. 8 shows the effects of using more dilute buffer on columns of type II. Using M instead of the usual 2 M phosphates the separation between the first two acids (*n*-caproic and *iso*valeric) is no longer com-

plete, though the separation between the second pair (*iso*valeric and *n*-butyric) is still very good. In 0·5M buffer the second separation is also impaired. Essentially similar results were obtained with the other columns.

sensitive to loads of 0·01 mmol. or less, so that small amounts of one acid can be detected and estimated in the presence of larger amounts of others. Excellent separations can be obtained with loads up to about 1 mmol. of each acid by the use of columns

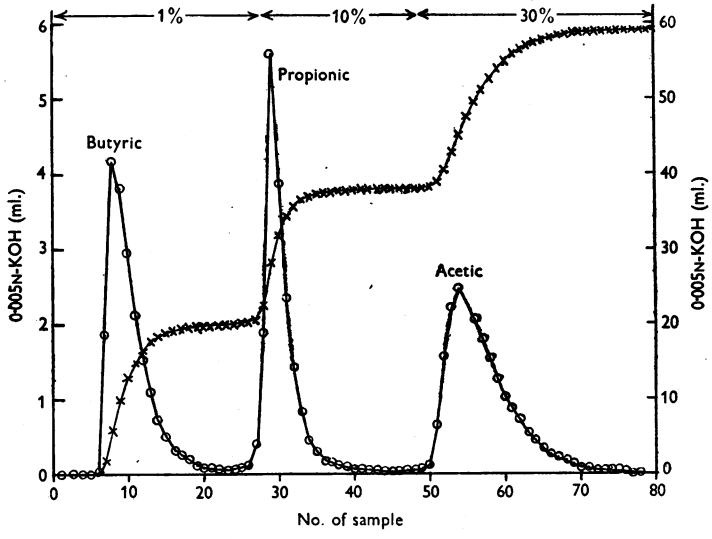


Fig. 3. Separation of *n*-butyric, propionic and acetic acids (column I). The butanol content of the developing solvent was changed at the points indicated. Acids: approx. 1 ml. 0·1N of each acid. Ordinates: titres of successive samples, o individual, × aggregate.

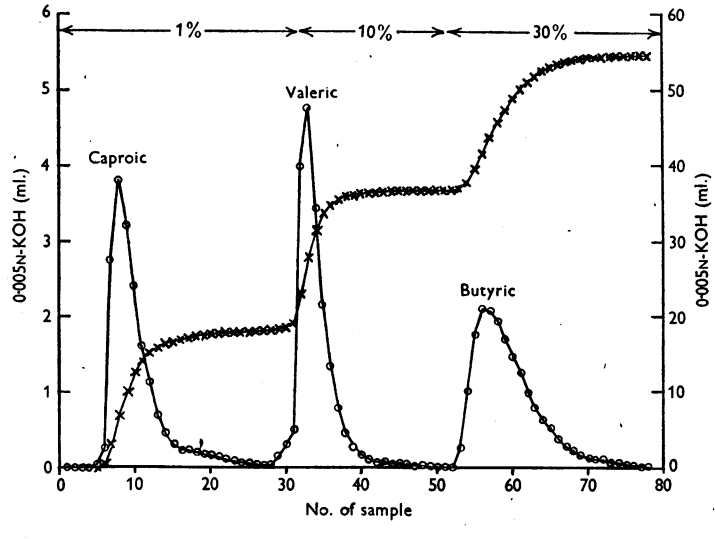


Fig. 4. Separation of *n*-caproic, *n*-valeric and *n*-butyric acids (column II). Details as for Fig. 3.

*Effect of load.* The optimal load for the columns is about 0·1 mmol. (≡20 ml. 0·005N) of each acid in the mixture, corresponding to about 6 mg. acetic or 10 mg. valeric acid. The method is, however, still of similar length, but about five times as large a cross-sectional area.

*Volume of sample.* In the method of Elsden (1946*a*) it is necessary to introduce the acids to be analyzed

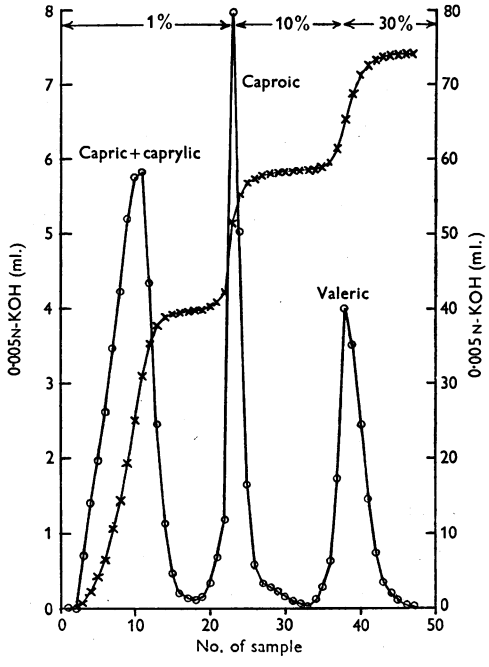


Fig. 5. Separation of *n*-capric + *n*-caprylic, *n*-caproic and *n*-valeric acids (column III). Details as for Fig. 3.

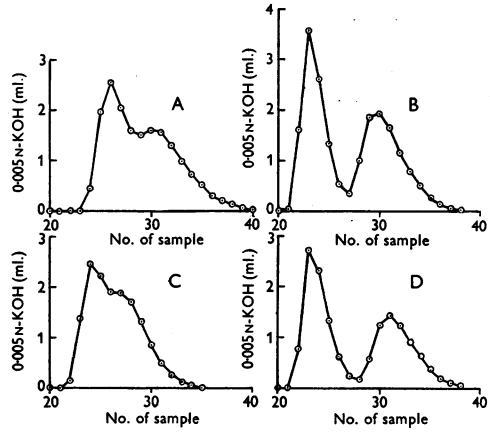


Fig. 6. Behaviour of isomeric valeric acids (column III). A, 0·5 ml. 0·1 N-*n*-valeric + 0·5 ml. 0·1 N-*iso*valeric acids. B, 0·5 ml. 0·1 N-trimethylacetic + 0·5 ml. 0·1 N-DL-methylethylacetic acids. C, 0·5 ml. 0·1 N-*n*-valeric + 0·5 ml. 0·1 N-trimethylacetic acids. D, 0·5 ml. 0·1 N-trimethylacetic + 0·5 ml. 0·1 N-*iso*valeric acids. Solvent: 30% butanol in chloroform throughout.

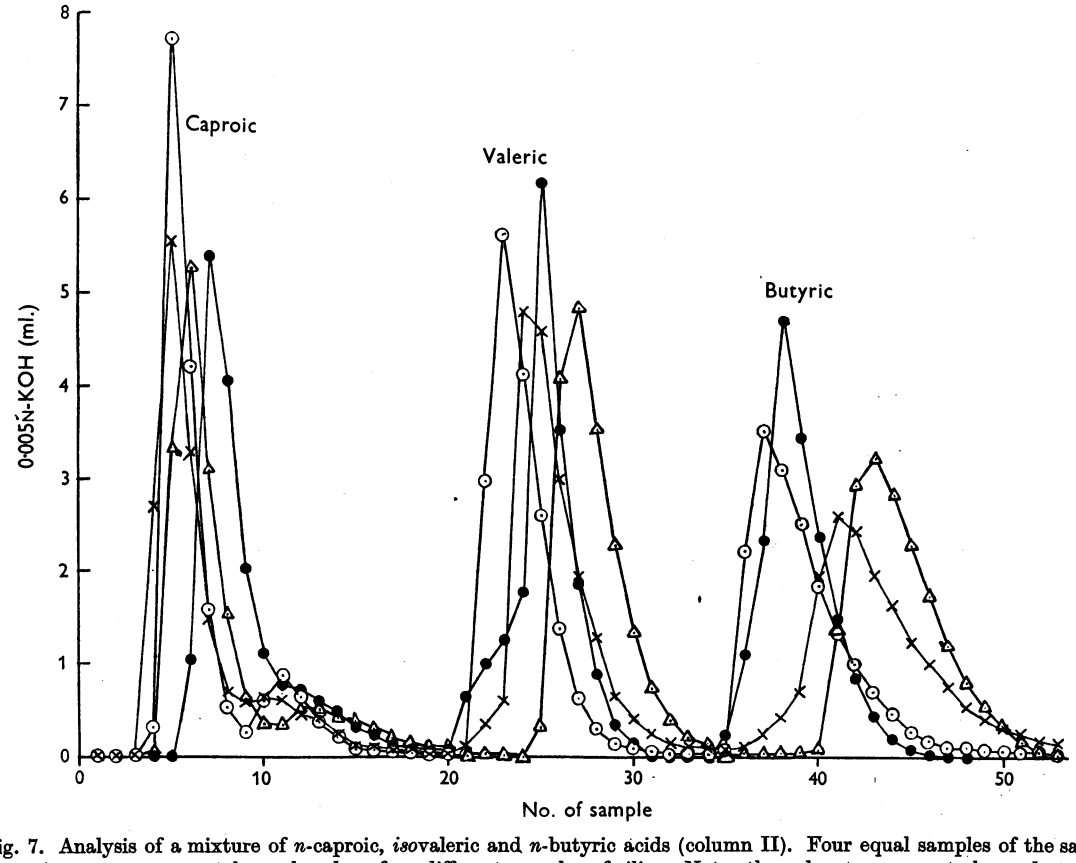


Fig. 7. Analysis of a mixture of *n*-caproic, *iso*valeric and *n*-butyric acids (column II). Four equal samples of the same mixture were separately analyzed on four different samples of silica. Note: the solvents were not changed at the same point in each case. Acids: approx. 1 ml. 0·1 N of each acid.

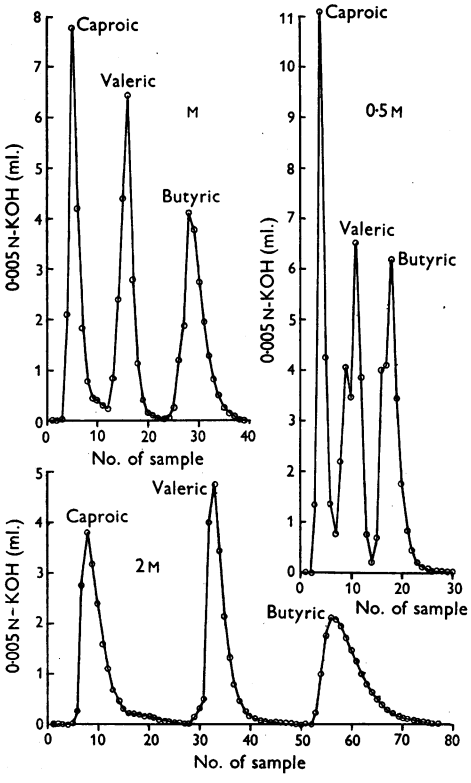


Fig. 8. Influence of concentration of buffer on separation of *n*-caproic, *iso*valeric and *n*-butyric acids on column II. Acids: 1 ml. 0·1 N approx. of each acid.

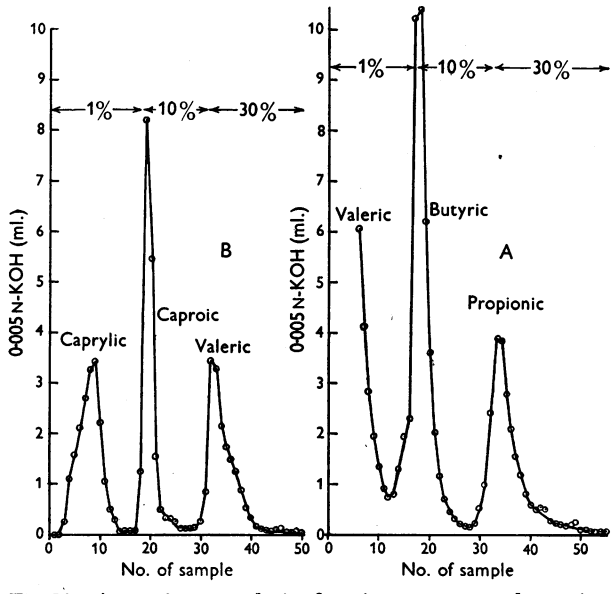


Fig. 10. Approximate analysis of a mixture on two columns (see text). Sample, 1 ml. A, Column IV; the first five samples were massed and placed on B (column III).

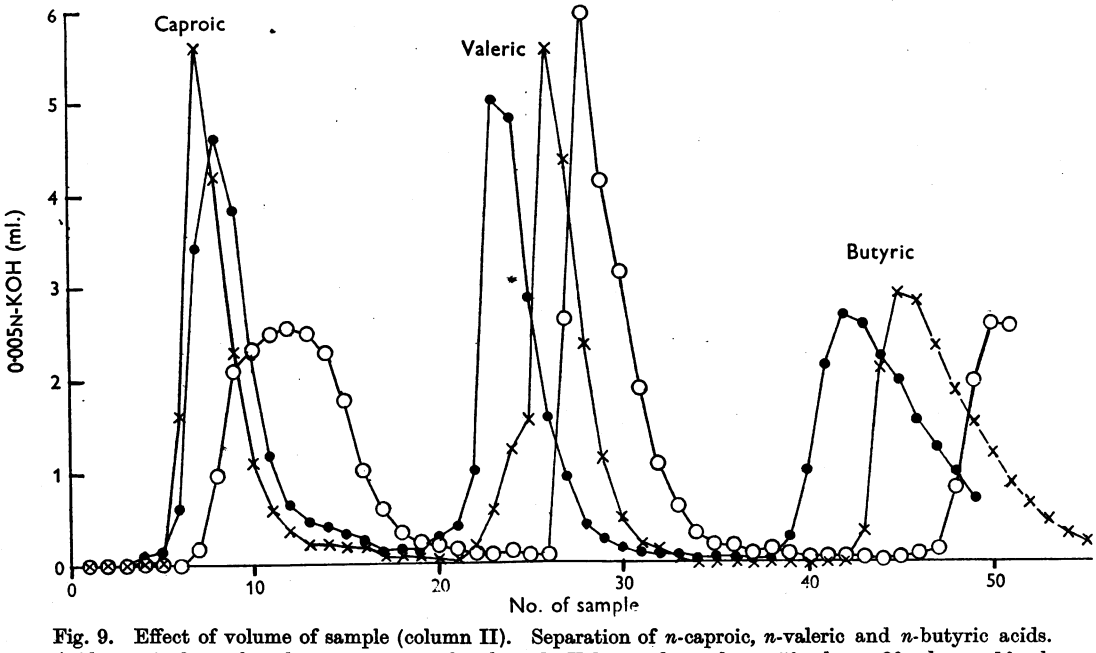


Fig. 9. Effect of volume of sample (column II). Separation of *n*-caproic, *n*-valeric and *n*-butyric acids. Acids: equivalent of 1 ml. 0·1 N approx. of each acid. Volume of sample: o, 50 ml.; •, 20 ml.; ×, 10 ml.

in a total volume of not more than 3 ml. With the 5 g. buffered columns, even when samples of 50 ml. are taken, there is still excellent resolution, though the first band is considerably broadened (Fig. 9).

*Applications of the method*

*Identification and approximate assay of components of a mixture.* Each acid shows strongly characteristic behaviour on the columns. Thus the valeric acids are by-passed in the first five to six samples of 1 % butanol by column I, eluted rapidly by 10 % and only very slowly by 1 % butanol from column II, and rapidly eluted only by 30 % butanol from column III. So characteristic is the behaviour of each member of the series of acids studied that it may be taken as evidence of identity, with the reservation that only certain pairs of the isomers we have tested proved to be distinguishable (p. 311), even under carefully controlled conditions.

Before proceeding to exact analysis it is often desirable to carry out an approximately quantitative analysis of an unknown mixture in order to discover what acids are present and in what concentrations. This can be done by taking advantage of the large sample volumes that the columns can handle. For this purpose an additional column (IV) is of great service: this is buffered with 2M-$KH_2PO_4$ alone, and by-passes caproic and higher acids, yields up valeric with 1 %, butyric with 10 % and propionic with 30 % butanol. Acetic acid is retained but can be approximately assayed by difference.

A small, measured sample of the mixture to be analyzed is placed on a column of type IV and the first five samples, which contain some of the valeric acid together with practically the whole of the higher acids, are not titrated but massed together and reserved. The column is then developed in the usual manner. A column of type III is then set up, the five reserved samples are placed upon it and the column then developed. The results of such an experiment are shown in Fig. 10: the acids taken in this case consisted of a 1 ml. sample containing about 0·1 mmol. each of acetic, propionic, *n*- and *iso*butyric, *n*- and *iso*valeric, *n*-caproic and *n*-caprylic acids.

Only approximate results can usually be obtained in this manner, but the procedure gives reliable indications of the nature of the acids present and approximate values for their respective concentrations. It is then possible to select columns suitable for exact analysis under optimal conditions.

*Routine analyses of mixtures.* Where routine analyses are to be carried out, and the identities of the acids present have been established, it may not be necessary to employ all three of the standard columns. In the analysis of rumen contents, for example, in which only acetic, propionic and butyric acids are usually assayed (Elsden, 1946*b*; Elsden *et al.* 1946, for example), only column I will be required. In a hypothetical case where, say, only valeric, butyric and propionic acids were to be determined, a column intermediate between types I and II could be specially designed for the purpose. It is reasonable to assume that intermediate columns would yield results as quantitative as those obtained on the standard columns used in the present experiments. In addition, higher acids by-passed by one column might be put directly on another that is more alkaline.

*Detection and estimation of impurities: purification of acids.* As the method is sensitive even to very small loads, it is not difficult to detect the presence of traces of contaminants in supposedly pure specimens of acids. We were able, for example, to demonstrate the presence of approximately 4 % of a valeric and 6 % of propionic acid in a redistilled sample of 'pure' *iso*butyric acid. In another experiment an allegedly pure specimen of *n*-caproic acid was found to contain no less than 17 % of an unknown impurity.

Since the columns can handle loads up to about 0·2 mmol. of acid, specimens of this order can be freed from traces of impurities by passage through an appropriate column and subsequent recovery by neutralization and evaporation to dryness.

*Preparation of characteristic derivatives.* Considerable amounts of purified acids can be obtained by the procedure outlined in the preceding paragraph and are of great value for the preparation of characteristic derivatives, such as the piperazonium salts or phenylhydrazides, for purposes of identification. The main difficulty likely to be encountered here lies in the removal of the indicator, but we avoid this by proceeding in the following manner.

A suitable column is set up and loaded with about 0·2 mmol. of each of the acids to be characterized. Samples are collected and titrated in the usual manner until the first acid begins to come through. The next six samples, which contain the bulk of this acid, are massed together, without titration, for subsequent recovery. Further samples are then titrated until the second acid begins to come through and this is likewise collected in six samples, again without titration, and so on. When more than 0·2 mmol. of an acid is required it is an easy matter to run more than one column and mass the corresponding eluates or to work with wider columns.

*Limitations of the method*

As the foregoing discussion indicates, this new method possesses numerous advantages over its predecessors. Certain drawbacks remain, however, in particular the inability of the columns completely to resolve mixtures of isomeric acids and of acids higher than caprylic.

It seems to be well established (Hilditch, 1947) that, with the exceptions of propionic and *iso*valeric acids, fatty acids containing either a branched chain or an odd number of carbon atoms occur but rarely in nature. Our procedure is therefore capable of separating and analyzing all the naturally occurring steam-volatile saturated fatty acids as far as $C_8$. We have shown that oenanthic ($C_7$) acid can be completely separated from caproic acid ($C_6$) but not from caprylic ($C_8$), while pelargonic ($C_9$) is inseparable from caprylic ($C_8$) and capric ($C_{10}$). While for biochemical purposes this limitation is perhaps not a serious one, for purely chemical work, in which odd-numbered acids are more likely to be encountered, the present method will break down in cases where acids containing more than seven carbon atoms have to be dealt with.

## SUMMARY

1. A new method is described for the identification, separation and estimation of most of the steam-volatile members of the saturated series of fatty acids. The procedure is based on the principle of partition chromatography: heavily buffered silica gels, without indicator, form the stationary phase of the partition columns, the moving phase consisting of mixtures of chloroform and butanol.

2. Naturally occurring fatty acids from acetic to caprylic can be identified, quantitatively separated and estimated. Caprylic cannot, however, be separated from higher acids.

3. Of the non-natural acids, oenanthic can be separated from lower but not from higher acids.

4. Partial separation has been achieved between isomeric forms of valeric acid but isomeric acids cannot in general be distinguished or separated.

5. Acids can be recovered from the columns in quantities large enough to permit the preparation of characteristic derivatives.

6. Optimal results are obtained with quantities of the order of 0·1 mmol. of each acid, whether taken separately or in mixtures.

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*Note.* The range of the method has been improved by the use of a column buffered with 9 parts of 2 N-KOH to 1 part of a 2 M-solution of glycine and KCl. Caprylic ($C_8$) and higher acids are eluted with 1 % butanol, oenanthic ($C_7$) with 10 % butanol and caproic ($C_6$) with 30 % butanol.

## REFERENCES

Elsden, S. R. (1946*a*). *Biochem. J.* **40**, 252.
Elsden, S. R. (1946*b*). *J. exp. Biol.* **22**, 51.
Elsden, S. R., Hitchcock, M. W. S., Marshall, R. A. & Phillipson, A. T. (1946). *J. exp. Biol.* **22**, 191.
Friedemann, T. E. (1938). *J. biol. Chem.* **123**, 161.
Goodall, R. R. & Levi, A. A. (1946). *Nature, Lond.*, **58**, 675.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
Hilditch, T. P. (1947). *The Chemical Constitution of Natural Fats*, 2nd ed. London: Chapman and Hall.
Isherwood, F. A. (1946). *Biochem. J.* **40**, 688.
Sato, Y., Bary, G. T. & Craig, L. C. (1947). *J. biol. Chem.* **170**, 501.
Scarisbrick, R., Baldwin, E. & Moyle, V. (1948). *Biochem. J.* **42**, xiv.
Smith, E. Lester (1942). *Biochem. J.* **36**, xxii.
Synge, R. L. M. (1946). *Analyst*, **71**, 256.