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Quantitative Paper Chromatography of Fatty Acids

I. The Influence of Residual Mobile Phase

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Use of test mixtures of ^{14}C labelled acids and scanning of both radioactivity and colour has enabled a detailed study of the correlation between photometric readings and the corresponding true amounts of fatty acids in connection with the method for quantitative evaluation of paper chromatograms developed by Kaufmann *et al.*

It is shown that residual mobile phase influences the conversion of fatty acid to copper soap in a manner which forwards the linearity of the photometric response curves. When scanning narrow cross-sections of the spots true linearity following Beer's law exists up to spot contents of about 20 μg.

In a previous paper[1] it has been shown that direct quantitation on paper chromatograms of radioactivity from ^{14}C labelled fatty acids was not biased by spot size or type of fatty acid. By using test mixtures of ^{14}C labelled acids and scanning both radioactivity and colour, it is thus possible in a relatively simple manner to study in detail the correlation between photometric readings and true amounts of fatty acids on paper chromatograms. This has been done in the following in connection with the method for quantitative evaluation of paper chromatograms which was developed by Kaufmann *et al.*[2,3]

According to Ref.[3] the chromatograms are coloured by treating the strip with a solution of cupric acetate which transforms the fatty acids to copper soaps, washing off the excess of the copper reagent and then in turn again releasing the added cupric ions by treatment with an aqueous solution of dithio-oxamide which forms a green copper complex. The acids are thus coloured indirectly and this means that the final photometric readings depend not only on the colouring step but also on the result of an intermediate chemical process. The latter can be written as,

$$Cu^{2+} + 2RCOOH \rightleftharpoons Cu(RCOO)_2 + 2\ H^+$$

Fig. 1. A chromatographic analysis of six equal samples of a mixture of stearic and myristic acid. After development V was dried for about an hour at about 100°C; VI was dried overnight at room temperature.

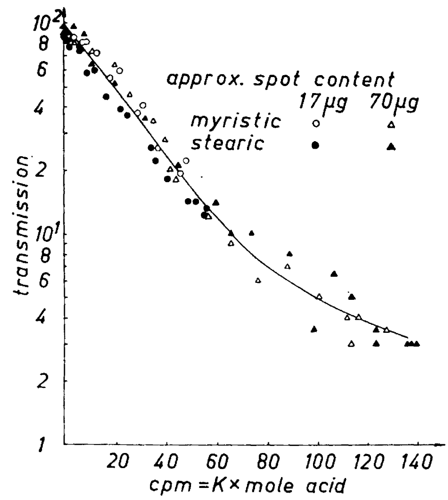

Fig. 2. A quantitative correlation between photometric and radioactivity measurements along a narrow cross-section of 4 chromatographic spots. The drying conditions correspond to V in Fig. 1.

In view of the fact that the mobile phase used to develop the chromatograms consists of 90 % acetic acid, this leads to certain considerations. The above reaction is influenced by the hydrogen ion concentration in the copper bath; addition of acetic acid will result in a lower conversion from fatty acid to copper soap depending on the fatty acid in question. According to Ref.[2] the chromatogram is dried for 1.5 h at 120°C before treatment with cupric acetate. The question is then: Is there enough acetic acid left on the paper after drying, to influence the amount of copper soap formed during treatment in the copper bath?

EXPERIMENTAL

Fig. 1 shows the result of a chromatographic analysis of six equal samples of a mixture of stearic and myristic acid. The mixture contained equal amounts of each of the two acids and each sample consisted of about 35 μg substance. All the chromatograms were developed simultaneously on the same sheet of paper. The final treatment with dithiooxamide was also alike for all the spots. The treatment of V and the treatment of VI differed only as far as the conditions during the drying step were concerned. V was dried as prescribed [2] for about 1 h at about 100°C. VI was dried at room temperature hanging in the hood overnight. The prolonged drying of VI resulted in no residual acetic acid; pH in the copper bath was 6.0 before and after treatment of the strip. The "normal" dyring of V, however, did not remove all acetic acid from the paper; pH in the copper bath was reduced from 6.0 to 5.7 by the paper treatment.

The same phenomenon as in Fig. 1 is shown quantitatively in Figs. 2 and 3. Fig. 2 corresponds to V; Fig. 3 corresponds to VI. In each case the shown curves are based on

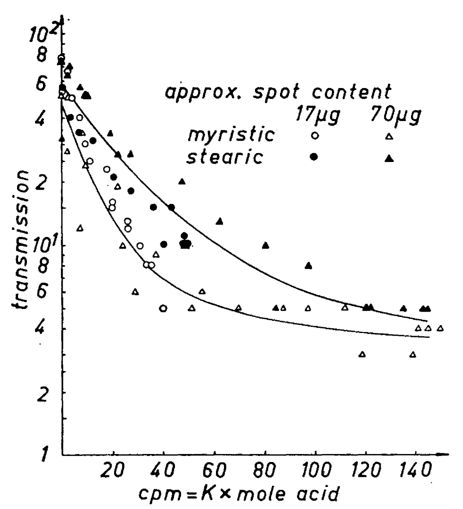

Fig. 3. A quantitative correlation between photometric and radioactivity measurements along a narrow cross-section of 4 chromatographic spots. The drying conditions correspond to VI in Fig. 1.

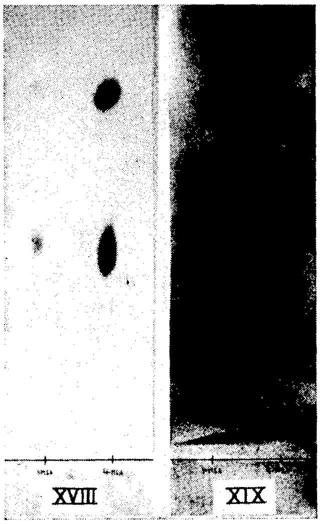

Fig. 4. Two chromatographic strips each based on the same two amounts of the stearic and myristic acid mixture from Fig. 1: addition of acetic acid made pH be 5.0 in the copper bath corresponding to XVIII; addition of sodium hydroxide made pH be 6.5 in the copper bath corresponding to XIX.

two chromatograms with sample sizes of 35 and 140 µg, respectively. The scanning of radioactivity as well as of colour was done with all of the paper except for a 2 mm wide slit across the center of the spots covered by brass foil thick enough to stop all ^{14}C β radiation and the plotted values refer thus only to a lengthwise cross-section of the chromatograms. Together with 2 mm wide counting and photo-scanning windows this ensured corresponding radioactivity and light transmission values from practically homogeneous 2 × 2 mm spot areas. The radioactivity units along the abscissa are proportional to "mole fatty acid" with the same proportionality factor for both the myristic and stearic acids.

A final experiment to illustrate the influence of the hydrogen ion concentration in the copper bath is shown in Fig. 4. In this case pH in the copper bath was altered directly by addition of acetic acid and sodium hydroxide, respectively. All further treatments were alike for both strips. The common drying time was long enough to remove all residual mobile phase. XVIII was treated with cupric acetate at pH = 5.0; XIX was treated with cupric acetate at pH = 6.5. Normally pH in the copper bath is 6.0 before treatment. The two chromatograms on each strip represent samples of 35 and 140 µg of the same mixture as in Fig. 1. A scanning of radioactivity showed no difference between the amounts of fatty acids present on XVIII and XIX.

DISCUSSION

Both the qualitative and the quantitative results show that residual mobile phase has a major influence on the final amount of the copper soaps due to the latter's dependence on the hydrogen ion concentration in the copper bath.

The found pH-effect is not simple. The only thing that can be evidently explained is the fact that the total amount of soap is decreased when the hydrogen ion concentration is increased as is seen when comparing XIX ($pH = 6.5$) to VI ($pH = 6.0$) to V ($pH = 5.7$) to XVIII ($pH = 5.0$) and Fig. 3 to Fig. 2. Concerning the ratio of the final amount of myristic soap to the corresponding amount of stearic soap which should have been the same in all cases since all the samples were taken from the same mixture, it is seen, again following the above sequence of chromatograms and curves, that it varies from a value above 1 to a value below 1. This leads to no evident explanation. The factors involved are the solubility and dissociation of the fatty acid and the solubility and dissociation of the copper soap together perhaps with an association between the fatty acid or soap and the paper itself. Only further investigations can explain how these factors interact whith the hydrogen ion concentration. For the present it can only be concluded that the shown anomaly is the result of what happens in the copper bath and not just a random phenomenon based on the various washing steps, since radioactivity measurements prove that the sum of factty acid and copper soap is not affected.

A study of Figs. 2 and 3 is also of practical interest. Fig. 2 represents a "normal" chromatographic assay. It is seen that the colour on the paper spectrophotometrically seems dissolved just like in a solution; Beer's law of light absorption being valid at least up to a spot content of about 20 μg. At higher spot contents the curve somewhat flattens out. It is also seen that the correlation between light transmission and true amount of acid is independent of the total spot content since the circles and triangles fit the same curve. The latter is also the case within a somewhat larger standard deviation in Fig. 3, which represents a chromatographic assay with prolonged drying.

As far as the enhanced standard deviations of the plotted values in Fig. 3 are concerned this can be explained by looking at Fig. 1. With no residual mobile phase the paper background appears smudged thus making quantitative readings difficult. Quantitative evaluations of the extra NaOH treated XIX in Fig. 4 would have been practically impossible. Some residual mobile phase is thus not only helpful in making Beer's law more valid but also in keeping the paper background clean.

Concerning the shape of the curves in Fig. 3, it is worth mentioning that these in reality are more "true" than in the "normal" Fig. 2, since at least the myristic curve is based on very near 100 % conversion to copper soap. Had the colouring been done directly with some organic dye one also should have expected the saturated type of curve from Fig. 3. This is, *e.g.* generally the case when paper electropherograms are coloured. One might thus stress that the larger popularity of quantitative fatty acid paper chromatography according to Kaufmann *et al.* as compared to other methods involving direct photometric scanning of paper chromatograms can be traced back to a "mistake" built-in in the method.

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REFERENCES

1. Hansen, H. J. M. *Acta Chem. Scand.* **15** (1961) 670.
2. Kaufmann, H. P. *Analyse der Fette und Fettprodukte* Springer-Verlag, Berlin 1958.
3. Kaufmann, H. P., Schnurbusch, H. and Shoeb, Z. E. *Fette Seifen Anstrichsmittel* **62** (1960) 1.

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