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Paper Chromatographic Separation of Volatile Fatty Acids

A Study of a Number of Factors Involved

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Several authors [1–4] have described methods for the paper chromatographic separation of volatile fatty acids, without prior formation of derivatives. These methods appear to have been developed relatively independently, and they thus differ in certain details, although the principle used for the detection of the acid spots is common to all.

In the course of other investigations, we desired to identify, and if possible determine quantitatively [5], small amounts of volatile acids, and it thus became necessary to study the techniques for their chromatographic separation more closely, in order to find the most sensitive and reproducible method. The method evolved has subsequently been used successfully for the determination of volatile acids in cheese [7].

In principle, the analysis is carried out as follows: The acid mixture in the form of a salt solution is placed on the paper and development is carried out with, for example, butanol/water.

As the R_F values of the alkali salts are very low, ammonium or ethylamine salts must be used.

Because of the volatility of the acids and their tendency to undergo esterification, an excess of base must be present throughout the entire process. When the chromatogram is ready it is dried and the excess of base evaporates. By spraying with a suitable indicator, it is possible to detect the position of the acids as coloured spots on a background of another colour. Whether the acids spots appear in the acidic or basic colour of the indicator depends on the concentration of the alkali and the pK value of the indicator.

Brown and Hall [1] and Brown [2] start with the sodium salts which are subsequently converted to the ammonium salts in the high concentration of ammonia. Butanol or ethanol/butanol saturated with 1.5 N or 3 N ammonia is used as the solvent, and drying is carried out for one hour at room temperature, or for five minutes at 100°. The paper is

sprayed with a 0.04 % aqueous solution of bromothymol blue adjusted to pH 7.5 with NaOH, and the acid spots appear yellow on a blue background. 5 μg of acid can be detected.

Hiscox and Berredge [3] use a solution of the acids in butanol neutralised to pH 6—7 with ethylamine. Water-saturated *n*-butanol is used as solvent, and the bottom phase consists of a butanol-saturated ethylamine solution. The concentration of the ethylamine is said to be "not critical". The paper is dried in front of a fan and sprayed with bromocresol green, the acids then appearing as blue spots on a yellow-green background. 4 μl of 0.001 *N* acid can be detected, corresponding in the case of acetic acid to 0.24 μg.

Kennedy and Barker [4] use the ammonium salts of the acids. In common with the previous authors they use Whatman No. 1 paper, but wash it with oxalic acid and a large volume of water. This gives a purer background and more distinct spots.

Ethanol/ammonia, water/acetone/ammonia or water/butanol/ammonia are used as solvents, the papers are dried for 5 minutes at 100°, and sprayed with an aqueous solution of bromophenol blue containing citric acid.

These authors do not give the lower limit of sensitivity, but state that they worked with 1—2 μmolar solutions, *i.e.* with very much larger amounts than the other workers.

Experience gained in a number of preliminary experiments taken in conjunction with the fact that the Hiscox-Berredge procedure permits the use of lower concentrations of acids than the other methods, convinced us that this method would be the most satisfactory in respect of both sensitivity and reproducibility. We therefore concentrated on developing this particular method for reasons which will become more apparent from the following discussion.

EXPERIMENTAL

An initial series of failures showed clearly that the success of the analytical procedure was critically dependent on obtaining the right balance between a number of factors such as the type of paper, the ethylamine concentration, the method of drying the papers, the indicator concentration, the solvent for the indicator, the method of spraying etc. Each of the individual factors was found to exert a very great influence on the final result. Consequently it was necessary to investigate the influence of each variable separately, and repeated experiments were required, before it became possible to decide on the most satisfactory procedure.

Paper Quality. The following types of paper were tested:

Munktell OB (unwashed, washed with butanol, and washed with oxalic acid) [4]
Munktell 20
» 20: 150 g
Whatman No. 1
» » 4
» » 54
Schleicher and Schüll 1101 L
» » » 2043 a
» » » 2043 b
» » » 602 H:P
» » » 1507

It became apparent that the above papers behaved in very different ways. At a given ethylamine concentration, some papers such as S.S. 602 H:P and Munktell OB washed with oxalic acid gave a dark blue background on which no acid spots were discernible, while other papers such as Whatman 54, S.S. 2043b and Munktell 20:150 g gave very light backgrounds, but at the same time only very faint acid spots appeared.

Munktell 20, S.S. 1101 L, S.S. 2043a and S.S. 1057 could not be used because of their very bad capillarity.

In certain cases impurities from the paper collected in the neighbourhood of the advancing solvent front, and these partly or completely masked caprylic acid. This applied especially in the case of the fastest running paper, Munktell OB and although this difficulty could be overcome by washing the paper with butanol before use this treatment is time consuming and troublesome.

Whatman No. 1 paper gave a clean solvent front, a pure background and good contrast, and the chromatograms ran at a suitable speed, so that in the main this paper was employed. The solvent front advanced the full length of a 57 cm strip in 24 hours, but the chromatograms could be taken off after 16 hours, and thus it was convenient to let them run overnight.

For the separation of formic, acetic and lactic acids, which will be described later, the faster running paper Munktell OB could be used. By using a special technique, the impurities at the solvent front could be eliminated, and the background remained pure although somewhat darker in colour than in the case of Whatman No. 1 paper.

The Concentrations of Ethylamine and the Indicator. The purity of the background depends on both the kind of paper and on the concentration of ethylamine present. Even with Whatman No. 1 paper the background is quite blue if 0.1 *N* ethylamine is used and the subsequent spraying is carried out with too weak an indicator. The indicator which is an acid naturally neutralises the excess of ethylamine if the concentration of the indicator is sufficiently high, but at the same time one runs the risk that the salts produced will buffer the acid spots to such an extent that they will not be visible.

Thus it is not desirable to have more ethylamine than is necessary to prevent evaporation or esterification of the acids, and a suitable indicator concentration must be chosen. With an ethylamine concentration in the bottom phase of 0.5 %, an indicator concentration of approximately 2 % is appropriate if ethanol is the solvent. With 0.1 % ethylamine, the indicator concentration should be about 0.5—0.7 %. With Whatman No. 1 paper the best combination is 0.010—0.025 *N* ethylamine in the bottom phase and 0.25 % indicator. For Munktell OB paper the indicator concentration should be *ca.* 0.4 %.

If the concentration of ethylamine is below 0.005 *N* a number of curious effects such as yellowish streaks behind the spots, a diminished spot size etc. become noticeable. In this connection it is of interest to decide whether ethylamine should be present solely in the bottom phase or in the solvent as well.

Experiments showed that considerably better results were obtained if ethylamine was not present in the solvent but only in the bottom phase. When ethylamine was present in the solvent the R_F values were lower, the spots were deformed and the background was considerably darker. In order to get the normal shade of colour in the background the ethylamine concentration must be reduced considerably. For this reason it is more difficult to make use of the ascending technique which is normally more simple. The descending technique proved to be more satisfactory.

Sprayer and Solvent for the Indicator. When the indicator solution is transferred to the paper by means of a compressed air sprayer, its concentration is increased by an amount

which depends on the solvent, the size of the drops, the ratio of air to liquid, the distance from the sprayer to the paper etc. Thus the indicator concentration given is only applicable with the sprayer and solvent which was used.

In order to avoid evaporation as much as possible a high boiling solvent should be used. Butanol has not a sufficiently great solvent power to be used alone, but if the indicator is first dissolved in ethanol and the solution obtained is diluted with an equal volume of butanol good results are obtained.

The requirements of a suitable sprayer are very great. It should deliver a relatively rapid stream of small uniform droplets distributed evenly over a relatively large surface. If the droplets are large, or if there is a mixture of large and small droplets, "moth-ea ten" spots appear, *i.e.* the amount of indicator on the paper is so high in certain places that the blue colour of the acid spots is masked. If the spraying area of the spray is small it is difficult to spray large areas uniformly, and uniform spraying is essential if certain amounts of acids are to give spots of a definite size. Local overdosing or underdosing decreases or increases the area of the spots since the concentration of the acid in the spot is minimal at the perifery which is therefore especially easily masked. If the sprayer delivers a very slow stream of indicator it takes too long a time to cover a large area.

In all, seven different sprayers were tested. All were of the usual fixed jet type, but they differed in detail. The sprayer which proved to be the best was one of a type made at the Department of Biochemistry at Uppsala, and shown diagramatically in Fig. 1. The most suitable sprayer must be selected by testing a batch.

Using the technique described here, bromocresol green, bromocresol purple and bromophenol blue were the only indicators which could be used. This is in agreement with the findings of the authors referred to above.

Drying the Paper. When the chromatogram is dried after development the excess ethylamine is evaporated with the water/butanol mixture. After the drying has proceeded to a certain stage the acids also can theoretically be evaporated. It is therefore important that the drying should proceed until the excess of ethylamine has been removed, but no further. It is not easy to decide exactly when this stage is reached.

In actual practice the paper is dried for the length of time which gives rise to the greatest contrast between the acid spots and the background on subsequent spraying. If the chromatogram is dried in the air at room temperature about one hour is required: if the time is prolonged to two hours the spots become somewhat smaller and fainter. Nevertheless the time of drying is less critical than the other factors. In our experiments it proved more satisfactory to dry the chromatograms in air at room temperature than at 100° or in circulating air at 37°.

The Concentration of the Acid and the Volume of the Sample. The Degree of Separation. In our experiments it has been possible to detect 0.005 μ eq. of acid. The volume of the drops has been varied between 1 and 8 μl without any disadvantages becoming apparent. Thus the amount of acid which is most readily used is 0.10—0.30 μeq. *i. e.* 5—20 μg in the case of acetic acid. Larger amounts of acid can of course be analysed provided that the distance between the spots is sufficiently large. The separation is however not as good as the length of the paper becomes inadequate.

Lactic, formic and acetic acids move most slowly and form a single spot near the starting line. Caprylic, capric and lauric acids also form a single spot but the remaining acids move at intermediate rates and are well separated. Capric and lauric acids are normally separated during the preparation of the sample as they are insoluble in water so that caprylic acid can be distinguished even in their presence.

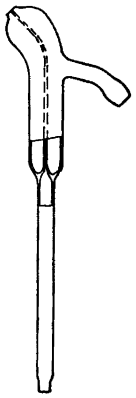

Fig. 1. Sprayer developed in the Department of Biochemistry, Upsala University.

In theory at least it should be possible to separate lactic, formic and acetic acids by virtue of the differences in R_F values. (See Table 1). Thus if the solvent front is allowed to run off the end of the paper the acids can move greater distances and differences in R_F values are more effectively utilised.

Our experiments on this line met with only partial success. It was sometimes possible to distinguish lactic acid and formic acid from one another if the single acids were used and were run side by side. Less frequently it was possible to separate formic acid and acetic acid.

As it seemed that unintential variations in the concentration of the ethylamine and the amount of acid might effect the results, these factors were subjected to a closer scrutiny. The effect of the water content of the butanol was also investigated. It transpired from these experiments that the separation is improved by increasing the concentration of the ethylamine, although it is still not entirely satisfactory. (See Fig. 2).

Even if the individual acids showed greater differences in R_{ac}* values, this was not so when they were mixed.

Clearly the acids are mutually soluble to some extent and this means that the R_{ac} values are not constant.

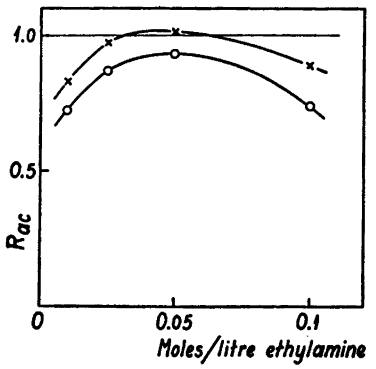

Fig. 2. The rates of travel of lactic and formic acids relative to acetic acid, as a function of the concentration of ethylamine.

——————— *Acetic acid*
—×——×— *Formic acid*
—O——O— *Lactic acid*

R_F value referred to acetic acid instead of the solvent front.

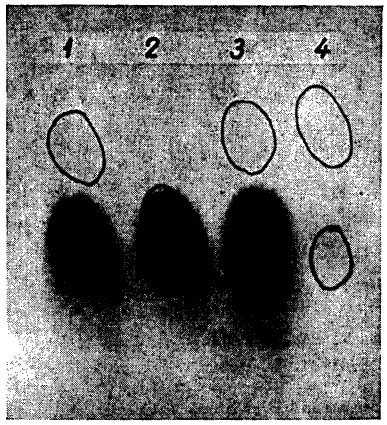

Fig. 3. Separation of lactic, formic and acetic acids using 0.1 N ethylamine in the bottom phase.

1) a mixture of all three acids.
2) » » » formic acid and acetic acid.
3) » » » » » » lactic acid.
4) » » » acetic » » » »

In the presence of large amounts of acids the R_F value of acetic acid decreases, as is apparent from the fact that the acetic acid spots in 1—3 are above those in 4.

However by making use of the technique below it is possible to detect the presence of lactic, formic and acetic acids in a mixture, even although the acids do not separate completely.

The acids are placed in the form of spots containing 0.5—1.5 μ eq. (total) on the quick running paper Munktell OB. A pad of Munktell paper or some other porous paper is attached to the lower end of the chromatogram as described by Miettinen and Virtanen [6] and the paper is run in parallel with an ordinary chromatogram. By virtue of the greater speed of running of the former, the separation of the acetic acid group is improved.

The paper is sprayed in the normal manner with indicator and the positions of the spots due to the lactic acid, the formic acid, and the acetic acid are marked round the outer edges. The paper is then sprayed on both sides with a silver nitrate reagent (equal parts of 0.1 N $AgNO_3$ and 5 N NH_3) and heated at 105° for 1—2 hours. After this treatment the location of the formic acid is indicated by a brown spot. By considering the position of this spot in relation to the outline of the spot due to the acid mixture as revealed when the indicator is washed away it is possible to decide if both acetic and lactic acids are present. Figs. 3 and 4 indicate the application of this technique.

It follows from Fig. 2 that it is advantageous to use 0.1 N or more concentrated ethylamine in order to obtain the best separation, but often the normal 0.025 N ethylamine is satisfactory with this technique.

When the concentration of the formic acid is high, the spot due to this material diffuses over the acetic acid spot and it may become difficult or impossible to decide whether both formic and acetic acids are present or not. (Fig. 3). This diffusion is

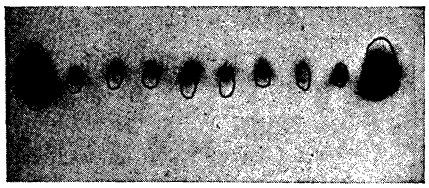

Fig. 4. The separation of formic and acetic acids in the distillate from various types of cheese. (Lactic acid is present only in very small amounts.) Ethylamine concentration in bottom phase, 0.025 N. Because of the low concentration of lactic acid, the acetic acid travels further than the formic acid, as is shown by the "empty space" under the formic acid spots. The controls, a mixture of all three acids seen at the edges, show an "empty space" above the formic acid spots due, in this case, to the lactic acid.

probably only apparent, being caused by the fact that the silver reaction is more sensitive than the indicator at the lowest acid concentrations.

The R_F values for the various acids should depend on several factors. It has already been mentioned that the concentration of the ethylamine and the amount of acid present effect the R_F values of formic and acetic acids, and this probably also applies in the case of the other acids. Table 1 lists the R_F values and R_{ac} values normally obtained.

Table 1. R_F and R_{ac} values of the acids

Acid	R_F, Whatman N:o 1	R_{ac}, Munktell OB
Lactic		0.75—0.94
Formic		0.82—1.00
Acetic	0.17	1.00
Propionic	0.25	
n-Butyric	0.37	
n-Valeric	0.49	
n-Caproic	0.63	
n-Caprylic	0.74	
n-Capric	0.75	
n-Lauric	0.73	

DISCUSSION

The method of detecting the fatty acids on the developed chromatograms is based on the fact that the acids are present in the form of their ethylamine salts which have a higher pH value, or at least a higher buffer capacity than their surroundings. When the indicator is sprayed on the paper this buffering capacity is utilised and the spots retain their pH while the surroundings assume a pH value closer to that of the acidic indicator. Thus the background appears in the acidic colour of the indicator and the spots in the basic tint.

The smaller the amount of indicator used the more clearly the spots stand out. If a large amount of indicator is used in order to keep the pH of the background low, the intensity of the colour in the spots decreases simultaneously. For this reason it is desirable to keep the acidic character of the background or its salt content low. All papers are more or less acidic and some contain salts as impurities. These papers retain ethylamine and thus acquire a high buffer capacity or already have such a high buffer capacity by virtue of their salt content that the background becomes blue. To eliminate this by adding acid to the indicator is wrong in principle, as by so doing one diminishes the chance of detecting small concentrations of acid. It would be better though still not satisfactory to increase the concentration of the indicator, as then one would at least increase the contrast to some extent.

The best way is to use clean paper and an ethylamine concentration which is no higher than absolutely necessary to avoid irreversible adsorption and to adjust the concentration of the indicator accordingly. Brown and Hall use such a large excess of alkali that the background acquires a higher pH than the spots. This is not very desirable as it implies that the relationship between the buffer capacities of the spots and the background is not as favourable as possible, and the sensitivity must be decreased.

From the foregoing remarks one is led to the conclusion that, *a priori*, the method developed by Hiscox and Berredge offers the greatest possibility for the detection of minimal amounts of acids.

If one wishes to work with higher concentrations, or wishes to suppress the appearance of traces of acids, there is no reason to suppose that the methods developed by Brown and Hall or by Kennedy and Barker will not give satisfactory results.

RECOMMENDED TECHNIQUE

In order to attempt quantitative experiments, it is essential that a standardised technique be employed. The following detailed procedure has been developed partly with this end in view, and partly in order to eliminate accidental irregularities which occasionally arise.

Preparation of the sample. The acids in the sample should be isolated by distillation neutralisation, evaporation, acidification with $NaHSO_4$ solution and extraction with butanol. The butanol solution is finally neutralised to bromophenol blue (pH 7) with 33 % ethylamine.

The concentration of the individual acids should be 0.025—0.050 *N*, and the amount of butanol used for the extraction is decided upon with the help of titration data and a knowledge of the origin of the material (the number of acids present must be known or estimated).

Solutions etc. 1) *n*-Butanol. Commercial 98—100 % primary *n*-butanol treated with *ca.* 10 g of KOH per litre and fractionated through an efficient column.

The fraction b.p. $\leqq$ 116° contains water and can be reworked. The fraction b.p. 116—118° is used for the chromatography. The fraction b.p. 118—119° can be used for preparing the sample.

2) Water-saturated butanol. The fraction of b.p. 116—118° is shaken vigorously with about 25 % of distilled water. The butanol is kept over water and can be used as soon as it is completely clear.

3) Butanol-saturated ethylamine. 33 % ethylamine is diluted to 0.025 *N* with butanol-saturated water, and the strength is checked by titration with 0.1 *N* HCl using methyl red as indicator.

4) Indicator solution. 2.5 g of bromocresol green is dissolved in 500 ml of ethanol and 500 ml of butanol (b.p. > 118° is suitable) is added.

5) Standard solutions of the acids. Approximately 0.1 eq. of acid is weighed into a 50 ml measuring flask, which is then filled to the mark with analytically pure methanol. The solution so obtained is titrated with 0.1 *N* alkali using phenolphthalein as indicator, and an appropriate amount to give a solution which will be eventually exactly *N* is transferred to a 50 ml flask. One drop of 1 % bromothymol blue is added followed by 33 % ethylamine until the colour turns blue, and the solution is diluted to the mark with butanol. (Lactic acid gradually decomposes and solutions of it change in concentration over a few weeks.) 0.1 *N*, 0.050 *N* and 0.025 *N* solutions are prepared from the *N* solutions by mixing the various acids and diluting with ethylamine-neutralised butanol. These figures refer to the individual acids, not to the total concentration. It is convenient to make two mixtures, one containing lactic, formic and acetic acids, and the other acetic, propionic, butyric, valeric, capric and caprylic acids.

6) Silver reagent. 50 ml 0.1 *N* $AgNO_3$ + 50 ml 5 *N* NH_4OH. The solution should be freshly prepared each day.

Method. 1) Ethylamine solution is placed in the bottom of the tank to a depth of about two cm and the lid is replaced.

2) The papers are cut and lined. A sheet of Whatman N:o 1 is used for the separation of the higher acids and a sheet of Munktell OB for the separation of formic, acetic and lactic acids. A pad of filter paper is attached to the lower edge of the latter sheet. This pad should weigh about five times as much as the chromatogram itself, and is folded together tightly and attached by means of a number of staples.

3) About 3 ul of the sample is spotted on to the paper, its position being marked with a small cross. The two outer positions on the paper are reserved for standard solutions, usually 0.050 *N* and 0.025 *N*.

4) The paper is hung in the through and kept in place with a glass rod, and the water-saturated butanol is run in. After 16—32 hours the papers are removed and allowed to dry in the air for one hour. (The pad should be removed immediately after the paper is removed from the tank.)

5) The papers are sprayed evenly with the indicator solution.

6) The acid spots apparent on the Munktell paper are marked around the edges, and the paper is sprayed with the silver reagent, and heated for 1—2 hours at 100—125°, during which time it should be allowed to hang freely. The indicator and the excess of silver reagent is removed by repeated washing with distilled water and the paper is dried in the usual way.

Preservation of the papers. The chromatograms obtained as above can only be kept for a few hours. In order to preserve them for longer periods they should be sprayed with a 5 % solution of paraffin in pure benzene. When the benzene has evaporated the paper is warmed until the paraffin melts and spreads evenly over the surface. The contrast is diminished by this procedure and unfortunately the weakest spots may disappear entirely.

If the treated chromatograms are kept between plain paper they may be preserved for up to several months but this method is really only satisfactory when large amounts of acids have been used. If a record is to be kept it is much better to photograph the chromatograms using panchromatic film or plates and a dense red-violet or orange filter.

SUMMARY

The methods available for the paper chromatographic analysis of volatile fatty acids have been investigated with a view to ascertaining which factors affect the sensitivity, the contrast between the spots and the background etc.

It has been shown that several factors must be carefully balanced one against the other if the best results are to be obtained. The variables concerned are the following: the quality of the paper, the concentration of volatile alkali in the atmosphere, the method of drying and the time of drying the paper, the concentration of the indicator and the solvent used, the construction of the sprayer, and finally the manner in which the spraying is carried out.

Detailed directions have been given of the most satisfactory procedure.

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