SEPARATION OF SATURATED MONO-HYDROXAMIC ACIDS BY PARTITION CHROMATOGRAPHY ON PAPER

By Adrienne R. Thompson

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Summary

Separation of simple saturated hydroxamic acids with a chain length of one to nine carbon atoms has been achieved by partition chromatography on paper. The relative merits of a number of solvents are discussed.

It is shown that efficient humidification of the system is important when using a solvent such as benzene, which dissolves very little water. Interference caused by metals in the filter paper can be overcome by preliminary treatment.

I. INTRODUCTION

This paper is concerned with the separation by partition chromatography on paper of the lower simple saturated hydroxamic acids \( \text{C}_n \text{H}_{2n+1} \text{CO.NHOH} \). The technique was developed for use in an investigation of volatile substances produced by fresh apples.

Carboxylic acids (both free and esterified), primary alcohols, and aldehydes can all be converted to hydroxamic acids and the separated hydroxamic acids can readily be detected as the coloured iron complexes.

After the methods of chromatographic separation described in this paper had been worked out, the work of Fink and Fink (1949) came to our notice. These authors obtained useful separations of hydroxamic acids derived from simple monocarboxylic acids with one to five carbon atoms; above this, their series was incomplete and not well separated. They also studied hydroxamic acids from a number of substituted and polycarboxylic acids.

The principles on which the paper chromatographic technique is based have been adequately discussed by Consden, Gordon, and Martin (1944) and, with respect to the separation of acids, by Lugg and Overell (1948). The technique of ascending chromatography, as described by Williams and Kirby (1948), has been used in the present investigations.

II. DEVELOPMENT OF TECHNIQUE FOR CHROMATOGRAPHIC SEPARATION OF HYDROXAMIC ACIDS

(a) Formation of Hydroxamic Acids

Pure samples of esters of simple saturated fatty acids containing from one to ten carbon atoms were converted to the corresponding hydroxamic acids by a method similar to that previously described (Thompson 1950). The reaction mixture contained 2 ml. ester or mixture of esters in ethanol (total

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concentration $4 \times 10^{-2}$M), 30 ml. ether, and 1.0 ml. hydroxylamine solution (prepared from equal volumes of 5 per cent. hydroxylamine hydrochloride and 12.5 per cent. sodium hydroxide in methanol, the sodium chloride being removed by filtration).

The mixture was allowed to stand at 25°C. for 30 minutes and 0.08 ml. glacial acetic acid added. The sodium acetate was filtered off and the ether removed under reduced pressure.

(b) Description of Chromatographic Technique

Portion of the solution of hydroxamic acids (containing about $10^{-5}$ mole of total hydroxamic acids) was applied as a spot about 1.5 in. from the edge of a filter paper sheet (Whatman No. 1). The paper was formed into a cylinder and the chromatographic separation carried out at 20°C. for 16-20 hours as described by Williams and Kirby (1948). In this time the front had travelled 10-20 in., the rate being fastest with benzene as mobile solvent and slowest with octyl alcohol.

The non-aqueous solvent was shaken with water and acid to obtain a mixture of two phases. The acid was used to repress ionization and prevent tailing (see Lugg and Overell 1948 and Section III (b) below). After allowing the solvent mixture to come to equilibrium at 20°C.,* the aqueous phase was placed in the bottom of the tank outside the dish carrying the paper and the mobile water-poor phase, and, initially, no other means of humidifying the air of the tank was used. In later experiments the air was humidified by lining the tank with paper soaked with the aqueous phase.

Owing to the instability of the hydroxamic acids, the papers were dried at room temperature. The spots were revealed by spraying the paper either with ferric chloride solution (10 per cent.) when the purple spots appeared against a yellow background, or with ferric perchlorate solution (containing approximately 2.7 g. iron and 250 g. perchloric acid per litre) when the background was white. With the perchlorate spray the colours began to fade after a few hours, except the colour from formhydroxamic acid, which faded noticeably in a few minutes. Using the ferric chloride spray the colour was stable for one to two weeks. In either case, a permanent photographic record was obtained (on plain process film using a medium yellow filter).

Table 1 shows the $R_f$ values of a number of unbranched hydroxamic acids for various acidified solvent mixtures. Photographs of some of the corresponding chromatograms are given in Plate 1, Figures 1-6.

III. Factors Affecting the Chromatographic Separation

(a) Composition of Mobile Phase

Separation of hydroxamic acids containing one to five carbon atoms was possible with butanol (Plate 1, Fig. 1) or amyl alcohol (Plate 1, Fig. 2), as was also shown by Fink and Fink (1949). With octyl alcohol (Plate 1, Fig. 3), the separation of hydroxamic acids containing from two to six carbon atoms was achieved. The useful range of separation for benzene-acetic acid (Plate 1,
### Table 1

**R<sub>F</sub> Values of Unbranched Hydroxamic Acids**

<table>
<thead>
<tr>
<th>Non-aqueous Solvent</th>
<th>Butanol 40 ml</th>
<th>Amyl Alcohol 40 ml</th>
<th>Amyl Alcohol 75 ml</th>
<th>Amyl Alcohol 100 ml</th>
<th>Octyl Alcohol 75 ml</th>
<th>Octyl Alcohol 50 ml</th>
<th>Octyl Alcohol 35 ml</th>
<th>Benzyl Alcohol 75 ml</th>
<th>Benzene 100 ml</th>
<th>Benzene-Octyl Alcohol 37.5 ml</th>
<th>Phenol 50 g</th>
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<table>
<thead>
<tr>
<th>Acid</th>
<th>Acetic 10 ml (glacial)</th>
<th>Acetic 10 ml (glacial)</th>
<th>Formic 25 ml</th>
<th>Sulphuric 0.3 ml (36N)</th>
<th>Formic 25 ml</th>
<th>Oxalic 0.5 g (hydrate)</th>
<th>Sulphuric 0.15 ml (36N)</th>
<th>Acetic 20 ml (glacial)</th>
<th>Formic 75 ml</th>
<th>Acetic 75 ml</th>
<th>Formic 100 ml</th>
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<tr>
<td>Water</td>
<td>50 ml</td>
<td>50 ml</td>
<td>75 ml</td>
<td>100 ml</td>
<td>75 ml</td>
<td>50 ml</td>
<td>50 ml</td>
<td>45 ml</td>
<td>75 ml</td>
<td>100 ml</td>
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<th>5</th>
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<tr>
<td>Formhydroxamic Acid C1</td>
<td>0.42</td>
<td>0.26</td>
<td>0.08</td>
<td>0</td>
<td>0</td>
<td>0.03</td>
<td>0.52</td>
<td></td>
<td></td>
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<tr>
<td>Acethydroxamic Acid C2</td>
<td>0.50</td>
<td>[0.53]</td>
<td>0.34</td>
<td>[0.35]</td>
<td>0.30</td>
<td>[0.30]</td>
<td>0.11</td>
<td>[0.14]</td>
<td>[0.10]</td>
<td>[0.11]</td>
<td>[0.49]</td>
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<td>0.01</td>
<td>[0]</td>
<td>0.05</td>
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<td>0.62</td>
<td>[0.66]</td>
<td>0.51</td>
<td>[0.53]</td>
<td>0.50</td>
<td>[0.51]</td>
<td>0.25</td>
<td>[0.30]</td>
<td>[0.23]</td>
<td>[0.25]</td>
<td>[0.62]</td>
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<td>0.05</td>
<td>[0.05]</td>
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<td>Butyrohydroxamic Acid C4</td>
<td>0.72</td>
<td>[0.77]</td>
<td>0.67</td>
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<td>Valerohydroxamic Acid C5</td>
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<td>[0.84]</td>
<td>0.78</td>
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<td>0.81</td>
<td>[0.82]</td>
<td>0.67</td>
<td>[0.69]</td>
<td>[0.69]</td>
<td>[0.69]</td>
<td>[0.79]</td>
<td>0.11</td>
<td>0.32</td>
<td>[0.30]</td>
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<tr>
<td>Hexanohydroxamic Acid C6</td>
<td>0.84</td>
<td>[0.91]</td>
<td>0.86</td>
<td>[0.86]</td>
<td>0.83</td>
<td>[0.85]</td>
<td>0.81</td>
<td>[0.80]</td>
<td>[0.81]</td>
<td>[0.82]</td>
<td>[0.89]</td>
<td>0.26</td>
<td>0.61</td>
<td>[0.55]</td>
<td>0.73</td>
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<tr>
<td>Heptanohydroxamic Acid C7</td>
<td>0.86</td>
<td>[0.94]</td>
<td>0.89</td>
<td>[0.88]</td>
<td>0.85</td>
<td>[0.85]</td>
<td>0.88</td>
<td>[0.90]</td>
<td>[0.86]</td>
<td>[0.91]</td>
<td>0.51</td>
<td>0.87</td>
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<td>Octanohydroxamic Acid C8</td>
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<td>0.89</td>
<td>0.90</td>
<td>0</td>
<td>0.77</td>
<td>0.95</td>
<td>[0.90]</td>
<td>0.89</td>
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<tr>
<td>Nonanohydroxamic Acid C9</td>
<td>0.89</td>
<td>[0.92]</td>
<td>0.89</td>
<td>[0.90]</td>
<td>0.90</td>
<td>[0.86]</td>
<td>0.90</td>
<td>[0.94]</td>
<td>[0.85]</td>
<td>[0.91]</td>
<td>0.89</td>
<td>0.97</td>
<td>[0.93]</td>
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<tr>
<td>Decanohydroxamic Acid C10</td>
<td>0.90</td>
<td>0.90</td>
<td>0.92</td>
<td>0.92</td>
<td>0.97</td>
<td>[0.94]</td>
<td>0.93</td>
<td></td>
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</tr>
</tbody>
</table>

*RF* values enclosed in square brackets were obtained when there was no humidification of the system other than the aqueous phase on the bottom of the tank. Otherwise the tank was humidified by lining the tank with paper soaked with the aqueous phase. The lines indicate the limits of usefulness of the separation.
Fig. 5) was three to eight and usually nine carbon atoms. With benzene-
formic acid (Plate 1, Fig. 4) the useful range was from four to nine and some-
times ten carbon atoms. Good separations of two to seven carbon atoms were
obtained with a mixture of benzene, octyl alcohol, and formic acid (Plate 1,
Fig. 6).

Separations from two to four carbon atoms were obtained with benzyl
alcohol, but the spots were rather indiscrete. Light petroleum and xylene were
of no value. Phenol gave comparatively high RF values and had a tendency
to distort the spots. Although of no use for separating the higher members
of the series, it gave a useful separation of the one and two carbon atom
hydroxamic acids, as previously found by Fink and Fink (1949). These workers
found phenol especially useful for separating hydroxamic acids derived from
dicarboxylic acids, which, in general, have lower RF values.

(b) Acid

The acid was added to the solvents with the object of suppressing the
ionization of the hydroxamic acids and thus preventing “tailing” (Lugg and
Overell 1948). This is a desirable precaution, although satisfactory chromato-
grams have sometimes been obtained, both by Fink and Fink (1949) and the
author, after applying the hydroxamates in approximately neutral solution and
without addition of acid. Fink and Fink (1949) did not use additional acid,
and their RF values indicate that the un-ionized hydroxamic acids travelled
forward on the paper. The author has omitted the addition of acid and ob-
tained satisfactory chromatograms with butanol but never with benzene.

It is evident that the ionization of the weak hydroxamic acids is sup-
pressed at a comparatively low hydrogen ion concentration, which may be
already attained in the approximately neutral spot applied to the paper. How-
ever, it is unwise to rely on this, as the effect may be due to a very slight excess
of the acid used for neutralization and it is better to ensure an adequate excess
by adding acid to the solvents.

Provided the composition of the mobile phase was not markedly altered
by the addition of acid, i.e. if only a small quantity was used, the RF values
were practically independent of the particular acid used. On the other hand,
by marked changes in the proportions of acetic acid and benzene, resulting
in marked alteration of the composition of the mobile phase, considerable
changes in the RF values were obtained (Table 2).

The author has used a large variety of acids. The choice has not been
limited, as in the separation of carboxylic acids by Lugg and Overell (1948),
to using a volatile acid since the test used for the detection of hydroxamic
acids is specific for these substances in the presence of other acids.

(c) Humidification

Hanes and Isherwood (1949) have adopted special measures to ensure
that the paper is allowed to become approximately saturated with respect to
the aqueous phase. In this laboratory, it has been found that when the non-
aqueous solvent was benzene, which dissolves very little water, the amount of
water vapour in the system has a very important effect on the forward movement of hydroxamic acid spots. If no additional means were adopted to humidify the air in the tank when benzene-formic acid was used, the chromatogram on development frequently showed a continuous streak starting from the point of application of the spot and fading off in the direction of solvent flow without differentiation into individual spots (Plate 2, Fig. 7). This "streaking" is easily distinguished from the "tailing" occurring in the absence of acid in which the spots fade off in the opposite direction.

This phenomenon was always encountered when large papers or a number of concentric papers were used under these conditions. If the air in the tank was humidified by lining the sides of the tank with filter paper soaked in the aqueous phase, this trouble could be avoided (Experiment 12, Plate 1, Fig. 4). The effect of this treatment, designed to assist equilibration between paper, gas phase, and liquid aqueous phase, was to reduce the R\textsubscript{F} values slightly.

<table>
<thead>
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<th>Table 2</th>
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<tr>
<td>EFFECT OF ALTERING THE PROPORTIONS OF BENZENE AND ACETIC ACID ON THE R\textsubscript{F} VALUES OF UNBRANCHED HYDROXAMIC ACIDS</td>
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<table>
<thead>
<tr>
<th>Solvent Proportions</th>
<th>R\textsubscript{F} Values of Hydroxamic Acids with the Number of Carbon Atoms shown</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Benzene (ml.)</td>
<td>Acetic Acid (glacial) (ml.)</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
</tr>
</tbody>
</table>

(d) Impurities in the Filter Paper

With benzene and acetic acid a peculiar effect was noticed. Before developing the chromatogram, wedge-shaped, faintly yellow areas were observed on the paper which, on spraying with iron solution, appeared as pale purple areas lying immediately in front of each hydroxamic acid spot in the direction of solvent flow (Plate 2, Fig. 8). This "shadow" effect was most intense in colour with the hydroxamic acid spot of highest R\textsubscript{F} value in the sample and most elongated with valerate and other members of the series located near the middle of the paper.

By extracting the paper before use with 50 per cent. acetic acid, washing with water, pressing it between filter paper sheets and drying, the "shadow" effect was almost entirely eliminated.

Two samples of filter paper (Whatman) had an iron content of 19 and 6.3 p.p.m. A polarographic analysis of an extract of the second sample of filter paper showed the following other metals to be present: copper (3.0 p.p.m.), lead (11.4 p.p.m.), zinc (4.2 p.p.m.), nickel (1.4 p.p.m.). The chromium concentration was too low to be analysed polarographically and cobalt and cadmium were not detected.
The "shadowing" was reintroduced by soaking the acetic-extracted filter paper in 10⁻¹ molar ferric chloride solution, pressing between filter paper sheets, and drying. With ferric chloride solution of 10⁻² molar concentration the effect was also observed, but it was less typical, with a large area of shadow compared with the spot area. Moreover, the shadow in this case was purple in colour even before spraying with iron solution. Of the other metals present in the filter paper, only copper and nickel form coloured hydroxamate complexes. Neither of these metals reproduced "shadowing" when introduced into acetic-extracted paper in the same way from 10⁻¹ molar solutions. The effect is explained by the ferric ions in the paper forming complexes with the hydroxamic acids with somewhat higher partition coefficients between benzene and water than the free hydroxamic acids and thus preceding them along the paper.

It is of interest that Hanes and Isherwood (1949) have described a somewhat similar shadowing in the separation of phosphoric esters on paper. In this case the shadows lagged behind the main spot. They were attributed to the presence of heavy metals in the paper as the interference could be removed either by extraction of the paper with aqueous alcoholic 8-hydroxyquinoline or by exposing the paper to a small amount of hydrogen sulphide gas before running the chromatogram. It was not removed by extraction with hydrochloric acid. In contrast, the shadowing experienced with the hydroxamic acids could be removed by extraction with hydrochloric acid, but acetic acid was preferred as having less effect on the strength of the paper.

IV. DISCUSSION

To the author's knowledge, the separation of hydroxamic acids containing one to nine carbon atoms represents the largest number of consecutive members of a homologous series (differing only by a —CH₂— group) which have been separated by the paper chromatographic technique. No particular advantage was obtained by using the two-dimensional chromatographic technique for the separation of the homologous series of hydroxamic acids, and two separate one-dimensional chromatograms, with solvents that separate the lower and higher members of the series respectively, were more convenient.

The method described permits the identification of the lower members of a homologous series of any substances that can be converted to hydroxamic acids—esterified acids, acids, alcohols (after oxidation), and aldehydes. The latter react directly with toluenesulphonhydroxamic acid to form hydroxamic acids. Details of these procedures will be given in a subsequent paper describing the use of this technique for identifying apple volatiles.

V. ACKNOWLEDGMENTS

The author wishes to thank Dr. F. E. Huelin for his interest in this work, and Miss Heather Smith for very able assistance.
VI. REFERENCES


Chromatograms illustrating the separation of hydroxamic acids in various solvents. The numbers beside the spots indicate the number of carbon atoms in the hydroxamic acids. The point of application of the solute is indicated at the bottom of the sheet and the solvent front at the top.
Fig. 7.—Benzene-formic acid. Chromatogram showing “streaking” due to insufficient humidification. Spot contained hydroxamic acids with two to ten carbon atoms.

Fig. 8.—Benzene-acetic acid. Chromatogram showing “shadows” caused by impurities in the filter paper. (The total quantity of hydroxamic acids in samples B, C, and D was twice that in sample A.)
