used because of its volatility). Slowly add a concentrated solution of potassium permanganate with a pipet, stirring constantly, until the color disappears approximately 1 minute after the drop is added. When the oxidation is completed, add an excess of pure calcium carbonate to neutralize the acid and place the dish on a steam bath. (Complete neutralization and thorough drying are essential.) Add to the dry precipitate on the steam bath 10 ml. of distilled water, 10 ml. of standard 0.1*N* silver nitrate solution, and 15 ml. of 50% ethyl (or methyl) alcohol. Cool and filter the contents of the dish through a paper filter. Wash the precipitate several times with 50% alcohol, adding the washings to the filtrate. Add ferric sulfate indicator to the latter and titrate the excess silver ion with standard 0.1*N* ammonium thiocyanate solution. The volume of silver nitrate consumed is used to calculate the quantity of succinic acid.

The results of control tests in which various amounts of fumaric and malic acids were added to 40 mg. of succinic acid showed that these acids did not interfere. In each case the determination indicated the presence of approximately 40 mg. of succinic acid; the greatest deviation was never more than ±1 mg. and was probably due to incomplete drying and washing of the precipitate.

DETERMINATION OF MALIC ACID

Transfer 10 ml. of the fermented substrate to a porcelain dish on a steam bath, neutralize with an excess of calcium carbonate, and evaporate to dryness. Add 10 ml. of distilled water, 10 ml. of standard 0.1*N* silver nitrate solution, and 15 ml. of 50% alcohol. After cooling, filter and wash the precipitate with 50% alcohol. Add ferric ion indicator and titrate with standard 0.1*N* thiocyanate solution.

The titration gives the sum of all three acids (malic, fumaric, and succinic); calculation is made on the basis of fumaric acid, since the molecular weights of the three acids are similar and fumaric acid occurs in far greater quantities in the mixture. The sum of the fumaric and succinic acids as previously determined is subtracted to obtain the malic acid.

PRESENCE OF RIBOFLAVIN IN FUMARIC ACID FERMENTATION

As indicated, the direct determination of fumaric acid in fermented substrate gives satisfactory results. However, crystals of mercurous fumarate obtained from pure fumaric acid solution are colorless, while those from the fermented substrate range in color from yellow to orange. The supernatant liquid is decolorized by the crystallization of mercurous fumarate, indicating that some dye is absorbed by the crystals of mercurous fumarate. The technical glucose in the tablets (weighing 6 to 8 pounds) used for these experiments is brownish, but of a different shade from that of the fermented substrate. Precipitation from nonfermented substrate after the addition of pure fumaric acid gave colorless crystals. Thus, it seems likely that some dye is produced in this fermentation. Crystallization of the mercurous fumarate occurs in a solution containing 10% nitric acid; this concentration of acid at 100° C. (steam bath temperature) destroys most natural dyes except riboflavin.

Analytical tests on numerous samples of fermented substrates showed riboflavin to be present in quantities from 4 to 8 γ %, the amount corresponding to the quantity of fumaric acid produced and to the deepening of the color of the fermented substrate.

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Chromatographic Separation of *p*-Phenylazophenacyl Esters on Silicic Acid

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THE separation and identification of small amounts of fatty acids either in the free state or from the hydrolysis of esters have been necessary in investigations of the identity of flavor materials in grapes and wines which are under way in this laboratory. The chromatographic separation of the acids as *p*-phenylphenacyl esters often leaves much to be desired, as the bands are invisible under ordinary light and must be viewed in a dark room under ultraviolet light, where they exhibit a pale blue fluoresence. The bands are often difficult to distinguish and to separate, particularly with the compounds of higher molecular weight. Highly colored *p*-phenylazophenacyl esters have been reported by Masuyama (*2*) and by Sugiyama (*4*). The chromatographic separation of a number of these bright orange colored *p*-phenylazophenacyl esters on silicic acid is described in the present paper. Other attempts in this laboratory to separate these derivatives on alumina columns were not successful.

MATERIALS

p-Phenylazophenacyl Bromide. *p*-Phenylazoacetophenone was prepared according to the method of Angeli (*1*) by the condensation of equimolar quantities of *p*-aminoacetophenone and nitrosobenzene in glacial acetic acid at room temperature. Recrystallization of the crude product from ethyl alcohol gave orange crystals of *p*-phenylazoacetophenone, melting point 114.5° to 116° C. An equimolar quantity of bromine was added dropwise over a period of an hour to a stirred solution of *p*-phenylazoacetophenone in glacial acetic acid maintained below 20°. The reaction mixture was poured into ice water and the precipitate was filtered, washed with water, and dried over calcium chloride in a vacuum desiccator. The *p*-phenylazophenacyl bromide was purified by chromatography on silicic acid using 50% benzene in Skellysolve B as the developing solvent by the technique described. Three small bands and one major band appeared as the column was developed. The major band was eluted to give *p*-phenylazophenacyl bromide, melting point 104–105° C. Analysis: calculated for $C_{14}H_{11}N_2OBr$: carbon, 55.46; hydrogen, 3.66; found: carbon, 55.12; hydrogen, 3.68. The yield in the bromination step was 15%. Various attempts to brominate *p*-phenylazoacetophenone with *N*-bromosuccinimide using a variety of conditions were unsuccessful.

Adsorbent. Silicic acid (Mallinckrodt, analytical reagent, No. 2847, 100 mesh) was activated by pumping in a vacuum desiccator over phosphorus pentoxide for 8 minutes at full capacity of a Cenco Hyvac pump. The activated adsorbent was stored in a tightly capped bottle until used.

Developing solvents. Thiophene-free benzene and Skellysolve B were used without further purification.

p-Phenylazophenacyl esters. The derivatives were prepared from the various acids and *p*-phenylazophenacyl bromide using the method of Shriner and Fuson (*3*) for the preparation of *p*-phenylphenacyl esters. Each derivative was purified by chromatography on silicic acid as described.

Table I. *p*-Phenylazophenacyl Esters

	Obsd. M.P., ° C.	Literature Melting Point, ° C. By Masuyama (2)	By Sugiyama (4)	Combustion Analyses Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
Acetate	125–7	117–8	125–5.5	68.07	68.13	5.00	5.07
Propionate	104–5	98–9	103–3.5	...	...	...	...
Butyrate	98–9	92–3	97.5–98.2	...	...	...	...
Isobutyrate	101.5–2	...	...	...	...	...	...
Valerate	69.5–70	67–9	71.5–72	70.35	70.13	6.22	6.08
Caproate	83–4	62–4	83–3.5	70.98	70.76	6.55	6.56
Caprylate	83–3.5	72–4	...	72.10	72.12	7.15	7.12
Caprate	84.5–5.5	64–6	84–5	73.06	72.97	7.66	7.48
Laurate	87–7.5	84–6	86–6.5	...	...	...	...
Myristate	92–3	91–3	...	...	...	...	...
Palmitate	95–6	97–9	94.5–5	75.27	74.51	8.86	8.94
Stearate	99–100	102–3.5	97.5–8	...	...	...	...

Table II. Chromatographic Separation of *p*-Phenylazophenacyl Esters of 12 Aliphatic Acids

G = complete. P = partial. O = no separation.

	Propionate	Butyrate	Isobutyrate	Valerate	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate	Reagent
Acetate	G											
Propionate	..	G	G									
Butyrate		..	O	G								
Isobutyrate			..	P	G							
Valerate				..	G							
Caproate					..	G	G					
Caprylate						..	G			G		G
Caprate							..	P	G			
Laurate								..	P	G		
Myristate									..	P	G	
Palmitate										..	P	G
Stearate											..	O

APPARATUS

The chromatographic column consists of an 8 × 250 mm. tube with female 18/9 ball joints on each end. The bottom of the column is fitted to a male 18/9 ball joint drawn down to a drip tip. This male joint is ground down sufficiently to permit the introduction of a thin Witt plate and circle of filter paper which support the adsorbent in the column. A 250-ml. separatory funnel with a built-in pressure-equalizing line and a male 18/9 joint at the bottom is used at the top of the column as a solvent reservoir.

PROCEDURE

The adsorbent is placed in the column as a slurry composed of the desired amount of activated silicic acid mixed with 2 parts of the solvent mixture to be used in developing the chromatogram. Any air bubbles are removed by tapping the column and stirring the slurry in the column by means of a length of wire. The adsorbent is allowed to settle for a short while and then packed tight by the application of 8 to 10 pounds' pressure until the excess solvent has been expelled from the column. The pressure is released when the solvent level is less than 1 cm. above the top of the adsorbent. When prepared in this manner the column is opalescent in appearance, which greatly facilitates the detection of the bands as the chromatogram is developed. The mixture of *p*-phenylazophenacyl esters dissolved in the minimum amount of the developing solvent is placed on the top of the column by means of a pipet and the chromatogram is developed under 5 to 8 pounds' pressure applied at the top of the solvent reservoir. The bright orange bands are clearly visible on the column. Development was continued until the bands were completely separated, whereupon they were either washed on through the column and collected separately or the column was dried out and the bands were dug out with a spatula and then eluted with acetone.

RESULTS AND DISCUSSION

Table I shows the corrected melting points of the *p*-phenylazophenacyl esters prepared in this investigation, as well as the melting points previously reported in the literature. The melting point range listed is from the first visible softening to the temperature at which the last trace of solid melted. Combustion analyses have been included in the table in most of those cases where a marked discrepancy in the reported melting points exists.

Table II shows the results obtained from the adsorption of 19 pairs of *p*-phenylazophenacyl esters of 12 aliphatic acids and 3 pairs involving the derivatives and *p*-phenylazophenacyl bromide. The pairs marked *G* in the table were completely separated into two zones on the column and the eluted components differed in melting point by not more than a degree from that of the original crystalline derivative. The pairs marked P gave only a single zone on development, but when the band was arbitrarily divided into three portions, the upper and lower portions gave materials of different melting points, indicating at least a partial separation. In the pairs marked O no observable separation was obtained, as the material from various parts of the band showed no significant difference in melting point. The developing solvent which was used for those pairs involving the derivatives from acetate through valerate consisted of 75% benzene and 25% Skellysolve B by volume, while derivatives of higher molecular weight were best separated using 50% benzene–Skellysolve B mixtures.

The blank spaces in the upper right part of Table II represent combinations which were not investigated but which should be completely separable without difficulty, as they represent pairs which have a greater difference in molecular weight than pairs which are shown to be separable.

Any excess reagent passes through the column first and is readily separable from all the *p*-phenylazophenacyl esters up through palmitate. However, essentially no separation was possible between the stearate ester and the reagent.

The derivatives listed in this work are mainly those of the straight-chain homologous fatty acids and they pass through the column in the reverse order of their molecular weights, as expected. The only example of a branched-chain acid derivative investigated, *p*-phenylazophenacyl isobutyrate, showed no separation from a mixture with the *n*-butyrate derivative and only poor separation from a mixture with the *n*-valerate derivative. The acid derivatives of higher molecular weight showed only poor separation with a difference of two carbons in the acid but good separation for a four-carbon difference.

The results of such investigations in this laboratory indicate that under the conditions used the chromatographic separations of mixtures of *p*-phenylazophenacyl esters of the higher molecular weight acids are somewhat better than similar separations of mixtures of *p*-phenylphenacyl esters. Highly colored acid derivatives which can be separated chromatographically are also of value where dark-room and ultraviolet light facilities are unavailable.

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