Results and Discussion

The light absorption spectrum of the purified product was determined on a Beckman DU spectrophotometer, using chloroform as a solvent, and compared with a similar spectrum determined on pure dianilinogossypol. Both curves, Figure 1, practically coincide except between 249 and 253 mμ. From these spectra, it is concluded that the material extracted from pig liver and crystallized as an aniline derivative was the aniline derivative of gossypol. Substantiating data were provided by comparison of the infrared spectrum of the crystalline material with that of pure dianilinogossypol. Almost identical curves, Figure 2, indicate positively that the crystalline product obtained from pig liver is pure dianilinogossypol.

The isolation of gossypol from animal tissue opens the way for further study on the metabolic fate of gossypol in the animal organism and the mode of action of this compound in damaging tissue.

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Quantitative Analysis of Short Chain Fatty Acids Using Gas Liquid Chromatography[1]

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Abstract

The short-chain fatty acids, propionic to pelargonic, have been analyzed by gas liquid chromatography, as their butyl, phenacyl, and decyl esters to overcome losses due to their volatility. The three methods are compared and the decyl ester procedure offers the most advantages.

Introduction

THE GAS LIQUID chromatographic analysis (GLC) of short-chain fatty acids and their methyl esters has been described by James and Martin (10), Van de Kamer et al. (18), McInnes et al. (7,12), Brandenberger and Müller (2), Hunter et al. (9), Vorbeck et al. (22), Böttcher et al. (1), and Gehrke and Lamkin (6). The main problem in quantitative analyses of these materials is the possibility of losses due to the volatility of the acids or their methyl esters during extraction, removal of solvents and transfer to the GLC unit. Unique techniques have been developed to overcome these problems in using the free acids (8,6). Ostle and Ahrens (15) made use of 2-chloroethanol esters to reduce losses due to volatility in order to obtain quantitative results.

This laboratory has been particularly interested in the quantitative analysis of the acids resulting from oxidation of unsaturated acids with reagents such as permanganate-periodate (21). This publication describes the analysis of the acids as their butyl, phenacyl, and decyl esters, procedures which largely overcome losses due to volatility.

Materials and Methods

Commercial samples of the short chain fatty acids were purified by distilling the methyl esters in a Podbielniak Heli-Grid column. The esters were converted to free fatty acids and the purity of each acid was checked by GLC analysis of the methyl esters prepared from the free acids with diazomethane.

The gas liquid chromatographic unit was of conventional design using thermal conductivity cells for detection. A 4 ft by ¼ in. OD stainless steel column packed with 60–80 mesh, acid washed Celite 545 coated with silicone grease prepared according to the procedure of Cropper and Heywood (4,5) in the ratio of 6:1 w/w was used to separate the phenacyl esters. The unit was operated at 200C, bridge current 200 ma, injector temperature 235C, and helium flow rate 60 ml per minute. The butyl esters of the C_3 to C_9 fatty acids were separated on a 6 ft by ¼ in. stainless steel OD column similarly packed with silicone on Celite and operated at 180C. The butyl esters of the dicarboxylic and long chain monocarboxylic acids were analyzed on the same column at 225C. The decyl esters were separated on an 8 ft by 3/16 in. OD copper column with the same packing and operated at 185–200C.

N-n-butyl-N-nitroso-N′-guanidine, synthesized according to the procedure of McKay (13), was used to prepare diazobutane (14) in ethereal solution. The butyl esters were formed by adding an ethereal solution of diazobutane to an ethereal solution of the fatty acids. The excess diazobutane and ether were carefully removed by evaporation using an air cooled reflux condenser.

The α-bromoacetophenone was prepared as outlined by Vogel (19) and was stored in a brown glass stoppered bottle. Phenacyl esters of the pure acids were made according to the technique outlined in Vogel (20) and were recrystallized from a mixture of ethyl ether and petroleum ether (Skellysolve "F") using low temperature crystallization where necessary.

The phenacyl derivatives of mixtures of fatty acids or soaps were prepared as follows: A mixture of fatty acids (30–100 mg) in 5–10 ml of methanol was saponified with potassium hydroxide. Two drops of bromthymol blue indicator were added and the solu-

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TABLE I

Analysis of Monocarboxylic Acids as Butyl Esters

		Acid wt %	Butyl ester wt %	GLC area %	
Propionic	C_3	13.3	14.4	14.2	14.6
Butyric	C_4	23.5	25.0	24.7	25.2
Valeric	C_5	12.4	13.1	13.1	13.0
Caproic	C_6	10.9	11.4	11.3	11.5
Heptanoic	C_7	11.4	11.7	11.8	11.5
Caprylic	C_8	13.5	12.0	12.2	12.0
Pelargonic	C_9	15.0	12.4	12.7	12.2

tion was titrated to a faint acidity with methanolic hydrogen chloride. The solution was filtered if excessive quantities of salt were present. One half gram of α-bromoacetophenone was added to the filtrate and the mixture was refluxed for 1 hr. The methanol was evaporated on a rotary evaporator under water aspirator vacuum at a temperature of 50–60C. Two ml of pyridine was added to the cooled solution and the mixture was heated on the steam bath for a few minutes. Ethyl ether was added to dissolve the phenacyl esters and the solid pyridine-phenacyl salt was removed by filtration. The ether was evaporated and 0.5–1.0 μl of the sample was injected into the GLC unit with a hypodermic syringe. The reagent, α-bromoacetophenone, is a lachrymator and all reactions should be carried out in a fume hood.

Pure decyl esters of propionic, caproic, pelargonic, and lauric acids were prepared by the general method of Ruhoff and Reid (16). To prepare the decyl esters from a mixture of short chain acids such as would be

TABLE II

Analysis of Dicarboxylic Acids as Butyl Esters

		Acid wt %	Butyl ester wt %	GLC area %	
Succinic	C_4	18.5	22.8	23.4	22.2
Glutaric	C_5	9.3	10.7	10.8	10.6
Adipic	C_6	18.3	19.8	19.8	19.8
Pimelic	C_7	19.8	18.8	18.5	19.0
Azelaic	C_9	17.2	14.0	13.8	14.3
Sebacic	C_{10}	16.9	13.9	13.7	14.1

obtained by oxidizing an unsaturated fat the procedure used was as follows: An aqueous solution of the potassium salts of the acids is evaporated to dryness on the steam bath and a sample (5–100 mg) is shaken for 1 hr at room temperature with 1 ml of decyl alcohol containing 10% by weight of dry hydrogen chloride and 4 ml of petroleum ether (bp 60–80C). The reaction mixture is washed several times with water to remove acid and separated from excess decyl alcohol by means of a column of aluminum oxide (25 g, anionotropic, activity grade I, M. Woelm, Eschwege) made up in ethyl ether. The esters are eluted with ether, the first 300 ml being collected and evaporated on a rotary evaporator at 45C under slight vacuum. A pressure of 12 mm can be used when most of the solvent has been taken off without loss of decyl propionate. Decyl esters were also prepared from a solution of fatty acid soaps obtained by steam distilling a fatty acid mixture into potassium hydroxide solution using the micro-Kjeldahl steam distillation apparatus described by Steyermark et al. (17).

TABLE III

Analysis of Mixed Mono- and Dicarboxylic Acids as Butyl Esters

	Acid wt %	Butyl ester wt %	GLC area %	
Adipic	26.4	33.1	32.9	32.9
Azelaic	15.2	17.1	16.6	17.0
Palmitic	30.2	26.0	27.1	26.1
Stearic	28.2	23.8	23.4	24.0

TABLE IV

Quantitative Analysis of Phenacyl Esters of Fatty Acids

		Wt %	GLC analysis	
Propionic	C_3	10.4	10.1	10.1
Butyric	C_4	8.9	9.4	9.3
Valeric	C_5	12.3	12.6	12.3
Caproic	C_6	8.8	9.0	8.8
Heptanoic	C_7	19.9	20.6	20.7
Caprylic	C_8	18.4	18.1	17.4
Pelargonic	C_9	21.3	20.2	21.4

Results

Butyl Esters of Fatty Acids. A mixture of purified fatty acids from propionic to pelargonic made up by weight was converted to butyl esters. The separation is adequate and butyl acetate should be resolved between the solvent and butyl propionate peaks. The quantitative results based on areas of triangulated peaks are given in Table I. The response of the thermal conductivity detectors is on a weight basis and the percent of acids in the original mixture are calculated to butyl esters for comparison with GLC results. There is reasonable agreement between the actual and GLC values.

A mixture of dicarboxylic acids with chain lengths of C_4 to C_{10} was converted to butyl esters with diazobutane and analyzed by GLC. The results are given in Table II with the appropriate conversion of acid to ester. A mixture of adipic, azelaic, palmitic, and stearic acid was similarly analyzed (Table III). The separation of the butyl esters of long chain monocarboxylic acids such as palmitic and the dicarboxylic acids is adequate on the silicone column.

Ensuing work with diazobutane as an esterification reagent revealed the complication of peaks appearing on the GLC chart corresponding to C_4 and C_8 acids when pure samples were used in which these acids were absent. The peaks were evidently due to formation of polymers in the ethereal solution of diazobutane on standing at room temperature or in a refrigerator. It was necessary to use freshly prepared diazobutane to limit these peaks to trace amounts.

Phenacyl Esters of Fatty Acids. A mixture was made up from the individual phenacyl esters and analyzed by GLC. The results are shown in Table IV, and the response of the thermal detectors was found to be on a weight basis with respect to the phenacyl esters. A mixture of propionic, valeric, heptanoic, and pelargonic acids was made up, converted to phenacyl esters and analyzed by GLC (Table V). It was necessary to convert the acid composition on a weight basis to ester composition by use of molecular weights for comparison to GLC results.

The technique was developed for use in analyzing the short chain acids produced by oxidation of unsaturated acids. The acids, pelargonic, caproic, and propionic which would result from the oxidation of oleic, linoleic, and linolenic acids, were added to the permanganate-periodate reagent (21). Two mixtures

TABLE V

Analysis of Acids as Phenacyl Esters

		Acid wt %	Phenacyl ester wt %	GLC area %	
Propionic	C_3	12.9	16.8	16.9	16.2
Butyric[1]	C_4	.....	.....	1.2	1.2
Valeric	C_5	19.1	20.8	20.3	19.6
Heptanoic	C_7	31.0	29.9	30.6	30.8
Pelargonic	C_9	37.0	32.5	31.0	32.2

[1] Present as impurity in valeric acid.

TABLE VI

Analysis of Fatty Acids as Phenacyl Esters in Oxidation Media

	Wt mg	Acid wt %	Phenacyl ester wt %	GLC area %
Test at 0 time				
Propionic	36.4	33.0	40.1	39.4
Caproic	39.2	35.7	33.6	35.2
Pelargonic	35.4	32.2	26.3	25.4
Test after 24 hr				
Propionic	35.2	29.5	36.5	36.0
Caproic	43.9	36.8	35.4	38.0
Pelargonic	40.1	33.7	28.1	26.0

were made up and one was worked up immediately and the second was allowed to stand for 24 hr. The mixtures were reduced in the usual manner, extracted into ethyl ether and the acids were converted to phenacyl esters. The results (Table VI) show good agreement between the composition of the mixture added to the oxidation medium and that of the recovered products.

Decyl Esters of Fatty Acids. Two mixtures of the decyl esters of propionic, caproic, pelargonic, and lauric acids were made up and analyzed in triplicate by GLC. The results are shown in Table VII. Since the work on butyl and phenacyl esters was carried out the GLC apparatus had undergone a number of minor modifications and it was now found that the factors shown were necessary for satisfactory results (3). In this way good agreement was obtained on a weight basis, between the calculated results and those obtained by GLC. Similar, though smaller, factors have also been used by other workers (8). Good results were also obtained when a mixture of propionic, caproic, and pelargonic acids was converted to decyl esters and analyzed by GLC using the same factors (Table VIII). Steam distillation of the mixture of acids and conversion to decyl esters also gave similar figures (Table VIII).

Discussion

The use of diazobutane to form butyl esters of the short chain fatty acids decreased the volatility involved with propionic and butyric acids. The acids can be recovered as non-volatile soaps from the medium in which they exist and can be converted to free acids in ethereal solution with sodium hydrogen sulfate according to the technique of McInnes (11). The use of diazobutane in ether facilitates removal of solvent from the ester without loss of short chain fatty acid esters. However, diazobutane tends to form polymers and it is necessary to prepare fresh diazobutane for each reaction since the dimeric product can be confused with butyl butyrate on the GLC chart.

The phenacyl method has several advantages over esterification with diazobutane for quantitative analyses. The acids may be handled as the non-volatile soaps. Thus in recovering the acids from an aqueous medium by continuous extraction with ether a small amount of an aqueous solution of potassium hydroxide may be placed in the flask with the refluxing ether so that the volatile acids are converted to soaps immediately after extraction. The soaps can then be transformed directly into phenacyl esters by the addition of the phenacyl bromide in alcoholic solution. Solvents such as methanol and ether are more easily removed from phenacyl esters without loss of esters such as phenacyl propionate. However, the use of the phenacyl esters has some disadvantages. Besides the lachrymatory effects of phenacyl bromide, the conversion of soaps to phenacyl esters is not quantitative, but no preferential esterification was found in the C_3 to C_{10} monocarboxylic series. An additional precaution in the use of phenacyl esters is that GLC equipment such as injector, column, and detector must be stainless steel or glass since decomposition of the phenacyl esters occurs if units such as copper columns are used. If solid derivatives are desired for identification the p-bromophenacyl derivatives of the short chain acids may be used. These derivatives have slightly longer emergence times than the corresponding phenacyl esters.

The decyl ester procedure offers the same advantage as the others in the initial use of the non-volatile soaps. Further advantages in this method are that the reagents do not require special preparation, and no by-products are formed which interfere with the GLC analysis. This procedure offers promise for quantitative conversion of acids to esters, and since there is a considerable increase in molecular weight the method is well suited to the analysis of small quantities of the volatile acids.

TABLE VII

GLC Analysis of Mixtures of Decyl Propionate, Caproate, Pelargonate, and Laurate

Acid	Factors	Mixture 1 Actual	Mixture 1 Found[a]	Mixture 1 Difference	Mixture 2 Actual	Mixture 2 Found[a]	Mixture 2 Difference
C_3	1.000	6.0	5.4±0.4	−0.6	15.0	15.0±0.3	0.0
C_6	1.068	32.0	32.5±0.8	+0.5	25.6	26.0±0.4	+0.4
C_9	1.143	22.1	21.6±0.8	−0.5	39.0	38.8±0.3	−0.2
C_{12}	1.220	39.9	40.5±0.3	+0.6	20.4	20.2±0.3	−0.2

[a] Means of three determinations.

TABLE VIII

GLC Analysis of Mixtures of Short Chain Fatty Acids as Decyl Esters

	As soaps			Steam distilled
	Actual	Found		Found
C_3	24.4	23.4	23.8	24.6
C_6	37.7	38.1	37.8	37.7
C_9	37.9	38.5	38.4	37.7

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