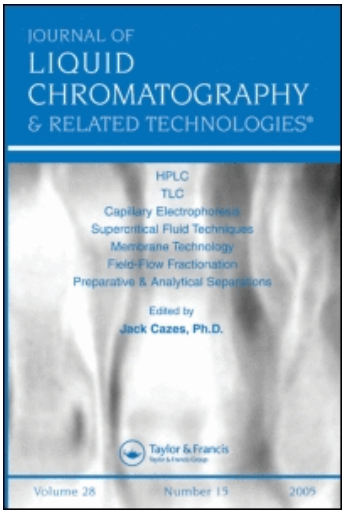

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Separation of Long And Short Chain Fatty Acids as Naphthacyl and Substituted Phenacyl Esters by High Performance Liquid Chromatography

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SEPARATION OF LONG AND SHORT CHAIN FATTY ACIDS AS NAPHTHACYL AND SUBSTITUTED PHENACYL ESTERS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatography of various C_2 - C_{24} fatty acids was performed with their p-bromophenacyl, p-nitrophenacyl, p-chlorophenacyl, and 2-naphthacyl esters. All separations were accomplished with two types of reversed phase columns with the eluent consisting of an acetonitrile:water gradient. For all derivatives tested, the separations were well defined and analogous, although certain esters eluted together as single peaks. Quantitative results indicate that the limit of detection in the present study is two picograms of n-caproic acid and 10 picograms of arachidonic acid as their p-bromophenacyl esters.

INTRODUCTION

Applications of high performance liquid chromatography (HPLC) have increased greatly during the last ten years. This increase in popularity is not very surprising in view of the vast power offered by modern HPLC for resolving various types of compounds of biochemical interest. While HPLC has been widely employed in the analysis

of nucleotides (1) and drugs (2), much less work has been attempted in the area of lipid and fatty acid analyses, particularly relating to biological systems. In the past this tendency was probably due to deficiencies of available detection devices. The UV detector has been, and still is, the most widely used detector available for quantifying samples of interest. While most lipids exhibit little or no useful absorption in the UV region, several methods have been developed for the formation of UV-absorbing derivatives of certain lipids, particularly fatty acids (3-9). These derivatives include, but are not limited to, the benzyl (3), p-nitrobenzyl (4), 2-naphthacyl (5), phenacyl (6), and p-bromophenacyl (7) esters.

In this paper HPLC analyses of p-bromophenacyl, p-nitrophenacyl, p-chlorophenacyl and 2-naphthacyl esters of C_2 - C_{24} fatty acids will be described. Ten micron particle size reversed phase columns were used to separate and to quantify many of the most difficult-to-separate fatty acids. The chromatographic properties of the above derivatives will be compared here with those in previously published work (5-7).

MATERIALS

Spectroquality distilled-in-glass acetonitrile, p-dioxane, and tetrahydrofuran, (Burdick and Jackson Laboratories, Muskegon, MI) were used for all HPLC separations. Triple-distilled, deionized water was filtered through a 0.45 μ membrane filter (Millipore Corp., Bedford, MA) to remove particulates which might clog the chromatographic column. Phenacyl bromide, p-bromophenacyl bromide, p-chlorophenacyl bromide, p-nitrophenacyl bromide, 3,4-dihydroxy-phenacyl chloride, and 2-naphthacyl bromide (Aldrich Chem. Co., Milwaukee, WI) were used prior to, and after recrystallization from pentane to determine whether or not recrystallization was needed. In all cases studied, no differences were noted. N,N-diisopropylethylamine was redistilled before use. Fatty acids were purchased from Sigma Chemical Co., St. Louis, MO, Applied Science Laboratories, State College, PA, and Calibiochem, San Diego, CA.

METHODS

Derivatization: A modified procedure of Moreland was used (11). Ten μ-moles of the fatty acid, 40 μ-moles of either N,N-diisopropylethylamine or Li_2CO_3, and 10-20 μ-moles of the UV-absorbing derivatizing agent were added to 1 ml of dimethylformamide. Following dissolution, the reaction mixture was heated at 65° for 15 minutes. An aliquot of the reaction mixture was then directly injected into the liquid chromatograph. This procedure works well with dry standards including chloroform-extracted tissues, etc. Traces of water will prevent quantitative derivatization, however, and, in such cases, the procedure of Durst et.al. should be used (8). Additionally, dicarboxylic acids require crown ether catalysis (9). Thus, if a Folch extraction with methanol and chloroform is used, then crown ether catalysis is required since the methanol will bring moisture with it (10,12).

Chromatographic Procedure: All analyses were performed with a Waters Associates Model ALC/GPC-244 Liquid Chromatograph equipped with a Model 660 Solvent Programmer, an auxilliary M6000A Solvent Delivery System, a Model 440 UV detector, and a variable wavelength UV detector, Waters Model 450. Although not reported in the present paper, the Model 450 was normally set to monitor absorbance at the absorbance maximum for the derivative being used while the Model 440 Detector continuously monitored at 254 nm. The columns of choice were a 3.9 mm x 30 cm μBondapak C_{18} and a μBondapak Fatty Acid Analysis column. Normally, two or three columns were used in series to achieve optimum separations. The initial eluent programs tested were gradients of THF:water, dioxane:water, and acetonitrile:water; the acetonitrile:water system proved to be the best, by far. A convex gradient (Curve #5 provided by the Model 660 Programmer), from 40% acetonitrile to 100% acetonitrile, was used. Flow rates between 0.5 and 2.0 ml/min were employed. Gradient profiles are superimposed upon the chromatograms of Figures 3 to 8. Thus, the concentration of acetonitrile in the eluent needed to elute a given ester under the gradient conditions used can be obtained by inspection of Figures 3-8.

RESULTS AND DISCUSSION

Reactions involved in the preparation of the fatty acid esters are shown in Figures 1 and 2. Resultant derivatives proved to be satisfactory for liquid chromatographic analysis in all cases (11,13). Attempts to prepare 3,4-dihydroxyphenacyl esters of the fatty acids from the chloride led to poor product yields; apparently the chloride ion is too poor a "leaving" group. On this assumption, it was decided to try using 3,4-dihydroxylphenacyl bromide. Unfortunately, this reagent is too unstable to store and, therefore, was inconvenient to use. The preparation of the 2-naphthacyl esters, not shown here, proceeded smoothly according to the procedure outlined in Figure 1 and described above. Table I lists the fatty acids separated in Figures 3-8 as their various ester derivatives. The standard numbers in Table I correspond to the numbers given in the figures.

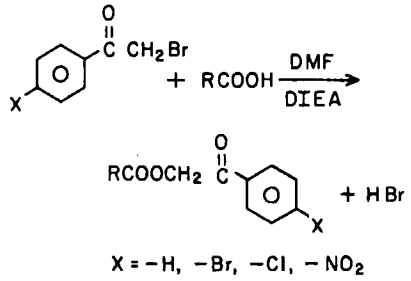

Figure 1. Formation of substituted fatty acid phenacyl esters for HPLC. See text for details.

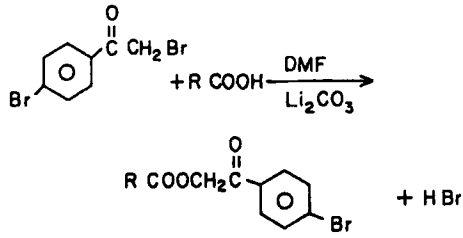

Figure 2. Formation of p-bromophenacyl esters for HPLC using Li_2CO_3 as the catalyst. See text for details.

TABLE I

FATTY ACID STANDARDS

STANDARD NUMBER	FATTY ACID		*	STANDARD NUMBER	FATTY ACID	*
1.	Acetic		2:0	23.	Lactic	5:0
2.	Propionic		3:0	24.	Valeric	5:0
3.	Butyric		4:0	25.	Heptanoic	7:0
4.	Caproic		6:0	26.	Nonanoic	9:0
5.	Caprylic		8:0	27.	Undecanoic	11:0
6.	Capric		10:0	28.	Tridecanoic	13:0
7.	Lauric		12:0	29.	Pentadecanoic	15:0
8.	Myristic		14:0	30.	Heptadecanoic	17:0
9.	Palmitic		16:0	31.	Nonadecanoic	19:0
10.	Stearic		18:0	32.	Heneicosanoic	21:0
11.	Arachidic		20:0	33.	Tricosanoic	23:0
12.	Behenic		22:0	34.	Myristoleic	14:1
13.	Lignoceric		24:0	35.	Palmitelaidic	16:1
14.	Palmitoleic		16:1	36.	Linoledaidic	18:2
15.	Oleic	cis	18:1	37.	Cis-5-Eicosenoic	20:1
16.	Elaidic	trans	18:1	38.	Erucic	22:1
17.	Vaccenic		18:1	39.	11,14,17-Eicosatrienoic	20:3
18.	Linoleic		18:2	40.	Brassidic	22:1
19.	α - Linolenic		18:3	41.	4,7,10,13,16,19-Docosahexaenoic	22:6
20.	Nervonic		24:1			
21.	Arachidonic		20:4	42.	5,8,11,14,17-Eicosapentaenoic	20:5
22.	γ - Linolenic		18:3			

*NOTE: The number to the left of the colon is the number of carbon atoms; the number to the right represents the number of double bonds present in the molecule.

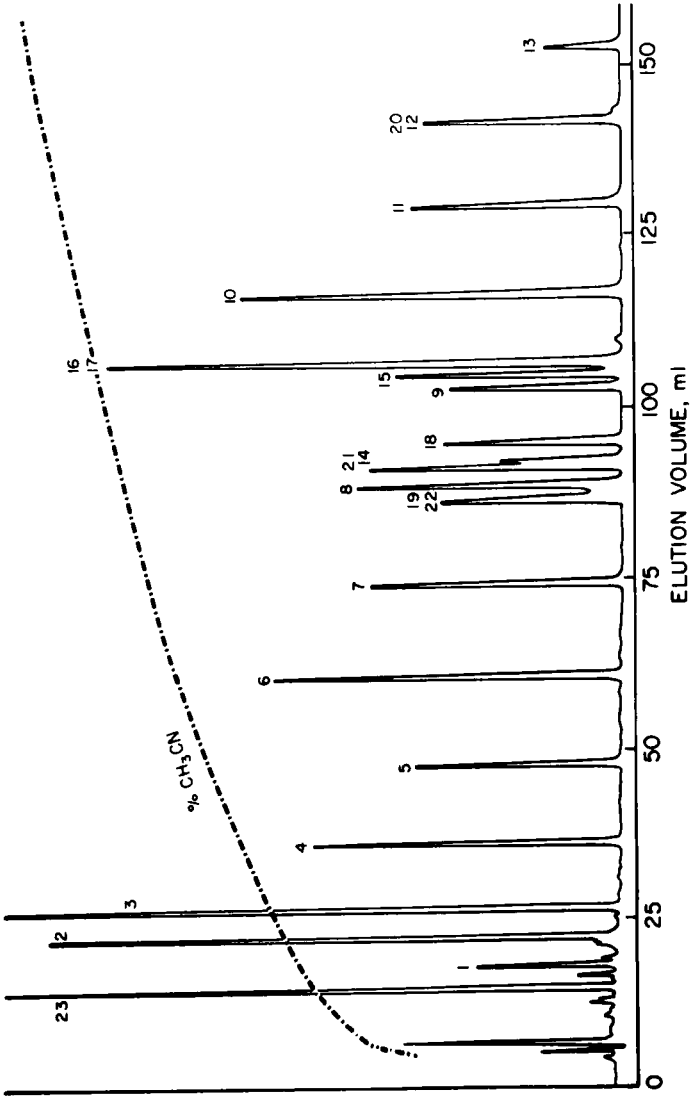

Figure 3. HPLC of fatty acid p-chlorophenacyl esters. Columns: Two 30 cm x 3.9 mm μBondapak C_{18}, eluent acetonitrile:water, gradient curve 5, 180 min, flow rate 1.0 ml/min; see Table 1 for peak identification. The gradient curve in this and subsequent figures reveals the solvent strength required to elute each ester, with the upper limit of the curve representing 100 percent acetonitrile and the lower limit 40 percent.

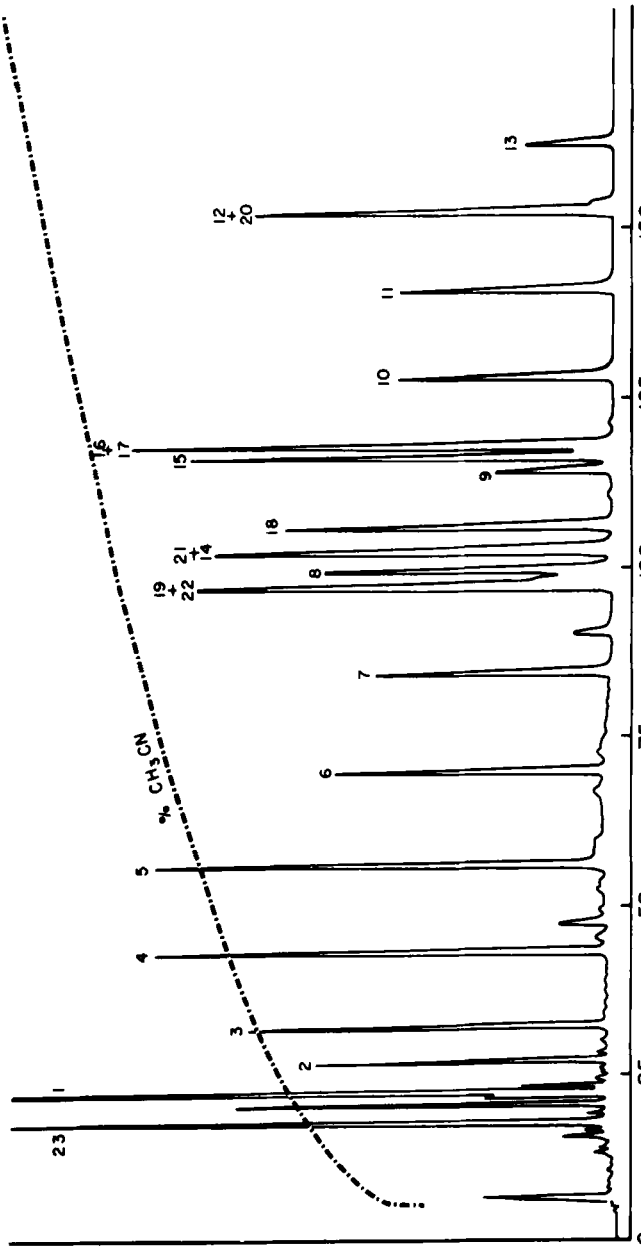

Figure 4. HPLC of fatty acid 2-naphthacyl esters. Columns: Two 30 cm x 3.9 mm μBondapak C18, eluent acetonitrile:water, gradient curve 5, 180 min, flow rate 1.0 ml/min, see Table 1 for peak identification.

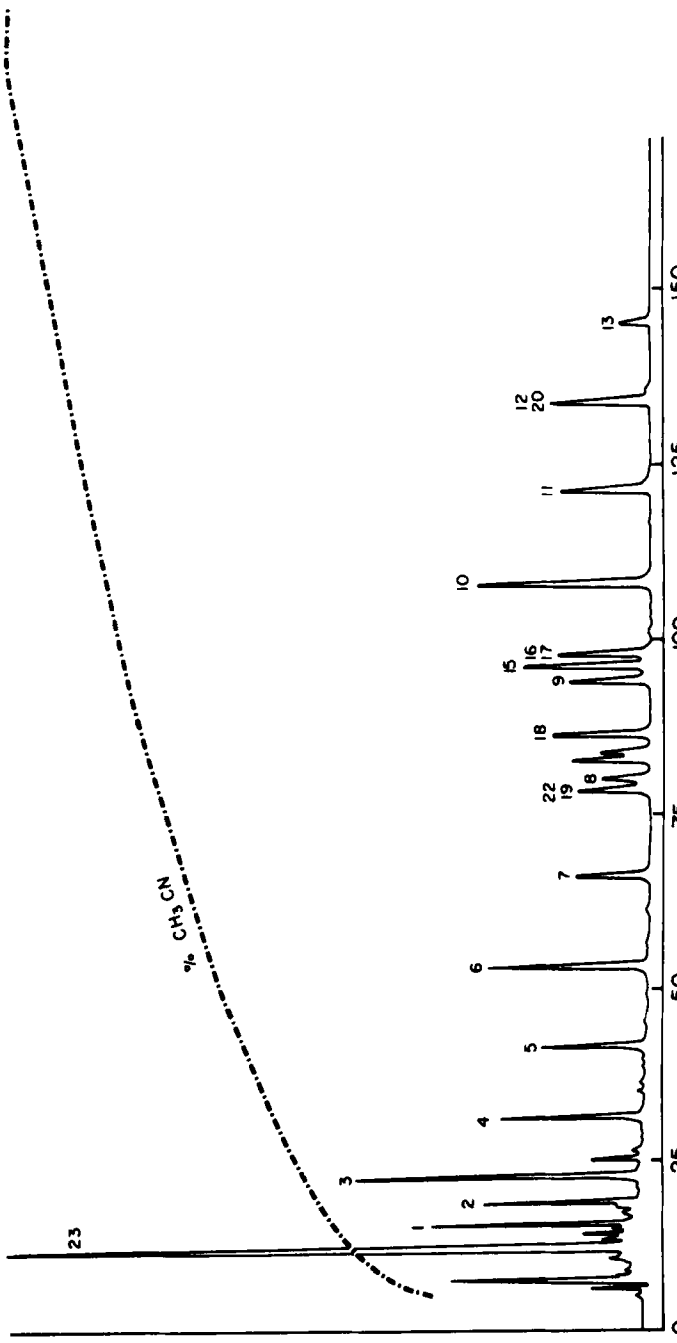

Figure 5. HPLC of fatty acid p-nitrophenacyl esters. Columns: Two 30 cm x 3.9 mm μBondapak C_{18}; eluent acetonitrile:water, gradient curve 5, 180 min, flow rate 1.0 ml/min; see Table 1 for peak identification.

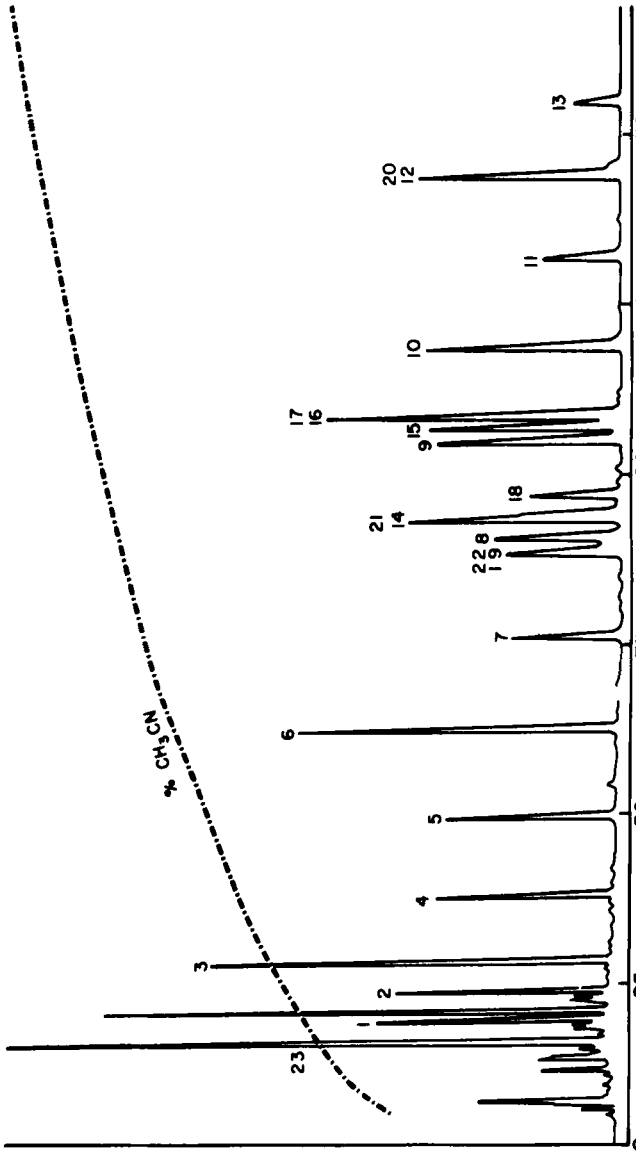

Figure 6. HPLC of fatty acid p-bromophenacyl esters. Columns: Two 30 cm x 3.9 mm μBondapak C_{18}, eluent acetonitrile:water, gradient curve 5, 180 min, flow rate 1.0 ml/min; see Table 1 for peak identification.

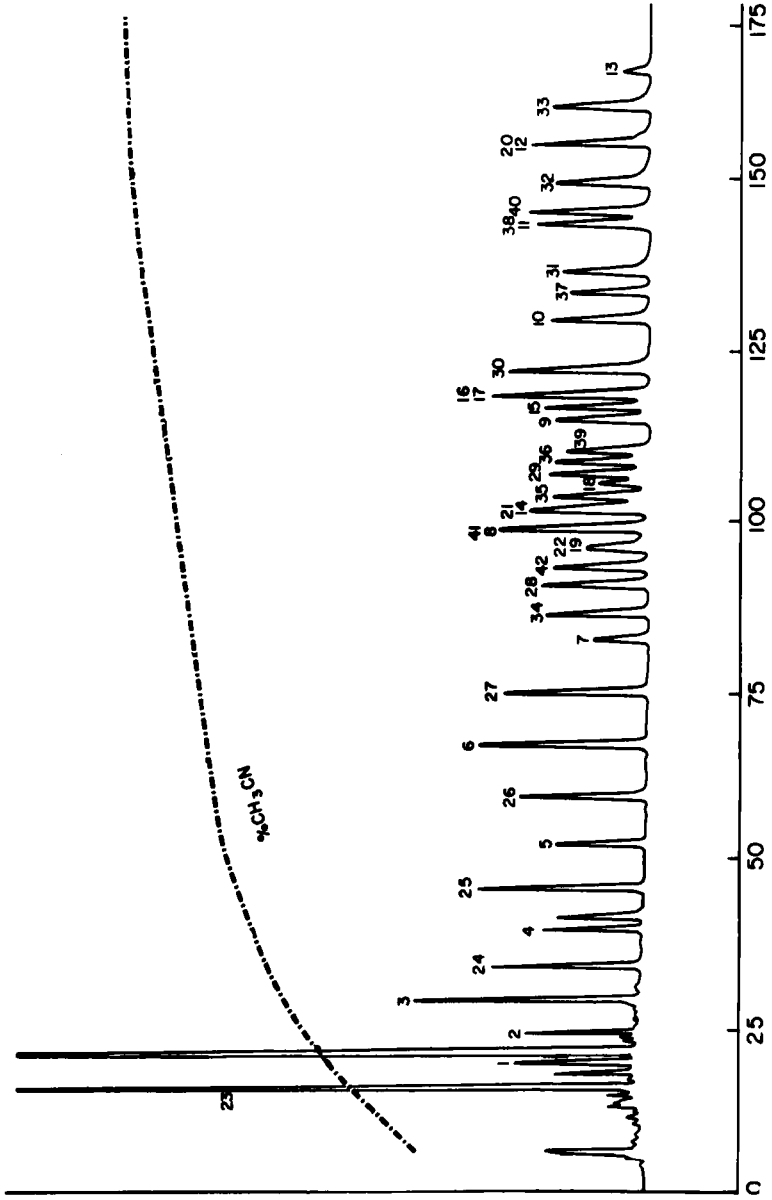

Figure 7. HPLC of fatty acid p-bromophenacyl esters. Columns: Two 30 cm x 3.9 mm μBondapak C_{18} eluent acetonitrile:water, gradient curve 5, 180 min, flow rate 1.0 ml/min; see Table 1 for peak identification.

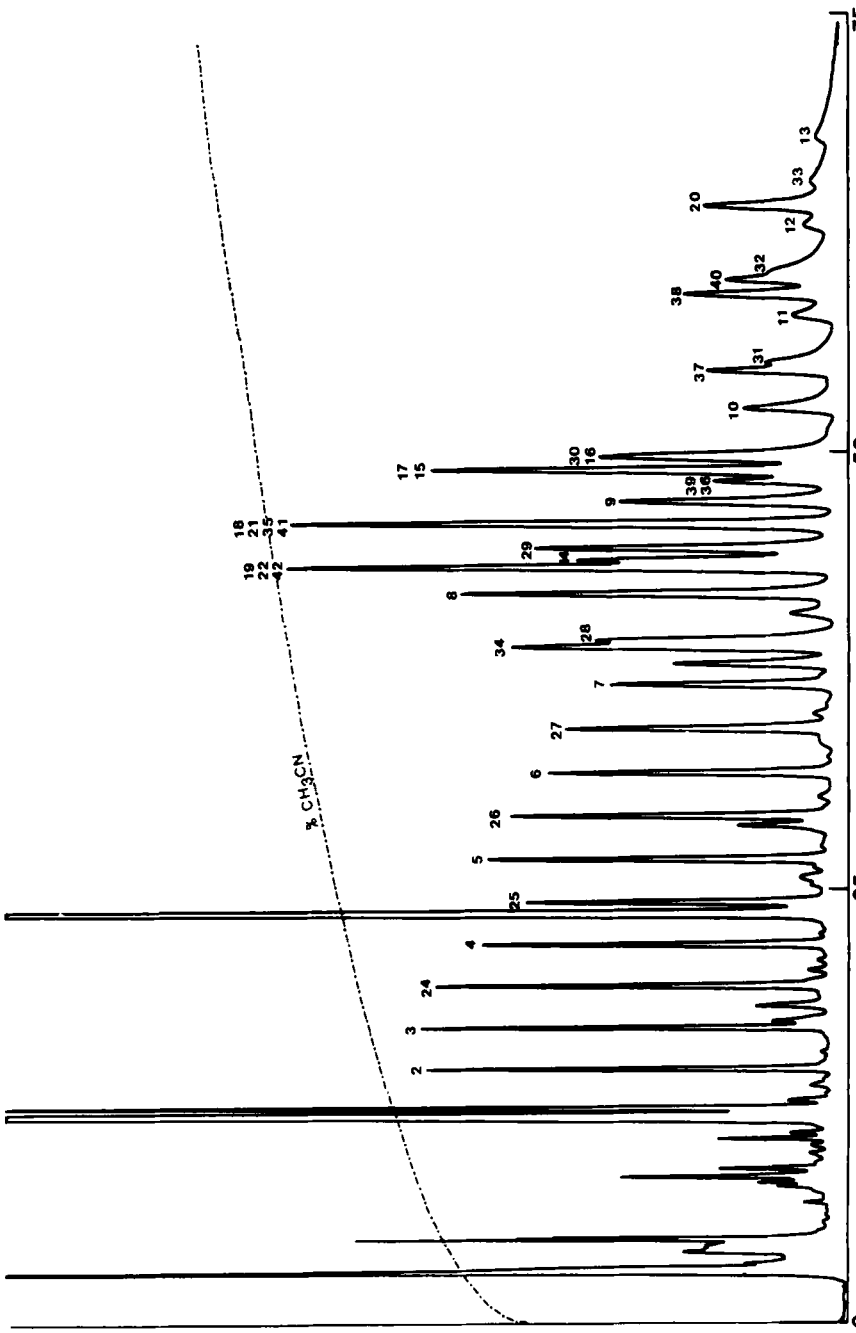

FIG. 8. HPLC of fatty acid p-bromophenacyl esters. Columns: Two 30 cm x 3.9 mm fatty acid analysis, eluent acetonitrile: water, gradient curve 5, 180 min, flow rate 1.0 ml/min; see Table 1 for peak identification.

Excellent chromatographic properties were observed for most of the esters studied. Figures 3-6 show the separations of components 1-23 for the p-chlorophenacyl, 2-naphthacyl, p-nitrophenacyl, and p-bromophenacyl esters respectively on μBondapak C_{18}. The chromatographic conditions were identical in all cases. Elution volumes of the saturated acid derivatives increased directly with increasing chain length, as would be expected in reverse phase separations, and inversely with the degree of unsaturation of the unsaturated fatty acid esters.

For example, examining Figure 3, we see that components 1 thru 13 (the saturated acid derivatives) elute in order of increasing carbon number, whereas components 10, 15, 18 and 19, which represent components that have identical carbon numbers, but increasing degree of unsaturation, elute in reverse order of their degree of unsaturation (i.e., for a given carbon number, a higher degree of unsaturation resulted in a lower elution volume).

Finally, it was noted that a trans-unsaturated eluted between the corresponding cis-unsaturated isomeric acid derivative and the fully saturated one. Thus, a cis-isomer must apparently be more polar than the trans-isomer with respect to its interaction with the reverse-phase column packing's surface. This is as one would expect on the basis of conformational changes induced by introduction of the double bond into the ester molecule. The trans-isomers are more linear than the cis-isomers and, hence, are able to interact more strongly with the reverse-phase surface. Close study of Figures 3-8 reveals that the above comparisons hold true for the 2-naphthacyl, p-nitrophenacyl, and p-bromophenacyl esters as well as for the p-chlorophenayl esters.

Similar resolution was also observed with the Fatty Acid Analysis column, as shown in Figure 8.

The Fatty Acid column is somewhat more polar than the μBondapak C_{18} and contains pi-electrons in its bonded phase. These pi-electrons impart to the column unique selectivity towards unsaturated fatty acid esters. For example, consider peaks 14 and 21 which rep-

resent arachidonic and palmitoleic acid esters, respectively. They are difficult to resolve on μBondapak C_{18} (see Figure 7, for example). However, as shown in Figure 8, they are easily separated with the Fatty Acid Analysis column. Another example is given by components 12 and 20 which are, respectively, behenic and nervonic acid esters. Although they are difficult to resolve on μBondapak C_{18} (see Figure 7) they are well resolved on the Fatty Acid Analysis column (see Figure 8).

Close inspection of Figures 7 and 8 also reveals that the selectivity of the Fatty Acid column towards long chain esters rapidly deteriorates above 18 carbons unless the longer chain esters contain one or more double bonds; in this case, the peaks sharpen considerably. Compare nervonic and lignoceric acids (24 carbons, saturated and unsaturated, respectively). This effect is probably due, at least in part, to the higher polarity of the Fatty Acid Analysis column relative to μBondapak C_{18}. Thus, for the very long chain esters there is a mismatch or incompatability in polarity between sample and column which leads to a loss in efficiency.

A second related cause for loss of efficiency is undoubtedly the decrease in mobile phase strength required to elute the long chain esters from the Fatty Acid Analysis column relative to μBondapak C_{18}. Thus, comparing Figures 7 and 8, essentially 100 percent acetonitrile is required to elute the lignoceric ester from μBondapak C_{18} but only about 80 percent to elute it from the Fatty Acid column. The solubility of lignoceric ester in 80/20 acetonitrile/water is marginal and severe tailing results.

From the above observations it would appear that the μBondapak C_{18} and Fatty Acid Analysis columns complement each other in terms of selectivity. Thus, many of the fatty acid esters that are most difficult to resolve via μBondapak C_{18} are easily separated from each other with the Fatty Acid Analysis column, and vice-versa.

These and related methods may be readily used for biological separations (12, 14, 15) and, in many cases, it should be possible to simplify the analyses when it is desired to monitor only a narrow

range of chain lengths such as would be the case, for instance, in prostaglandin analysis (14). In the separation of extremely non-polar samples such as mycolic acids from the H37Ra strain of mycobacterium tuberculosis which contains C_{70} - C_{90} fatty acids, only the μBondapak C_{18} column could be used due to the extreme non-polarity of the sample (15). Finally, other classes of compounds, such as bile acids, should be ideally suited to this sort of analysis since they too contain a free derivatizable carboxyl group.

The use of various substituted phenacyl and 2-naphthacyl groups as chromophores gives huge increases in sensitivity, particularly when monitored at the absorbance maximum. Figure 9 shows the results of a quantitative assay of the p-bromophenacyl ester of n-caproic acid monitored at 254 nm. A Varian CDS-101 integrator was used to calculate peak areas as a function of concentration. The standard error in each case is contained within the data point. Ten microliters of each standard solution was injected and the limit of de-

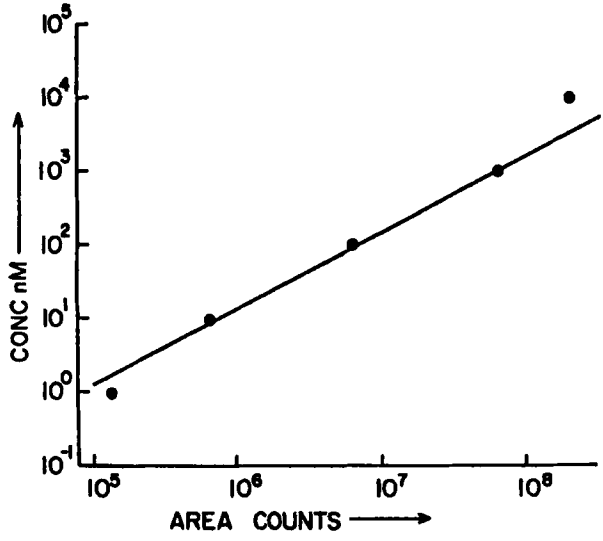

Figure 9. Quantitative HPLC assay of p-bromophenacyl ester of n-caproic acid. Columns: Two 30 cm x 3.9 mm μBondapak C_{18}, eluent acetronitrile:water, gradient curve 5, 180 min, flow rate 1 ml/min, 10 μl injections of 10^0, 10^1, 10^2, 10^3, and 10^4 nM standard solutions; see text for details.

tection approached 2 picograms. Above 10 ng, the peak areas from the CDS-101 integrator were no longer linear with concentration.

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