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THE DETERMINATION OF FATTY ACIDS BY HPLC AND LIQUID-LIQUID EXTRACTION

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ABSTRACT

Five free fatty acids (even carbon numbers, C_{10}-C_{18}) were separated using reversed-phase liquid chromatography and detected using a post-column ion-complexation system in which the acids were extracted by chloroform as copper-ion complexes from the aqueous acetonitrile mobile phase (gradient: 70-87%; flow rate: 0.8 mL/min) after post-column addition of copper-buffer reagent. The chloroform phase containing copper-ion complex, after separation by a membrane phase-separator, was mixed with sodium diethyldithiocarbamate and monitored with a high degree of sensitivity by a UV/VIS detector at 440 nm.

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LIST OF SYMBOLS

SYMBOL	DEFINITION	UNITS OR REFERENCE
mL	milliliter	$1x10^{-3}$ liter
µL	microliter	$1x10^{-6}$ liter
mg	milligram	$1x10^{-3}$ gram
mm	millimeter	$1x10^{-3}$ meter
nm	nanometer	$1x10^{-9}$ meter
L	liter	
g	gram	
v	volume	
w	weight	
min	minute	
UV	ultraviolet	
VIS	visible	
I.D.	internal diameter	
C_8	octyl	
C_{18}	octadecyl	
GC	gas chromatography	
MS	mass spectrum	
HPLC	high performance liquid chromatography	
ODS	octadecyl silane	
GLC	gas liquid chromatography	

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CHAPTER 1

INTRODUCTION

Fatty Acids

The fatty acids are a large group of aliphatic carboxylic acids. The term "fatty acid" comes from the widespread occurrence of these carboxylic acids in natural fats and oils. Sometimes fatty acids are referred to as free fatty acids, nonesterified fatty acids, or unesterified fatty acids. The fatty acids are a homologous series of compounds with the general formula RCOOH, where R represents an alkyl chain. In saturated fatty acids, the R group is a straight chain group that is characterized as $CH_3(CH_2)_x$. The fatty acids range from volatile liquids to waxy solids as their molecular weight is increased. Fatty acids that contain from six to nine carbon atoms are oily liquids. Those that have ten or more carbon atoms are solids that increase in waxlike properties with increasing molecular weight. The carboxylic acid group of a typical fatty acid has a pK_a between 4.85 to 4.77.

In the human body, free fatty acids exist in an ionized form bound to albumin in plasma and intracellular fluid. They are readily taken up by most tissue to satisfy the energy requirements of the body[1].

In mammalian tissues, such as brain, the fatty acids are present in their free form in very small quantities[2]. Mainly, they are found esterified to glycerol as a part of triacylglycerols of phospholipids[3]. The chain length and the

number of double bonds in these lipids influences the fluidity of the cell membrane, while phosphate-based groups influence the polarity or ionic charge of the membrane[4]. Fatty acids thus play a crucial role in determining the lipidic environment of the cell membrane, both in the plasma membrane and in the interior membranes.

The most common method of classification of fatty acids is based upon chain length: short chain (2-4 carbon atoms); medium chain (6-10 carbon atoms); and long chain (12-26 carbon atoms). In human nutrition and metabolism, the fatty acids of the long-chain class and even numbers of carbon atoms are important. That is also why C_{10} to C_{18} were chosen in this research. Tables 1 and 2 indicate the major properties and solubilities of these fatty acids.

Capric acid is a minor component of many the naturally occurring fats and oils. It occurs in vegetable oils, especially coconut oils and oils of the palm family. It is also found in milk fat and is present in small quantities in other vegetable and animal fats. Capric acid is very slightly soluble in water and easily soluble in ethanol, ether, and in most organic solvents.

Lauric acid is found abundantly in nature and it is a major constitutent of the component acids of several oils, particularly coconut palm oils. It is also present in butter fats, the percentage varying with the source of the fat. Lauric acid is very slightly soluble in boiling water and appreciably soluble in acetone, ethanol, and ether.

Table 1: Major properties of the fatty acids.

Common name	Formula	Melting Point (°C)	Boiling Point (100 mm Hg)	Refractivity (N_d^{70})
Capric	$CH_3(CH_2)_{10}COOH$	31.3	NA	1.4169
Lauric	$CH_3(CH_2)_{12}COOH$	44.0	225.0	1.4230
Myristic	$CH_3(CH_2)_{14}COOH$	54.4	250.5	1.4273
Palmitic	$CH_3(CH_2)_{16}COOH$	62.8	268.0	1.4309
Stearic	$CH_3(CH_2)_{18}COOH$	69.6	291.0	1.4337

Table 2: The solubility of fatty acids[5].

(Solubility at 20°C expressed in g per 100g of solvent)

Acid	H_2O	C_6H_6	MeOH	EtOH	$COMe_2$	$CHCl_3$	MeCN
C_{10}	0.015	398	510	NA	407	326	66
C_{12}	0.0055	93.6	120	105	60.5	83	7.6
C_{14}	0.0020	29.2	17.3	23.9	15.9	32.5	1.8
C_{16}	0.0007	7.3	3.7	7.2	5.4	15.1	0.4
C_{18}	0.0003	2.5	0.1	2.3	1.5	6.0	<0.1

Myristic acid is widely distributed in animal and vegetable fats and forms a major component of the fatty acids of the myristicaceae and the seeds fats of the palm family and butter fat. It is insoluble in water, soluble in ethanol to the extent of 44 g in 100 mL, and soluble in ether, acetone, glacial acetic acid and chloroform.

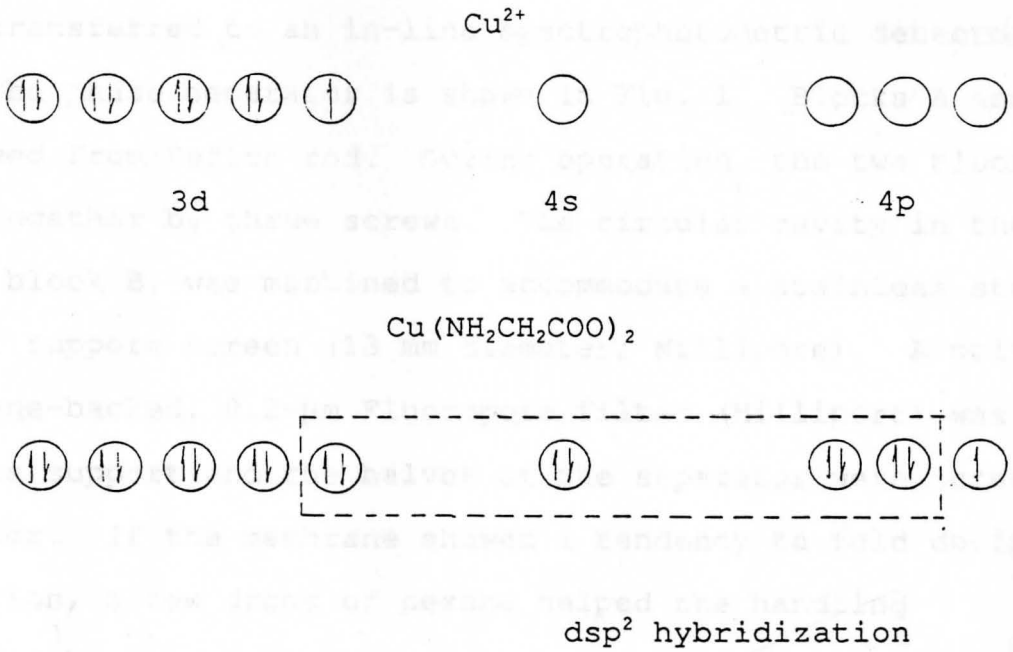
Palmitic acid occurs more abundantly than any of the other saturated acids. It is present in nearly all of the oils and fats of animal, vegetable, or marine origin. It is the major acid in a large number of fats. It is insoluble in water, sparingly soluble in cold ethanol, soluble in hot ethanol and ether and chloroform, and sparingly soluble in petroleum ether.

Although stearic acid does not occur as abundantly in nature as palmitic acid, it is generally referred to as the "characteristic fatty acid". It occurs as the principal saturated acid in only a few fats. However, it is widely distributed in animal and vegetable fats, and its ease of preparation by the reduction of its many unsaturated counterparts makes it the most widely known and most important of the saturated fatty acids. It is very insoluble in water, but is also readily soluble in ether, benzene, and carbon disulfide and chloroform. It is only slightly soluble in petroleum ether.

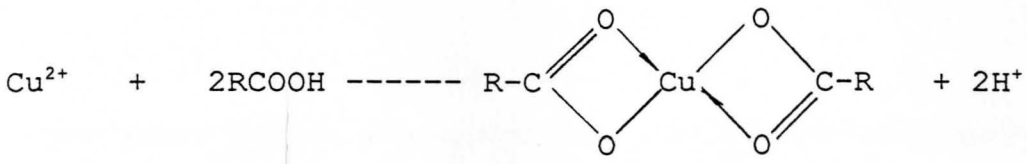
Copper-Ion Complexation

On the basis of valence bond theory of coordination compounds, electron pairs from the ligands are considered as being shared with the metal ion. Each of these electron pairs occupies both a molecular orbital on a ligand and one of several equivalent hybrid orbitals on the metal.

In the 4-coordinate complexes of copper with a square planar configuration, the four bonds of the central atom arise from dsp^2 hybridization because dsp^2 hybridization is more stable than sp^3 hybridization. This is due to placing as many d electrons as possible in the lower energy orbitals, thereby reducing the average energy of the d electrons. For example, the distribution of electrons in the square planar copper-glycine complex $Cu(NH_2CH_2COO)_2$[6] is represented diagrammatically as follows:



Copper complexes with fatty acids can be formed in a similar manner. The reaction equation is illustrated as follows:



On-Line Extraction

Liquid-liquid extraction is one of the most versatile techniques for separation and concentration of analytes. In this project, the post-column sample stream effluent from the HPLC was mixed (and thereby segmented) with a stream of chloroform and was passed through a mixing coil to facilitate extraction. It was then passed into a phase separator where the segmented stream was separated into its constituent phases. The phase of interest was then transferred to an in-line spectrophotometric detector.

The phase separator is shown in Fig. 1. Blocks A and B were machined from Teflon rod. During operation, the two blocks were held together by three screws. The circular cavity in the lower part, block B, was machined to accommodate a stainless steel filter support screen (13 mm diameter; Millipore). A polyethylene-backed, 0.2-μm Fluoropore filter (Millipore) was placed on this support and the halves of the separator were screwed together. If the membrane showed a tendency to fold during this operation, a few drops of hexane helped the handling considerably.

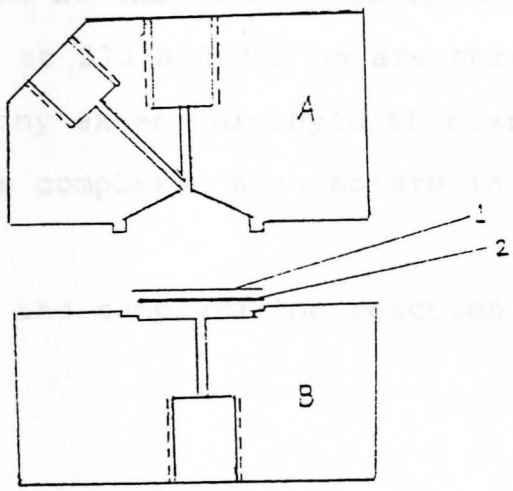


Fig. 1. Membrane phase separator.

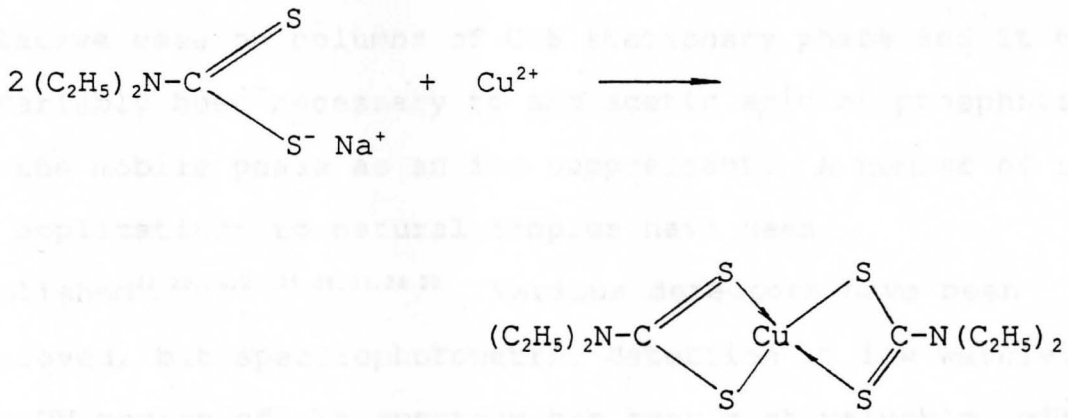
1: Fluoropore membrane; 2: Teflon-coated filter support screen.

Copper-Diethyldithiocarbamate Complex

Salts of diethyldithiocarbamic acid have been widely used as analytical reagents. In the laboratory, sodium diethyldithiocarbamate has been reacted with copper ion in low concentration to produce a brown colloidal suspension of the copper-diethyldithiocarbamate complex, which is soluble in organic liquids. The best solvents are pyridine and chloroform[7,8,9].

The suspension can be analyzed spectrophotometrically at 440 nm[10,11,12,13,14,15]. Maxima at 270 and 295 nm are stronger, but are not useful since any excess diethyldithiocarbamate and other diethyldithiocarbamate complexes also absorb in this region[16,17,18,19,20].

The equation for the complexation reaction is represented as:



CHAPTER 2

HISTORICAL

Analysis of Fatty Acids by Reversed-phase HPLC

Reversed phase HPLC is of considerable value for the separation of free fatty acids and derivatives of those fatty acids encountered most often in animal and plant tissues. In the following sections, separations are discussed in terms of the various types of derivatives of fatty acids that have been examined and used for changing the polarity and/or aiding the detection of fatty acids.

1. Free Fatty Acids

Fatty acids can be separated in the unesterified form with relative ease on columns of ODS stationary phase and it has invariably been necessary to add acetic acid or phosphoric acid to the mobile phase as an ion suppressant. A number of reports of applications to natural samples have been published[21,22,23,24,25,26,27,28,29]. Various detectors have been employed, but spectrophotometric detection at low wavelengths in the UV region of the spectrum has been most valuable, although two novel detection systems, i.e., nephelometry and electrokinesis, have also been tried.

Many kinds of organic aqueous mixtures have been used as the mobile phase. Acetonitrile/aqueous phosphoric acid (pH=2)

mixtures were used by Aveldano et al.[21] for the separation of free fatty acids, on a column of Zorbax™ ODS with spectrophotometric detection at 192 nm for unsaturated acids or at 205 nm for saturated fatty acids. They found that the longer the aliphatic chain, the greater was the increase in the capacity factor with decreasing concentrations of acetonitrile in the mobile phase. Thus as the solvent strength increased, the retention times of the shorter-chain fatty acids decreased faster than for the long-chain components.

In an equally systematic study with methanol/water/phosphoric acid mixtures as the mobile phase, very similar conclusions were drawn[25].

If the separated compounds are to be collected for analysis by other methods, it is best to use a mobile phase containing acetic rather than phosphoric acid. Thus some useful separations have been attained with tetrahydrofuran/acetonitrile/water/acetic acid (3:67:30:0.1 by volume)[22], and with methanol/water/acetic acid (89:11:0.2 by volume)[23] as mobile phases.

2. Methyl and Other Alkyl Esters

Fatty acids can be separated as methyl esters that can be detected via their refractive indexes[30,31,32] or by UV absorption. Again, ODS phases have been preferred for the HPLC separation with acetonitrile/water or methanol/water mixtures as the mobile phase.

Perhaps the most systematic study of the separation of

methyl ester derivatives with real samples was that of Aveldano et al.[21]. The mobile phase was acetonitrile/water (7:3 by volume) at 2 mL/min, changed to acetonitrile alone over 20 min. towards the end of the separation. As described earlier for unesterified fatty acids, the order of elution of specific components was greatly dependent on the relative proportions of acetonitrile and water in the mobile phase. In another study using model systems, methanol/water mixtures were employed as the mobile phase[33].

Relatively simple mixtures of the methyl ester derivatives of fatty acids, including those from fungal and animal tissues in addition to model mixtures, have been separated on a number of occasions both on an analytical or small scale[34,35,36,37,38,39], and with up to gram quantities with suitable equipment[40,41,42]. Esters of short-chain fatty acids, such as those in milk fat, have been separated[43], as have those of much longer chain-length than are normally encountered (up to C_{32})[21,44,45]. While the capacity to utilize gradients in the mobile phase can shorten analysis times appreciably, some excellent separations have been achieved with isocratic elution conditions over a wide range of chain-length[35,43,46,47]. Increasing the flow rate after the 16:0 and 18:1 fatty acid derivatives have eluted, for example, can speed-up the elution of stearic acid, which is often the last component to elute in many natural samples.

3. Derivatives Suitable for UV Detection

By preparing fatty acid derivatives that have a good

absorption band in the UV region of the spectrum, it is possible to detect components separated by HPLC, and to quantify them spectrophotometrically with great sensitivity. Many types of derivatives have been prepared for this purpose, but none appears to have any clear advantage in terms of separation over the others. The choice may be governed by a need for higher than normal sensitivity if the sample size is particularly small, or it may simply be a question of the convenience or availability of a particular derivatization reagent. Because of the ease of preparation and their relatively high molar extinction coefficients in the UV region of spectrum, phenacyl or related derivatives, such as p-bromo- or p-methoxyphenacyl esters, appear to be gaining favor.

One of the first attempts at a comprehensive separation of phenacyl ester derivatives was by Borch[48], who used a column of micro Bondapak™C_{18}, eluted in a stepwise fashion with acetonitrile/water mixtures (from 67:30 to 97:3 by volume) at 2 mL/min. The factors governing the order of elution are similar to those discussed earlier for methyl esters.

Most analysts have used UV detection at 254 nm in the analysis of phenacyl esters, probably because this is suited to inexpensive fixed-wavelengths detectors. However, it has been shown that an appreciable increase in sensitivity is possible if the detector can be operated at 242 nm, which is the absorption maximum for such compounds[49].

Phenacyl ester derivatives have been employed in the analysis, by means of reversed-phase HPLC, of the fatty acids of

butter fat and related samples[50,51,52], of the cellular lipids of the microorganism, Vibrio parahaemolyticus[53], and of the phosphoglycerides of amniotic fluid[54].

Naphthacyl ester derivatives appear to have been used with standard mixtures only[55,56,49]. Electron-impact and chemical-ionization mass spectra of a few derivatives were obtained by introduction of the sample into the mass spectrometer via a solid probe, but they were of little value for structure elucidation[55]

Substituted phenacyl derivatives, which tend to have higher molar absorptivities, can be detected with much higher sensitivity than is possible with the unsubstituted compounds; p-bromophenacyl esters have been employed for the separation or analysis of fatty acids of biological interest[57,58,59,60], of grain and feed extracts, of the minor C_{20} to C_{30} fatty acids in the serum of patients with adrenoleukodystrophy, and of the C_{30} to C_{56} fatty acids from Mycobacterium tuberculosis[61].

p-Methoxyphenacyl esters were used in some comprehensive studies of a wide range of fatty acids of bacterial origin[62,63,64]. The fatty acids of a few seed oils have been quantified in the form of phenylazophenacyl esters, which were detectable at 330 nm[65].

Others have separated fatty acids with UV detection in the form of isatinyl methyl esters[66], anthrylmethyl esters[67,68], p-nitrobenzyl ester[69,70], benzyl ester[71], pentafluorobenzyl esters[72,73], naphthylamides[74], isopropylidene hydrazides[75], 2-nitrophenylhydrazides[76,77,78], and methoxy-and nitro-anilides[79,80].

4. Derivatives Suitable for Fluorescence Detection

The use of derivatives of fatty acids that can be detected by fluorescence spectroscopy has allowed for large increases in the sensitivity of HPLC analysis. It is now possible to quantify amounts that would not be possible with GLC on capillary columns or even with GC/MS. The first and most widely-used derivatives of this kind were anthrylmethyl esters. These derivatives are prepared simply by reaction of the free fatty acids with 9-diazomethylanthracene in an inert solvent[67,81], although this has not always given acceptable results and other methods have been described[68,82,83]. Optimum sensitivity was obtained by fluorescent excitation at 360 nm and emission at 440 nm (UV detection at 256 nm could also be used). Although the improvement in detectability was somewhat dependent on the chromatographic conditions, it was at least 10-fold better than with UV detection, and was in the low picogram range.

Various mobile phase mixtures have been employed to separate anthrylmethyl esters[67,84,85,86,87,88,89,90]. In addition, the value of a C_8 phase was confirmed by Baty[82], who obtained a particularly good resolution with a complex acetonitrile/water gradient as the mobile phase.

HPLC/MS has been used with these derivatives to give distinct molecular ions that allowed the detection of minor components by ion-monitoring techniques[68].

A number of workers have made use of coumarin derivatives, including 4-methyl-7-methoxycoumarin[91,92,93,94], 4-methyl-6,7-

dimethoxycoumarin[95] and 4-methyl-7-acetoxycoumarin[96] in analysis of fatty acids by reversed-phase HPLC with fluorescence detection. Because of the relatively small size of the alcohol moiety, coumarin esters have tended to give better resolution than other types of fluorescent derivatives. In one study[96], fluorescence was determined in a post-column reaction chamber following hydrolysis of the ester linkage. The mobile phase was a gradient of methanol/water (9:1), while the column containing the ODS phase was maintained at 40°C.

9-Aminophenanthrene[97], 5-(dimethylamino)-1-naphthalenesulphonylsemipiperazide[98,99], 2-naphthacyl[100], phenanthrimidazole[101], and methyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinonone[102,103,104] derivatives have also been used in the HPLC analysis of fatty acids using fluorescence detection. Of these, the last compound seems to hold promise in terms of sensitivity, since it permitted the analysis of fatty acids in amounts as low as 0.3 femtomole.

Because derivative reactions are often extremely slow in aqueous solution and, in most cases, additional equipment is needed, Wolf[105] and Van Der Horst[106] performed the automated HPLC determination of fatty acids using on-line derivatization with either 4-bromomethyl-7-methoxycoumarin or the fluorophore 9-bromomethylacridine respectively.

5. Derivatives Suitable for Electrochemical Detection.

Shimada et al.[107] reported a new derivatization method using

ferrocene reagents that was developed for the determination of fatty acids by HPLC with electrochemical detection. Fatty acids were reacted with 3-bromoacetyl-1,1'-dimethyl ferrocene in the presence of 18-crown-6 ether and potassium fluoride. The resulting derivatives showed satisfactory sensitivity at +0.60v vs. an Ag/AgCl reference electrode, because the ferrocene derivative undergoes facile oxidation and the product is in turn readily reduced by using a twin electrode electrochemical detector. Some similar methods have been employed by Shimada et al.[108,109,110,111,112,113] and Ikenoyo[114].

Separation of Fatty Acids by Reversed-phase HPLC with Silver-Ion Complexation

Silver ion chromatography has been used in three ways, i.e., with silver gel adsorbents impregnated with silver nitrate, with silver ion bonded to ion-exchange resins, and in the reversed-phase mode with silver ions in the mobile phase (acetonitrile is an excellent solvent for silver nitrate).

One of the first separations using the reversed-phase mode was reported by Schomburg et al[115]. In this instance, methyl oleate was separated from methyl elaidate on a column of LiChrosorb™ RPB, eluted with isopropanol/1.5% aqueous silver nitrate (5:4). A similar approach was used to separate the p-bromophenacyl ester derivatives of these and related fatty acids for UV detection[116], while another[117] employed dansyl-ethanolamine derivatives in the same way for fluorometric detection. Later, a

wide variety of unsaturated fatty acids were separated as methyl ester derivatives on a column packed with an ODS phase, using methanol/water (5:1), containing 0.01M silver perchlorate, as the mobile phase[118]. These separations look to be only slightly better than could have been achieved by reversed-phase chromatography alone. In addition, the cost of silver salts would be a negative factor against frequent use of this technique, which appears only to have been tried with model mixtures. Whether the silver ions in the eluent could corrode the HPLC equipment would also be a cause for concern. In one of these reports, no bad effects were seen over a 2-year period[118]; in another, silver metal was found to be deposited in the connecting tubing and in the detector cell, and it was necessary to remove it periodically by flushing with dilute nitric acid[115].

Analysis of Fatty Acids Using Copper-Ion Complexation

The first investigation of a colorimetric method for long-chain fatty acids was conducted by Ayers[119], who measured the extinction of a chloroform solution of their copper soaps, prepared by shaking a solution of the potassium soaps with copper nitrate and chloroform.

Iwayama[120] improved this method by using a copper nitrate-triethanolamine reagent shaken with a chloroform solution of the free fatty acids, and greater sensitivity was obtained.

Duncombe[121] significantly increased the sensitivity by adding sodium diethyldithiocarbamate to Iwayama's copper-containing

chloroform solution, which was inexpensive and fairly sensitive, so that it was possible to perform a microdetermination of long chain fatty acids in chloroform solution. Similar studies had been performed by Barreto[122], who used sodium diethyldithiocarbamate as a color developer, but with an ammoniacal copper sulfate reagent which gave much lower sensitivity than Iwayama's solution.

Another color developer was demonstrated by Hlynczak et al.[123] using the reagent dithizone. Long-chain fatty acids were determined on paper chromatograms by treating with copper acetate. The problem was that it was not applicable when the radioactivity of the fatty acids was to be measured.

On-line Extraction of Fatty Acids

Only one paper has been published on the application of on-line extraction in reversed-phase HPLC for the determination of free fatty acids by Lawrence et al.[124] in which the acids are extracted as ion pairs with chloroform from the aqueous acetonitrile mobile phase after the post-column addition of aqueous methylene blue solution. The chloroform phase containing the ion pairs was monitored with an absorbance detector at 651 nm. One problem associated specifically with the system as set up for fatty acids was the need to routinely backflush the detector cell with acetonitrile to remove deposits which caused fatty acid adsorption and thus loss of resolution and sensitivity.

This sample approach was successfully applied to the direct determination of the artificial sweeteners, cyclamate, saccharin and acesulfam K in diet beverages using the dye methyl violet 2B[125]. In addition, sodium dioctylsulfosuccinate was also easily detected in beverage powders, by applying a similar approach[126].

CHAPTER 3
STATEMENT OF THE PROBLEM

Current interest in the metabolism of fatty acids necessitated the initiation of the development of a highly sensitive method for their determination in biological samples. Although the analysis of fatty acids by HPLC has become increasingly popular, the analysis of fatty acids has been hampered and complicated by the lack of a strongly ultraviolet-absorbing or fluorescent chromophore[127]. The poor ultraviolet absorptivity or refractive index detection[128] also makes the direct LC separation and quantitation of fatty acids difficult due to interferences, particularly if the acids are present only at parts per million levels. Thus, the majority of carboxylic acid-containing compounds of biochemical interest are typically derivatized with ultraviolet-absorbing or fluorescent compounds. Despite such a large repertoire of derivatization agents (over 47 different pre-column labeling agents developed to produce a suitable response for HPLC analysis), many existing methods suffer from excessively long run times[129] or require isolation of fatty acids with minicolumns[130]. These methods are currently unsuitable for providing a complete resolution and quantitative determination within a single chromatographic separation of the free fatty acid ester derivatives containing the acyl chain lengths of free fatty acids (C_4 and C_8)[131,132].

The use of post-column copper-ion complex on-line extraction in this investigation may have begun to satisfy the needs of

developing a sensitive and specific HPLC method that uses inexpensive reagents for determination of fatty acids.

CHAPTER 4

MATERIALS AND APPARATUS

Materials

All chemicals used in this laboratory were of analytical quality, or of the highest quality available. All fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile (HPLC), Methanol (HPLC) and 1-Propanol were obtained from J.T.Baker Chemical Co. (Phillipsburg, NJ); Cupric Nitrate and Acetic Acid were obtained from GFS Chemicals (Columbus, OH); Triethanolamine and Sodium diethyldithiocarbamate were obtained from Aldrich Chemical Co. (Milwaukee, WI); Chloroform was obtained from Fisher Scientific (Fair Lawn, NJ).

Apparatus

The HPLC system equipped with an on-line post-column extraction process is shown in Fig. 2. Samples were analyzed using a System Gold Chromatography System (Beckman), including two Model 110B pumps for the mobile phase, a Model 406 analog interface module, a Model 166 programmable UV/VIS detector, and a Model 210A injector equipped with a 100 µL loop. An Econosphere C_8 column measuring 4.6 mm I.D. x 150 mm with a particle size of 5 µm (Alltech Associates Inc.) was used for separation of the fatty acids. Copper buffer and sodium diethyldithiocarbamate reagents were pumped using a Model 6000 chromatography pump (Water's Associates). Chloroform was pumped using a peristaltic

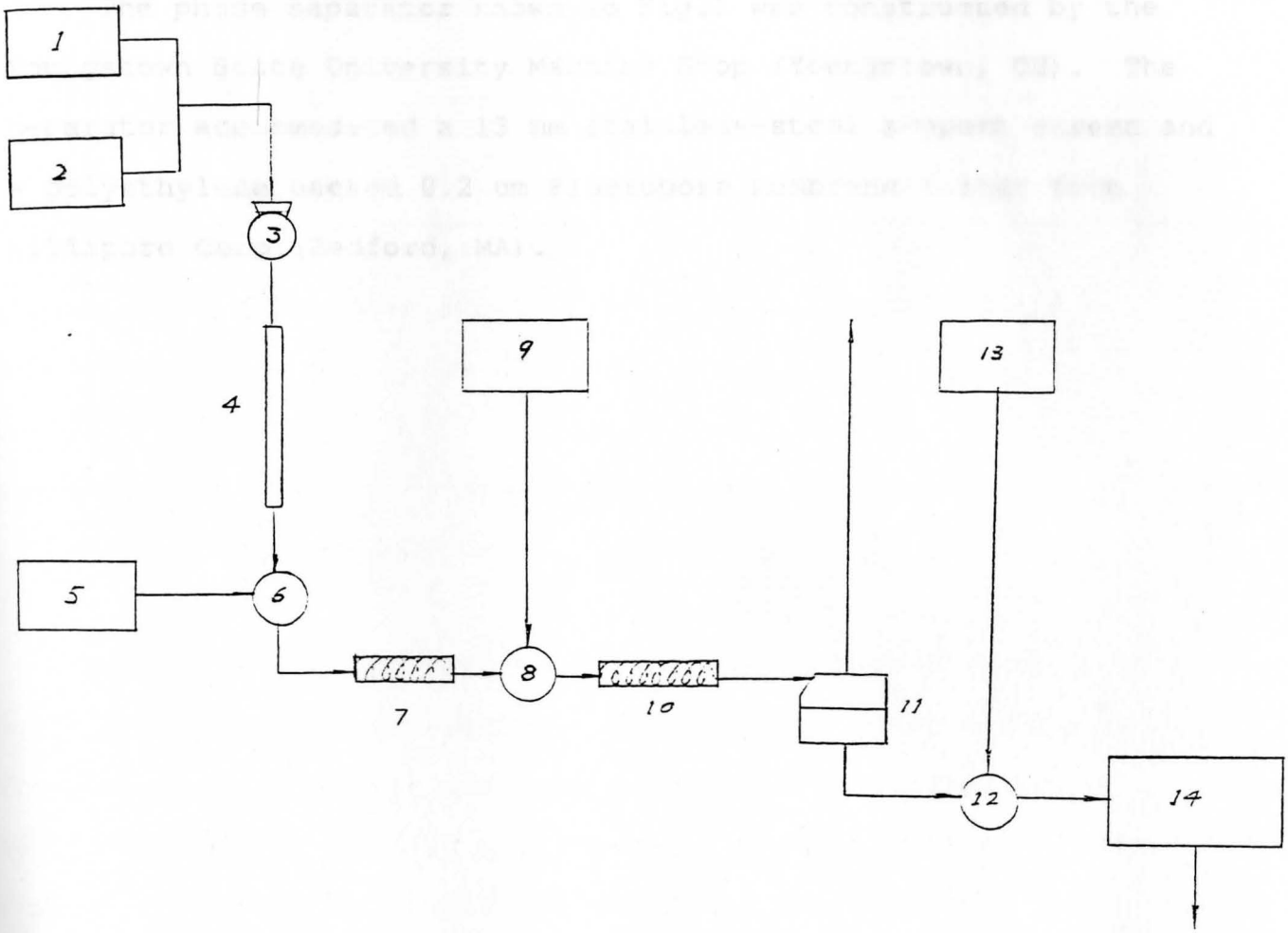


Fig. 2. Scheme of analytical system.

1 and 2: 110B pumps; 3: Injector valve; 4: C_8 column

5 and 13: Water's pumps;

6,8 and 12: Mixing tee A,B and C; 7: Reaction coil;

9: Proportioning pump; 10: Extraction coil

11: Phase separator; 14: UV detector

proportioning pump (Technicon). Chloroform resistant tubing, used to deliver chloroform, was obtained from Cole-Parmer Instrument Co. Mixing tees were obtained from Alltech Associates (Deerfield, IL).

The phase separator shown in Fig.1 was constructed by the Youngstown State University Machine Shop (Youngstown, OH). The separator accommodated a 13 mm stainless-steel support screen and a polyethylene backed 0.2 um Fluoropore membrane filter from Millipore Corp.(Bedford, MA).

CHAPTER 5

EXPERIMENT

Reagents

The fatty acid standard was a 1.0 mg/mL solution of each fatty acid dissolved in methanol. The copper reagent[120] consisted of 9 volumes of aqueous 1M triethanolamine, 1 volume of 1N acetic acid and 10 volumes of 3.20% (w/v) $Cu(NO_3)_2$ $3H_20$. The diethyldithiocarbamate reagent was a 0.1% (w/v) solution of sodium diethyldithiocarbamate in 1-propanol. Both the reagents were stored in the refrigerator and used within 7 days.

Procedure

1. Liquid chromatography

Separation of the fatty acids was accomplished using two model 110B pumps controlled by a 406 analog interface module. The mobile phase linear gradient range was 70 to 87% aqueous acetonitrile from 0 to 10 min, then returned to 70% acetonitrile from 10 to 11 min and equilibrated for 20 min before the next run. The mobile phase flow rate was set at 0.8 mL/min. An injector equipped with a 100 µL loop was used for injection of the samples onto an Econosphere C_8 column. Injection volumes were 10 µL of samples.

2. Post-Column Copper-Ion Complex Extractor

The post-column extractor was assembled as shown in Fig.2. All components were of stainless steel except the phase separator. The copper reagent was mixed with mobile phase at mixing tee A. The effluent flowed through a 45.0 cm x 0.3 mm I.D. tubing to mixing tee B where it was mixed with chloroform. The two-phase mixture flowed through a 90.0 cm x 0.3 mm I.D. coiled tubing which enabled the extraction of fatty acid-copper-ion complex into the chloroform phase. The organic phase containing the extracted complex was then separated using the membrane phase separator. The organic phase effluent, after mixing with sodium diethyldithiocarbamate reagent at mixing tee C, flowed through the 166 programmable UV/VIS detector set at 440 nm.

The copper buffer and sodium diethyldithiocarbamate reagents were pumped by the Water's pumps at 0.5 mL/min and 0.3 mL/min respectively. The chloroform flow was set at 2.0 mL/min using the proportioning pump. The chloroform pump was the first to be turned on and the last to be turned off each day. This was necessary in order to properly wet the Teflon membrane in the phase separator with the organic phase.

CHAPTER 6

RESULTS AND DISCUSSION

HPLC Separation of Fatty Acids

According to the spectroscopic analysis of individual fatty acids and mixtures of fatty acids by the 8452A Diode Array Spectrophotometer (Hewlett Packard), the maximum absorbance was at the wavelength of 198 nm. A 220 nm wavelength was chosen in order to minimize the effects of other absorbing interferences. Fig.3 through 7 shows the wavelength scan reports of the individual fatty acids.

There are two types of reversed-phase columns that have been employed successfully for the separation of fatty acids: the octyl (C_8) and octadecyl (C_{18}) columns. Here the C_8 column was used because of the lower degree of non-polarity as compared to the C_{18}. There were less avid retention times for the very highly non-polar fatty acids, thus offering an advantage of shorter analysis time compared to the C_{18} column.

A large number of solvents or solvent combinations were tried in both isocratic and gradient modes in order to separate these fatty acids. A chromatogram that illustrates the separation of five fatty acids is presented in Fig.9. The mixture was separated using a gradient mobile phase from 70 to 87% aqueous acetonitrile and detected by means of UV absorbance at 220 nm. The analysis was rapid and the compounds were well resolved. The separation was somewhat improved over that obtained earlier[21,52] and similar to other work which employed two

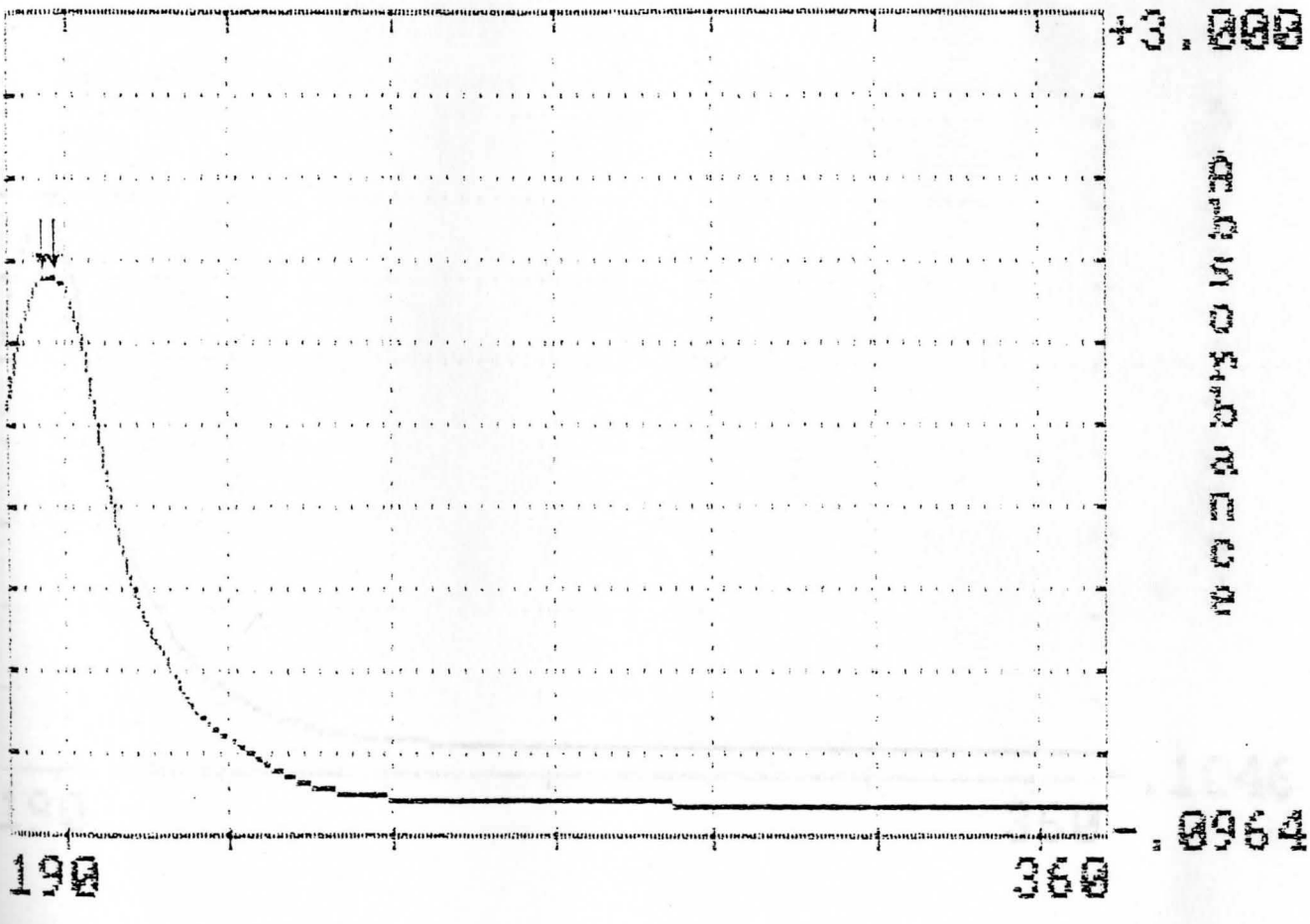


Fig. 3. Wavelength scan of capric acid.

1.0 mg/mL solution of capric acid dissolved in methanol.

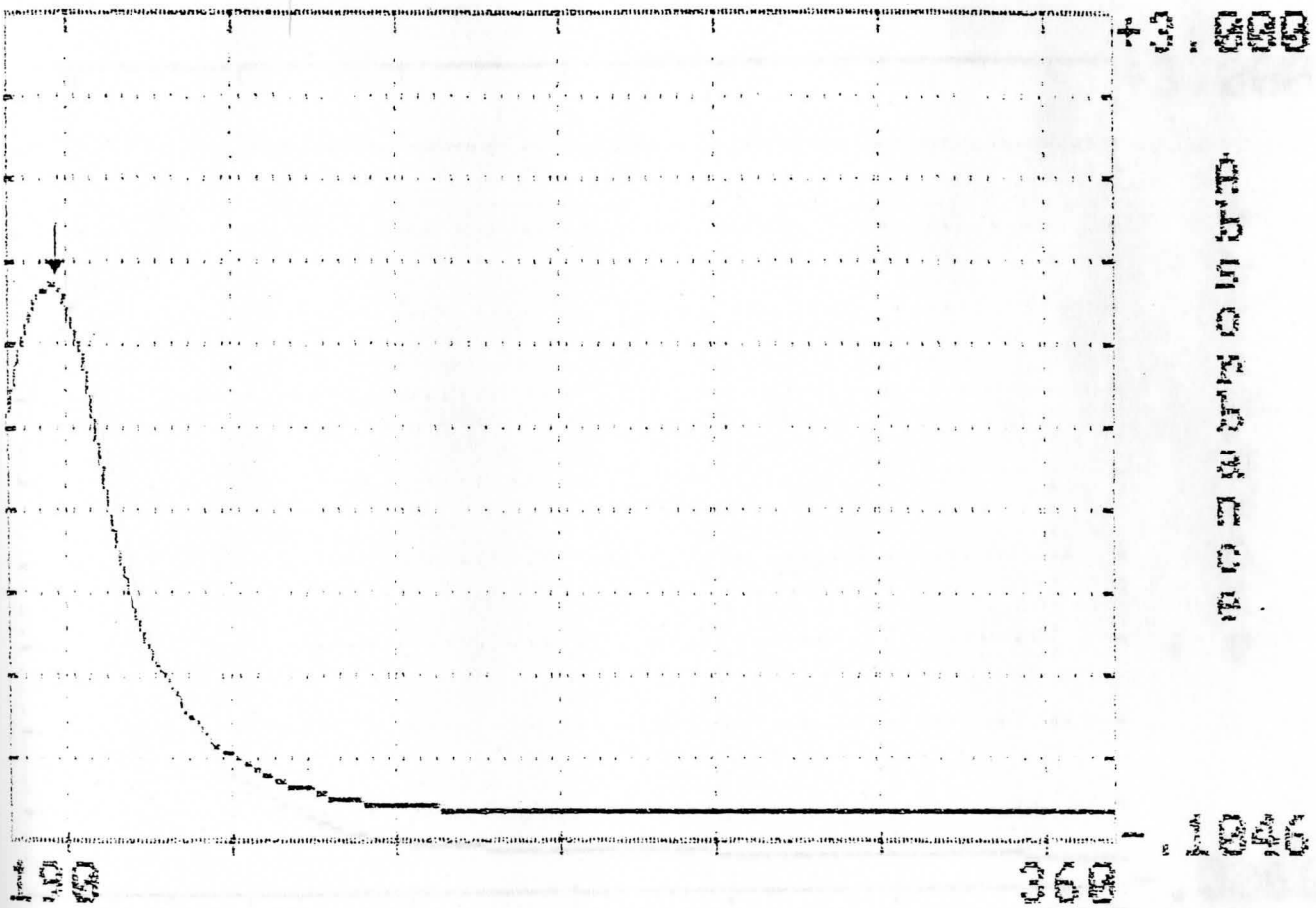


Fig. 4. Wavelength scan of lauric acid.

1.0 mg/mL solution of lauric acid dissolved in methanol.

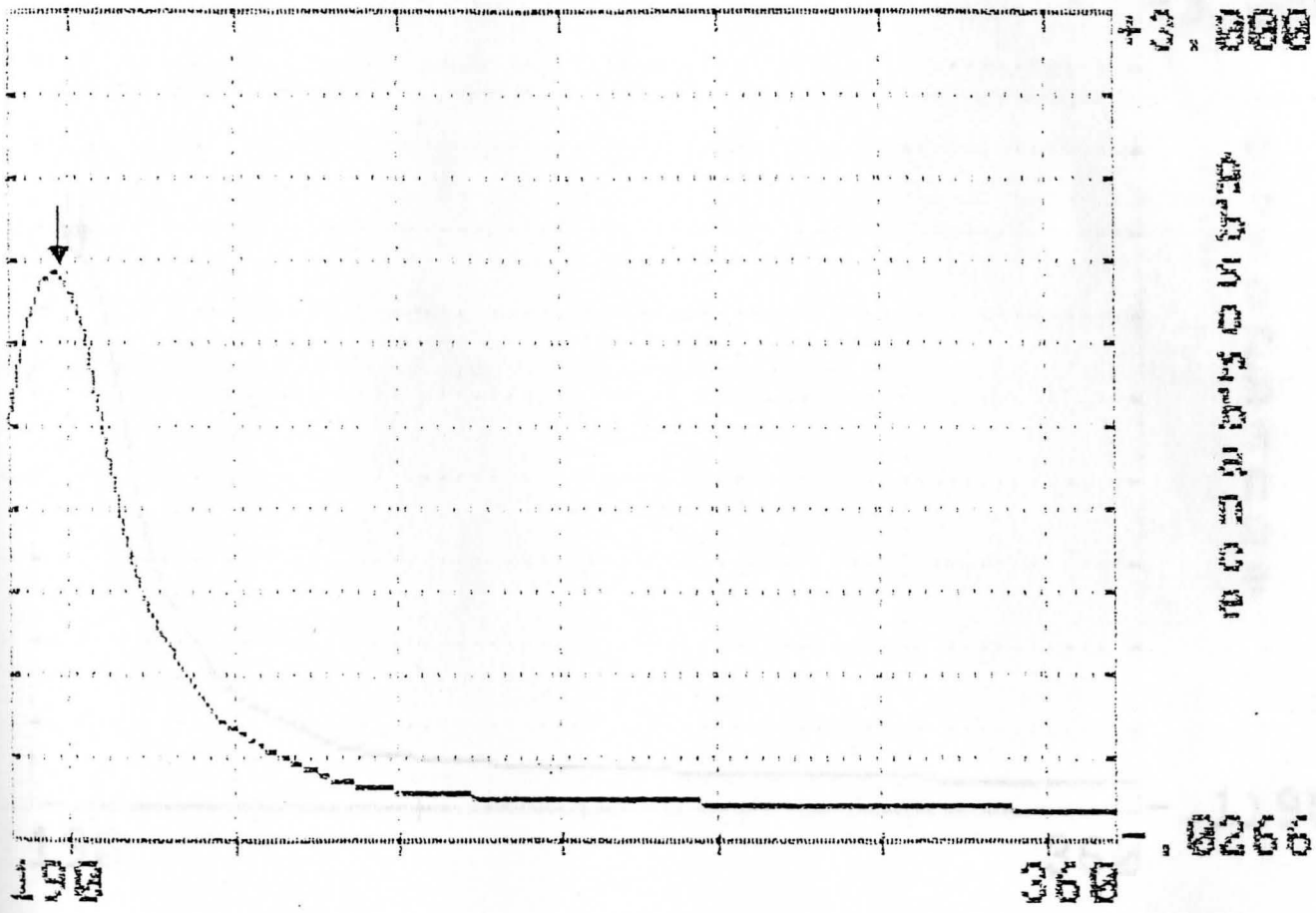


Fig. 5. Wavelength scan of myristic acid.

1.0 mg/mL solution of myristic acid dissolved in methanol.

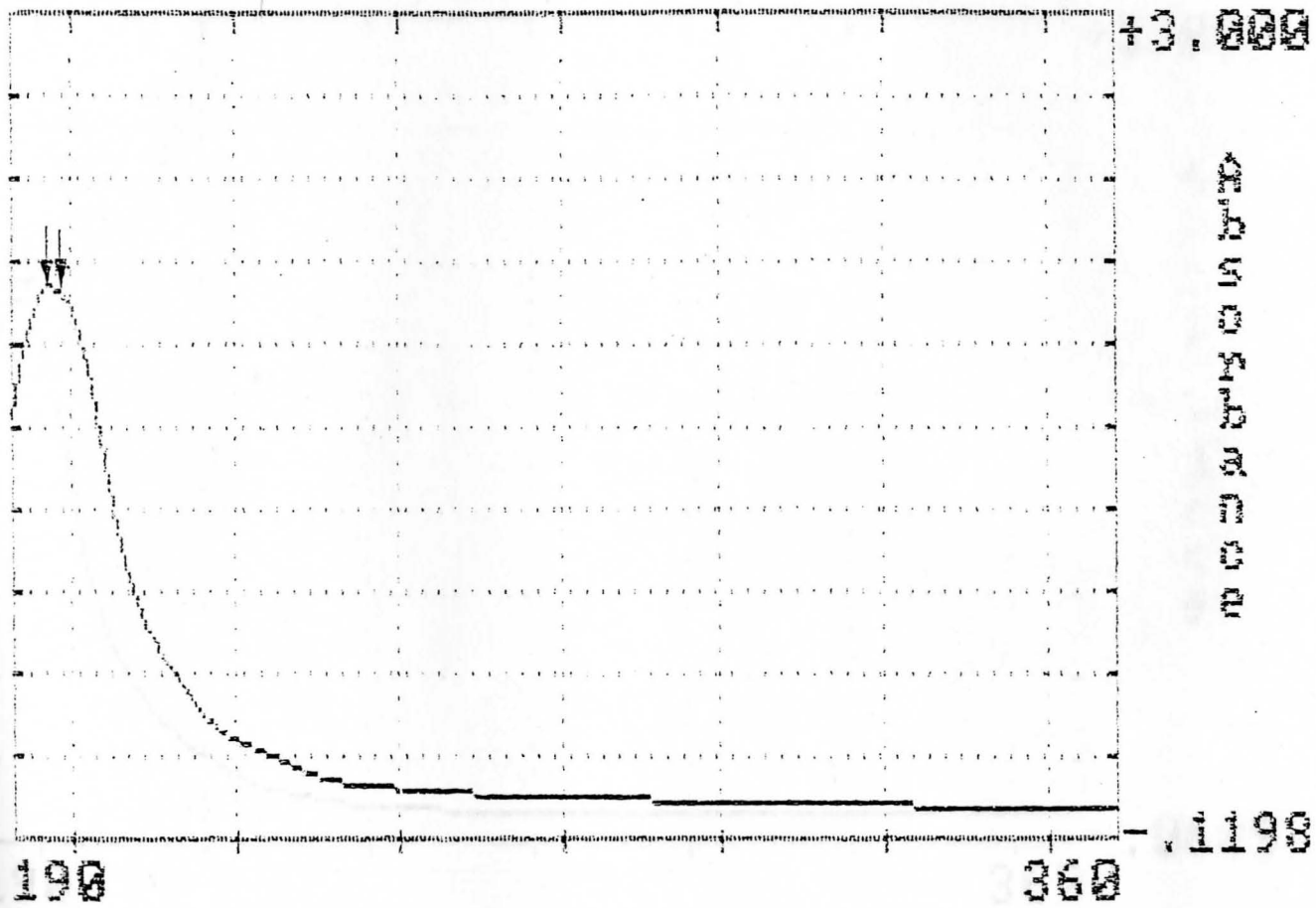


Fig. 6. Wavelength scan of palmitic acid.

1.0 mg/mL solution of palmitic acid dissolved in mathanol.

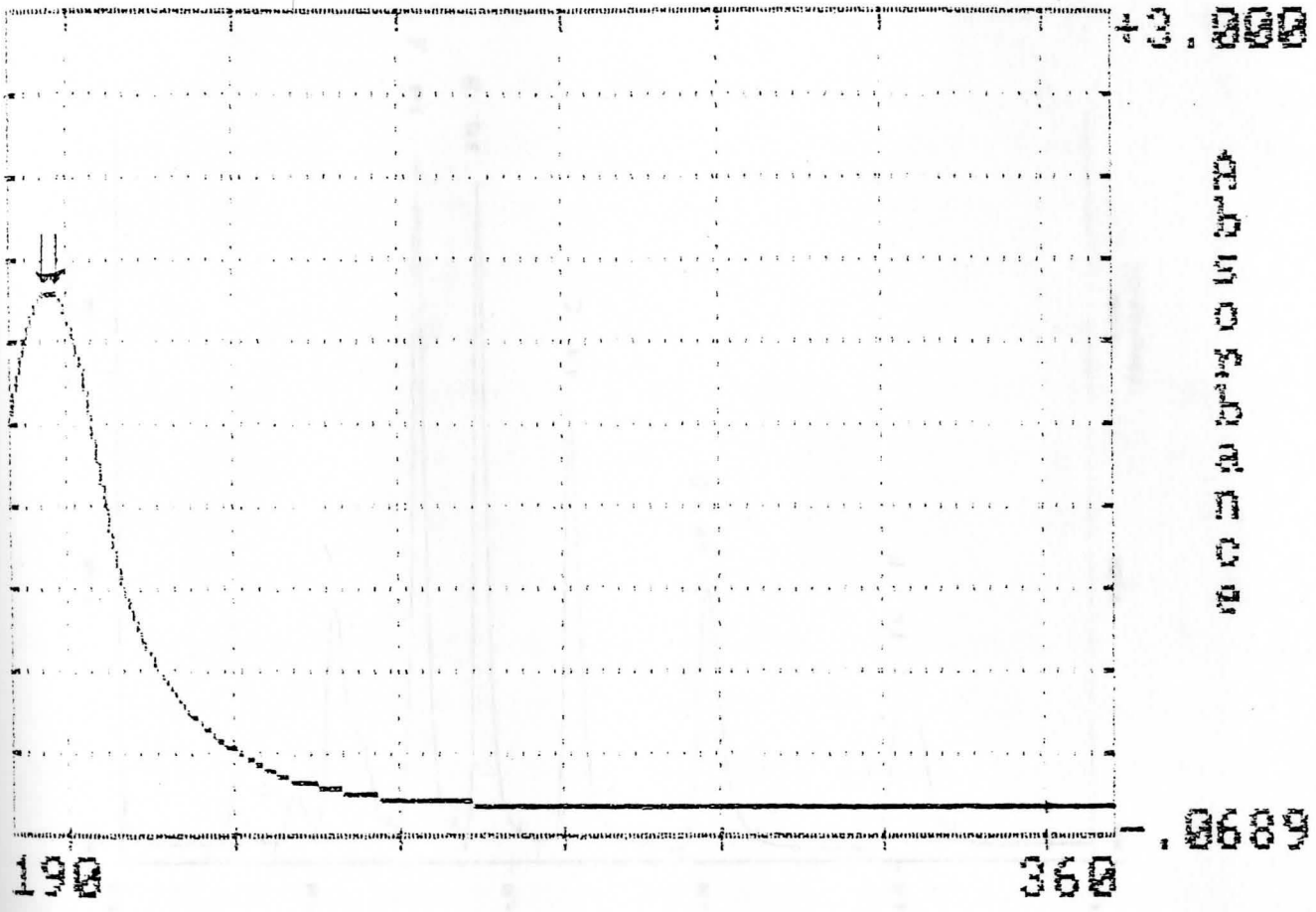


Fig. 7. Wavelength scan of stearic acid.

1.0 mg/mL solution of stearic acid dissolved in methanol.

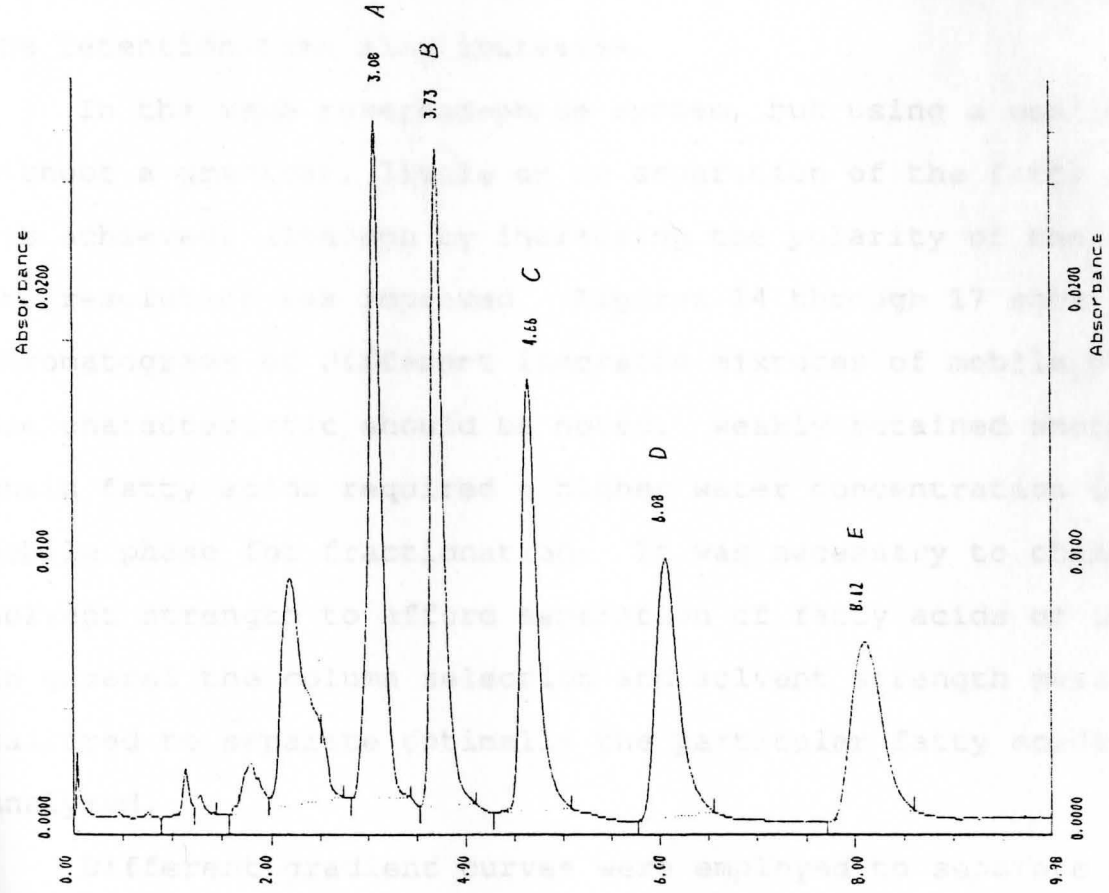


Fig. 8. Chromatogram of separation of fatty acid mixture. Flow rates: 0.8 mL/min; gradient mobile phase from 70 to 87% aqueous acetonitrile; A: capric acid; B: lauric acid; C: myristic acid; D: palmitic acid; E: stearic acid.

columns in series[64,56].

Because the elution order of fatty acids in the mixture will vary with the acetonitrile concentration, the individual fatty acids were run under the same conditions in order to identify each peak in the mixture. Figures 9 through 13 show the retention time of each fatty acid. From these chromatograms, it can be seen that as the carbon number of fatty acids increases, the retention time also increases.

In the same reversed-phase system, but using a mobile phase without a gradient, little or no separation of the fatty acids was achieved, although by increasing the polarity of the solvent, the resolution was improved. Figures 14 through 17 show the chromatograms of different isocratic mixtures of mobile phase. One characteristic should be noted: weakly retained shorter-chain fatty acids required a higher water concentration in the mobile phase for fractionation. It was necessary to change the solvent strength to afford separation of fatty acids of interest, In general the column selection and solvent strength must be tailored to separate optimally the particular fatty acids being analyzed.

Different gradient curves were employed to separate the mixture. From Figures 18 through 20, it can be seen that the gradient curve 0 shows the best separation.

The direct separation of copper(II) fatty acid complexes was tried. The results, however, were not satisfactory and the column was contaminated severely.

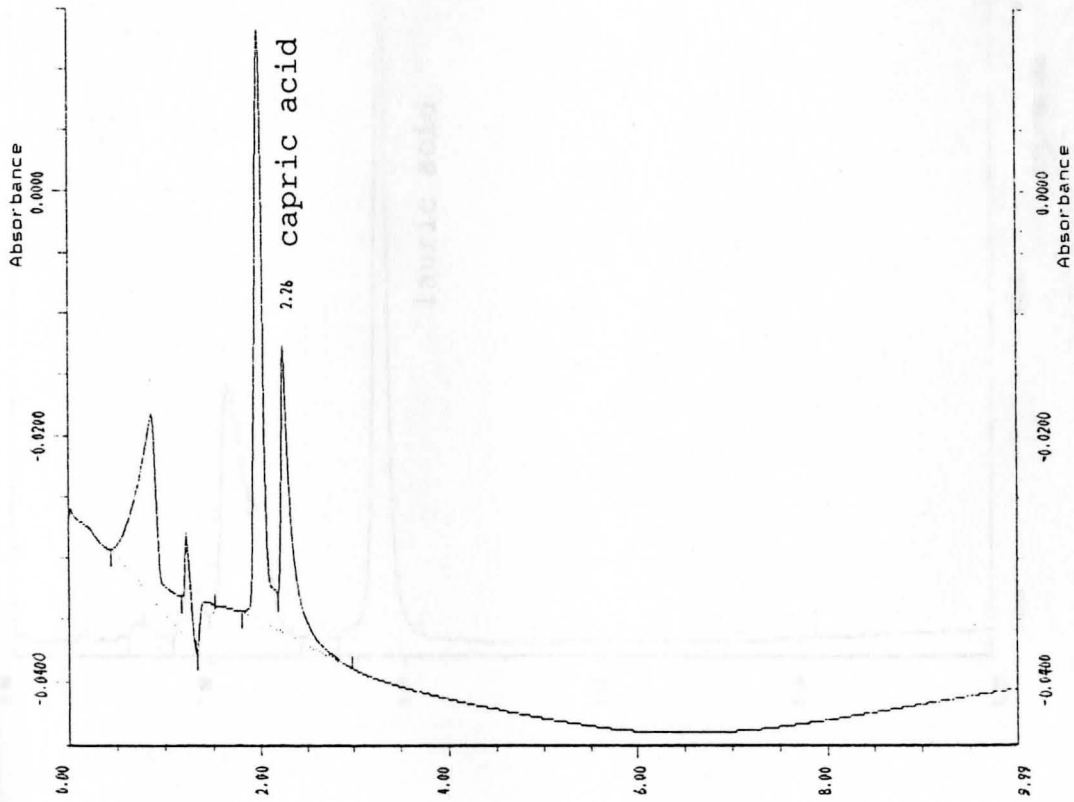


Fig. 9. Chromatogram of capric acid.

Flow rates: 0.8 mL/min; gradient mobile phase from 70 to 87% aqueous acetonitrile.

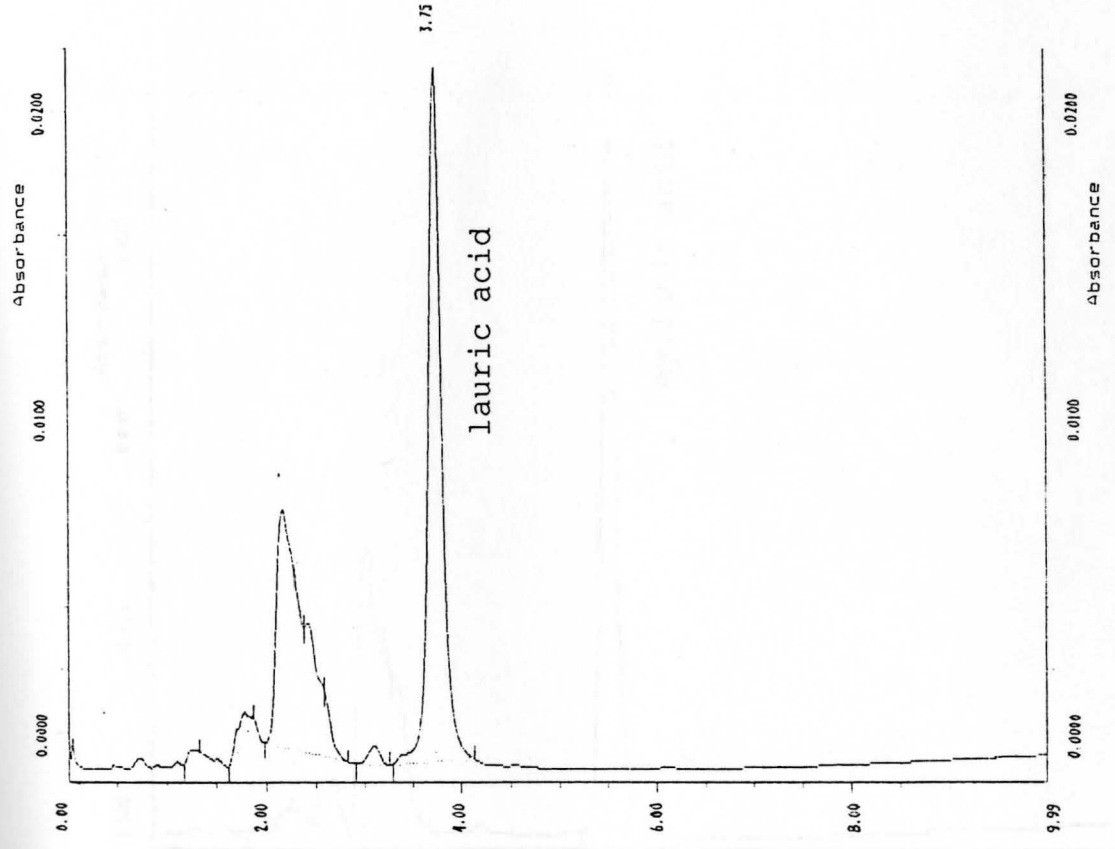


Fig. 10. Chromatogram of lauric acid.

Flow rates: 0.8 mL/min; gradient mobile phase from 70 to 87% aqueous acetonitrile.

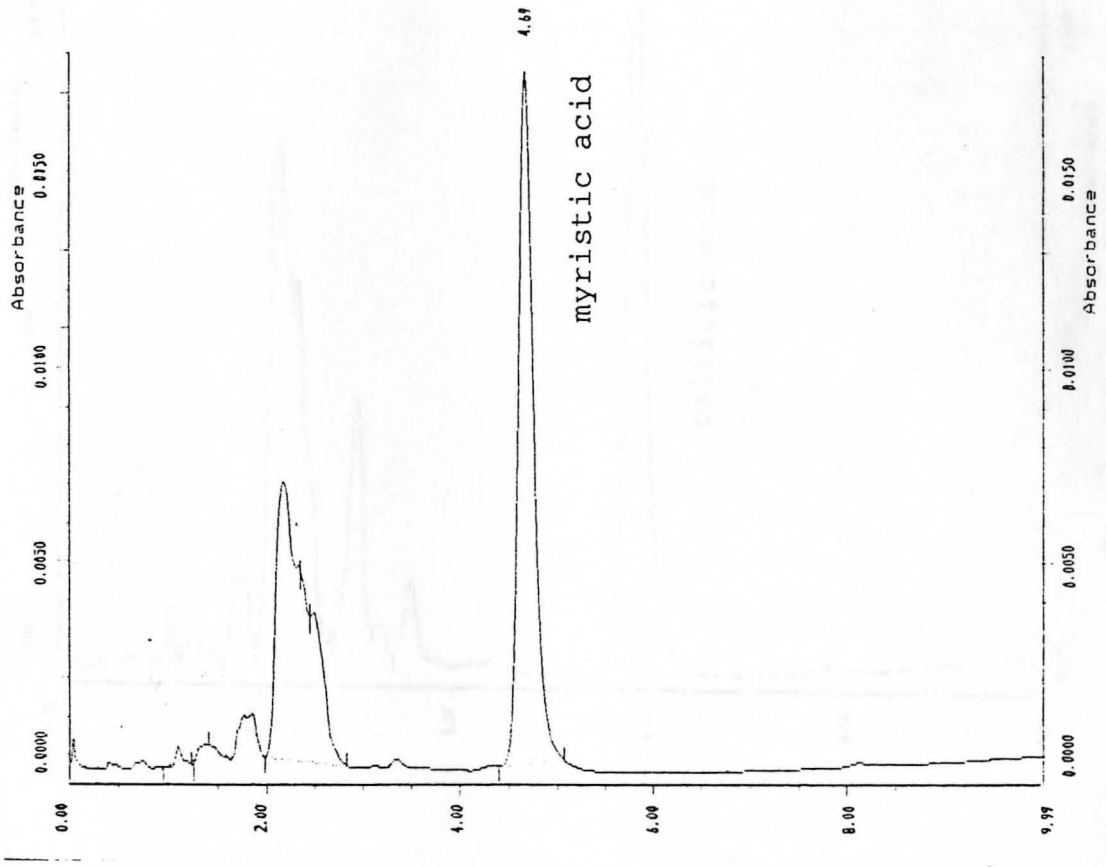


Fig. 11. Chromatogram of myristic acid.

Flow rates: 0.8 mL/min; gradient mobile phase from 70 to 87% aqueous acetonitrile.

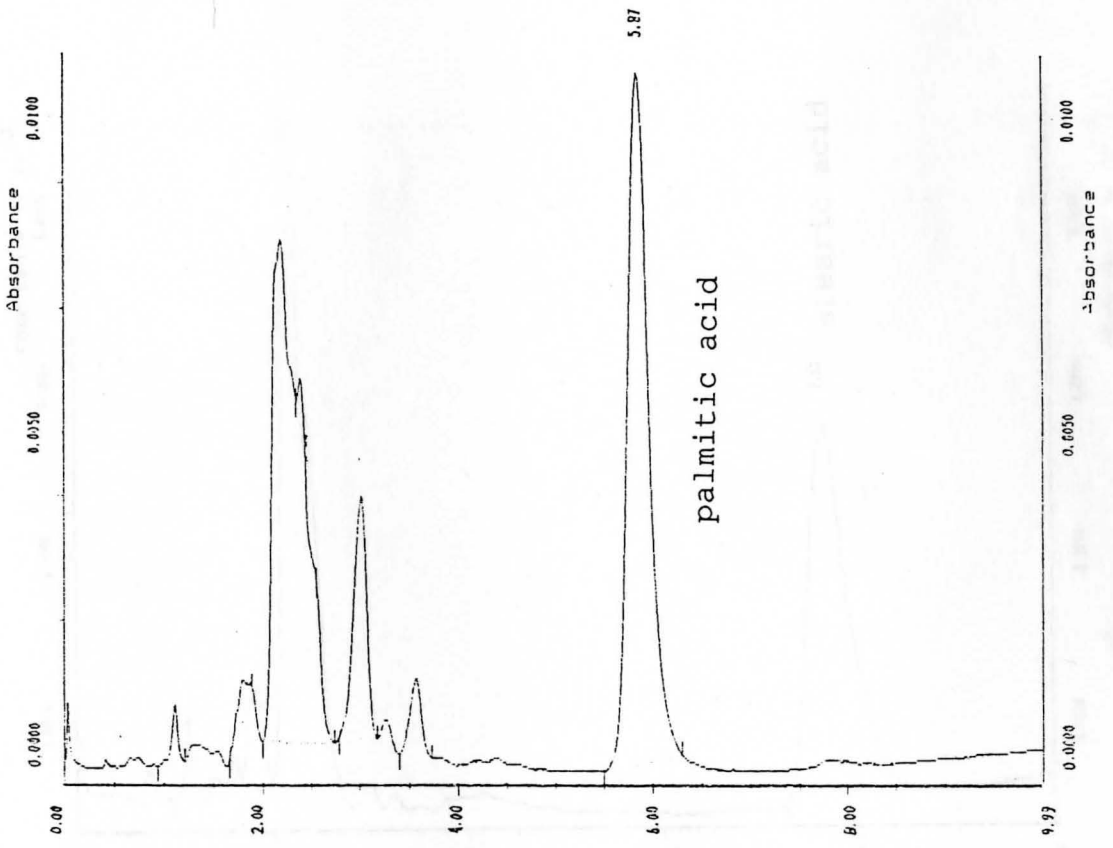


Fig. 12. Chromatogram of palmitic acid.

Flow rates: 0.8 mL/min; gradient mobile phase from 70 to 87% aqueous acetonitrile.

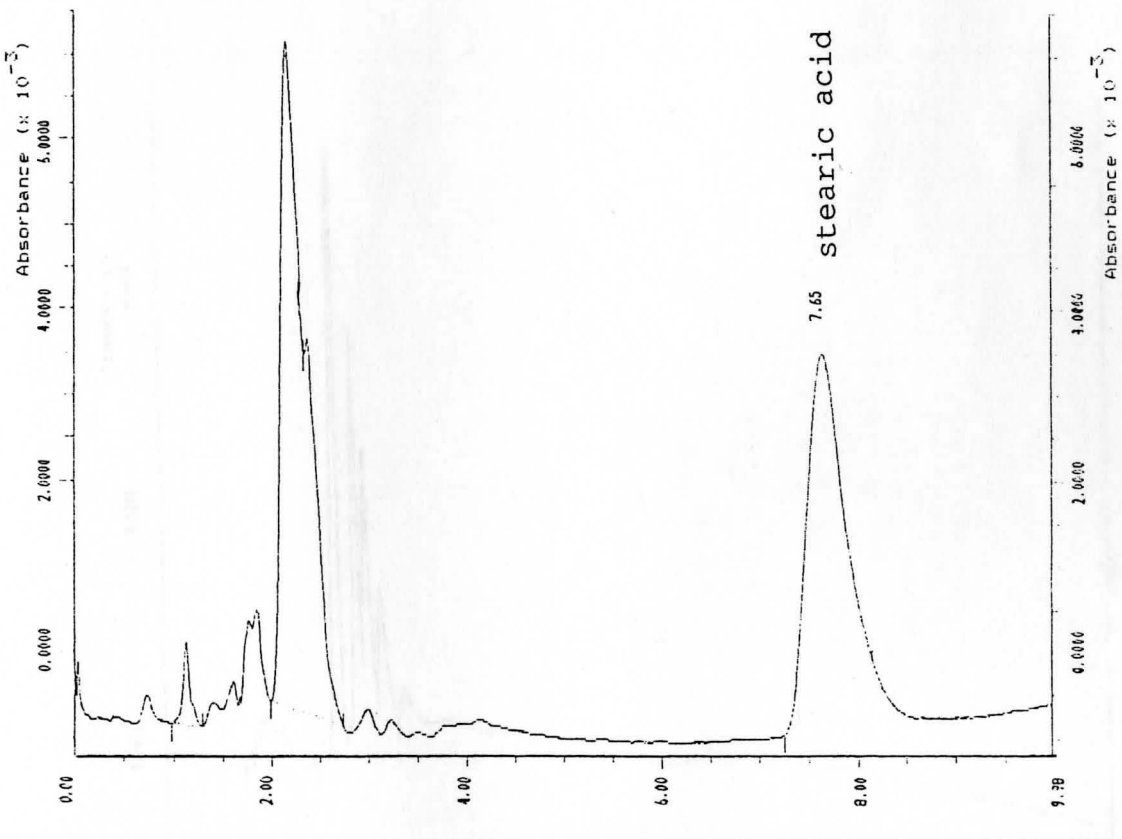


Fig. 13. Chromatogram of stearic acid.

Flow rates: 0.8 mL/min; gradient mobile phase from 70 to 87% aqueous acetonitrile.

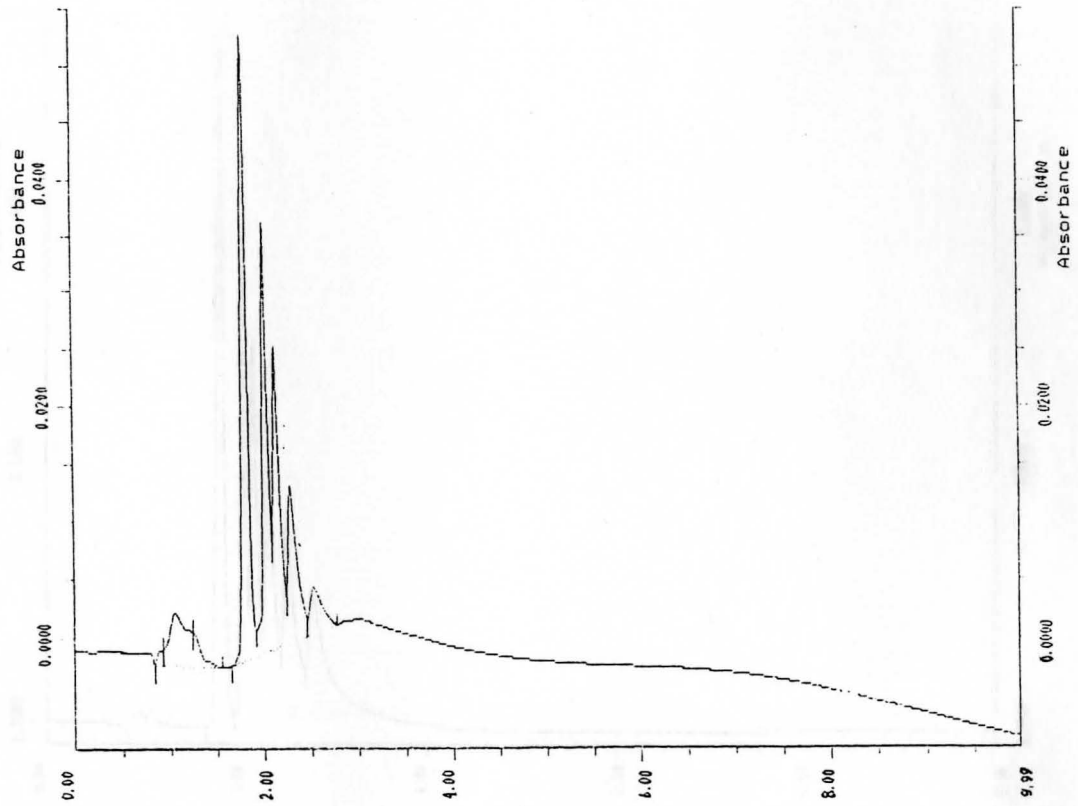


Fig. 14. Chromatogram of mixing fatty acids at isocratic mobile phase of 100% acetonitrile. Flow rates: 0.8 mL/min.

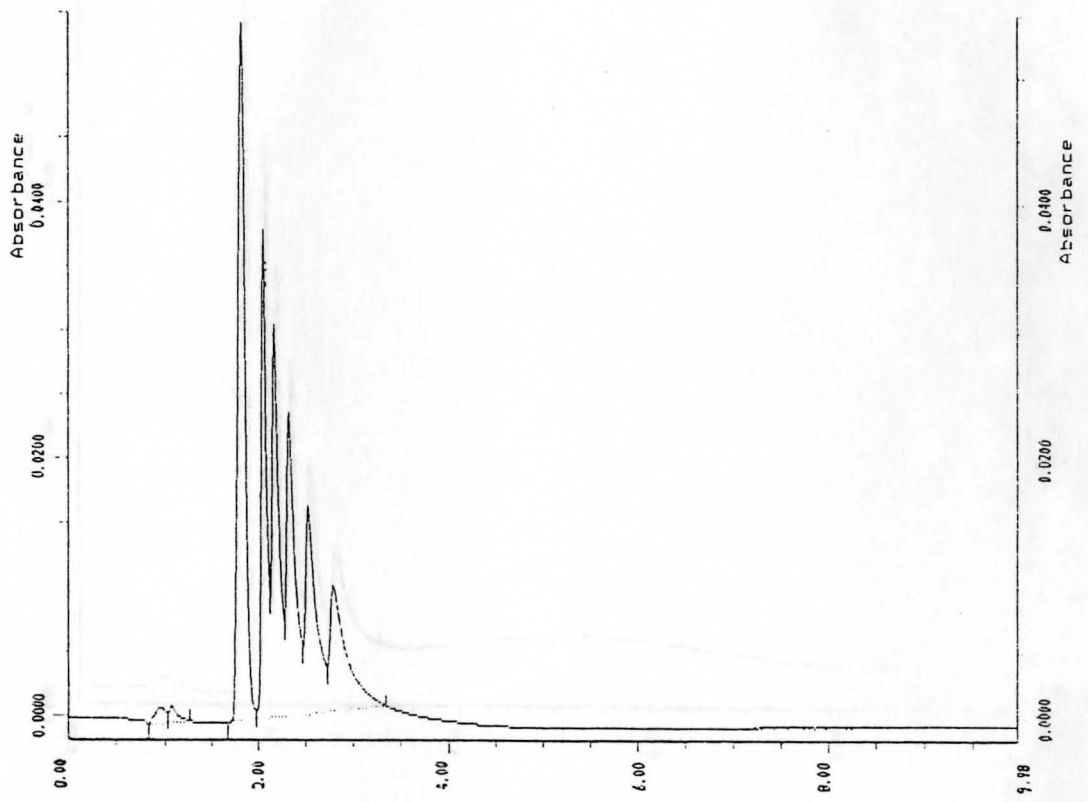


Fig. 15. Chromatogram of fatty acid mixture at isocratic mobile phase of 90% acetonitrile. Flow rates: 0.8 mL/min.

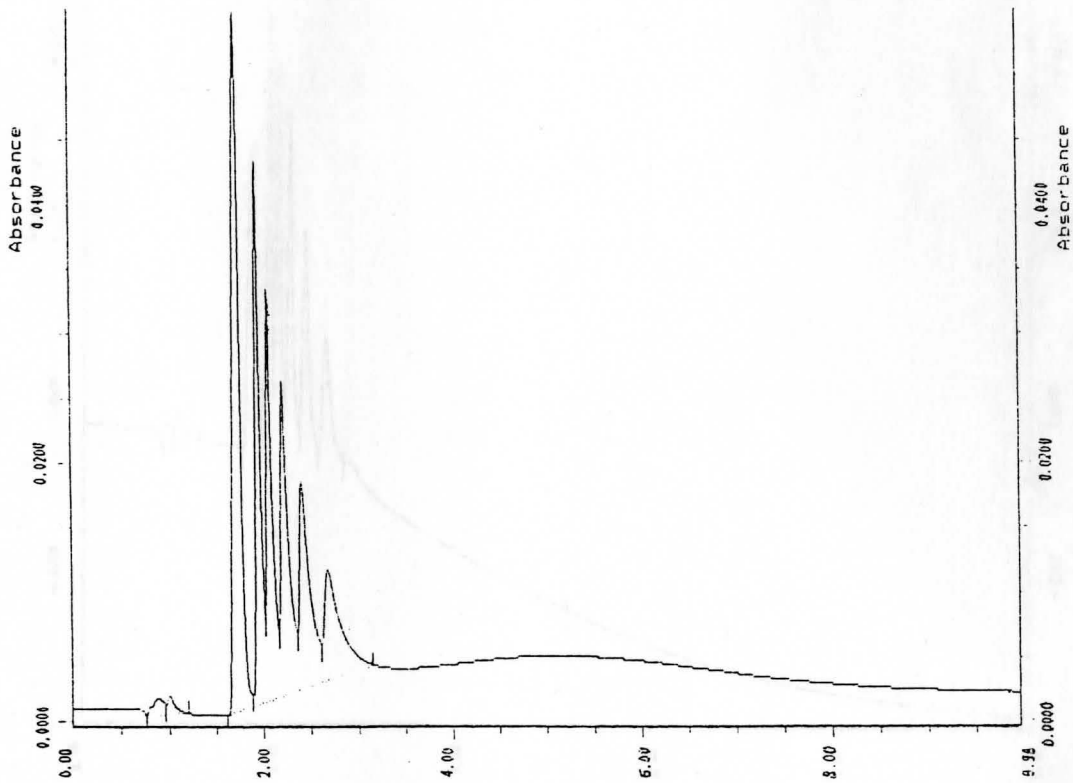


Fig. 16. Chromatogram of fatty acid mixture at isocratic mobile phase of 85% acetonitrile. Flow rates: 0.8 mL/min.

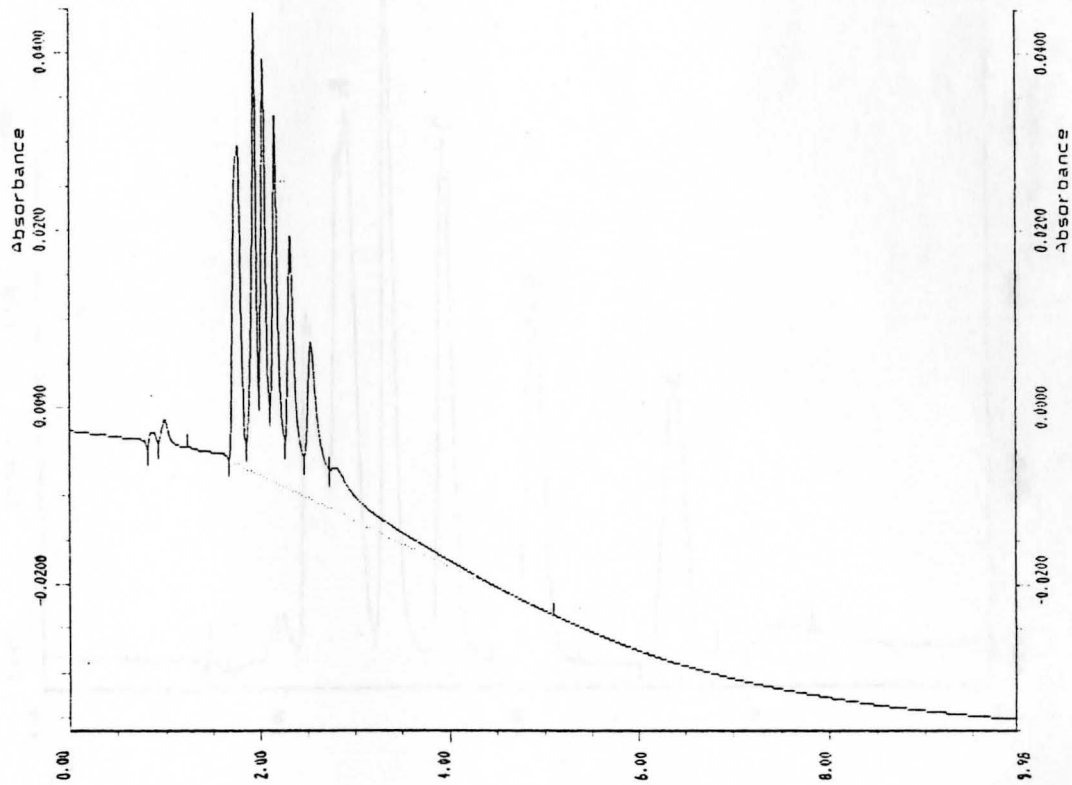


Fig. 17. Chromatogram of fatty acid mixture at isocratic mobile phase of 80% acetonitrile. Flow rates: 0.8 mL/min.

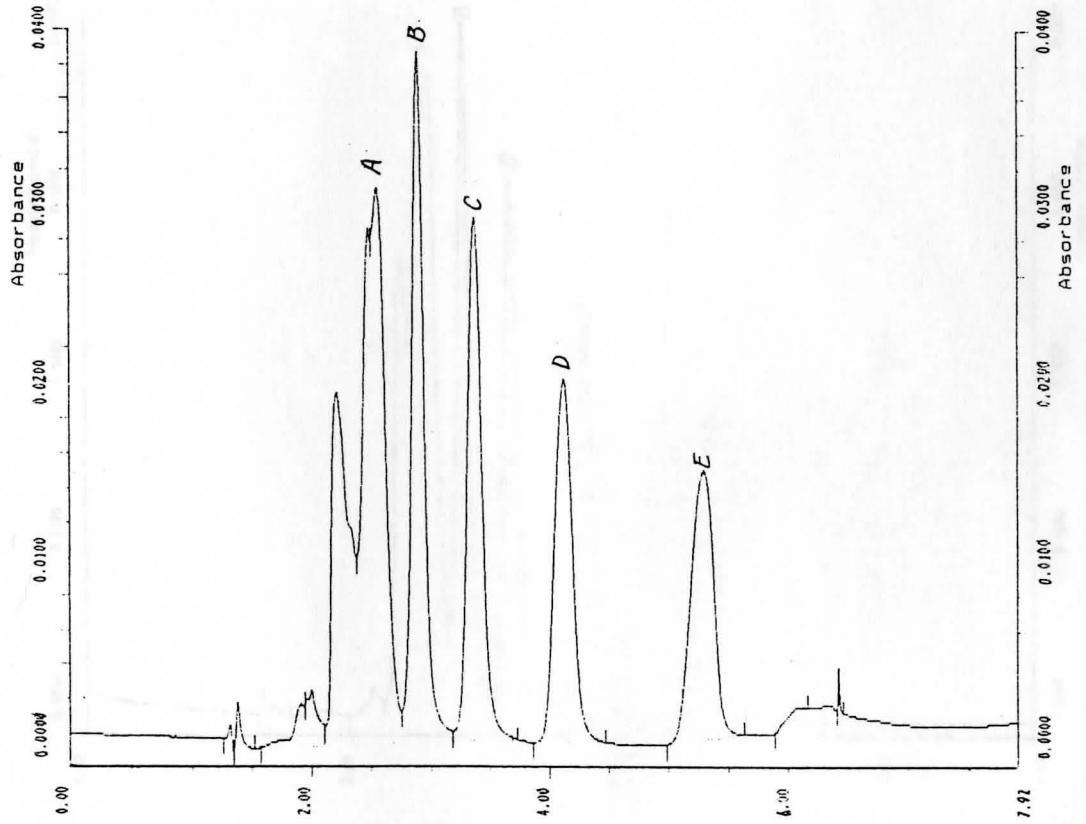


Fig. 18. Chromatogram of fatty acid mixture at gradient curve 5. Flow rates: 0.8 mL/min; 70 to 87% aqueous acetonitrile. A: capric acid; B: lauric acid; C: myristic acid; D: palmitic acid; E: stearic acid.

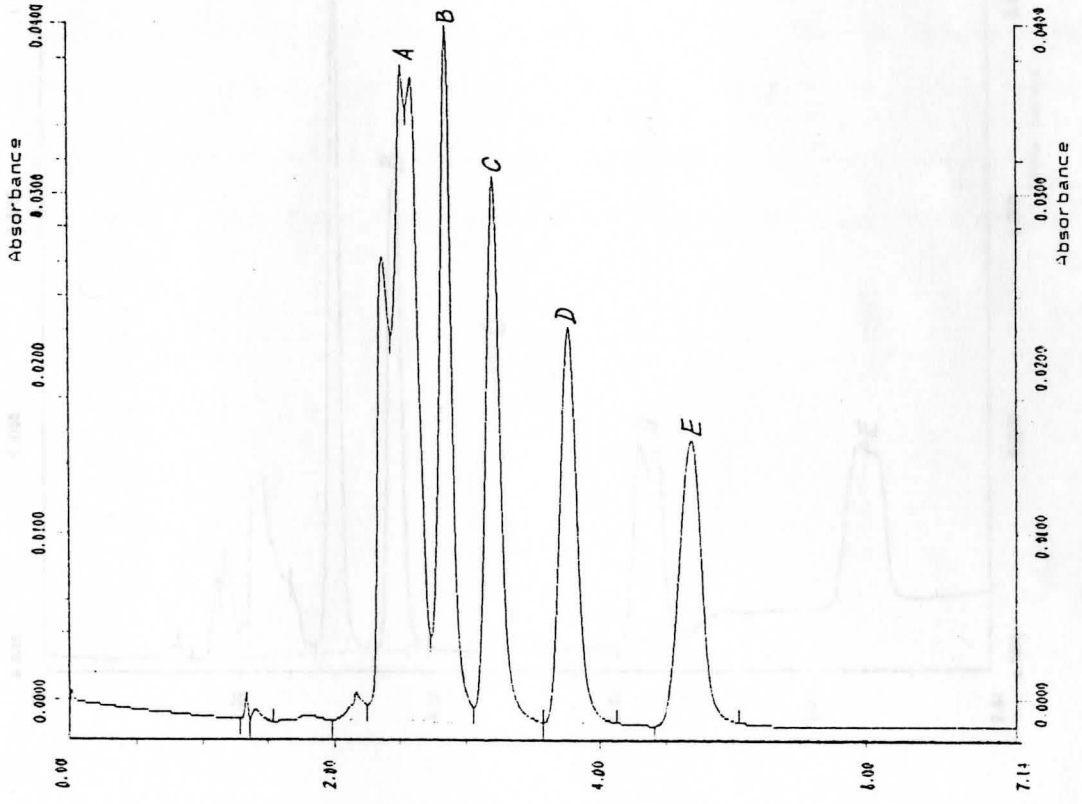


Fig. 19. Chromatogram of fatty acid mixture at gradient curve 2. Flow rates: 0.8 mL/min; 70 to 80% aqueous acetonitrile. A: capric acid; B: lauric acid; C: myristic acid; D: palmitic acid; E: stearic acid.

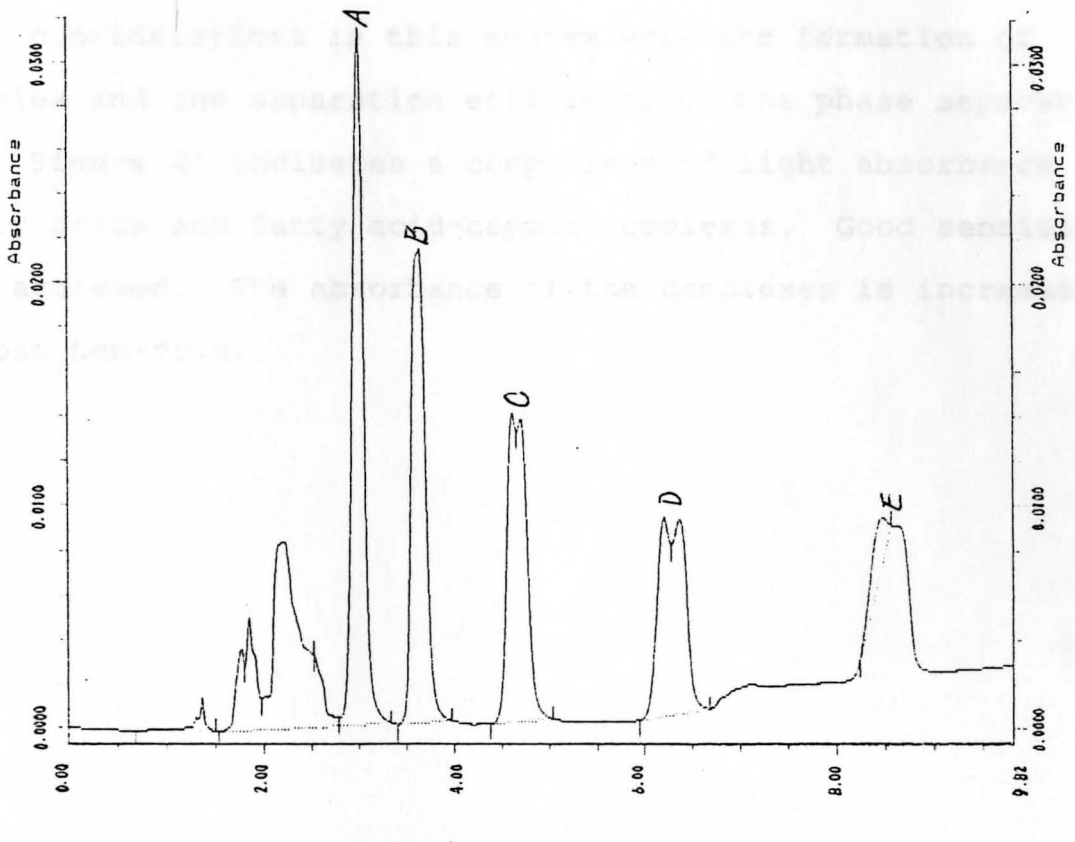


Fig. 20. Chromatogram of fatty acid mixture at gradient curve 0. Flow rates: 0.8 mL/min; 70 to 87% aqueous acetonitrile. A: capric acid; B: lauric acid; C: myristic acid; D: palmitic acid; E: stearic acid.

Post-Column Extractor

A post-column extraction system containing a reaction coil, an extraction coil and a membrane phase separator were linked to the HPLC system in order to improve the specificity and sensitivity of the analysis of fatty acids (see Figure 2). The main considerations in this system were the formation of the complex and the separation efficiency of the phase separator.

Figure 21 indicates a comparison of light absorbance between fatty acids and fatty acid-copper complexes. Good sensitivity was achieved. The absorbance of the complexes is increased almost ten-fold.

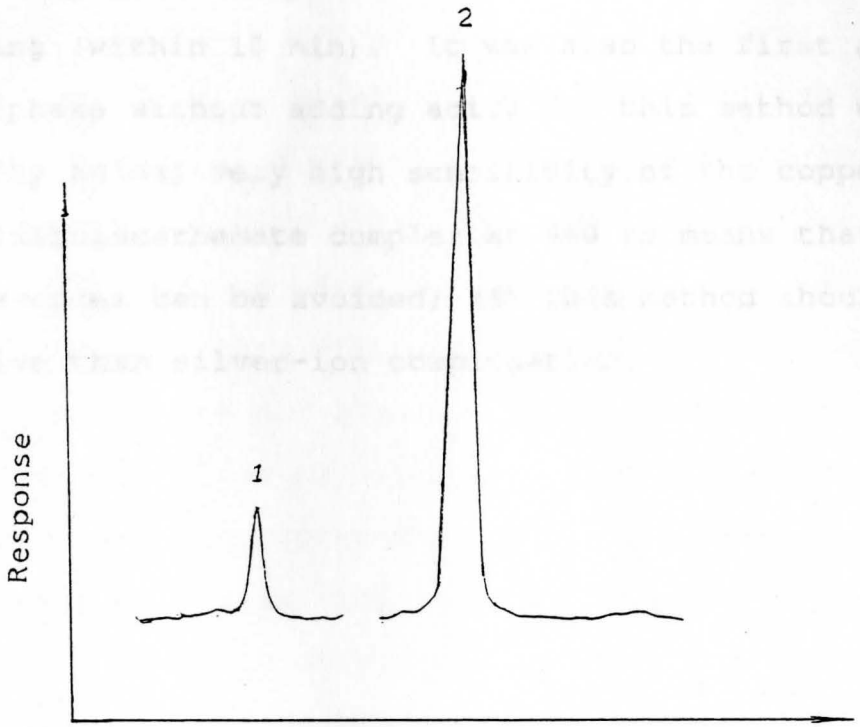


Fig. 21. Comparison of detector response of fatty acids.

1: Response without complex; 2: Response with complex.

CHAPTER 7

CONCLUSIONS

From this study, the following conclusions may be reached: (1) The work done in this investigation was believed to be the first application of analysis of fatty acids using HPLC with copper-ion complexation plus on-line extraction; (2) the method used to separate fatty acids was very simple and much less time-consuming (within 10 min). It was also the first application of mobile phase without adding acid; (3) this method was specific for fatty acids; very high sensitivity of the copper-diethyldithiocarbamate complex at 440 nm means that potential interferences can be avoided; (4) this method should prove less expensive than silver-ion complexation.

CHAPTER 8

FUTURE WORK

Although the method to determine fatty acids using post-column copper-ion complexation was demonstrated successfully, there is more work that must be done.

(1) The most important study is to improve chromatographic resolution of fatty acids with the reaction system attached. Different lengths of reaction coil for complex formation and the chloroform extraction coil have been tried to run the sample, but no significant improvement was obtained. It seems that the problem mainly comes from the membrane phase separator. The design of the present separator was based on a reported membrane phase separator design[133]. This design possessed many advantages: the start and stop procedures were not as critical to the membrane plugging as other designs[134,135], and high separation efficiency could be obtained even under different phase volume ratios and varying back pressures. However the internal phase separator volume was too large, which resulted in tremendous increases in band broadening. The internal volume of the separator used in this study was determined to be 46 μL. A modification of the phase separator is suggested that would decrease the internal volume of the separator.

(2) Although the first step of separation of fatty acids was very successful, a mixture of acetonitrile/methanol/water mobile phase is worth a try in order to improve the separation or to

separate many more fatty acids using this method. Incorporation of acetonitrile definitely facilitates the separation of both unsaturated and saturated fatty acids. According to Glajch et al.[136], in order to optimize mobile phase selectivity, the addition of a proton donor solvent (such as methanol) to a proton acceptor solvent (such as acetonitrile) leads to improved resolution, primarily due to hydrogen bonding and dipole-dipole interaction.

(3) A study of various flow rates and ratios of the extraction solvent to the mobile phase needs to be conducted, because these parameters have an effect on both the extraction efficiency and the chromatographic resolution. The phenomenon of permeation of the aqueous phase to the organic phase was observed if the flow rate of chloroform was lower than 1.0 mL/min.

(4) Since residence time of an analyte in a post-column system influences band broadening, different complexation reactions, extraction coil configurations, and lengths of coil should be investigated. Teflon tubing can be coiled, crocheted and knitted in a three-dimensional configuration in order to conduct these studies.

(5) Different concentrations and flow rates of copper buffer reagent determine reaction times and reaction efficiency of copper ion with free fatty acids. The choice of the optimum concentrations and flow rate of copper reagent can obviously improve the resolution and sensitivity.

(6) Some studies of control condition have to be conducted, such as the optimum flow rate of sodium diethyldithiocarbamate

reagent, the pressure in the post-column extraction system, and the temperature of reaction of copper ion with fatty acids. If the flow rate of sodium diethyldithiocarbamate is too high, the organic phase effluent from the separator will go back into the separator, which can cause serious contamination of the extraction system, requiring a long cleaning period. The environment of the post-column system, especially the phase separator, was very sensitive to the pressure, which also had effects on the chromatographic resolution. The optimum pressure could be regulated by using a screw clamp to squeeze the aqueous phase outlet.

(7) Other colorimetric reagents for copper should be investigated. There are some other kinds of reagents suggested as color developer with copper for colorimetric determination. Table 3 shows the relative sensitivities of reagents for microdetermination of copper[137]. Usually, five reagents are used. They are: sodium diethyldithiocarbamate used in this study, dithizone, 2.2′-biquinoline, neocuproine, and hydrobromic acid[138].

Table 3: Relative Sensitivities of Reagents for Microdetermination of Copper.

Reagent	Wavelength (nm)	Molar Absorptivity
Diethyldithiocarbamate(aqueous)	440	8,000
Diethyldithiocarbamate(pentanol)	440	12,700
2,2′-biquinoline	540	5,490
2,9-Dimethyl-1,10-phenanthroline	454	7,950
4,7-Diphenyl-1,10-phenanthroline	420	12,140
2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline	480	14,100
Biscyclohexanoneoxalyldihydrazone	595	16,000
Oxalyldihydrazide	542	22,000
2-Carboxy-2′-hydroxy-5′-sulfoformazylbenzene	600	22,500
Dithizone	508	24,600
1,5-Diphenylcarbazide	495	158,800

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