THE ANALYSIS OF EXPERIMENTAL DIETS FOR LONG CHAIN FATTY ACIDS

by

Jeanette (Colona) Gorman

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CHAPTER I

INTRODUCTION

Atherosclerosis and associated embolisms have now emerged as the leading cause of death in the United States, Northern Europe, and in many other countries (1). Consequently, a considerable amount of research has been devoted to its cause and prevention. It is becoming increasingly apparent that atherosclerosis accompanies abnormalities in the metabolism of various lipids, and the number of lipids involved in the process seems to be growing. At this stage of our knowledge, it cannot be stated dogmatically that abnormalities of lipid metabolism are the cause of the disease, but imbalances induced in lipid metabolism have led to lesions resembling atherosclerosis in many respects (2).

Since an increased level of cholesterol in the blood has been recognized as one of the factors having a positive relationship to vascular aging, more attention has been directed toward factors regulating the concentration of cholesterol in the blood and tissues. Evidence has shown that dietary fatty acids can influence serum cholesterol levels markedly (3).

One of the difficulties encountered in lipid research is the lack of a standard analytical method for fatty acid analysis. This is due to the difference in the physical and chemical properties of foods and the nature of the physico-chemical properties of lipids.

It is necessary to adapt methods to meet the requirements of the particular type of tissue or tissues to be investigated. One of the problems in fatty acid analyses has been solved since the development of the gas-liquid chromatograph and its application to fatty acid analysis. This instrument has provided a very effective tool for the separation of fatty acids and fatty acid esters (4).

CHAPTER II

LITERATURE REVIEW

Lipids

Lipids are one of the three classes of material that make up the bulk of the organic matter of living cells. The general characteristics of lipids are: insolubility in water and solubility in the so-called fat solvents; actual or potential relationship to esters; and at the same time, metabolic utility to the animal organism (5).

Lipids are commonly classified into three groups: (1) simple lipids - esters of fatty acids and various alcohols, (2) compound lipids - esters of fatty acids and glycerol which also incorporate other chemical groups, and (3) derived lipids - substances derived from simple and compound lipids by hydrolysis (6).

Fatty Acids

There is considerable variation in the chemical composition of fatty acids. Often the identity of the lipid is determined by the type of fatty acid present in the molecule. The fatty acids found in natural food fats and oils are chiefly composed of an even number of carbon atoms. Furthermore, they consist almost exclusively of straight-chain acids rather than branched-chain acids (7).

The fatty acids that occur most frequently in food fats and oils are generally classified as either saturated or unsaturated fatty acids. The unsaturated fatty acids are further subdivided into the monoethenoid acids (characterized by one double bond), the diethenoid acids (characterized by two double bonds), and the polyethenoid acids (characterized by three or more double bonds (7). From the nutritional standpoint and for this thesis, it has been found convenient to classify the diethenoid acids as polyethenoids.

The polyunsaturated fatty acids are of considerable importance to nutrition. These acids are required in diets of higher animals and man because they are not synthesized at rates to meet the physiological needs (7). According to Holman (8) "the term essential fatty acid should include only those substances which are active both for growth and for maintenance of dermal integrity, limiting the term to linoleic and arachidonic acids and to such other acids as may be derived metabolically from them." Linoleic and linolenic acids are the precursors of the more highly unsaturated acids in animal tissue. Arachidonic acid is synthesized from linoleic acid (8).

Linoleic and linolenic acids are quite prevalent in plants, but occur in low amounts in animal tissue unless the animal has ingested liberal amounts of these fatty acids. Linoleic acid is the chief fatty acid of the common vegetable oils and often occurs in

amounts of fifty percent or more. Linolenic acid occurs in small amounts in most vegetable oils. Arachidonic acid is not present in vegetable fats, but is normally found in all animal fat. The glandular tissues contain a greater amount of this acid than the muscle tissue (7).

In most cases the vegetable fats have fewer varieties of fatty acid components than do those from animal sources. Palmitic and oleic acids are the predominant fatty acids in most plant and animal lipids (9). Oleic acid is the most abundant fatty acid found in nature. It is seldom present in amounts less than ten percent and usually comprises forty percent of the total fatty acids (10).

Stearic acid, prevalent in animal fat, is absent or present in only small amounts in most of the vegetable fats. The plant lipids that contain appreciable quantities of stearic acid are mostly of tropical origin. Myristic acid is found in small quantities in nearly all animal and plant lipids. The long-chain saturated fatty acids (arachidic, behenic, and lignorceric) are found in many animal fats, but are seldom present in plant oils. While the saturated fatty acids are abundant in animal fats, they usually comprise only about ten percent of the fatty acids of plant origin (7).

Physical and Chemical Properties of Fatty Acids

The melting and boiling points of fatty acids are a function of the length of the carbon chain and the number of double bonds present. The melting point of the saturated fatty acids increases with an increase in the length of the carbon chain. The introduction of a double bond causes a considerable decrease in the melting point, and further decrease occurs with the presence of additional double bonds (9).

In general the fatty acids are insoluble in water and soluble in the so-called fat solvents, i.e., ethyl ether, petroleum ether, benzene, hot alcohol, chloroform. The solubility of fatty acids in water decreases as the length of the carbon chain increases. While the fatty acids are generally insoluble in water, their alkali metal soaps are quite soluble in water and insoluble in the fat solvents (9).

The fatty acids are easily oxidized under favorable conditions. Oxidative deterioration of the fatty acids involves reactions that form peroxides and hydroperoxides that are further decomposed to other components such as aldehydes, ketones, and shorter chain fatty acids. The unsaturated fatty acids are very susceptible to oxidation and the susceptibility increases with increase in the number of double bonds. Oxidation originates at the site of the double bond (11).

Saturated fatty acids are considerably less susceptible to oxidation; if oxidation does occur, it can be at any point along the

carbon chain, but predominates at the beta position. However, when oxidation does occur in the saturated fatty acids, it is at a very slow rate. Fortunately, there are present in animal and plant tissue a number of natural antioxidants that deter or delay the oxidation of the fatty acids (11).

Extraction of Fatty Acids

Many methods have been used for the extraction of fatty acids from plant or animal tissue. The precise method depends on the nature of the material to be studied.

Lipids are often linked in some form of combination with carbohydrate or protein in tissue. In order to break this linkage, it is usually necessary to employ some type of dehydrating agent such as ethanol, methanol or acetone (12). Hot alcohol is a useful solvent for liberating much of the protein bound lipid from tissue, except the cephalin group (13). Folch *et al.* (14) developed a method, using chloroform-methanol as the solvent, that is very effective for the extraction of lipids from brain and other nerve tissue. With a few adaptations this method has been successfully applied to other types of tissue.

The extraction of triglycerides from fat-rich tissue, e.g. adipose tissue and seed oil, involves very little difficulty because

these fats are not generally bound into lipoprotein or lipocarbohydrate, and they are easily soluble in practically all fat solvents (13). Lipids are more efficiently extracted if dry tissue rather than wet tissue (13) is used, and if the tissue is ground into very fine particles (15).

In the selection of a solvent, consideration must be given to the fact that some fat solvents are also good solvents for certain non-lipid constituents of tissue. For example, ethyl ether will extract some non-fat substances (13).

To prevent oxidation of lipids during storage and extraction it is advisable to maintain an absolute minimum exposure to air, light and elevated temperatures (11).

Saponification

Saponification or alkaline hydrolysis is the cleavage of the ester linkages that connect the fatty acids to other lipid components to yield the salts of the fatty acids (soaps) (16). Alkaline hydrolysis is effective for splitting the fatty acid ester linkages of the triglycerides, glycerophosphatides, sterol esters, and waxes. It is also effective in splitting the ester linkages of choline, ethanolamine, or serine in glycerophosphatides. Alkaline hydrolysis is effective against amide-linked fatty acids (sphingolipids), glycosidic linkages

(cerebrosides), or the phosphoryl choline linkage in sphingomyelin. It is quite ineffective against the acetal linkage of the plasmalogens and the phosphoric ester linkage in glycerophosphoric acid (13).

Saponification is usually carried out after the lipid sample has been isolated in a solvent. The solvent is then evaporated and the dried product hydrolyzed under reflux for three to eighteen hours, depending upon the resistance of the lipid to hydrolysis, in a solution of alcoholic-potassium hydroxide (17).

A "cold" saponification method is recommended for the heat labile unsaturated fatty acids. In this method the lipid sample is left to stand for one to several days at room temperature in a solution of potassium hydroxide and alcohol (4). Buttery et al. (18) in their method extracted and saponified the fatty acids simultaneously in a solution of potassium hydroxide-ethanol.

The general procedure at the end of the hydrolysis period is evaporation of the alcohol and addition of water to the sample. The non-saponificable material is then removed by washing two or more times with petroleum ether or hexane. The aqueous solution is acidified and the free fatty acids extracted three or more times with a fat solvent (17).

Methylation

For the separation of the fatty acids with more than ten carbon atoms their methyl esters are recommended for gas-liquid chromatography because the esters of the fatty acids have a much lower boiling point

than the free fatty acids. The temperatures necessary to volatilize the long chain free fatty acids often cause decomposition of the fatty acids (17).

A number of methylation procedures are available. Vorbeck et al. (19) compared four procedures that are most frequently used for the preparation of methyl esters for gas-liquid chromatography. The methods compared were diazomethane, methanol-hydrochloric acid with sublimation, methanol-hydrochloric acid on ion exchange resin, and methanol-boron-trifluoride. Results showed that the greatest variation among the methods occurred in the mixture of lower molecular weight acids. The methods gave comparable results with the higher molecular weight fatty acids.

Gas-Liquid Chromatography

Gas-liquid chromatography is a method for separating components of mixtures. The separation depends upon the difference in the distribution coefficients of the components between a fixed phase and mobile phase (4).

In gas-liquid chromatography a sample is administered, flash evaporated, and swept by a constantly-flowing stream of carrier gas into one end of the column. The components are carried through the column at different rates, that are governed by their partition coefficient between the gas phase and the stationary phase. Ideally, they emerge from the other end of the column at different times.

Their presence in the emerging carrier gas is detected by physical or chemical means, and the response of the detector is fed into a strip chart recorder. Each peak on the chart represents a discrete chemical compound or a mixture of compounds. The area under the peak is proportional to the concentration of the compound or a mixture of compounds in the sample (4).

The most useful substrate for the separation of the fatty acid esters is the apiezon greases and the polyesters. The polyesters have the advantage of greater selectivity on the basis of degree of unsaturation of the fatty acids. One of the polyester substrates that has been demonstrated to be effective in the resolution of the esters of fatty acids is polyethylene glycol adipate. It has good temperature stability up to 200° C, is suitable for fatty acid esters up to twenty-six carbons in length, and gives good separation of all fatty acids except isomers of the monenes (17).

Southern Regional Nutrition Research Study

Experiments were conducted at the Virginia Agricultural Experiment Station in 1962 to study nutrient balance in preadolescent children. The experiments were a part of the Southern Regional Nutrition Research Project Number S-28, entitled "Metabolic Patterns in Preadolescent Children." For approximately seven weeks twelve girls whose age ranged from seven years, ten months to nine years, five months, served as subjects (20).

The experimental diets were planned to provide two levels of protein intake and two levels of riboflavin intake, with other nutrients approximately at recommended allowances. These diets were designed to include only foods from plant sources, and to consist of natural foods and vitamin-mineral supplements as needed to meet objectives of the nutrition study (20).

The experiment was divided into seven periods each six days in length. During period I, the preliminary or adjustment period, all subjects were given Diet 13. During the remaining periods (II through VII) the subjects were assigned to a different dietary regime (Diets 9, 10, 11, and 12). Diets 9 and 10 differed only in riboflavin and niacin; this was true also for diets 11 and 12. The lipid components of diets 9 and 10 were the same, as were the lipid components of diets 11 and 12 (20).

One of the primary purposes of the experiment was to relate the lipid components of the diets to the serum lipids of the subjects. The Human Nutrition Research Division of the USDA, one of the cooperating agencies in the Regional Project, analyzed the serum lipids of the subjects. The objective of the study reported in this paper, part of the responsibility of the Virginia Station in the project, was to determine the fatty acids in the diets (20). This involved the following:

1. development of a method for analyzing the long chain fatty acids, particularly those of sixteen and eighteen carbons, in plant food composites.
2. quantitative determination of the various long chain fatty acids in the experimental diets. Diets from Period V were selected because all minor adjustments in the diets had been made.

CHAPTER III

METHODS AND PROCEDURES

Preparation of Food Composites

A food composite, representing the food served to the subjects, was prepared for each of the six days of Period V from each of the two experimental diets (Diets 9 and 10 and Diets 11 and 12). Margarine and vitamin-mineral supplements were not included in any of the twelve food composites. Menus for this period are given in the Appendix, P. i - vi. Each of the food composites was made to 2000 grams, by the addition of water, then thoroughly blended. An aliquot of each of the food composites was freeze-dried, and the percent of moisture was determined. The lyophilized samples were then placed in screw-cap jars and stored in a dessicator over calcium chloride in a freezer at -20° C for fifteen months before being analyzed for fatty acids (20).

Extraction and Saponification of the Fatty Acids

A freshly prepared solution of 5 percent potassium hydroxide in 90 percent ethanol was added to a flask containing two grams of lyophilized food composite and 8 mg. of standard heptadecanoic acid and mixed gently. The stoppered flask was placed in the dark for three days and was shaken occasionally during this time. At the end

of this period the mixture was heated in a 70° C water bath for one hour and then filtered. The flask and the residue on the filter paper were washed first with 95 percent ethanol, then 50 percent ethanol, and finally with tap water. The sample was transferred quantitatively to a separatory funnel with 75 ml. of tap water. The unsaponifiable material was removed by washing twice with petroleum ether. One hundred ml. of water was added to the soap solution before freeing the fatty acids with 2 N hydrochloric acid added in excess. The acidified solution was extracted three times with petroleum ether to remove the free fatty acids. The petroleum ether extracts were dried over anhydrous sodium sulfate, filtered and evaporated in a 60° C water bath. The final traces of petroleum ether were removed by a small stream of nitrogen.

Methylation

The fatty acids were methylated by the methanol-boron-trifluoride method of Metcalfe and Schmitz (21). The methyl esters of the fatty acids were then diluted to a known volume with benzene. A detailed description of the fatty acid analysis is given in the Appendix, vii - xi.

Apparatus

The methyl esters of the fatty acids were separated by a Beckman GC-2A gas chromatograph. A Bristol strip chart recorder, operated at a speed of one-half inch per minute, recorded the response

of the detector. The coiled copper column, 8 feet long and one-fourth inch in diameter, was packed with 15 percent ethylene glycol adipate on chromosorb W, 80/100 mesh.[1] The helium carrier gas was operated in an inlet pressure of 35 PSIG. The column temperature was held at 190° C and the filament current at 300 milliamperes. The sample size injected with a Beckman liquid sampler was 0.01 ml. The peaks on the chromatogram were identified by comparison of retention time of the unknown samples to a known standard[2] separated under the same conditions.

Calculations

Dilutions of a quantitative standard mixture containing methyl myristate (C_{14}), methyl palmitate (C_{16}), methyl palmitoleate ($C_{16:1}$), methyl stearate (C_{18}), and methyl oleate ($C_{18:1}$) were partitioned on the gas chromatograph under the same conditions as were the unknown samples. A polar planimeter was used to measure the area under the curves. Standard curves were prepared, and the amount of each fatty acid ester present in the unknown sample was extrapolated from its respective curve. In the case of methyl linoleate ($C_{18:2}$) and methyl linolenate ($C_{18:3}$) no quantitative standard was available. The quantities of these methyl esters were extrapolated from the oleate standard curve. Methyl heptadonate (C_{17}), the internal standard

[1]Fisher Scientific Company, Silver Spring, Maryland.

[2]Applied Science Laboratories, Inc., State College, Pennsylvania.

was extrapolated from the palmitate and stearate standard curves. At the concentration in which heptadonate was present these two standard curves were identical.

The efficiency of the extraction procedure was determined by the quantitative recovery of heptadecanoic acid added to the sample at the start of the extraction procedure. The values for the fatty acids in the diets given in Table 1 have been corrected for the percentage recovery of heptadecanoic acid.

CHAPTER IV

RESULTS AND DISCUSSION

The length and condition of storage of the lyophilized samples indicated that it is possible that autoxidation of the fatty acids may have occurred. Since unsaturated fatty acids form sparingly soluble polymers on autoxidation, it is possible that an unoxidized fatty acid molecule could be held in such a polymer through the glycerol linkage and thus would not be removed by solvent extraction. Direct saponification would split these linkages and allow quantitative removal of the unoxidized fatty acids (18).

The chromatographic column and operating condition of the gas-liquid chromatograph were selected on the basis that they would separate efficiently the majority of the fatty acids present in the food composites. The methylation procedure and the operating controls used on the gas-liquid chromatograph would permit separation of the fatty acid esters of more than ten carbon atoms, but less than twenty carbon atoms. Limitations imposed on the analysis of the fatty acids of the food samples by the substrate of the chromatographic column are shown in the Appendix, p. xii. This table shows the general effect of a change in molecular structure of the fatty acids on their retention time on a polyester column (17).

The nature of the fatty acid extraction procedure resulted in some loss of the fatty acids. To determine the degree of loss an internal standard was added at the initial step of the extraction procedure. Heptadacanoic acid was selected because its presence was not detected in the food composites and because of the similarity of its chemical and physical properties to the fatty acids being analyzed. The percent recovery of this acid for each food composite sample is shown in Table 1. For the twelve food samples analyzed the average percent recovery of heptadacanoic acid was 89.1 percent.

In the preparation of the fatty acid esters for separation on the gas-liquid chromatograph the methyl esters of the fatty acids for samples of Diets 9 and 10 were diluted with benzene to a final volume of 3 ml. This dilution was equivalent to 6.66 mg. of freeze-dried food composite per 0.01 ml. of solvent. For samples from Diets 11 and 12 the final dilution was 4 ml. This was equivalent to 5.0 mg. of freeze-dried food composite per 0.01 ml. of benzene. Photographs of the fatty acid chromatograms are shown in the Appendix, p. xiii-xvi.

The amounts and kinds of fatty acids found in the food sample composites are shown in Table 1. Other fatty acids were detected in the food samples, but they occurred in amounts too small to be measured. The most abundant fatty acid found in the food samples was oleic acid, present in amounts of approximately 50 percent of the total fatty acids. Linoleic acid, next in abundance, ranged

TABLE 1

The Amount and the Percent of Fatty Acids in the Food Composites for Period V

Acid	Diets 9 and 10 Day 1 gm.	Day 2 gm.	Day 3 gm.	Day 4 gm.	Day 5 gm.	Day 6 gm.
Myristic	.12	.12	.18	.07	.07	.09
Palmitic	4.43	3.90	6.91	3.28	3.62	3.21
Palmitoleic	.22	.15	.23	.18	.18	.21
Stearic	1.82	1.78	2.66	1.46	2.45	1.78
Oleic	12.01	11.49	16.52	9.14	12.53	9.32
Linoleic	5.74	5.94	13.12	6.28	5.19	5.61
Linolenic	.18	.24	.35	-	.23	.53
Total Fatty Acids	24.52	23.62	39.97	20.41	24.27	20.75
% Polyunsaturated	24.14	26.16	33.70	30.77	22.33	29.59
% Unsaturated	74.02	75.44	75.61	76.43	74.70	75.52
% Saturated	25.98	24.56	24.39	23.57	25.30	24.48
P:S Ratio	.93	1.07	1.38	1.31	.88	1.21
% C_{17} Recovered	101.3	86.3	83.3	74.6	79.5	80.3

Acid	Diets 11 and 12 Day 1 gm.	Day 2 gm.	Day 3 gm.	Day 4 gm.	Day 5 gm.	Day 6 gm.
Myristic	.09	.19	.21	.08	.11	.12
Palmitic	5.99	9.17	10.40	6.09	7.28	6.10
Palmitoleic	.42	.42	.38	.35	.46	.29
Stearic	3.34	4.76	4.30	3.11	4.10	3.25
Oleic	22.88	29.64	30.64	20.13	26.10	23.26
Linoleic	10.53	14.19	19.29	12.46	12.21	12.71
Linolenic	.21	.27	.27	-	-	.42
Total Fatty Acids	43.46	58.64	65.49	42.22	50.26	46.15
% Polyunsaturated	24.71	24.66	29.87	29.51	24.29	28.45
% Unsaturated	78.32	75.92	77.23	78.02	77.14	79.48
% Saturated	21.68	24.08	22.77	21.98	22.86	20.52
P:S Ratio	1.14	1.02	1.31	1.34	1.06	1.39
% C_{17} Recovered	92.5	93.5	94.0	91.0	103.0	93.0

from 21.4 percent to 32.8 percent for Diet 9 and 10 and for Diet 11 and 12 the range was 24.2 percent to 29.5 percent.

The amount of fatty acids present in the food composites of Diet 9 and 10 was similar except for Day 3, which was much higher than the other days. The percent of saturated and unsaturated fatty acids was very similar for each of the six days for this diet. The P/S ratio (polyunsaturated:saturated fatty acids) ranged from 1.38 for Day 3 to 0.88 for Day 5. In this study the polyunsaturated fatty acids are defined as those acids having two or more double bonds.

For Diet 11 and 12 the amount of fatty acids found in the food composites ranged from 42 grams for Day 4 to 65 grams for Day 3. The percent of saturated and unsaturated fatty acids was very similar for all six days of this diet. The P/S ratio was highest for Day 6 (1.39) and lowest for Day 2 (1.02).

The average amount of the total calculated fat and the average amount of fatty acids present in the food composites for the six days of Diet 11 and 12 was twice that of Diet 9 and 10 as shown in Table 2. Comparing the averages of Diet 9 and 10 to Diet 11 and 12 the percent of saturated, unsaturated, and polyunsaturated fatty acids are nearly identical (Table 2). The average of the P/S ratio is similar for the two diets. The P/S ratio found for Diet 9 and 10 was 1.13 and for 11 and 12, 1.21.

TABLE 2

The Average Values of the Analyzed Fatty Acids and Calculated Fat for The Food Composites from The Two Diets

	Calculated	Analyzed				
	Fat gm.	Fatty Acids gm.	Saturated %	Unsaturated %	* Polyunsaturated %	P/S ratio
Diet 9 - 10	30.9	25.6	24.71	75.29	27.78	1.13
Diet 11 - 12	60.6	51.0	22.32	77.69	26.92	1.21

* Polyunsaturated are the fatty acids having two or more double bonds.

TABLE 3

Comparison of the Fatty Acid Distribution in Various Dietaries

	Saturated %	Unsaturated %	Polyunsaturated %	P/S ratio
This study	23.5	76.5	27.4	1.17
Hardinge et al.(22)				
vegetarian	20.1	79.9	24.3	1.30
lacto-ovo-vegetarian	36.1	64.0	14.5	.41
non-vegetarian	41.0	59.0	12.1	.30
Osborn and Ohlson (23) (Mixed diet)	46.1	53.9	9.6	.20

Experiments have demonstrated repeatedly that a dietary substitution of natural fats rich in polyunsaturated fatty acids for those rich in saturated fats will result in a decrease in serum cholesterol levels in a majority of subjects (3). Harding et al. (22) studied the fatty acids in diets of pure vegetarians, lacto-ovo-vegetarians and non-vegetarians in relationship to serum cholesterol levels. They demonstrated that strict vegetarians have lower cholesterol levels than the general population who eat a diet of animal and vegetable foods. These studies showed in the older age group a highly significant inverse relationship between the ratio of dietary polyunsaturated: saturated fatty acids and serum cholesterol. In the adolescent group serum cholesterol levels did not differ significantly between the vegetarians and non-vegetarians.

It is of interest to compare the fatty acid distribution in these diets with findings from other studies (Table 3). Hardinge, et al. (22) showed a slight difference in the P/S ratio of the non-vegetarians' diets (0.30) and lacto-ovo-vegetarians' diets (0.41), and a high P/S ratio for the pure vegetarians (1.30). The P/S ratio of the "all" vegetable diet fed the preadolescent children in this experiment compares favorably with the P/S ratio of the fatty acids of the pure vegetarians' diet in the study reported by Hardinge et al. Osborn and Ohlson (23) studied the pattern of fatty acids in meals served in the staff dining room of a hospital during eighty-four days

The meals were considered to represent the usual diet eaten by a group of healthy individuals. They found these meals to be high in saturated fatty acids and low in polyunsaturated fatty acids with a P/S ratio of 0.20. The P/S ratio would be expected to be low for these diets, since the menu contained a liberal amount of animal food, high in saturated fats.

CHAPTER V

SUMMARY AND CONCLUSION

The Southern Regional Nutrition Research Project S-28 has as its objective the study of metabolic patterns in preadolescent children. In the summer of 1962, twelve preadolescent girls were housed and cared for in one of the women's residences at the Virginia Polytechnic Institute. During a period of almost seven weeks the subjects consumed controlled diets; excreta were collected and blood samples were obtained for future analysis. The experimental diets were designed to include only foods from plant sources. This part of the metabolic study was planned to determine the amount and distribution of the fatty acids in those diets (20).

Methods and procedures were developed for extracting fatty acids from a lyophilized food composite composed of plant products only. The lipids were extracted from the samples and saponified by the "cold" alcohol-potassium hydroxide method. Methyl esters of the extracted fatty acids were prepared and separated by gas-liquid chromatography. Qualitative, quantitative, and internal standards were used to identify and determine the amount of fatty acids present in the food composites.

The seven fatty acids measured were myristic, palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic. The total amount

of fatty acids in the food composites for Diet 11 and 12 (average 51.0 gm.) was twice that of Diet 9 and 10 (average 25.6 gm). Oleic acid was the most abundant fatty acid accounting for nearly fifty percent of the total fatty acids for the food composites. Linoleic acid was the next most plentiful fatty acid present in the diets. Only very small amounts of myristic, palmitoleic and linolenic acids were present in these diets.

The fatty acid pattern was similar for each of the six days of Diet 9 and 10 and for each of the six days of Diet 11 and 12. The average values for the distribution of fatty acids for Diet 9 and 10 were almost identical to those of Diet 11 and 12. The P/S ratio ranged from 0.88 to 1.39 for the twelve composites.

Low values for stearic acid and high amounts of linoleic acid in these diets were the major factors contributing to an average P/S ratio of 1.17, extremely high in comparison to that of 0.20, found by Osborn and Ohlson (23) for a mixed diet considered representative of American eating habits.

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VITA

Jeanette Colona Gorman was born in Prince George County, Virginia on June 24, 1931. Her earlier education was in the public schools of Hopewell, Virginia. In 1949 she entered Radford College, Radford, Virginia, and completed her first two years of college on this campus before transferring to Virginia Polytechnic Institute, Blacksburg, Virginia. She graduated from V.P.I. in 1953 with a Bachelor of Science degree in Home Economics. The same year she married Joseph Vincent Gorman, Jr. During the following years she was a homemaker and laboratory technician. In 1962 she entered graduate school at V.P.I.

APPENDIX

MENU - DIETS 11 AND 12 - PERIODS IV - VII

Day 1

Breakfast: gm.

Orange Juice- - - - - - - - -100
Bread - - - - - - - - - - - - 40
Margarine - - - - - - - - - - 10
Apple Butter- - - - - - - - - 20
Applesauce Cupcake- - - - - - 30

A. M.

Lemonade- - - - - - - - - - -120
Cashew Nuts - - - - - - - - - 20

Lunch:

Jelly - - - - - - - - - - - - 20
Bread - - - - - - - - - - - - 40
Margarine - - - - - - - - - - 10
50% Fat Peanut Butter - - - - 20
Canned Apricots - - - - - - - 80
Apricots Juice- - - - - - - - 20
Pineapple - - - - - - - - - -100
Raw Apple Wedges- - - - - - -100
*Choc. Peanut Butter Krisp - - 15

P. M.

Apple Juice - - - - - - - - -100
Saltines- - - - - - - - - - - 20
Orange Ice- - - - - - - - - - 75
Ground Almonds- - - - - - - - 10

Supper:

Heinz Baked Beans - - - - - - 70
Green Beans - - - - - - - - - 50
Margarine - - - - - - - - - - 15
Orange Ice- - - - - - - - - - 75
Peanut Butter Fondant - - - - 20
Grape Juice - - - - - - - - -100
Applesauce Cupcake- - - - - - 30
*Choc. Peanut Butter Krisp - - 15

* Made with 50 percent fat peanut butter.

Day 2

Breakfast: gm.

Apple Juice- - - - - - - - - - - 100
Bread - - - - - - - - - - - - - - 40
Margarine - - - - - - - - - - - - 5
Apple Butter- - - - - - - - - - - 20
Applesauce Cupcake- - - - - - - - 30

A. M.

Cashew Nuts - - - - - - - - - - - 30
Apple Juice - - - - - - - - - - -100

Lunch:

Soy Tomato Soup - - - - - - - - -100
Celery Sticks - - - - - - - - - - 10
Pineapple Chunks- - - - - - - - - 40
Pineapple Juice - - - - - - - - - 10
Cherry Tapicoa- - - - - - - - - -150
Graham Crackers - - - - - - - - - 20
Ground Almonds- - - - - - - - - - 10
*Choc. Peanut Butter Krisp - - - - 30

P. M.

50% Fat Peanut Butter - - - - - - 10
Jelly - - - - - - - - - - - - - - 20
Bread - - - - - - - - - - - - - - 40
Margarine - - - - - - - - - - - - 10
Grape Juice - - - - - - - - - - -150
Ground Almonds- - - - - - - - - - 10

Supper:

Meatless "Meat Loaf"- - - - - - - - 50
Boiled Potato - - - - - - - - - - - 65
Margarine - - - - - - - - - - - - - 10
Canned Green Asparagus- - - - - - - 50
Raspberry Ice - - - - - - - - - - - 75
†Choc. Peanut Butter Krisp - - - - 20
Potato Chips- - - - - - - - - - - - 20
Plain Fondant - - - - - - - - - - - 20

† Made with ordinary peanut butter.

MENU - DIETS 11 AND 12 - PERIODS IV - VII

Day 3

Breakfast:	gm.
Pineapple Juice	70
Orange Juice	50
Bread	40
Margarine	5
Jelly	20
Applesauce Cupcake	30
A. M.	
Limeade	100
Banana	100
Lunch:	
Jelly	20
Bread	40
Margarine	10
50% Fat Peanut Butter	20
Canned Pears	40
Pear Juice	10
Applesauce Cupcake	30
Apple Juice	100
Cashew Nuts	20
Carrot Sticks	5
Choc. Peanut Butter Krisp	30
Ground Almonds	
P. M.	
Orange Ice	75
Graham Crackers	20
Supper:	
Kidney Bean Loaf	50
Ground Brazil Nuts	10
Heinz Spanish Rice	50
Margarine	15
Raw Cabbage	40
Raw Carrots, grated	10
Salad Dressing	12
Peanut Butter Fondant	20
Ground Almonds	10
Pineapple Juice	100

Day 4

Breakfast:	gm.
Pineapple Juice	70
Orange Juice	65
Bread	40
Margarine	5
Raspberry Jelly	20
Cashew Nuts	10
A. M.	
Lemonade	140
Banana	100
Lunch:	
Heinz Veg. Soup (condensed)	100
Sliced Tomato	40
Ground Almonds	10
Saltines	20
Raspberry Ice	75
*Choc. Peanut Butter Krisp	15
Plain Fondant	20
P. M.	
Jelly	20
Bread	40
Margarine	10
50% Fat Peanut Butter	20
Apple Juice	100
Supper:	
Lentil Patty	50
Brazil Nuts	20
Boiled Potato	65
Green Beans	40
Margarine	10
Apple sauce	100
Peanut Butter Fondant	20
Grape Juice	100
Ground Almonds	10
*Choc. Peanut Butter Krisp	15

*Made with 50 percent peanut butter.

MENU - DIETS 11 AND 12 - PERIODS IV - VII

Day 5

Breakfast:	gm.
Orange Juice	100
Bread	40
Strawberry Jelly	20
Applesauce Cupcake	30
A. M.	
Limeade	100
Cashew Nuts	30
Lunch:	
Jelly	20
Bread	40
Margarine	10
50% Fat Peanut Butter	20
Canned Peaches	60
Peach Juice	10
Apple Juice	100
Peanut Butter Fondant	20
Raw Apple Wedges	50
*Choc. Peanut Butter Krisp	30
Ground Almonds	10
P. M.	
Saltines	20
Raw Apple Wedges	50
Grape Juice	120
Supper:	
Spaghetti w/tomato Sauce	50
Boiled Potato	50
Green Lima Beans	50
Margarine	10
Orange Ice	75
†Choc. Peanut Butter Krisp	20
Ground Almonds	10
Lemonade	170
Plain Fondant	20

†Made with ordinary peanut butter.

Day 6

Breakfast:	gm.
Orange Juice	120
Bread	40
Margarine	5
Currant Jelly	20
Applesauce Cupcake	30
A. M.	
Raw Apple Wedges	50
Ground Almonds	10
Plain Fondant	10
Lunch:	
Corn Soy Soup	150
Canned Pears	80
Pear Juice	20
Ground Almonds	10
Saltines	20
Jelly	20
Bread	40
Margarine	10
50% Fat Peanut Butter	20
*Choc. Peanut Butter Krisp	30
P. M.	
Raw Apple Wedges	50
Applesauce	30
Supper:	
Meatless "Meat Loaf"	50
Catsup	10
Sweet Potato, boiled	60
Margarine	15
Sliced Tomato	40
Lime Ice	75
Prune Juice	100
Peanut Butter Fondant	20
Ground Almonds	10
Plain Fondant	10

*Made with 50 percent peanut butter.

MENU - DIETS 9 AND 10 - PERIODS IV - VII

Day 1

Breakfast:	gm.
Orange Juice	100
Bread	40
Margarine	10
Apple Butter	30
Applesauce Cupcake	30
A. M.	
Lemonade	120
Cashew Nuts	10
Plain Fondant	20
Lunch:	
Bread	40
Margarine	20
Strawberry Jelly	30
Canned Apricots	80
Apricot Juice	20
Plain Fondant	30
Pineapple Juice	100
Raw Apple Wedges	100
P. M.	
Apple Juice	100
Cashew Nuts	10
Orange Ice	75
Supper:	
Heinz Baked Beans	70
Green Beans	50
Margarine	15
Orange Ice	75
Grape Juice	150
Potato Chips	30
Applesauce Cupcake	30
Jelly	10

Day 2

Breakfast:	gm.
Apple Juice	100
Bread	40
Margarine	15
Apple Butter	30
Applesauce Cupcake	30
A. M.	
Lemonade	150
Cashew Nuts	10
Plain Fondant	20
Lunch:	
Soy Tomato Soup	100
Celery Sticks	10
Pineapple Chunks	40
Pineapple Juice	10
Cherry Tapioca	150
Apple Juice	100
P. M.	
Bread	40
Margarine	20
Raspberry Jelly	30
Raspberry Ice	75
Supper:	
Meatless "Meat Loaf"	50
Boiled Potato	65
Margarine	15
Canned Green Asparagus	50
Raspberry Ice	75
Plain Fondant	30
Grape Juice	150
Potato Chips	20

MENU - DIETS 9 AND 10 - PERIODS IV - VII

Day 3

Breakfast:	gm.
Pineapple Juice	70
Orange Juice	50
Bread	40
Margarine	10
Apple Jelly	20
Applesauce Cupcake	30
A. M.	
Limeade	-100
Banana	-100
Lunch:	
Jelly	20
Bread	40
Margarine	20
Canned Pears	80
Pear Juice	20
Applesauce Cupcake	30
Cashew Nuts	20
Carrot Sticks	5
Apple Juice	-100
Plain Fondant	20
P. M.	
Orange Ice	75
Graham Crackers	20
Grape Juice	-100
Jelly	20
Supper:	
Kidney Bean Loaf	50
Brazil Nuts	10
Heinz Spanish Rice	50
Margarine	15
Raw Cabbage	40
Raw Carrots, grated	10
Salad Dressing	12
Plain Fondant	30
Pineapple Juice	-100
Orange Ice	75

Day 4

Breakfast:	gm.
Pineapple Juice	70
Orange Juice	65
Bread	40
Margarine	10
Raspberry Jelly	30
Cashew Nuts	10
A. M.	
Lemonade	-140
Banana	-100
Lunch:	
Heinz Veg. Soup (condensed)	-100
Sliced Tomato	40
Pineapple Tapioca	-100
Raspberry Ice	75
Plain Fondant	30
P. M.	
Jelly	20
Bread	40
Margarine	20
Apple Juice	200
Supper:	
Lentil Patty	50
Brazil Nuts (ground)	20
Boiled Potato	65
Green Beans	40
Margarine	20
Applesauce	-100
Plain Fondant	20
Grape Juice	-100
Raspberry Ice	75

MENU - DIETS 9 AND 10 - PERIODS IV - VII

Day 5

Breakfast:	gm.
Orange Juice	100
Bread	40
Margarine	15
Strawberry Jelly	30
Applesauce Cupcake	30
A. M.	
Limeade	100
Cashew Nuts	30
Lunch:	
Bread	40
Margarine	20
Jelly	30
Canned Peaches	60
Peach Juice	10
Apple Juice	100
Raw Apple Wedges	50
Plain Fondant	30
P. M.	
Raw Apple Wedges	50
Grape Juice	120
Orange Ice	75
Supper:	
Spaghetti w/Tomato Sauce	50
Boiled Potato	50
Green Lima Beans	50
Margarine	20
†Choc. Peanut Butter Krisp	20
Lemonade	170
Orange Ice	75

Day 6

Breakfast:	gm.
Orange Juice	120
Bread	40
Margarine	10
Currant Jelly	30
Applesauce Cupcake	30
A. M.	
Raw Apple Wedges	50
Dates	25
Plain Fondant	30
Lunch:	
Margarine	20
Corn Soy Soup	150
Dates	25
Canned Pears	80
Pear Juice	20
Plain Fondant	20
Bread	40
Raspberry Jelly	30
P. M.	
Raw Apple Wedges	50
Grape Juice	150
Lime Ice	75
Supper:	
Meatless "Meat Loaf"	50
Catsup	10
Sweet Potato, boiled	60
Margarine	15
Sliced Tomato	40
Lime Ice	75
Prune Juice	100
Applesauce Cupcake	30

Detailed Procedures Used for the Determination of Fatty Acids in a Lyophilized Plant Food Composite

A. Preparation of heptadcanoic acid standard

1. Add 100 mg. of heptadecanoic acid to a 250 ml. volumetric flask and dilute to volume with 95 percent ethyl alcohol.

B. Saponification and extraction of fatty acids

1. Add the following to a 125 ml. Erlenmeyer flask:
   a. 2.0000 gm. of freeze-dried sample.
   b. 20 ml. of the heptadecanoic acid standard (8 mg/20 ml.).
   c. 10 ml. of freshly prepared potassium hydroxide-ethanol solution, (5 gm. of KOH dissolved in 10 ml. of 70 percent ethanol).
   d. 20 ml. of 95 percent ethanol.
2. Stopper and shake flask gently.
3. Let solution stand in dark for 3 days. Shake occasionally during this period.

C. Filtration and washing

1. At the end of the three-day period remove stopper and heat sample flask in 70° C water bath for one hour.
2. Filter sample through Whatman No. 1 filter paper into a vacuum flask.
3. Wash Erlenmeyer flask and residue on the filter paper with the following solutions, in order:
   a. 20 ml. of warm 95 percent ethanol.
   b. 30 ml. of warm 50 percent ethanol.

c. 20 ml. of tap water (warm).

(a slight vacuum is necessary to complete the filtration).

D. Removal of unsaponifiable material

1. With 75 ml. of tap water transfer quantitatively the solution in the vacuum flask to a 500 ml. separatory funnel.
2. Add approximately 60 ml. petroleum ether. Stopper and shake vigorously.
3. Let stand for approximately three hours for separation of the solvent layers.
4. Drain aqueous layer (bottom layer) to another separatory funnel. Discard petroleum ether layer (top layer).
5. To the separatory funnel containing the aqueous layer add approximately 60 ml. of petroleum ether. Shake vigorously and let stand for approximately two hours.
6. Repeat step D-4.

E. Freeing the fatty acids

1. Add 100 ml. of distilled water to the separatory funnel containing the aqueous layer.
2. Add 2 N HCl (approximately 42 ml.) until the solution is acid. Add HCl in slight excess. Test for acidity with litmus paper.
3. Shake and let stand for approximately 15 minutes.

F. Extracting the fatty acids

1. Add 60 ml. of petroleum ether to the acid solution and shake vigorously. Let stand overnight for the separation of the two layers.
2. Drain the aqueous layer into another 500 ml. separatory funnel.
3. Drain the petroleum ether layer into a 125 ml. Erlenmeyer flask that contains approximately 5 gm. of anhydrous sodium sulfate. Mix and let stand for 10 minutes or longer. Then filter through Whatman No. 1 filter paper into a 50 ml. Erlenmeyer flask in a 60° C water bath. Evaporate the petroleum ether to about 10 ml.
4. Wash separatory funnel (just emptied) with 60 ml. of petroleum ether, and add this washing to the separatory funnel containing the aqueous solution. Shake and let stand for approximately two hours.
5. Repeat step F-2.
6. Repeat step F-3, but add 5 more grams of sodium sulfate.
7. Repeat step F-4.
8. Discard aqueous layer.
9. Repeat step F-2, but once again add 5 more grams of sodium sulfate.
10. Wash separatory funnel (just emptied), the 125 ml. flask containing the sodium sulfate, and the filter paper with 30 ml. or more of petroleum ether.

11. In the 60° C water bath evaporate the petroleum ether to about 20 ml. Stopper and store in refrigerator overnight.

G. Methylation of the long chain fatty acids.

1. Evaporate the petroleum ether on a 60° C water bath, and remove the last traces of petroleum ether with a slight stream of nitrogen gas.
2. Add 3 to 5 ml of boron trifluoride solution to the flask containing the fatty acids, and boil for two minutes on a boiling water bath.
3. Transfer quantitatively to a 125 ml. separatory funnel with 20 ml. of distilled water and 30 ml. of petroleum ether. Shake vigorously and let stand for about 20 minutes for the two layers to separate.
4. Discard aqueous layer (bottom layer).
5. Add about 3 gms. of anhydrous sodium sulfate to Whatman No. 1 filter paper and filter the petroleum through this into a 20 ml. beaker in a 60° C water bath.
6. Wash the separatory funnel and the filter paper (containing the sodium sulfate) with 20 ml. or more of petroleum ether. Evaporate the petroleum ether, removing final traces with a slight stream of nitrogen.
7. Transfer the methyl esters of the fatty acids quantitatively with benzene to a volumetric container and dilute to volume with benzene.

8. Stopper sample and store in refrigerator until ready to separate components on the gas chromatograph.

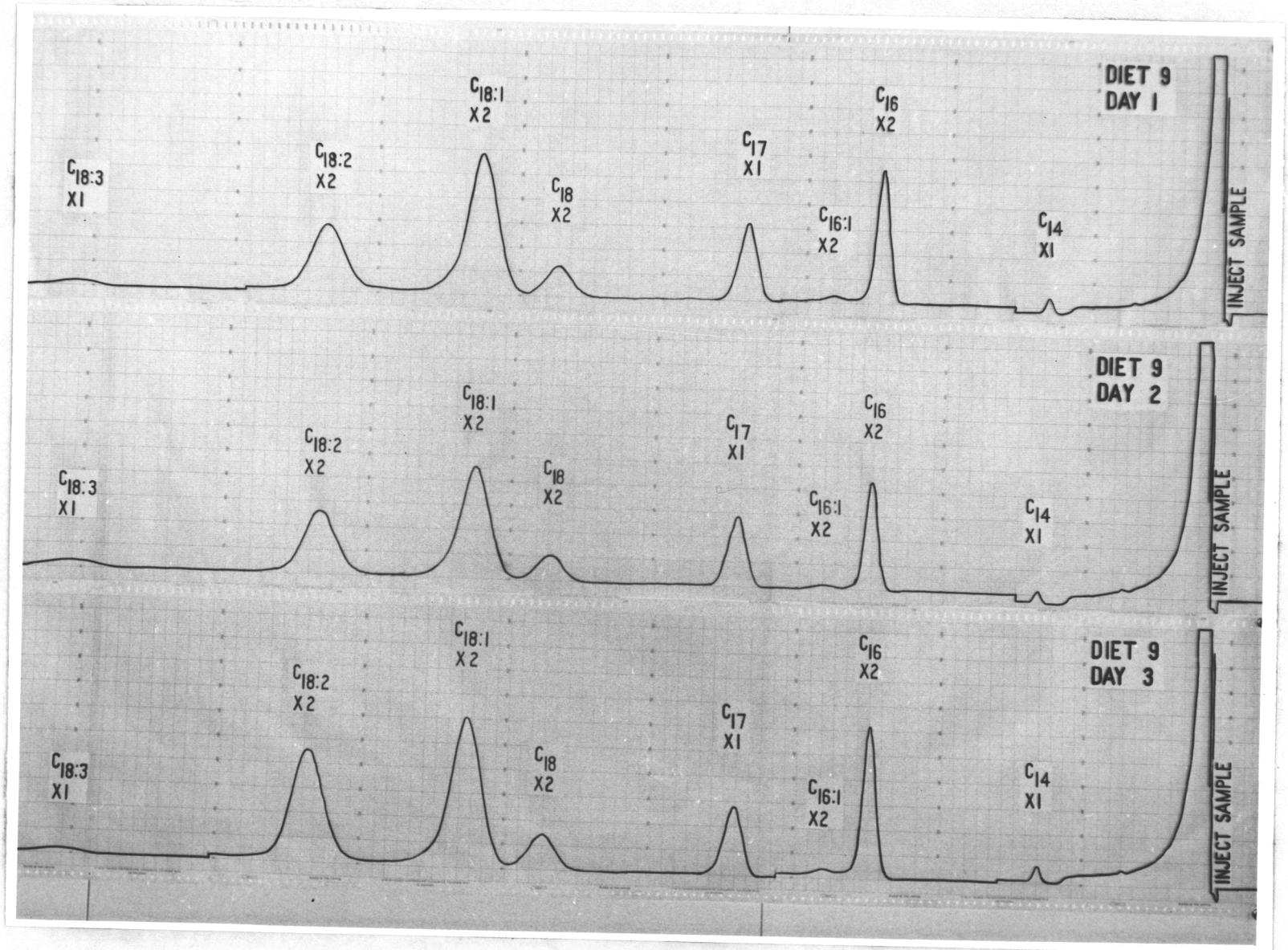
Notes:

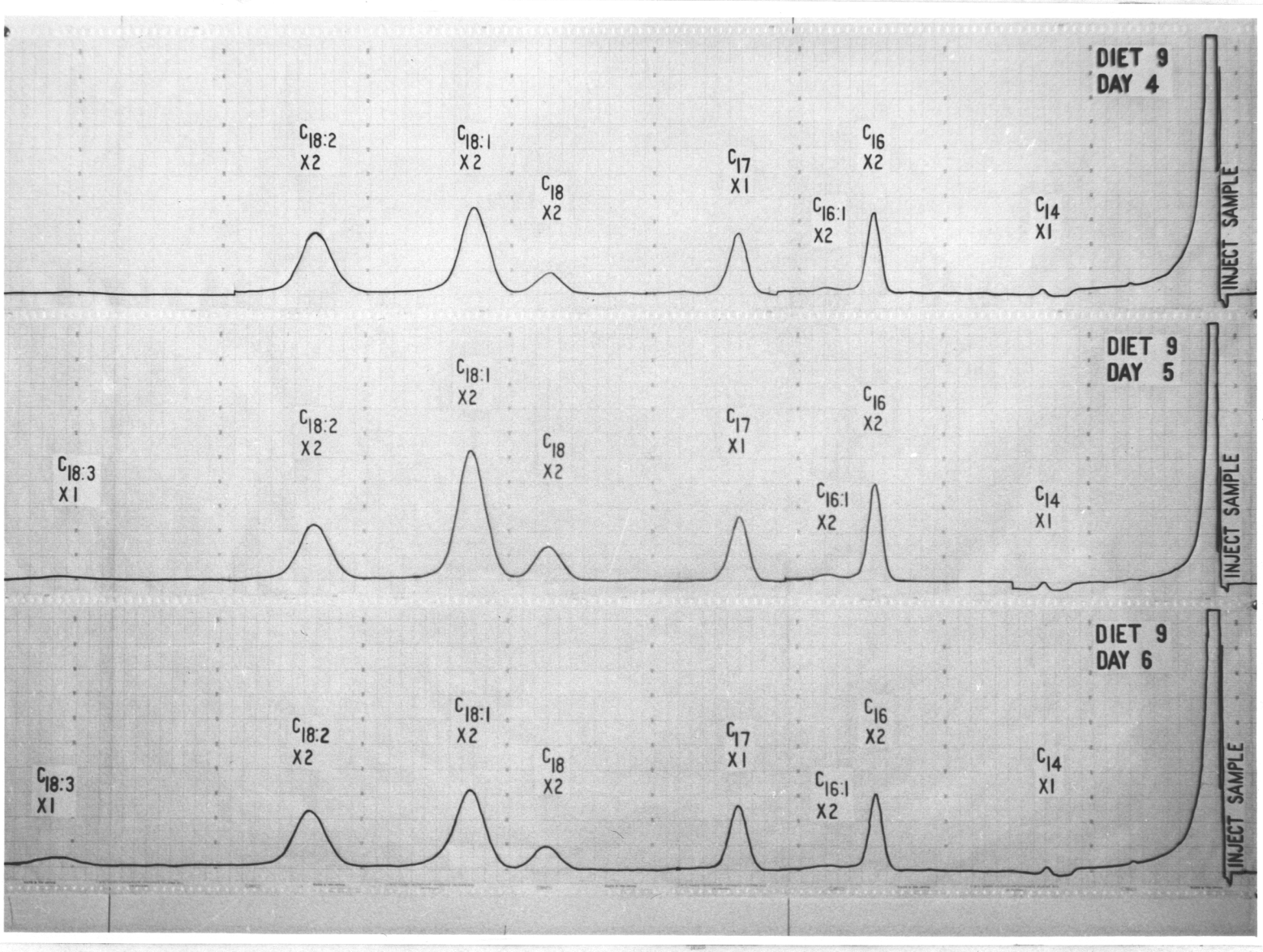
1. Redistilled petroleum ether should be used.
2. General precautions for handling and storing fatty acids and esters of fatty acids: All flasks and other types of containers should be sealed or closed with glass stoppers, plastic caps, plastic liners and aluminum foil liners. Cork or rubber stoppers should be avoided, since impurities are extracted by solvent vapor and contaminate the sample.

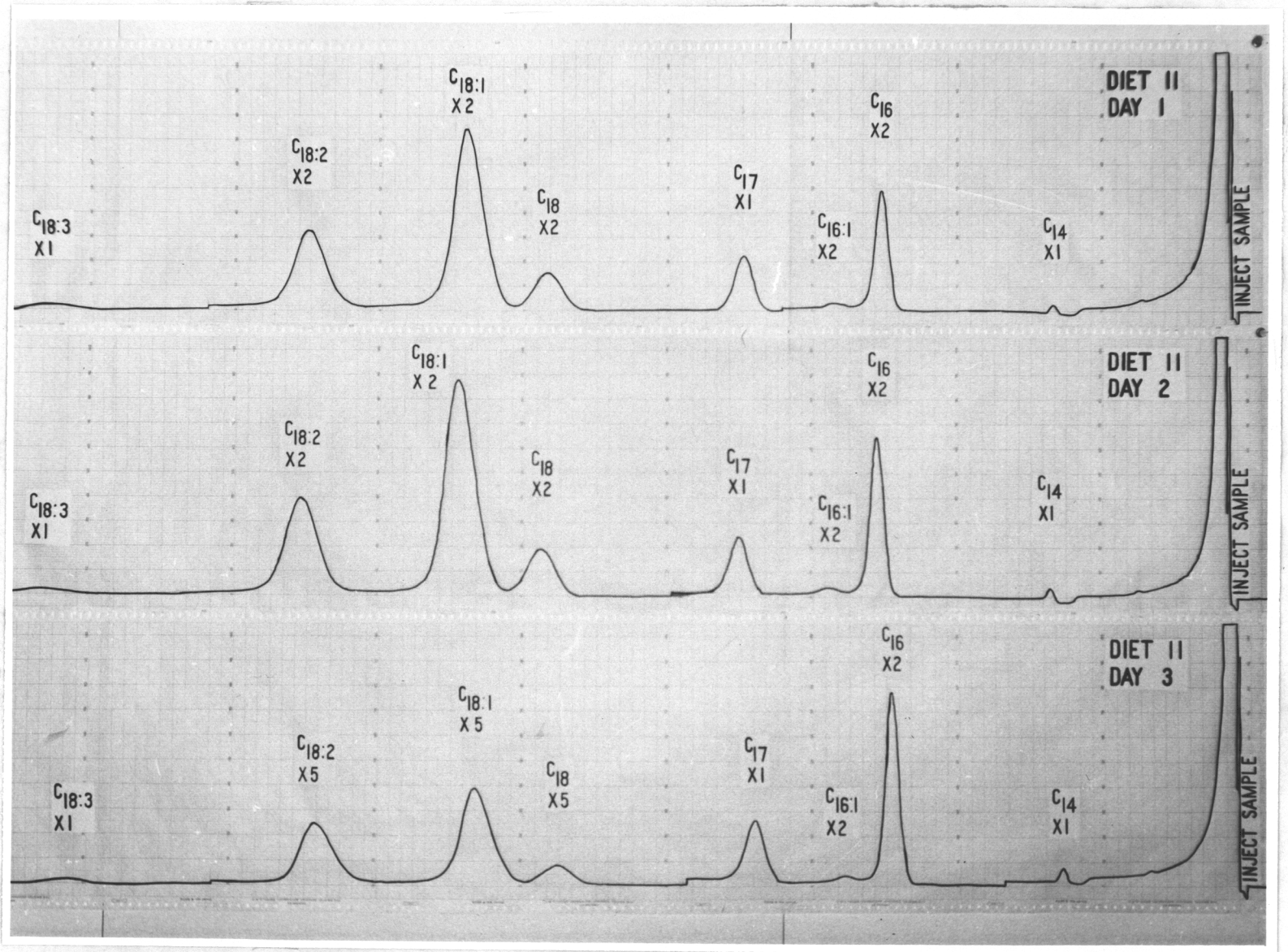
   To prevent oxidation, especially of the polyunsaturated fatty acids, the lipids, fatty acids, and fatty acid esters should not be exposed to laboratory air for more than a few minutes. They should never be stored solvent free except in sealed ampoules containing an atmosphere of nitrogen. They can be stored safely for months in the dark at low concentration in any petroleum ether at 4° C or lower.

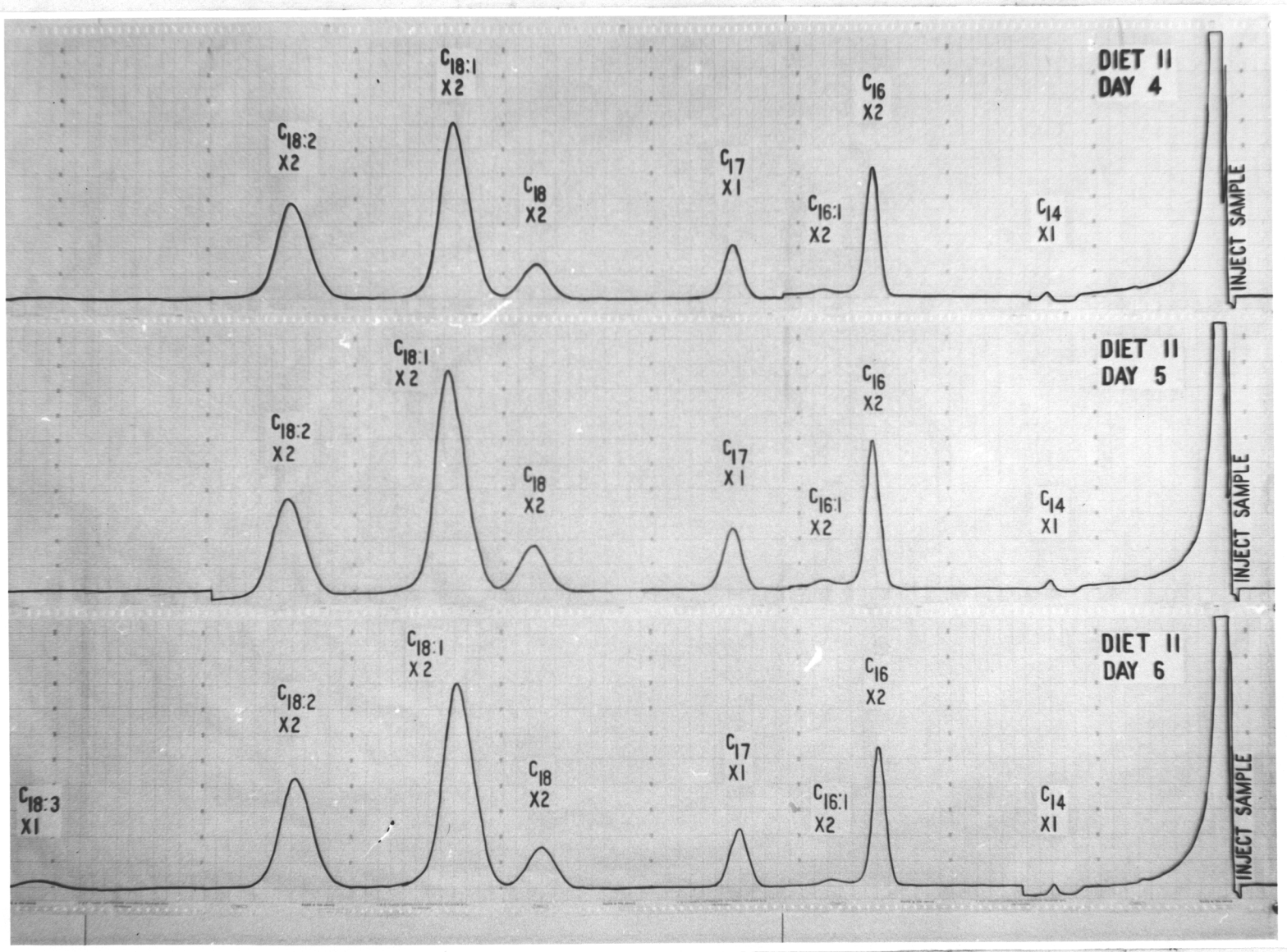
The general effect of a change in molecular structure of the fatty acids on retention volume on polyester columns at 180° C.

Structural Change	Change in Retention Volume
Increase in chain length by one $-CH_2-$ group	Increase in retention time by a factor of 1.46
Introduction of a single methyl side chain	Substance moves ahead of corresponding straight chain by a factor of 0.9.
Introduction of one double bond at 9,10-position	Substance moves slower than corresponding saturated acid by a factor of 1.15 - 1.2
Change in configuration of double bond from cis to trans	No effect (cis and trans move together)
Change in position of double bond	No effect (isomers move together)
Introduction of two double bonds	Substance moves slower than the corresponding monounsaturated acid by a factor of 1.22
Changes in configuration about the double bonds in dienes	Not known
Conjugation of double bonds in dienes	Not known
Introduction of three double bonds	Substance moves slower than the corresponding diene by a factor of 1.3
Introduction of four double bonds	Substance moves slower than the corresponding saturated acid by a factor of 1.7
Introduction of five double bonds	Slower and well separated from the corresponding tetraene
Introduction of six double bonds	Slower and well separate from the corresponding pentaene


DIET 9
DAY 1
C18:3
X1
C18:2
X2
C18:1
X2
C18
X2
C17
X1
C16:1
X2
C16
X2
C14
X1
INJECT SAMPLE
DIET 9
DAY 2
C18:3
X1
C18:2
X2
C18:1
X2
C18
X2
C17
X1
C16:1
X2
C16
X2
C14
X1
INJECT SAMPLE
DIET 9
DAY 3
C18:3
X1
C18:2
X2
C18:1
X2
C18
X2
C17
X1
C16:1
X2
C16
X2
C14
X1
INJECT SAMPLE


DIET 9
DAY 4
C18:2
X2
C18:1
X2
C18
X2
C17
X1
C16:1
X2
C16
X2
C14
X1
INJECT SAMPLE
DIET 9
DAY 5
C18:3
X1
C18:2
X2
C18:1
X2
C18
X2
C17
X1
C16:1
X2
C16
X2
C14
X1
INJECT SAMPLE
DIET 9
DAY 6
C18:3
X1
C18:2
X2
C18:1
X2
C18
X2
C17
X1
C16:1
X2
C16
X2
C14
X1
INJECT SAMPLE


DIET 11
DAY 1
C18:3 X1
C18:2 X2
C18:1 X2
C18 X2
C17 X1
C16:1 X2
C16 X2
C14 X1
INJECT SAMPLE
DIET 11
DAY 2
C18:3 X1
C18:2 X2
C18:1 X2
C18 X2
C17 X1
C16:1 X2
C16 X2
C14 X1
INJECT SAMPLE
DIET 11
DAY 3
C18:3 X1
C18:2 X5
C18:1 X5
C18 X5
C17 X1
C16:1 X2
C16 X2
C14 X1
INJECT SAMPLE


DIET 11
DAY 4
C18:2 X2
C18:1 X2
C18 X2
C17 X1
C16:1 X2
C16 X2
C14 X1
INJECT SAMPLE
DIET 11
DAY 5
C18:2 X2
C18:1 X2
C18 X2
C17 X1
C16:1 X2
C16 X2
C14 X1
INJECT SAMPLE
DIET 11
DAY 6
C18:3 X1
C18:2 X2
C18:1 X2
C18 X2
C17 X1
C16:1 X2
C16 X2
C14 X1
INJECT SAMPLE

ABSTRACT

The Southern Regional Nutrition Research Project Number S-28 has as its objective the study of metabolic patterns in preadolescent children. In the summer of 1962, twelve preadolescent girls were housed and cared for in one of the women's residences at the Virginia Polytechnic Institute. During a period of almost seven weeks the subjects consumed controlled diets; excreta were collected and blood samples were obtained for future analysis. The experimental diets were designed to include only foods from plant sources. This part of the metabolic study was planned to determine the amount and distribution of the fatty acids in those diets.

Methods and procedures were developed for extracting the fatty acids from the lyophilized food composite composed of plant foods only. The fatty acids were extracted and methylated. Their methyl esters were separated and determined quantitatively by gas-liquid chromatography. The total amount of fatty acids in Diet 11 and 12 was twice that of Diet 9 and 10. The most abundant fatty acid was oleic, accounting for nearly 50 percent of the total fatty acids in the food composites. Linoleic acid was the next most abundant fatty acid present in the diets. The average values for the six days of the two diets had almost identical fatty acid patterns. As would be expected, the ratio of polyunsaturated to saturated fatty acids was very high for these two diets (1.17) as compared to ratios found for representative American diets of plant and animal foods (0.20 - 0.41).