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Determination of Animal Fat in Margarine by High Pressure Liquid Chromatography of the Sterols

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DETERMINATION OF ANIMAL FAT IN MARGARINE BY HIGH PRESSURE LIQUID CHROMATOGRAPHY OF THE STEROLS

BY

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ABSTRACT

The utility of high pressure liquid chromatography as a routine method to detect animal fats in vegetable margarines on the basis of sterol content was investigated. Cholesterol, which makes up almost 100% of the total sterol in animal fats, constitutes only 0-4% of the total sterols in vegetable oils.

The major phytosterols in vegetable oils currently used for margarine production are B-sitosterol, stigmasterol and campesterol. Brassicasterol is less common but in rapeseed oil it is present in high proportion. The minor phytosterols are: Δ^5-avenasterol, Δ^7-stigmasterol, Δ^7-avenasterol, 24-methyl cholest-7-enol and cholesterol.

A uBondapak C_{18} column and a variable wavelength ultraviolet detector along with a variety of mobile phases, have been used throughout this investigation. A method was developed for isolating, extracting and derivatizing sterols from biological material. Optimum chromatographic conditions are described. Lard, soy bean, corn, cottonseed, safflower, sunflower, olive, walnut and rapeseed oils were analyzed. A good separation was obtained for free sterols from vegetable oils. Free cholesterol could be separated from the major phytosterols but not from the whole group and in a

blend of 25% lard and 75% corn oil, cholesterol did not show up clearly.

With benzoate derivatives a good separation was obtained for cholesterol from major phytosterols, but brassicasterol from rapeseed oil and some minor phytosterols from other oils interfered with the cholesterol peak. Because of this interference, the detectable ratio of animal fat in vegetable margarine is dependent on the raw material. Fifty percent of animal fat can be easily detected in all kinds of margarines. In soy bean oil margarine, 25% animal fat can be easily detected. The method is reproducible, rapid, and worth further investigation to find a column and solvent system capable of separating cholesterol completely from all phytosterols, so that very low levels of animal fat could be detected in vegetable oil.

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INTRODUCTION

Ghee is the name of butter fat obtained from cow's or sheep's milk, to which some flavors are added. That was the multipurpose butter in our country (Saudi Arabia). Then, the margarine expansion overwhelmed our market and consumers preferred the margarine for its convenience. Most of the imported margarine was labeled as "Substitute Ghee." Some labels specified that it had a vegetable and/or animal origin and under the name of "Substitute Ghee" a lot of animal fat was dumped on the country, especially when the prices of vegetable oils were high. To prevent such commercial fraud, to avoid the health problems of cholesterol, and due to the fact that lard is not allowed to enter the country on religious grounds, the Saudi Arabian government has prohibited the import of dripping fat and permits only margarine of vegetable origin. Usually, the margarine manufacturer has pre-set criteria for the finished fats and oils he uses (Anderson and Williams, 1969 and Massiello, 1978), and different formulas geared to the cost of the oils which are the major ingredient cost in margarine. Prices of oils and fats change a good deal, like all commodity prices. The premium or special margarines are not subject to such adjustments. The table following shows the fluctuations in lard use as an ingredient of margarine (Anderson and Williams, 1969).

Fats and Oils Used in Margarine. Million Pounds

Year	Vegetable Oils						Animal Fats		
	Soya	Corn	Cotton	Saf-flower	Peanut	Other	Lard	Beef	Total
1964	1139	150	101	12	10	11	64	13	1500
1965	1112	161	114	10	4	19	100	14	1535
1968	1240	179	70	42	4	17	153	15	1720

Source: U.S. Dept. of Agriculture (Riepma, 1970).

Due to the fact that every imported margarine must be checked for the presence of animal fats, our Food Quality Control Laboratory is in need of a quick routine method for the detection of animal fat in vegetable margarine.

Measuring cholesterol, a sterol formed predominantly in animals, is one of the most frequently performed assays in the laboratory, for which a wide variety of methods are available and used. The classical method is colorimetric assay based on the photometric measurement of the color formed when cholesterol reacts with Lewis acid (Abell et al., 1958). However, because of the hazards associated with using the strong acid medium in which the color is formed, alternate methodologies have been and are being developed. Most of the newer methods are based on enzymatic hydrolysis and oxidation (Gray et al., 1977 and Anonymous, 1979), or on chromatographic analysis (Driscoll et al., 1971). The enzymatic reactions are followed by colorimetric or electrochemical analysis. By gas chromatography alone, the determination of steroids in biological samples cannot usually be achieved. It is necessary to employ other procedures to separate crude

fractions and isolate sterols by preparative methods. The isolation of such sterols from a biological material is commonly established by fractionation of lipid extracts by column chromatography or thin layer chromatography (TLC) and in many cases by silver nitrate-silica gel TLC of either the free sterols or their acetates (Rees, 1976). Although liquid chromatography (LC) has been used in analysis of lipids in general (Aitzetmuller, 1975), its specific application in cholesterol methodology has been limited because cholesterol and related compounds absorb very little UV radiation in the relatively high wave length in which most UV detectors used in LC operate. Consequently, LC has been used in assays in which column chromatography is followed by chemical analysis of collected chromatographic fractions (Shin, 1963) or in which the eluate is monitored on-line with other types of detectors. These detectors include the refractive index detector (Werthessen et al., 1970), the moving-wire flame ionization detector (Worth and MacLeod, 1969), and a laser infrared detector (Freeman, 1974). However, there are problems associated with these instruments including slow analysis time and lack of sensitivity.

With recent advances in LC-detector technology, high performance spectrophotometers allow chromatographic eluates to be monitored at wavelength as low as 200nm. By coupling a high-performance reversed phase chromatographic column and such a detector and by eluting with the appropriate solvents,

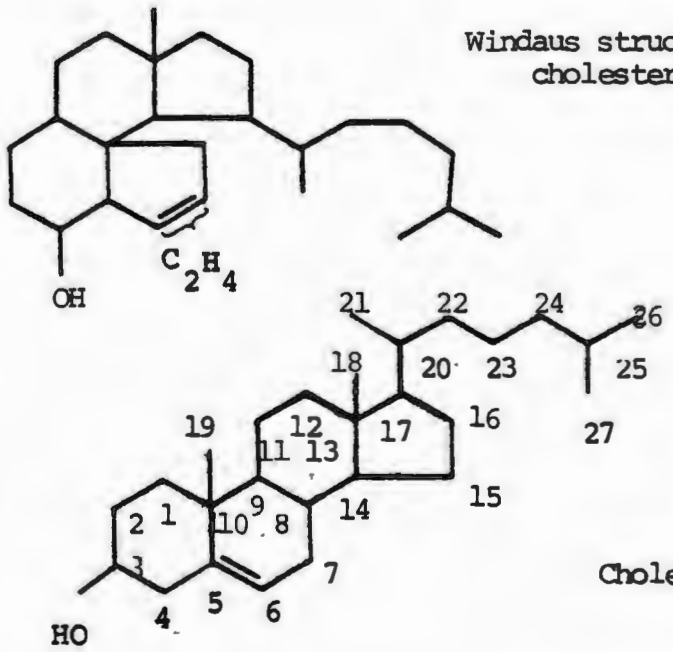
it is possible to measure cholesterol as it is eluted from the chromatographic column.

The analysis in various mixtures of natural origin of slightly polar free sterols related to cholesterol and phytosterol is of a great practical importance since the identification of these sterols will allow knowledge of the origin of food products or biological materials. The topic of this research is the investigation of cholesterol and the major phytosterols in margarine and its related oils and fats using high pressure liquid chromatography (HPLC) for the assay after extracting the sterols and, if necessary, making derivatives.

REVIEW OF LITERATURE

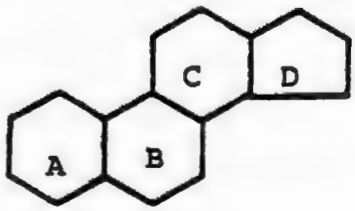
The history of sterols began in 1815 when a French chemist named Chevreul observed that a white crystalline compound could be obtained from the unsaponifiable material of certain animal fats. He found it to be identical with a compound isolated from gallstones some 45 years earlier by Poulletier de la Salle and in recognition of the initial source material named it cholesterine (Greek: chole=bile and stereos-solid) (Dam, 1958). In 1824, cholesterol was found by Chevreul in human and animal bile, by Lecanu in 1838 in human blood and by Couverbe in 1838 in brain. Then the investigations into the occurrence of cholesterol and other sterols in the unsaponifiable portion of fats followed rapidly (Kritchevsky, 1963). The term "cholesterol" was introduced in the beginning of the 20th century when it was proved that cholesterine, like alcohols, could form esters and the generic term "steroids" was derived from it (Nes and McKean, 1977).

In 1928, Adolf O. Windaus (1876-1959), the father of steroid chemistry, was awarded the Nobel prize in chemistry for work that he had done during a quarter of acentury on the structure of cholesterol. The interesting thing is that Windaus had gotten it wrong. His structure for cholesterol, structure (1) in Figure 1, was later shown to be in

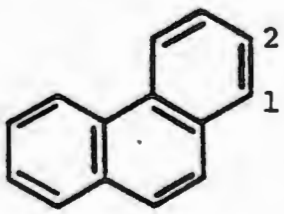


Windaus structure for cholesterol

Cholesterol



common saturated steroid tetracyclic 1,2-cyclopentanoperhydrophenanthrene.



phenanthrene

Figure 1. Structures related to cholesterol

error, for in 1932 J. D. Bernal in England published an X-ray examination of ergosterol, another steroid that could be related chemically to cholesterol. This was one of the first applications of the new X-ray technique to an organic structure problem. By the end of the year, the evidence accumulated in such papers led quickly to structure (2) as the correct formula for cholesterol (Dence, 1980).

Other unsaponifiable compounds similar to cholesterol have been found in the lipids of a variety of plant and animal sources. The collective name of sterols has been adopted for all crystalline unsaponifiable alcohols with properties resembling those of cholesterol. The term phytosterols was proposed for all plant sterols. It came from the name phytosterin which was introduced for a sterol isolated from Calabar beans by Hess in 1878 (Kritchevsky, 1963).

Steroids of some kind are apparently present in all living organisms, with the possible exception of bacteria. They include sterols, certain sapogenins, alkaloids, heart poisons and hormones. Each steroid in common possesses a characteristic tetracyclic carbon skeleton, namely, the skeleton of the perhydrocyclopentanophenanthrene molecule (Figure 1) (perhydro = fully hydrogenated (Dence, 1980). In completely saturated steroids, rings A, B, and C are cyclohexane rings, and ring D is a cyclopentane ring. Cyclohexane rings are not planar, for if they were, the C-C-C bond angles would be 120° instead of the observed values

which lie much closer to the tetrahedral angle of 109-110°. Aromatic rings are planar. In naming steroids, we begin by orienting the molecule as shown in Figure 2 for cholestane, a saturated C_{27} steroid. The numbering is as indicated. According to the International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB) 1967, the use of a steroid name implies that groups at positions 8, 9, 10, 13, and 14 are oriented in the 8B, 9a, 10B, 13B and 14a configurations, respectively, unless stated to the contrary. We then need only to specify in the name the configuration at position 5 and 17 and any other positions of asymmetry. By long standing tradition, the terms a and ß are also applied as shown in structure of Figure 3. Many steroids have common or trivial names such as cholesterol. Systematic names, however, are based on certain parent hydrocarbon systems. Elaboration is described using principles from organic nomenclature and expressing this by means of prefixes and suffixes. An example for sterols is given in Figure 4 (Dence, 1980).

The reactions to be expected of steroids are determined by their structures and by the functional groups that are present. Some of the principal functional groups are: -OH, $\overset{H}{\diagdown}C=O$, CH_3, C_2H_5, $-NH_2$, -COOH, -OR, $-C\equiv C-H$, $\overset{-O}{\underset{R}{\diagdown}}C=O$, -F, -Cl, -Br, -I, $\diagdown C=O$, $-NO_2$. As a class, the steroids are almost entirely solids. Steroids are generally insoluble in water but reasonably soluble in many organic solvents. Ethanol, acetone, chloroform and dioxane are

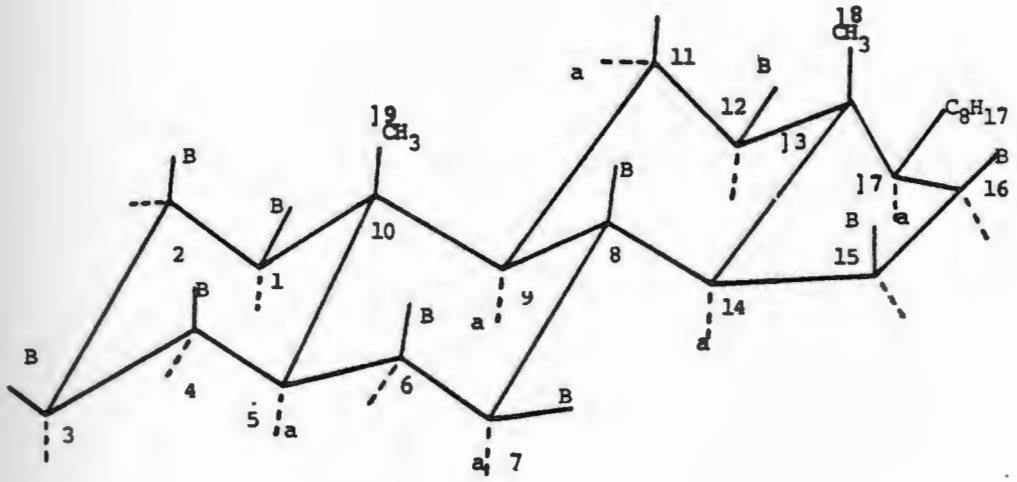


Figure 2. Cholestane

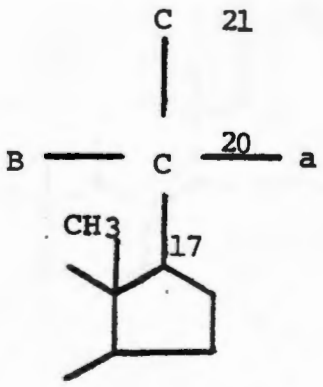
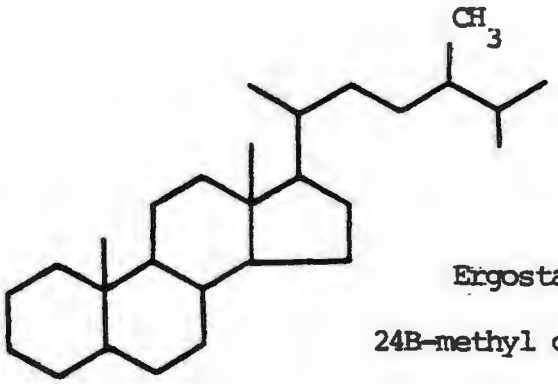
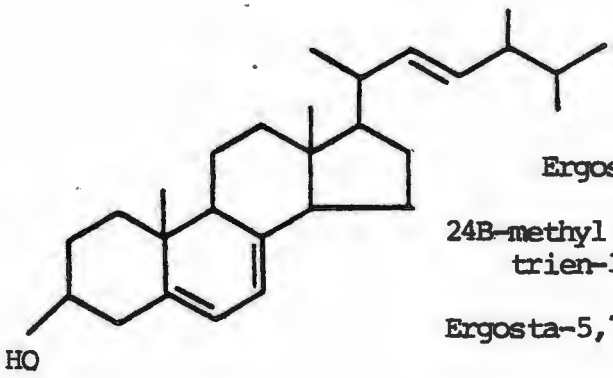


Figure 3. Orientation at carbon 20.



Ergostane

24B-methyl cholestane



Ergosterol

24B-methyl cholest-5,7,22-trien-3B-ol

Ergosta-5,7,22-trien-3B-ol.

Figure 4. Example of nomenclature of ergosterol.

frequently used solvents. One very unusual solubility property of steroids has been known for a long time. This is the fact that cholesterol and other 3B-hydroxy steroids are precipitated from solution by digitonin, itself a glycoside of a 3B-hydroxy-spirostan, whereas 3a-hydroxy steroids are not so precipitated. The 3B-hydroxy steroid is precipitated as a complex of the glycoside. Other saponins will work also but digitonin was found to give nearly quantitative precipitation (Cook, 1958). For ultraviolet and visible spectroscopy, the C=C and C=O bonds are the ones most important in steroids of natural origin. The C=N also plays an important role in derivatives used for analysis. The commonly measurable region is from 210 to 800nm of which the region from 400 to 800 is regarded as the visible and lower wavelength as the near ultraviolet region. Pure cholesterol, for instance, has no significant absorption from 210 to 800 nm. On the other hand. 5,6-dehydrocholestanol (cholesterol) exhibits end-absorption because of a peak from the Δ^5 bond near 200nm (Nes and McKean, 1977). That is due to the fact that only certain transitions are allowed and they are dependent on the environment, extent of conjugation, degree of substitution and nature of the atoms joined by pi electrons (Pomeranz and Meloan, 1971).

Sterols in mammals

Mammalians and plants synthesize steroids from acetic acid, whereas insects lack the enzymes for steroid biosynthe-

sis and thus depend on phytosteroids as a cholesterol source. The overall picture is shown in Figure 5. The mechanism of cholesterol biosynthesis in mammalians has been clarified in detail through numerous elegant studies employing precursors labelled with C^{14} and/or H^3. Mevalonic acid isolated and characterized from dried distiller solubles and shown to possess the R configuration at C-3, is the key intermediate for all terpenoid compounds including cholesterol (Crouch et al., 1978). The nature of the squalene oxide cyclase constitutes a bifurcation in the steroid pathway yielding lanosterol as the first cyclized intermediate in nonphotosynthetic organisms and cycloartenol in place of lanosterol in photosynthetic organisms. The existence of the bifurcation is now unequivocal, although the association with organismic types could bear additional definition. At some stage after cyclization into the the cycloartenol series, the three-membered ring is opened. The mechanism has not been investigated, but reasonable hypothesis is that protonation-deprotonation is involved, in which case the enzume would be an isomerase. The isomerization can be experimentally bypassed by the administration of lanosterol which proceeds in about the same yield to Δ^5 sterols as does cycloartenol (Figure 6). For steric reasons, lanosterol is thermodynamically more stable than cycloartenol; any isomerization of one to the other would be expected toward lanosterol and this has been observed chemically (H^+ catalyst) (Nes and McKean, 1977).

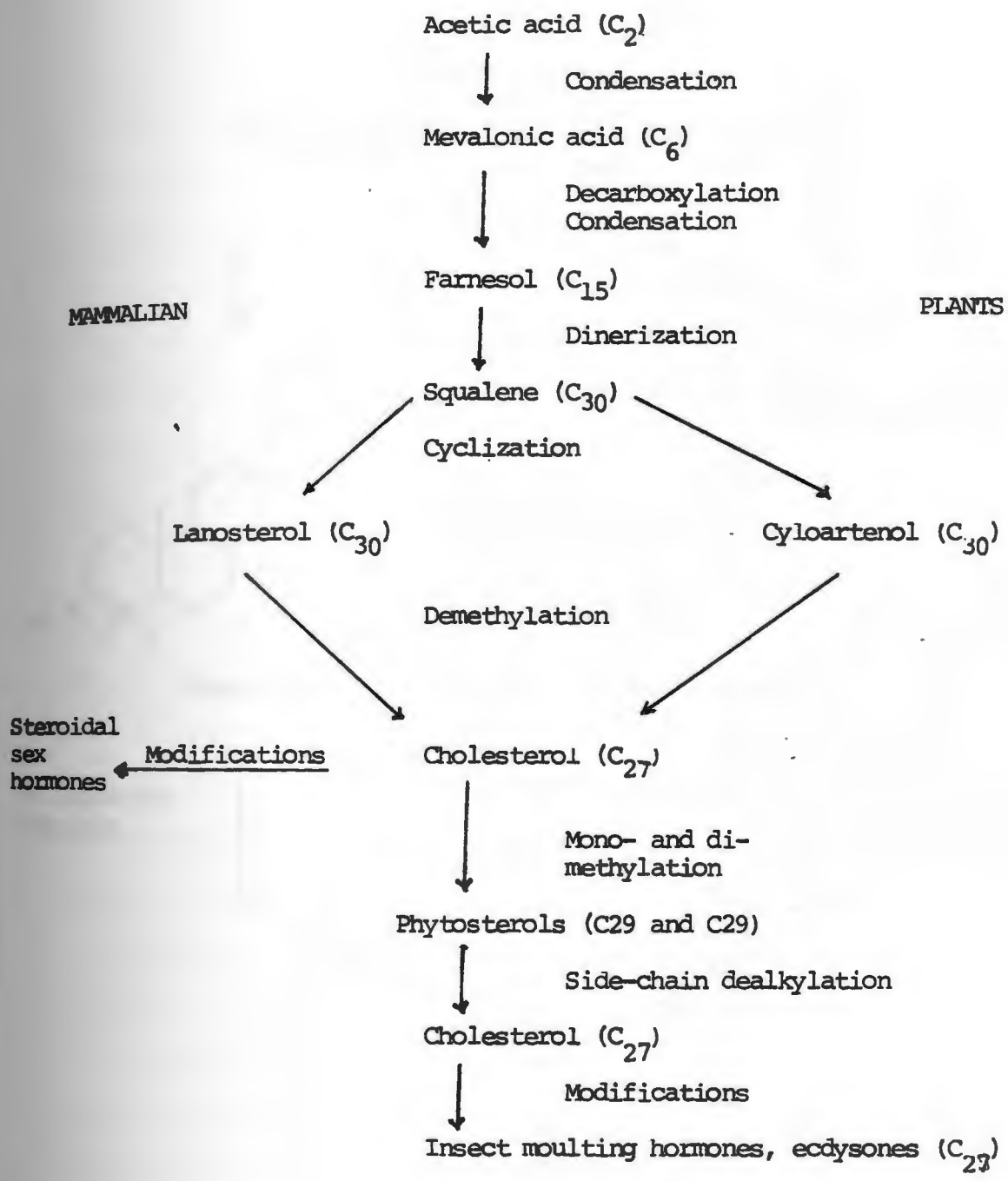


Figure 5. Outline of steroid biosyntheses (Crouch et al., 1978).

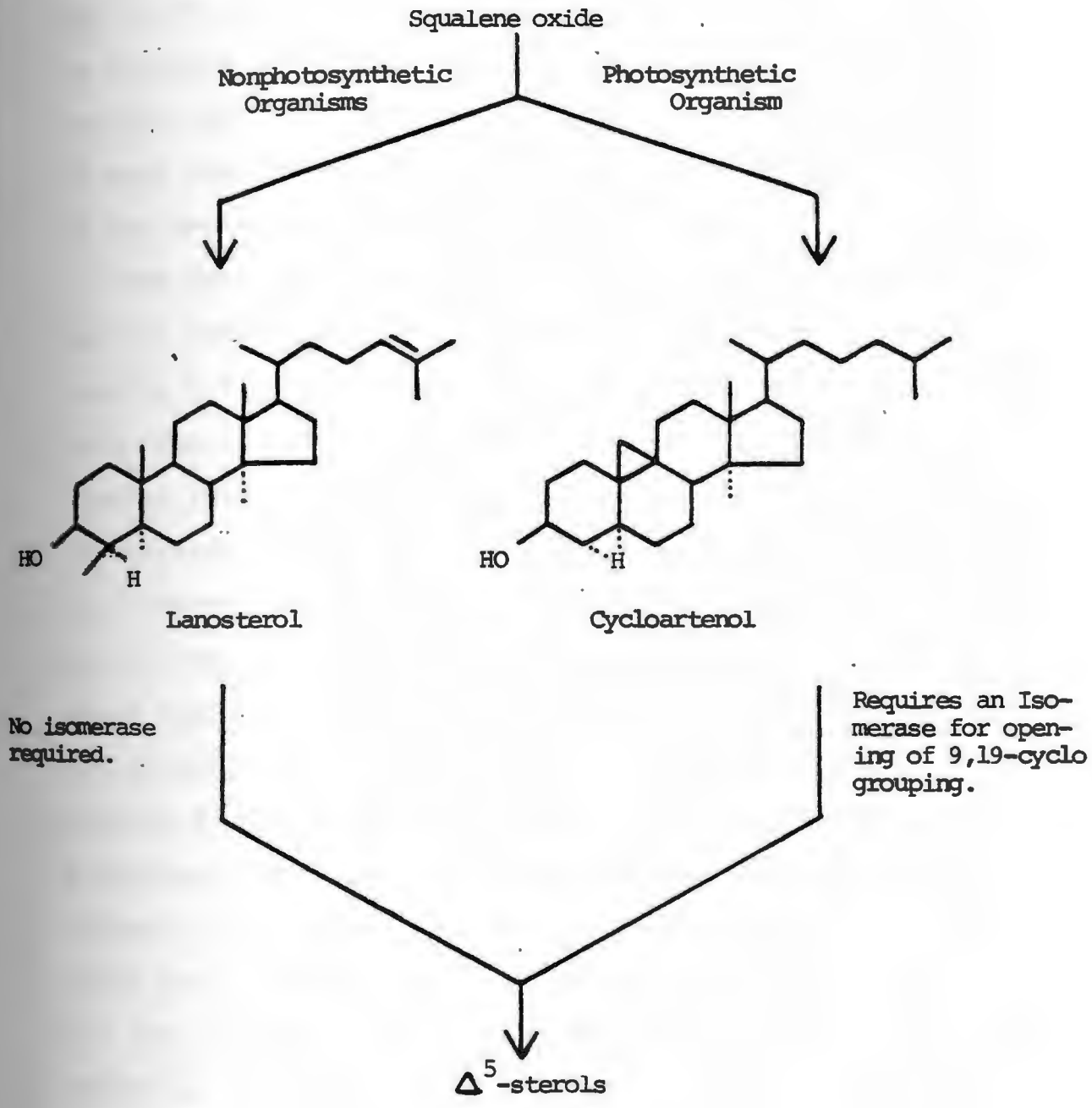


Figure 6. The lanosterol-cycloartenol bifurcation and its apparent association with organismic type.

Human beings and other mammals investigated usually do not absorb enough dietary sterol (other than cholesterol) for it to appear consequentially in their tissues. There is a strong suggestion that this discrimination against sterols with modified side chains begins with the vertebrates. In many subsequent investigations cholesterol was found to be the dominant sterol of all higher vertebrates. Except for the gastrointestinal tract, mammalian sterol is nearly but not completely pure cholesterol. Vertebrate tissues usually not only contain no sterols with side chains which have been alkylated, shortened or enlarged, but also no Δ^{22}-sterols (Nes and McKean, 1977). However, the sterols of vertebrates are by no means always absolutely pure cholesterol. Common animal sterols include small amounts of the Δ^{0}- and Δ^{7}-analogs (cholestanol and lathosterol), the Δ^{24}-derivative (demosterol), $\Delta^{5,7}$-derivative (7-dehydrocholesterol) and others (Nes and McKean, 1977). Cholesterols and lanosterols contain a hydroxyl group at carbon 3 of ring A and a branched aliphatic chain of eight or more carbon atoms at carbon 17. They occur either as free alcohols or as long-chain fatty acid esters of the hydroxyl group at carbon 3. All are solids at room temperature. Cholesterol is the precursor of many other sterols in animal tissues, including the bile acids, detergent like compounds that aid in emulsification and absorption of lipids in the intestine, the androgens, or male sex hormones; the estrogens, or female sex hormones, the progestational hormone progesterone and the adrenocortical hormones (Kritchevsky, 1963).

Plant sterols

Plant sterol biosynthesis has been extensively investigated only since about 1965 (Goad, 1977). Apart from the additional mechanisms required for the introduction of the extra carbon atoms into the side chains of typical plant sterols, there was probably the expectation that the pathway in plants would be much the same as the animal route leading from acetyl-Co A through mevalonic acid, squalene and lanosterol to cholesterol. It soon became apparent, however, that such is not the case and the studies of phytosterol biosynthesis have been rewarded by the recognition of several intriguing mechanistic differences not only between animals and plants but also between particular classes of plants (Goad, 1977).

Cholesterol is the major sterol of animals. The typical plant sterols are: campesterol, sitosterol and stigmasterol (Figure 7). The addition of a C at C_{24} of the side chain distinguishes phytosterols from animal sterols. It is now recognized that cholesterol (cholest-5-en-3B-ol) is widespread in the plant kingdom, but it usually constitutes only a few percent of the sterol mixture (Goad, 1977). Weinrauch and Gardner (1978) have compiled data on the sterol composition of 39 oils, 16 margarines and a variety of fruits, vegetables, seeds and nuts, spices, cereals and legumes. Almost without exception, the major sterol in plants is B-sitosterol (24-ethyl cholest-5-en-$_3$B-ol) and in the majority

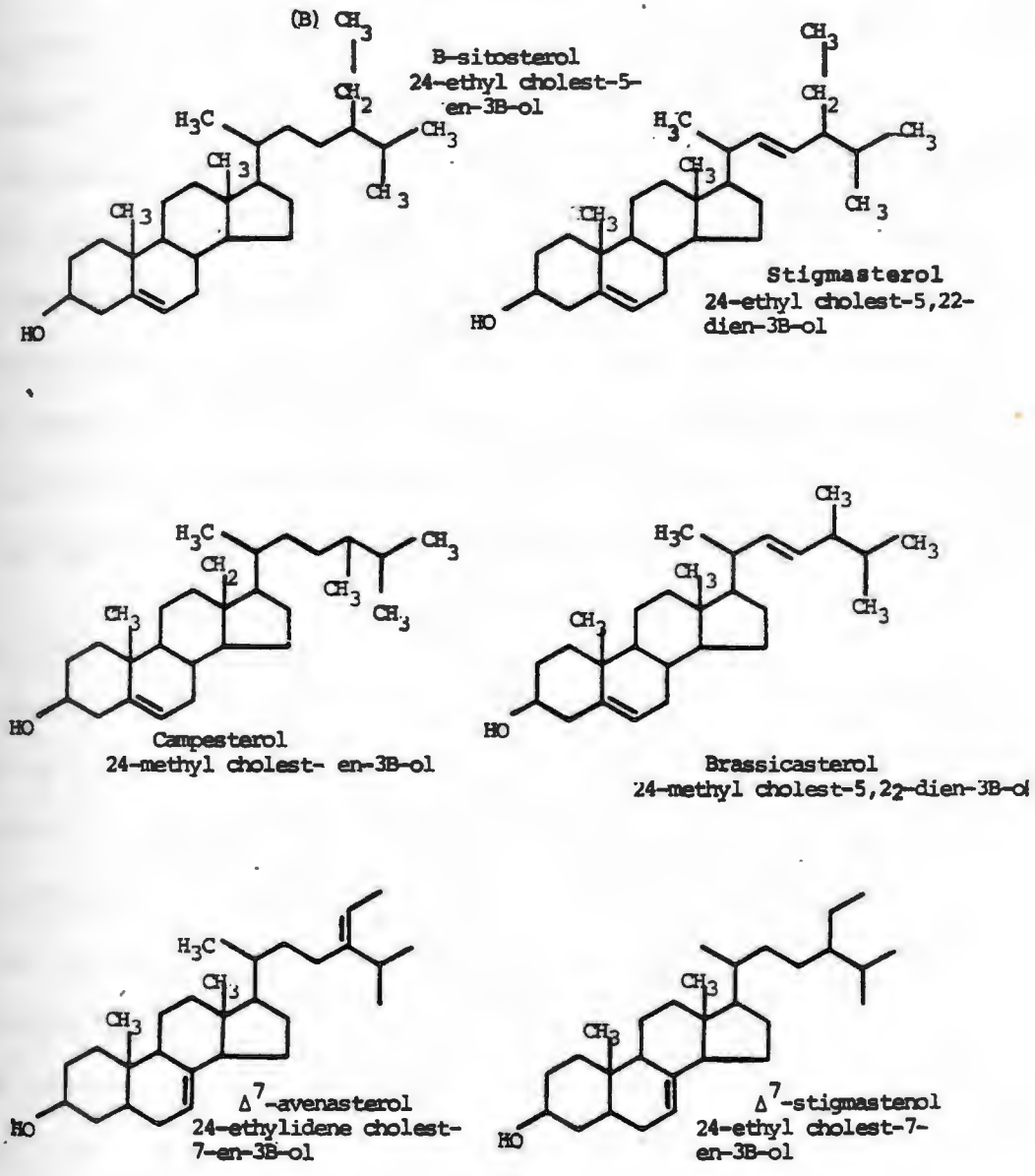


Figure 7. Structure of six common plant sterols

of plant oils campesterol (24-methyl cholest-5-en-3Bol) and stigmasterol (24-ethyl cholest-5,22-dien-3B-ol) are second and third highest in concentration, while certain oils, such as pumpkin seed oil, rapeseed oil, rice bran oil, safflower oil, sesame seed oil and sunflower oil, contain relatively high concentrations of one or more of the less common phytosterols Δ^5-avenasterol (24-ethylidene cholest-5-en-3B-ol), Δ^7-stigmastenol (24-ethyl cholest-7-en-3B-ol), Δ^7-avenasterol (24-ethylidene cholest-7-en-3B-ol) and brassicasterol (24-methyl cholest-5,22-dien-3B-ol). The sterol composition of some oils of commercial importance are listed in Table 1 (p73) and the structures of 6 plant sterols are shown in Figure 7.

Margarine

Margarine was first prepared a century ago. It was devised by a French chemist, Hippolyte Mege-Mouries, who had responded to an offer by Louis Napoleon III of a prize for the production of a satisfactory substitute for butter. In the new product Mege had used margaric acid, a fatty acid component isolated in 1813 by Michael Eugene Chevreul and named because of the lustrous pearly drops that reminded him of the Greek word for pearl - margarine (Riepma, 1970).

Types of margarines

First and most typical are margarines composed of vegetable oils (Massiello, 1978). A second is blend margarines which combine animal and vegetable fats in one

proportion or another. They are commonly referred to as "AV" or "VA" products, depending on the predominance of animal ("A") or vegetable ("V") fat. The "AV" types are required by the Standards to be between 50 and 90 percent animal fat. A typical "AV" weight composition would be 90 percent pure lard and 10 percent soybean oil. The third is that of margarines composed wholly of animal fats. Usually the fat content is slightly hardened pure lard (Riepma, 1970). Today there are ten different types of margarine produced. There are regular, whipped and polyunsaturated margarines in both stick and soft forms. There are diet margarines, liquid margarines and new 60% vegetable oil spreads. These products cater to the needs of many different segments of the population (Massiello, 1978). In recent years, medical research findings have suggested the advisability of increasing the proportion of polyunsaturated fatty acids in the diet and reducing the intake of cholesterol. Parodi (1975) determined the sterol content of some margarines. In a margarine made of blended animal and vegetable fats, he found by GC that cholesterol constituted 94% of the sterol content which indicated the presence of animal fat at high proportion in that margarine.

U.S.A. regulations for margarine

Animal fats used in margarine are covered by the margarine regulations found in the Code of Federal Regulations, No. 21 Food and Drugs, Part 166, revised as of April, 1980,

a section of which reads, "The rendered fat or oil, or stearin derived therefrom (any or all of which may be hydrogenated), of cattle, sheep, swine or goats, or any combination of two or more such articles - to be declared by the name of the specific animal fat, oil or stearin, for example, "beef fat." If the animal fat or oil is hydrogenated the name should include the word (hydrogenated) or hardened. Where combinations are used, the names are to be arranged in order of predominance, with the animal fat, oil or stearin present in greatest proportion named first.

Raw materials

The grease is collected from slaughtered animals. Then it is washed, minced and put into autoclaves with double jackets. Steam is blown in at normal pressure. The fat is drawn off, while the residue or greaves are collected on the screens of the autoclave. The fat is then crystallized slowly at about 30°C. The crystallized product, which is a granular mass, is filtered and pressed yielding about 60 percent liquid with melting point between 28 and 34° C.

The vegetable oils are obtained from oil-bearing seeds or fruits. The seed is crushed, heated moistened with a little steam and then pressed. The oil after that is refined, degummed, deodorized and hydrogenated. Hydrogenation consists of adding very pure hydrogen to oil, with a finely divided nickel catalyst (Van Stuyvenberg, 1969).

Margarine manufacture

The fats and oils in proper proportions are heated to 30°C or more and oil soluble ingredients (emulsifiers, coloring agents, flavoring agents and vitamins) added. An aqueous phase containing milk or dried protein and water is prepared separately. After pasteurizing and cooling, salt and preservatives are added. The two phases are blended and the resulting emulsion is rapidly chilled in heat exchangers and allowed to stand in crystallizing chambers for 2 minutes until the product is sufficiently stable to be extruded and packaged. For soft tub margarine, the fluid melt is agitated during chilling to prevent fat crystals from growing into a firm network. The emulsifying agents (mono and diglycerides, sometimes lecithin) help to keep the water dispersed as fine droplets within the oil phase. Whipped margarines are produced by introducing nitrogen gas just prior to chilling and maintaining vigorous agitation during the crystallizing step. The low fat spreads with a higher water content require more emulsifying agents and more careful control of production conditions in order to maintain the water-in-oil emulsion (Brekke, 1980).

Analysis of sterols

Measuring cholesterol is one of the assays that have passed through several laboratory investigations and critical discussions since it was discovered and until now. Generally, any method for sterol determination involves one

or more of the following stages:

1. Extraction of lipids from tissue
2. Saponification
3. Fractionation of lipid classes and isolation of sterols
4. Derivative formation
5. Measurement of the sterols.

Extraction of lipids from tissue.

Both free sterols and esterified sterols will be extracted from biological tissue by common lipid solvents such as ethylether and chloroform-methanol (Bligh and Dyer) and the alcohol and ester forms can be separated to some extent from other neutral lipids by silicic acid column chromatography and thin layer chromatography (Kates, 1972 and Heftman, 1975). Most methods for sterols involve saponfication of an oil, fat or lipid extract, extraction of the free sterols into a nonpolar solvent and measurement of the sterols.

Saponification

Saponification of a fat may be accomplished in a number of different ways and the precise manner in which it is accomplished depends to some extent on the nature of the original material and the object to be obtained. Most fats are saponifiable by means of potassium and sodium hydroxide, alkali alcoholates or some other base in methanol, ethanol, butanol or other organic solvent. Fats may also be hydrolyzed with steam under pressure or by the action of a

catalyst such as Twitchell reagent or some other aromatic sulfonic acid (Markley, 1947). For purposes of quantitative identification, it is essential that all operations involved in the saponification and subsequent steps be conducted in such a manner that no alteration occurs.

For ordinary fats and oils, Hilditch (1941), has observed that saponification is readily accomplished by refluxing for six hours 100 parts of fat with 60 parts of potassium hydroxide and 500 parts of 95% to 100% ethanol. Abell et al. (1958) used alcoholic potassium hydroxide solution by adding 6 ml of 33% w/w KOH in water to 94ml of the absolute alcohol, while Kates (1972) recommends 1-2 hour reflux with 90% methanolic sodium hydroxide (0.3 N). In the AOAC (1980) procedure for unsaponifiable residue, 2g of fat is boiled for 30 minutes under an air reflux with 25 mls 95% ethanol and 1.5 ml 60% KOH in water.

Isolation of sterols

On saponifying the lipids with an alkali in alcoholic solution, the esterified fatty acids of the lipid, including the steryl esters, form alkali salts (soaps) and the liberated alcohols constitute the neutral fraction. After dilution with water, the higher alcohols and sterols are separated from the hydrolyzed material by extraction with an organic solvent such as petroleum ether (Kates, 1972) or ethyl ether (AOAC, 1980) which is immiscible in water. The material recovered from the solvent forms the unsaponifiable matter.

For quantitative determination, the chemical properties of the sterol molecule have been utilized in the below-mentioned methods:

Digitonin and glycoside precipitation; in which an alcoholic solution of cholesterol is mixed with an alcoholic solution of the steroid digitonin. The result is a precipitation of 1:1 molecular complex of cholesterol and digitonin, known as cholesterol digitonide (Cook, 1958). Digitonin is used frequently for the separation of 3B-hydroxy (precipitable) and 3a-hydroxy (not precipitable) steroids.

After the precipitation is complete, it is necessary to split the digitonide into its components and to recover the unprecipitated steroid and digitonin from the supernatant solution. This latter part of the method passed by several modifications. Schoenheimer and Sperry (1934) dissolved the digitonide in a cold pyridine-ether mixture to precipitate the digitonin at which time the steroid could be recovered from the solution. Bergmann (1940), improved this method by applying dry pyridine and keeping the solution at 70-100° for one hour. The pyridine is then removed by distillation in vacuum.

The evaluation and criticism of digitonide formation method is as follows:

A) The advantages are:

(1) The low solubility of the complex makes the test very sensitive

(2) The reaction has been used directly as a granimetric method for the determination of cholesterol.

(3) Immediate precipitation occurs.

B) The disadvantages are:

(1) The formation of insoluble complex is not restricted to digitonin but saponins, tiyonin and digitonin and the alkaloid formation give precipitates with cholesterol. Commercial digitonin frequently contains gitonin as impurity.

(2) Variable amounts of water of crystallization of the digitonide may affect the gravimetric results.

(3) Digitonin forms molecular complexes with many nonsteroid substances, e.g. phenols and terpene alcohols.

(4) Besides cholesterol, most naturally occurring sterols having a 3B-hydroxy group yield insoluble digitonides

(5) The samples vary in purity.

(6) The digitonin is expensive.

Measurement of sterols

Measuring cholesterol is one of the most frequently performed assays in the laboratory, for which a wide variety of methods are available and used. Digitonide procedures include:

1) Gravimetric determination based on digitonide formation, in which relatively small amounts of sterol may be estimated according to the fact that the weight of digitonide is more than four times (4.18 for cholesterol) greater than that of the sterol.

2) Microdetermination: with small amounts of digitonide estimations may be made by oxidation methods using chromic acid. The remaining chromic acid may be determined by titration with thiosulfate, or the CO2 liberated from the digitonide on oxidation is estimated gasmetrically (Kirk et al., 1934).

Colorimetric methods

Many steroids when treated with acid reagents (Lewis acids) give intense colors. The actual color produced with a steroid depends on the acid used and on the steroid concentration. First, Liebermann in 1885 described the color changes taking place when sulfuric acid is added to a concentrated solution of cholesterol in acetic anhydride. The color shifts through red to blue. Later, Burchard in 1889 applied the reaction to a solution of cholesterol in chloroform or certain other water-free solvents and noted that a green color developed, the red and blue stages being bypassed when the solution of cholesterol is dilute.

Among the colorimetric methods of cholesterol determination based on Liebermann-Burchard reaction are the following:

1) In 1934, Schoenheimer and Sperry presented their procedure for the determination of free and total cholesterol in blood or other biological material. In principle, the procedure consists of the precipitation of cholesterol with digitonin followed by the application of a color reaction to the precipitate. They first extracted the blood with acetone-

absolute alcohol solution which then was heated and cooled. After that, digitonin solution was added to precipitate the free cholesterol. The dried precipitate of cholesterol digitonide was dissolved in acetic acid. Next, the temperature of the water bath was adjusted to 25° and acetic anhydride was added followed by concentrated sulfuric acid. The intervals between the addition of reagents was so timed that not less than 27 minutes nor more than 37 minutes elapsed between the addition of H_2SO_4 and reading the color.

2) In 1943, Sperry and Brand recognized that most of the methods in general use for the determination of cholesterol, based on the Liebermann-Burchard color reaction, were unsatisfactory and the principal sources of inaccuracy were known. To avoid these sources of error, they described their modification as follows:

a. Saponification is necessary before the estimation of total cholesterol in bloood serum or tissues containing esterified cholesterol, because in the combined form it develops considerably more color at a faster rate than does free cholesterol.

b. Interference by extraneous colors may be avoided by the use of mild conditions for saponification and a weak acid (acetic acid) for acidification and by extraction of the sterol from a water-alcohol solution of the acidified residue.

c. The development of color may be influenced to a considerable extent by small and uncontrollable variations

in the procedure of adding acetic anhydride and sulfuric acid. This source of error may be avoided by mixing these reagents beforehand.

d. Small variations in the temperature at which color is developed have a large effect on the time at which maximum color is reached and on the color intensity at the maximum. The temperature and time of reading should be selected so that the reading is carried out at the maximum, and should be the same for each sample in a series of determinations. The common practice of reading several unknown samples against one cholesterol standard violates this requirement.

3) In 1953, Pearson et al. found out that such methods mentioned before, are tedious and subject to possible errors as a result of incomplete extraction of cholesterol and of manipulative processes required by the methods. So, they tried to eliminate or greatly reduce these sources of error in a newer procedure which requires only the addition of reagents to serum and direct measurement of the density of the resultant color. They found that the results of their method agree with those obtained by the Schoeheimer-Sperry procedure. The procedure of Pearson et al. involves the addition of p-toluenesulfonic acid, glacial acetic acid and acetic anhydride to the serum, followed by concentrated sulfuric acid. After the solution has stood at room temperature the optical density of the color is measured at 550nm.

In the same year, 1953, Zlatkis et al. did the same but used ferric chloride solution for the color development instead of p-toluenesulfonic acid.

4) In 1963, Shin applied microcolumn chromatography using aluminum oxide or silicic acid for the separation of minute quantities of free and esterified cholesterol from human cerebrospinal fluids. However, the cholesterol measurement was based on color according to the Zaltkis et al. procedure (1953).

5) In 1972, Fasce and Vanderlinde presented results of an interlaboratory survey of cholesterol assays in which methodology, standardization technique, type of standard, reagent source and control sera were considered. In their surveys conducted during 1969 and 1970 one sample from each of four lots was mailed to each of the 380 hospitals and independent laboratories engaged in clinical chemistry testing in upstate New York. The following analytical methods were used:

- Saponification, Extraction, H_2SO_4 - acetic anhydride.
- Extraction, H_2SO_4 - acetic anhydride ($CHCl_3$).
- Extraction, H_2SO_4 - acetic anhydride (acetic acid).
- Extraction, H_2SO_4.
- Extraction, H_2SO_4 - FeCl3.
- Direct H_2SO_4 - FeCl3.
- Direct H_2SO_4 - acetic anhydride.
- Direct p-toluenesulfonic acid.

Results of these analytical methods were interpreted with great trepidation. It was concluded that methodology, standardization technique, type of standard, reagent source, control sera, reagent kits and interlaboratory precision, all have effects on the results. Finally, they recommended a computer program capable of processing results by various combinations and permutations of variables. This left the surveyor with the sole task of considerating what variable to evaluate, a task best accomplished by comprehensive review of potential influences within his own laboratory.

Enzymatic methods

Enzymes, the polypeptides designed by nature to catalyze the reactions necessary for life, are prsent in all living things. These catalysts increase the rate of reaction by many orders of magnitude at or near room temperature and atmospheric pressure. Furthermore, the number of enzymes known by man has increased from less than 100 in 1930 to about 2000 today. The gradual replacement of classical wet chemistry techniques by enzyme-catalyzed reactions is particularly evident in the clinical laboratory.

In 1974, Allain *et al*. introduced a procedure for the enzymatic determination of total serum cholesterol which requires no prior treatment of the sample. Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase (Figure 8). The free cholesterol produced is oxidized by cholesterol oxidase to cholest-4-en-3-one

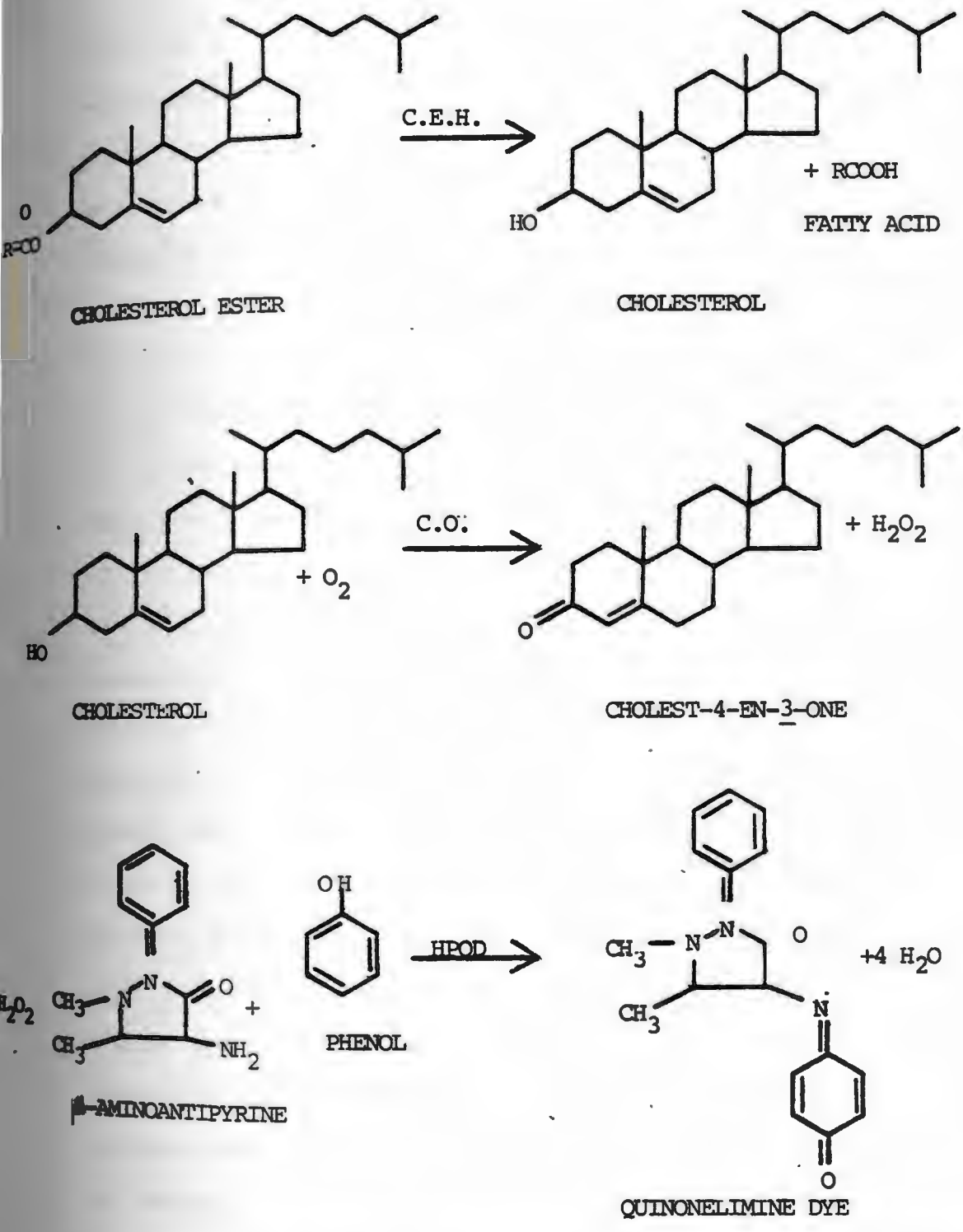


Figure 8. Reaction scheme for the enzymatic measurement of total serum cholesterol.

- Nonstandard abbreviations used: C.E.H. cholesterol ester hydrolase (EC 3.1.1.13); C.O., cholesterol oxidase (cholesterol: oxygen oxidoreductase, EC 1.1.3.6.); HPOD (horseradish source; donor: H_2O_2 oxidoreductase, EC 1.11.1.7).

with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500nm. The results correlated well with those obtained by Liebermann-Burchard procedures.

Rat pancreatic juice cholesterol ester hydrolase (EC 3.1.1.13) has been purified by Hyun et al. (1969) through sequential precipitation with 35% aceton, diethyl aminoethyl cellulose chromatography and hydroxyl apatite chromatography. They found the final preparation and intermediate factors during purification have the same ratios of ester synthesizing and hydrolyzing activities as the original juice and, accordingly, they suggested that these activities in pancreatic juice are a function of the same enzyme. The disadvantages of this procedure are: 1) Enzyme purity and activity may vary widely by manufacturer and even from lot to lot; 2) in aqueous solution, the catalytic activity is usually lost fairly rapidly, making the use of enzyme reagents both troublesome and expensive; 3) sometimes, the soluble enzyme can be oxidized by oxygen in air or its structure destroyed at the air-water interface; 4) it is sensitive to heat and microbiological attack. Consequently, the use of enzyme catalyzed reactions has been slow largely because of the undesirable properties associated with soluble enzyme reagents.

For negating or at least minimizing the difficulties mentioned above, the technique of enzyme immobilization was

described alongside the technique of electrochemical sensors which gave rise to most of the analytical instrumentation used in this field. Enzymes are immobilized or insolubilized by attachment to any inert support material. These supports have ranged from stainless steel balls to feather protein. The advantages of enzyme immobilization are:

- The enzymes are rendered insoluble with the resulting derivative that retains the catalytic activity.
- Once immobilized, an enzyme is often stable for weeks or even months.
- It is easy to separate the insoluble enzyme derivative from reactants and/or products for reuse.
- The derivative can be packed in a column similar to those used in liquid chromatography or it can be used in a stirred tank with the product.
- The enzyme derivative has different catalytic properties from the enzyme and these properties can be controlled by immobilization conditions.

In 1979, Boehringer Mannheim GMBH, Biochemica (Anonymouse, 1979) introduced their three-bottle-kit-enzymatic-colorimetric method for the determination of cholesterol in foodstuffs. In this method, the cholesterol is oxidized by cholesterol oxidase to cholestenone. In the presence of catalase, the hydrogen peroxide produced in this reaction oxidizes methanol to formaldehyde. The latter reacts with acetylacetone forming a yellow lutidine dye in the presence of NH_4^+ ions.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{chol. oxidase}} {}^{4}\text{-cholestenone} + H_2O_2$$

$$\text{Methanol} + H_2O_2 \xrightarrow{\text{catalase}} \text{formaldehyde} + 2\ H_2O$$

$$\text{Formaldehyde} + NH_4^+ + 2\ \text{acetylacetone} \longrightarrow \text{lutidine} + 3\ H_2O$$

The concentration of the lutidine dye formed is stoichiometric with the amount of cholesterol and is measured by the increase of optical density in the visible range at 405nm. Each test-combination contains:

- Bottle (1) with ca. 95 ml of solution consisting of ammonium phosphate buffer 0.8 mol/l - pH 7.0, methanol 2.6 mol/l and catalase 220.000 U.
- Bottle (2) with 60 ml of solution consisting of: acetylacetone 0.5 mol/l and stabilizers.
- Bottle (3) with 0.8 ml of suspension consisting of: cholesterol oxidase 12 U.

It should be noted that the procedure is not specific for cholesterol since cholesterol oxidase oxidizes any sterols in which the hydroxyl group at carbon 3 is in the B-position. Therefore, phytosterols, such as stigmasterol and sitosterol, also react in the assay.

Paper chromatography.

This is no longer used in steroid chromatography, except for some polar steroid conjugates. Other chromatographic sheets have also lost the race, because they cannot resist the aggressive reagents and solvents used for sterols. Glass-fiber paper is unsuitable because its coarse texture produces excessive diffusion of chromatographic zones (Heftmann, 1975).

[redacted] layer chromatography.

Thin layer chromatography of steroids has reached a state of perfection where one can hardly expect to devise major improvements in developing and detection methods. Evaluation of chromatography by computer methods is simply a device for exploiting the wealth of information provided by TLC (Johnson, 1973). Silica gel plates with gypsum binder have been standard equipment for TLC of steroids. The modified silica gel plates with silver nitrates are used for unsaturated steroids with digitonin for Δ^5-3 B-hydroxy steroids. Most of the reagents used for detecting steroids by destructive methods contain sulfuric acid, but sulfuric acid without any additives also produces characteristic color and fluorescence responses as well as permanent black zones. The elution of steroids from thin layer plates for subsequent analysis by GLC requires quantitative techniques. It also requires nondestructive methods of detection. Iodine vapors, fluorescent sprays and marker dyes have been advocated (Heftmann, 1975).

Specifically, for TLC of sterols, silver nitrate-impregnated absorbents offer advantage that sterols and their acetates are separated in accordance with the degree of unsaturation and acyl esters of the same sterol according to the unsaturation of the fatty acids. Hydrophobic hydroxy alkyl Sephadex LH-20 is a promising new absorbent, capable of separating sitosterol from campesterol by recycling a

methanol-hexane (19:1) mixture three times (Hyde and Elliott, 1972). Jeong et al. (1974) had used thin layer chromatography (TLC) for fractionating the unsaponifiable material of vegetable oils. They found four separated zones, containing less polar compounds, triterpene alcohols, 4-methylsterols and sterols, respectively. The sterol fraction was cut off, extracted with ether, dried and purified further by repeated preparative TLC for subsequent GLC analysis. The plates were coated with 0.5mm layer of Wakogel B-10 (Wak Pure Chemical Industries, Osaka, Japan) and developed with hexane-ether (7.5:2.5) for one hour. Sweeney and Weihrauch (1977) used two-way development. The first elution mixture was isopropanol-acetic acid 96:4 and the second mixture was petroleum ether-acetic acid-ether 90:1:10.

Gas chromatography

Gas chromatography is a process by which a mixture is separated into its constituents by a moving gas phase passing over a sorbent. It has produced sweeping changes in many fields of research since its introduction in 1952. Basically, it is a separation process capable of extremely high resolving power. Its direct applicability is limited to compounds which can be volatilized without decomposition, such as thermally stable, nonionic compounds with a maximum molecular weight of around 400-500. This powerful analytical method has contributed significantly to advances in many fields - for example, catalyst research, biochemical studies and flavor analysis. Scaled-up in size, gas chroma-

graphytography also offers a method for preparing very pure compounds.

Grunwald (1970) used OV-101, a liquid dimethylsilicane polymer as a stationary phase for phytosterols separations. Since the collected individuals were to be used in further biological experiment, he did not use the often used stationary phase, dimethylsiloxane polymer SE-30 because with SE-30, free sterols tail excessively and poor component resolution may result even when an acid-washed silanized support in a glass column is used. Therefore, derivatives such as esters and ethers including trimethylsilyl (TMS) ethers are generally used. Nordby and Nagy (1973) had determined the relative retentions of twelve acetylated plant sterols on fourteen gas-liquid chromatographic liquid phases. The modified Carbowax SP-1000 adequately resolved three critical pairs of sterols.

By gas chromatography alone, the determination of sterols in biological samples cannot usually be achieved. It is necessary to employ other procedures to separate crude fractions and isolate sterols by preparative methods such as column chromatography and thin layer and in many cases by silver-nitrate silica gel. Phytosterols are typically characterized by the presence of either an extra C_1 or C_2 alkyl group at C-24. However, the above chromatographic methods will not separate different C-24 homologues of sterols, e.g. cholesterol (C-27) campesterol (C-28) and

sitosterol (C-29). Such sterols can be readily separated by gas liquid chromatography (GLC) on analytical scale, but present certain problems on a preparative basis.

Gas chromatography suffers from one major limitation in the analysis of steroid in that many of the biologically important steroids either have low volatility or are too thermally labile for direct gas chromatographic analysis. For these steroids, derivatization techniques are required for satisfactory separations, but there are several problems inherent to the derivatization of mixtures of steroids. Some of the problems include: (a) known yield of derivatives can be difficult to achieve without careful study; (b) either different conditions for derivatization or different derivatives may be necessary for the various steroids present in a mixture; (c) multifunctional steroids can give mixtures of derivatives; (d) prior knowledge of the composition of the mixture is needed to ensure proper derivatization; (e) derivatization techniques are time-consuming and often require a recovery or purification step prior to injection.

The normally used detectors in gas chromatography are nonspecific. The only parameter obtained is a retention time expressed in some form such as relative retention time. Therefore, it is clear that different retention data demonstrate nonidentity, but identical data may be ambiguous. This is best shown for the case of sterols epimeric at C-24, because so far all analysis of such epimeric pairs of compounds by GLC have produced identical retention data for

both isomers (Ikekawa, 1968). However, GLC separates compounds primarily by difference in volatility and therefore by difference in molecular weight.

[redacted] pressure liquid chromatography

In 1971, Henry et al., presented a survey of applications of high speed liquid chromatography to the analysis of steroids. He mentioned that studies had been underway in his laboratory for some time to develop optimum methods for determining steroids and related compounds by high speed liquid chromatography (HSLC). He found that not only can this liquid chromatography overcome limitations of earlier methods for determining steroids such as poor resolution, matrix interference, long analysis time and thermal decomposition, but it can also be extended into the area of steroid conjugates, such as glycosides and phospnates.

Although high pressure liquid chromatography has been used in analysis of lipids in general (Aitzetmuller, 1975) and in reference sterols separation (Colin et al., 1979), its specific application in sterols methodology has been limited because cholesterol and related compounds absorb very little at UV radiation in the wavelength range (254 nm) which most UV detectors used in LOC operate. The maximum absorption comes at 191 nm for cholesterol, 190 nm for cholesterol acetate and 230 nm for cholesteryl benzoate. The maximum absorption for phytosterols is 206 nm for B-sitosterol and 188 nm for stigmasterol (Grasselli and Ritchey, 1975).

The fact that some sterols do not have an appreciable extinction at 254 nm, the operating wavelength of commercially available detectors a few years ago, has created difficulties in terms of sensitivity and minimum amount detectable. Longer path cells to increase sensitivity are impractical because of extra-column band spreading effects which diminish resolution when the cell volume is increased. Compensation by decreasing the cell cross-section would involve problems in light transmission and baseline noise. However, the UV detector is the most convenient and sensitive detector for high speed LC when the compounds of interest have a reasonable extinction coefficient at the detecting wavelength. The UV detector equilibrates rapidly after a change in mobile phase compoisition and is ideal for gradient elution where the mobile phase is continually changing during the separation. The R.I. detector, on the other hand, equilibrates slowly after a mobile phase change and cannot be conveniently used in a gradient elution experiment. It has an advantage, however, in that it can be used with mobile phases that have a high UV background (Henry et al., 1971).

With recent advances in high pressure liquid chromatography (HPLC), its high performance spectrophotometer equipped with low volume flow cells allow chromatographic eluates to be monitored at wavelengths as low as 200 nm. The speed, versatility, and reliability of HPLC have been

major factors for its acceptance both as a research tool and for routine analysis. HPLC has a number of advantages over other analytical techniques. These include:

- Analysis of sample components is not limited by their thermal stability or volatility. In fact, HPLC is the method of choice for a variety of thermally labile or high molecular weight compounds.
- HPLC provides a means for determination of multiple components in a single analysis.
- Both aqueous and nonaqueous samples can be analyzed with little or no sample pretreatment.
- A variety of solvents and column packings are available, providing a high degree of selectivity for specific analysis.
- Separation times are short. Normally analyses are completed in a few minutes, with excellent precision and accuracy.
- Separated components can easily be collected and isolated from the mobile phase for further analysis of characterization.

The number of technical publications on the use of HPLC has increased significnatly over the past few years. In 1973, about 140 articles were published, of which only 16 were related to food and agricultural applications. In 1979, the number of articles increased to about 1500, including 188 devoted to these applications (Tweeten and Euston, 1980).

With the current interest in human nutrition and product adulteration, food chemists are constantly searching for new methods of analysis. Because of the variety of column packings and solvents available, HPLC can be used to perform a number of different types of food analysis. HPLC methods have been developed for determination of sample composition, sample purity and sample component stability in a broad range of foods and beverages. Among the recent applications of high pressure liquid chromatography to sterol analysis are:

1 - Duncan, et al. (1979(have described a method for measuring free and total cholesterol in blood serum using uBondapak C_{18} column. They found that the correlation of his results with others was excellent. They used a variable wavelength detector.

2 - Hunsbury and Scallen (1978) substituted a method that requires several days with a method that takes only 10 minutes using high pressure liquid chromatography. They found that their method is capable of measuring the enzymatic conversion of demosterol to cholesterol.

3 - Hunter et al. (1978) used high pressure liquid chromatography as a preparative separation tool for minute quantities of sterols from a large excess of closely related sterols. They used a dual column assembly with uBondapak C_{18}-Porasil B with mobile phase acetonitrile hexane. Cholesterol was eluted after seven hours.

4 - Rees, et al. (1976) applied the HPLC to sterols separation. They recommended that the reversed phase separation of sterols on uBondapak C_{18} can be applied to the preparative separation of sterols mixtures isolated from biological materials by the conventional techniques of columns and thin layer chromatography.

5 - Colin, et al. (1979) have described a method for separating standard free sterols by high pressure liquid chromatography, using pyrocarbon modified silica gel column. Due to the limitation of their detector, they could not measure cholesterol at wavelength lower than 254nm, which is quite higher than cholesterol maximum absorption region for UV radiation. However, they recommended using this detector rather than Refractive Index detector.

6 - Newkirk, et al. (1981) have used high pressure liquid chromatography for determining cholesterol in foods. They eluted cholesterol as its benzoate ester on uBondapak C_{18} column. Detection was carried out with variable wavelength detector. When they compared their results with results obtained from gas chromatographic analysis, it was found that HPLC is a favorable technique.

Conclusions

Paper, thin layer, conventional column and gas chromatography are used extensively in the separation of steroids. However, each of these chromatographic methods as well as conventional multistep extraction techniques, suffers from one or more limitations for the fast analysis of steroid mixtures. Since many of these limitations can be eliminated by the use of high pressure liquid chromatography, this technique should be the method of choice for the analysis of many steroid mixtures.

EXPERIMENTAL

Apparatus

(1) Liquid chromatograph: Waters Associates Inc., Milford, MA, with Model 6000A solvent delivery system, U6K universal LC injector and Model 450 variable wavelength detector. An Omniscribe (Houston Instruments) recorder and Hewlett Packard 3351B Laboratory Automation System were used to record and integrate chromatographic peaks.

(2) Columns: uBondapak C_{18}, 3.9mm x 30cm, and u-Porasil, 3.9mm x 30cm, Waters Associates.

(3) Pre-column: Co:Pell ODS, 2mm x 5cm, Whatman.

Reagents

(1) HPLC solvents; acetonitrile, methanol, isopropyl alcohol, dichloromethane, and hexane were either Nanograde (Mallinckrodt) or Distilled in Glass (Burdick and Jackson). UV-grade acetonitrile and isopropanol were used in some experiments. All solvents, including distilled water, were prefiltered through millipore or glass fiber filters.

(2) Benzoyl chloride (Matheson Coleman & Bell) was used without purification.

(3) Pyridine (Fisher) reagent grade, redistilled, stored over potassium hydroxide.

(4) Acetic anhydride, reagent grade.

(5) Petroleum ether and dichloromethane were Nanograde (Mallinckrodt).

Materials

(1) Beef fat, pork fat, vegetable oil and margarines were purchased at local supermarkets. Rapeseed oil was extracted from crushed rapeseed.

(2) Cholesterol, stigmasterol, campesterol and a plant sterol mixture were purchased from Applied Science, Supelco and Steraloids.

(3) β-sitosteryl, stigmasteryl and cholesteryl acetates and cholesteryl benzoate were obtained from Steraloids.

Methods

Fats and Oils

The samples of margarines were first melted and filtered through Whatman No. 1 filter paper containing anhydrous sodium sulphate. The vegetable oils were used directly. The beef and pork fat were dissolved in petroleum ether (PE), filtered, dried over anhydrous sodium sulphate and the solvent evaporated.

Saponification

One gram of oil and/or margarine was weighed into a 50 ml glass tube equipped with Teflon-lined cap and 15 ml of 1.5 M KOH in methanol was added. The tube was then sealed, kept in a water bath at 100°C for 20 minutes and allowed to cool to room temperature. After cooling, 10 ml water and 15 ml petroleum ether were added. The tube was shaken for one minute and put in a centrifuge for three minutes at 2000 rpm to separate the PE layer from the soap.

The PE layer was then pipetted to a small beaker and evaporated to near dryness on a water bath and then under a stream of nitrogen.

LC of free sterols

The residue of sterols obtained from PE evaporation was dissolved in 5 ml dichloromethane (DCM), dried over anhydrous sodium sulphate and concentrated to 1 ml, from which 10 ul was injected into the LC.

Acetylation

The sterol residue was dissolved in 1.2 ml acetic anhydride:pyridine, 2:1 (v/v) and transferred to a glass tube equipped with a Teflon-lined cap. The reaction solution was kept on a water bath at 37°C for 15 minutes and then on an ice bath for 2 minutes. After that, 0.6 ml PE, 0.6 ml acetone and 0.6 ml water were added. The tube was capped, shaken and put again in the 37°C water bath for 5 minutes. Then the reaction solution was transferred to a small separatory funnel and washed four times with 2 ml water each. The PE layer containing the sterols acetates was transferred from the separatory funnel, dried over anhydrous sulphate and concentrated to 1 ml, from which 10 ul was injected.

Benzoylation

The sterol residue was dissolved in 1 ml acetone and transferred to a 20 ml glass tube equipped with Teflon-lined cap and 10 ml of 7.5 M sodium hydroxide in water and 0.2 ml benzoyl chloride were added. The tube was capped and shaken vigorously on a mixer for 3 minutes. The reaction

mixture was then transferred to a separatory funnel containing 25 ml water and extracted two times with 15 ml DCM each. The DCM extract was collected in another separatory funnel and washed two times with 20 ml of saturated sodium carbonate and one more time with 20 ml water. The DCM layer was then dried over anhydrous sodium sulphate and concentrated to 1 ml. Benzoates from standards were made by benzoylating 5 mg of the free sterol and diluting the final DCM extract to 10 ml.

Chromatographic conditions

(1) Free sterols. A uBondapak C_{18} column was eluted with solvent system (a) methanol:methylene chloride:water (71:12:17) at flow-rate 1 ml/min.; (b) acetonitrile:methanol-methylene chloride-water (1:1) at flow rate 2 ml/min.; (c) methanol:isopropanol (1:0.1) at flow-rate 1.1 ml/min. The detector wavelength was adjusted at 200 and 215 nm.

(2) The chromatographic conditions for sterols acetates were: uBondapak C_{18} column, eluted with acetonitrile: isopropanol (1.4:0.1) at flow rate 1.5 ml/min., detection at 200 nm.

(3) For sterols benzoates: uBondapak C_{18} eluted with solvent acetonitrile (100%) at flow-rate 2 ml/min, detection at 230 nm.

(4) The majority of runs were made with an attenuator setting of 0.1 and recorder chart speed of 0.5 cm/min.

RESULTS

Initial trials

To choose the column for this work both uPorasil and uBondapak C_{18}, the two most common HPLC columns were tested for the resolution of free sterols and their benzoates. A variety of solvent systems were employed. All sterols showed good elution individually with mixtures of isopropanol, acetonitrile, methanol, hexane and/or dichloromethane, but the separation of mixtures of sterols was better in the uBondapak C_{18} column as shown later. Figure 9 shows the best separation obtained for free sterols on uPorasil. Peaks 1 and 2 are solvent peaks, while Peaks 3 and 4 are unresolved sterols. Colin et al. (1979) also tried the normal phase for free sterols and found that lanosterol eluted first, then a whole group of mono- and di-unsaturated sterols and finally ergosterol which was well separated from others. The resolution of sterol benzoates on uPorasil was also poor (Figure 10). Accordingly, the use of such a column is not effective for routine analysis and the uBondapak C_{18} (Figure 14) was the choice for this work.

The determination of sterols in unsaponified oil was attempted by dissolving 0.5 g of rapeseed oil or corn oil in 10 ml dichloromethane and injecting 10 ul into the column. Many peaks followed this injection and so the

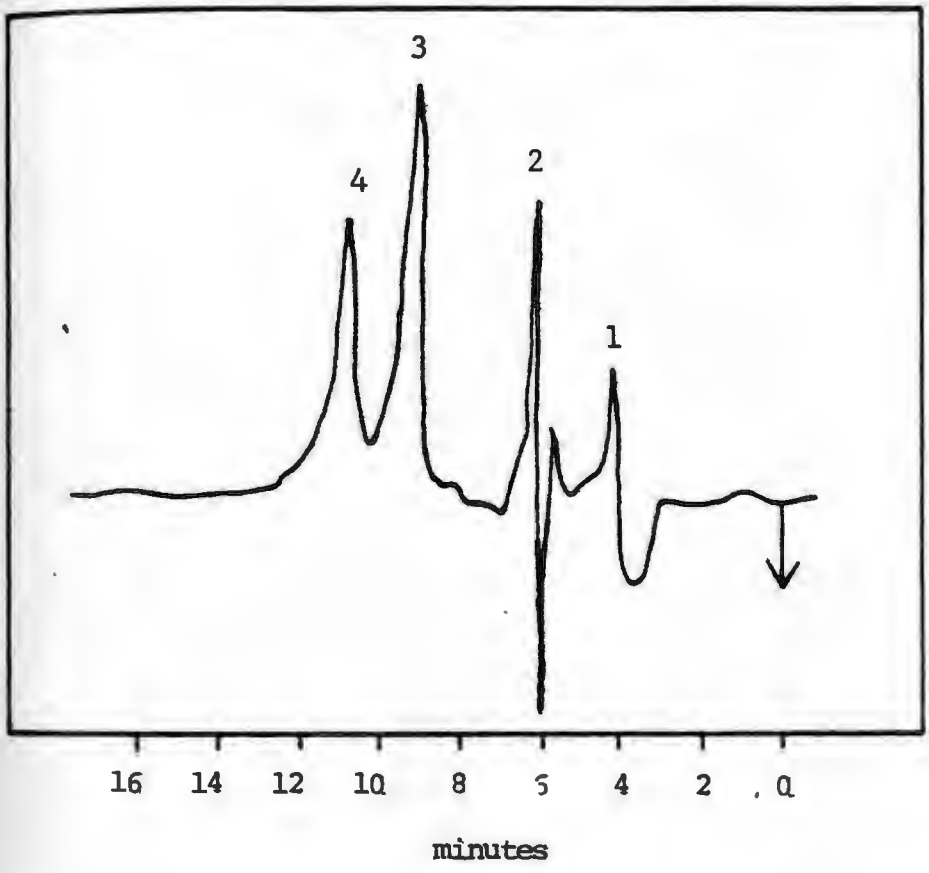


Figure 9. Separation of corn oil sterols on uPorasil column, eluted with mobile phase acetonitrile:hexane, 90:10, and detected at 200nm. Peaks 1 and 2 are for solvent and peaks 3 and 4 are for sterols.

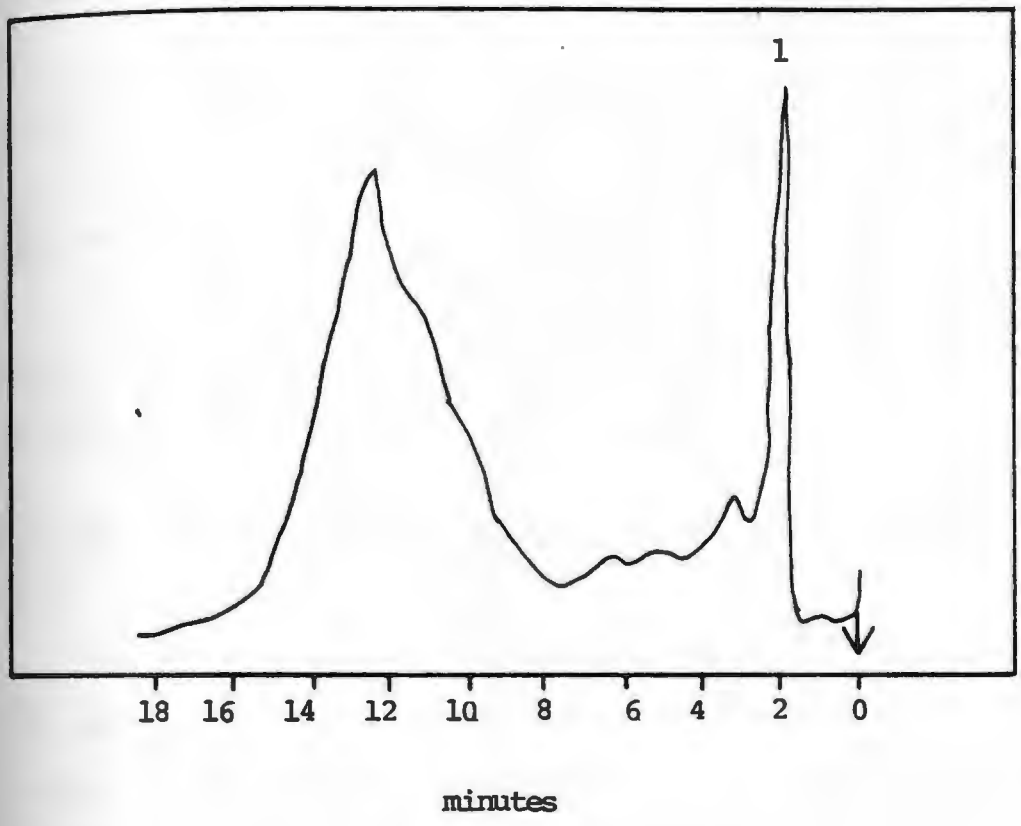


Figure 10. Corn oil sterol benzoates on uPorasil column, eluted with acetonitrile:dichloromethane, 90:10, detected at 230nm. Peak No. 1 is the solvent.

chromatogram was confusing, as shown in Figure 11. Not only are the small amounts of free or esterified sterols (1% of the total lipid) masked by other lipid components but injection of crude oil is impractical because it would require extensive analysis time to allow elution of all peaks before injecting another sample. Therefore, saponification was adopted for sterol determination.

Direct injection of a diluted (isopropanol) saponification mixture did not give good results due to the large solvent peak, as shown in Figure 12.

Chromatography of free sterols with different solvent systems

The separation of free sterols isolated from soya oil and eluted by solvent system (a); methanol:dichloromethane: water, is illustrated in Figure 13. By this mobile phase, cholesterol could not be separated from stigmasterol which is a major sterol in vegetable oils. In addition, it is difficult to work with this solvent at low wavelengths such as 200 nm where maximum absorption of cholesterol occurs. Adding isopropyl alcohol improved the detector responses but the separation of cholesterol from stigmasterol was not improved.

To prepare mobile phase (b) acetonitrile was added 1+1 to solvent system (a). Figure 14 showed a good separation for corn oil sterols but stigmasterol unfortunately still had a retention time similar to cholesterol.

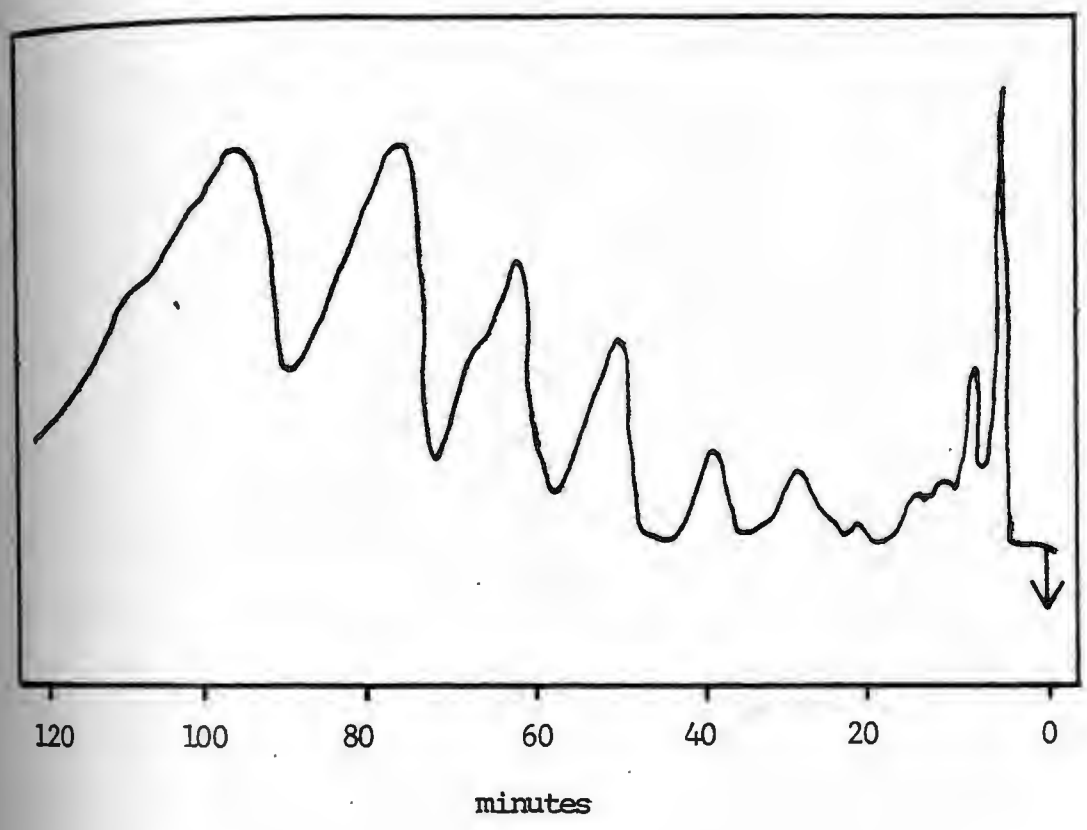


Figure 11. Separation of unsaponified rape seed oil dissolved in dichloromethane on uBondapak C_{18}, eluted with mobile phase acetonitrile 100% at flow-rate 2ml/min.

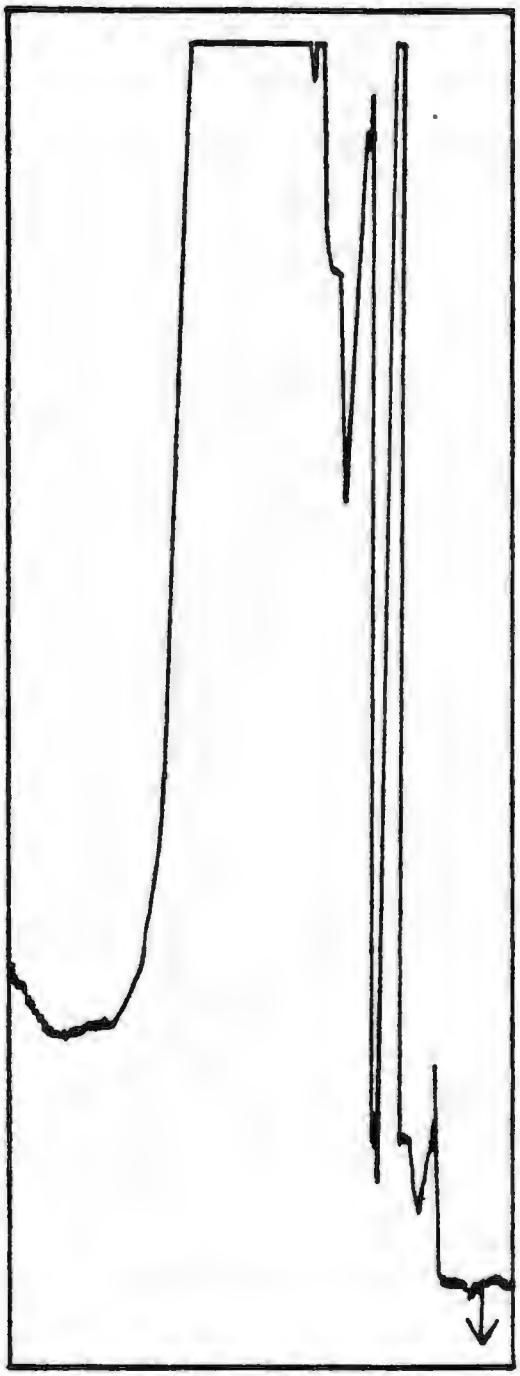


Figure 12. Separation of saponified oil after dilution.

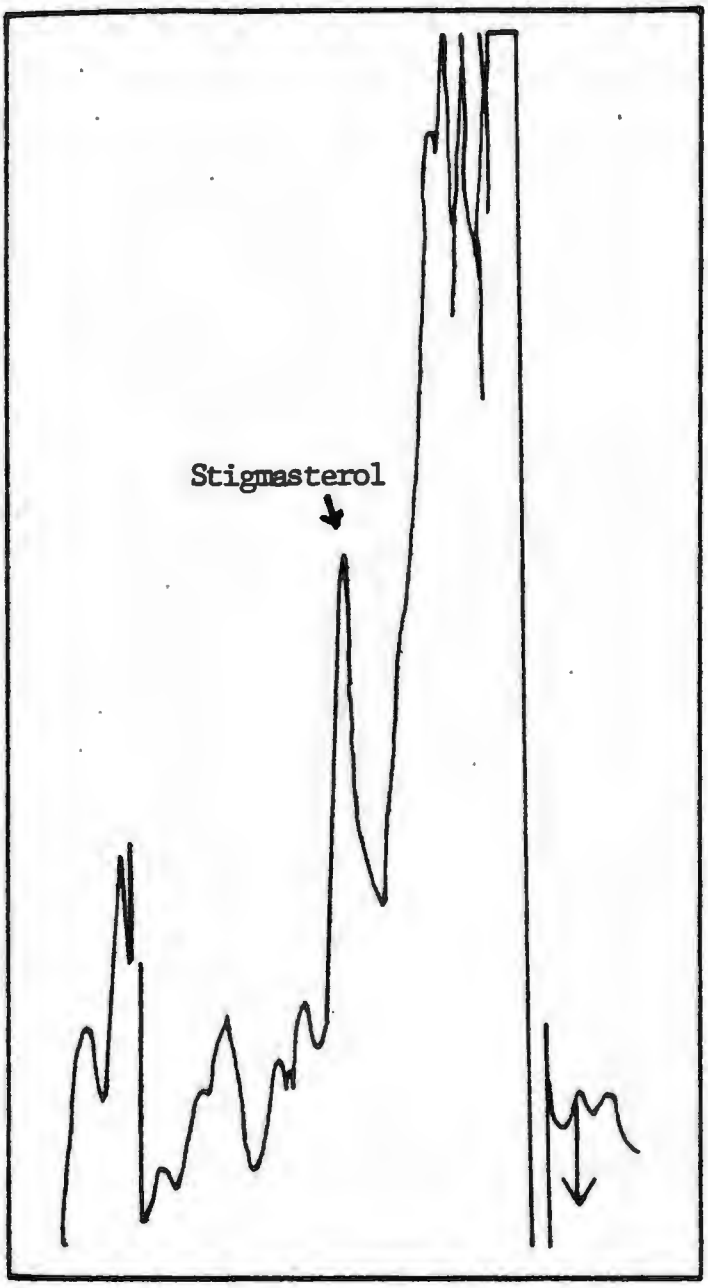


Figure 13. Corn oil sterols on uBondapak C_{18}, eluted with mobile phase methanol:dichloromethane:water, 71:12:17 (solvent a), detection at 200 nm.

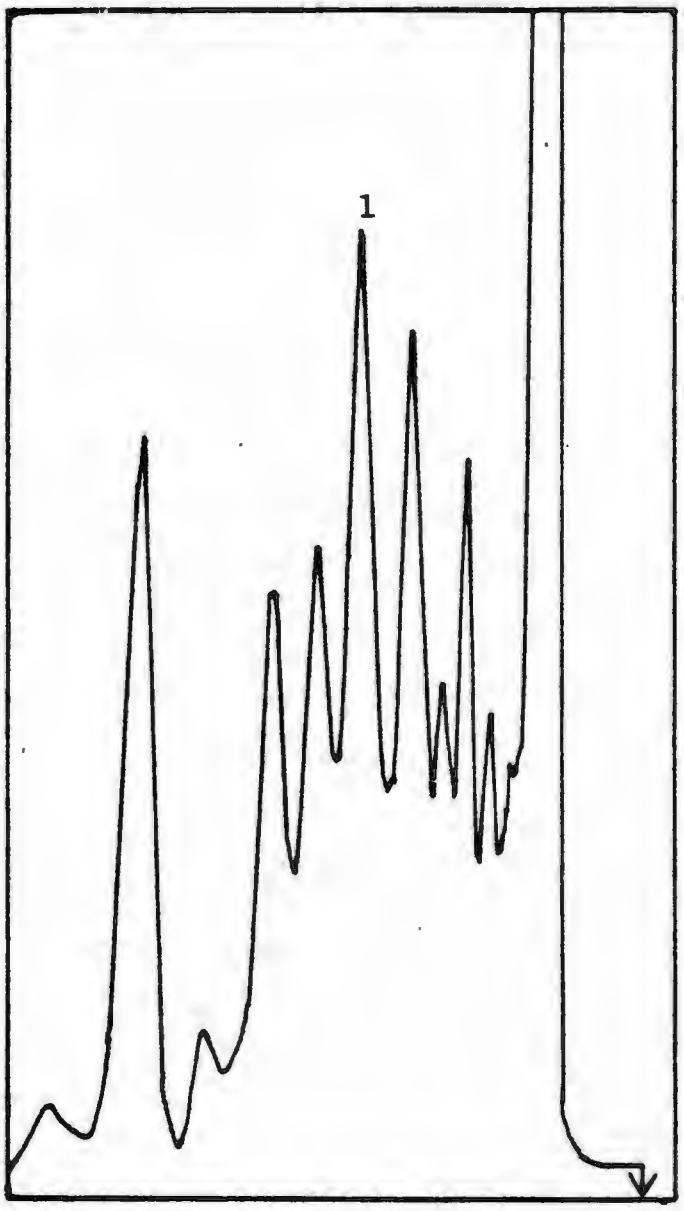


Figure 14. Corn oil sterols on uBondapak C_{18}, eluted with mobile phase (acetonitrile):(methanol-dichloromethane-water), 1:1 (solvent b) at flow rate 2 ml/min. Peak No. 1 is stigmasterol.

For mobile phase (c) more polar solvents were used: methanol:isopropanol:water. The elution was better here and the detector was easily monitored at 200 nm. But, when cholesterol was injected with corn oils sterols, the cholesterol peak chromatographed with two minor peaks, as shown in Figure 15.

Mobile phase (d), isopropanol:acetonitrile, gave better separation (Figure 16) with only one minor peak in the cholesterol region. On chromatographing mixtures of lard and corn oil, the cholesterol coming from lard did not show up clearly, perhaps due to the fact that the proportion of phytosterols in corn oil (1%) is much higher than that of cholesterol in lard (0.1%) (USDA, 1979). In Figure 17, 25% lard in corn oil could barely be detected.

A mixture of plant sterols was obtained from Applied Science Co. They reported that the mixture contained four plant sterols; B-sitosterol, stigmasterol, campesterol and brassicasterol. When the mixture was analyzed (Figure 18) three peaks came out. The third peak was identified as B-sitosterol, the second one was identified as stigmasterol plus campesterol, by comparison with individual standards of these sterols. The first peak is presumed to be brassicasterol, but this could not be verified because a brassicasterol standard was not available. Under the same conditions cholesterol came out at the same retention time as the first peak. Therefore, it was assumed that brassicasterol was the phytosterol interfering with cholesterol.

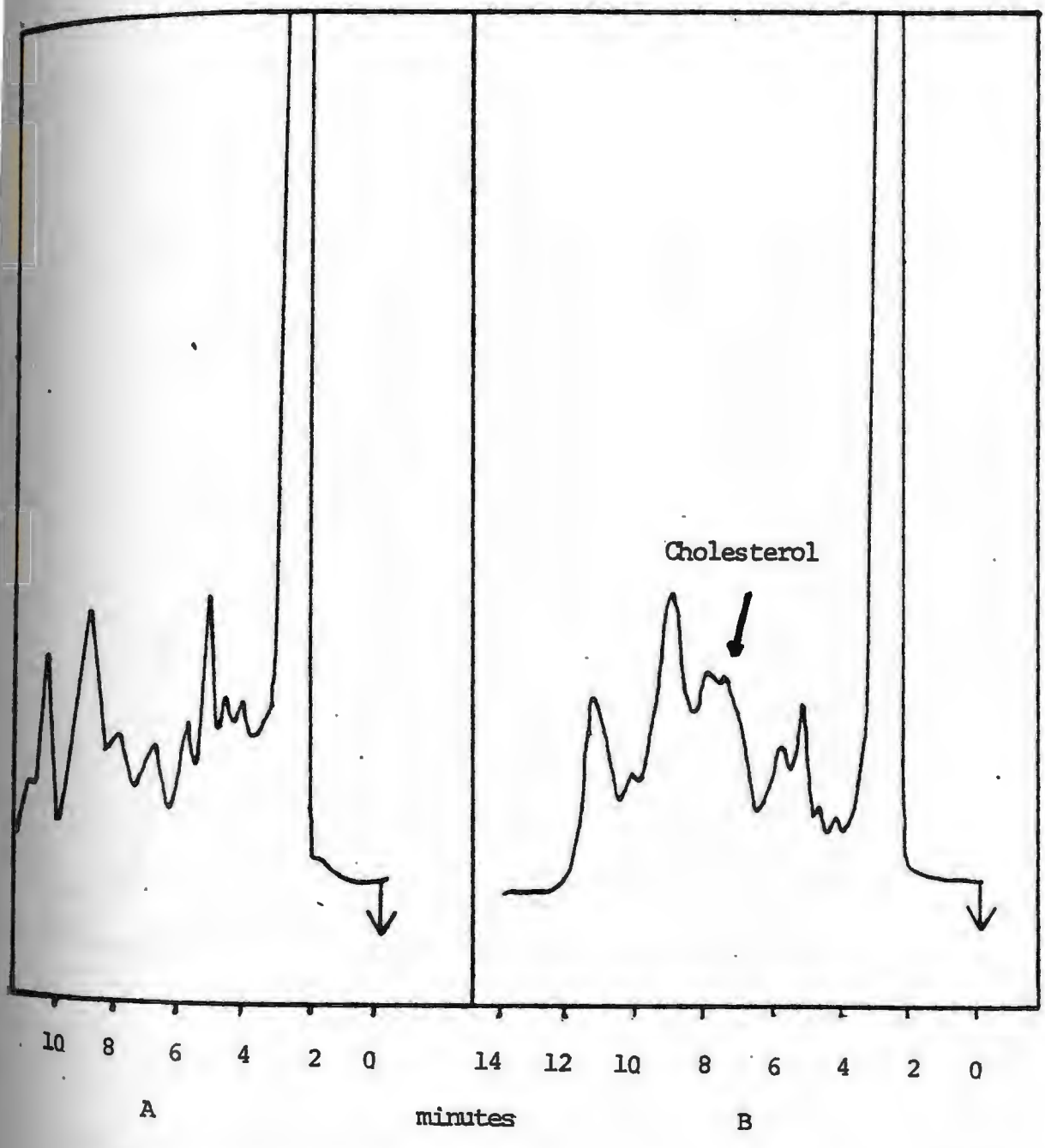
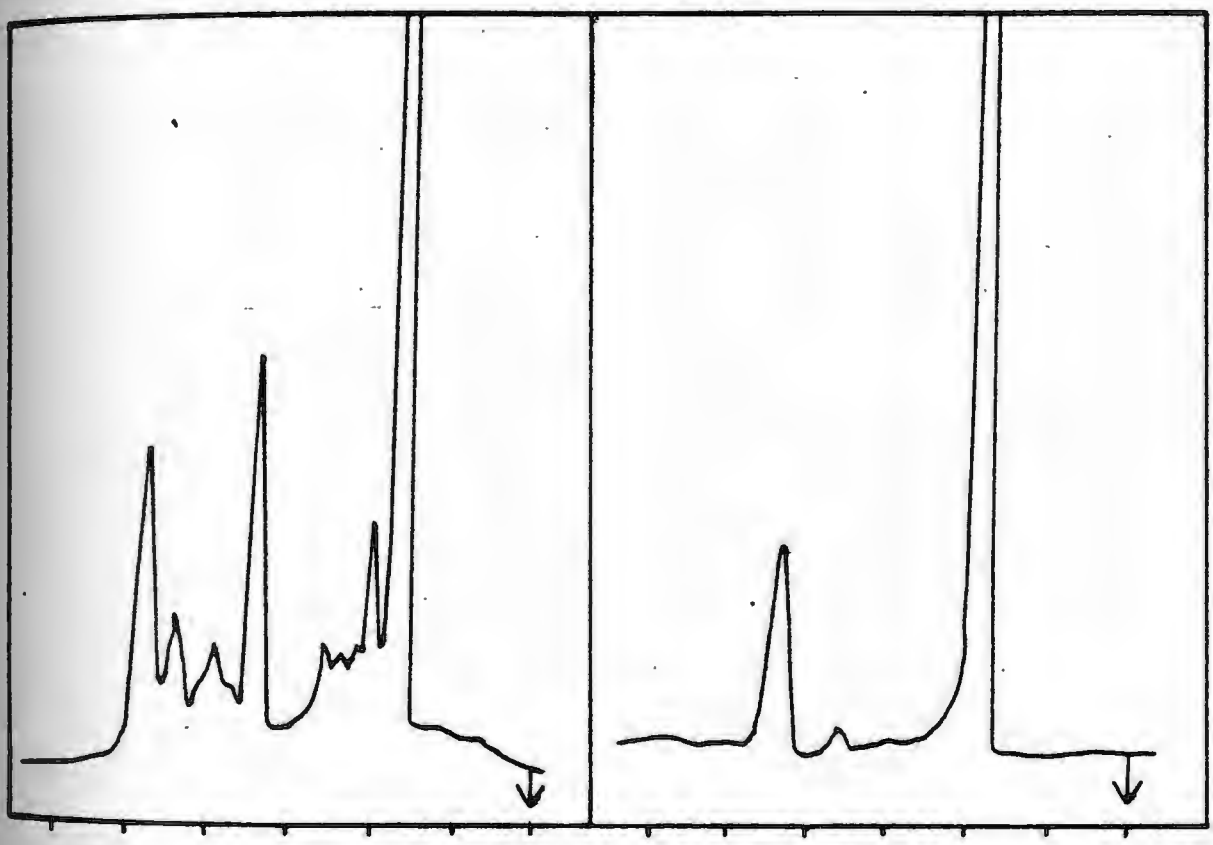


Figure 15. A: Corn oil sterols on uBondapak C_{18}, eluted with mobile phase methanol:isopropanol:water (solvent c), and detected at 200 nm. B: Same corn oil sterols containing standard cholesterol under the same conditions.



A B

Figure 16. A: Corn oil sterols on uBondapak C_{18}, eluted with acetonitrile:isopropanol, 1.0:0.5 (solvent d) detected at 200 nm. B: Elution of standard cholesterol under the same conditions.

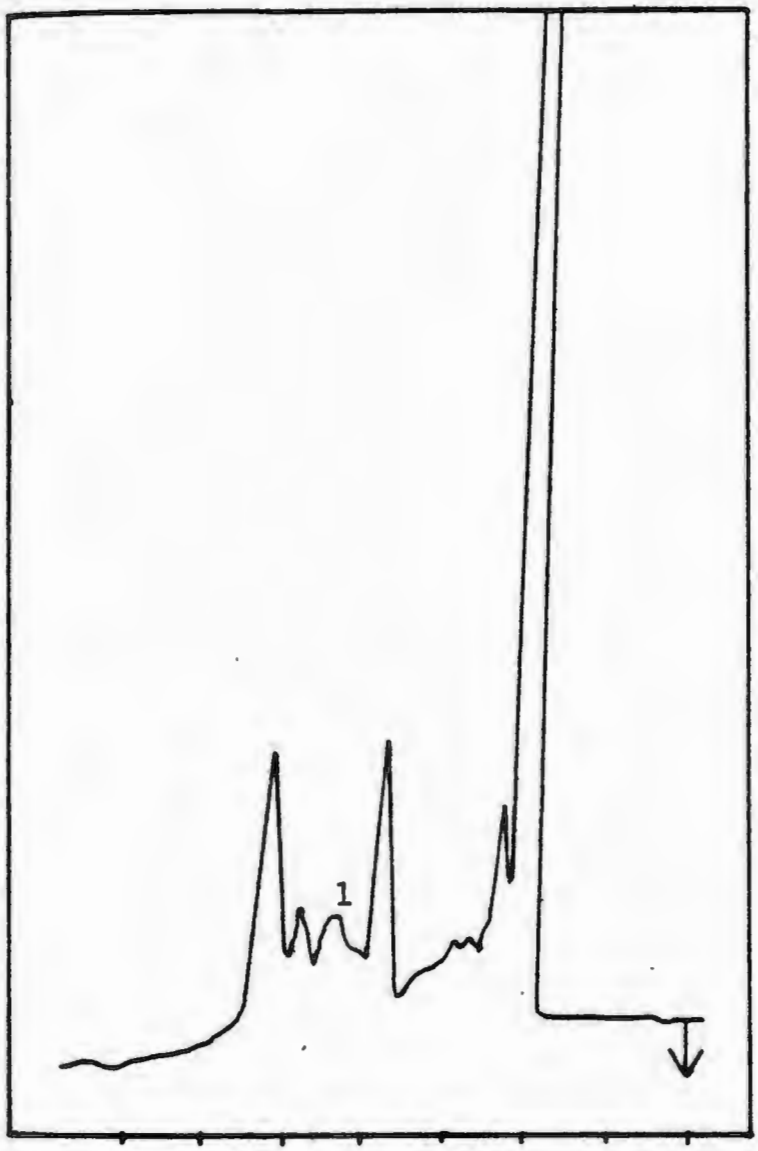


Figure 17. Free sterols extracted from a mixture of lard and corn oil (25:75), on uBondapak C_{18} eluted with 1.0:0.5 acetonitrile:isopropanol (solvent d). Peak No. 1 contains cholesterol.

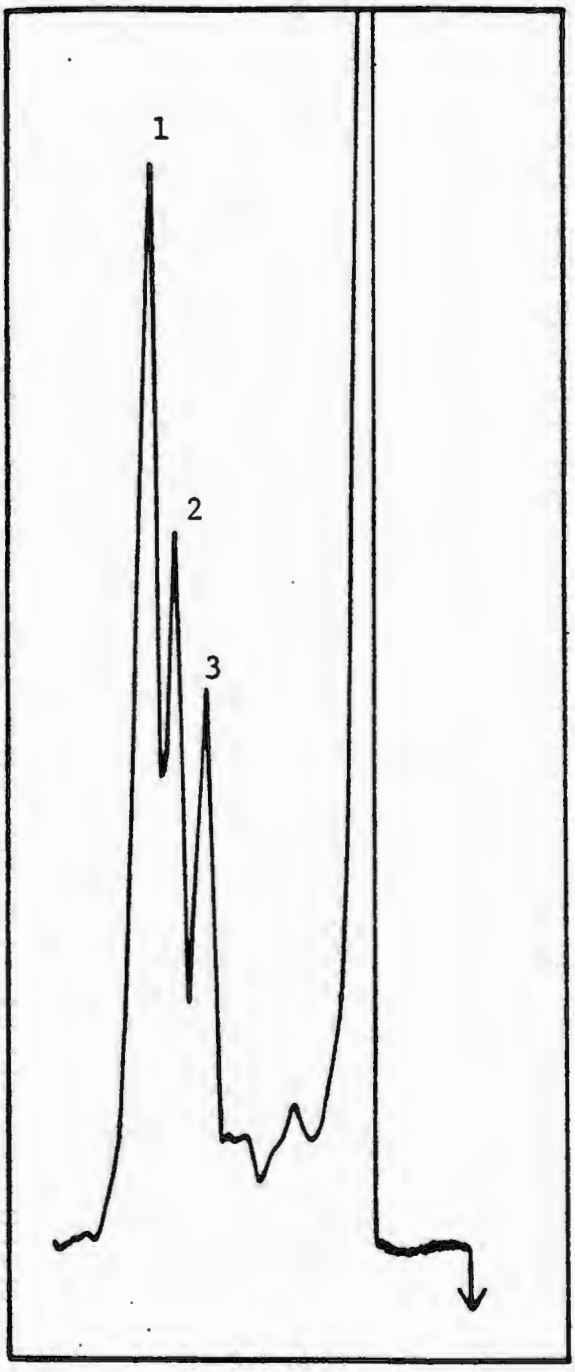


Figure 18. Separation of a plant sterol mixture containing B-sitosterol, campesterol, stigmasterol and brassicasterol, on uBondapak C_{18}, eluted with acetonitrile:isopropanol, 1.4:0.1, detected at 200 nm. Peak No. 1 is B-sitosterol, No. 2 contains stigmasterol and campesterol, and No. 3 is for brassicasterol.

Acetylation and sterol acetates

Standard cholesteryl acetate could be separated from B-sitosteryl acetate and stigmasteryl acetate, as shown in Figure 19. Acetates of cholesterol and stigmasterol were successfully prepared by heating cholesterol and stigmasterol for 15 minutes in a water bath with acetic anhydride alone, but extracts of saponified oils did not chromatograph well after acylation. This approach was not pursued further for the following reasons: (1) The acetates of sterols absorb UV radiation at very low wavelength (Grasselli and Ritchey, 1975) and were less sensitive than the benzoate derivatives, which could be analyzed at 230 nm, a more convenient wavelength, and (2) cholesteryl acetate could not be separated from some phytosteryl acetates. Rees et al. (1979) had used solvent (a), methanol:chloroform:water, to separate sterols acetates on Bonadapak C_{18}. His results showed that cholesteryl and brassicasteryl acetate have the same retention time and that stigmasteryl and campesteryl acetate were not separated from each other at the same conditions.

Benzoylation and sterol benzoates

For the above reasons, the investigation was shifted to benzoylation. The only commercially available standard sterol benzoate which could be obtained was cholesterol benzoate. Therefore, the other sterol benzoates were made from standard free sterols. The benzoates of sterols gave

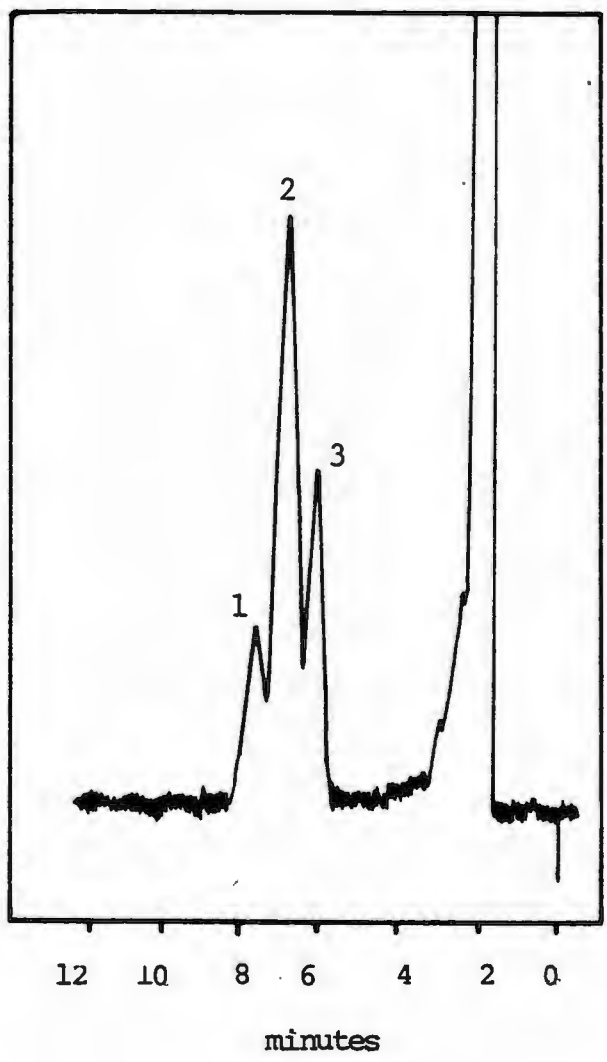


Figure 19. Separation of standard acetates of cholesterol, stigmasterol and B-sitosterol, on uBondapak C_{18}, eluted with 100% acetonitrile at flow-rate 2 ml/min., detected at wavelength 200 nm. Peak No. 1 contains B-sitosteryl acetate, Peak No. 2 contains stigmasteryl acetate and Peak No. 3 is cholesteryl acetate.

a sensitive detection at wavelength 230 nm and the benzoylation was reproducible. Fitzpatrick and Siggia (1973) used pyridine as solvent for the benzoylation reaction, while Blau and King (1978) describe a procedure using sodium hydroxide. Both procedures were attempted and use of sodium hydroxide was adopted for the following reasons: (1) Pyridine can form a compound with benzoyl chloride that might interfere in the chromatogram, (2) a lot of washings especially with hydrochloric acid are needed when pyridine is there. These are laborious and may cause loss of sample, (3) pyridine has a sharp smell which persists in the lab and could be harmful to health in the long term, and (4) the longer time consumed by pyridine method.

Plant oils

Both reference sterols and sterols isolated from some oils and fats were benzoylated for the purpose of this work. A variety of mobile phases were tried to achieve the best separation for cholesteryl benzoate from the benzoates of major phytosterols (B-sitosterol, stigmasterol and campesterol). Figures 20 and 21 show the interference between stigmasteryl benzoate and cholesteryl benzoate and how the separation improved when the solvent ratio was shifted toward acetonitrile and finally the best resolution which was obtained with 100% acetonitrile. Campesteryl benzoate was not separated from stigmasteryl benzoate (Figure 21). When a mixture of cholesteryl benzoate and major phytoster-

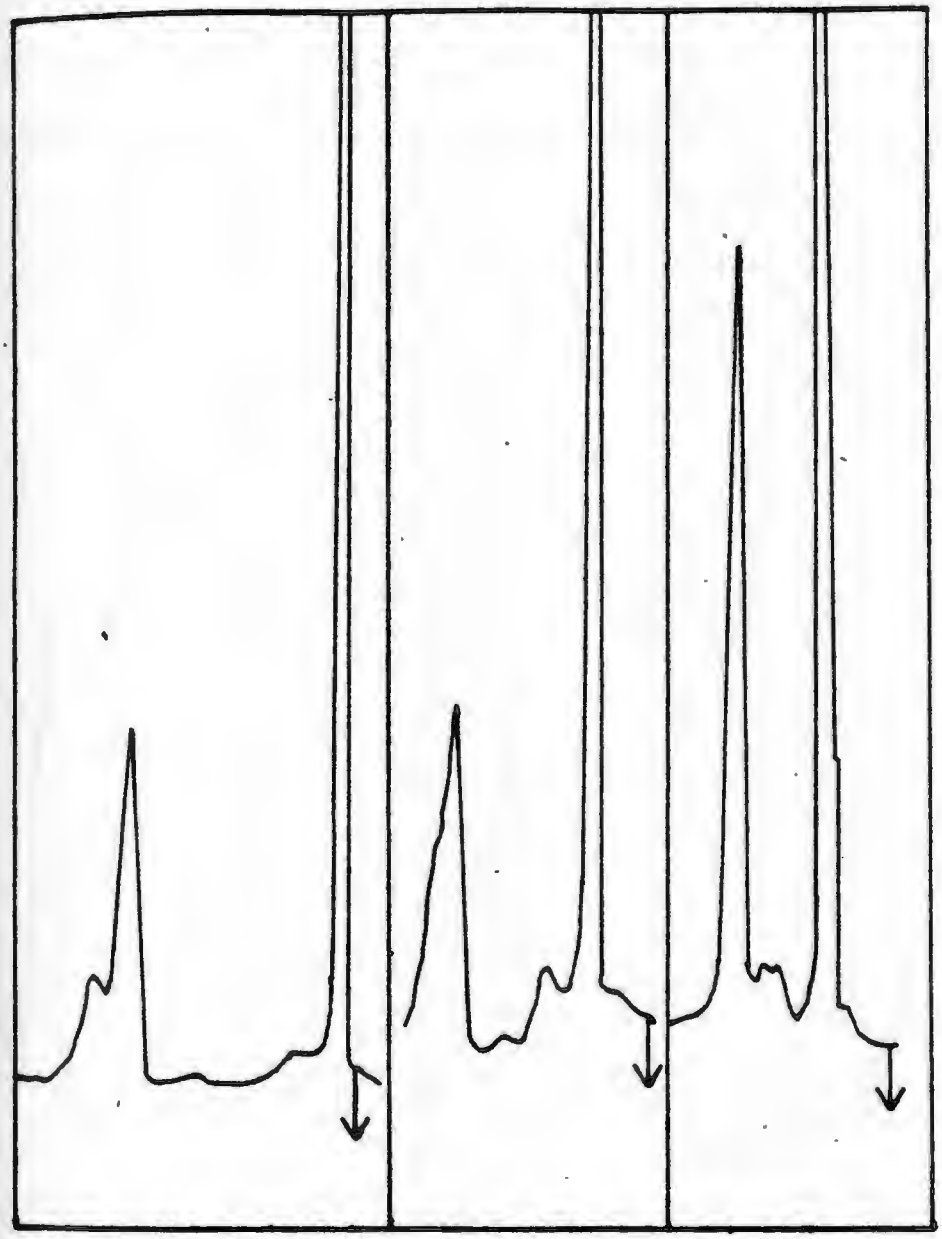


Figure 20. The effect of mobile phase ratio on the separation of a mixture containing cholesteryl benzoate and stigmasteryl benzoate, on uBondapak C_{18}, eluted with acetonitrile:isopropanol, 1:0.5 in solvent A, 1.5:0.2 in solvent B and 1.9:0.1 in solvent C, detection at 230 nm.

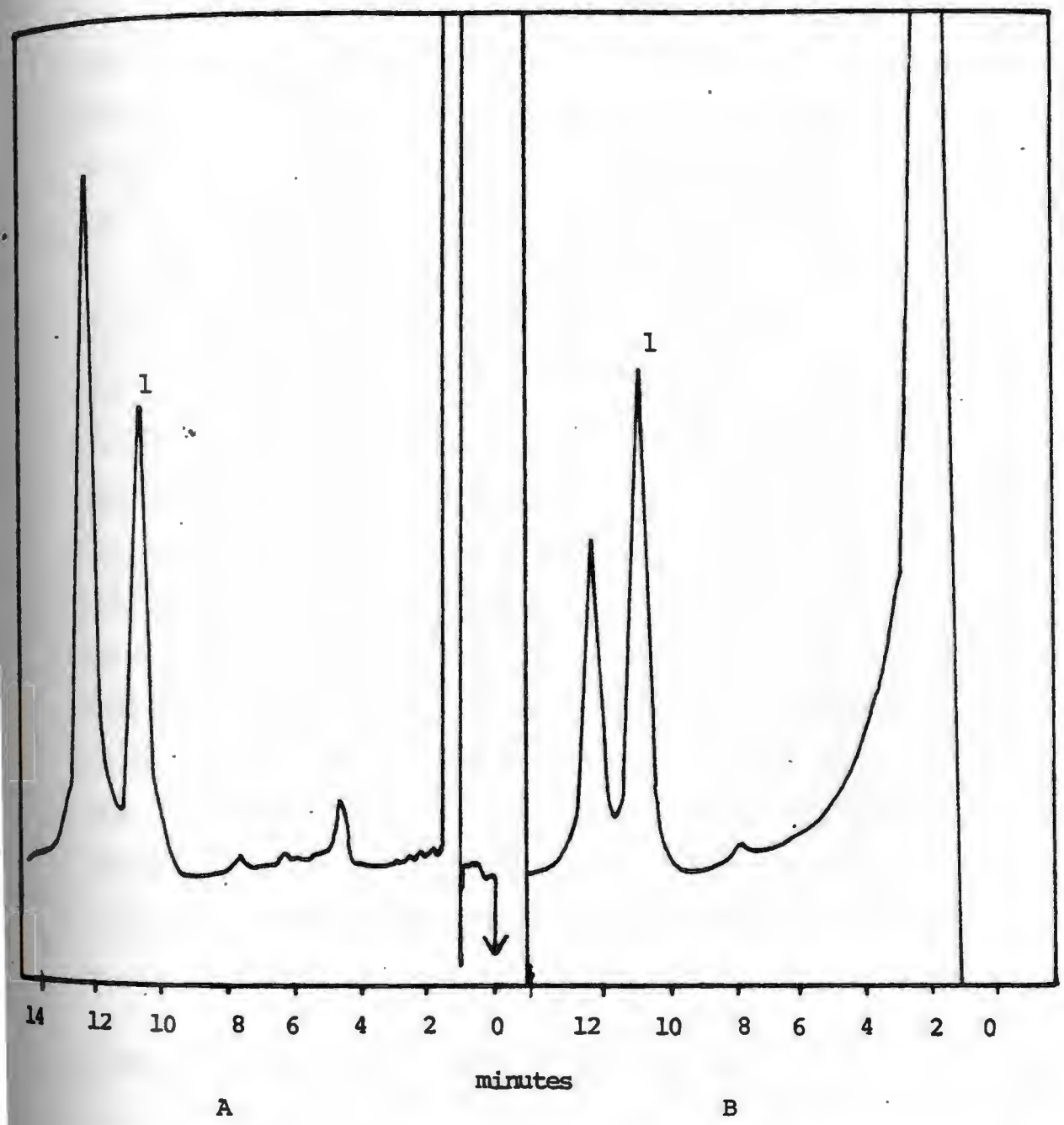


Figure 21. A: Separation of cholesteryl benzoate and stigmasteryl benzoate. B: Separation of cholesteryl benzoate and campesteryl benzoate. The benzoates were eluted in uBondapak C_{18} column with mobile phase acetonitrile 100%, at flow-rate 1 ml/min. Peak No. 1 is cholesteryl benzoate.

ol benzoates was injected, the mixture gave a chromatogram with three peaks; the first peak was cholesteryl benzoate, the second peak contained both stigmasteryl and campesteryl benzoate and the third peak was B-sitosteryl benzoate (Figure 22). Thus, cholesteryl benzoate was separated from the benzoates of major phytosterols of vegetable oils.

The patterns of sterol benzoates for some oils and fats which are related to margarine are shown in Figures 23, 24, and 25, and Table 2. Peak No. 1 in each oil is B-sitosterol, Peak No. 2 is stigmasteryl + campesteryl benzoates, while the third peak (No. 3) contains other phytosterol benzoates that have the same retention time of cholesteryl benzoate. Rapeseed oil has a higher "cholesteryl" peak due to the fact that it contains brassicasterol whose benzoate interferes with cholesteryl benzoate. So, that should be borne in mind before taking a decision about the margarine sterol content. Also, the peak which is smaller than 10% in the other plant oils and interferes with cholesteryl benzoate is expected to include Δ^5-avenasterol.

Comparison of vegetable oil results with literature

The numbers in parenthesis in Table 2 are percentages expected under the three peaks of interest based on the vegetable oil compositions (Table 1) compiled by Weihrauch and Gardner (1978). The agreement with the theoretical is reasonably good. Unfortunately, the peak of major interest, the "cholesterol peak," shows the most discrepancies.

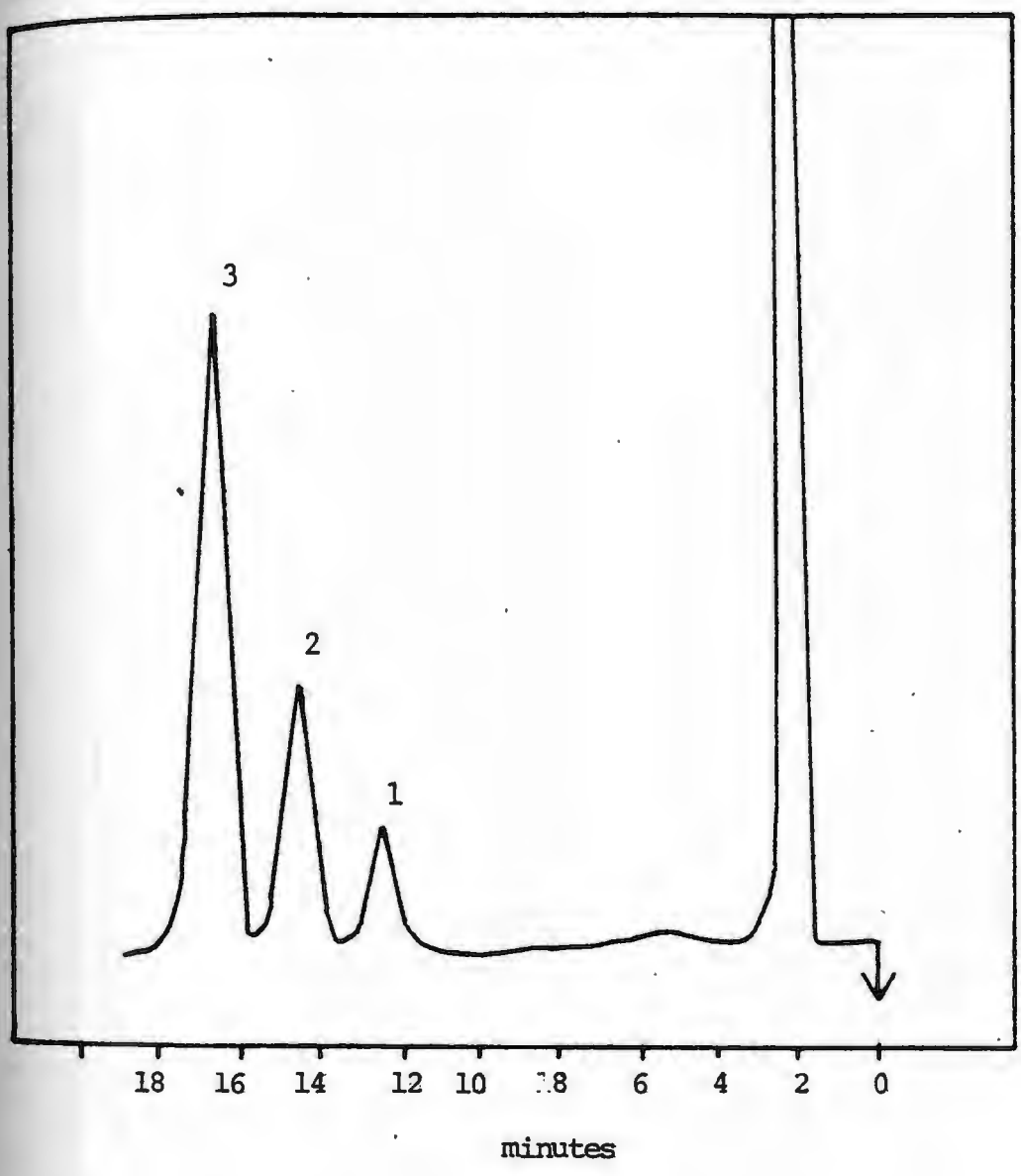


Figure 22. Separation of a mixture of benzoates of cholesterol, stigmasterol, campesterol and B-sitosterol, on uBondapak C_{18}, eluted with mobile phase acetonitrile 100%, at flow-rate 1 ml/min, detected at 200 nm. Peak No. 1 is cholesteryl benzoate, Peak No. 2 contains stigmasteryl benzoate plus campesteryl benzoate, and Peak No. 3 is for B-sitosteryl benzoate.

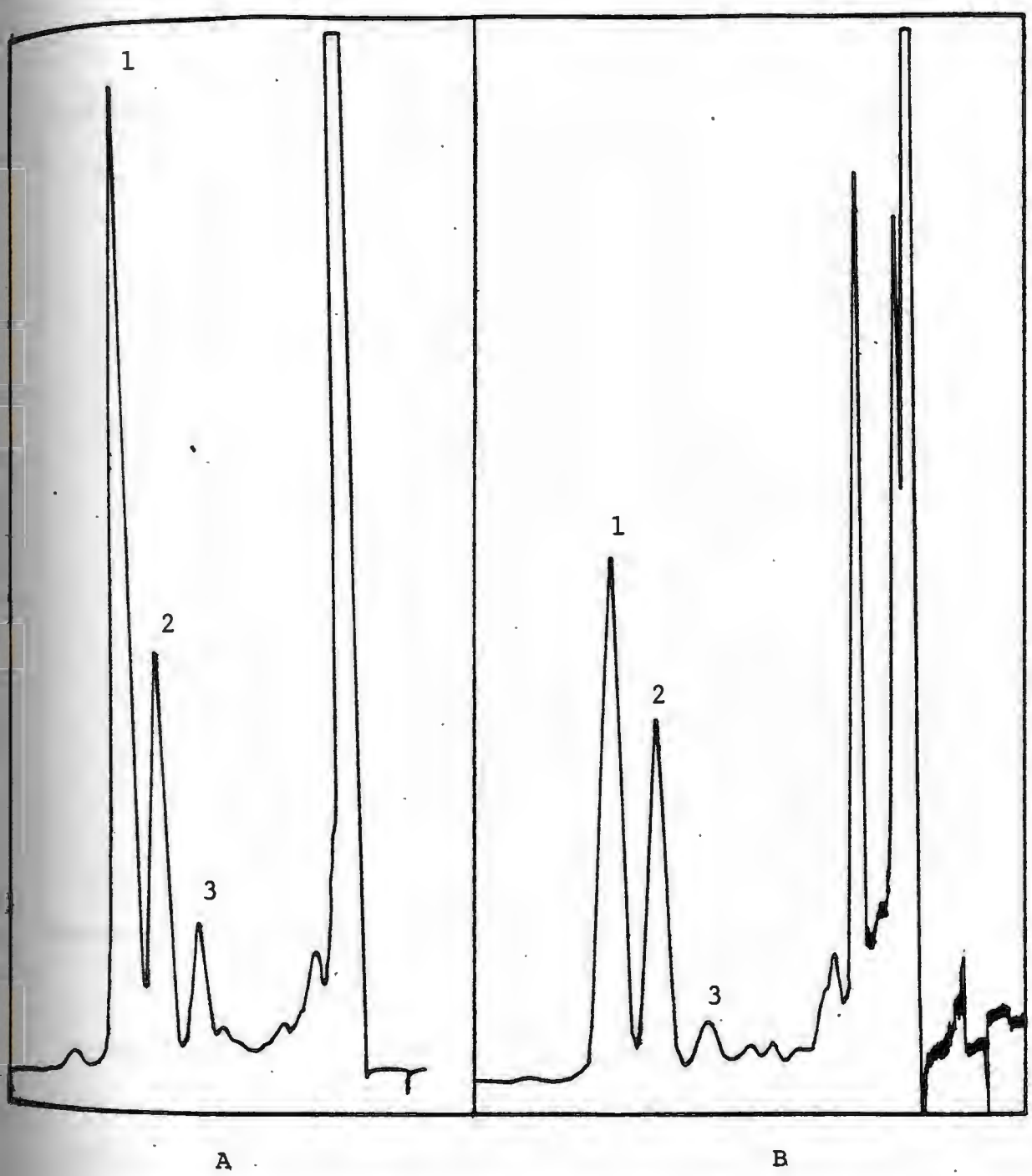


Figure 23. Sterol benzoates from (A) corn oil and (B) soya bean oil. They were eluted on uBondapak C_{18} column, with mobile phase acetonitrile 100% at flow rate 2 ml/min, and detected at 230 nm.

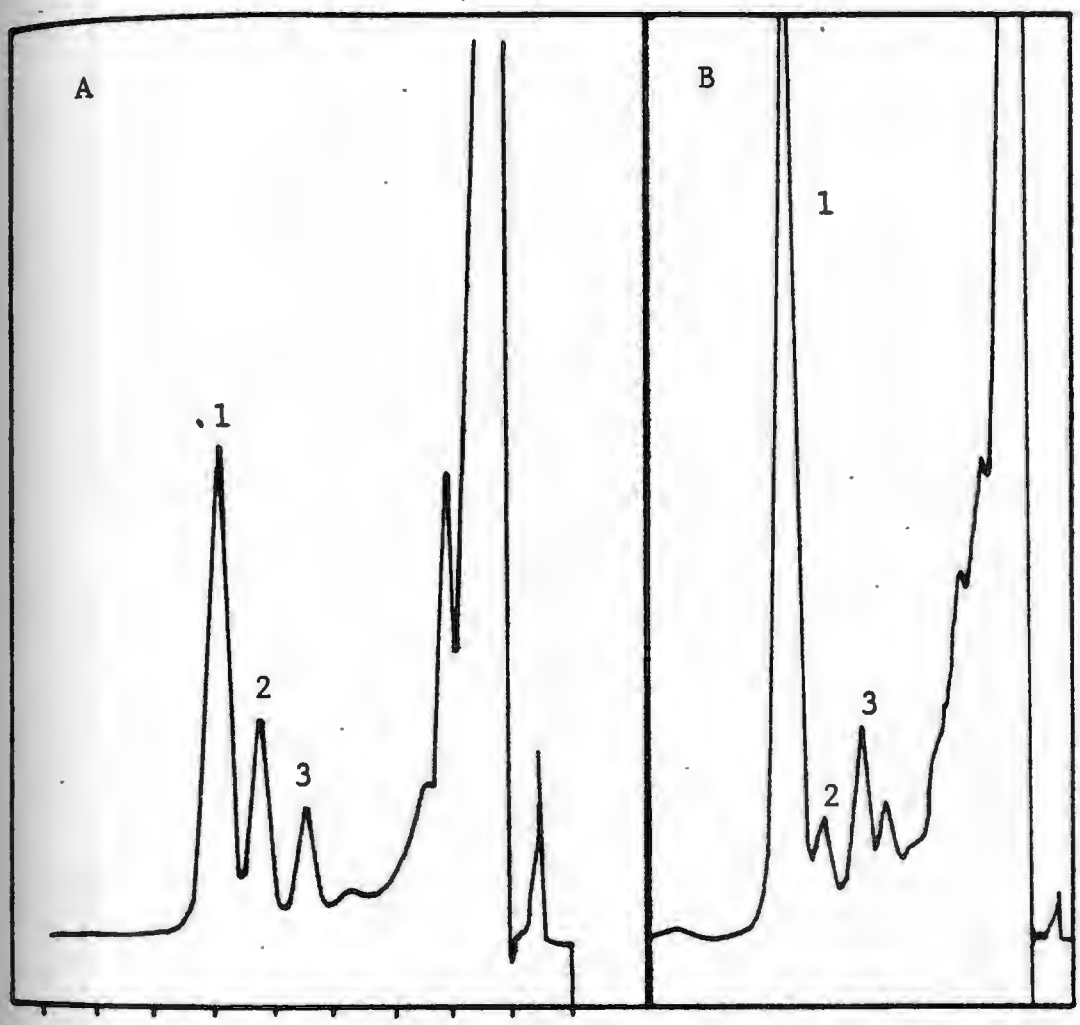


Figure 24. Sterol benzoates from (A) Peanut oil, (B) Olive oil. They were eluted on uBondapak C_{18} column with mobile phase acetonitrile 100% at flow rate 2 ml/min. and detected at 230 nm.

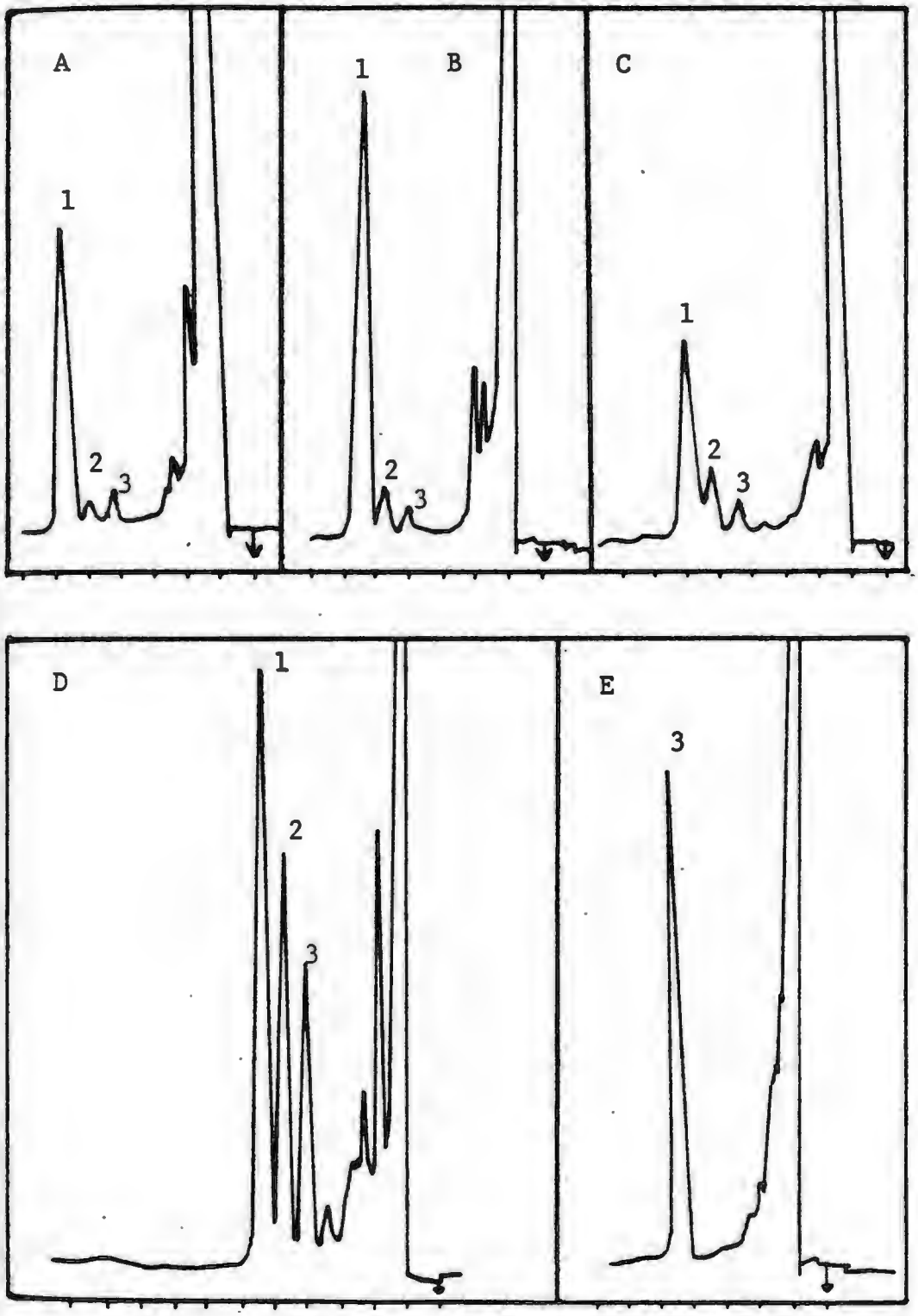


Figure 25. Sterol benzoates from (A) Walnut oil (B) Cotton seed oil, (C) Safflower oil, (D) Rapeseed oil and (E) Lard. They were eluted on uBondapak C_{18} column with mobile phase acetonitrile 100% at flow rate 2 ml/min, and detected at 230 nm.

TABLE 1. Sterol composition of some common plant oils.[a]

	B-sitosterol	Campesterol	Stigmasterol	Avenesterol	Stigmastanol	Brassicasterol	Cholesterol
	%	%	%	%	%	%	%
Soy bean oil	55.7	21.3	21.3	0.5	0.5	-	-
Corn oil	72.4	16.5	7.9	2.3	0.6	-	-
Safflower Oil	52.0	11.0	9.0	6.0	21.4	-	-
Cottonseed Oil	92.6	6.1	tr	1.2	tr	tr	-
Olive Oil	86.9	3.4	1.1	8.5	tr	-	-
Rapeseed Oil	55.2	30.4	0.4	2.4	-	10.8	0.8
Sunflower Oil	64.1	9.5	10.3	6.9	8.3	-	tr

[a] Calculated from Weihrauch and Gardner, 1978.

TABLE 2. Area percent of each peak to the total of the three major phytosterols peaks found in vegetable oils.[a]

	Cholesteryl[b] +phytosteryl benzoates	Stigmasteryl +campesteryl benzoates	B-sitosteryl benzoate
	found	found	found
Soya bean oil	4.4 (0.5)[c]	36.0 (43.6)	59.6 (56.4)
Corn oil	8.4 (2.3)	26.1 (24.4)	65.5 (72.5)
Cotton seed oil	4.7 (1.2)	6.1 (6.1)	86.9 (92.6)
Safflower oil	10.0 (7.8)	19.7 (25.6)	70.2 (66.6)
Olive oil	9.7 (8.5)	5.0 (4.5)	85.2 (86.9)
Rapeseed oil	14.4 (13.3)	31.3 (31.0)	54.3 (55.6)

a Single determinations.

b Cholesterol figures include cholesterol, brassicasterol and other minor sterols such as Δ^5-avenasterol and Δ^7-avenasterol because their benzoates are expected to elute at the same retention time.

c Calculated from data compiled by Weihrauch and Gardner, 1978.

However, the patterns in different samples for the same kind of oil were shown to be reproducible (Figure 26 and 27). Table 3 shows the results after analysis of several oil samples.

Analysis of mixtures of vegetable oils and lard

Cholesterol constitutes around 95% of the lard sterol content as shown in Figure 28 and in the literature (Dence, 1980). Cholesterol is present in vegetable oils but at small amounts, 0-4% of the total sterol (Weihrauch and Gardner, 1978 and Appelqvist and Ohlson, 1972). Vegetable sterols are not present in lard according to Figure 28 and Nes and McKean (1977). For the purpose of detecting animal fat in vegetable margarines, mixtures of lard and vegetable margarines were prepared at the ratios of 0, 25, 50, 75, and 100% lard. The sterols were extracted, benzoylated and analyzed by HPLC. Figures 29 and 30 and Tables 4 and 5 show the trend of cholesteryl benzoate peak to increase in accordance with the amount of lard added to soya bean oil margarine or corn oil margarine. From these results we can deduce that the cholesterol value is a good indication for the presence of lard in a vegetable margarine. If the peak exceeds 15%, it is highly likely that the margarine contains more than 25% animal fat. If the cholesterol is higher than 30%, the sample certainly contains over 50% animal fat.

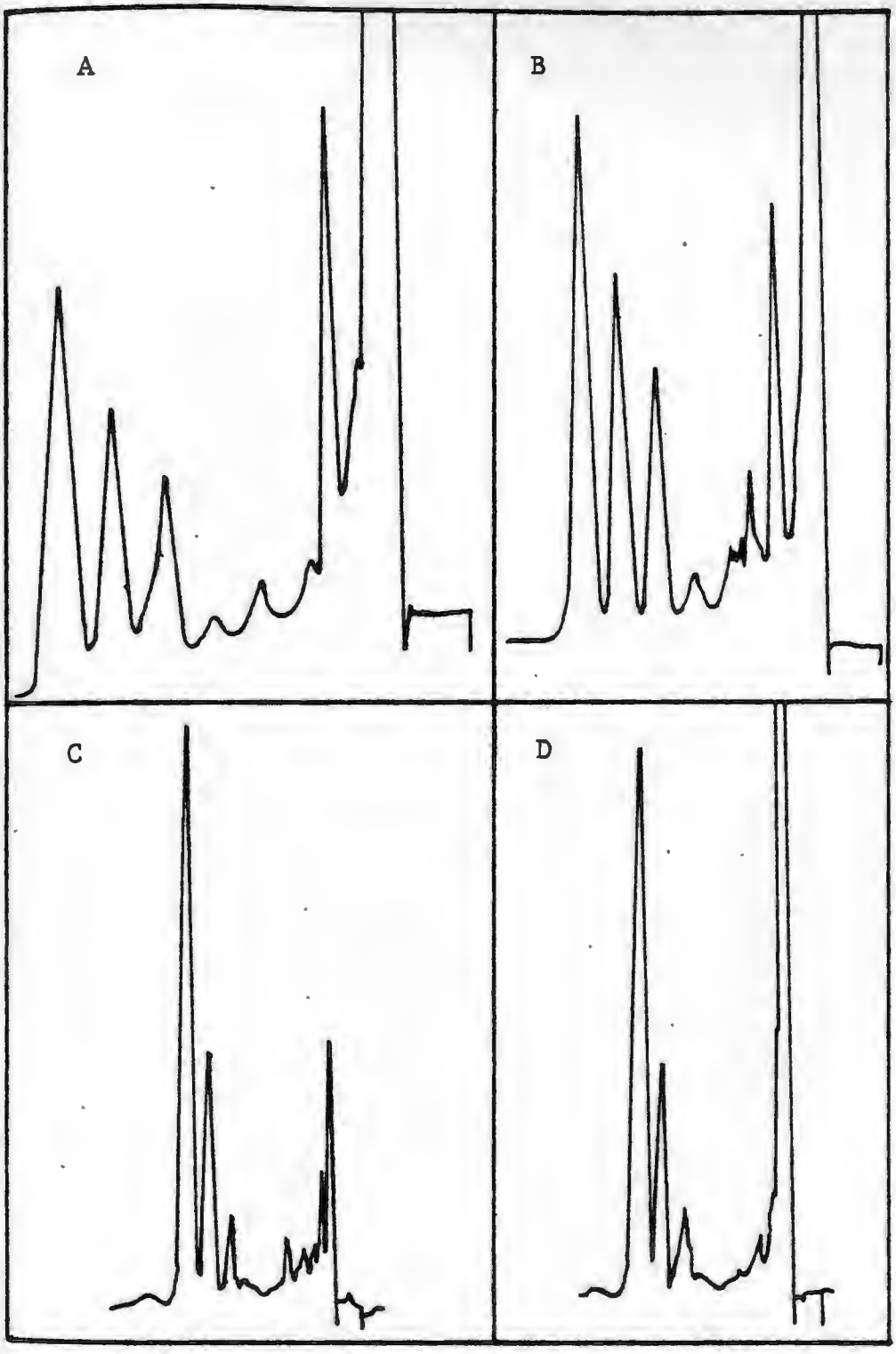


Figure 26. Sterol benzoates from different samples of two kinds of oils. A and B are rapeseed oils and C and D are corn oils.

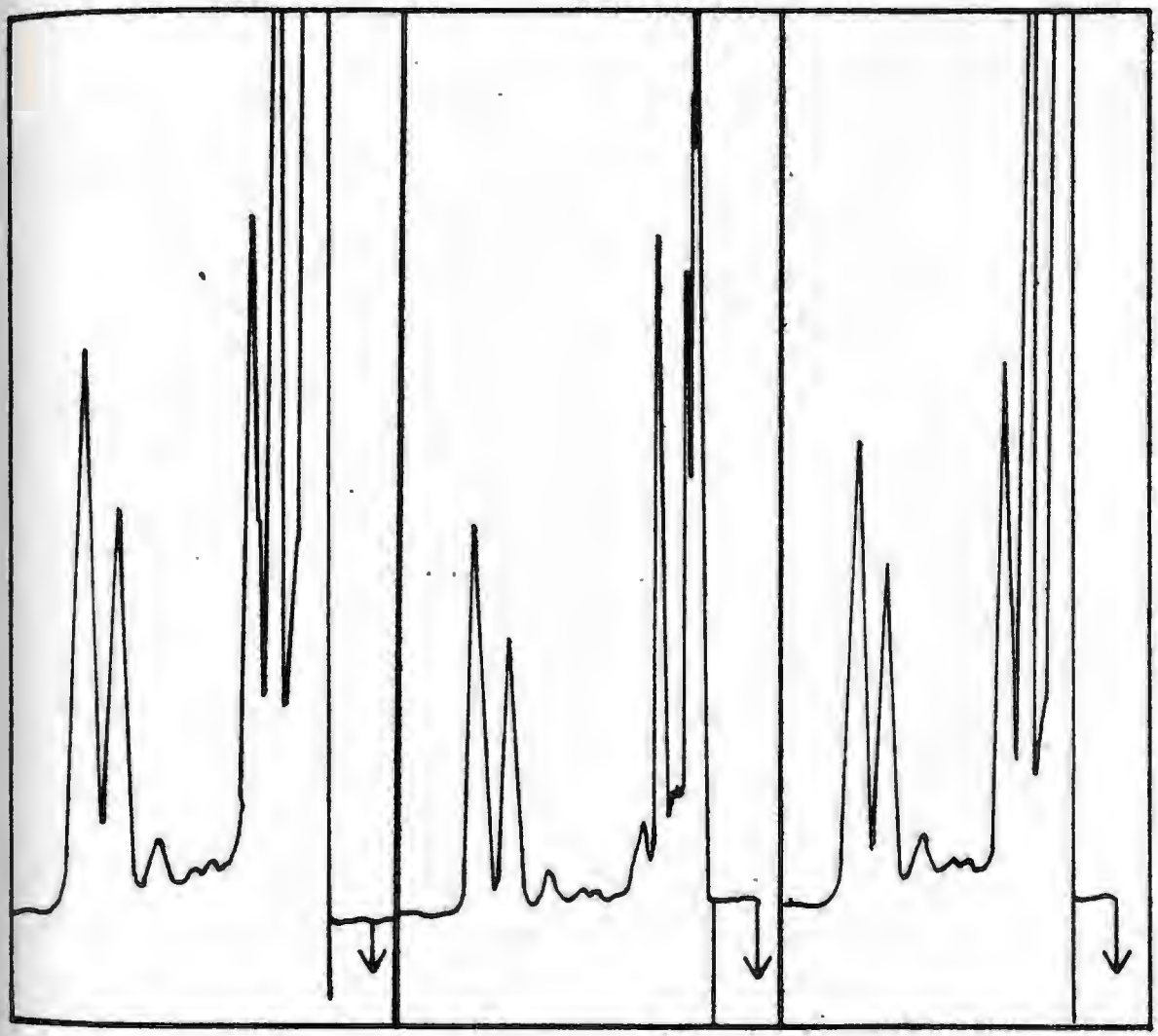


Figure 27. Sterol benzoates from three different brands of soya bean oil, eluted on uBondapak C_{18} column with mobile phase acetonitrile 100%, detected at 230 nm.

TABLE 3. Repeat analysis for samples of four oil types

Kind of oil	Cholesteryl benzoate peak[a]	Stigmasteryl + campesteryl benzoate peaks	B-sitosteryl benzoate peak
	%	%	%
Soya bean oil	4.4	36.0	59.6
-do-	4.7	37.0	58.4
-do-	4.4	37.1	58.4
Olive oil	9.7	5.0	85.2
-do-	9.3	5.3	85.3
Corn oil	8.4	26.1	65.5
-do-	6.5	24.5	68.9
Rapeseed oil	21.2	29.2	49.6
-do-	20.5	29.2	50.3

[a] Includes phytosterols such as brassicasterol.

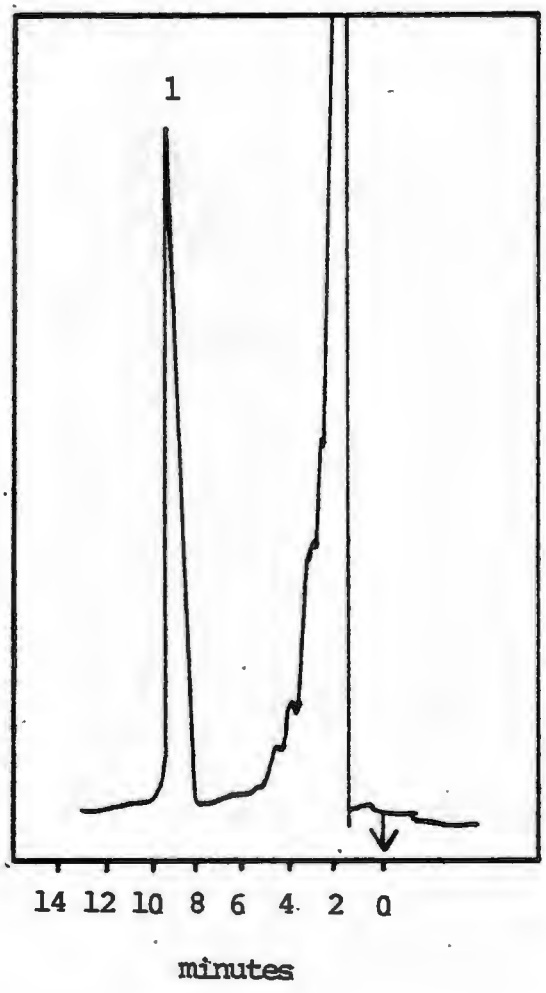


Figure 28. Separation of lard sterol benzoates on uBondapak C_{18} column 30cm x 3.9mm ID, mobile phase acetonitrile 100%, flow-rate 2 ml/min, 1 = cholesteryl benzoate.

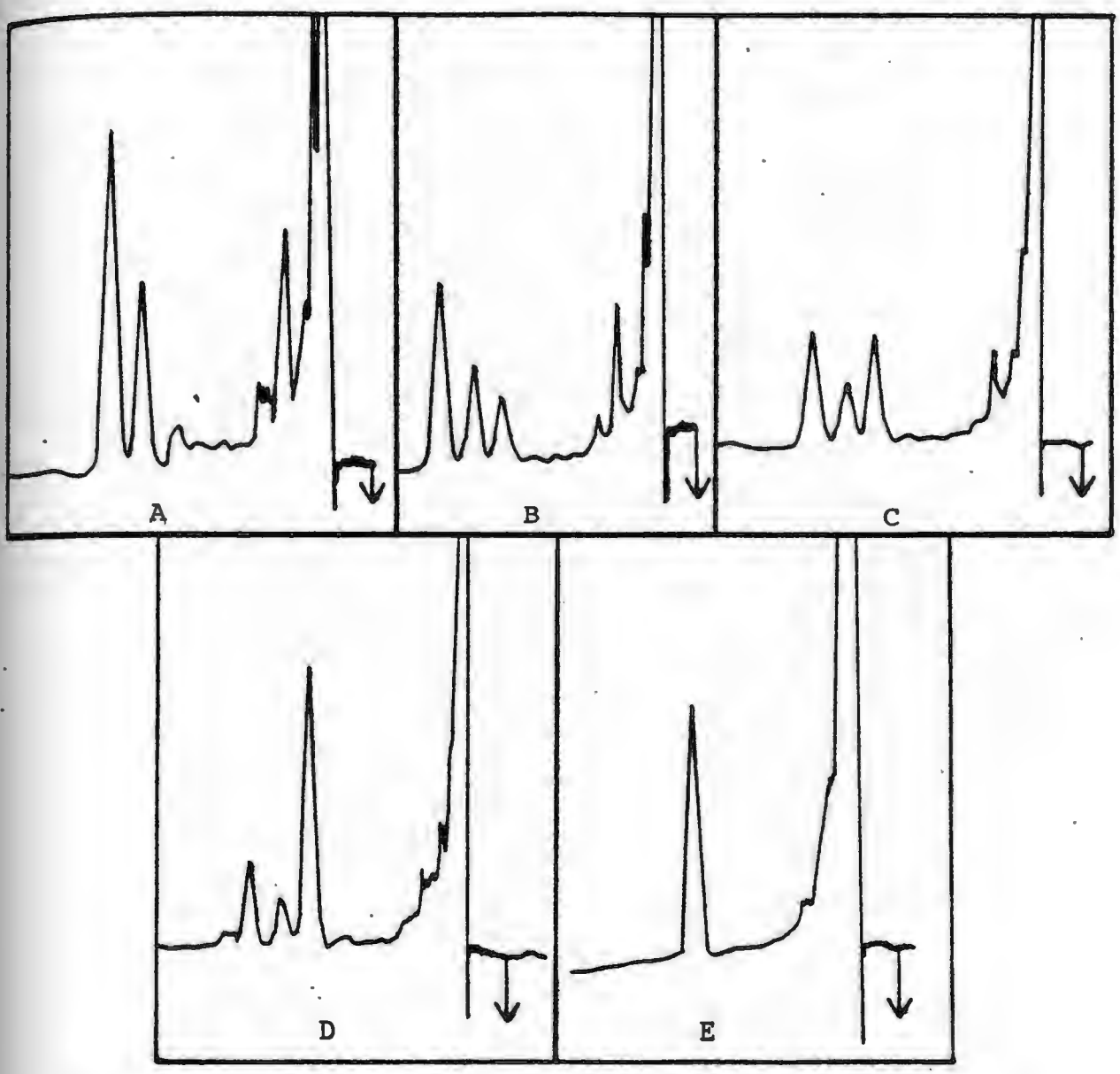


Figure 29. Sterol benzoates from mixtures of soya bean oil margarine and lard at different ratios. Chromatogram (A) 100% margarine, (B) 25% lard and 75% margarine, (C) 50% lard and 50% margarine, (D) 75% lard and 25% margarine, and (E) 100% lard. uBondapak C_{18} column with mobile phase acetonitrile 100% at flow-rate 2 ml/min and detection at 230 nm.

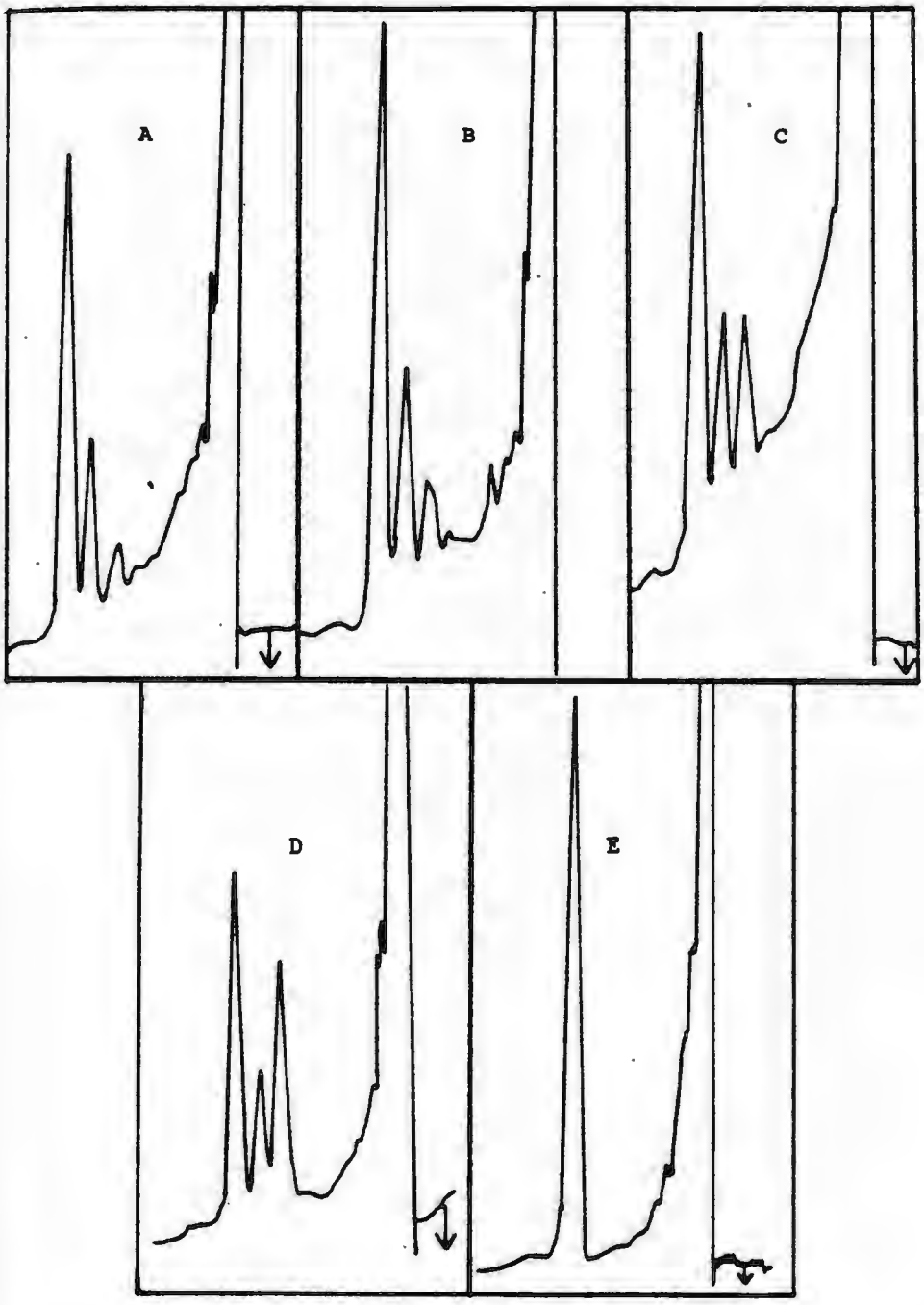


Figure 30. Sterol benzoates from mixtures of corn oil margarine and lard at different ratios. Chromatogram (A) 100% margarine, (B) 25% lard, (C) 50% lard, (D) 75% lard and 25% margarine and (E) 100% lard.

TABLE 4. Response of lard addition to soya bean oil margarine, on the basis of benzoylated sterols

Mixture		Cholesterol +phytosterol %	Stigmasterol +campesterol %	B-sito sterol %
Soya bean oil margarine	(1)[a]	7.2	31.9	60.7
	(2)	6.4	32.5	61.1
25% lard added	(1)	16.1	26.9	57.0
	(2)	18.3	28.3	53.4
50% lard added	(1)	36.1	22.0	42.0
	(2)	35.4	22.4	42.3
75% lard added	(1)	58.1	14.9	26.9
	(2)	67.5	11.9	20.8
Lard 100%	(1)	100.0	-	-
	(2)	100.0	-	-

[a] (1) and (2) = Two different margarines. Single determination of each.

TABLE 5. Area percent of mixtures of corn oil margarine and lard at different ratios.

Ratio	Cholesteryl benzoate peak	Stigmasteryl + campesteryl benzoates peaks	B-sitosteryl benzoate peak
Corn oil 100%	6.7	22.8	70.4
25% lard + 75% margarine	11.6	22.6	65.8
50% lard + 50% margarine	18.1	20.4	61.4
75% lard + 25% margarine	32.2	17.9	49.8

Conclusion

The results obtained through this work have proved that high pressure liquid chromatography is a good tool for the analysis of sterols. Although, the separation was difficult between cholesterol and some minor phytosterols, the detection of animal fat was possible. The utility of HPLC is practical when the fraudulation is more than 25% and critical at less proportions, especially if rapeseed oil were present in the margarine. However, HPLC performs separation and detection at the same time without any preparative procedures such as thin layer chromatography or digitonin precipitation, which is a good factor for obtaining more accurate results. It is convenient for such routine analysis and time-saving. Of its economical disadvantages, the deuterium lamp of the detector is expensive and it has only short life. What is recommended for achieving better results for this work is a column that can separate cholesterol from the entire vegetable oil sterol group. Sterols standards were not abundantly available and their purity was often poor. So, it is recommended to have more advanced preparative procedures for obtaining less expensive pure sterols, a task in which HPLC also could be a good tool.

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