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CHROMATOGRAPHIC STEROL ANALYSIS AS APPLIED TO THE INVESTIGATION OF MILK FAT AND OTHER OILS AND FATS

J. W. COPIUS PEEREBOOM

Government dairy station (Rijkszuivelstation)
Leiden

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FOREWORD

This book on the analytical chemistry of the sterols particularly deals with chromatographic separation and identification methods of sterols and with the application of sterol analysis to the examination of fats. The matters discussed are for the greater part founded on the author's own investigations and most of it is published now for the first time. Amongst others, this holds for the thin-layer chromatography of sterols. His description of this analytical technique should be welcome to the several kinds of chemists who have to do with sterols in their respective fields, such as organic chemistry, fat chemistry, food chemistry and physiological chemistry.

Also for the first time a detailed and complete survey of the analysis of fats by means of sterols is provided by this work. At the Government Dairy Station (Rijkszuivelstation) in Leiden this system of analysis has been built up during well over fifty years of research and development. The publication of this book gives an occasion to pay attention now to these activities of the Government Dairy Station.

According to the Code of Principles concerning Milk and Milk Products, drawn up under the auspices of the Food and Agriculture Organization and of the World Health Organization of the United Nations, and accepted by the governments of fifty-seven countries, the term "milk" shall mean exclusively the normal mammary secretion obtained from one or more milkings without either addition thereto or extraction therefrom. The provisions of the Code are equally applicable in the same sense to milk products therein considered and they evidence the importance attached now throughout the world to the genuineness of milk and dairy products, implying that of the milk fat in all these products. By this, no small burden is laid on the shoulders of the analytical chemist to whom it falls to give a judgment in this respect. Apart from the well-known difficulties of fat analysis, it may be worth-wile to recall that a lack of evidence of adulteration does not necessarily mean a proof of genuineness, just as failing to prove the genuineness does not necessarily imply the admixture of foreign components. In this situation he may be charged by interested parties of quite different attitude; such for instance as Dame Justice who, averse as she is from sentencing an innocent, is not eager to deal out the blame of adulterator; or the Customer who, disliking the unfamiliar, readily suspects an adulteration if he comes across a product whose properties deviate from those known to him.

Dealing as we are with a product underlying biological variations, too suspicious customers can only be appeased by a system of official inspection in which the raw material, the milk in the present case, is analyzed periodically and its data, such as the refractive index of the milk fat, are registered and compared with those of the finished products the date of production of which is known also. In this way the composition of exported dairy products is guaranteed by the Netherlands Government since the

beginning of this century. It was the rise of the margarine trade that lead to this drastic action, as part of which the Government Dairy Station started its work in 1903.

Before the first world war an adulteration occurred now and then and had to be proved in court. Figures are whimsical wittnesses for the prosecution, easily evoking as they do discussions on propability, and even statisticians may enter the court as witnesses for the defence. Sterol crystals however — originating from vegetable fats — put on the Table of Justice as corpus delictum, proved convincing at that time. Such events have initiated the exploration of the field of sterol chemistry half a century ago by the Government Dairy Station, in which investigation it has been active since then and to which Tulleken, Den Herder, Roos, Riemersma and the author of this book have contributed. *The analytical application of the sterols proved of much wider utility in analytical chemistry than the detection of the adulteration of butterfat.* Of recent practical importance in food control is its adoption by Roos for the detection of refined inedible lard in pure lard and for the analysis of egg products, and that by Riemersma and by Copius Peereboom for the detection of animal fats in oils and fats, the vegetable origin of which must be warranted, f.i. for dietary purposes. Den Herder has laid the basis of the system. He simplified the method of precipitation of the sterol digitonides in avoiding preliminary extraction of the unsaponifiable fraction. He studied the crystal forms of sterols and their conduct in polarized light; the melting curves of mixtures of different sterol acetates; the proportion of free and bound sterols in various fats. He introduced the technique of increasing the proportion of sterols originating from vegetable fats in mixtures with butterfat, by a preceding separation of the bound sterols. He developed the sterol content, either total or free or bound, to a very characteristic value for the identification of fats and for their quantitative analysis in mixtures. In those days, when quantitative analysis of fats was for the greater part on a rather rough basis of estimation, his results in detecting even very small admixtures to butterfat were striking and worked so effectively that, even under intensive control, no adulteration of butter with foreign fats has been revealed since 1914.

There is a comprehensive literature on the analytical chemistry of sterols, but the analysis of fats *by means of* sterols is rather poorly dealt with. Now that the author has presented us a report of his own investigations as well as a description of the methods applied and the results obtained previously, he has provided the analytical chemist with a reliable guide for the application of this analytical technique which has received too little attention up till now. Enlarging the scope of the investigations he has also given a line of approach to the structural analysis of several unknown sterol types. For this reason not only chemists engaged in analytical work will take note of this book with advantage.

J. G. VAN GINKEL
Director of the Government Dairy Station

CONTENTS

The author was awarded the degree of Doctor of Science, University of Amsterdam, The Netherlands, on a thesis with the same title and contents, on 23st October 1963.

1 CLASSICAL ANALYTICAL METHODS FOR NATURAL STEROLS, APPLIED TO FAT ANALYSIS

1.1 Fat analysis based upon characteristic values

As a consequence of the very complex composition of natural fats the analytical chemistry of lipoids forms a distinct field with special problems. Besides multifarious types of triglycerides as quantitatively most important fraction, a minor part consists of an enormous variety of other lipoid substances: monoglycerides, diglycerides, phosphatides, tocopherols, sterols, sterol esters, hydrocarbons, carotenoids and traces of several other compounds. The triglycerides forming the main fraction of the fat consist of a glycerol molecule esterified with three fatty acids. The triglyceride fraction largely varies in composition, numerous combinations of the fatty acid constituents in their molecules being conceivable. Evidently, the great number of possible triglycerides still further enhances the complexity of this type of analysis.

One of the purposes of fat chemistry is the determination of all these individual types of lipoids in a fat, but in the past appropiate analytical techniques were lacking. At the end of the nineteenth century a complete analysis of a natural fat thus was by no means possible. Nevertheless, fat analysis had to differentiate between the various types of oils and fats and to determine the proportion of the constituents of commercially available fat mixtures. To that end physical data were introduced as characteristic numbers e.g. refractive number and density. For practical reasons a chemical approach was usually made by saponification of all glycerides into glycerol and fatty acids, leaving a minor fraction withstanding this procedure viz. the *unsaponifiable fraction*. The saponification thus brings about a first experimental simplification of the problem of analysing such complex mixtures. Fat chemistry, therefore, was perforce principally engaged with the fatty acid composition of the fat mixture. In this procedure the actual structure of the triglycerides was neglected. Subsequently, chemical characteristics derived from the fatty acid composition of the fat mixture were introduced as empiric values. These values, further on denoted as "*fatty acid values*" were widely applied in practice as characteristics for the identification of the various types of fat. The same procedure could be applied to unravel the composition of commercial fat mixtures.

The features of either the triglycerides as such or alternatively those of the unsaponifiable fraction could be applied as well for the purpose, but because of analytical imperfections they were in the past only sparingly used in practice. Considering these three essentially different analytical techniques, we can divide the methods of fat analysis into three main groups, based upon the properties of:

1. the *fatty acids*, viz. fat analysis through the fatty acid composition.
2. the *triglyceride fraction* as such, viz. fat analysis through the triglyceride composition or "glyceride (fat) analysis".
3. the *unsaponifiable fraction*, viz. fat analysis through guide substances in this fraction or "guide (fat) analysis".

1.1.1 Fatty acid values and special applications

In the past the fatty acid composition of a fat sample could be typified only approximatively by the procedures of the above group 1. All fatty acid values are in fact rough indications of the actual fatty acid composition. They had to be introduced since reliable and preferably also quantitative techniques for the determination of all fatty acids separately were lacking at the time. A variety of fatty acid features was applied for the characterization of the fatty acid composition. They can be divided into three sub-groups (KAUFMANN 1958).

The first group essentially uses the chain length of the fatty acids as a characteristic e.g. the saponification value, determining the mean molecular weight of the total fatty acid fraction. The REICHERT-MEISSL-WOLLNY (R.M.W.) value is a measure for the group of *lower fatty acids*, which are both volatile and water soluble. Other instances are the POLENSKE value, A and B values etc. The unsaturated nature of some fatty acids forms the basis of the second sub-group e.g. iodine value, hydrogen value, thiocyanogen value etc. With the data of the third group of fatty acid values special oxygen containing functional groups are evaluated e.g. hydroxyl, acetyl, and carbonyl values. KAUFMANN (1958) has denoted these groups as acidimetric, enometric, and oxidimetric fatty acid values (German "Fettsäurekennzahlen").

A more simple situation is met when a type of fat contains a *specific fatty acid.* Experimental data concerning the percentage of that special fatty acid then can be applied as a characteristic. Instances are a.o. the specific occurrence of erucic acid in rapeseed oil, of isovaleric acid in dolphin fat, and of butyric acid in butter fat. Of old an approximation of the butyric acid content of butter fat is given by the R.M.W. value. It has been applied in many countries as an empiric fatty acid value in checking the purity of butter. More exact evaluations of the actual butyric acid content were devised afterwards, leading to an accurate quantitative determination by means of chromatographic methods. In an ideal situation the percentages of all fatty acids of a fat like butter fat may be determined quantitatively e.g. by paperchromatography or gas-liquid chromatography. These data then could be applied to the control of the genuineness of that special type of fat. Especially by applying gas-liquid chromatography, the complete fatty acid composition of a fat like butter fat can be evaluated at present in a relatively simple and rapid way.

However, the exact knowledge of the complete fatty acid composition does not necessarily solve all problems encountered when analysing complex fat mixtures. In fact we always have to reckon with the *natural variability in fatty acid composition*

shown by several samples of the same type of fat. Natural circumstances like climate, season, soil composition, and especially breed and feeding in the case of animal fats exert great influence upon the fatty acid composition. Because of this natural variability the detection of small amounts of one type of fat in another often will meet with difficulties. This is illustrated clearly by the varying composition of butter fat. The R.M.W. value of Dutch butter changes during the year within the extreme limits of 18 and 36, with a mean value of about 25–26 in September–October and 29–30 in April–May (VAN RIJN 1899, SWAVING 1939), these differences being mainly due to the lactation period and feeding of the cattle.

In the RIJKSZUIVELSTATION (GOVERNMENT DAIRY STATION) at Leiden this variation in R.M.W. value was studied by TULLEKEN AND VAN SILLEVOLDT (cf. HOTON 1909). They could establish a statistical correlation between R.M.W. value and refractive number of Dutch butter fat, originally based on the analysis of 91,077, later on followed by the investigation of 745,194 butter samples.

Because of the great range in R.M.W. value, adulteration of butter by foreign fats (especially by those having high R.M.W. values) cannot be detected unambiguously in this way. Therefore, the guarantee of the genuineness of Dutch butter is given by a special procedure of control of the manufacturing process. For individual lots of cream in the butter factories R.M.W. values and refractive numbers are determined. The corresponding values of the manufactured butter should agree. A special number of the National Quality Mark is conferred upon every consignment. In this way the serial numbers of this mark are linked up with the chemical data of the butter sample, thus guaranteeing its genuineness. However, upon the mere examination of a butter sample of unknown origin the *chemical proof* of an adulteration with moderate amounts e.g. 20% of vegetable oil cannot be furnished by the R.M.W. value. The same restricted applicability in detecting butter adulterations is inherent to other fatty acid values. This restriction is particularly valid when the fatty acid value for butter fat exhibits a great natural variability and the same characteristic for the foreign fat is rather near one of the *limits* of this variability.

On the other hand an example of a fatty acid value low for butter fat and high for some vegetable fats, is given by the POLENSKE value, by which the volatile but water insoluble fatty acids can be determined. This value amounts to 16–17 for coconut fat; 9.0–10.5 for palmkernel oil; 1.5–3.5 for butter fat; and less than 1.0 for most other oils and fats. Consequently, the POLENSKE value is applicable in detecting gross adulterations of butter with coconut and palmkernel fat. Reviewing the above statements, we must conclude that the natural variability in the butter fat composition may hamper a reliable detection of foreign fats by means of fatty acid values.

1.1.2 Glyceride (fat) analysis

Some attempts have been reported to imitate high priced and valued fats like butter fat by mixing foreign fats or special fractionated parts thereof; by transesterification

of low priced fats with coconut fat, tributyrin etc.; or by complete synthesis from glycerol and fatty acid fractions (TÄUFEL 1929, 1958). The so-called "*Bolzella*" fat has the same fatty acid values as genuine butter (GUEX 1957). Consequently, adulteration of butter with Bolzella fat cannot be detected at all by means of any fatty acid value. However, we should always keep in mind that fatty acid values serve as a rough substitute for the actual features of the triglyceride fraction. In this case the possible deviating characteristics of the Bolzella triglycerides should be inspected by the methods of glyceride (fat) analysis. Indeed the amount of ether-insoluble glycerides has yielded a significant difference between genuine butter fat and Bolzella fat (HANSSEN 1960). The use of the BÖMER value in detecting admixture of beef tallow to lard is another well-known instance of the applications of this glyceride analysis.

The experimental difficulties encountered in determining the various triglyceride types, however, have limited the applications of glyceride analysis in the past. Furthermore, intramolecular and intermolecular exchanges are known to occur during analytical operations; therefore, some caution is required in the interpretation of experimental results. Modern methods such as lipase hydrolysis (V. D. WAL 1960); reversed-phase paperchromatography (KAUFMANN 1959, 1961a); reversed-phase thin-layer chromatography (KAUFMANN 1960, 1961b, 1962); thin-layer chromatography with additional procedures e.g. ozonisation-reduction techniques (PRIVETT 1961); molecular distillation; and gas-liquid chromatography (HUEBNER 1961) now enable a simple and rapid analysis of the various triglycerides. In the future a more extensive application of "glyceride values" in fat analysis is to be expected.

1.1.3 Guide (fat) analysis

Because of the present-day limits of the glyceride analysis and the fundamental limitations of the application of many fatty acid values, nowadays much attention is paid to the third main group of fat analysis, based on characteristics of constituents of the small unsaponifiable fraction. Such a component may be used to advantage as a kind of *natural marker, guide substance, or guide*, characteristic of certain oils and fats, thus enabling the analysis of commercial fat mixtures. We have denoted this third type of fat analysis as "guide substance analysis" or abbreviated as "guide (fat) analysis" (German "Leitsubstanzanalyse"). The application of this guide analysis is based essentially upon the fact that the proportion and types of the minor constituents in a special type of fat were generally found to be constant.

We propose also to introduce a subdivision, based on the types of minor constituents of this group such as sterols, tocopherols etc. present in the unsaponifiable fraction, which are applied as natural markers in practice. The largest and most significant group, viz. the sterol group is the subject of this investigation. However, we shall first mention some applications of other minor components to problems of fat analysis reported in the literature.

Several classical colour reactions actually belong to this type of guide analysis e.g.

the identification of sesame oil by the red colour produced with a furfural-hydrochloric acid reagent, according to BAUDOUIN and VILLAVECCHIA. A minor phenolic component of the unsaponifiable fraction, namely sesamolin causes this specific colour reaction.

A group of substances of the unsaponifiable fraction, important for their physiological activity (vitamin E), are the *tocopherols* comprising several isomers a.o. α-, β-, γ-, δ-tocopherol (α-tocopherol = 5,7,8-trimethyltocol; β = 5,8-dimethyl; γ = 7,8-dimethyl; δ = 8-monomethyl; ζ = 5,7-dimethyltocol; ε = 5,8-dimethyltocotrienol ; η = 7-monomethyltocol).

Several authors e.g. ANGLIN *et al.* (1955), MAHON *et al.* (1954, 1955) WINDHAM (1957), and NAZIR *et al.* (1959) have attempted the detection of vegetable oils in butter by means of the total content of these tocopherols. Whereas the edible vegetable oils contain amounts of tocopherol between 1000 and 16000 μg/g (LANGE 1950, BLAIZOT 1956, HERRAIZ 1949, VITAGLIANO 1958, BIEFER 1956), the tocopherol content of butter fat is extremely low, viz. about 10–50 μg/g. According to KRUISHEER (1955) the tocopherol content of Dutch butter fat varies from 10–40 μg/g.

In an extensive study dealing with the tocopherol content of American butter ANGLIN *et al.* (1955) concluded that for summer butter fat 99% of the samples have tocopherol contents less than 52 μg/g, 99% of the winter butter fat samples presenting values even below 30 μg/g. Usually, adulteration of butter with 10% of vegetable oil will result in a significant increase in the tocopherol content (ANGLIN 1955, NAZIR 1959). Samples of butter fat containing 50–70 μg/g of tocopherol are to be considered as "suspicious"; contents above 70 μg/g positively indicating adulteration with vegetable fat. The Canadian Food and Drug Regulations (1954) dictate for pure butter a tocopherol content not exceeding 50 μg/g.

The isomer α-tocopherol is the only form occurring in animal fats (BROWN 1952), the vegetable oils containing mostly also considerable amounts of the other isomers. Therefore, detection of the other isomers in butter might furnish even more conclusive evidence of adulteration with vegetable oils than does the total-tocopherol content (SHIPE 1954). The method of ANGLIN *et al.* has restricted application since small amounts of some vegetable fats e.g. olive oil and coconut fat containing tocopherol contents of only 10–100 μg/g (HERRAIZ 1949, BLAIZOT 1956) cannot be detected with certainty. No more can this total-tocopherol method reveal the presence of small amounts of animal fat in mixtures with vegetable oils. Furthermore, tocopherol mixtures are widely used as antioxidants nowadays. Too high a percentage of tocopherols, therefore, indicates the addition of foreign matter, but not necessarily of a foreign fat.

By paperchromatography the tocopherol group can be separated into the various isomers: a.o. α-, β-, γ-tocopherol. While cottonseed oil and peanut oil have nearly equal amounts of α-, and γ-tocopherols, olive oil and sunflower oil predominantly contain the α-isomer. In sesame oil and linseed oil only the γ-isomer was found, whereas the presence of soybean oil in mixtures with other oils can be evidenced by

its specific, large amount of δ-tocopherol (BIEFER 1956). An evaluation of the percentages of these isomers can thus be applied to the analysis of mixtures of oils and fats.

The presence of several higher *hydrocarbons* has been demonstrated in the unsaponifiable fraction, of which the most abundant one: squalene ($C_{30}H_{50}$) is found in olive oil in quantities of 1000–4900 μg/g with a mean value of about 3000 μg/g. Other vegetable oils mostly contain much smaller percentages of squalene, viz. 40–360 μg/g (HADORN 1950). The presence of olive oil in other edible oils is detected by a significant increase in the squalene content (PHILIPPE 1944, HADORN 1949, 1950). However, the so-called *rectified B olive oil*, i.e. "synthetic olive oil" prepared by re-esterifying extracted olive oils or distilled fatty acids, is conspicuous by extremely low squalene value (WITTKA 1960).

Other minor components e.g. compounds containing phosphorus, coloured carotenoids, anthocyanins are also applicable as natural markers. KUZDZAL *et al.* (1960) have described an identification of ordinary cheese substance in the more special types of cheese manufactured from goat's milk. In the latter only small quantities of carotene: about 0.5 μg/g fat are found, whereas the cow's milk contains about 6.5 μg carotene/g fat. Consequently, the most likely type of adulteration can be detected by a quantitative spectrophotometric determination of the carotene content.

The formerly troublesome analysis of the constituents of the unsaponifiable fraction is facilitated by modern chromatographic methods. By column chromatography on silicic acid these constituents are separated, showing elution curves that are characteristic of the oil. The curves of olive oil and other oils e.g. teaseed oil are entirely different so that adulteration of virgin olive oil with e.g. teaseed oil is detectable (JACINI 1960, CAPELLA 1960). By the more simple method of thin-layer chromatography the unsaponifiable fraction can be fractionated into several components. The spots on a chromatoplate constitute a definite pattern, often characteristic of the type of fat (COPIUS PEEREBOOM 1961). MCGUGAN (1959) describes the pattern of the unsaponifiable fraction of butter fat, obtained after separation in a mixture of hexane–ethyl acetate (95 : 5). Samples of butter fat adulterated with 10% of vegetable or marine animal oils are characterized by spots appearing in areas where butter samples show none, or by an increased intensity of spots normally shown by butter fat.

Among the minor constituents of the unsaponifiable fraction the sterols are quantitatively as well as qualitatively the most significant group. This group will be the subject of this investigation.

1.2 THE PRINCIPAL NATURALLY OCCURRING STEROLS

The most significant substance of the group of sterols i.e. cholesterol has been known since the eighteenth century as the chief constituent of human gall stones. Cholesterol

is the principal sterol of the higher animals; it is abundant in nerve tissue, fat, and skin. Usually it is accompanied by traces of other sterols, which in a few instances have been identified as dihydrocholesterol and 7-dehydrocholesterol.

Cholesterol ($C_{27}H_{46}O$) contains a C_{27} carbon skeleton and a hydroxyl group. The cholesterol molecule (fig. 1) contains 8 asymmetric carbon atoms, resulting in a great number of possible stereoisomers. In cholesterol and related sterols the secondary hydroxyl group at C_3 projects to the front of the molecule. The two angular methyl groups at C_{10} and C_{13}, the C_8 hydrogen atom, and the C_{17} side chain all lie at the same side of the ring system as the C_3 hydroxyl group. By convention constituents projecting to the front of the molecule are described as β-oriented (FIESER 1959). They

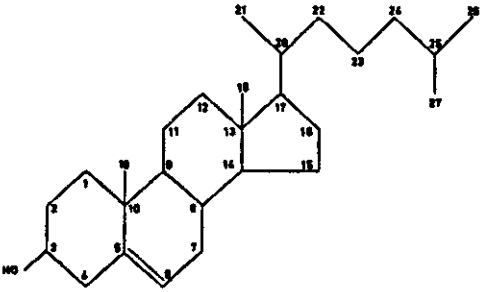
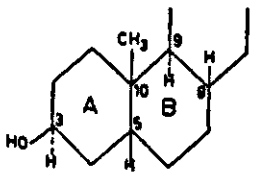
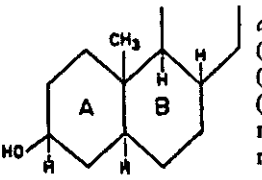


FIG. 1. *Cholesterol* ($C_{27}H_{46}O$) ($\Delta 5$-cholestene-3β-ol) melt. p.: 148°C, melt. p. acetate: 115°C

are joined to the system of the four rings A, B, C, and D by solid lines. Constituents projecting to the rear or underside of the molecule e.g. the C_9 and C_{14} hydrogen atoms are α-oriented, being represented by broken lines. After hydrogenation of the double bond at $C_{5(6)}$ two stereoisomers are formed with different conformation at C_5. In *dihydrocholesterol* (fig. 2) the juncture between the rings A and B is trans as in trans-decalin. Sterols belonging to this group are designated as 5α. The rings A, B, and C of the dihydrocholesterol molecule are of the more stable chair conformation; the ring junctures B/C and C/D also being trans. Dihydrocholesterol has been detected in small percentages in animal fats. In the C_5 epimer viz. *coprostanol* the ring juncture A/B is cis as in cis-decalin (5 β compound). Coprostanol has been found in nature e.g. in human faeces.



coprostanol ($C_{27}H_{48}O$)
5β(-cholestane-3β-ol)
(A/B: cis)
melt. p.: 101°C
melt. p. acetate: 90°C



dihydrocholesterol
($C_{27}H_{48}O$)
(5α-cholestane-3β-ol)
(A/B: trans)
melt. p.: 142°C
melt. p. acetate: 110°C

FIG. 2.

Animal fats contain predominantly cholesterol with small percentages of dihydro- and dehydrocholesterol. In vegetable oils and fats the presence of several closely related sterols has been demonstrated; these are known as phytosterols (HESSE 1878). They are structurally related to dihydrocholesterol, but vary in the degree of unsaturation and the length of the C_{17} side chain. ANDERSON *et al.* (1926) separated a crude corn oil phytosterol mixture into three fractions called: α-, β-, and γ-sitosterol. The most important of these sitosterols viz. *β-sitosterol*, is found widely distributed in plants and is present in all vegetable oils and fats. The structure of the molecule differs from that of cholesterol by an ethyl group attached to C_{24}. By introducing this group, a new asymmetric C-atom is formed; obviously two configurations (viz. 24-a and 24-b) are conceivable (fig. 3). The structure of β-sitosterol, having the b-configuration has been established as: 24-b-ethyl cholesterol (Δ5-stigmastene-3β-ol).

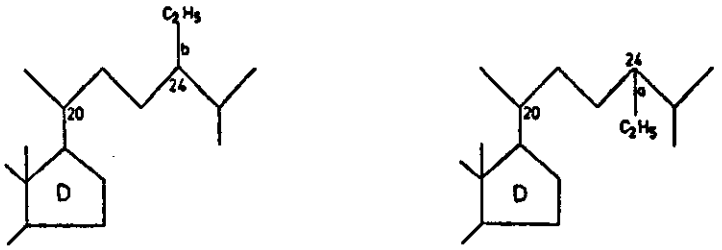


FIG. 3.

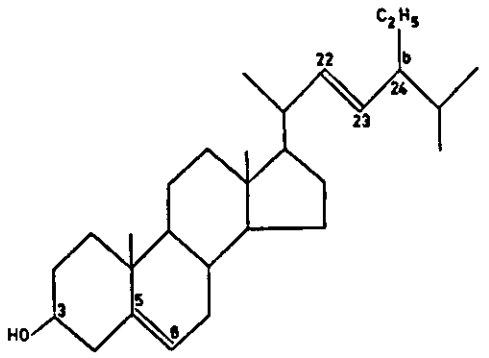
β-sitosterol ($C_{29}H_{50}O$)
24-b-ethyl cholesterol
(Δ5-stigmastene-3β-ol)
melt. p.: 137°C
melt. p. acetate: 126–127°C

clionasterol (γ-sitosterol (?)) ($C_{29}H_{50}O$)
24-a-ethyl cholesterol
(Δ5-stigmastene-3β-ol)
melt. p.: 139°C
melt. p. acetate: 137°C

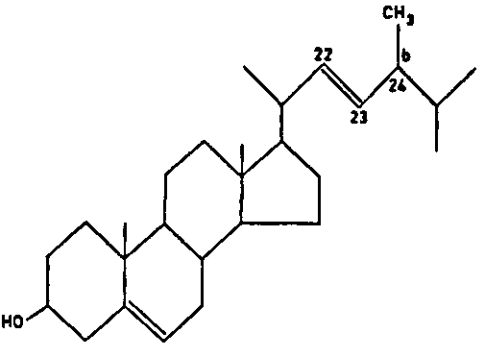
A sterol named *clionasterol* was isolated from the sponge: *Cliona celata* and has been shown to be the C_{24} epimer of β-sitosterol viz. 24-a-ethyl cholesterol (fig. 3). *γ-Sitosterol* is one of the principal sterols of soybean oil (BONSTEDT 1928) and a minor component of the sterol mixtures from many other oils and fats (ICHIBA 1935). As is stated most often in the literature, the structure of γ-sitosterol is identical with clionasterol and hence its structure should be 24-a-ethyl cholesterol (BERGMANN 1947, ELSEVIER'S ENCYCLOPAEDIA 1952).

Another phytosterol belonging to the 24-a series: *campesterol* ($C_{28}H_{48}O$) has been isolated from rapeseed oil (FERNHOLZ 1941), it is also present in many other oils. The structure has been proved to be 24-a-methyl cholesterol (Δ5-ergostene-3β-ol). By hydrogenation of these mono-unsaturated sterols the saturated *stanols* are formed e.g. β-sitostanol, γ-sitostanol ($C_{29}H_{52}O$), structurally related to the 5α-oriented dihydrocholesterol. These stanols are minor components of the sitosterol mixtures of several oils (BONSTEDT 1928, ICHIBA 1935).

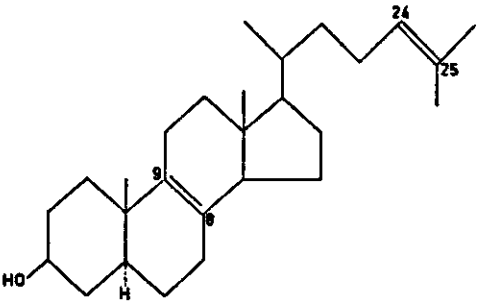
Important di-unsaturated phytosterols are *stigmasterol* and *brassicasterol*, of which the former was first isolated from Calabar bean phytosterols as the sparingly soluble acetate tetrabromide (WINDAUS 1906b) and is present in many vegetable oils, especially soybean oil. The structure differs from that of β-sitosterol by a second double bond at C_{22} having the trans configuration. Brassicasterol has been isolated from rapeseed oil by WINDAUS (1909b) via its insoluble acetate tetrabromide. Like stigmasterol, brassicasterol belongs to the 24-b series, possessing a methyl group attached to C_{24} instead of the ethyl group in the stigmasterol molecule (fig. 4).



stigmasterol ($C_{29}H_{48}O$)
Δ5,22-stigmastadiene-3β-ol)
melt. p.: 170°C
melt. p. acetate: 144°C



brassicasterol ($C_{28}H_{46}O$)
(Δ5,22-ergostadiene-3β-ol)
melt. p.: 148°C
melt. p. acetate: 158°C



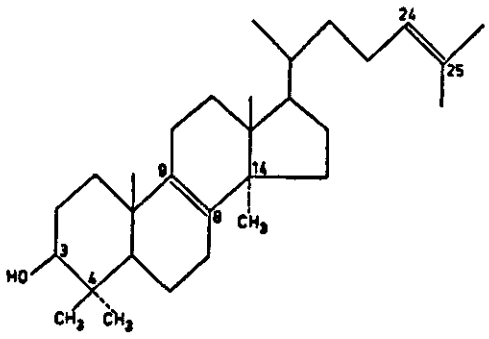
zymosterol ($C_{27}H_{44}O$)
(Δ8(9),24-cholestadiene-3β-ol)
melt. p.: 110°C
melt. p. acetate: 107°C

FIG. 4.

A third di-unsaturated sterol: *zymosterol*, quantitatively the second sterol of yeast fat, has a carbon skeleton resembling that of dihydrocholesterol, but with two double bonds at $C_{8(9)}$ and $C_{24(25)}$ (Δ8(9),24-cholestadiene-3β-ol). It has not been found in the common edible oils and fats.

Besides β-, and γ-sitosterol crude sitosterol preparations contain also a small fraction,

better soluble in ethanol which has been denoted as "*α-sitosterols*". Further recrystallization of this fraction has yielded more homogeneous preparations: α_1-, α_2-, and α_3-sitosterols, of which the α_1- and α_3-sitosterols contain two non-conjugated double bonds and are isomers of the formula: $C_{29}H_{48}O$, whereas α_2-sitosterol is believed to be a homologue: $C_{30}H_{50}O$ (WALLIS 1936, BERNSTEIN 1939). The structures of these α-sitosterols are not established firmly, but nowadays are supposed to be related to the triterpenoid alcohol: lanosterol, having two methyl groups attached at C_4 (ELSEVIER'S ENCYCLOPAEDIA 1952) (fig. 5). However, a recent investigation by MAZUR (1958) suggested the possibility that α_1-sitosterol is identical with citrostadienol, isolated from grape fruit peel oil. The latter substance has been shown to be 4α-methyl-24-ethylidene-Δ7-cholestene-3β-ol.



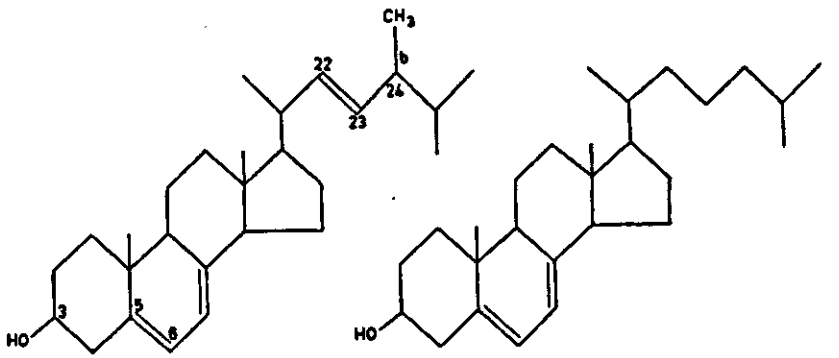
lanosterol ($C_{30}H_{50}O$) (4,4,14α-trimethyl Δ8(9),24-cholestadiene-3β-ol)
melt. p.: 140°C
melt. p. acetate: 141°C

FIG. 5.

In addition to these di-unsaturated, non-conjugated phytosterols, several sterols having at least two conjugated double bonds are found in nature. The most important sterol of this group: *ergosterol*, the provitamin of vitamin D_2, was first isolated from ergot of rye by TANRET (1890); it has been shown to be present in several fungi, yeasts, the yolk of hen eggs etc. In addition to the double bond at C_{22} in the side chain, the B ring of the skeleton contains a system of two conjugated double bonds, which causes a specific ultraviolet absorption spectrum with pronounced maxima at: 261, 272, 282, and 294 nm. (fig. 6). The same conjugated system is found in the provitamin of vitamin D_3: *7-dehydrocholesterol* that occurs in small percentages in sterol mixtures of animal origin. In some marine invertebrates a.o. the Gastropoda *Buccinum undatum* considerable quantities, up to 27%, of provitamin D-like substances, supposedly 7-dehydrocholesterol, have been found in the sterol mixtures (BOCK 1938).

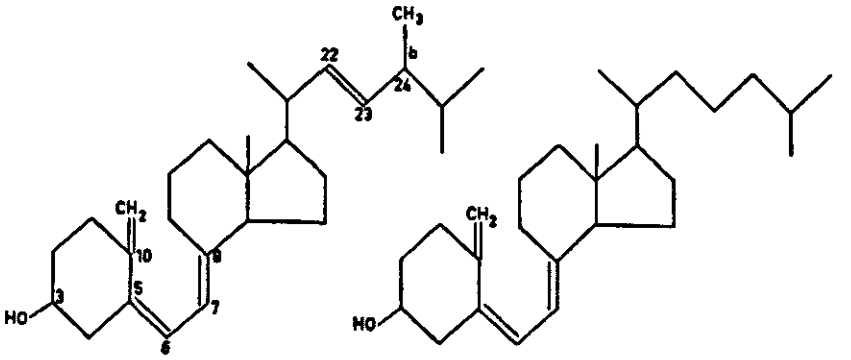
Under the influence of U.V. radiation ergosterol and 7-dehydrocholesterol can be transformed into several substances, of which the vitamins D_2 and D_3 are the prominent ones. In this reaction the B ring of the skeleton is opened, forming a system of three conjugated double bonds and a methylene group (fig. 6). Because of this structure

the vitamins D_2 and D_3 do *not belong* to the group of the sterols, but they are mentioned because of their structural relationship. These vitamins possess great antirachitic activity. The tuna and halibut liver oils e.g. contain large amounts of vitamin D_3.



ergosterol ($C_{28}H_{44}O$)
(Δ5,7,22-ergostatriene-3β-ol)
melt. p.: 165°C
melt. p. acetate: 181°C

7-dehydrocholesterol ($C_{27}H_{44}O$)
(Δ5,7-cholestadiene-3β-ol)
melt. p.: 150°C
melt. p. acetate: 130°C



vitamin D_2 ($C_{28}H_{44}O$)
melt. p.: 116°C
melt. p. acetate: 88°C

vitamin D_3 ($C_{27}H_{44}O$)
melt. p.: 82°C

FIG. 6. Conjugated sterols

Several sterols and steroids can be isolated by means of their sparingly soluble molecular compounds with digitonin, a glycosidic saponin. The presence of a free 3β-OH group and a C_{10}-methyl group in the β configuration in the sterol molecule is required for the formation of the molecular compound i.e. the sterol digitonide. When e.g. the configuration at C_{10} is inverted (lumisterol) or with 3 α-OH sterols no

precipitation occurs. In peculiar contrast with the specificity of digitonin in the group of sterols, is the unspecific behaviour as to a variety of substances like n-amylalcohol, geraniol, α-naphtol, p-bromophenol etc., also precipitatable by digitonin (FIESER 1959). Of course in fat analysis no difficulty whatsoever need be feared from this situation.

In the course of this investigation of the sterols present in the common edible oils and fats, only those which can be precipitated by digitonin solution have been studied.

1.3 STEROL VALUES IN FAT ANALYSIS

Roughly half of the unsaponifiable fraction of oils and fats consists of substances belonging to the group of sterols precipitatable with digitonin. In quantitative as well as in qualitative respect the sterol group is one of the most important classes of constituents of the unsaponifiable fraction.

Whereas cholesterol is a natural marker characteristic for fats of animal origin, vegetable oils contain a diversity of phytosterols such as β-sitosterol, stigmasterol, γ-sitosterol, and brassicasterol. This differentiation in sterol composition has found application in fat analysis, especially in the detection of small amounts of animal fat in vegetable fat and vice versa.

Generally, mixtures of oils and fats can be analysed by a thorough investigation of the composition of the sterol mixture isolated with digitonin. Physicochemical properties of these sterols form the basis of a special type of guide analysis. They result in a number of characteristic values, the so-called "sterol values" (German "Sterinkennzahlen"). We distinguish:

I. Sterol content of the fat, denoted as: a;
II. Melting point of the sterol acetates, denoted as: m;
III. Composition of the sterol mixtures, e.g. percentage of cholesterol, denoted as: c.

In oils and fats the sterols are present in the free state as well as esterified with several fatty acids, viz. the sterol esters. The fractions of these so-called free sterols and bound sterols were isolated and investigated separately. The above-mentioned three sterol values were determined not only for the total-sterol fraction, but also for both fractions of the free and bound sterols separately.

Further on we shall call the complex of sterol values the "sterol (fat) analysis" and we propose the following notation for the sterol values of these three fractions:

1. Sterol values of the total-sterol fraction are denoted as: a_0, m_0, c_0 etc.
2. Sterol values of the free-sterol fraction are denoted as: a', m', c' etc.
3. Sterol values of the bound-sterol fraction are denoted as: a'', m'', c'' etc.

Table 1 presents a survey of this system of sterol values.

TABLE 1 Notations of the sterol values applied to fat analysis

		Total-sterol values	Free-sterol values	Bound-sterol values
I	content	a_0	a'	a''
II	melting point of the acetates	m_0	m'	m''
III	composition of the sterol mixture viz. cholesterol percentage	c_0	c'	c''
	β-sitosterol percentage	b_0	b'	b''
	stigmasterol percentage	s_0	s'	s''
	etc.	etc.	etc.	etc.

On account of this system of characteristic values sterol analysis now has become an important tool in fat analysis and has gained a place among the other methods. Although in the past some of these sterol values were applied successfully to special problems of fat analysis, no general system of sterol (fat) analysis has been described in the literature. The various applications of this system of sterol analysis will be illustrated in the following sections of this investigation.

1.4 APPLICATION OF THE STEROL CONTENT TO FAT ANALYSIS (sterol value *a*)

1.4.1 Gravimetric methods

The isolation of sterols from natural sources is facilitated by WINDAUS' discovery (1909a) of the sparingly soluble compound given by cholesterol and the glycosidic saponin: digitonin $C_{56}H_{92}O_{29}$, from *Digitalis purpurea*. The sterol digitonide formed by this reaction has recently been proved to be a real molecular compound (BEHER 1957).

$$\underset{\text{digitonin}}{C_{56}H_{92}O_{29}} + \underset{\text{cholesterol}}{C_{27}H_{46}O} \longrightarrow \underset{\text{cholesterol digitonide}}{C_{56}H_{92}O_{29} \cdot C_{27}H_{46}O}$$

Applying this reaction KLOSTERMANN (1913, 1914a, 1914b) has devised a quantitative method for the determination of the sterol content of oils and fats. In the case of a determination of the *total* sterol content the method starts with saponification of the sample. By adding an excess of hydrochloric acid the fatty acids are separated. This is followed by extraction with an ether–petroleum ether mixture, washing this extract with water. A 1% ethanolic digitonin solution is added; the precipitate of the sterol digitonides is collected by filtration and the sterol content is calculated from the amount of dried precipitate. This procedure has been improved by many authors a.o. SPRINKMEYER *et al.* (1914) and KÜHN *et al.* (1914, 1915).

The method is far superior to the older procedure of BÖMER (1898), who isolated the unsaponifiable fraction of a fat by successive extractions of the soap solution with ether. The sterols are purified by repeated crystallizations of the unsaponifiable fraction. According to MARCUSSON *et al.* (1914) and BERG *et al.* (1914c) the sterols can be isolated from the unsaponifiable fraction by applying digitonin precipitation. Theoretically, no reliable sterol contents can be obtained in this way, since even after eight extractions some cholesterol still remains in the soap solution (KLOSTERMANN 1914b). Surprisingly, this troublesome method is apparently preferred in several even recent publications (SCHRAMME 1939, HADORN 1954b, WOLFF 1953). TULLEKEN (cited by SCHOORL 1909, CODEX ALIM. NED. 1914) devised a simplification of the procedure by replacing the numerous extractions by perforation.

A significant improvement of the gravimetric procedures for the determination of the sterol content of oils and fats has been introduced by DEN HERDER (1950, 1954, 1955). Adding the digitonin *directly* to the soap solution, the sterol digitonides precipitate quickly. The precipitate is filtered and contaminating soaps are removed by washing with water. This so-called "precipitation in the soap" method of DEN HERDER, has been recognized and standardized internationally (I.U.P.A.C. 1954). Later identical results were published by CANNON (1955, 1956, 1957, 1958).

The total-sterol contents of oils and fats, mentioned in this investigation, have been determined by this method (cf. also NED. NORM 1961).

Experimental procedure:

Weigh about 15 g of fat in a conical flask of 300 ml. Add 10.5 ml of a 40% potassium hydroxide solution and 20 ml of 96% ethanol. Connect an air cooled condensor to the flask, heat on a water bath (while shaking) until the mixture has become clear. Continue boiling during ½ h. Then add 60 ml of water, 180 ml of 96% ethanol and 30 ml of a 1% solution of digitonin (R. BRUNSCHWIG) in ethanol 96% at a temperature of about 40°C. Allow to cool and keep at room temperature during 16 hrs. Collect the precipitate by filtering through a filter paper in a Büchner funnel. Wash with water until free of soap, then once with 96% ethanol and once with ether. Transfer the precipitate, dried at 30–40°C, into a tared weighing bottle.

Total sterol content: $a_0 = f \times \frac{b}{10 \, . \, a}$ %

wherein:
a = weight of fat in g
b = weight of digitonide in mg

The sterol content is calculated from the weight of the digitonide by multiplying with a factor f: the quotient of the molecular weights of the sterol and the digitonide.

In the case of cholesterol, f amounts to: $\frac{386.67}{1616.01} = 0.2393$. The factor: 0.241, mentioned in many papers and handbooks of fat chemistry, is based on the old incorrect formula of digitonin $C_{55}H_{94}O_{28}$. When the fat sample consists of pure vegetable fat a factor: $f = \frac{414.72}{1652.04} = 0.2510$, based upon the mean molecular weight of a β-sitosterol–stigmasterol mixture (1 : 1), applies (cf. BAUER 1943). In routine

analysis of fat mixtures good results are obtained with mean values of 0.245 or even of 0.25 (DEN HERDER 1953), as was already shown by WINDAUS (1910). According to a practical interpretation of the NEDERLANDSE NORM NEN 1046 (1961) the following quantities of fat are recommended:

30 g of fats with a sterol content of about 0.05% e.g. palm oil.

15 g for sterol contents of 0.05–0.20% e.g. coconut fat, palmkernel oil, lard, tallow etc.

10 g for sterol contents of 0.20–0.50% e.g. butter, White grease and several liquid oils.

5 g for sterol contents exceeding 0.50% e.g. maize oil.

The quantities of solvents and reagents are proportionally reduced on the basis of the above procedure for 15 g of fat. Obviously, the quantity of digitonin solution, however, always is 30 ml. The purity and precipitating properties of the digitonin will have to be examined periodically (SCHOENHEIMER 1933). The necessity of a correction: 0.02% for the solubility of the sterol digitonides in the soap solution as advocated by DEN HERDER (1955) is questioned (ROOS 1961b).

The sterols of oils and fats occur partly in the free state, partly esterified with fatty acids. A portion of the sterols is associated with carbohydrates forming glycosides denoted as sterolins or phytosterolins, which have been detected in many vegetable oils, e.g. in soybean oil in percentages of about 0.005–0.03. The commercial soybean lecithins are extremely rich in these phytosterolins: 1.1–2.8% and contain also about 0.50% of free and 0.65% of esterified sterols (JANTZEN 1934, THORNTON 1940). The free-sterol fraction has been isolated for the first time by MARCUSSON *et al.* (1913), by precipitation of the free sterols with an ethanolic digitonin solution. After some hours an excess of ether is added and the precipitate is filtered. This rapid and efficient procedure has been altered by several authors e.g. FRITZSCHE (1913), SPRINKMEYER *et al.* (1914), OLIG (1914), the latter proposing the addition of warm chloroform or benzene before the filtration of the precipitate; by HAWLEY (1933) and finally by DEN HERDER (1955), who recommends the addition of enough of a chloroform–ethanol mixture to retain the fat in homogeneous solution.

In this investigation the content of free sterols is determined according to DEN HERDER:

Weigh 50 g of fat in a 500-ml conical flask. Add 135 ml of a 1% ethanolic digitonin solution and 67 ml of chloroform. Then add such a quantity of a chloroform–ethanol (1 : 2) mixture that the fat dissolves at 25–40°C, the solution remaining homogeneous. Swirl occasionally or mix by a mechanic mixer and allow the mixture to stand during 16 hrs at such a temperature that the solution remains clear. Collect the precipitate by filtering through a paper filter in a Büchner funnel and wash out successively with 50 ml of chloroform–ethanol (1 : 1) and 50 ml of ether. If the precipitate is not free from fat, extract the filter and precipitate during 20 min in an extraction vessel with ether. The precipitate, dried at 30–40°C, is transferred quantitatively into a tared bottle.

Free sterol content: $a' = f \times \frac{b}{10.a}\,\%$

wherein:
a = weight of fat in g
b = weight of digitonide in mg
f = 0.25 in routine analysis of fat mixtures.
When analysing very hard fats, the solution is stirred at a temperature of about 40°C. The filtration has to be carried out with a Büchner funnel, warmed with water of about 50°C. The above procedure is suitable for fats having a free-sterol content of about 0.06–0.15%. In the case of much higher free-sterol contents the quantity of the fat sample can be reduced to about 25 g. When analysing fats with low free-sterol contents e.g. palm oil an amount of fat of 100 g has to be saponified.

Recently a method has been described by which the precipitation of the digitonides is accelerated by the addition of an aluminium chloride solution (BROWN 1954, VAHOUNY 1960a). Furthermore, some promising procedures were reported, by which the free sterols are separated from the bound sterols by column chromatography before their quantitative determination (TRAPPE 1942, KRUCKENBERG 1948, WYCOFF 1957). A similar micro device based on a paperchromatographic separation of cholesterol and cholesterol esters on silicic acid coated paper is described by QUAIFE *et al.* (1959).

KLOSTERMANN *et al.* (1914a, 1914b) discovered that 80–90% of the cholesterol of animal fats and especially of butter fat occurs in the free state. The phytosterols of vegetable oils, however, are present in the free and the esterified state in nearly equal amounts. These authors have determined the total-sterol and free-sterol contents of several vegetable and animal fats and have calculated the percentages of bound sterols by difference.

The bound-sterol contents given in this investigation were determined experimentally according to the following procedure.

The combined filtrate and washings,obtained after filtering the free-sterol digitonides are freed from volatile solvents by heating on a water bath. The bound sterols present in the remaining fat are determined quantitatively after saponification according to the above "precipitation in the soap" procedure.

1.4.2 Other procedures

Several other methods have been proposed for a reliable quantitative determination of cholesterol, as will be discussed now. Sterols form precipitates with several other agents e.g. lithium chloride (ZWIKKER 1917), calcium or manganese chlorides (HACKMANN 1950), perchloric acid (LANGE 1949), and also with other saponins e.g. nagitine (DELSAL 1943) and the more specific tomatine (KABARA 1961), but these methods have not found much application in practice. DREKTER *et al.* (1936) have devised a method for the precipitation of cholesterol from blood serum by a pyridine–sulfur trioxide reagent. The sample is dissolved in benzene and a pyridine–acetic anhydride mixture is added. The cholesterol is then converted by pyridine–sulfur trioxide to its pyridinium sulfate, which precipitates after addition of petroleum ether. By this method, however, a lower content of free cholesterol is obtained as compared with the digitonin procedure.

The following methods have been proposed, but these are seldom used in actual practice: 1. a titrimetric method based on the digitonin precipitation (THUESEN 1943); 2. a nephelometric method for cholesterol digitonide suspensions (POLLAK 1952); 3. a peculiar method based on the inhibition by cholesterol of the erythrocyte haemolysis produced by digitonin (SCHMIDT-THOMÉ 1942); and 4. a titrimetric method, determining the amount of chromic acid required for the oxidation of cholesterol (STAUB 1947, WAGHORNE 1952). Especially in the clinical literature several colour reactions suitable for the determination of the cholesterol content of blood serum are described. These methods are mostly based on the LIEBERMANN-BURCHARD reaction, given by an acetic anhydride–sulfuric acid reagent. The absorption of the blue-green coloured reaction mixture is determined colorimetrically. In many clinical laboratories the experimental procedure, described by SCHOENHEIMER *et al.* (1934) and SPERRY *et al.* (1950) is applied.

Unfortunately, the absorption depends not only upon the sterol content but also upon the *structure* of the sterol molecule. When the sample contains exclusively cholesterol, the SCHOENHEIMER-SPERRY method gives accurate results. By a modification of this method the cholesterol content of pure animal fats and derived food products thus can be determined correctly (RIFFART 1934, ROOS 1958). The presence of considerable amounts of other sterols like $\Delta 7$-stenols, 7-dehydrocholesterol or 24-dehydrocholesterol (desmosterol), however, necessitates special, more complicated measurements (MOORE 1952, IDLER 1953, AVIGAN 1960). The rate of the LIEBERMANN-BURCHARD reaction with $\Delta 7$-sterols or poly-unsaturated sterols is much higher than with normal $\Delta 5$-mono-unsaturated sterols like cholesterol. The former sterols are denoted as fast-acting, the latter as slow-acting sterols. There is also a difference in colour intensity between the sterols of the slow-acting group. For the phytosterols from vegetable oils extinction coefficients of 50–80% of that of cholesterol have been observed (IDLER 1953, COOK 1961) (see fig. 31). Because of these differences an application of the LIEBERMANN-BURCHARD reaction to the analysis of mixtures of animal and vegetable fat encounters many difficulties.

Other colour reactions, mentioned in the literature, e.g. with TSCHUGAEFF'S reagent: acetyl chloride and zinc chloride in acetic acid, or with iron(III)chloride–sulfuric acid might present similar problems. Because of this diversity of extinction coefficient, the time-honoured gravimetric method, by which the isolated sterol digitonide remains available for a subsequent preparation of the sterol acetates and sterols, is to be preferred in fat analysis. In recent papers two original methods are proposed. WEBSTER *et al.* (1959) and VAHOUNY *et al.* (1960a, 1960b) describe a colorimetric determination of the digitonin part of the digitonide molecule by anthron reagent. By MICHAELS (1958) an analogous method using an orcinol–iron(III)chloride reagent is mentioned. In these procedures the sterol part of the digitonide molecule remains intact and available for further processing.

The spectrophotometric determination of cholesterol at 200–220 nm, proposed by WEIGENSBERG *et al.* (1959) appears also very promising.

TABLE 2 Mean values of the contents of total, free, and bound sterols (a_0, a', and a'') and of the melting points of the corresponding sterol acetates (m_0, m', and m'') of some fats

Fat	Total sterols		Free sterols		Bound sterols	
	content %	melting point** of acetates in °C	content %	melting point of acetates in °C	content %	melting point of acetates in °C
VEGETABLE OILS						
castor oil	0.23	130.5	0.16	129.0	0.07	126.0
cocoa butter	0.24	130.0	0.17	130.0	0.07	128.0
coconut fat	0.10	127.5	0.06	128.5	0.04	127.0
cottonseed oil	0.31	123.0	0.20	124.0	0.11	121.0(?)
linseed oil	0.43	127.5	—	—	—	—
maize oil	0.85	129.0	0.25	129.5	0.60	130.0
olive oil	0.11	118.0	0.06	121.0	0.05	116.5
palm oil	0.04	129.5	0.03	131.0	0.01	128.0
palmkernel oil	0.08	125.5	—	—	—	—
peanut oil	0.24	128.0	0.15	128.5	0.09	127.0
hydrogenated peanut oil	about 0.07	127.5	0.03	128.0(?)	0.04	129.0(?)
pumpkin oil	0.38	163.0	0.22	168.0	0.16	157.5
rapeseed oil	0.62	136.0	0.27	138.0	0.35	135.0
safflower oil	0.31	121.0	0.22	121.5	0.09	122.5
sesame oil	0.50	128.0	0.21	129.0	0.29	—
soybean oil	0.34	130.0	0.22	132.0	0.12	122.0
hydrogenated soybean oil	about 0.30	130.0	—	—	—	—
sunflower oil	0.35	119.0	0.16	127.5	0.19	117.0
ANIMAL FATS						
butter fat	0.31	114.5	0.28	114.5	0.03	114.5
lard	0.08	115.0	0.06	114.5	0.02	114.5
beef tallow	0.08	115.0	0.06	114.5	0.02	114.5
hydrogenated whale oil	about 0.22	115.0	0.16	115.0	0.06	115.0
hydrogenated herring oil †	0.60	—	0.25	—	0.35	—
hydrogenated menhaden oil †	0.40	114.5	0.32	114.5	0.08	114.5
egg-yolk fat (hen)	3.2	115.0	—	—	—	—
INEDIBLE ANIMAL FATS a.o.						
"White grease"	0.30	115.0	0.21	114.5	0.09	114.0
"Fancy tallow"	0.25	115.5	0.21	114.5	0.04	114.0

** The sterol acetates were recrystallized twice, after cooling at room temperature. The melting points are end-melting points, determined with short Anschütz thermometers. The mean values given in this table are rounded off to the nearest half degree. † cf. THUESEN (1953)

1.4.3 Applications

Mean values of the percentages of the total-, free-, and bound-sterol fractions in several oils and fats, gathered from the literature (KAUFMANN 1941a, LANGE 1950, VITAGLIANO 1958, DEN HERDER 1955) and from numerous own experiments, are given in table 2.

The total-sterol content i.e. the sterol value a_0, has already been applied to many problems of fat analysis. Obviously, adulteration of fats with a low sterol content by a fat of high sterol content produces a significant increase in the sterol content of the mixture. Samples of palm oil e.g. contain only 0.03–0.05% of phytosterol. When a sterol content exceeding 0.06% is found, most likely the sample of palm oil is admixed with another oil or fat. In the same way adulteration of a first quality, pure lard ($a_0 = 0.08\%$) with refined inedible animal fat, e.g. the so-called White grease ($a_0 = 0.2$–0.4%) is detectable. Consequently, "lard", presenting a sterol content exceeding 0.2%, is adulterated. Theoretically, it is also possible that parts of inferior quality of the animal tissues like brains and intestines were melted out during the manufacture of the fat (ROOS 1956). However, this procedure has also to be considered as an adulteration of the fat. According to MEYER (1961) a differentiation between pressed and extracted cocoa butter can be based on the percentage of sitosterols. Whereas pressed cocoa butter contains about 0.27–0.42% of sitosterols, the sitosterol content of cocoa extraction fat, as measured with the LIEBERMANN-BURCHARD reaction, is much higher viz. 1.0–2.0% (cf. chapter 7, 5).

Another example of the usefulness of the total-sterol content in fat analysis is given by the analysis of the "synthetic butter fat" *Bolzella*, of which all fatty acid values were equal to those of pure butter fat. However, a differentiation of this fat from butter fat is enabled by a determination of the sterol content, which showed a much lower value ($a_0 = 0.08\%$) than butter fat with $a_0 = 0.3\%$ (HANSSEN 1960, ROOS 1958).

By prolonged heating or in processing a fat, e.g. refining, hydrogenation, the sterol content may change. Some examples are given of a small decrease in sterol content during the refining processes e.g. of rapeseed oil (KAUFMANN 1950), hog greases (ROOS 1956, 1958), and olive oil (VITAGLIANO 1958) (see table 3).

TABLE 3 Changes in sterol content of fats during the refining processes, according to KAUFMANN *et al.* (1950), ROOS (1958), and VITAGLIANO *et al.* (1958) respectively.

	Rapeseed oil %	Hog grease %	Olive oil "rettificato B" %
crude oil	0.60	0.37	0.46
neutralized	0.58	0.30	0.39
bleached	0.27	0.29	0.36
deodorized	0.15	0.30	0.36[5]

During the hydrogenation process the sterol content decreases gradually. MARCUSSON *et al.* (1914) followed this decrease during the hydrogenation of a whale oil sample: 0.13–0.10–0.07–0.07–0.05 and 0.02%. Part of the cholesterol may be hydrogenated to the dextro-rotatory isomers coprostanol and dihydrocholesterol, which causes a decrease in the originally higher levo-rotation of the sterol mixture. In another part of the cholesterol the structure of the 3β-hydroxyl group is changed; therefore this fraction is no more precipitable with digitonin.

During the heating of oils and fats e.g. in frying chips a considerable decrease in sterol content results (LARSEN 1943). The sterols are changed to oxidized or polymerized steroids like cholestadienes etc., which do not precipitate with digitonin. In the LIEBERMANN-BURCHARD reaction these steroids may produce strong colours. After excessive heating the sterol content of the fat may diminish to low values or even to zero. Furthermore, the structure of the remaining sterols sometimes can be altered considerably (see chapter 4).

1.5 MELTING POINT OF THE STEROL ACETATES AS A CHARACTERISTIC VALUE (sterol value *m*)

1.5.1 Procedures

In 1901 BÖMER (1901b, 1902) discovered that the presence of small amounts of phytosterol acetates from vegetable oil resulted in a significant increase in the melting point of pure cholesterol acetate prepared from butter fat. Later on this well-known phytosterol acetate test ("Phytosterinacetatprobe") was introduced in several countries in checks on the purity of butter samples, having suspiciously low R.M.W. values viz: 18–25.

The melting point diagram (fig. 8) demonstrates the increase in melting point of cholesterol acetate (melt.p.: 114.3°–115.2°C) upon addition of even small amounts of the higher melting phytosterol acetates (melt.p.: about 118°–135°C). Crystallization from 96% ethanol enriches the phytosterol acetate content in the sterol acetate mixture. Consequently, still higher melting points are obtained after this procedure. If after several crystallizations a melting point exceeding 116–117°C is obtained, the presence of phytosterol acetates and, therefore, of vegetable oil in the butter sample has been proved. Amounts of 2% of cottonseed oil in cod liver oil and 1% of sesame oil in butter could be detected after three respectively five crystallizations (BÖMER 1901b, 1902). When analysing a mixture of butter with 2½% of margarine ($a_0 = 0.1$%), DEN HERDER (1950) attained a sterol acetate melting point of 117°C after *ten* crystallizations.

Theoretically, this phytosterol acetate test only proves the presence of a foreign sterol viz. phytosterol in the butter fat. Strictly speaking, the presence of the major constituents of the vegetable fat viz. the triglycerides is *not* demonstrated. This restriction has to be considered in all methods of guide analysis.

The sterol acetate melting point, being an important characteristic value, has found application in several other problems of fat analysis. The sterol digitonides isolated via the method of "precipitation in the soap" are converted into the acetates by the acetylation procedure of WINDAUS (1909a), altered by several authors e.g. KLOSTERMANN (1913), OLIG (1914), KÜHN *et al.* (1914, 1915), STEUART (1923), HADORN *et al.* (1954a), and DEN HERDER (1955). In this investigation the method described by the last mentioned author has been used throughout.

Dissolve 100 mg of sterol digitonide in 1 ml of acetic anhydride and heat during 15–20 min in a glycerol bath at 145°C. Cool to about 80°C and add 4 ml of 96% ethanol. Heat to boiling and filter the warm solution. Carefully bring the liquid to gentle boiling and add dropwise so much water (about 0.5–1.5 ml) that the sterol acetate is on the verge to precipitate but at the boiling point still remains in solution. Cool during 2 hrs at room temperature and then during ½ hr in ice water. Filter the precipitate in a micro Büchner funnel (fig. 7) and wash with 1 ml of 80% ethanol. Purify this crude sterol acetate by two crystallizations from 1 ml of hot 96% ethanol. Wait till the solution has reached room temperature and then cool during ½ hr in ice water. Dry the precipitate during 1 hr at about 30°C and then during 10 min at 100°C. Determine the end-melting point of this precipitate carefully.

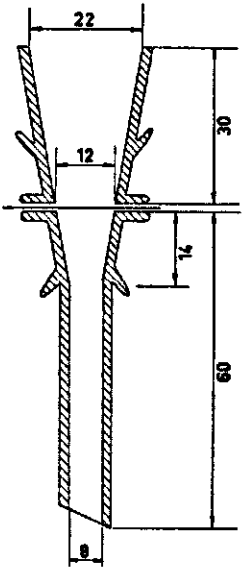


FIG. 7. Glass micro Büchner funnel for sterol acetate precipitates, used at the RIJKSZUIVELSTATION, mentioned by OLIG (1914), and SPRINKMEYER *et al.* (1914) and described in detail by DEN HERDER (1955), (cf. CANNON 1958).

Some authors prefer a simple crystallization of the sterol acetate from the acetic anhydride solution (HADORN 1954a). In table 4 the melting points of the phytosterol acetates from a variety of oils and fats are compared. It should be noted, however, that the various authors have not used the same procedure in preparing the sterol acetates. Table 2 presents mean values of the melting points of the acetates of the total-, free-, and bound-sterol fractions, calculated from our own experimental data. The melting points are not affected considerably by the quality of the fat sample, as indicated by the percentage of free fatty acids and the peroxide number (VITAGLIANO 1961).

TABLE 4 Melting points in °C of the sterol acetates prepared from edible fats.

	BÖMER (1901b)	SPRINKMEYER (1914)	KOFLER (1935)	STEUART (1923)	VITAGLIANO (1957a)	DEN HERDER (1955)	Own experiments
number of crystallizations	0	1	2	0	3	2	2
palm oil		128.6		127		129.2	129.4
palmkernel oil		126.2		128/125.5		124.8	125.7
coconut fat		127.0	128.5	127		127.3	128.0
soybean oil		131.5	129	132	127.4	132.5	129.9
maize oil		128.4			123.0	125.9	129.1
sunflower oil			119.5			118.5	118.9
cottonseed oil	124.0	124.8	125.5	122.5	122.9	122.2	123.9
rapeseed oil	134.5	135.4	128		132.0	136.5	135.5
sesame oil	128.5		127		130.8	127.8	128.1
olive oil		122.2	125		117.6–119.7	116.2	119.2
linseed oil	128.5	129.4	131	129/132		127.3	
peanut oil		126.3	128	125	126.1		126.8
pumpkin oil						167.0	163.4
safflower oil							122.1
castor oil			128				130.4
cocoa butter		128.8					130.4
Mowrah fat		162.5					
shea nut fat		165.5					161.1
butter fat	112.5–113.5		114	114.0	113	114.9	
lard		113.0	114.5	114		115.2	
White grease							115.2
beef tallow				114.5			
hardened whale oil		112.8	113	below 114		114.2	

In the literature of about 1913–1915 some controversy existed as to the appropriateness of the isolation procedures of KLOSTERMANN (total-sterol fraction) and MARCUSSON (free-sterol fraction) in BÖMER'S phytosterol acetate test. KLOSTERMANN *et al.* (1914) established, however, that the cholesterol of animal fats exists for 80–90% in the free state, the quantity of free phytosterols in vegetable fats only amounting to about 50% of the total. Because of this difference between animal and vegetable fat the application of the free sterol fraction to the phytosterol acetate test had to be regarded as an incorrect procedure. The method of KLOSTERMANN therefore was preferred many years as an official method suitable for the isolation of sterol mixtures intended for the phytosterol acetate test.

From the above data it will be obvious that a still better method of determining small amounts of vegetable oil in butter would be to perform this test with the bound-sterol fraction. The principle of this procedure was given by DEN HERDER (1955).

1.5.2 Melting point diagrams

In contrast to cholesterol acetate, having a constant melting point, the phytosterol acetates exhibit a great variety of melting points viz. from about 118°C (for olive oil) to about 163°C (for pumpkin oil) (table 2). Because of this diversity the shape of the melting point diagrams cholesterol acetate–phytosterol acetates depends upon the type of the phytosterols (fig. 8, 9, 10, 16). JAEGER (1907), constructing for the first time a complete melting point diagram with phytosterol acetates from Calabar fat, discovered a maximum at about 70% of phytosterol acetate (fig. 8A). The phytosterol acetates prepared from other vegetable oils present analogous curves, differing in curvature at high percentages of phytosterol acetate. Generally, there exists a more or less pronounced maximum between 60–80% of phytosterol acetates. A quantitative evaluation of the percentage of vegetable oil in butter fat, therefore, is only justified at low percentages of phytosterol acetate and preferably if the type of the vegetable oil and the curvature of the corresponding melting point diagram are completely known. Even when these difficult conditions are fulfilled, incorrect values for the percentage of vegetable oil are calculated because of the following phenomenon.

The melting point diagram A of fig. 9 was acquired by carefully preparing mixtures of recrystallized cholesterol acetate and soybean oil phytosterol acetate preparations. When, however, the sterol *digitonides* isolated from butter fat and soybean oil are mixed in the same ratios, slightly *higher* melting points are found for the corresponding acetates, thus producing curve B of fig. 9* (cf. ROOS 1961b).

A correct quantitative evaluation of the percentage of a vegetable fat in butter, therefore, also requires known standard curves prepared by mixing the digitonides, and a reliable, standardized crystallization procedure (ROOS 1961b).

Some vegetable oils contain phytosterol mixtures presenting aberrant melting point diagrams. The phytosterol acetates from olive oil and sunflower oil have low melting

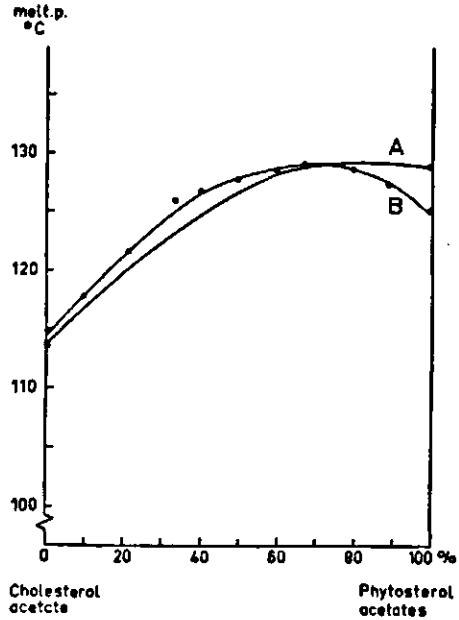


FIG. 8. Melting point diagram of cholesterol acetate with phytosterol acetates from Calabar fat (A) according to JAEGER (1907) and from cottonseed oil (B)*.

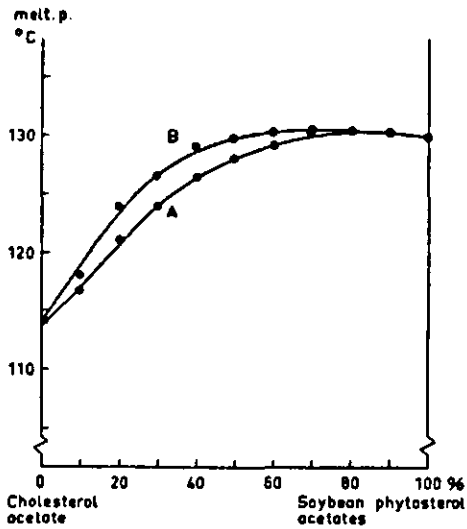


FIG. 9. Melting point diagrams of cholesterol acetate with soybean oil phytosterol acetates, obtained by mixing the acetates (A) and the digitonides (B)*.

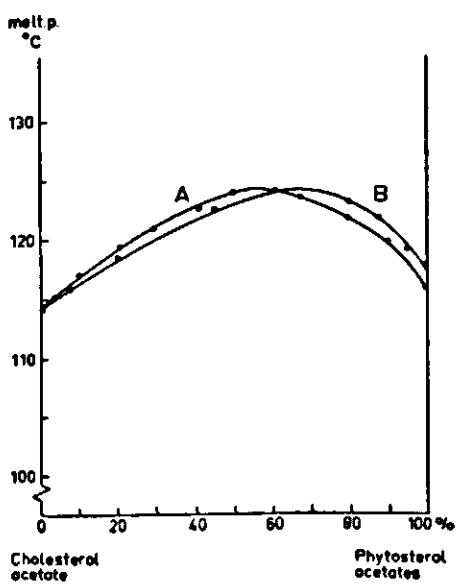
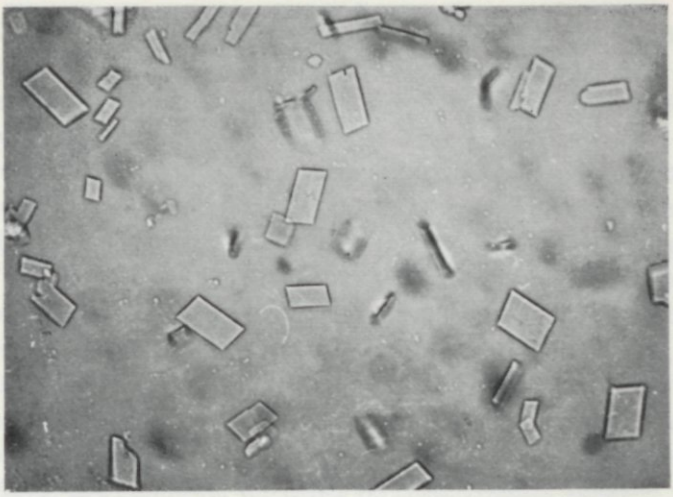
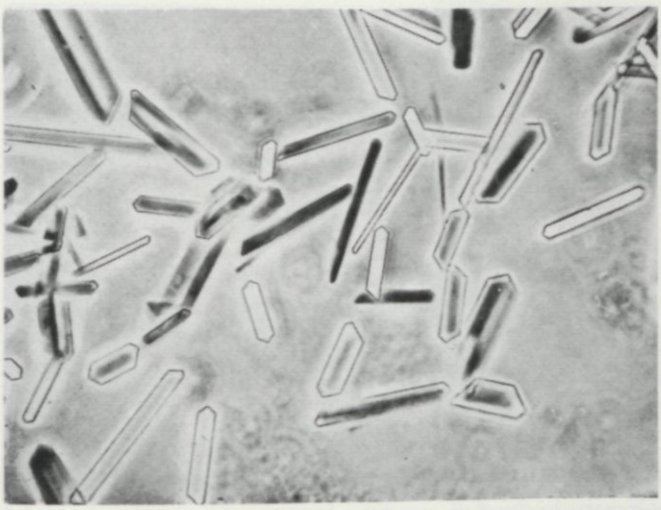


FIG. 10. Melting point diagrams of cholesterol acetate with phytosterol acetates from olive oil (A) and sunflower oil (B)*.

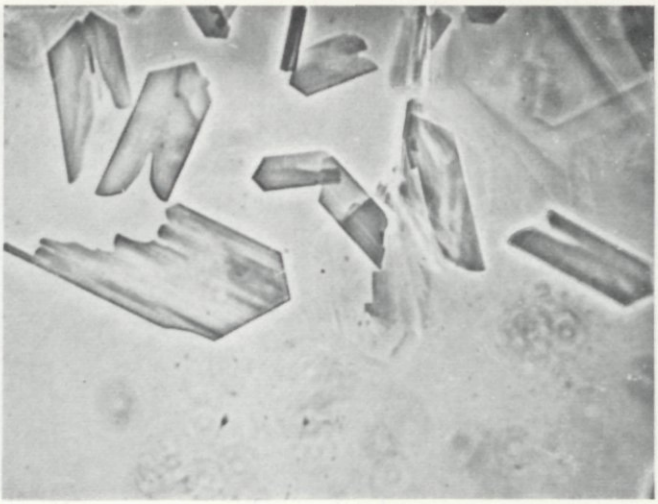


A

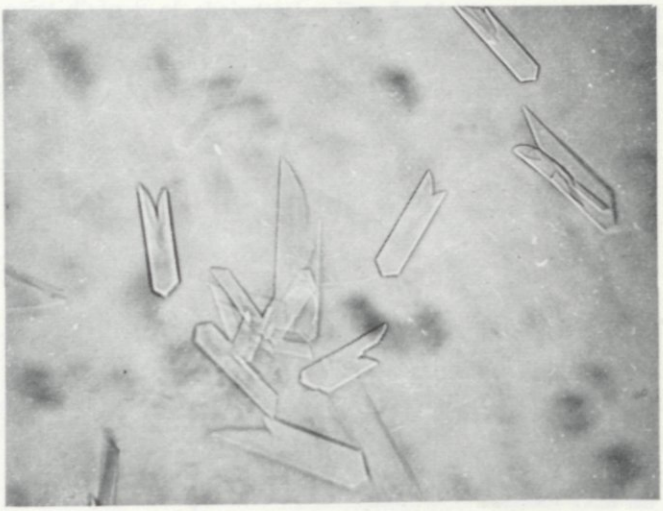


B

FIG. 11. Shape of crystals of cholesterol (A), phytosterols (B), and mixtures of cholesterol with at least 7% of phytosterol (C, turn over) at a linear magnification of 450 times (DEN HERDER 1955).



C



C

points viz. 117.6°–119.7°C (VITAGLIANO 1957a) and 118.5°–119.5°C (ALLAN 1927, KOFLER 1935) respectively. In contrast to the flat curves shown by most phytosterol acetates, the curves of olive oil and sunflower oil phytosterol acetates exhibit a steep slope at high percentages (fig. 10).
The minimum value at 90% of sunflower oil phytosterol acetates described by KOFLER *et al.* (1935) could be confirmed neither by DEN HERDER nor in our own experiments. On account of this nearly linear decrease at the right side of the diagram, small percentages of cholesterol acetate can be estimated in mixtures with this type of phytosterol acetates (table 5, A). Small amounts of vegetable oils yielding high melting sterol acetates can be detected as well (table 5, B). Admixture of e.g. 5% of soybean oil or 5% of lard to olive oil results in an increase in melting point of 1.9° and 2.8°C respectively. In some special cases mixtures of two vegetable oils can be analysed by determining their sterol acetate melting points e.g. mixtures of olive oil–soybean oil (VITAGLIANO 1957a) and sunflower oil–maize oil (DEN HERDER 1954).

TABLE 5 Increase in the sterol acetate melting points of samples of olive oil adulterated with lard (A) or soybean oil (B)

	Melting points of the sterol acetates in °C
A (COPIUS PEEREBOOM en ROOS 1960)	
Olive oil, sample 1	120.0
Olive oil 1 + 5% lard	122.8
Olive oil 1 + 10% lard	124.0
B (VITAGLIANO 1957a)	
Olive oil, sample 2	118.4
Olive oil 2 + 5% soybean oil	120.3
Olive oil 2 + 10% soybean oil	122.1

1.5.3 Reliability of BÖMER's phytosterol acetate test

The reliability of the original phytosterol acetate test of BÖMER for detecting vegetable oils in butter fat was investigated by several authors. Indeed adulterations of butter with common edible vegetable oils always could be detected by an increase in the sterol acetate melting point. However, amounts of 10–20% of some special fats i.e. shea nut fat and Mowrah fat or illipé butter in butter did *not* result in an increase in the sterol acetate melting point (SPRINKMEYER 1914, OLIG 1914, BERG 1914a, KAUFMANN 1941b). These special fats could be detected in butter fat by the characteristic dextro-rotation of their sterol-free unsaponifiable fraction of: + 38.7° and + 35.5° respectively (BERG 1914b, 1914c). The structure of the phytosterols from these fats, will be discussed in chapter 6.

Consequently, in the presence of these phytosterols the original phytosterol acetate test, will give no reliable results. DEN HERDER (1955) therefore stressed the necessity of confirming positive results of the phytosterol acetate test by subsequent examination of the sterols reobtained by saponification of their acetates (fig. 11).

The difference in the microscopic characteristics of cholesterol and phytosterols was for the first time applied to the analysis of oils and fats by SALKOWSKI (1887). He

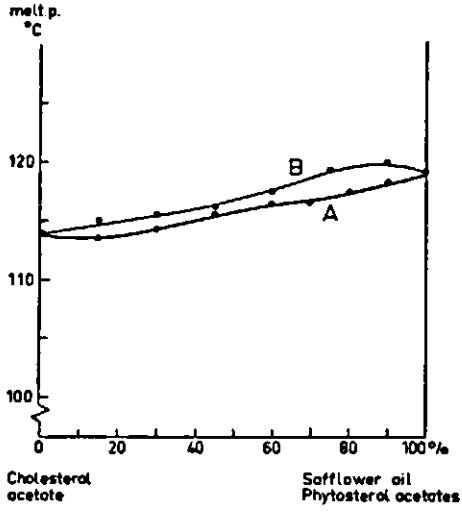


FIG. 12. Melting point diagram of cholesterol acetate with the total (A) and free (B) phytosterols from safflower oil*.

described the shape of animal fat sterols as rhombic plates, while the sterols of vegetable oil crystallize in needles. This distinction enabled the detection of e.g. cottonseed oil in cod-liver oil. SALKOWSKI even has mentioned a special type of crystals, showing a re-entrant angle. Previously, HESSE (1878) had discovered in Calabar fat a vegetable sterol, which he designated as phytosterol. SALKOWSKI erroneously believed cholesterol to be present in vegetable seed oils and phytosterols in butter fat. The difference in melting point between cod-liver sterol (146°C) and vegetable oil sterols (132°–134°C) was applied to the analysis of fat mixtures.

BÖMER (1898, 1901a, 1901b) established the fundamental difference between cholesterol occurring in animal products and the phytosterols of vegetable origin. The different habits of the crystals of these two sterol types were investigated thoroughly. In mixtures of cholesterol and the phytosterols the crystal structure of the latter prevails. The presence of peculiar telescope-shaped crystals in mixtures of cholesterol and phytosterol (e.g. ratio 3 : 1) observed by BÖMER has not been applied in practice. ZETZSCHE (1898) has described in detail another characteristic type of crystals shaped like a swallow's tail, showing a considerable difference with the normal types of

cholesterol and phytosterol crystals. Probably the special habit of these crystals would be the same as that mentioned before by SALKOWSKI.

Cholesterol crystals have the shape of a parallelogram with an obtuse angle of 100.5°; showing oblique extinction in polarized light. Phytosterol crystals appear as hexagons with top-angles of 108° or as chisels with top-angles of 72°. In polarized light they show straight extinction. The swallow-tail crystals are characterized by straight ex-

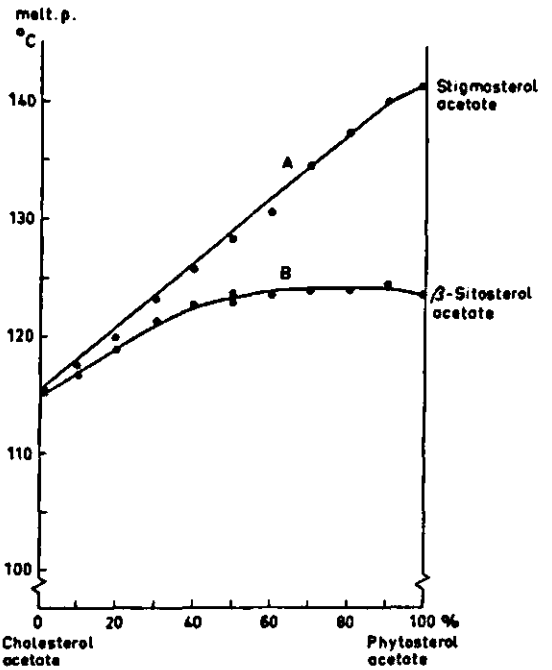


FIG. 13. Melting point diagram of cholesterol acetate with stigmasterol acetate (A) and β-sitosterol acetate (B).

tinction and an obtuse top-angle of 108°, similar to those of pure phytosterol (BÖMER 1898, DEN HERDER 1954). These crystals occur only in mixtures of cholesterol with moderate amounts of phytosterols (exceeding 7%). According to DEN HERDER (1955) the presence of swallow-tail crystals is applied as a characteristic for the presence of phytosterols and therefore of vegetable fat. Because of the high percentage of free sterols in butter fat, the phytosterol acetate test should not be carried out exclusively with the total-sterol fraction, but preferably also with the fraction of the bound sterols. Furthermore, the test should always be concluded by a microscopic examination of the sterols obtained by saponification of the acetates.

Only if the melting point of the sterol acetate mixture exceeds 116°–117°C, and some swallow-tail crystals have been found as well, a definite proof of adulteration of butter with vegetable oil is established.

We shall show in chapter 6 of this investigation that the high-melting pumpkin oil phytosterols (from *Cucurbita Pepo*), contain considerable percentages of some special phytosterols, having a $\Delta 7$-sterol structure. The melting point diagram of these pumpkin oil phytosterol acetates with cholesterol acetate does not exhibit the normal curvature but has a pronounced *minimum* at about 77% of cholesterol acetate. Consequently, a mixture of cholesterol acetate with 25% of pumpkin oil phytosterol acetates shows nearly the same melting point as pure cholesterol acetate.

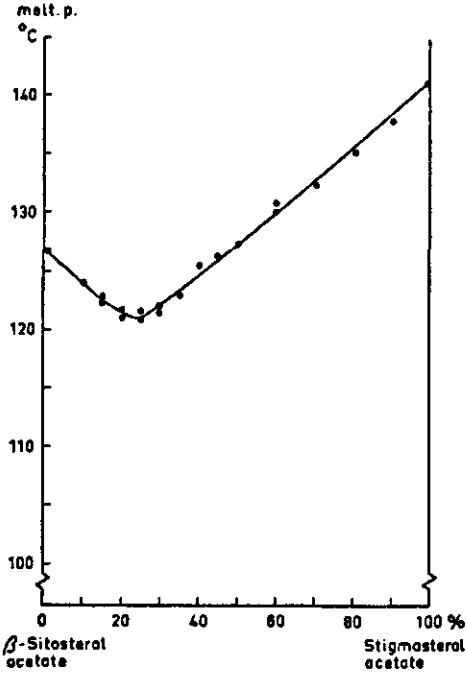


FIG. 14. Melting point diagram of β-sitosterol acetate with stigmasterol acetate.

As in the case of admixture of shea nut or Mowrah fats, admixture of this pumpkin oil to butter fat cannot be detected by the normal phytosterol acetate test. Furthermore, we have found that the melting point diagrams of the total and free phytosterol acetates from safflower oil show a peculiar, flat curve without any maximum value (fig. 12). The melting points of butter fat–safflower oil mixtures therefore only exceed 116°C at about 50% of phytosterol acetate, as will be discussed more in detail in chapter 6.

The diversity in curvature of the melting point diagrams shown by the various natural phytosterol acetates might be caused by differences in the sterol composition of these mixtures. Approximatively we can assume the phytosterol acetate mixtures to consist only of β-sitosterol acetate and stigmasterol acetate. A thorough knowledge of the

binary melting point diagrams of cholesterol acetate, β-sitosterol acetate, and stigmasterol acetate, and of all possible ternary mixtures is a clue to the shape of the curves exhibited by the natural phytosterol acetates. The melting point diagrams of binary mixtures of cholesterol acetate and pure preparations of β-sitosterol acetate (m.p. 123.9°C) and of stigmasterol acetate (m.p. 141.3°C) were determined*. A nearly linear increase in melting point is shown (fig. 13). Surprisingly, we have found an unexpected minimum value in the melting point diagram of β-sitosterol acetate with stigmasterol

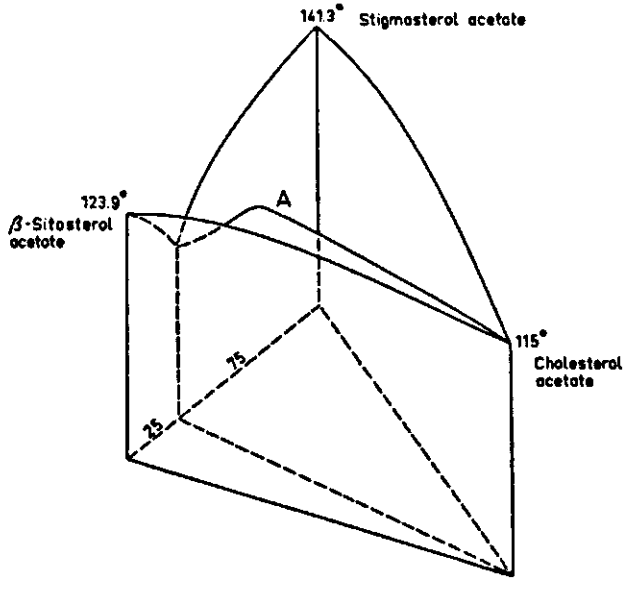


FIG. 15. Ternary melting point diagram: cholesterol acetate–β-sitosterol acetate–stigmasterol acetate*.

acetate at 25% of stigmasterol acetate (fig. 14). This minimum might be one of the causes of the occurrence of maximum values at about 75% of phytosterol acetate in the curves of the naturally occurring (ternary) mixtures. The three curves of fig. 13 and 14 together constitute a ternary system of which the frame is presented in fig. 15. The ternary intersection between cholesterol acetate and the eutectic composition viz. stigmasterol acetate–β-sitosterol acetate (25 : 75) was determined (fig. 15,A and 16). A pronounced maximum was obtained at 30% of cholesterol acetate (fig. 16,B).

The anomalous melting point diagrams of e.g. olive oil, safflower oil, sunflower oil, and pumpkin oil phytosterol acetates with cholesterol acetate might be caused by the presence of peculiar types of phytosterols differing from stigmasterol, β-, and γ-sitosterol.

The importance of the sterol acetate melting point in fat analysis has been shown in the investigation of the already mentioned "synthetic butter fat" Bolzella. Although HANSSEN *et al.* (1960) have investigated samples of Bolzella fat by determining

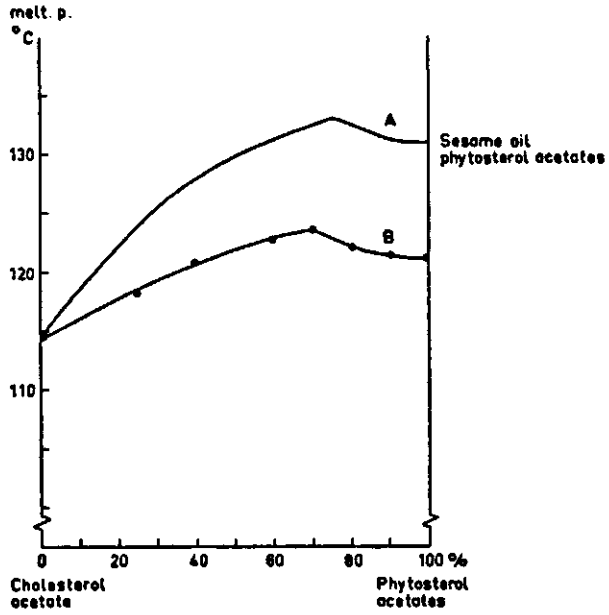


FIG. 16. Melting point diagrams of cholesterol acetate with sesame oil phytosterol acetates: A (DEN HERDER 1950) and with a synthetic mixture of stigmasterol acetate–β-sitosterol acetate (25 : 75): B*.

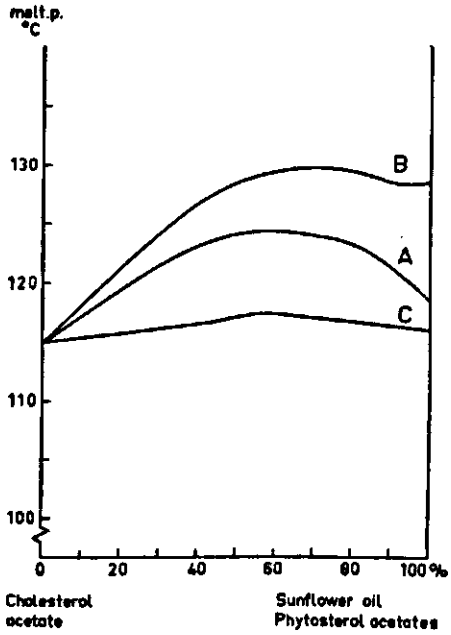


FIG. 17. Melting point diagrams of cholesterol acetate with the total (A), free (B), and bound (C) phytosterol acetates isolated from sunflower oil (DEN HERDER 1955).

numerous, non-characteristic fatty acid values, the melting point of the sterol acetates was omitted. However, already in 1956 a simple and irrefutable identification of a sample of Bolzella fat was accomplished by Roos by means of sterol analysis. The high sterol acetate melting point of 127°C and the presence of swallow-tail crystals proved an adulteration with vegetable fat beyond doubt. Thus, sterol analysis enables a simple and rapid differentiation between this Bolzella fat and genuine butter fat.

The melting points of the free-sterol fractions mostly show higher values than those of the bound-, and total-sterol fractions (see table 2). These differences must be taken into account, when applying this sterol value in fat analysis. Therefore, a knowledge of the data of the tables 2, 4, and 20 is necessary. Thus, the melting point of the free-sterol acetates of sunflower oil is 128.5°C, whereas those of the total-, and bound-sterol fractions are much lower: 118.6° and 116.1°C respectively (DEN HERDER 1955). The melting point diagrams with these sunflower oil phytosterol acetates show steep slopes for the total-, and free-sterol fractions; but a flat curve for the bound sterols (fig. 17). When analysing butter fat adulterated with sunflower oil, the isolation of the bound sterols therefore would be unsuitable. In such a case the phytosterol acetate test should be performed preferably with the *free* sterols.

1.6 CHOLESTEROL PERCENTAGE AS A CHARACTERISTIC OF STEROL MIXTURES (sterol value *c*)

1.6.1 Detection of vegetable fat in animal fat and vice versa

Quantitative evaluation of the cholesterol content of sterol mixtures would solve many problems of fat analysis. Although the presence of vegetable oils in butter can be detected qualitatively by the phytosterol acetate test and subsequent microscopic examination of the sterols, a quantitative approach of this problem was badly needed. Nowadays a quantitative determination of the amount of vegetable fats in products manufactured from skimmed milk powder and vegetable fats e.g. coconut fat, is of practical importance. Partly hydrogenated vegetable fats are exported as the so-called "vegetable ghee" to countries a.o. of the Near and Middle East. Much work has been done to devise a reliable method of detecting refined inedible fats of animal origin in this "vegetable ghee" (ROOS 1956). In this case admixture of animal fat is undesirable especially because of religious prohibitions. As will be shown in chapter 4 and 7 the purity of a vegetable ghee sample can be tested by sterol analysis. We even could establish a differentiation between admixture respectively of pure lard and of the above-mentioned inedible animal fats. The similar problem of analysing margarines labeled "pure vegetable" also had to be solved.

The percentages of conjugated and non-conjugated tetraenoic fatty acids, determined by U.V. spectrophotometry might be of use in some of these problems. LEMBKE *et al.* (1953) devised a method of detecting margarine fats in butter based on the absorption

at 302 nm. Absorption at this wavelength is due to conjugated tetraenoic systems. These are present in butter fat in amounts of about 0.0046–0.0072%. In the case of margarine fats even much lower percentages are found. MORRIS *et al.* (1952) have reported a similar method using the absorption at 316 nm. According to these authors butter fat contains 0.001–0.004% of conjugated tetraenoic systems as determined from these absorptions, whereas margarine fats contain essentially none. Just like many fatty acid values, however, the natural variability of this absorption hampers the detection of adulterants. Generally, absorption data and numbers, showing high values with pure butter fat and low ones with margarine fats, are not suitable for detecting butter adulterations.

The content of all non-conjugated tetraenoic fatty acids, the so-called arachidonic acid content, determined after alkali-isomerisation, has been applied to the detection of animal fats in vegetable oils. Whereas in the latter only negligible amounts of "this acid" ($\leqslant 0.04\%$) are found, the animal fats have arachidonic acid contents of 0.1 to about 0.6% (ROOS 1958, COPIUS PEEREBOOM EN ROOS 1960). Therefore, samples of vegetable fat containing more than 0.04% of this "acid" should be suspected of admixture with animal fat. In the presence of hydrogenated animal fats, however, the method fails.

Hydrogenated fats can be detected in butter fat by the occurrence of high quantities of the trans-octadecenoic acids, determined according to the TWITCHELL procedure (GROSSFELD 1938). By using differential infrared spectrophotometry BARTLET *et al.* (1961) found that in butter fat the percentages of cis-trans conjugated dienes and trans non-conjugated dienes are nearly equal. Partly hydrogenated fats are particularly rich in the trans isomers of oleic acid (detectable at 967 cm^{-1}), thus changing the above-mentioned ratio. This difference enabled the detection of about 7% of hydrogenated foreign fat in butter. The presence of marine animal oils can be detected by the TORTELLI-JAFFÉ colour reaction with bromine solution, or by means of a trichloroacetic acid reagent according to the procedure of BERTRAM (1937). High percentages of some other animal fats like tallow and butter fat also exhibit positive results in the latter test. This BERTRAM reaction, however, is not specific for animal fats, there being animal fats yielding negative results on testing; on the other hand there are some vegetable fats giving positive results, e.g. soybean oil.

WINDAUS (1906a) devised a method of differentiating cholesterol and the phytosterols based on the differences in solubilities of their sterol dibromides. The sterols are dissolved in ether and are treated with bromine, giving a precipitate of cholesterol dibromide, while under these circumstances phytosterol dibromides do not precipitate. This procedure is satisfactory with relatively large quantities of sterol mixtures containing at least 20% of cholesterol, but fails with smaller values (HOLDE 1906, WERNER 1911). By microscopic examination of the cholesterol and phytosterol ethers ($(C_{27}H_{43})_2O$) HOLDE (1906) demonstrated about 30% of cholesterol in mixtures with phytosterols. WERNER (1911) questioned the value of this method.

1.6.2 Detection of cholesterol from the melting point diagram

Upon adding 20% of cholesterol acetate to palm oil phytosterol acetates a small increase in melting point viz. from 128.5° to 130.0°C (points P and B in fig. 18) is observed. However, a subsequent decrease to about 129.6°C (point C in fig. 18) is

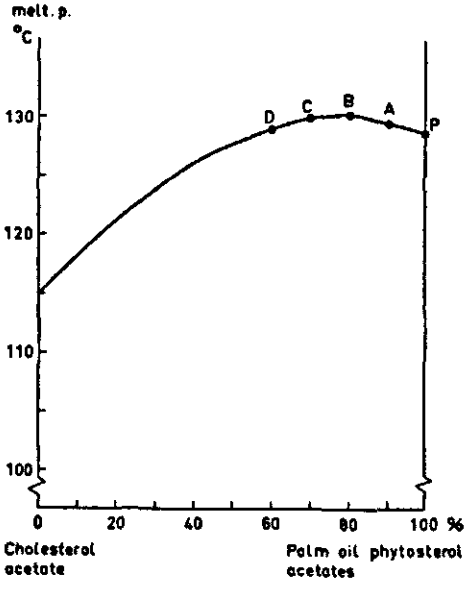


FIG. 18. Melting point diagram of cholesterol acetate with palm oil phytosterol acetates. Addition of 10, 20, 30, and 40% of cholesterol acetate extra.

found when another 10% of cholesterol acetate is added to the mixture of melting point B. Considering this difference DEN HERDER (1954) devised a qualitative, and in special cases even semi-quantitative test for detecting small amounts of cholesterol acetate. Adding three successive 10% portions of cholesterol acetate to a phytosterol acetate mixture, containing already 10% of cholesterol acetate (A), the melting points B, C, and D of fig. 18 are obtained. Starting at the phytosterol side of the diagram, point B still lies on the rising part; points C and D already lie on the descending part of the melting point diagram.

If the original phytosterol mixture had contained no cholesterol, the point C would be found still on the increasing section. In that case a decrease in melting point would just have started after the addition of 20% of cholesterol acetate. From the further decrease in melting point upon the addition of 10% of cholesterol acetate extra, we must conclude to the presence of about 10% of cholesterol acetate in the original phytosterol acetate mixture. Instead of the laborious preparation of these mixtures, in a more simple procedure only one portion of 20% of cholesterol

acetate is added. When a decrease in melting point due to this addition is observed, original admixture of cholesterol acetate indicating adulteration with animal fats will be probable.

The type of the vegetable fat and the curvature of the corresponding melting point diagrams being known, a semi-quantitative evaluation of the cholesterol content viz. C can be obtained by the formula:

C = 25% minus the percentage of cholesterol acetate which is required to achieve the maximum in the melting point diagram (if the normal place of this maximum would be at about 25% of cholesterol acetate). Because of the lack of information about the amount of animal fat already present, the calculations of the total cholesterol acetate percentage remain inaccurate. Considering the already mentioned difference between the melting point diagrams of the sterol acetates and the corresponding digitonides (fig. 9), it is recommended to mix the phytosterol digitonide from the vegetable fat with pure cholesterol digitonide in the above ratios prior to the preparation of the sterol acetates (Roos 1961b).

From microscopic examination

Microscopic examination of the swallow-tail crystals of cholesterol acetate with about 7–50% of phytosterol acetates has been applied by Roos and Van Dijk (1961a) to a qualitative identification test. After isolation of the free sterols the sterol acetates are recrystallized from 96% ethanol. Because of the higher solubility of cholesterol acetate as compared with that of the phytosterol acetates, the former is enriched in the mother liquor, whereas the precipitate will contain relatively more phytosterol acetate. After sufficient enrichment of the cholesterol acetate in the mother liquors and upon subsequent saponification, the sterols are examined microscopically. Observation of the characteristic swallow-tail crystals forms evidence of the presence of cholesterol and therefore indicates admixture of a fat of animal origin. With this procedure we could detect about 3% of the refined inedible animal fat White grease in palm oil.

1.6.3 Other procedures

According to Lada (1954) after oxidation of the phytosterols with chromic acid small amounts of propionic acid and some other acids are found. Under these circumstances pure cholesterol would yield no propionic acid. The value of this method is questionable.

Obviously, many physicochemical data of cholesterol and the phytosterols do not have significant differences. The molecular extinctions of cholesterol, stigmasterol, and β-sitosterol in U.V. spectrophotometry appeared to be nearly equal, the values of $\varepsilon_{max.}$ for $\lambda = 203$ nm respectively amounting to: 3400, 3800, and 2800 (Bladon 1952). Optical rotation values and the curves obtained by optical rotatory dispersion measurements of the xanthates of cholesterol and β-sitosterol, given in fig. 19, exhibit no differences* (cf. Dirkx 1961).

The value of infrared spectrophotometry for solving this problem is questioned by BEHER *et al.* (1957). According to these authors and to GATTORTA *et al.* (1961) cholesterol and the phytosterols can be differentiated by their X-ray diffraction powder diagrams, as is also shown by our own experiments (see fig. 20).

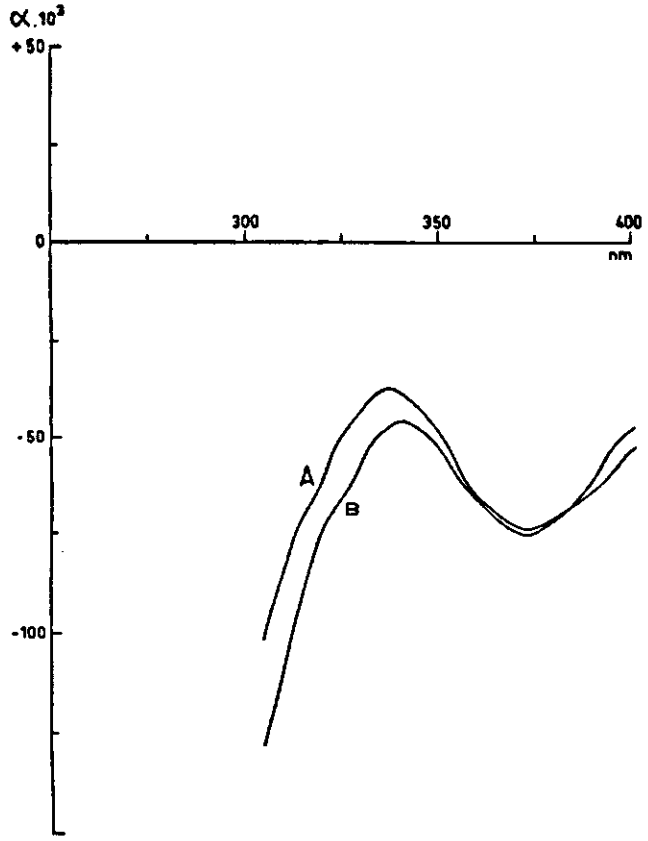


FIG. 19. Rotatory dispersion curves of cholesterol xanthate (A) and β-sitosterol xanthate (B).

Reviewing the methods mentioned in this chapter, as applied to the detection of vegetable oils in butter, the tocopherol method fails with some oils like olive oil and coconut fat. As has been demonstrated, even BÖMER's original phytosterol acetate test meets with difficulties in adulteration of butter fat with a.o. shea nut fat and pumpkin oil.

An identification of foreign *animal* fats in butter is not possible with the procedures of sterol analysis, but will have to be accomplished by other methods, preferably by those of glyceride fat analysis (HORWITZ 1954, BHALERAO 1956a, 1956b).

No procedure for a quantitative determination of the percentages of cholesterol and phytosterols in their mixtures was available at the start of this investigation. The few

methods, which were described for the detection of animal fats in mixtures with vegetable fats, are not always reliable and are not suitable for quantitative evaluation.

Knowledge of the percentages of cholesterol and phytosterols, would enable us to make evaluations about the amounts of animal and vegetable fats present in their

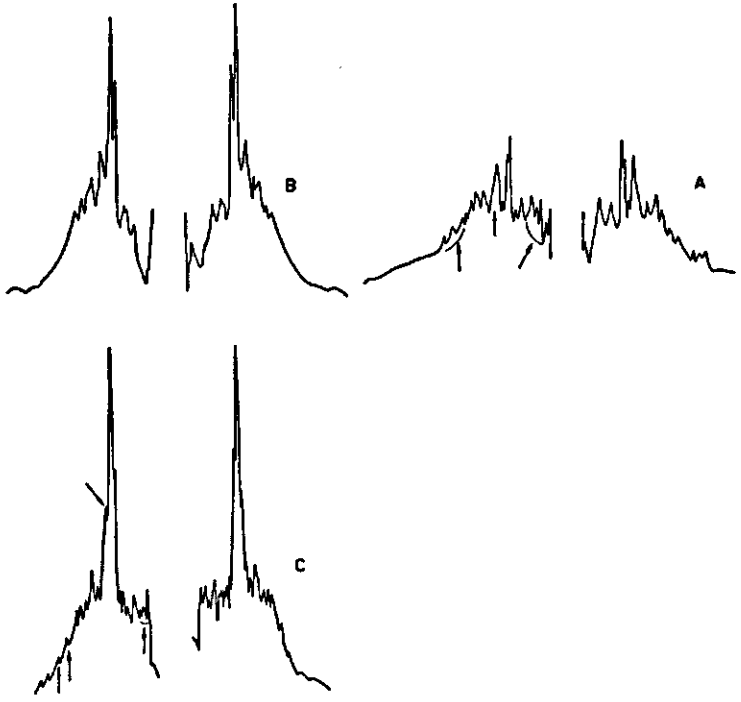


FIG. 20. Densitometric curves of X-ray diffraction powder diagrams[1] of cholesterol (A), soybean oil phytosterols (B), and a mixture cholesterol–soybean phytosterols (4 : 6) (C). Characteristic diffraction peaks of cholesterol are indicated by arrows*.

mixtures. The semi-quantitative analysis of special fat mixtures like butter–vegetable oil, palm oil–lard mixtures, and a check on the purity of vegetable fats then would become possible. Some examples of the more elaborate calculations based on the cholesterol content of sterol mixtures, and on the total-sterol contents of the fats will be given in chapter 7.

The structure of cholesterol and some phytosterols like campesterol and stigmasterol only differs in one or two methyl groups or a double bond in the side chain. Therefore, a separation of these sterols based on different physicochemical properties in dependance of the nature of their side chains ought to be attempted. Higher fatty acids, also differing only in the number of methyl groups and double bonds, were separated

[1] I thank DRS. A. DE VRIES (PHILIPS, Eindhoven) for his help and advice.

with reversed-phase partition chromatography, making use of their different solubilities and partition coefficients. Analogously to these separations, the various techniques of chromatography might possibly also succeed in fractionating these closely related sterols.

The aim of this investigation, therefore, was to examine the present possibilities of chromatographic analysis of sterols in order to achieve reliable procedures for both reciprocal problems viz. the detection of vegetable oils in butter and of animal fats in vegetable fats. Furthermore, to obtain quantitative data for the percentages of cholesterol and phytosterols in these mixtures. The application of these data to several problems of fat analysis will be discussed. Some chromatographic separations of steroids and sterols were known from the literature. However, not long ago R. P. Cook in his well-known textbook: "Cholesterol" (1958) stated "it is difficult to separate a mixture of C_{27}, C_{28}, and C_{29} sterols with a double bond in position 5".

2 THE CHROMATOGRAPHIC SEPARATION OF STEROLS

2.1 Adsorption chromatography

Many substances belonging to the group of the polar steroids can be separated by adsorption chromatography on columns of aluminium oxide, silicic acid etc. (Neher 1958). Results with the lipophilic, apolar sterols, however, were not encouraging. Generally, it was found that only those sterols which show marked structural differences can be separated by adsorption methods. The type and number of the polar substituents like hydroxyl and keto groups, further the systems of conjugated double bonds are of importance. The normal $\Delta 5$-mono-unsaturated sterols such as cholesterol, β-sitosterol, and the group of conjugated sterols e.g. ergosterol and 7-dehydrocholesterol are readily separated on aluminium oxide columns (Bock 1938, Winterstein 1933, Windaus 1936, Boer 1936). Because of the difficult detection of mono-unsaturated sterols in these procedures, some authors have studied the chromatographic behaviour of specially selected coloured sterol esters.

A separation of some sterol esters of p-nitrobenzoic acid was described by Brockmann *et al.* (1947); of esters of azobenzene carboxylic acid by Ladenburg *et al.* (1938) and by Idler *et al.* (1952); and of ^{131}I labeled *p*-iodobenzoic acid esters by Stokes *et al.* (1955, 1957). Sterols differing in the degree of unsaturation or in the position of a nuclear double bond (e.g. $\Delta 7$-cholestenol and $\Delta 5$-cholestenol) were found to be separable by these methods. Also sterols showing steric differences in the shape of their molecules e.g. in the conformation at the junctures of the rings A and B and the configuration of the hydroxyl group are most readily separated by adsorption methods.

Examples are the separations of the closely related sterols: cholesterol (3β-OH) – epi-cholesterol (3α-OH) by Galinovsky *et al.* (1948); of dihydrocholesterol (3β-OH, A/B: trans) – epi-dihydrocholesterol (3α-OH, A/B: trans) by Galinovsky *et al.* (1948); of coprostanol (3β-OH, A/B: cis) – epi-coprostanol (3α-OH, A/B: cis) by Lederer *et al.* (1946); of coprostanol – dihydrocholesterol by Von Christiani *et al.* (1944) and of cholesterol–lanosterol a.o. by Daniel *et al.* (1945). No separations were reported in the group of the $\Delta 5$-mono-unsaturated sterols, differing only in the length of the side chain and the presence of a double bond. These small structural differences usually do not affect the adsorption affinity of the sterol for the adsorbent. Consequently a separation in this group of sterols is not possible.

Differences in adsorption affinity are also employed in a special type of paperchromatography, in which the paper is coated with an adsorbent like silicic acid or aluminium oxide. Several classes of lipoid substances are analysed in this way (ROUSER 1961). QUAIFE *et al.* (1959) described the separation of cholesterol and the cholesterol esters on zinc carbonate coated paper. In blood serum the percentages of free and esterified cholesterol can be determined quantitatively. Similar separations on paper coated with silicic acid, upon elution with ether–light petroleum mixtures, were performed by CORMIER *et al.* (1957). On papers coated with aluminium oxide, SHULL *et al.* (1952) separated several sterols e.g. cholesterol, ergosterol, and vitamin D_3 using mixtures of hexane and ether. Although the R_F values of pure cholesterol and β-sitosterol are slightly different, no *separation* of these sterols has been reported (table 6 no. 1). Applying these methods to the analysis of cholesterol and the related phytosterols no separation in this group could be accomplished (RIEMERSMA 1957).

Instead of coating the paper with an adsorbent, KRITCHEVSKY *et al.* (1952) impregnated it with the waterrepelling agent: Quilon (a commercial stearato chromic-(III)complex. With alcohol–water mixtures e.g. ethanol–water (80 : 20) (see table 6 no. 2) or methanol (see table 6 no. 3) conjugated sterols such as ergosterol and 7-dehydrocholesterol were separated from cholesterol and the phytosterols. No separation was obtained between the sterols of the latter group. DAVIS *et al.* (1952), using similar mobile phases viz. methanol–water (95 : 5) and methanol–water–ethylene glycol monomethylether (62 : 19 : 19), did only succeed in separating the vitamins D_2 and D_3 from the other sterols. The differences in R_F values between the $\Delta 5$-sterols and the conjugated sterols like ergosterol even appeared to be relatively small (table 6 no. 4, 5). Applying these Quilon systems to the analysis of phytosterol mixtures, VITAGLIANO (1957a, 1957b) could distinguish the phytosterols of olive oil with R_F values of 0.33–0.41 from those of the other vegetable oils with R_F values of 0.41–0.58 and from pure cholesterol ($R_F = 0.56$). In some cases this paperchromatographic analysis thus enabled the detection of an adulteration of olive oil with other vegetable oils or with animal fat (table 6 no. 6). KISS *et al.* (1956) described the separation of some steroids and sterols a.o. cholesterol, ergosterol, and cholesterol acetate on paper impregnated with the aluminium soaps of palmitic and stearic acid, applying mixtures of alcohols, water, and carbon tetrachloride as mobile phases (table 6 no. 7).

The polar properties of the paper may be modified by changing the structure of the cellulose molecules e.g. by introducing acetyl groups. Papers having a high degree of acetylation enabled RITTER *et al.* (1958) to analyse several steroids. Mixtures of benzene, methanol, and water have been applied as mobile phases. No separation of cholesterol and the phytosterols could be accomplished in our own experiments with commercial acetylated papers (a.o. Schleicher and Schüll no. 2043b "vollacetyliert").

From the preceding instances it has to be concluded, that the structural differences

between cholesterol and the phytosterols are too small to enable separation by these methods, just as in the case of adsorption column chromatography. Better results are likely to be obtained with pure liquid–liquid partition paperchromatography, where the migration rates are determined by the partition coefficients for two immiscible phases: the stationary and the mobile phase. In the "oldest" type of paperchromatography the stationary phase is constituted by a thin water layer attached to the paper, the mobile phase consisting of mixtures of e.g. polar alcohols, acids, and water. When the solubilities of substances in the mobile phase are very high, as compared with those in the stationary phase, polar organic liquids have to be introduced as stationary phase e.g. formamide, propylene glycol etc. When separating substances of a very low polarity, stationary phases of apolar nature have to be chosen e.g. paraffin oil, silicone oil, high petroleum fractions etc. In the analysis of lipoid substances this third type of paperchromatographic technique, known as *reversed-phase paperchromatography*, has become of great importance by the work of many authors a.o. by KAUFMANN c.s. and MANGOLD c.s.

Only a few authors have studied the paperchromatography of sterols using the normal systems, in which the stationary phase is constituted by a cellulose–water complex. MCMAHON *et al.* (1950) succeeded in separating cholesterol and 7-dehydrocholesterol in such a system (R_F values: 0.0 and 0.9) by means of a phenol–methanol–water (13.5 : 30 : 56.5) mixture (see table 6 no. 8). LATA *et al.* (1952) separated cholesterol and the cholesterol esters in n-butanol saturated with water, whereas KAISER *et al.* (1956) mentioned a separation of the lipoids: vitamin A alcohol and vitamin A acetate in an analogous system viz. isopropanol–water (1 : 1) (see table 6 no. 15).

To achieve better separations of steroids and sterols, the water–cellulose complex had to be substituted by apolar organic liquids like formamide, dimethylformamide, propylene glycol, phenyl cellosolve etc. As mobile phase a great variety of apolar solvents: hexane, benzene, chloroform etc. has found application. With these so-called ZAFFARONI systems various types of polar steroids and a.o. cholestenediols (SMITH 1954) are analysed. Separations of apolar sterols by these systems were reported in a few cases (NEHER 1958). NEHER *et al.* (1952) described the separation of ergosterol, stigmasterol, and cholesterol in the system: phenyl cellosolve (ethylene glycol monophenylether)/heptane by a multiple descending technique consisting of several elutions with interjacent periods of drying. Using the same system LINKS (1955) succeeded in separating ergosterol and 7-dehydrocholesterol at the low temperature of —8°C. However, RIDDEL *et al.* (1955), also applying this phenyl cellosolve system, could observe no difference between the R_F values of these three sterols (table 6 no. 10). Our own experiments with this system as well as with similar systems a.o. dimethylformamide/hexane–chloroform–methanol mixtures also produced discouraging results.

Analogously to the successful separation of the higher fatty acids on papers impregnated with petroleum fractions, paraffin or silicone oils (KAUFMANN 1954), these

reversed-phase systems have been applied to the analysis of sterols. Although the naturally occurring fatty acids with even numbers of carbon atoms are readily separated, the analysis of the entire sequence of both the even and the odd numbers could only be accomplished with great effort. Expectantly, the analysis of the closely related Δ5-sterols would meet with more difficulties than does the separation of naturally occurring fatty acids.

KODICEK AND ASHBY (1954) have studied the separation of ten sterols on such paraffin impregnated papers, developing ... se with mixtures of ethylene glycol monoethyl-ether–n-propanol–methanol–water (35 : 10 : 30 : 25); n-propanol–methanol–water (15 : 82 : 3); and methanol–water (95 : 5) (see table 6 no. 11, 12, 13). On account of the R_F values for pure sterol preparations, the first system would theoretically enable a separation of cholesterol–β-sitosterol. Analogous results using these systems were reported by SWELL (1956). MARTIN *et al.* (1955) and MARTIN (1957) have separated various steroids and sterols a.o. cholesterol acetate, coprostanol, dihydrocholesterol, cholesterol, and 7-ketocholesterol in the system: petroleum (bp 180–220°C)/n-propanol–water (60 : 40), whereas KUČERA *et al.* (1957) have reported different R_F values for cholesterol and β-sitosterol (0.54 and 0.65) in the system petroleum/n-propanol–water (88 : 12). However, when analysing natural sterol mixtures no reliable separations could be realized in such systems* (RIEMERSMA 1957, GRACIAN 1959).

In the laboratory of the RIJKSZUIVELSTATION (GOVERNMENT DAIRY STATION) at Leiden the system: paraffin/methanol–water (95 : 5) was studied by RIEMERSMA (1957). He obtained sufficient difference between the R_F values of cholesterol and the phytosterols even when analysing the sterols isolated from mixtures of animal and vegetable fat. The method was applied in practice to the identification of small amounts of animal fats in vegetable fat. Within the group of phytosterols, however, no separation was achieved. Later on GRACIAN *et al.* (1959) reported a difference between the R_F values of cholesterol and β-sitosterol using papers impregnated successively with silicone oil and paraffin oil. The mobile phases consisted of mixtures of ethanol–isopropanol (90 : 10 or 80 : 20) and ethanol–isopropanol–cyclohexane (60 : 30 : 10) (table 6 no. 16). Mixtures of both sterols, however, show elongated spots with intermediate R_F values.

In the reversed-phase chromatographic analysis of substances differing in minor details e.g. number of methyl groups, in some cases good results are achieved by masking the reactive, polar groups of the molecule. The effect of an increase in chain length by one apolar methyl group then might produce a relatively more pronounced effect on the polarity of the molecule. The polarity of reactive groups viz. double bond and hydroxyl group is changed e.g. by preparing the halogen addition compounds or the acetates. GRACIAN *et al.* (1959) separated the bromine addition compounds of cholesterol and β-sitosterol on papers impregnated with silicone oil and paraffin oil. After elution with acetic acid–water (85 : 15) the cholesterol dibromide remains at the starting point and is thus separated from the moving β-sitosterol dibromide. However,

this method fails in the presence of stigmasterol, since the sparingly soluble stigmasterol tetrabromide also remains at the starting point. Separations of the "doubly masked" sterol acetate dibromides in the systems: paraffin/acetic acid–water (80 : 20) or ethanol–water (95 : 5) were studied by RIEMERSMA *et al.* (1958). Although no separation of cholesterol acetate dibromide and β-sitosterol acetate dibromide could be achieved, this method enabled the identification of the di-unsaturated stigmasterol in mono-unsaturated C_{28} and C_{29}-phytosterol mixtures. After bromination of the sterol acetate mixture by WINDAUS' procedure, the solution is directly spotted on the chromatogram. The insoluble stigmasterol acetate tetrabromide remains at the starting point, while the other sterol acetate dibromides move a small distance. In this procedure no detection of the sterols by phosphomolybdic acid is possible. The antimony(III)chloride reagent as applied by RIEMERSMA (1958) could advantageously be substituted by a 50% ethanolic cadmium chloride solution. In U.V. radiation of 365 nm the sterol acetate dibromides become visible as fluorescent spots*.

In the literature separations of the esters of cholesterol with several fatty acids viz. the C_1–C_{18} sequence on paraffin impregnated papers are mentioned e.g. by ZIMMERMANN *et al.* (1956) and MICHALEC *et al.* (1960), using the systems: paraffin/acetic acid–chloroform–paraffin oil (65 : 25 : 10 or 80 : 15 : 5) and paraffin/acetic acid. Between the acetates of cholesterol and β-sitosterol no reliable separations could be achieved in these as well as in many other systems e.g. paraffin or silicone oil/alcohols, acetic acid, water mixtures; formamide, dimethylformamide or phenyl cellosolve/with mixtures of hexane, benzene, chloroform* etc. The comparatively best results were obtained with the system: paraffin/ethanol–water (95 : 5), yielding R_F values of 0.51 and 0.46 for cholesterol acetate and β-sitosterol acetate*.

In the study of TUNMANN (1956) dealing with the composition of the sterol mixture from *Potentilla anserina*, the acetates from β-, and γ-sitosterol, ergosterol, and the constituents of the Potentilla sterol mixture were clearly separated in the system: paraffin/ethanol or acetic acid. However, no experimental details of the procedure were given.

The very best results known at the start of this investigation had been obtained by SULSER AND HÖGL (1957), in separating cholesterol and the phytosterols. By a special technique of horizontal semi-circular chromatography using the system: paraffin/acetic acid–water (84 : 16) natural sterol mixtures containing cholesterol, stigmasterol, and β-sitosterol were separated into three distinct semi-circular bands (table 6 no. 18). By this procedure cholesterol is differentiated with certainty from the phytosterols, thus enabling the detection of small amounts of animal fat in vegetable oils. The phytosterol mixtures of a few vegetable oils were investigated by these authors.

Reviewing all paperchromatographic systems, the system of SULSER AND HÖGL was chosen as the starting point of this investigation. With the semi-circular technique ("Halbkreisverfahren"), described by these authors, generally the maximum degree of separation of closely related substances can be accomplished. However, the method

appeared to be less suitable for routine work, permitting only the analysis of one or two sterol mixtures with one piece of paper. Furthermore, the solvent front proceeds only slowly and after an accommodation during 16 hrs the elution required at least 70–80 hrs. Since a considerable reduction of this period was desirable, the normal ascending technique was attempted. Just as experienced by the above-mentioned investigators, with this procedure no separation between cholesterol and the phytosterols could be achieved. By MATTHIAS (1954) a method was designed combining the advantages of circular technique and ascending method, respectively high degree of separation and rapid development. Small wedge-shaped paper strips (in German denoted as: Keilstreifen) are mounted in a frame by means of two glass rods. Small semi-circular bands are formed on the paper strip; thus enabling a clear separation of complex mixtures.

We have attempted to apply the technique of MATTHIAS to the separation of sterols but have devised a slightly modified form of the paper. Hexagonal holes are made in a sheet of paper (about 20 × 50 cm), leaving bridges of 1 cm width (fig. 21). With this technique we were able to obtain even better separations than with SULSER AND HÖGL'S method. Furthermore, the time of run was decreased to about 48 hrs. This technique enabled the simultaneous analysis by paperchromatography of many sterol mixtures.

The degree of impregnation with paraffin oil appeared to exert great influence. On account of the experiences by KAUFMANN *et al.* (1954), who investigated the behaviour of higher fatty acids in similar systems, a degree of impregnation of about 0.15–0.16 g paraffin oil/g of paper was applied. After their isolation from the fat mixture, the sterols are dissolved in chloroform (0.1% solution). A quantity of about 20 μl is then spotted on the 1 cm wide bridges between the hexagonal holes of the chromatogram (see fig. 21). By this arrangement the mobile phase is now forced to flow through the spot, directly eluting the sterols from it. In the normal ascending technique the solvent front often surrounds the spot during the first moments of elution. This phenomenon is caused by impurities at the starting point. The migration rates of the substances can thereby be affected considerably.

By the MATTHIAS' technique, however, this anomalous behaviour is avoided. After elution the dried chromatogram is sprayed with phosphomolybdic acid reagent. When analysing a cholesterol–phytosterol (2 : 8) mixture three clearly separated blue-green coloured bands are produced (fig. 21).

Under these experimental circumstances the R_F values of the three bands are respectively 0.25; 0.30; and 0.34. The first band contains β-sitosterol, the second band the pair: campesterol-stigmasterol and the third band is produced by cholesterol (last column no. 22, table 6). The degree of separation of these bands depends upon many experimental conditions, which will be discussed more in detail in chapter 3. Using the high degree of impregnation of about 0.15 g paraffin oil/g paper, the R_F values appear to be very reproducible. Considering the R_F values of a great number of

sterols some relationships between structure and migration rate were established, as will be shown in the next chapter.

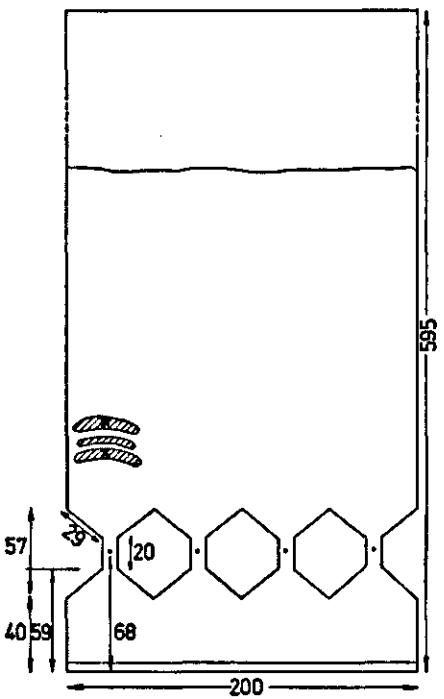


FIG. 21. MATTHIAS paperchromatogram with three separated sterol bands. System: paraffin/acetic acid–water (84 : 16). Dimensions in mm.

Further experiments established that the degree of separation of the three bands still could be increased by decreasing the amount of paraffin oil. The best separations have been achieved using a degree of impregnation of 0.09–0.10 g paraffin oil/g paper. The reproducibility of this degree of impregnation and of the resulting R_F values, however, appeared to be reduced as compared with that using 0.15 paraffin oil/g paper. The low degree of impregnation was applied further on in routine analysis of sterol mixtures. The application of this chromatographic analysis of sterols to several problems in fat analysis, especially in the detection of vegetable fat in animal fats, and vice versa, will be discussed more in detail in chapter 4.

During our investigation two other studies dealing with the paperchromatography of sterols have been published. Experiencing difficulties with the paraffin/acetic acid systems, DE ZOTTI *et al.* (1959) have devised a separation of cholesterol and β-sitosterol in the analogous system: petroleum (bp 220–240°C)/pyridine–water (85 : 15). In addition to the separation of cholesterol and β-sitosterol, a separation of the several types of phytosterols into two or three spots has likewise been obtained. Some sterol mixtures e.g. from the unsaponifiable fraction of rapeseed oil were separated into three spots. The acetates of cholesterol, β-sitosterol, and ergosterol were fractionated

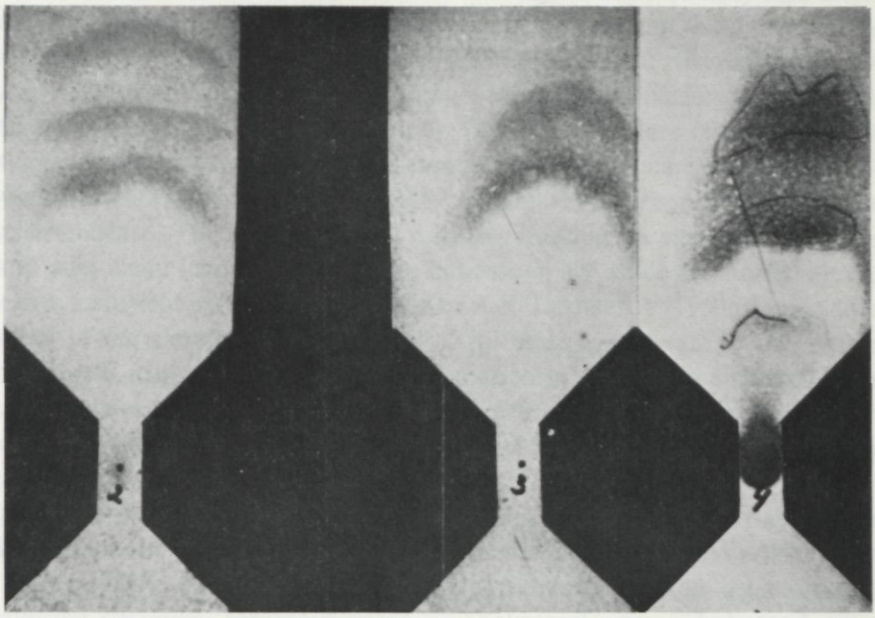


FIG. 22. Paperchromatographic analysis of sterols.
spot 2. mixture of palm oil with 10% of lard.
spot 3. soybean oil phytosterols.
spot 4. unsaponifiable fraction of a palm oil–lard (7 : 3) mixture.

in the system: petroleum (bp 220–240°C)/acetic acid–water (98 : 2) with a time of run of 63 hrs. The stenones prepared from the corresponding sterols by the OPPENHEIMER procedure were separated in the same system.

A variety of even-numbered C_2–C_{22} saturated and C_{18} unsaturated fatty acid esters of β-sitosterol, γ-sitosterol, stigmasterol, ergosterol, stigmastanol, and γ-sitostanol were separated by KŮKSIS AND BEVERIDGE (1960a) in the systems: paraffin/methanol–chloroform–water–acetic acid (45.5 : 45.5 : 4.5 : 4.5); propionic acid–water (90 : 10); and acetic acid–chloroform–water (75 : 20 : 5). The high R_F values of all free sterols were not conducive to enable a good separation to be made. A nearly linear relationship between R_F value and molecular weight (or number of carbon atoms) of these esters was established. The introduction of a double bond in the sterol side chain or in the fatty acid chain resulted in the same increase in R_F value as shortening the latter by two methylene units. Later on KŮKSIS AND BEVERIDGE (1960b) reported a fractionation of the sterols from a corn oil sterol ester hydrolysate in the system paraffin/propionic acid–water (88 : 12). The separation in this system appeared to be similar to those in the above-mentioned paraffin/acetic acid–water systems. The corn oil sterols were fractionated into three clearly discernable bands, thus producing chromatograms analogous to those presented in figs. 21 and 22.

The application of *gas-liquid chromatography* at high temperatures to the analysis of steroids and sterols has been reported for the first time in 1960 by BEERTHUIS (1960a, 1960b). Using very sensitive detection methods viz. argon detectors and flame ionisation detectors, separated peaks of cholesterol esters, cholesterol, sitosterols, and of pairs e.g. cholesterol–dihydrocholesterol can be obtained (BEERTHUIS 1960b, EISNER 1962, FRANCO 1962). It is therefore to be expected that the introduction of gas-liquid chromatography in steroid and sterol analysis will yield good results.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 Preparation of the sterol mixture

SULSER AND HÖGL (1957) analysed the total unsaponifiable fraction of fat mixtures by paperchromatography without any previous purification. Other constituents of the unsaponifiable fraction such as hydrocarbons, tocopherols etc., however, are coloured as well by the phosphomolybdic acid reagent. When large quantities are spotted on the paper e.g. to detect small amounts of phytosterols in cholesterol, bands caused by these substances may hamper the analysis (see fig. 22, spot 4). Therefore, a previous purification procedure of the sterols present in the unsaponifiable fraction is necessary. The sterols are isolated as their digitonides according to the method of "precipitation in the soap" mentioned in chapter 1. The subsequent preparation of the sterols from their digitonides can proceed according to three alternative methods.

A. The digitonides are converted into the sterol acetates according to the procedure mentioned in chapter 1. The sterol acetates are saponified into the sterols as follows: 2–10 mg of sterol acetate is dissolved in 1 ml of 96% ethanol and saponified by addition of two drops of 40% potassium hydroxide. The saponification is continued till the acetates are completely dissolved as evidenced by the clearness of the solution. Add 10 ml of water and extract this solution twice with 10 ml of ether. Wash the combined ethereal extracts four times with water, dry over anhydrous sodium sulfate, and filter. After evaporating the ether, the residue is dissolved in chloroform p.a. (0.1% solution) and spotted on the paperchromatogram.

B. Since the old WINDAUS' procedure of splitting the digitonides in boiling xylene has some disadvantages e.g. discolouration of the sterols, SCHOENHEIMER (1933) devised the so-called pyridine splitting procedure:

About 15 mg of digitonide is dissolved in 1 ml of dry pyridine. After a reaction time of 16 hrs at room temperature the digitonide is splitted and the digitonin is precipitated by adding 50 ml of ether. After centrifugal separation the digitonin is washed with another 50 ml portion of ether. After a second centrifuging the ethereal extracts are combined, washed with a saturated sodium bicarbonate solution and dried. After evaporating the ether, the residue is dissolved in chloroform. This procedure has been modified by BERGMANN (1940). The pyridine solution of the digitonides is heated during 1 hr at 70–100°C and evaporated to dryness in vacuo. The residue is ground and the sterols are extracted with ether.

With the method of SCHOENHEIMER good results can be obtained. We have modified this procedure by replacing the solvent pyridine, which has some disadvantages, by dimethylformamide.

C. An amount of 50 mg of sterol digitonide is triturated with 500 mg of sodium acetate according to a procedure of LIFSCHÜTZ (1935). The digitonide is gradually dissolved by heating the mixture during ½ hr in 5 ml of 96% ethanol. After addition of 25 ml of ether the digitonin and the salt are precipitated. The precipitate is filtered and washed with ether. The sterol is recovered by evaporating the solution. A sample of coconut fat and a mixture of soybean oil with 20% of a refined inedible animal fat (White grease) were analysed according to these three procedures. The data obtained by paperchromatographic analysis of the resulting sterol mixtures are given in table 7. The sterol mixtures isolated according to the procedures B and C appeared to be somewhat less pure than those prepared via the sterol acetates.

In the routine procedure of sterol analysis the same portion of sterol acetates supplies material for the determination of the melting point and for the preparation of the sterols according to procedure A. When evaporating the ethereal solution care must be taken to avoid excessive heating. The 0.1% solution of sterols in chloroform is autoxidized easily a.o. to hydroxy compounds. Therefore, the sterol solution has to be spotted on the chromatogram at once. Applying procedure A on 15 g of fat with a sterol content of 0.2% the yield of purified sterols will be about 15 mg. With the

methods B and C nearly quantitative yields can be obtained. The difference in paperchromatographic analysis of the *total* unsaponifiable fraction and the *purified sterol mixture* is shown in fig. 22.

TABLE 7 Separation of the sterols from rapeseed oil, coconut fat and from a soybean oil–White grease mixture. Isolation according to the sterol acetate method (A), or to the dimethylformamide (B) or sodium acetate (C) splitting procedures.

Rapeseed oil phytosterols	A %	B %	C %	Coconut fat phytosterols	A %	B %	C %	Soybean oil–White grease (8 : 2) mixture	A %	B %	C %
band 1	49	51	56	band 1	47	41	54	band 1	51	50	45
band 2	33	32	27	band 2	11	23	14	band 2	30	29	33
band 3	18	17	17	band 3	42	36	32	band 3	19	21	22

2.3.2 Procedure of paperchromatography

The reversed-phase paperchromatography of sterols in the system paraffin/acetic acid–water (84 : 16) is executed as follows:

Three hexagonal holes with sides of 29 mm and 20 mm are made in a Schleicher and Schüll no. 2043b mgl paper (preferably the "washed" quality) of 20 × 59.5 cm, according to the procedure of MATTHIAS. Between two hexagons a "bridge" of 10 mm width is formed. At both sides of the paper only half a hexagon is cut out (see fig. 21). The distance from the centre of the bridge to the bottom of the paper is 68 mm. A pencil line is drawn at 5 mm from the bottom of the paper. The direction of the paper fibres should be parallel to the direction in which the mobile phase moves. Beforehand a quantity of 300 ml of an acetic acid (Analar. B.D.H.)–water (84 : 16) mixture has been shaken vigorously with 30 ml of paraffinum liquidum medicinale in a separatory funnel. After a contact period of 65 hrs at a temperature of 23°C the two layers are completely clear and are separated. The amount of paraffin in the acetic acid layer was found to be approximately 0.02%, whereas the acetic acid percentage in the paraffin layer amounts to about 1.9. The paraffin layer is dissolved in petroleum ether (bp 60–80°C); a 7% solution thereof serving for the impregnation procedure. The acid layer is used as mobile phase. The best paperchromatographic separations are obtained when using freshly prepared stationary and mobile phases.

The paper is taken at both ends and is dipped with a to and fro movement three times in the 7% paraffin oil solution in the way a photographic film is treated in dish development. The movement is started at the bottom of the paper marked by the pencil line. Care must be taken that during the immersion the solvent front remains parallel to the origin line. After the impregnation procedure the chromatogram is dried during 10 min in the air, by hanging it on a plastic line upside down. The degree

of impregnation measured from the weight of the paper before and after impregnation amounts to about 0.09–0.10 g paraffin oil/g of paper. In investigations of the relationship between structure and R_F values the higher degree of impregnation viz. 0.15–0.16 g/g paper was applied. The R_F values obtained by the latter procedure appeared to be reproducible to 0.02 units.

At the centre of the bridges (fig. 21) 20 μg of the sterol mixture, preferably 0.02 ml of a 0.1% solution in chloroform p.a., is spotted by a micro pipette. After an accommodation during 16 hrs in a chromatographic vessel, the chromatogram is dipped into the mobile phase viz. acetic acid–water (84 : 16) to the pencil line. The chromatogram is then developed during 48 hrs by the ascending technique at a temperature of 21–25°C. The length of run should be: 25–30 cm. After the development procedure the chromatogram is dried during 2–3 hrs in the air. Upon heating during 1–2 hrs in an oven at 80°C some conjugated sterols like ergosterol become visible as fluorescent spots under U.V. radiation (365 nm). The chromatogram is then sprayed with a 10% ethanolic solution of phosphomolybdic acid (MERCK) and heated during 1–4 min at 80°C. Blue-green bands are developed on a rapidly darkening light green background. Care must be taken that the spots are coloured to a maximum intensity, but that the background is not darkened too much by excessive heating. After some hours especially when exposed to sunlight the spots merge into the background, the whole paper then showing a dark blue colour.

The R_F values are calculated for the centre of the bands. Large quantities (> 100 μg) of saturated sterols like coprostanol and dihydrocholesterol are visible as yellow spots. With these substances a blue-green colour is only attained upon further spraying with a mixture of ether–sulfuric acid (2 : 1) and subsequent heating during 5–10 min at 80°C. However, the paper is badly damaged by this treatment. Near the front of the mobile phase some bands appear caused by oxidation products e.g. hydroxy sterols. These substances might be formed during the saponification, acetylation or chromatography of the samples.

Small quantities of accompanying sterols, which normally would not be detected, are visualized by spotting higher amounts of the sterol mixtures (up to 150 μg) on the paper. Still higher quantities viz. 200–600 μg, can be applied on Whatmann no. 3 paper. More specific colour reactions that can be used for the structural analysis of some unknown sterols, will be discussed in chapter 3.

2.4 QUANTITATIVE EVALUATION OF THE PAPERCHROMATOGRAPHIC ANALYSIS

A quantitative determination of the percentages of sterols belonging to the first, second, and third bands can be carried out in several ways a.o. by planimetric evaluation of the spot areas and by densitometry of the paperchromatogram.

2.4.1 Quantitative evaluation by planimetry

Directly after the staining procedure the blue-green bands are outlined with a pencil. The spot areas are determined by following the outline of the bands with a planimeter. Each determination is repeated twice. The areas are recalculated to percentages of their total. A series of synthetic mixtures of cholesterol and refined soybean phytosterols was analysed in this way; each mixture was determined in sixfold by one person. The percentage areas for the various bands are given in table 8.

TABLE 8 Quantitative evaluation of some cholesterol–soybean phytosterol mixtures. Spotted amount of the sterol mixture: 30 μg.

Percentage area of the three bands								
band 1	band 2	band 3	band 1	band 2	band 3	band 1	band 2	band 3
chol.–phyto. (9 : 1)			*chol.–phyto.* (8 : 2)			*chol.–phyto.* (7 : 3)		
12	—	88	21	—	79	32	2	66
10	—	90	18	—	82	37	2	61
8	—	92	20	—	80	30	5	65
13	—	87	23	—	77	28	5	67
16	—	84	25	—	75	26	6	68
10	—	90	23	—	77	29	8	63
chol.–phyto. (6 : 4)			*chol.–phyto.* (5 : 5)			*chol.–phyto.* (4 : 6)		
32	7	61	38	14	48	42	18	40
36	8	56	41	10	49	37	20	43
29	9	62	37	15	48	42	16	42
33	7	60	32	18	50	40	18	42
30	11	59	34	20	46	35	21	44
31	11	58	42	17	41	38	22	40
chol.–phyto. (3 : 7)			*chol.–phyto.* (2 : 8)			*chol.–phyto.* (1 : 9)		
44	23	33	48	28	24	51	34	15
41	26	33	49	28	23	54	35	11
48	23	29	51	25	24	51	34	15
41	22	37	46	32	22	48	38	14
38	25	37	48	29	23	48	37	15
36	29	35	48	27	25	58	31	11
chol.–phyto. (0 : 10)								
65	35	—						
62	38	—						
68	32	—						
55	45	—						
64	36	—						
54	46	—						

The percentage areas of the first and second band are added, thus giving the experimental percentage of phytosterols. From these data the "experimental proportion" of cholesterol–phytosterol was derived. The data indicate an unequivocal correlation between this proportion and the actual composition of the analysed cholesterol–phytosterol mixture. Therefore, a satisfactory quantitative determination could be obtained. The deviations from linearity of this relationship were tested by statistical treatment.

The actual phytosterol content (μ_x), the mean experimentally determined phytosterol content ($\bar{x}$), based on six determinations, the values of $|\bar{x}-\mu_x|$, and the value of STUDENT's t of these means are given in table 9. Applying STUDENT's t-test five of these t-values* fall outside the critical limits at the 5% level of significance. Therefore, the corresponding $\bar{x}$ values can be considered as not belonging to the original μ_x population.

TABLE 9 Statistical interpretation of the paperchromatographic analyses

Mixture of					
% phytosterol	*% cholesterol*	$\bar{x}$	var.	$\bar{x}$-μ_x	t
10	90	11.5	7.9	+1.5	+1.517
20	80	21.7	6.27	+1.7	+1.685
30	70	35.0	6.8	+5.0*	+5.056*
40	60	40.7	4.67	+0.7	+0.674
50	50	53.0	10.4	+3.0*	+3.033*
60	40	58.2	2.57	—1.83	—1.854
70	30	66.0	9.2	—4.0*	—4.044*
80	20	76.5	1.1	—3.5*	—3.539*
90	10	86.5	3.9	—3.5*	—3.539*

At low phytosterol percentages too high data of $\bar{x}$ are found, whereas at high phytosterol percentages too low data were evaluated. Nevertheless, by using a calibration line relating the "experimental proportion" cholesterol–phytosterol to the actual composition of the sterol mixture, reasonable quantitative evaluations can be made. In this procedure the standard deviation within the groups, as calculated from the experimental data, amounted to 2.42%.

In some chromatographic analyses, described in the literature, the area of round and ovoid spots has been found to increase with the logarithm of the actual spot content. The above-mentioned correlation clearly indicates that this relation does not hold in our experiments.

2.4.2 Densitometric evaluation

Chromatograms sprayed with the colour reagent phosphomolybdic acid are not stable. After some time, especially upon exposure to light, the background assumes the same

colour as the sterol spots. Such chromatograms are unsuitable for densitometric measurements. Upon testing a variety of other colour reagents, we found the chromatograms visualized with phosphotungstic acid to be satisfactorily stable. The dried chromatogram is sprayed with a 15% ethanolic solution of phosphotungstic acid and kept at 60°C during 2–6 min. Quantities of about 20 μg of sterols such as cholesterol produce distinct red bands. The intensity of the colour of the bands is less than with the phosphomolybdic acid reagent, but remains stable during ½ hr.

The chromatograms were scanned according to the "transmittance procedure" using a registering densitometer of DR. B. LANGE.

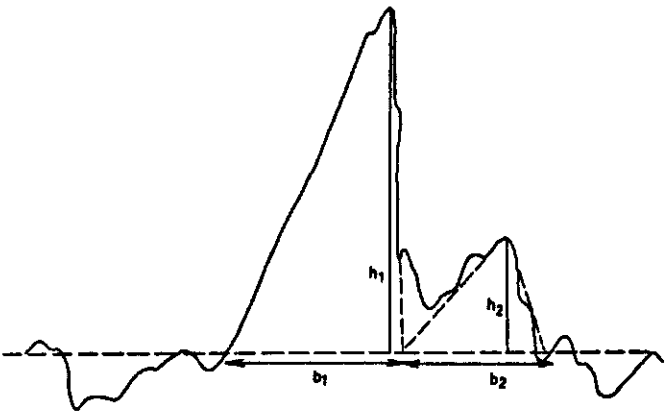


FIG. 23. Quantitative evaluation of a chromatogram with a DR. LANGE densitometer. Areas determined by ½ h × b. Colour reagent: phosphotungstic acid.

After testing the various experimental conditions good results were obtained with slit width 10 mm, green filter, sensitivity 4.0 (2.5). Because of the inconstancy of the optical recording instrument ("Nachlaufschreiber" of DR. B. LANGE) the densitometric curve showed a great noise, which hampers an accurate evaluation of the areas of the peaks by planimetry. Therefore, the areas were determined approximatively by multiplication of half the peak height with the base, a procedure in use in gas-liquid chromatography. Scanning the bands of two chromatograms (actual proportion cholesterol–phytosterol 5 : 5) the following percentages were found: *band* 1 : 31.6 (31.0); *band* 2 : 12.1 (12.0); *band* 3 : 56.3 (57.0). Three chromatograms of a 7 : 3 mixture gave the experimental data: 77.0%–23.0%; 76.7%–23.3%; and 74.1%–25.9% (cf. fig. 23).

Applying this procedure, the accuracy of the results appeared to be slightly inferior to that obtained with planimetry. Some drawbacks of the scanning technique are a.o. inhomogeneity of the paper, uncertainty of the stoichiometry of the colour reactions, deviations from BEER's law at high optical densities and non-linearity of the calibration curves (BUSH 1961). In routine analysis of sterol mixtures therefore the planimetric procedure was preferred.

3 A STUDY OF THE SYSTEM: PARAFFIN/ACETIC ACID–WATER (84 : 16)

3.1 Principles of paperchromatography

3.1.1 Fundamental equations

Before investigating the relationship between structure and migration rates of a group of sterols, we have to give first a short survey of some theoretical aspects. The relationship between partition coefficient and R_F value in liquid-liquid partition chromatography was elucidated by Martin and Synge (1941). If paperchromatography may be considered as a process of ideal liquid-liquid partition chromatography, the concentration variation of a solute in a chromatographic column will closely follow the normal frequency distribution. For a given solute the position of the maximum concentration is indicated by the formula:

$$R_F = \frac{A_L}{A_L + \alpha \,.\, A_S} \qquad [1]$$

or

$$\alpha = A_L/A_S \; (1/R_F - 1) \qquad [2]$$

where:

α = partition coefficient of the solute.

A_L = cross sectional area occupied by the mobile phase.

A_S = cross sectional area occupied by the stationary phase.

This equation governs the correlation between partition coefficient and migration rate of a substance. In an ideal solution of the solute A the following equations apply:

$$\mu_A^S = \mu_A^{S0} + RT \ln N_A^S \qquad [3]$$

$$\mu_A^L = \mu_A^{L0} + RT \ln N_A^L$$

where:

μ_A^S and μ_A^L are the chemical potentials of A in the stationary and mobile phases respectively.

μ_A^{S0} and μ_A^{L0} are chemical potentials in some defined standard state.

N_A^S and N_A^L are the molar concentrations of A in the phases S and L.

When supposing equilibrium between the phases S and L, the chemical potentials μ_A^S and μ_A^L will be equal. Hence:

$$\mu_A^S - \mu_A^L = \mu_A^{S0} - \mu_A^{L0} + RT \ln N_A^S - RT \ln N_A^L = 0 \qquad [4]$$

According to MARTIN (1948) the free energy $\Delta \mu_A$, required to transport 1 Mol of the solute A from phase S to phase L is given by:

$$\Delta \mu_A = \mu_A^{So} - \mu_A^{Lo} = RT \ln N_A^L / N_A^S \quad [5]$$

Since $N_A^L/N_A^S = \alpha$ according to NERNST's distribution law:

$$\Delta \mu_A = RT \ln \alpha$$

With equation 2:

$$\Delta \mu_A = 2.303\ RT \log A_L/A_S\,(1/R_F - 1) \quad [7]$$

The distribution of A between the two phases is governed by the value of this $\Delta \mu_A$. In a series of homologous compounds we should not compare the R_F values, but the data of the $\Delta \mu_A$ functions given by this equation.

BATE-SMITH *et al.* (1950) therefore introduced the R_M function, defined as:

$$R_M = \log (1/R_F - 1) \quad [8]$$

Hence equation 7 gives:

$$\Delta \mu_A = k \,.\, R_M - k \,.\, P \quad [9]$$

where $k = 2.303\ RT$

P = paper constant (= $\log A_s/A_L$) (cf. HAIS 1958).

By a first approximation the $\Delta \mu_A$ can be considered as the sum of the free energies of the various parts x, y, z etc., constituting the molecule.

$$\Delta \mu_A = x.\ \Delta \mu_{Ax} + y.\ \Delta \mu_{Ay} + \ldots . \Delta \mu_{A0} \quad [10]$$

Accordingly, for a given pair of phases the introduction of a new group in the molecule changes the R_M value by an amount depending on the nature of the group, but *not* on the rest of the molecule. From equations 9 and 10:

$$R_M = x/k \,.\, \Delta \mu_{Ax} + y/k.\ \Delta \mu_{Ay} + \ldots . \Delta \mu_{A0}/k + P \quad [11]$$

Introducing the so-called *group constants*:

$$G_0 = \Delta \mu_{A0}/k;\ G_x = \Delta \mu_{Ax}/k\ ;\ G_y = \Delta \mu_{Ay}/k \text{ etc.},$$

we obtain the equation:

$$R_M = x \,.\, G_x + y \,.\, G_y + \ldots . \underbrace{G_0 + P}_{Z} \quad [12]$$

Usually, the constant concerning the basic skeleton of the molecule viz. G_0 and the paper constant P are combined into the fundamental constant Z (MACEK 1955). If we may assume that there is no interaction between the various parts of the molecule, the R_M values should be additive. In a series of homologues the R_M value for any member then will be a linear function of its number in the series. Because of the

difficulties encountered in estimating all group constants G_x, G_y, and G_0, many authors have restricted their investigations to the calculation of ΔR_M values due to various substituents e.g. $\Delta R_M^{CH_3}$ and ΔR_M^{OH} values.

The validity of this concept and especially of equation 1 and its underlying suppositions were tested experimentally a.o. by CONSDEN *et al.* (1944). From the experimental R_F value of glycine and the partition coefficient of glycine known from the literature, the A_L/A_S for a given chromatogram was determined. Using this A_L/A_S value and the experimental R_F values of other amino acids on the same chromatogram, they were able to calculate the corresponding partition coefficients. These values were compared with those known from the literature (table 10). Testing some groups of sugars and biologically important acids MULVANY *et al.* (1951) and BENSON *et al.* (1950) confirmed the validity of the theory of MARTIN c.s. (table 10). As a consequence of the R_M concept the diagrams of R_M value versus number of dientical substituents should present straight lines. This linear relationship was tested in various series of homologues a.o. peptides by PARDEE (1951), phenolic hydroxyl compounds by BATE-SMITH *et al.* (1950), and steroids by BUSH (1960, 1961).

TABLE 10 Comparison between R_F values and partition coefficients

CONSDEN *et al.* (1944)

System: n-butanol saturated with water

$A_L/A_S = 3.25$

	α exp.	α calc.
glycine	70.4	70.4
alanine	35.9	42.3
valine	12.2	13.8
norvaline	8.7	9.5
leucine	4.5	5.5
norleucine	3.5	3.2

MULVANY *et al.* (1951)

	α exp.	R_F exp.	α calc.
xylose	17.1	0.12	16.0
arabinose	21.5	0.10	20.6
mannose	21.6	0.10	20.6
glucose	30.5	0.07	ref.
galactose	36.2	0.06	36.0

BENSON *et al.* (1950)

System: n-butanol–propionic acid–water (46 : 23 : 31).

	α exp.	R_F exp.	R_F calc.
alanine	3.65	0.33	ref.
fumaric acid	0.50	0.72	0.78
succinic acid	1.02	0.65	0.64
glycolic acid	2.06	0.51	0.47
malic acid	2.40	0.45	0.43
isocitric acid	2.75	0.41	0.40
glyceric acid	2.95	0.38	0.38
tartaric acid	3.20	0.32	0.36
glucose	5.75	0.20	0.24

VAN DUIN (1961) has demonstrated that the true retention volumes of dinitrophenylhydrazones of several series of carbonyl compounds on columns of Celite or silica gel also obey to the above additivity rule. The principles of paperchromatography were studied subsequently a.o. by REICHL (1956) and SCHAUER *et al.* (1955, 1958). In some paperchromatographic systems the values of the various group constants were given. Table 11 presents the group constants of parts of the amino acid molecules, determined in the system: phenol saturated with water and 0.1 % cupron on Whatman no. 1 paper (SCHAUER 1955, HAIS 1958).

TABLE 11 Group constants in the system: phenol saturated with water and cupron 0.1 % for different parts of the amino acid molecules. Comparison of experimental and calculated R_F values

			R_F exp.	R_F calc.
C atom	—0.27			
branching	+0.07			
benzene ring	—1.01	aspartic acid	0.17	0.17
prim. OH group	+0.36	alanine	0.59	0.56
sec. OH group	+0.38	serine	0.36	0.36
phenolic OH group	+0.91	lysine	0.48	0.48
—COOH	+1.07	cystine	0.29	0.29
α —NH_2	+0.20	methionine	0.81	0.81
imidazole	—0.25	tyrosine	0.62	0.62
—S—	+0.02	histidine	0.69	0.70
—S—S—	+0.04			
paper constant (on Whatman 1)	—0.57			

The paper constant was derived from the equation:

$$2R_M \text{ (methionine)} - R_M \text{ (cystine)} = \log A_S/A_L\ (= P) + 4 \log \Delta\mu^{CH_2}/RT$$

$$P = 2 \times (-0.63) - 0.39 - 4 \times (-0.27) = -0.57\,.$$

From these group constants "theoretical" R_M and R_F values of some amino acids were calculated. The agreement between these R_F values and the experimental ones appeared to be excellent.

However, there may be some interaction between adjacent parts of the molecule. MACEK *et al.* (1955) have demonstrated that the *place* of a hydroxyl group in some alkaloids influences the R_M value. To account for this effect he introduced *position factors* A_x, A_y etc.

$$R_M = A_x\,.\,x\,.\,G_x + A_y\,.\,y\,.\,G_y + \ldots .\, Z \qquad [13]$$

FRANC *et al.* (1956) have found that in some cases this location effect may also be accounted for by introducing a correction factor, based on the known dipole moment $(\vec{\mu})$ of the substance $\left(\text{viz. } + 2.3 \log \frac{1 + 0.1\,\vec{\mu}}{1 - 0.1\,\vec{\mu}} \right)$.

This entire theory concerning R_M values etc. was tested mainly in systems with water or polar liquids like formamide as stationary phase. Theoretically, it should also hold in reversed-phase systems, but not many investigations on such systems are reported (cf. HAIS 1958, KAUFMANN 1958b, DE ZOTTI 1959).

3.1.2 Applications of the R_M concept

Instead of restricting the R_M concept to one solvent system, the same procedure can be applied to the difference of the R_M values of one compound in *two* solvent systems. BUSH (1960) has denoted these values as ΔR_{Ms} values, as contrasted with the ΔR_{Mg} and ΔR_{Mr} values. The former deals with the substitution of a hydrogen by the group g, the latter (ΔR_{Mr}) with the interchange of two groups or of two steric configurations of the same group. Determining the R_M values for a number of related substances with known structure, the various group constants and the fundamental constant can be derived. In special cases only the ΔR_{Mg} values are calculated. The R_M value of a related compound, of which the number of substituents is not yet precisely known, then can give additional indication as to the correct structure of that substance. The most complete evidence is obtained when a set of suitable solvent systems each with known group constants etc. is available.

The ΔR_{Ms} values then will already give a good impression of the structure of unknown compounds, especially when supplemented with ΔR_{Mr} values obtained after simple microchemical reactions e.g. acetylation, bromination (BUSH 1961). In this respect solvent systems resulting in large ΔR_{Ms} values for some groups (or preferably for one group only) and small values for the other groups would be very suitable. Unfortunately, at present not many systems which satisfy these requirements, are available. If the group constants and position factors in a given system are derived for compounds of known structure, "theoretical" R_F values can be calculated for related substances. In a still further simplified concept the "theoretical" R_F values are calculated on the basis of average ΔR_M values. The latter are obtained from a graph representing R_M versus number of identical groups e.g. methylene units.

Comparing the respective sets of group constants G^1_x, G^1_y etc.; G^2_x, G^2_y etc. for some solvent systems with those of a reference system (viz. G^0_x, G^0_y etc.), MACEK (cf. HAIS 1958) has introduced so-called ΔG values.

($\Delta G^1_x = G^1_x - G^0_x$; $\Delta G^2_x = G^2_x - G^0_x$ etc.). These ΔG values depend on the physicochemical properties of the respective systems and hence are suitable for identification purposes.

An elaborate example of an application of the R_M concept to structural analysis of steroids was given by KABASAKALIAN *et al.* (1960). They have determined the ΔR_{Mg} values of several substituents in a group of steroids, applying some ZAFFARONI systems (e.g. propylene glycol/toluene). $\Sigma \Delta R_{Mg}$ values were calculated for a number of steroid molecules. The ΔR_M value of the basic group viz. the pregnane nucleus was obtained in the $\Sigma \Delta R_{Mg}$ versus R_M graph as the intercept. Knowing these ΔR_M values,

"theoretical" R_F values of some steroids in a number of ZAFFARONI systems were calculated. The agreement between these calculated and the experimental R_F values was reasonable. Small remaining differences are readily explained by interaction between the various groups and also by the influence of spacial configurations.

3.1.3 The occurrence of critical pairs

A problem in structural analysis by means of paperchromatography is the occurrence of substances having the same R_F values. Dealing with the chromatography of steroids BUSH (1960) has mentioned some general rules to avoid the interference of such "steroid mimics" in structural analysis. Usually, the mimic substance will differ in at least two groups from the studied compound, both groups exerting equal but opposite influences on the R_M value (e.g. two keto groups instead of one hydroxyl group). Selecting a suitable set of solvent systems and applying chemical-modification steps (i.e. ΔR_{M_T} effects), the risk of confusing the steroid with its mimics usually can be minimized.

Still greater difficulties are encountered in the analysis of the higher fatty acids in reversed-phase systems. With systems like undecane (bp 190–220°C)/acetic acid–water (95 : 5) or acetic acid–acetonitrile (25 : 75) and paraffin/acetic acid–water (90 : 10), KAUFMANN *et al.* (1954, 1958a) and others e.g. SPITERI (1954) have separated the series of saturated fatty acids. The unsaturated fatty acids were also fractionated, but the introduction of a double bond appeared to exert an influence on the R_F value quite similar as a shortening of the chain length with two methylene units. Hence in natural mixtures several fatty acids were found with the same R_F values, constituting the so-called "critical pairs" (e.g. palmitic acid–oleic acid; myristic acid–linoleic acid). To characterize these critical pairs KAUFMANN *et al.* (1960) have introduced a paperchromatographic value ("Papierchromatographische Wertzahl: pcW"), defined as the difference between the number of carbon atoms (n) and twice the number of double bonds (m) of the molecule. Hence pcW = n — 2m. Palmitic acid and oleic acid both have the pcW number 16, which is thus indicative of this critical pair of fatty acids.

Generally, a separation and subsequent quantitative determination of both members of a critical pair has to involve a chemical-modification step, e.g. separation before and after hydrogenation of the fatty acid mixture or oxidation of the unsaturated fatty acids. It is to be regretted that the terms: mimic compounds, members of a critical pair, and critical partners are hardly descriptive for the general conception they actually represent. A terminology like "equidistant" or "equiproportional" compounds is to be considered more appropiate.

The additivity of R_M values should have been applied universally to problems of structural analysis, as has been done with analogous physicochemical constants such as molecular refraction, optical rotation etc. However, the R_M concept has not yet been actualized in practice to a great extent, a.o. as a consequence of the inconstancy of ΔR_M values in some "normal" paperchromatographic systems with water as

stationary phase. BUSH (1960, 1961) has given a thorough study of the significance of R_M and ΔR_M values in different solvent systems, applying these to problems of structural analysis and especially to the identification of "critical pair" steroids.

3.2 EXPERIMENTAL CONDITIONS IN THE SYSTEM: PARAFFIN/ACETIC ACID–WATER (84 : 16)

3.2.1 Influence of external circumstances

The standardized chromatographic procedure, described in paragraph 2.3.2, is based upon the results of many experiments testing a variety of external conditions. Several factors influence the migration rate and the separation of the common sterols. In the literature not much attention has been paid to the fact that *mutual saturation of both phases is essential for obtaining reproducible R_F values.* It is *absolutely* necessary to attain equilibrium between both phases under the experimental conditions. This is promoted by proper choice and standardization of the relative amounts of both phases and their time of contact, which governs the mutual saturation as well as the thorough demixing.

TABLE 12 Variation of experimental conditions. Mobile phase: acetic acid–water (84 : 16).

No	g paraffin oil/g paper	Distance of solvent front in cm	R_F values of			Time of run in hrs	Temperature in °C
			cholesterol	stigmasterol	β-sitosterol		
A (time of demixing 65 hrs)							
1	0.05	37.5	0.53	0.48	0.48	48	23
2	0.08	35.2	0.49	0.44	0.39	48	23
3	*0.09*	*35.0*	*0.42*	*0.39*	*0.35*	48	23
4	0.11	36.0	0.36	0.33	0.29	48	23
5	0.13	32.5	0.34	0.30	0.28	48	23
6	0.17	33.5	0.26	0.24	0.20	48	23
(time of demixing 48 hrs)							
7	0.10	26.6	0.56	0.51	0.44	48	23
8	0.16	25.6	0.39	0.36	0.29	48	23
B (time of demixing 65 hrs)							
1	0.09	12.1	0.52	0.46	0.42	8	23
2	0.09	23.8	0.45	0.41	0.36	24	23
3	0.09	28.4	0.42	0.38	0.33	32	23
4	0.09	33.5	0.45	0.41	0.36	48	23
5	0.09[5]	31.0	0.33	0.30	0.25	48	14
6	0.10	31.9	0.36	0.32	0.27	48	18
7	0.10	33.8	0.42	0.39	0.34	48	23

Comparing chromatograms obtained with contact periods of both phases of 48 hrs and 65 hrs respectively, in the former case a shortening in the length of run and consequently an increase in R_F value was observed (table 12, A no. 7, 3). With the longer period viz. 65 hrs better separations of cholesterol and the phytosterols were obtained. Still longer periods did not result in any significant improvement in the separation of these sterols. The contact period therefore was standardized to 65 hrs. The quantities of paraffin oil dissolved in the acetic acid layer and of acetic acid in the paraffin layer then amount to 0.04 and 1.9% respectively. The data obtained with varying degrees of impregnation (table 12, A no. 1–6) imply that the best separation of cholesterol and the major phytosterols was accomplished at an impregnation of 0.09–0.10 g paraffin oil/g of paper. An accommodation of the paper with vapours of the mobile phase during 16 hrs gave better reproducible results than after shorter periods of e.g. 8 hrs.

At longer times of development of a chromatogram (e.g. from 8 to 48 hrs) the length of run is increased proportionally and the R_F values are decreased slightly. The best possible separation of the major sterols is obtained after a time of development of 48 hrs (table 12, B no. 1–4). With still longer periods the sterol bands become diffuse, thus hampering a clear separation. The migration speed of the solvent front is also increased at higher temperatures. A rise in temperature from 14° to 25°C gives rise to a small increase in R_F value (table 12, B no. 5–7). A time of run of 48 hrs and a constant temperature of 23°C were selected as the best and most practicable conditions.

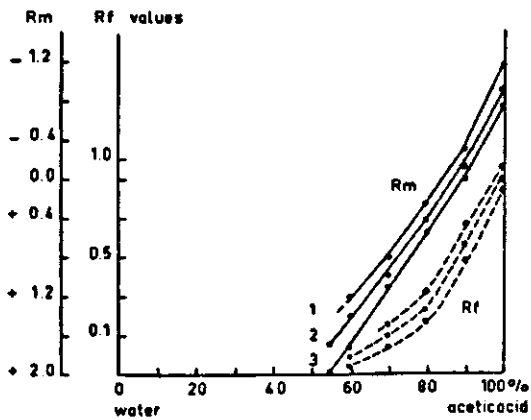


FIG. 24. Influence of water content of the mobile phase on the R_F and R_M values of ergosterol (1), cholesterol (2), and β-sitosterol (3).

The water content of the mobile phase considerably affects the R_F values. The graph of R_F value versus water content presents a rapidly decreasing curve. A predominantly linear relation is found between the corresponding R_M values and the water content

(fig. 24). According to SOCZEWINSKI *et al.* (1962) in ideal partition chromatography a linear relation between R_M value and composition of the mobile phase is found, when investigating systems that consist of a two-component solvent practically immiscible with the third component (an organic liquid). The studied system on the whole fulfils this requirement.

Cholesterol and the two major phytosterols are most clearly separated at a water content of the mobile phase of about 16%. The distance between the two phytosterol bands then amounts to about 0.6 cm. Using different water contents e.g. of 23% and 9%, this distance is reduced respectively to about 0.1 and 0.3 cm. On the basis of the above data the following conditions were selected: time of demixing 65 hrs; time of accommodation 16 hrs; time of development 48 hrs; degree of impregnation 0.09–0.10 g paraffin oil/g; temperature 23°C (see also experimental procedure, paragraph 2.3.2).

With these conditions the highest "degree of separation" (German: Trennschärfe) of sterols such as cholesterol, stigmasterol, and β-sitosterol has been accomplished. The eminent suitability of the studied system can be illustrated by comparing the separation of these sterols with that of the higher fatty acids on the same chromatogram. The bands of the three sterols are all situated in between those of palmitic and stearic acid, which differ in only two methylene units. The distance between the bands of cholesterol and β-sitosterol amounts to about 2.5 cm.

3.2.2 Features of the partition process

The degree of approximation of the ideal partition process in our system was studied more in detail. In only a few instances the behaviour of both phases in reversed-phase paperchromatography has been investigated. KAUFMANN *et al.* (1958b) have described the characteristics of the system: undecane (0.31 g/g)/acetic acid–water (85 : 15), which has been applied to the fractionation of the higher fatty acids. They found that farther from the starting point the amounts of both the stationary and the mobile phase decrease gradually. DE ZOTTI *et al.* (1959) have studied in the same way the system petroleum (0.06 g/g)/acetic acid–water (90 : 10 and 98 : 2). With the mobile phase acetic acid–water (90 : 10) they likewise observed a decrease in the amount of petroleum in the several zones of the chromatogram, but significantly smaller than in the system studied by KAUFMANN *et al.* However, using the mobile phase acetic acid–water (98 : 2) a slight *increase* in the petroleum content for the various zones was reported. In both cases they found a decrease in the A_L/A_S values on the zones of the chromatogram respectively from 5.27 to 3.3 and from 25.4 to 8.8.

In our system (0.10 g paraffin oil/g) the partition process was studied by determining the amounts of paraffin oil and of mobile phase present in six subsequent zones of the chromatogram, each representing 5 cm of the distance (table 13). The amount of paraffin oil increases slightly along the chromatogram (with about 10%).

On the other hand the amount of mobile phase (determined as acetic acid) present in the zones closer to the solvent front is significantly lower. As a result of both effects

TABLE 13 Amounts of stationary and mobile phase in the subsequent zones of four chromatograms

Zone cm	Amount of paraffin/100 cm² in mg				Amount of 84% acetic acid/100 cm² in mg				A_L/A_S value				Mean values
	I	II	III	IV	I	II	III	IV	I	II	III	IV	
0–5	91.0	77.2	111.0	93.4	1373	1370	1351	1376	15.1	17.7	12.2	14.7	14.6
5–10	83.6	86.0	107.4	103.0	1255	1323	1277	1321	15.0	15.4	11.9	12.8	13.3
10–15	94.2	89.2	110.8	109.6	1181	1205	1075	1000	12.5	13.5	9.7	9.1	10.0
15–20	97.0	92.2	115.6	113.6	988	963	833	713	10.2	10.4	7.2	6.3	8.5
20–25	98.2	91.6	110.8	113.4	705	624	322	260	7.0	6.8	2.9	2.3	4.6
25–30	98.2	97.6	117.2	119.0	315	283	132	131	3.2	2.9	1.1	1.1	2.0
front													

the A_L/A_S values for the various zones decrease linearly from about 14.6 (start) to 2.0 (front) (see the data of table 13). The slight increase in amount of stationary phase along the chromatogram is in contradiction with the results obtained by KAUFMANN and partly also with those of DE ZOTTI.

Consequently, in our system no constant value for the A_L/A_S factor on a chromatogram applies. However, we still tried to ascertain the validity of the formula $\alpha = A_L/A_S$ $(1/R_F - 1)$ by applying an average of the A_L/A_S values, belonging to those zones of the chromatogram to which the sterol bands have ascended. The partition coefficients of two sterols have been determined experimentally.

After the standardized demixing period of 65 hrs both liquid phases of an acetic acid–water–paraffin oil (volume ratio: 336 : 64 : 30) mixture are separated. An amount of about 10 mg of sterol is dissolved in 10 ml of the paraffin layer. This solution is shaken vigorously with 10 ml of the acetic acid layer. After 65 hrs the two phases are separated again and their sterol contents are determined by the LIEBERMANN-BURCHARD colour reaction.

From total amounts of 20.0 and 20.1 mg of β-sitosterol partitioned between both phases, quantities of 19.7 and 19.4 mg were found in the paraffin layer and 0.99 and 1.0 mg in the acetic acid layer respectively. From these data partition coefficients of 19.9 and 19.4 are derived. Similarly, 20.0 mg of cholesterol was partitioned, yielding quantities of 18.6 mg and 1.32 mg in the paraffin and acetic acid layer respectively. This indicated a partition coefficient for $\alpha_{\text{cholesterol}}$ (exp.) = 14.1. Supposing the mean R_F values of β-sitosterol and cholesterol in this system to be 0.31 and 0.43 respectively, the sterol bands would be situated on the third zone (10–15 cm) of the chromatogram. The average A_L/A_S value of the first three zones amounts to 12.6. Substituting the above data into MARTIN's formula $\alpha = A_L/A_S$ $(1/R_F - 1)$ the following "theoretical" partition coefficients were derived.

$\alpha_{\beta\text{-sitosterol}} = 12.6\ (1/0.31 - 1) = 28.0$

$\alpha_{\text{cholesterol}} = 12.6\ (1/0.43 - 1) = 16.7.$

The reasonable agreement between these roughly evaluated "theoretical" and the experimentally estimated partition coefficients is indicative of the applicability of MARTIN'S formula in reversed-phase chromatography. This also gives some indication as to the near-ideal nature of the partition process in the studied system.

3.3 CORRELATIONS BETWEEN STRUCTURE AND R_M VALUES

3.3.1 R_F and R_M values of a group of sterols

In order to study the relationships between structure and migration rates, the R_F values of a number of sterols, provitamins, and tetracyclic triterpenoids were determined in the studied system. The procedure described in chapter 2, with a degree of impregnation of 0.15–0.16 g paraffin/g paper, was adhered to. Although this procedure was closely standardized, deviations in the external conditions, especially in the degree of impregnation might produce small variations in the R_F value. These disturbing effects were partly eliminated by spotting a reference compound viz. cholesterol on every chromatogram. The relative R_S values of the sterols etc. are expressed as the fraction of the distance travelled by this standard steroid (table 14, column 1). Each value is based on at least four independent determinations. The mean R_F value of cholesterol 0.34 was derived from numerous experiments. In an earlier communication (COPIUS PEEREBOOM 1961) we have determined the R_F values of the various sterols by taking the arithmetic average. When a chromatogram showed an R_F value of cholesterol deviating from this mean value, the R_F values of the other sterols were corrected by multiplication with the factor:

$$\frac{\text{mean } R_F \text{ chol.}}{\text{exp. } R_F \text{ chol.}}.$$

Although this procedure is applied often in paperchromatographic studies, it is theoretically incorrect. It is based upon the supposition that by calculating R_S values a sufficient correction is obtained for varying experimental conditions. This is not substantiated for mathematical treatment of R_M and ΔR_M values in studies concerning the correlation between R_M values and sterol structure.

We therefore have followed the advice given by BUSH (1961) and have corrected our experimental R_F values by way of their R_M values. In this procedure the R_F values of the reference viz. cholesterol and of the other compound are converted *directly* into their R_M values. The R_M of the compound is then corrected by multiplication with the factor:

$$\frac{\text{mean } R_M \text{ chol.}}{\text{exp. } R_M \text{ chol.}}.$$

The mean of these corrected R_M values is given in table 14, column 2. The corresponding R_F and R_S values then are derived by calculation. However, we found only minor differences between these corrected R_S values (column 4) and the experimental ones (column 1). The R_F values are based on the distances to the centre of the blue-green coloured bands.

Pure preparations

The studied compounds were pure preparations having chemical characteristics on the whole corresponding with those reported in the literature. Generous gifts of pure compounds were made by several scientists and companies viz. sterol no. 13 (table 14) from PROF. D. H. R. BARTON F.R.C. (London), no 7 from DR. A. KUKSIS (Canada), no 11 from PROF. W. KLIJNE (British Medical Research steroid collection), the no's 10, 14, 16 from N.V. PHILIPS-DUPHAR (Weesp), the no's 4, 19–27, 36 from ORGANISCH CHEMISCH LABORATORIUM DER RIJKSUNIVERSITEIT at Leiden, no 10 from the N.V. KONINKLIJKE NEDERLANDSCHE GIST- EN SPIRITUSFABRIEK (Delft), the no's 28–31 from DR. M. J. D. VAN DAM (N.V. KON. SAJET- EN VIJFSCHACHTFABRIEK, Veenendaal), the no's 2, 18, 39, 43 from the UNILEVER RESEARCH LABORATORIUM (Vlaardingen), and the no's 35, 41 from the LABORATORIUM VOOR ANORGANISCHE EN FYSISCHE CHEMIE DER RIJKSUNIVERSITEIT at Leiden. The no's 33, 34, 37, 38 were purchased from ALDRICH CHEMICAL Co., Δ 3-cholesterol acetate (no 3) from CALIFORNIA CORPORATION FOR BIOCHEMICAL RESEARCH, and no 6 from LIGHT AND CO.

The brassicasterol (7-dihydroergosterol) preparation was of high purity (melt. p. 149°C, $\alpha_{D(CHCl_3)} = -60°$). Its isomer 5-dihydroergosterol has been prepared synthetically. Besides these dihydroergosterol isomers, the coconut fat phytosterols belonging to the third band are also listed in the table. The structure of these third-band phytosterols is supposed to be isomeric with that of brassicasterol, as will be discussed in chapter 4. The sample of parkeol was isolated from shea nut fat.

Besides the major bands, coloured with phosphomolybdic acid, the lumisterol, epilumisterol, and 7-dehydrocholesterol samples show bands with $R_F = 0.60$–0.63, which cannot be coloured with this reagent, but can be visualized as fluorescent bands under U.V. radiation of 365 nm. The sample of Δ 4,6,8(14)-cholestatriene exhibits two fluorescent bands, a small one with $R_F = 0.21$ and the major spot with $R_F = 0.60$. Both bands produce no colour upon treatment with phosphomolybdic acid. With this reagent a blue band with $R_F = 0.12$ and a strong spot at the starting point are shown. Comparing these R_F values with those of e.g. cholestane and lumisterol, the main band ($R_F = 0.60$) should be attributed tentatively to cholestatriene. Most of the substances were visualized by spraying with an ethanolic phosphomolybdic acid solution. With this reagent cholestane-3-one, "Δ3-cholestenol", coprostanol, dihydrocholesterol and their acetates yield yellow bands on the chromatogram (at amounts of about 100 μg). Upon spraying with an acidified 2,4-dinitrophenylhydrazine solution the samples of cholestane-3-one and "Δ3-cholestenol" both showed a yellow band at

TABLE 14 Experimental and corrected R_F, R_S and R_M values of some sterols, provitamins, triterpenoid alcohols, and related compounds in the system: paraffin/acetic acid–water (84 : 16) using the standardized chromatographic procedure (degree of impregnation 0.15 g/g)

Compound	Formula	Abbr.* form.	Double bonds Δ	1 R_S value, exp.	2 R_M value, exp.	3 R_F value, corr. from 2	4 R_S value, corr. from 3	5 R'_M value, calculated
MONO-UNSATURATED STEROLS								
1 cholesterol	$C_{27}H_{46}O$	FC_{27}	5	≡1.0	0.288	0.34	≡1.0	0.25
2 Δ7-cholestenol (lathosterol)	$C_{27}H_{46}O$	FC_{27}	7	1.07	0.253	0.36	1.06	0.23
3 Δ3-cholestenol	$C_{27}H_{46}O$	FC_{27}	3	0.58	0.618	0.19	0.57	
4 epi-cholesterol	$C_{27}H_{46}O$	FC_{27}	5	0.79	0.417	0.28	0.82	
5 campesterol**	$C_{28}H_{48}O$	FC_{28}	5	0.88	0.360	0.30	0.88	0.40
6 Δ7-ergostenol	$C_{28}H_{48}O$	FC_{28}	7	0.93	0.321	0.32	0.94	0.38
7 γ-sitosterol	$C_{29}H_{50}O$?	FC_{29} ?	5	0.91	0.325	0.32	0.94	
8 β-sitosterol	$C_{29}H_{50}O$	FC_{29}	5	0.75	0.503	0.24	0.71	0.55
DI-UNSATURATED STEROLS								
9 7-dehydrocholesterol	$C_{27}H_{44}O$	$2FC_{27}$	5,7	1.25	0.128	0.43	1.26	0.17
10 zymosterol	$C_{27}H_{44}O$	$FC_{27}F$	8(9),24	1.33	0.046	0.47	1.38	0.08
11 desmosterol (24-dehydro-cholesterol)	$C_{27}H_{44}O$	$FC_{27}F$	5,24	1.40	0.091	0.45	1.32	0.10
12 3-dehydrocholesterol	$C_{27}H_{44}O$	$2FC_{27}$	3(4),5	1.18	0.195	0.39	1.15	
13 brassicasterol (7-dihydro-ergosterol)	$C_{28}H_{46}O$	$FC_{28}F$	5,22	1.06	0.267	0.35	1.03	0.25
14 5-dihydroergosterol	$C_{28}H_{46}O$	$FC_{28}F$	7,22	1.02	0.276	0.35	1.03	0.23
15 third-band phytosterols of coconut fat	$C_{28}H_{46}O$?	$FC_{28}F$?	5,22 ?	1.08	0.244	0.36	1.06	
16 22-dihydroergosterol	$C_{28}H_{46}O$	$2FC_{28}$	5,7	1.06	0.259	0.36	1.06	0.32
17 stigmasterol	$C_{29}H_{48}O$	$FC_{29}F$	5,22	0.87	0.373	0.30	0.88	0.40
18 α-spinasterol	$C_{29}H_{48}O$	$FC_{29}F$	7,22	0.90	0.377	0.30	0.88	0.38
TRI-UNSATURATED COMPOUNDS								
19 ergosterol	$C_{28}H_{44}O$	$2FC_{28}F$	5,7,22	1.26	0.115	0.43	1.26	0.17
20 lumisterol	$C_{28}H_{44}O$	$2FC_{28}F$	5,7,22	1.09	0.229	0.37	1.09	
21 pyrocalciferol	$C_{28}H_{44}O$	$2FC_{28}F$	5,7,22	0.98	0.309	0.33	0.97	
22 isopyrocalciferol	$C_{28}H_{44}O$	$2FC_{28}F$	5,7,22	1.25	0.124	0.43	1.26	
23 epi-lumisterol	$C_{28}H_{44}O$	$2FC_{28}F$	5,7,22	1.24	0.129	0.43	1.26	

24 tachysterol	*$C_{28}H_{44}O$*	*3FC28F*	*10(5),6,8(9),22*	*3.36 ?*	*—0.504 ?*	*0.76 ?*	*2.24 ?*	
25 vitamin D_2	$C_{28}H_{44}O$	3FC28F	19(10),5,7(8),22	1.19	0.147	0.42	1.23	0.23
26 dihydrovitamin D_2	$C_{28}H_{46}O$	$3FC_{28}$	19(10),5,7(8)	0.91	0.354	0.31	0.91	0.38
27 vitamin D_3	$C_{27}H_{44}O$	$3FC_{27}$	19(10),5,7(8)	1.14	0.184	0.40	1.18	0.23
TRITERPENOID ALCOHOLS								
28 lanosterol	$C_{30}H_{50}O$	$FC_{30}F$	8(9),24	0.68	0.566	0.21	0.62	0.55
29 24-dihydrolanosterol	$C_{30}H_{52}O$	FC_{30}	8(9)	0.50	0.760	0.15	0.44	0.70
30 agnosterol	$C_{30}H_{48}O$	$2FC_{30}F$	7,9(11),24	0.56	0.686	0.17	0.50	0.47
31 24-dihydroagnosterol	$C_{30}H_{50}O$	$2FC_{30}$	7,9(11)	0.56	0.682	0.17	0.50	0.62
32 parkeol (from shea nut fat phytosterols)	$C_{30}H_{50}O$	$FC_{30}F$	9(11),24	0.75	0.403	0.28	0.82	
SATURATED STANOLS, HYDROCARBONS ETC.								
33 cholestane	$C_{27}H_{48}$			0.77	0.428	0.27	0.79	
34 Δ4,6,8(14)-cholestatriene	$C_{27}H_{42}$		4,6,8(14)	1.79	—0.178	0.60	1.77	
35 dihydrocholesterol (β-cholestanol)	$C_{27}H_{48}O$	C_{27}		0.76	0.485	0.25	0.73	0.40
36 coprostanol	$C_{27}H_{48}O$	C_{27}		0.22	1.168	0.07	0.30	
37 cholestane-3-one	$C_{27}H_{46}O$			0.56	0.644	0.19	0.57	
38 Δ5-cholestene-3-one	$C_{27}H_{44}O$		5	2.26	—0.405	0.72	2.12	
				2.76	—0.849	0.88	2.59	
39 7-hydroxycholesterol (Δ5-cholestene-3β, 7α diol)	$C_{27}H_{46}O_2$		5	2.64	—0.621	0.81	2.38	
STEROL ESTERS								
40 cholesterol acetate	$C_{29}H_{48}O_2$		5	0.23	0.896	0.11	0.22	0.80
41 dihydrocholesterol acetate	$C_{29}H_{50}O_2$			0.20	0.935	0.10	0.20	0.95
42 epi-cholesterol acetate	$C_{29}H_{48}O_2$		5	0.30	0.894	0.11	0.22	
43 cholesterol butyrate	$C_{31}H_{52}O_2$		5	0.13	1.224	0.06	0.12	
44 stigmasterol acetate	$C_{31}H_{50}O_2$		5,22	0.21	0.924	0.11	0.22	0.95
45 β-sitosterol acetate	$C_{31}H_{52}O_2$		5	0.22	0.960	0.10	0.20	1.10
46 ergosterol acetate	$C_{30}H_{46}O_2$		5,7,22	0.32	0.847	0.13	0.26	0.72
47 cholesterol dibromide	$C_{27}H_{46}O\ Br_2$		5	0.40	0.572	0.21	0.62	
48 dl α-tocopherol	$C_{29}H_{50}O_2$			0.35	0.858	0.12	0.24	

* The abbreviated formula $2FC_{28}F$ means a sterol structure with 28 C atoms. The F before and after C_{28} corresponds to the number of double bonds in the sterol nucleus and in the side chain of the molecule respectively.

** A pure preparation was not available. The R_F value is calculated from the second band of impure β-sitosterol preparations, from which the stigmasterol has been removed.

R_S value of 0.57. With the same reagent a chromatogram of Δ5-cholestene-3-one revealed two bands at high R_S values, the major at $R_S = 2.76$ and the minor one at $R_S = 2.26$.

Of every compound a sufficient quantity was spotted to ensure that after the colouring a small but clearly visible band appeared on the chromatogram. In the case of the normal Δ5-sterols such as cholesterol, stigmasterol etc. amounts of 5–15 μg were spotted.

3.3.2 Critical pairs of sterols

According to the additivity rule of MARTIN the relationship between R_M values and the number of carbon atoms in a homologous series generally should be linear. This relationship has been established in many cases e.g. in the separation of cholesterol esters in reversed-phase systems (a.o. ZIMMERMANN 1956) and the fractionation of esters of ergosterol and various phytosterols in a paraffin/propionic acid–water (90 : 10) system by KUKSIS *et al.* (1960a). The latter authors have observed that the introduction of a double bond in the sterol side chain or in the fatty acid chain (but not in the nucleus) decreases the R_F value to the same extent as a reduction in chain length by *two* methylene units. Thus the R_F for stigmasterol butyrate will be equal to that of β-sitosterol acetate. They estimated that the effect of the introduction of one *nuclear* double bond is equal in R_F value to shortening the fatty acid chain by only *one* methylene unit. Analogous to the behaviour of the higher fatty acids in systems such as undecane/acetic acid–water (95 : 5), the sterol esters thus are arranged in critical pairs. The compounds belonging to such a critical pair have nearly the same R_F values and cannot be separated by any normal reversed-phase system.

When separating sterols in the studied system a comparable situation is found. A nearly linear relationship is observed when the R_M values of Δ5 mono-, and di-unsaturated sterols are plotted against the difference of the number of carbon atoms (n) and that of the double bonds (m) (fig. 26). In this investigation this difference is denoted further on by the symbol N_c (viz. $N_c = n-m$). Its meaning is completely analogous to that of the "papierchromatographische Wertzahl" (pcW $= n-2m$), introduced by KAUFMANN *et al.* (1960) in their study concerning the separation of the higher fatty acids. Because of this nearly linear relation the sterols for the greater part follow the additivity rule of MARTIN (COPIUS PEEREBOOM 1961). The N_c values of other sterols a.o. those with conjugated systems are found experimentally by substituting their R_M values in this graph and determining the nearest N_c values. The introduction of a double bond in the molecule produces an increase in polarity (increase in R_F value and decrease in R_M value), which is approximately equal to that caused by shortening the length of the carbon chain by one methylene unit. Therefore, 7-dehydrocholesterol ($2FC_{27}$), ergosterol ($2FC_{28}F$)^2, zymosterol ($FC_{27}F$), and desmosterol ($FC_{27}F$) have nearly equal R_M values. On the basis of the above definitions these

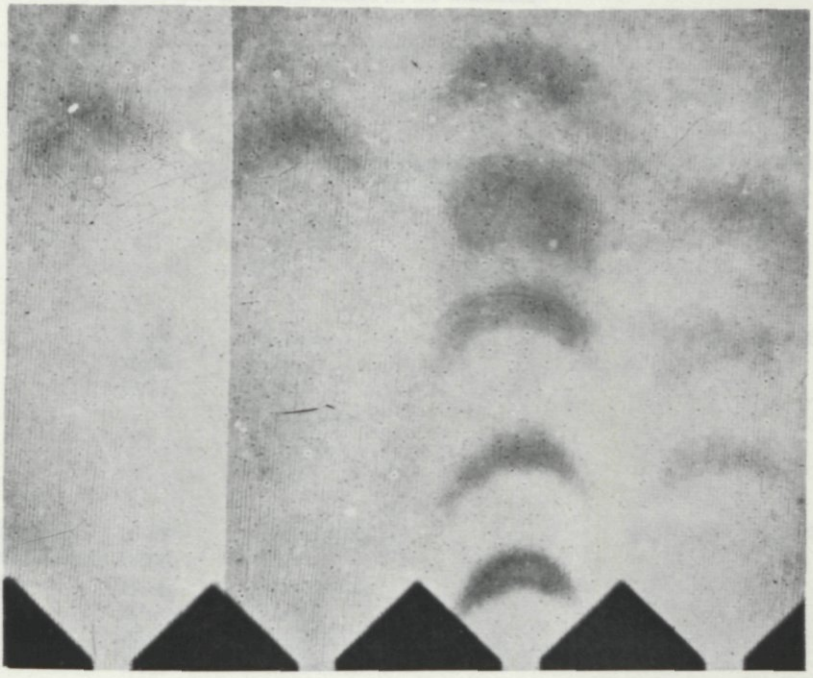


FIG. 25. Paperchromatography of sterols in paraffin/acetic acid–water (84 : 16).

spot 1. 20 μg of vitamin D_2.

spot 2. 40 μg of lumisterol.

spot 3. 50 μg of a mixture of cholesterol acetate, dihydrolanosterol, β-sitosterol, stigmasterol, cholesterol, and ergosterol.

spot 4. 250 μg of technical wool-fat alcohols.

sterols all have the same N_c value viz. 25 and thus belong to a "critical pair".

TABLE 15 "Critical pairs" of sterols, classified by their N_c values; $N_c = n - m$ (n is the number of carbon atoms and m the number of double bonds in the molecule).

N_c	$m = 0$	$m = 1$	$m = 2$	$m = 3$	No. band	R_F values	R_M values
25			zymosterol, $FC_{27}F$; 7-dehydrocholesterol, $2FC_{27}$	ergosterol, $2FC_{28}F$	4	0.40–0.47	0.05–0.18
26		cholesterol, FC_{27}	brassicasterol, $FC_{28}F$; 22-dihydroergosterol, $2FC_{28}$		3	0.33–0.37	0.23–0.31
27	dihydrocholesterol, C_{27}	campesterol, FC_{28}; $\Delta 7$-ergostenol, FC_{28}	α_1-sitosterol, $FC_{29}F$; stigmasterol, $FC_{29}F$; 7-dehydro-β-sitosterol, $2FC_{29}$		2	0.25–0.33	0.31–0.49
28	dihydrocampesterol, C_{28}	β-sitosterol, FC_{29}	α_2-sitosterol, $FC_{30}F$; lanosterol, $FC_{30}F$		1	0.20–0.24	0.50–0.60
29	dihydrositosterol, C_{29}	dihydrolanosterol, FC_{30}				0.14–0.17	0.70–0.80
30	tetrahydrolanosterol, C_{30}; tetrahydro α_2-sitosterol, C_{30}						

[a] The abbreviated formula $2FC_{28}F$ means a sterol structure with 28 C atoms. The 2F before and the F after C_{28} corresponds to the number of double bonds in the sterol nucleus and in the side chain respectively.

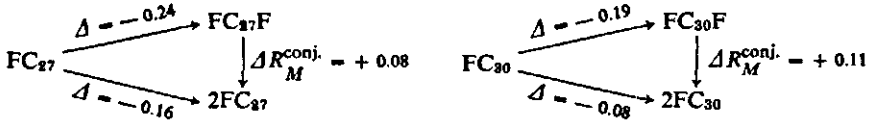
Other critical pairs are constituted by cholesterol (FC_{27}), brassicasterol ($FC_{28}F$), 22-dihydroergosterol ($2FC_{28}$) — with $N_c = 26$ — and by campesterol (FC_{28}), stigmasterol ($FC_{29}F$) with a N_c of 27. Dihydrocholesterol (C_{27}) is at the margin of the latter critical pair. In table 15 sterols with equal N_c values are arranged horizontally, thus indicating the various "critical pairs".

Sterols belonging to the same band still may exhibit some difference in their R_M values. However, this difference is too small to permit a separation under normal conditions. Besides the separation of cholesterol and the two major phytosterols the studied system also enabled separation of the tetracyclic triterpenoid alcohols present in wool fat viz. lanosterol ($FC_{30}F$) and 24-dihydrolanosterol (FC_{30}), as is shown in fig. 25. It is, however, impossible to separate the other triterpenoid alcohols agnosterol ($2FC_{30}F$) and 24-dihydroagnosterol ($2FC_{30}$) both having a system of two conjugated double bonds viz. $\Delta 7(8), 9(11)$. Furthermore a separation of vitamin D_2 ($3FC_{28}F$) and dihydrovitamin D_2 ($3FC_{28}$) appeared to be impossible. Both substances have a system of three conjugated double bonds, while the former has an additional, non-conjugated double bond in the side chain. The presence of such conjugated systems might be of great influence. These conjugation effects, therefore, were studied more in detail.

3.3.3 Calculation of ΔR_M values

The incorporation of an ethylenic linkage can be accomplished in several parts of the molecule. We can distinguish the introduction of a double bond either in the side chain or in the nucleus of the molecule. In the former case the double bond will be non-conjugated, whereas in the latter case the double bond may form part of a system of two or even three conjugated double bonds. In table 16 several instances of all three alternatives and of the resulting $\Delta R_{M\,g}$ values are given. Since generally the introduction of another double bond causes a decrease in R_M value ($R_{M2} < R_{M1}$), the resulting $\Delta R_{M\,g}$ value mostly is negative. ($R_{M2} - R_{M1} = \Delta R_M^{C=C} = \ln \Delta\mu_A^{C=C}/RT$). The ΔR_M values of group A of table 16 vary between —0.05 and —0.24 with a mean value of —0.15. If the double bond forms part of a system of two conjugated double bonds, the mean of the ΔR_M values in this group viz. B is slightly smaller viz. about —0.08. As compared with the ΔR_M value of group A viz. —0.15, in this case a *correction term* of +0.07 due to conjugation effects has to be taken into account. An exceptionally high *positive* value is found for the conversion lanosterol → agnosterol.

By U.V. irradiation of 7-dehydrocholesterol and ergosterol the B-ring of the nucleus opens and a system of three conjugated double bonds in the resulting vitamins D_3 and D_2 is produced. The ΔR_M value for this conversion amounts to +0.06. It was found that in a critical pair, sterols with conjugated double bonds generally have higher R_M values than those having only non-conjugated double bonds. Thus the R_M values of 7-dehydrocholesterol ($2FC_{27}$) and dihydroagnosterol ($2FC_{30}$) are higher than those of zymosterol ($FC_{27}F$) and lanosterol ($FC_{30}F$) respectively.



In this case a value of $\Delta R_M^{conj.}$ of +0.10 is obtained, which should be correlated with the correction term of +0.07 due to conjugation effects.

The introduction of a methyl group or the lengthening of the side chain with one methylene unit gives rise to an increase in R_M value. From the data of table 17 a

TABLE 16 Effect of the introduction of another double bond on the R_M value

A The double bond, introduced into the molecule, is *not* a part of a conjugated system

$$C=C+C=C \longrightarrow C=C-C=C$$

	$\Delta R_{M\theta}$
cholesterol (FC_{27}) → zymosterol ($FC_{27}F$)	−0.24
campesterol (FC_{28}) → brassicasterol ($FC_{28}F$)	−0.09
β-sitosterol (FC_{29}) → stigmasterol ($FC_{29}F$)	−0.13
dihydrocholesterol (C_{27}) → cholesterol (FC_{27})	−0.20
dihydrolanosterol (FC_{30}) → lanosterol ($FC_{30}F$)	−0.19
22-dihydroergosterol ($2FC_{28}$) → ergosterol ($2FC_{28}F$)	−0.14
Δ7-ergostenol (FC_{28}) → 5-dihydroergosterol ($FC_{28}F$)	−0.05
mean value	−0.15

B The second double bond becomes part of a system of *two* conjugated double bonds

$$C=C+C=C \longrightarrow C=C-C=C$$

	$\Delta R_{M\theta}$
cholesterol (FC_{27}) → 7-dehydrocholesterol ($2FC_{27}$)	−0.16
dihydrolanosterol (FC_{30}) → dihydroagnosterol ($2FC_{30}$)	−0.08
lanosterol ($FC_{30}F$) → agnosterol ($2FC_{30}F$)	+0.12
brassicasterol ($FC_{28}F$) → ergosterol ($2FC_{28}F$)	−0.15
campesterol (FC_{28}) → 22-dihydroergosterol ($2FC_{28}$)	−0.10
mean value	−0.08

C The double bond forms part of a system of *three* conjugated double bonds

$$C=C+C=C-C=C \longrightarrow C=C-C=C-C=C$$

	$\Delta R_{M\theta}$
ergosterol ($2FC_{28}F$) → vitamin D_2 ($3FC_{28}F$)	+0.03
7-dehydrocholesterol ($2FC_{27}$) → vitamin D_3 ($3FC_{27}$)	+0.06
22-dihydroergosterol ($2FC_{28}$) → dihydrovitamin D_2 ($3FC_{28}$)	+0.10
mean value	+0.06

mean value of this $\Delta R_M^{CH_2}$ of +0.15 has been found. These $\Delta R_M^{CH_2}$ values agree almost completely with those found when separating the higher fatty acids. The R_F (and R_M) values of stearic, palmitic, myristic, and lauric acid, as determined in the same system, respectively are 0.21(0.58); 0.33(0.31); 0.50(0.00); and 0.66(—0.29). The $\Delta R_M^{C_2H_4}$ value amounts to about 0.29, nearly equal to twice the $\Delta R_M^{CH_2}$ value in the sterol series. Comparing the separation of the sterols with that of the higher fatty acids on the same chromatogram, the bands of a series of three sterols viz. β-sitosterol, stigmasterol, and cholesterol all are situated in between those of palmitic acid and stearic acid.

TABLE 17 Effect of the introduction of a methyl or methylene group

	$\Delta R_M^{CH_2}$
campesterol (FC_{28}) → β-sitosterol (FC_{29})	+0.14
cholesterol (FC_{27}) → campesterol (FC_{28})	+0.07
zymosterol ($FC_{27}F$) → brassicasterol ($FC_{28}F$)	+0.22
brassicasterol ($FC_{28}F$) → stigmasterol ($FC_{29}F$)	+0.11
5-dihydroergosterol ($FC_{28}F, \Delta 7$) → α-spinasterol ($FC_{29}F, \Delta 7$)	+0.10
β-sitosterol (FC_{29}) → dihydrolanosterol (FC_{30})	+0.26
7-dehydrocholesterol ($2FC_{27}$) → 22-dihydroergosterol ($2FC_{28}$)	+0.13
vitamin D_3 ($3FC_{27}$) → dihydrovitamin D_2 ($3FC_{28}$)	+0.17
mean value	+0.15

The equality of the $\Delta R_M^{C=C}$ and $\Delta R_M^{CH_2}$ values (viz. —0.15 and +0.15) actually causes the occurrence of the various critical pairs.

There is no appreciable influence of the position of a double bond in the B-ring of the nucleus, as is indicated by table 18. The $\Delta R_M^{\Delta 5 \rightarrow \Delta 7,8}$ values show a considerable variation with a mean value of approximately —0.02.

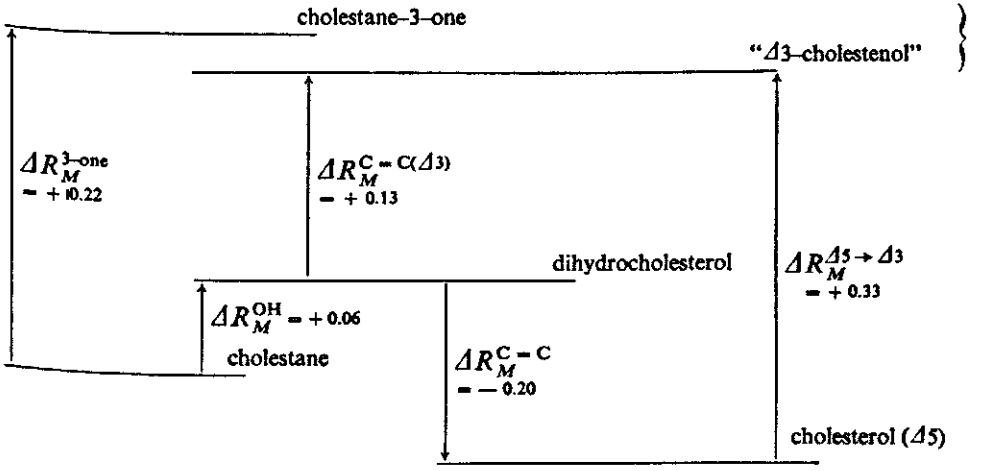
TABLE 18 Influence of the position of a double bond in the nucleus

	ΔR_M
1. cholesterol ($\Delta 5$) → $\Delta 7$-cholestenol	—0.03[5]
2. brassicasterol ($\Delta 5,22$) → 5-dihydroergosterol ($\Delta 7,22$)	+0.01
3. campesterol ($\Delta 5$) → $\Delta 7$-ergostenol	—0.04
4. stigmasterol ($\Delta 5,22$) → α-spinasterol ($\Delta 7,22$)	+0.004
5. desmosterol ($\Delta 5,24$) → zymosterol ($\Delta 8(9), 24$)	—0.04[5]
6. cholesterol ($\Delta 5$) → $\Delta 3$-cholestenol	+0.33
7. dihydrocholesterol → $\Delta 3$-cholestenol	+0.13

However, when the $\Delta 5$ double bond is shifted to the C_3-position, a high positive ΔR_M value viz. +0.33 is obtained (table 18, no 6). This high value is caused a.o. by the

positive ΔR_M value for the introduction of a double bond in the unstable $\Delta 3$-position viz. $\Delta R_M^{C=C(\Delta 3)} = +0.13$ (table 18, no 7). Obviously, in $\Delta 3$-cholestenol the degree of polarity of the HO—C(H)=C(H)— system does not agree with that based on the sum of both groups separately. We thus were able to confirm that in the mobile phase the $\Delta 3$-cholestenol molecules are isomerized to their tautomeric keto isomers viz. cholestane-3-one, thus exhibiting nearly the same R_F value. This statement was confirmed by the yellow colour of the $\Delta 3$-cholestenol band upon spraying with 2,4-dinitrophenylhydrazine.

The "$\Delta 3$-cholestenol" was freshly prepared by saponification of $\Delta 3$-cholestenol acetate. In the acetate the occurrence of such isomerization is not possible. Therefore, the R_F values of $\Delta 5$-cholesterol acetate and $\Delta 3$-cholestenol acetate in reversed-phase thin-layer chromatography are almost equal (cf. chapter 5). The $\Delta R_M^{3\text{-one}}$ value calculated from the R_M values of cholestane and cholestane-3-one viz. +0.22, therefore, corresponds with the sum of the values of ΔR_M^{OH}, and $\Delta R_M^{C=C(\Delta 3)}$ (respectively +0.06 and +0.13).



The $\Delta R_M^{acetate}$ value, derived from the R_M values of various sterols and their acetates, amounts to approximately +0.55. The $\Delta R_M^{3\beta\text{-OH}}$ value is calculated from the R_M values of cholestane and dihydrocholesterol viz. +0.06. Unlike the effect in normal systems, usually exhibiting high ΔR_M^{OH} values (KABASAKALIAN 1960), the substitution of a C_3 equatorial hydrogen atom by a hydroxyl group in this system apparently exerts only a small influence on the R_M value. The place where an alkyl group occurs in the molecule exerts no great influence on the R_M value. Therefore, the R_M values of related tetracyclic triterpenoid alcohols: lanosterol ($FC_{30}F$), dihydrolanosterol (FC_{30}), and parkeol ($\Delta 9(11),24(25)$-lanostadienol, $FC_{30}F$), all of which have two angular methyl groups at C_4, for the greater part agree with the graph of fig. 26.

3.4 Applications of the correlation between structure and R_M values

3.4.1 Rules concerning the correlation and ΔR_M values

From the above data the following rules concerning the relationship between sterol structure and resulting R_M value in the studied system can be formulated. They are valid in the case of sterols and related compounds, which for the greater part have a stereochemical structure related to that of dihydrocholesterol (equatorial hydroxyl group, ring juncture A/B trans).

1. The introduction of one double bond in the sterol molecule produces quite the same decrease in R_M value (of 0.15 units) as a shortening of the carbon chain with one methylene group.
2. When the ethylenic linkage forms part of a system of two conjugated double bonds, a correction term of +0.07 units has to be applied.
3. The introduction of another double bond, which then forms part of a system of *three* conjugated double bonds, again increases the R_M value (with about +0.06 units).
4. The place of non-conjugated double bonds and of alkyl groups in the molecule generally exerts no great influence on the R_M value.
5. Upon altering the steric configuration of the hydroxyl group at carbon atom 3, the R_M values are greatly affected.

In the above parts several ΔR_M values were derived:

$\Delta R_M^{CH_2} = +0.15$

$\Delta R_M^{C=C} = -0.15$

$\Delta R_M^{C=C-C=C} = 2x -0.15 +$ *correction term of* $+0.07 = -0.23$

$\Delta R_M^{C=C-C=C-C=C} = 3x -0.15 +$ *correction term of* $+0.28 = -0.17$

$\Delta R_M^{\Delta 5 \rightarrow \Delta 7} = -0.02$

$\Delta R_M^{3\beta\text{-OH}} = +0.06$

$\Delta R_M^{3\text{-one}} = +0.22$

$\Delta R_M^{ac.} = +0.55$

The linear relationship between R_M values and N_c numbers is shown in fig. 26. The following numerical form of this relation has been calculated by the least squares method: $R_M = 0.153\,(N_c - 24) - 0.05$.

By means of the above ΔR_M values the R'_M values of certain sterols and closely related compounds can be calculated. In these calculations the intercept $R_{M0} = -0.05$ can be used as an initial value (at $N_c = 24$). The calculated R'_M value of a sterol is then found as follows:

R'_M sterol $= R_{M0} + n\,\Delta R_M^{CH_2} + m\,\Delta R_M^{C=C}$ + correction terms for conjugation and position effects.

The R'_M values of a number of sterols were calculated and listed in column 5 of table 14. One of the best examples of such a calculation is:

R'_M dihydrovitamin D_2, $3FC_{28} = -0.05 + 4 \times 0.15 - 3 \times 0.15 + 0.28 = 0.38$ (experimental corrected value = 0.354).

On the whole the agreement between these calculated R'_M values and the experimental ones (listed in column 2) appeared to be satisfactory.

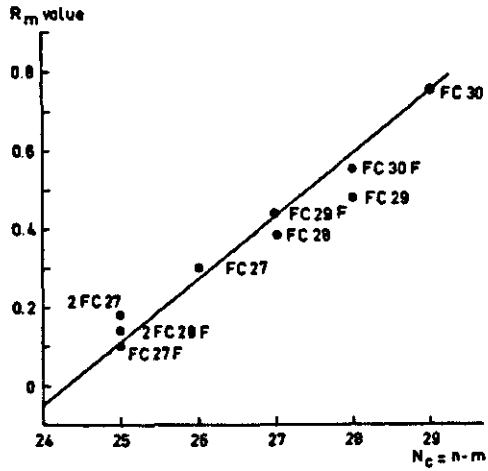


FIG. 26. R_M values of some sterols plotted against their N_c values (N_c = number of carbon atoms minus number of double bonds).

3.4.2 Identification of unknown sterols

By applying the above rules, governing the effect of various groups in the sterol molecule upon R_M values, important data concerning the structure of as yet unidentified sterols may be obtained. Studying the paperchromatography of steroids KABASAKALIAN *et al.* (1960) and BUSH (1961) have derived ΔR_M values for several substituents in the pregnane nucleus. For a number of steroids of known structure they have derived calculated R_F values based on these ΔR_M data. Good agreement between calculated and experimental values was observed. BUSH (1961) has given a thorough study of the application of such ΔR_M values to problems of structural analysis in the steroid group. Analogously, in the system: paraffin/acetic acid–water (84 : 16) we may gain some indication as to the structure of unknown phytosterols by using calculated ΔR_M values. On the basis of these data, we will suggest in chapter 4 a hypothetical structure of the "third-band phytosterols" isolated from coconut fat.

Obviously, the reversed-phase systems described by other authors are suitable for this purpose as well. The R_F–N_c graphs of the systems no. 12, 18, and 19 of table 6 show also nearly linear correlations (fig. 27).

3.4.2.1 *Colour reactions*

A tentative identification of unknown sterols may be facilitated by various colour reactions. In addition to the very sensitive spray reagent phosphomolybdic acid, more specific colour reagents suitable for the detection of sterols were reported in the literature a.o. antimony(III)chloride, antimony(V)chloride, phosphotungstic acid, silicotungstic acid, zinc(II)chloride with benzoyl chloride etc. An extensive review of

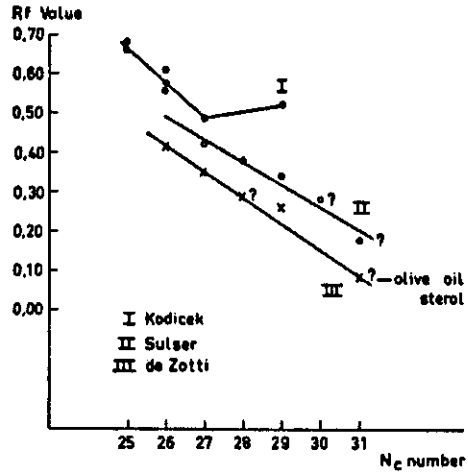


FIG. 27. R_P versus N_c graph in the reversed-phase systems, described by KODICEK (1954), SULSER (1957), and DE ZOTTI (1959) (cf. table 6).

various colour reactions used in the paperchromatography of steroids is given by NEHER (1958).

We have found several other reactions also suitable in identifying unknown sterols a.o. trichloroacetic acid, calcium chloride, bismuth(III)chloride, sodium periodate–permanganate, urea, and dimethyl-p-phenylene diamine–m-toluylene diamine. Table 19 gives a survey of the colours produced by nine sterols and related compounds upon spraying the chromatogram with these colour reagents.

These more specific colour reactions can furnish some indication as to the structure of as yet unidentified naturally occurring sterols. In special cases a differentiation between the sterols belonging to the same critical pair may be based on such colour reactions. The colours produced by ergosterol and 7-dehydrocholesterol in some reactions (a.o. IV, V, X) differ from those of zymosterol ($FC_{27}F$) and related compounds. This differentiation was applied to the structural analysis of the phytosterols of pumpkin oil, which show a distinct fourth band with $R_S = 1.15$ on the chromatogram. This band belongs to the ergosterol critical pair, but did not produce any of the colour reactions indicative of ergosterol and 7-dehydrocholesterol. Therefore, a non-conjugated $FC_{27}F$ structure was suggested tentatively, as will be discussed in chapter 6.

3.4.2.2 *Structure of γ-sitosterol*

As another example of the application of the above correlation between sterol structure and R_M values, the structure of γ-sitosterol was made a matter of critical consideration. On the basis of these rules it appeared likely that steric differences in the configuration of the alkyl group at C_{24} would have *no* influence on the R_M value. Sterols such as β-sitosterol, brassicasterol, and ergosterol have the 24-b configuration. On account of molecular rotation data and other characteristics the stereoisomeric 24-a configuration is attributed to campesterol and γ-sitosterol. The R_M value of campesterol probably fits into the linear correlation given by the other 24-b sterols. Since we had no sufficiently pure preparation of campesterol available, this statement is only tentative. It is based on the R_M value of the second band of purified soybean oil sterols of which the stigmasterol fraction has been removed previously. In view of these experiments and the general opinion that separations of such stereoisomers with reversed-phase partition chromatography can scarcely be attained if at all, it is most likely that the configuration at C_{24} has no influence on the R_M value. The R_F values of 24-b-ethyl cholesterol (β-sitosterol) and 24-a-ethyl cholesterol, which is the generally accepted structure for γ-sitosterol, therefore, should be equal. The γ-sitosterol preparation we received from DR. A. KUKSIS, still contained an impurity (β-sitosterol). Upon paperchromatographic analysis this preparation shows a predominant band with $R_F = 0.32$ (70% as evaluated by planimetry). A second minor band with $R_F = 0.24$ was attributed to the contaminating β-sitosterol. RECOURT (1962) has confirmed this estimation later on by gaschromatographic analysis of the same sample. The gaschromatogram revealed a major peak of 70% with a retention time considerably shorter than that of the accompanying β-sitosterol peak.

The band with an R_F value of 0.32 thus has to be attributed to γ-sitosterol, indicating a N_c of 27. In the case of normal Δ5-phytosterols this N_c value would evidence a structure with 28 carbon atoms. This is in peculiar contrast to the generally accepted $C_{29}H_{50}O$ formula and *would suggest a* $C_{28}H_{48}O$ *formula for γ-sitosterol.* In fact in the literature there has always remained some doubt as to the correct structure of γ-sitosterol.

OPPENAUER (1935) degraded γ-sitosterol to dehydro-epiandrosteron. Thus γ-sitosterol should have the same carbon skeleton as β-sitosterol. DIRSCHERL *et al.* (1943) found that oxidation of β-, and γ-sitosterol yielded d-, and l-6-methyl-5-ethyl heptanone-2 respectively. As the structure of β-sitosterol is 24-b-ethyl cholesterol, γ-sitosterol would be its stereoisomer 24-a-ethyl cholesterol. Most of the recent surveys in this field indeed represent the formula of γ-sitosterol as $C_{29}H_{50}O$. However, the statements of DIRSCHERL are not conclusive and the deviating physical constants of the available γ-sitosterol preparations give rise to some doubt.

SANDQVIST *et al.* (1931) already considered a formula lying *between* $C_{27}H_{46}O$ and $C_{29}H_{50}O$. On the basis of the differing molecular rotations of β- and γ-sitosterol viz. —149 and —178 respectively, BERGMANN *et al.* (1947) concluded, "that γ-sitosterol

TABLE 19 Colour reactions of some sterols on a paperchromatogram

Compound	U.V. radiation 365 nm	I $SbCl_3$	II Sb_2Cl_5	III $BiCl_3$	IV $CaCl_2$ in U.V. radiation	V trichloroacetic acid	VI phosphotungstic acid	VII silicotungstic acid	VIII dimethyl-p-phenylene diamine	IX MILLON'S reagent	X urea, U.V. radiation of 365 nm	XI $NaIO_4$–$KMnO_4$
cholesterol	—	violet	yellow-brown	violet	—	—	pink-violet	pink-orange	—	very faint yellow	—	—
β-sitosterol	—	violet	very faint yellow	violet	—	—	pink-violet	pink-orange	—	—	—	—
stigmasterol	—	faint violet	grey-purple	rose-violet	—	—	violet-brown	faint purple-brown	—	—	—	—
lanosterol	—	orange	yellow	orange	—	—	orange-yellow	orange-yellow	—	—	—365 nm / +254 nm	yellow
ergosterol	+	very faint purple	pink	—	++	faint purple	faint purple	faint purple-brown	very faint blue	yellow	++	—
zymosterol	—	pink-brown	orange-yellow	faint yellow	faint	—	yellow-brown	yellow	blue	yellow	faint 365 nm / +254 nm	yellow
7-dehydrocholesterol	+	purple-brown	brown-blue	faint purple-brown	+	green-purple	purple-brown	purple-brown	—	yellow	+	yellow
vitamin D_3	+	brown	brown-blue	grey-brown	+	—	brown	brown	blue	faint yellow	—	yellow
lumisterol	+	faint brown	purple	—	+	purple	purple-brown	purple-brown	blue	yellow	+	yellow

is not a C_{24}-epimer of 22,23-dihydrostigmasterol or more likely that the samples which have so far been described contain a more levo-rotatory component such as for example 22,23-dehydro-γ-sitosterol". BERGMANN (1953) further stated: "As yet however γ-sitosterol has not yet been degraded to an acid such as Δ5-3 hydroxynorcholenic acid which would prove beyond doubt that the configurations of all other carbon atoms are the same as those of β-sitosterol. The possibility, however unlikely it may be, has therefore not completely been excluded that γ-sitosterol may differ from β-sitosterol in the configuration of yet another carbon atom such as C_{20}."

BERGMANN *et al.* (1947) demonstrated clionasterol, occurring in certain sponges, to be a C_{24}-epimer of β-sitosterol. A considerable lack of agreement remained between the constants of γ-sitosterol (melt.p. 143–148°C; $a_D = -41°$ to $-45°$) and of clionasterol (melt.p. 139°C; $a_D = -37°$). Recently, KUKSIS *et al.* (1960c) have confirmed the existence of significant differences in the physical constants of γ-sitosterol and clionasterol. RIEMERSMA *et al.* (1958), of the RIJKSZUIVELSTATION, already assumed that the soybean phytosterol band with $R_F = 0.29$ also contains the γ-sitosterol fraction; later on this tentative statement was definitely proven by our experiments (COPIUS PEEREBOOM 1961). Consequently, the γ-sitosterol molecule would have a carbon skeleton of 28 instead of 29 atoms. KUKSIS *et al.* (1960a) have reported that the R_F values of γ-sitosterol and several γ-sitosterol esters in three reversed-phase systems are always slightly higher than those of β-sitosterol and its esters. Therefore,

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TABLE 19 (continued)

The colour reactions are carried out on spots of 100 μg of sterol on Whatman no. 3 paper. The degree of impregnation of the paper amounts to about 0.12 g/g of paper. The dried chromatogram can be dealt with in one of the following ways:

A. Viewing the chromatogram under U.V. radiation of 365 nm.

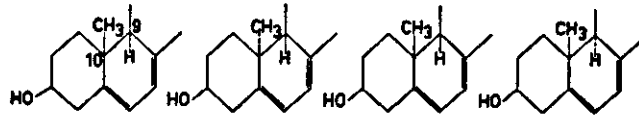
B. Spraying with the following reagents, noting the colours produced in daylight and U.V. radiation (365 nm).

I. A solution of antimony(III)chloride (50% in ethanol). Heat 5–10 min at 70°C.
II. A solution of antimony(V)chloride (20% in chloroform).
III. A solution of bismuth(III)chloride (33% in ethanol). Heat some seconds at 60°C.
IV. A solution of calcium chloride (50% in ethanol–water (1 : 1)). Heat 10–15 min at 80–90°C. Fluorescent spots under U.V. radiation of 365 nm.
V. Moisten the chromatogram with trichloroacetic acid, deliquesced with a few drops of water.
VI. A solution of phosphotungstic acid (15% in ethanol). Heat some min at 60°C.
VII. A solution of silicotungstic acid (25% in ethanol). Heat some min at 60°C.
VIII. A mixture of dimethyl-p-phenylene diamine and m-toluylene diamine (1 : 1) (1% of the mixture in water).
IX. MILLON's reagent (one part of mercury dissolved in two parts of concentrated nitric acid). Heat 2–4 min at 40–50°C; spray again and heat.
X. A solution of urea in water (50%). Heat 10 min at 80°C. Fluorescent spots under U.V. radiation of 365 nm and 254 nm.
XI. A solution of sodium periodate (1% in water). Spray after 15 min with a 1% solution of potassium permanganate.

they agreed with the hypothesis of RIEMERSMA. Considering the above statements and especially in view of the rules between structure and R_M values, we would suggest a C_{28} structure for γ-sitosterol to be the most probable.

3.4.3 Influence of different steric configurations

Compounds which, when activated properly, are converted into the various forms of vitamins D, are denoted as *provitamins D*. During the activation there occurs a change involving the rupture of the B-ring of the nucleus between carbon atoms 9 and 10, the C_{10} methyl group being converted into a methylene group. Ergosterol and 7-dehydrocholesterol are the principal provitamins D occurring in nature. Upon activation with U.V. radiation they yield vitamin D_2 and D_3 respectively. Some primary irradiation products, the lumisterols, still retain the sterol nucleus, although with a different stereoisomeric configuration. Other irradiation products such as the tachysterols already have lost the typical sterol ring structure. Under special experimental circumstances vitamin D_2 can be converted into two other substances viz. pyrocalciferol and isopyrocalciferol, both being stereoisomers of ergosterol. The steric configuration of ergosterol and of these C_9, C_{10} stereoisomers viz. lumisterol, isopyrocalciferol, and pyrocalciferol, are described in the literature as 10β, 9α; 10α, 9β; 10β, 9β; and 10α, 9α.



ergosterol lumisterol isopyrocalciferol pyrocalciferol

	Configuration of the molecule: 3β-OH; juncture A/B trans;			
	and 10β, 9α	10α, 9β	10β, 9β	10α, 9α
$R_F(R_M)$ values	0.43(0.11)	0.37(0.23)	0.43(0.12)	0.33(0.31)

The R_F and R_M values of these four stereoisomers in the studied reversed-phase system are: 0.43(0.11); 0.37(0.23); 0.43(0.12); and 0.33(0.31) respectively. These data show that the R_F values of provitamins possessing a β-configuration at carbon atom 10 are higher than those of the corresponding stereoisomers with a C_{10} α-configuration. The separation of provitamins with different steric configurations at carbon atom 10 i.e. ergosterol (10β, 9α) and lumisterol (10α, 9β) or isopyrocalciferol (10β, 9β) and pyrocalciferol (10α, 9α) can be accomplished. However, we were not able to achieve a separation of provitamins D with the same C_{10} configuration.

In the studied system the series of increasing R_F values (decreasing R_M values) and consequently of increasing polarity of these four stereoisomers is:

$$10\alpha, 9\alpha < 10\alpha, 9\beta < 10\beta, 9\beta < 10\beta, 9\alpha$$

The ΔR_{Mr} values connected with the difference in the steric configuration at the C_{10} methyl group (10α and 10β) are respectively:

$$9\alpha,\ 10\alpha \rightarrow 9\alpha,\ 10\beta \qquad \Delta R_{Mr} = -0.20$$
$$9\beta,\ 10\alpha \rightarrow 9\beta,\ 10\beta \qquad \Delta R_{Mr} = -0.11$$

Epi-lumisterol, having an axial hydroxyl group viz. 3α-OH, has an R_F value (0.43) higher than that of lumisterol, thus enabling a separation of both compounds. Consequently

$$3\beta\text{-OH}\ (10\alpha,\ 9\beta) \rightarrow 3\alpha\text{-OH}\ (10\alpha,\ 9\beta) \quad \Delta R_{Mr} = -0.10$$

If the steric configuration at carbon atom C_3 and at the juncture A/B is different from that of the dihydrocholesterol series, the R_F value deviates considerably. The R_M value of dihydrocholesterol (equatorial hydroxyl group: 3β-OH, juncture A/B trans viz. 3β-OH, 5α) is much lower than that of coprostanol (axial hydroxyl group: 3β-OH, A/B cis viz. 3β-OH, 5β).

In reversed-phase chromatography a lower R_M value corresponds with a higher polarity of the solute. Obviously, coprostanol, having a hydroxyl group in axial position, which generally is thermodynamically less stable than in equatorial position, has a lower polarity than dihydrocholesterol. Considering the STUART models of such chair shaped cyclohexane rings, the six equatorial hydrogen atoms project from the equator of the molecule. The six axial hydrogen atoms, however, are arranged together in two groups of three on the upper side and three at the underside of the molecule. The axial atoms or their polar substituents have less opportunity of contacting with the solvent molecules. The influence of a polar substituent with axial configuration, therefore, should be smaller than with equatorial substituents (HAIS 1958). According to BUSH (1961) an equatorial hydroxyl group thus has a larger ΔR_{Mg} value than an axial hydroxyl group.

The difference in polarity of coprostanol and dihydrocholesterol, as shown in this system, is indicated by:

$$3\beta\text{-OH},\ 5\beta < 3\beta\text{-OH},\ 5\alpha$$

and the corresponding ΔR_{Mr} value by:

$$3\beta\text{-OH},\ 5\beta \rightarrow 3\beta\text{-OH},\ 5\alpha \qquad \Delta R_{Mr} = -0.68$$

hydroxyl group: axial equatorial

This corresponds with the data reported in the literature concerning the polarity of related C_{19} and C_{21} steroids in some normal chromatographic systems.

In general it has been found (NEHER 1958):

$$\underset{\text{axial}}{3\alpha\text{-OH},\ 5\alpha} \leqslant 3\beta\text{-OH},\ 5\beta < \underset{\text{equatorial}}{3\alpha\text{-OH},\ 5\beta} \leqslant 3\beta\text{-OH},\ 5\alpha$$

This polarity sequence indicates that the polarity is dependent mainly on the axial or equatorial position of the hydroxyl group at C_3 and only subordinately on the conformations at the ring juncture A/B. In normal systems the C_{19} and C_{21} steroids, having an axial hydroxyl group, thus exhibit a lower polarity than those with an equatorial configuration. This difference usually enables a paperchromatographic separation to be made.

Epi-cholesterol (axial hydroxyl group: 3α-OH, Δ5) likewise has a lower polarity (and hence lower R_F value) than its stereoisomer cholesterol (equatorial hydroxyl group: 3β-OH, Δ5), the corresponding ΔR_{Mr} value amounting to:

$$\underset{\text{axial}}{3\alpha\text{-OH}, \Delta 5} \rightarrow \underset{\text{equatorial}}{3\beta\text{-OH}, \Delta 5} \qquad \Delta R_{Mr} = -0.13$$

In the above parts of this chapter several aspects of the relationships between structure and migration rate and of their application in practice has been reviewed.

4 APPLICATIONS OF THE CHROMATOGRAPHIC STEROL SEPARATION TO THE ANALYSIS OF FAT MIXTURES

4.1 Analysis of mixtures of animal and vegetable fats

The separation of sterols by paperchromatography has developed into a powerful tool in the analysis of mixtures of vegetable and animal fats. Occasionally, this method also gives some indication as to the composition of mixtures of vegetable fats.

4.1.1 Analysis of animal fats

The identification of vegetable oils in an animal fat like butter fat is carried out by the phytosterol acetate test, especially according to the modification of Den Herder. As this test was designed as a qualitative identification procedure, an extension to semi-quantitative evaluations met with difficulties. The paperchromatographic sterol analysis, however, enables a reliable quantitative determination of the amount of phytosterols and hence of the percentage of vegetable fat in butter. Nowadays the interest in such a procedure is aroused again a.o. due to the necessity of analysing artificial milk products like skimmed milk containing vegetable fat. Another application involves the quantitative evaluation of the percentage of vegetable products admixed to pure dried whole eggs (Roos 1961). Using the normal paperchromatographic procedure, as described in chapter 2, small amounts of phytosterols can be determined in cholesterol and vice versa.

The smallest percentage of cholesterol and of the phytosterols which can be detected depends on the amount of spotted sterol mixture and on the sensitivity of the colouring reagent. By staining with phosphomolybdic acid amounts as low as 0.5–1.0 μg of sterol still can be visualized. The sensitivity of the normal procedure thus enables the detection of about 8–10% of phytosterol or cholesterol in mutual mixtures. The smallest detectable percentage of vegetable fat depends in the first place on this sensitivity and secondly on the respective sterol contents of the animal and vegetable fats. In mixtures of butter fat and soybean oil, both having total-sterol contents of about 0.3%, an adulteration of butter with 12% of soybean oil can easily be detected (fig. 28, spot 4). Vegetable oils with high total-sterol contents like rapeseed oil ($a_0 = 0.55$) can be detected in even smaller amounts. A considerable increase in sensitivity is gained by performing the chromatographic analysis with the fraction of the free sterols instead of the total-sterol fraction. Most of the common animal fats contain sterol mixtures with about 90% of free sterols, the quantity of cholesterol esters in butter fat thus amounting to about 0.03%. In vegetable oils the free and bound sterols are distributed

more equally. In a mixture of vegetable and animal fats the percentage of phytosterols in the sterol mixture is increased by isolating the bound sterols. As the percentage of bound sterols in coconut fat is of the same order as that of butter fat (0.03%), the identification of 12% of coconut fat in butter fat is possible in that way. Rapeseed oil with a high total-sterol content and a high ratio of bound to free sterols was detected in amounts as low as 1–2%. In a more extensive calculation the following sterol balance is obtained:

amount of bound sterols in butter fat:	0.03%
amount of bound sterols in rapeseed oil:	0.32%

In a mixture of both fats

2% of rapeseed oil contributes:	0.0064% phytosterol
98% of butter fat contributes:	0.0294% cholesterol
Total-sterol content:	0.0358%

The percentage of phytosterol in this mixture amounts to $64/358 \times 100 = 18\%$, which is easily detectable by paperchromatographic analysis. For practical reasons we have to consider especially the adulteration of butter with margarine fats. Although the fat composition of commercial margarines varies considerably, we mostly found total-sterol contents of 0.10–0.25%, for a third to a half in the bound position. In spite of the presence of some animal fat in a sample of margarine, we were able to detect an amount of 8.5% of that margarine ($a_0 = 0.1\%$) in butter fat by analysing the bound-sterol fraction (fig. 28, spot 3). When spotting higher amounts on the chromatogram viz. 50–100 μg of sterols, the smallest amount of phytosterol detectable in mixtures with cholesterol was decreased to about 1.5%. The presence of 6% of coconut fat in butter thus was detected by analysis of the total-sterol fraction, whereas only 2.5% of the same fat was found by analysing the bound sterols (fig. 29, spot 2).

4.1.2 Analysis of vegetable fats

Apart from this sterol analysis, no reliable quantitative method for the detection of small amounts of animal fat in mixtures with vegetable fats was reported in the literature. Although the so-called BERTRAM colour reaction with trichloroacetic acid was believed to be indicative for the presence of all types of animal fats, lard gives a negative result, whereas on the other hand a positive colour reaction is obtained with soybean oil. Some other tests were already mentioned in chapter 1 part 6. Some indication as to the presence of animal fat could be obtained by classical sterol analysis viz. by the sterol content and the melting point of the acetates. The limitations of these methods were already discussed in chapter 1.

A reliable procedure for the detection of small amounts of animal fats and especially of lard was badly needed for analysing the so-called vegetable ghee exported from

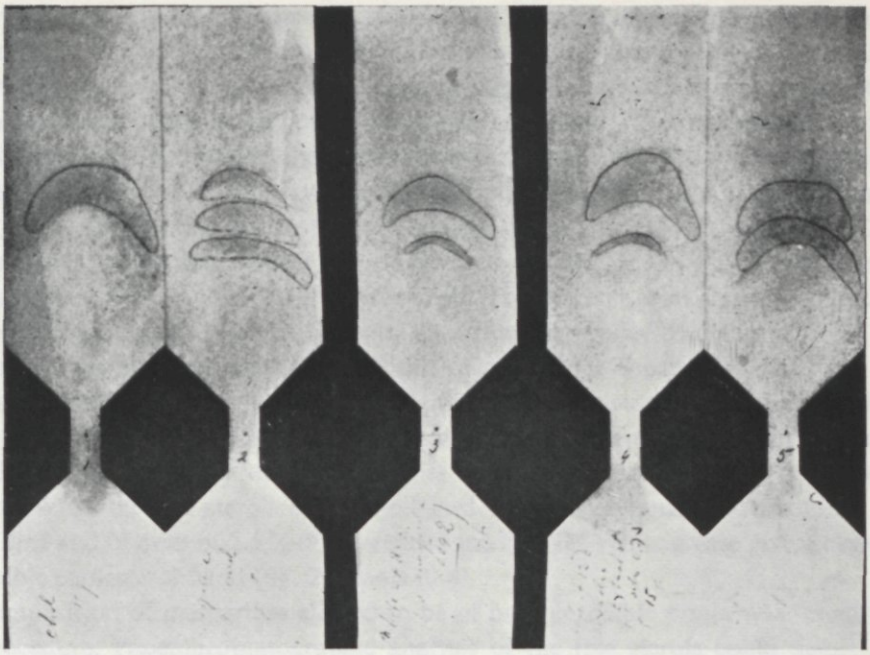


FIG. 28. Detection of animal and vegetable fats in mutual mixtures.

spot 1. cholesterol from lard.
spot 2. free sterols of margarine with 8% of lard.
spot 3. bound sterols of butter with 8.5% of margarine.
spot 4. total-sterol fraction of butter with 12% of soybean oil.
spot 5. soybean oil phytosterols.

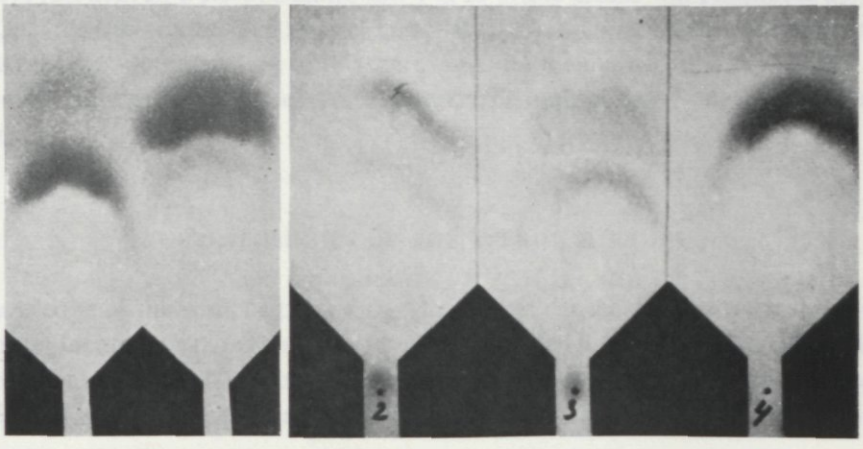


FIG. 29. Detection of animal and vegetable fats.

spot 1. free sterols of cottonseed oil with 2% of White grease.
spot 2. bound sterols of butter with 4% of coconut fat.
spot 3. free sterols of palm oil with 5 % of lard.
spot 4. free sterols of palm oil with 2.5% of White grease.
spot 5. butter cholesterol.

Western Europe a.o. to the Near and Middle East. Because of religious prohibitions, admixtures of these vegetable fats with the body fats of cows and pigs and especially with refined inedible animal fats e.g. White grease, are undesirable. For the greater part this problem could be grappled by a paperchromatographic detection of cholesterol in the sterol mixture. By the above procedure a percentage of 8–10% of cholesterol is detectable on the chromatogram. This enables the detection of an admixture e.g. of about 10% of lard ($a_0 = 0.08\%$) in palm oil ($a_0 = 0.03\%$) (fig. 22, spot 2). The sterols from this fat mixture consist for $1/10 \times 0.08 = 0.008\%$ of cholesterol and for $9/10 \times 0.03 = 0.027\%$ of phytosterols. The percentage of cholesterol in the mixture thus amounts to $8/35 \times 100 = 23\%$, which can easily be detected.

Obviously, the smallest detectable amount of animal fat depends on the limit of sensitivity of the paperchromatographic method and on the respective sterol contents of the animal and vegetable fats. Because of the high free-sterol content of most animal fats, the sensitivity of the method is increased by analysing the free-sterol fraction. When the free sterols of palm oil–lard mixtures are analysed, quantities of 5% of lard and of even of 2.5% of the refined inedible fat White grease give a clearly discernible cholesterol band (fig. 29, spots 3, 4)

The composition of margarines alleged to be of pure vegetable origin was tested by this procedure. Paperchromatographic analysis of the free sterols could detect an admixture of 8% of lard to the vegetable margarine fat (phytosterol content 0.1%), as is shown in fig. 28, spot 2. By spotting higher amounts of sterols i.e. 50–100 μg smaller amounts of animal fat will be detectable. Amounts of 4% of refined White grease in cottonseed oil ($a_0 = 0.37\%$) were detected by the total-sterol procedure, whereas the presence of only 1% of this fat can be established by analysis of the free-sterol fraction. In a sterol balance this 1% of White grease will contribute about 0.003% of free cholesterol. The 99% of cottonseed oil contains about 0.158% of free phytosterols. By spotting 50 μg of this sterol mixture a cholesterol band of $3/161 \times 50\ \mu g = 0.9\ \mu g$ may be expected, which exceeds the limit of sensitivity of the colour reaction.

4.2 Composition of the sterol mixture

The knowledge of the composition of sterol mixtures can be applied to many other problems of fat analysis than those described above. Therefore, a thorough knowledge of all sterol values of the common vegetable and animal oils appeared to be necessary (cf. table 2, chapter 1). We have analysed a great number of oils and fats[3], determining

[3] We gratefully acknowledge the assistance of the oil factories: Kon. Fabr. T. Duyvis Jz. N.V. at Koog aan de Zaan, N.V. Mij. tot Expl. der Ver. Oliefabrieken (V.O.Z.) at Zwijndrecht, N.V. Cacaofabriek "de Zaan" at Koog aan de Zaan, Gerkens' Olieraffinaderij N.V. at Wormer, and N.V. Oliefabrieken Crok en Laan at Wormerveer by procuring fat samples.

the following data of the total-sterol fraction and of the fractions of free and bound sterols viz. sterol content, melting point of the acetates, composition of the sterol mixture as evaluated by paperchromatography, and the arachidonic acid content. The data are compiled in table 20.

These data give an insight into the sterol values and their natural variability. The analysis of sterol mixtures then can give some clue to many problems of fat chemistry. In the past, a reliable analysis of naturally occurring phytosterol mixtures was only possible by laborious purification procedures, for the greater part of a qualitative character. Only a few quantitative methods, using special analytical techniques, have been published a.o. the determination of stigmasterol in crude soybean oil phytosterols by an isotope dilution technique (DONIA 1957) and by infrared spectrophotometry (JOHNSON 1957). The paperchromatographic sterol analysis enabled us to confirm many of the semi-quantitative data concerning phytosterol compositions that have been reported.

BONSTEDT (1928) and others (see ELSEVIER'S ENCYCLOPAEDIA 1952) have established the presence of about 25% of stigmasterol and 4% of campesterol in soybean oil sterols. The bulk of this sterol mixture, however, is made up of β-, and γ-sitosterol. Our paperchromatographic analysis reveals nearly equal amounts of first-band (mainly β-sitosterol) and second-band phytosterols (campesterol, γ-sitosterol, and stigmasterol). A separate chromatographic determination of the stigmasterol content could be accomplished by reversed-phase thin-layer chromatography of the sterol acetate bromides, as will be discussed in chapter 5. The percentage of stigmasterol on the chromatoplate was roughly evaluated at 30. The percentage of γ-sitosterol and campesterol then is approximately 20. The sterol balance of soybean oil thus amounts to approximately 50% of β-sitosterol, 30% of stigmasterol, and 20% of γ-sitosterol and campesterol. The absence of considerable amounts of stigmasterol in cottonseed oil and tall oil, as reported by WALLIS *et al.* (1937) and by HASSELSTROM (1949) respectively, was confirmed in our chromatographic procedure by the lack of any detectable second band.

In general sterol values are not affected by the degree of rancidity of the fat. Samples crude and refined soybean oil, oxidized during 8 hrs at 100°C in a SWIFT test tube, have similar amounts of first-, and second-band phytosterols as the untreated oil. VITAGLIANO (1961) found that the melting points of the sterol acetates isolated from vegetable seed oils and animal fats remained constant during oxidation of these fats. As an exception the phytosterols of olive oil were proved to be more sensitive to oxidation. High melting points of the sterol acetates were observed when the peroxide number of the olive oil exceeds 25. On the other hand excessive *heating of a fat* at high temperatures can have a great influence on the sterol composition. MARCUSSON *et al.* (1913) have already observed that after heating a vegetable oil at high temperatures a significant decrease in the free-sterol content and a discolouration of the free-sterol digitonides are found.

We have studied this phenomenon to some extent, restricting our experiments to the heating of soybean oil. Samples of refined soybean oil, containing 50% of first-, and 50% of second-band phytosterols, were heated at a temperature of 220°C. After a heating period of 10–16 hrs paperchromatographic analysis revealed a distinct third band. On prolonged heating this band disappeared again. During this treatment the sterol content of the oil decreased from 0.38% to 0.21%. Simultaneously, the levorotation of the sterol mixture is diminished from —33° to —21°. After heating the soybean oil at a still higher temperature, viz. 285°C during 10 hrs, a sterol content as low as 0.01% was observed.

Upon perusal of the data of table 20 several peculiar details are noticed. The cholesterol of animal fats ordinarily is present for 70–90% in the free state. The percentages of free sterols in common vegetable oils, however, vary to a great extent. Whereas in soybean oil the *free* sterols dominate (viz. 65% of the sterol mixture), the phytosterols of e.g. maize oil are esterified for the greater part, leaving only a small amount in the free state (viz. 35%). Usually, the acetate melting point of the total-sterol fraction is intermediate to the melting points of the free-, and bound-sterol fractions. The last mentioned fraction mostly shows the lowest data. The reported sterol acetate melting points vary from 114.6°C (grapeseed oil) to 164.0°C (pumpkin oil). However, for the common oils usually melting points in the range 125–135°C are found. The special properties of some *high* melting phytosterol acetates of e.g. pumpkin oil and shea nut fat will be discussed in chapter 6. The phytosterol mixtures of e.g. safflower oil, sunflower oil, crude avocado oil, and grapeseed oil have peculiarly *low* melting points for their sterol acetates viz. 120.7°; 119.4°; 119.4°; and 114.6°C respectively. After hydrogenation the sterol content of vegetable oils is decreased considerably. Because of the low sensitivity of the phosphomolybdic acid reaction in the case of hydrogenated sterols like dihydrocholesterol, the paperchromatographic detection of these so-called stanols in hardened fats meets with difficulties. However, these stanols can easily be detected by reversed-phase thin-layer chromatography, as will be discussed in chapter 5.

Regarding their phytosterol composition, the common vegetable oils can be divided into two main groups. In the first group the phytosterols display exclusively first and second bands in paperchromatographic analysis. Those of the second group also contain some amounts of third-band phytosterols. To the first group belong a.o. soybean oil, maize oil, unhydrogenated palm oil, cottonseed oil, safflower oil, grapeseed oil, wheatgerm oil, sunflower oil, and cocoa butter. The second group comprises: rapeseed oil, coconut fat, peanut oil, sesame oil, and less common oils like castor oil, pumpkin oil, almond oil, nigerseed oil, tobaccoseed oil, shea nut fat and occasionally also olive oil, palmkernel oil, hardened palm oil etc. All of them contain varying amounts of third-band phytosterols. Because of this difference, the admixture of oils belonging to the second group with those of the first group can be detected by sterol analysis. For example admixtures of high amounts of coconut fat to palm and palmkernel oil; of rapeseed and peanut oil to soybean oil; of shea nut fat to cocoa butter

are detectable by this method of analysis. In some less common non-edible vegetable oils we have found peculiar sterol mixtures. The sterols of some samples of crude avocado oil (from the fruits of the tree *Persea americana*) and of almond oil (oleum amygdalae expressum) have a remarkable composition. Only first-band and third-band phytosterols were found, but we were not able to detect any second-band phytosterols.

The alcoholic fraction of commercial wool-wax samples contains cholesterol, lanosterol, and dihydrolanosterol in quantities of about 20%, 10%, and 10% respectively. Cholesterol and these triterpenoids can be separated and determined quantitatively using the investigated paperchromatographic system (see fig. 25). The major compounds of the wool wax viz. several types of acids and alcohols are not coloured by phosphomolybdic acid and do not hinder the analysis. Generally, the method can be used for screening the composition of unknown phytosterol mixtures.

4.3 Probable structure of the third-band phytosterols

In several vegetable oils we have found considerable amounts of third-band phytosterols, presenting the same R_F value as cholesterol. The correlation between the structure of a sterol molecule and the R_F value in reversed-phase paperchromatography, derived in chapter 3, might give some indication concerning the structure of these third-band phytosterols. On the basis of this correlation sterol structures such as $FC_{28}F$, $2FC_{28}$, $2FC_{29}F$ etc. with $\Delta 5$ or $\Delta 7$ double bonds, all belonging to the same critical pair, should be considered as possible.

Especially the phytosterols of rapeseed oil, known to be rich in brassicasterol, appeared to contain high quantities of third-band phytosterols viz. 10–25%. According to the above-mentioned correlation, the R_F value of brassicasterol should be similar to that of cholesterol, as both belong to the same critical pair. A pure preparation of brassicasterol (melt.p. 149°C, $a_{D,\ CHCl_3} = -60°$) indeed showed an R_F value equal to that of cholesterol[4]. The phytosterols of the second group of vegetable oils (e.g. rapeseed oil, peanut oil, sesame oil, coconut fat) all contain considerable quantities of third-band phytosterols. Concerning these peculiar phytosterols only scarce references occur in the literature. On the basis of the following arguments we suppose them to have structures isomeric to that of methyl-cholestadienol ($FC_{28}F$) e.g. brassicasterol (cf. Copius Peereboom en Roos 1960a).

By applying a paraffin/propionic acid–water system, Kuksis and Beveridge (1960b) have also observed the presence of some "third-band phytosterols" in purified hydrolysates of corn oil phytosterol esters. They supposed the structures to be similar to those of the α-sitosterols. According to a statement of Barton (1945), the structure of the α-sitosterols is mostly described as similar to those of the group of tetracyclic

[4] We gratefully acknowledge the generous help of Prof. D. H. R. Barton F.R.S. (London), for putting a pure preparation of brassicasterol at our disposal.

triterpenoid alcohols: $C_{29}H_{48}O$ or $C_{30}H_{50}O$. The triterpenoid alcohol lanosterol ($C_{30}H_{50}O$, $FC_{30}F$), which largely fits in with the above relationships, shows an R_F value (viz. 0.22) lower than that of the second-band phytosterols. On the contrary the third-band phytosterols, detected in these reversed-phase systems, have R_F values higher than that of the second band. MAZUR (1958) has proposed another structure for α_1-sitosterol viz. 4α-methyl-24-ethylideneΔ7-cholestene-3β-ol ($FC_{30}F$). This structure as well as the triterpenoid structure for α_1-sitosterol would have a slightly lower polarity as compared with that of stigmasterol. This difference, however, would not result in a *higher* R_F value in these paperchromatographic systems, as stated by KUKSIS AND BEVERIDGE. It is rather likely that such sterols would have a low R_F value, similar to those of β-sitosterol and lanosterol. Therefore, it is improbable that the third-band phytosterols of common oils such as coconut fat are composed exclusively of sterols of these special types. Furthermore, these special sterol types mostly exhibit a pronounced dextro-rotation, as contrasted with the normal levo-rotation of coconut phytosterols.

In view of the paperchromatographic data we also have to consider the possibility that the third-band phytosterols (or major parts thereof) might be constituted of poly-unsaturated, conjugated sterols with structures such as $2FC_{28}$, $2FC_{29}F$ and R_F values similar to that of cholesterol. Concerning the presence in products of vegetable origin of such conjugated sterols, often denoted as provitamins D, many instances have been reported. Considering this possibility, we have investigated the properties of phytosterol mixtures isolated from vegetable oils with high percentages of third-band phytosterols. Because of the possible influence of the refining process, the sterols of both the crude and the corresponding refined oils were studied.

We have found indeed that the phytosterol digitonides of crude coconut, palm, and palmkernel oil have characteristic U.V. spectra, closely resembling those of ergosterol and 7-dehydrocholesterol with maxima at 261, 272, 282, and 294 nm (fig. 30). Like the greater part of the common edible oils, peanut oil showed the normal spectrum of Δ5 non-conjugated sterols with very low peaks at these wavelengths. The preparation of small amounts of sterols from the digitonides is troublesome. It is also somewhat risky in view of the possibility of changes in the composition during the process. Therefore, we have attempted to measure the U.V. spectra of the sterol digitonides as such, which is by no means easy because of their insolubility in common solvents. Finally we found that a mixture of dimethylformamide–methanol (1 : 9) was a good solvent for U.V. spectrophotometry of sterol digitonides. To obtain a clear solution an amount of e.g. 60 mg of sterol digitonide is dispersed in 1 ml of dimethylformamide and heated in a water bath. Then 9 ml of warm methanol is added and if necessary the solution is warmed on a water bath. Spectrophotometrically pure grades (MERCK) of both solvents were used. No differences were noticed between the shapes of the spectra of digitonides and the corresponding sterols e. g. cholesterol and ergosterol.

With this simple and rapid procedure we have investigated the properties of the phytosterol mixtures isolated from some vegetable oils. Comparing the $E^{1\%}_{1\,cm}$ values of the digitonides of crude coconut, palm, and palmkernel oil at the maximum of 282 nm with that of ergosterol, we deduced the presence of respectively 3.28–0.78–1.24% of conjugated sterols in these three sterol mixtures (table 21).

Thus the statements of HUNTER *et al.* (1942) and MELLIER (1951) concerning the occurrence of about 1.3% of conjugated sterols in crude palm oil were confirmed. The spectra of the sterol digitonides from crude palm and palmkernel oil closely resemble those of coconut fat. After refining the coconut fat, the spectrum of the sterol digitonides retains the four-band shape. However, the digitonides of the refined palmkernel oil exhibit a spectrum analogous to that of normal phytosterol mixtures e.g. from peanut oil. These normal phytosterol digitonides all show the sitosterol curve devoid of significant maxima at the above wavelengths.

TABLE 21 Composition of the phytosterol mixtures of some crude and refined oils

Sterols	Sterol content, gravimetric	Percentage of the phytosterols of the			$E^{1\%}_{1\,cm}$ of sterol digitonides at 282 nm	Percentage conjugated sterols in the sterol mixtures	Absorbance in the LIEBERMANN-BURCHARD reaction after 30 min
		first band	second band	third band			
	%	%	%	%		%	
crude coconut fat	0.09	46	22	32	0.220	3.28	0.05
refined coconut fat	0.09	41	11	48	0.100	1.49	0.14
crude palm oil					0.052	0.78	
crude palmkernel oil	0.11	56	35	9	0.083	1.24	0.09
refined palmkernel oil	0.09	54	22	24	0.045	0.67	0.11
crude peanut oil	0.29	62	24	14	0.016	0.24	0.09
refined peanut oil	0.29	55	31	14	0.016	0.24	0.11
cholesterol							0.23
ergosterol					6.7		ca. 0.30*
7-dehydrocholesterol					7.0		ca. 0.30*

* both fast-acting sterols.

The $E^{1\%}_{1\,cm,\,282\,nm}$ values and hence the amounts of conjugated sterols in the sterol mixtures of refined coconut fat and palmkernel oil are *decreased* to about 50% as compared with those of the crude oils. The percentage of the third-band phytosterols of these oils, however, is *increased* considerably after the refining process.

Another striking difference between the phytosterols of crude and refined oils is shown in the LIEBERMANN-BURCHARD colour reaction, using an acetic anhydride–sulfuric acid 9 : 1 reagent. The reaction rates of the sterol mixtures of nearly all vegetable oils are rather slow (viz. slow-acting sterols) as contrasted with fast-acting

sterols such as ergosterol, 7-dehydrocholesterol, and $\Delta 7$-sterols (fig. 31). The absorbance versus reaction time graph of a sterol mixture in this colour reaction is characteristic of the sterol composition.

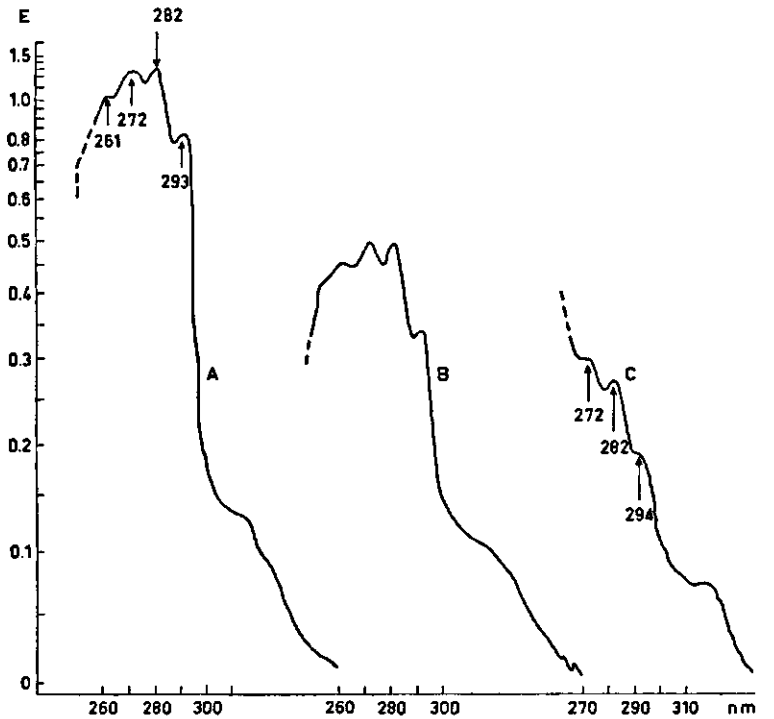


FIG. 30. U.V. absorption spectra of the sterol digitonides of crude coconut fat (A), crude (B), and refined (C) palmkernel oil, dissolved in dimethylformamide–methanol (1 : 9) and measured with an Unicam S.P. 700 spectrophotometer.

The curves of β-sitosterol, stigmasterol, and the sterol mixtures of many vegetable oils all lie below that of cholesterol. The optimum extinction coefficient shown by e.g. stigmasterol amounts to about 50% of that of cholesterol. Surprisingly, the extinction coefficients of the sterols of the refined oils were significantly higher than those of the corresponding crude oils (fig. 31). We may assume that this difference will be due to the presence of special phytosterol types in the refined oils. It has been reported (a.o. COOK 1961) that di-unsaturated, non-conjugated phytosterols such as brassicasterol and fucosterol have higher reaction rates than $\Delta 5$ mono-unsaturated sterols.

Consequently, it is likely that the increase in absorbance in the LIEBERMANN-BURCHARD reaction and the increase of the percentages of third-band phytosterols are correlated phenomena, probably caused by dehydrogenation of the sterols during the refining process. In view of the observed reaction rates of the crude and refined vegetable oils,

the presence of a considerable amount of fast-acting sterols like conjugated or $\Delta 7$-sterols is not probable. The amounts of conjugated sterols in the sterol mixtures of coconut fat etc., as given in table 21, are too small to account for the high percentage of third-band phytosterols. Furthermore, the amount of the latter sterols is *increased* after the refining process, while on the other hand the percentage of conjugated sterols is *decreased*. The major fraction of the third-band phytosterols, therefore, should have a *non-conjugated* structure. Since special structures like $\Delta 7$ nuclear bonds are excluded, a di-unsaturated C_{28}-phytosterol skeleton with a $\Delta 5$ nuclear double bond is the most probable.

Reviewing the above arguments, we suppose the third-band phytosterols of coconut fat and probably of other vegetable oils to consist of di-unsaturated, non-conjugated sterols of the methyl-cholesterol type ($FC_{28}F$). Further indications as to the validity of this hypothesis based on reversed-phase thin-layer chromatography of sterol acetates and of brominated sterol acetates will be given in chapter 5. However, a definite proof of this tentative statement by methods of *preparative* organic chemistry is beyond the scope of this investigation.

The separation of phytosterol mixtures by gas-liquid chromatography is comparable with that obtained by the studied paperchromatographic system. Besides the peaks of β-sitosterol and stigmasterol a small third peak at a shorter retention time (comparable with higher R_F value) is obtained (BEERTHUIS 1960b, EISNER 1962, FRANCO 1962).

On the basis of the above arguments, it is highly improbable that this third peak should be attributed to γ-sitosterol (with the formula $C_{29}H_{48}O$ (?)) or α-sitosterol (with the formula $C_{30}H_{50}O$), as has been stated by EISNER *et al.* (1962) and FRANCO *et al.* (1962) respectively. On the contrary we may suggest that the structure of this aberrant phytosterol type is identical with that of the above-mentioned third-band phytosterols.

4.4 DETECTION OF CHOLESTEROL IN MIXTURES WITH THIRD-BAND PHYTOSTEROLS

4.4.1 With the normal paperchromatographic procedure

The presence of considerable amounts of third-band phytosterols in common oils such as rapeseed, peanut oil, and coconut fat reduces the reliability of the paperchromatographic detection of animal fat in mixtures with vegetable oils. In calculations of the amount of animal fat based upon the respective sterol contents and the cholesterol percentage in the sterol mixture, the latter percentage could not always be approximated correctly on the basis of the experimental amount of third-band sterols. The significance of these calculations will be discussed more in detail in chapter 7. In paperchromatographic analysis of cholesterol, the following phenomena have to be considered, requiring some additional procedures.

Re. A: Vegetable oils of group I viz. devoid of third-band phytosterols

If the type of vegetable fat is known or has been identified by other methods of fat analysis and if the data of table 20 indicate that normally no third-band phytosterols are found, a third band on the chromatogram proves the presence of cholesterol and hence of animal fat. In this case the amount of third-band sterols in the mixture gives the percentage of cholesterol.

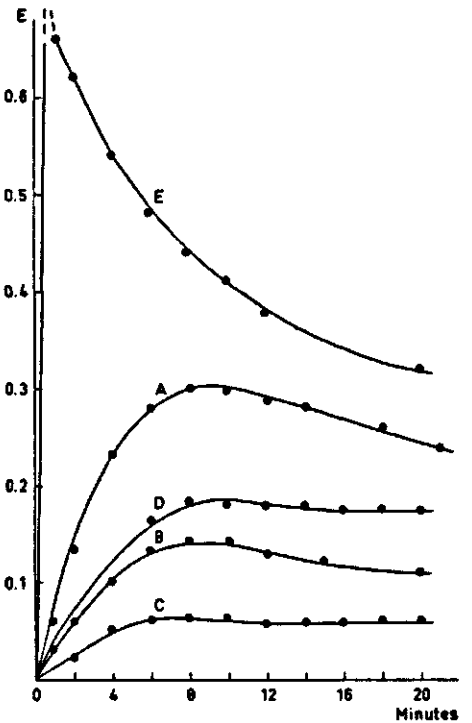


FIG. 31. Absorbance versus time graph in the LIEBERMANN-BURCHARD reaction of A: cholesterol; B: stigmasterol; C: crude and D: refined coconut sterols; E: ergosterol.

Re. B: Vegetable oils of group II viz. containing third-band phytosterols

If according to table 20, the vegetable fat contains a quantity of third-band phytosterols, the following statements will hold:

1. The amount of third-band phytosterols in the sterol mixtures of common edible oils, belonging to this group, usually does not exceed 25%. The analysis of numerous samples of commercial pure vegetable margarine fats established that these fats show an average content of 10–15% of third-band phytosterols. The highest amounts were encountered in some samples of refined coconut fat. *Sterol mixtures that comprise 30% of third-band sterols or more, therefore, probably contain cholesterol from admixture of animal fat.*

This statement has to be confirmed by subsequent microscopic detection of swallow-tail crystals in preparations of purified third-band sterols, as will be discussed below. An evaluation of the percentage of cholesterol can be obtained by diminishing the experimental percentage of third-band sterols with the normally occurring amount of third-band phytosterols, as given in table 20. A more detailed example of such a calculation will be discussed in chapter 7.

2. As a rule the percentages of third-band phytosterols in phytosterol mixtures are not considerably higher than those of the second-band phytosterols. Exceptions were only found in the aberrant phytosterol mixtures of almond oil and crude avocado oil. In addition to statement 1, the ratio of third-band to second-band phytosterols need be considered as well. *When the amount of third-band sterols greatly exceeds that of the second-band phytosterols, the presence of cholesterol will be highly probable.*

3. The amount of β-sitosterol (viz. the quantity of the first-band sterols) of a great number of common edible oils is higher than that of the second-band phytosterols. As a roughly estimated average value the ratio 3 : 2 may be assumed. Analysing synthetic mixtures of refined soybean phytosterols with cholesterol in increasing ratios, the distinct form of the second band fades away in the normal procedure at a cholesterol percentage of about 45. Therefore, a chromatogram with strong first and third bands but with a scarcely visible second band, furnishes a convincing proof of the presence of cholesterol and hence of animal fat in the fat mixture. A discussion of other analytical differences between cholesterol and the third-band phytosterols will be given in chapter 5.

4.4.2 Further examination of questionable samples

A further proof of the presence of cholesterol in sterols isolated from vegetable fat samples that are suspected of being mixed with animal fat can be obtained as follows. To obtain a relatively large portion of third-band sterols the sterol mixture is fractionated on a great number of chromatograms. The third bands of these chromatograms are cut out and eluted with ether. The paraffin in the extract is removed by thin-layer chromatography. The purified residue is examined microscopically as to the presence of cholesterol crystals or of swallow-tail crystals. The observation of crystals of these types then evidences the presence of cholesterol in the sterol mixture. In this way small amounts of animal fat e.g. 3% of inedible animal "White grease" are detectable in palm oil.

Details of the experimental procedure:

Amounts of 100 μg of the questionable sterol mixture are spotted on the six starting points of a chromatogram. Three chromatograms, containing a total amount of 1800 μg of sterols, are developed under the normal experimental conditions. The place of the third bands is determined by cutting off half a chromatogram at each side of the paper and spraying these with phosphomolybdic acid. The zone of the third bands is cut out and extracted with ether. The ethereal solution is evaporated down to 1 ml and then spotted on a chromatoplate (coated with silicic acid G, MERCK). The chromatoplate is developed twice with an iso-octane–ethyl acetate (85 : 15) mixture. By this procedure the con-

taminating paraffin oil is completely removed. In order to detect the sterol spots without spraying with a destructive colouring reagent, the silicic acid layer is provided with 0.01% of a water soluble fluorescent indicator e.g. Ultraphor W. T. Hochkonz. (B.A.S.F.) (cf. COPIUS PEEREBOOM 1960b). In U.V. radiation of 365 nm sterols are then visible as fluorescent spots. The sterol band is scraped off and extracted with ether. After evaporating the solvent carefully, the sterols are dissolved in ethanol (80%). After placing a small volume of this ethanolic solution on a microscope slide, the preparation is inspected for the presence of cholesterol crystals or of the specific swallow-tail crystals at a linear magnification of 80–200 times.

5 THIN-LAYER CHROMATOGRAPHY OF STEROLS

5.1 Introduction

The introduction of paperchromatography has given a practical way of performing liquid-liquid partition chromatography on a horizontal plane. Its experimental simplicity clearly contrasts with the more laborious technique of column partition chromatography. Column *adsorption* chromatography of old is of great value in analytical chemistry. However, in order to decrease the time of analysis a two-dimensional equivalent of this procedure was required. Such a technique is given by the method of thin-layer chromatography (abbreviated as T.L.C.). Izmailov and Schraiber (1938) were the first to describe such a technique, separating alkaloids on layers of aluminium oxide. The review of this paper in Chemical Abstracts states: "A drop-chromatographic method is proposed which permits observing the distribution of the substance in different zones in a drop of the substance placed on a flat layer of an adsorbent (CaO, MgO, Al_2O_3 etc.) 2 mm thick on an object glass..... The ultra-chromatogram is developed with several drops of the solvent." Williams (1947) has applied a layer of adsorbent sticking between two glass plates with a small hole in the upper one to enable the introduction of sample and solvent. The admixture of a binding agent to the adsorbent was introduced by Meinhard *et al.* (1949).

This technique was applied successfully to the analysis of terpenes, essential oils etc. by Kirchner *et al.* (1951) and by Reitsema (1954). They have employed respectively "chromatostrips" and "chromatoplates", coated with an adsorbent layer containing starch or plaster of Paris as binding agent. Remarkably, at first this technique has not been applied to other materials than essential oils and pharmaceuticals. Thin-layer chromatography (German: Dünnschichtchromatographie) has only gained general recognition through the work of Stahl. He established a considerable improvement in the experimental part of the technique, designing a suitable equipment and standardizing the adsorbent, which later on were manufactured commercially. The standardized technique was adopted by nearly all investigators working in this field. T.L.C. was then applied to the analysis of several groups of substances, especially lipophilic compounds such as glycerides, tocopherols etc. The chromatoplates can be eluted with a variety of solvents and solvent mixtures, mainly of apolar nature.

In the group of the glycerides a fractionation into the various sub-groups viz. mono-, di-, and triglycerides has been accomplished. Hitherto however the separation of the various types of triglycerides could not have been achieved with the normal procedure of T.L.C. Therefore, the technique of reversed-phase thin-layer chromatography (ab-

breviated rev-phase T.L.C.) was introduced especially by KAUFMANN c.s. and MANGOLD (1961). It has been applied to the separation of several closely related compounds belonging to the groups of the fatty acids, fatty alcohols, cholesterol esters, diglycerides, and triglycerides. In extensive studies KAUFMANN c.s. have described the procedure of impregnating layers of silicic acid, diatomaceous earth etc. with stationary phases such as undecane, silicone oil, and paraffin oil. For the greater part principles and results of this technique are analogous to those of reversed-phase paperchromatography. Its advantages are a.o. a tenfold reduction in the time of analysis; the appearance of smaller, less diffuse spots, enabling better separations; and the possibility of using more agressive colouring reagents.

The analogy between the reversed-phase techniques of paperchromatography and T.L.C. appears strikingly from the fact that in T.L.C. the behaviour of the various triglycerides can readily be expressed by their "pcW numbers" (papierchromatographische Wertzahl according to KAUFMANN). These are defined as the number of carbon atoms minus twice the number of double bonds. Using chromatoplates with a petroleum impregnated "kieselgur G" layer, developed in an acetone–acetonitrile (80 : 20) solvent, KAUFMANN AND MAKUS (1961) have accomplished the fractionation of saturated triglycerides, differing by a factor two in their "pcW numbers". In partition techniques the introduction of a double bond in the fatty acid molecule has the same influence on the polarity and migration rate of a compound as a shortening of the carbon chain with two methylene groups. This phenomenon gives rise to the occurrence of critical pairs. The same relationship is found in rev-phase T.L.C. Therefore, two triglycerides one with a palmityl group and the other with an oleyl group usually appear to have nearly the same R_F values. However, some triglycerides, although forming critical pairs and hence inseparable in paperchromatography, have proved to be separable in rev-phase T.L.C. in consequence of their different degree of unsaturation. As an example KAUFMANN AND DAS (1962b) by using a multiple development technique have succeeded in separating a mixture of the "critical" triglycerides: trioleine, palmitodioleine, dipalmito-oleine, and tripalmitine. Applying this technique to the analysis of natural oils and fats, they have accomplished a fractionation of the triglycerides into a number of spots. Obviously, in such complex mixtures each spot may contain more than one triglyceride.

Another method of separating the members of a critical pair consists in performing microchemical reactions which attack the double bonds of the molecule. By bromination and hydrogenation procedures, executed in situ on the chromatoplate, triglycerides with the same pcW numbers but with a different number of double bonds have been separated (KAUFMANN 1962a).

In the preceding chapters various applications of reversed-phase paperchromatography of sterols to the analysis of fat mixtures have been described extensively. However, a more general application of this sterol analysis to the routine procedures of biochemistry and fat chemistry is hampered by the time-consuming character of

paperchromatographic techniques. Therefore, we have applied the more rapid techniques of normal and rev-phase T.L.C. in combination with the above bromination procedure to the analysis of sterols and their acetates.

5.2 Normal thin-layer chromatography of sterols

Although T.L.C. of steroids has been investigated by many authors, only a few data concerning the separation of sterols, related provitamins, and triterpenoid alcohols were available at the start of this investigation in 1961. Janecke *et al.* (1960) have accomplished the separation of cholesterol and vitamin D_3 on silica gel chromatoplates with the solvent mixture hexane–ethyl acetate (90 : 10), but no other separations in the group of sterols were reported by them. Tschesche *et al.* (1960) have described the behaviour of several polar steroids and of cholesterol and β-sitosterol on silica gel chromatoplates, applying e.g. di-isopropylether and benzene as mobile phases. Cholesterol and β-sitosterol and their acetates appeared to have the same R_F values. Migration rates of several triterpenoids and triterpenic carboxylic acids in solvents such as benzene, di-isopropylether, mono-chlorobenzene–acetic acid (90 : 10) were studied (Tschesche 1961). In benzene lanosterol and β-amyrin have quite different R_F values. Nowadays a variety of adsorbents suitable for T.L.C. is commercially available. We have investigated the migration rates of a group of sterols and related compounds on chromatoplates prepared with some of these adsorbents.

Chromatoplates were prepared with 15 g of silica gel G, Merck (containing about 13% of plaster of Paris) and 30 ml of water and were dried during 20–30 min at 120°C. The layers are approximately 0.4 mm thick. Because of our good experiences with the Matthias procedure, we have employed this technique also in T.L.C. Placing an appropiate templet on the chromatoplate, hexagonal holes are made in the silica layer by scrubbing with a hard brush. By this equivalent of the Matthias technique a chromatoplate accomodated with four "chromatostrips" was obtained. An amount of 3–5 μg of sterols is spotted on the 8 mm wide "bridges". Several solvent mixtures were tested as mobile phases.

Good results were achieved with the mixtures hexane–ethyl acetate (80 : 20) and mono-chlorobenzene–acetic acid (90 : 10) (cf. table 22, column 1 and 2). Time of development is approximately 3 hrs. In the group of $\Delta 5$ mono-unsaturated sterols like cholesterol and β-sitosterol no separation could be accomplished. With the former mixture conjugated sterols such as ergosterol and 7-dehydrocholesterol are subject to strong adsorption by the silica gel and are thus retarded slightly as compared with cholesterol. In this way a synthetic mixture of cholesterol–ergosterol (1 : 1) is separated. Cholesterol and vitamin D_2, however, have nearly the same R_S values. The migration rates of the triterpenoid alcohols: lanosterol, dihydrolanosterol, and agnosterol are considerably higher than those of cholesterol (R_S lanosterol = 1.36). A

TABLE 22 R_S values (s = cholesterol) of some sterols and related compounds in T.L.C.

System / Compound	Silica gel G			Kieselgur G	Aluminium oxide G, (FLUKA)	Aluminium oxide, (WOELM)
	hexane–ethyl acetate (80 : 20)	mono-chlorobenzene–acetic acid (90 : 10)	cyclohexane–ethyl acetate (80 : 20)	cyclohexane–ethyl acetate (99.5 : 0.5)	hexane–ethyl acetate (80 : 20)	iso-octane–ethyl acetate (80 : 20)
	1	2	3	4	5	6
cholesterol	≡1.0	≡1.0	≡1.0	≡1.0	≡1.0	≡1.0
Δ7-cholestenol	0.94	0.97	0.93	—	—	0.95
β-sitosterol	1.00	1.01	1.00	1.00	0.97	1.00
stigmasterol	1.00	1.01	1.00	1.00	1.00	1.00
dihydrocholesterol	1.01	1.00	0.91	0.96	1.00	0.91
zymosterol	0.93	1.00	0.92	1.02	0.95	0.91
ergosterol	0.91	0.99	0.93	0.89	0.94	0.89
7-dehydrocholesterol	0.93	0.93	1.00	0.93	0.92	0.90
lanosterol	1.36	1.22	1.41	1.37	1.38	1.46
dihydrolanosterol	1.36	1.22	1.41	1.38	1.38	1.48
agnosterol	1.37	1.13	1.32	1.35	1.42	1.45
vitamin D_2	1.02	1.16	1.11	1.11	1.00	1.00
epi-cholesterol	1.18	1.21	1.23	1.23	1.45	1.41
pyrocalciferol	1.28	1.13	1.30	1.35	1.38	1.61
isopyrocalciferol	0.91	0.99	0.98	0.92	0.93	0.96

mixture of cholesterol–epi-cholesterol can be separated easily. In the solvent mixture mono-chlorobenzene–acetic acid (90 : 10) (table 22, column 2) the R_F values of cholesterol and ergosterol are about equal. The migration rate of vitamin D_2, however, is as high as that of the triterpenoid alcohols, thus enabling a separation of cholesterol and vitamin D_2. The mixture cyclohexane–ethyl acetate (80 : 20) (see column 3) allows nearly the same fractionation to be made.

We were able to demonstrate that excellent separations in the group of sterols were also achievable on layers prepared from the standardized kieselgur G (MERCK). The chromatoplates are prepared from 15 g of this kieselgur G and 30 ml of water using a drying period of 25 min at 120°C. Because of the small capacity of these thin kieselgur layers amounts of only 0.4–0.8 μg of sterols are spotted. Using the solvent mixture cyclohexane–ethyl acetate (99.5 : 0.5) a synthetic mixture of ergosterol–cholesterol–vitamin D_2 is separated into three distinct bands with R_S values of respectively 0.89, 1.00, and 1.11 (cf. table 22, column 4 and COPIUS PEEREBOOM 1962). The pairs cholesterol–lanosterol and cholesterol–epi-cholesterol are also separable.

The processing of chromatoplates coated with aluminium oxide encounters more difficulties than that of silica gel or kieselgur plates. We have tried samples of aluminium oxide G from MERCK, aluminium oxide G, and aluminium oxide with "Leuchtpigment" from FLUKA etc. Chromatoplates of reasonable quality were prepared from aluminium oxide FLUKA (15 g with 41 ml of water, heated during 30 min at 120°C). In the solvent mixture hexane–ethyl acetate (80 : 20) the R_F values of cholesterol and epi-cholesterol show a considerable difference. Even better results were accomplished with the alkaline type of aluminium oxide, containing no binding agent, which is manufactured by WOELM (table 22, column 6). An amount of 30 g of this aluminium oxide is mixed with 35 ml of water. The chromatoplate is dried during 16 hrs at room temperature and then heated during 30 min at 130°C. The R_S values of the tested sterol samples in the solvent mixture iso-octane–ethyl acetate (80 : 20) were mainly similar to those of column 5. The isomers pyrocalciferol and isopyrocalciferol were separated in all systems, the greatest difference in R_S values being observed with layers of aluminium oxide. With aluminium oxide a greater difference in migration rates of cholesterol and triterpenoid alcohols like lanosterol is found than with layers of the other adsorbents.

In most systems the R_S values of cholesterol and Δ7-cholestenol show some difference. The best separation of both sterols was obtained on kieselgur G plates with the solvent mixture: cyclohexane–ethyl acetate (99.9 : 0.1).

Reviewing these results we have to conclude that sterols, either differing in the shape of their carbon skeleton or in the types of conjugated systems, can be separated by T.L.C. However, no fractionation in the group of Δ5 mono-unsaturated and Δ5,22 di-unsaturated sterols could be accomplished.

5.3 Reversed-phase thin-layer chromatography of sterols and sterol acetates

Using the above-mentioned elaborate techniques of Kaufmann c.s., we have studied the rev-phase T.L.C. of a group of sterols and related compounds, and of their respective acetates. Chromatoplates are impregnated with a stationary phase, preferably with undecane (bp 190–220°C)[5], by dipping them into a 10–15% solution of this hydrocarbon in petroleum ether. The solvent is evaporated in the air. Because of the slow but marked volatilization of the stationary phase in the case of undecane, the degree of impregnation decreases proportionally to the drying period. Therefore, the prescribed period of drying should be strictly adhered to. After impregnation hexagonal holes are made in the layer with a brush, as has been described above. After drying the chromatoplate during 40–80 min, the solution of sterols or sterol acetates is spotted on the "bridges" and finally the chromatogram is developed with the mobile phase.

Preliminary experiments stressed the importance of complete mutual saturation of the mobile and stationary phase. The temperature in the chromatographic vessel during development should be fairly constant, preferably 22–24°C. A complete saturation of the vessel with vapours of the mobile phase is necessary. This is accomplished by lining the inside of the jar with filter paper. If these conditions have not been fulfilled, the correct state of equilibrium cannot be attained. Several causes of non-equilibrium are conceivably involved a.o. lowering of the temperature resulting in more restricted miscibility and solution of the main component of the mobile phase in the stationary phase to redress a possible unsaturation.

In the procedure for separating various saturated or unsaturated triglycerides Kaufmann and Makus (1961) prescribed a "partial saturation" of the mobile phase with the stationary phase by mixing the saturated mobile phase with amounts of e.g. 20% of unsaturated mobile phase. However, when analysing sterols or their acetates we found complete saturation of the mobile phase essential. Developing undecane impregnated layers with a mobile phase such as acetone–acetonitrile (70 : 30), to which an amount of 30% of unsaturated mobile phase was added, a secondary front line 6 cm above the starting points appeared. This distance was decreased to 4 respectively 2 cm, when decreasing amounts viz. 20 and 10% of the unsaturated mobile phase were added. Under saturation conditions the secondary front line disappeared completely. On account of the experiences of Kaufmann *et al.* (1961, 1962a, 1962b) and of our own experiments we have selected kieselgur G (Merck) as the most appropiate inert adsorbent. A variety of stationary phases such as undecane, tetradecane, paraffin oil, silicone oil, decalin etc. and of mobile phases such as acetone, acetonitrile, acetic acid, mono-chloroacetic acid, propionic acid, water mixtures, were tested as to the separation of sterols and their acetates.

[5] Available from J. Haltermann, Hamburg, Germany.

5.3.1 Separation of sterol acetates

The best results in separating a group of sterol acetates were accomplished with undecane as stationary and the solvent mixture acetic acid–water (92 : 8) (system A) respectively acetic acid–acetonitrile (25 : 75) (system B) as mobile phases. In the former system (A) the chromatoplate after having been coated with a 0.2 mm thick kieselgur G layer is dipped during 10 seconds in a 10% solution of undecane in petroleum ether (bp 40–60°C) (cf. COPIUS PEEREBOOM 1962). After a drying period of 60 min, at the end of which an amount of 4–6 μg of sterol acetates is spotted, the chromatoplate is developed in a chromatographic vessel of 19 × 7 × 30 cm with the acetic acid–water (92 : 8) mixture. The degree of impregnation is determined by collecting the kieselgur G – undecane layer from the chromatoplate and extracting the undecane with petroleum ether. From the difference in weight before and after extraction the degree of impregnation is calculated. After a drying period of 60 min on an average an amount of 0.30 g of undecane is left on the chromatoplate (viz. 0.09 g undecane/g kieselgur). The development lasts about 5 hrs. The chromatoplate is then dried during 2–3 hrs in the air. The undecane is evaporated nearly completely by subsequent heating during 45 min at 100°C in a drying oven having forced convection by means of a fan. After cooling the chromatoplate is sprayed with a 20% ethanolic solution of phosphomolybdic acid and heated during 5–10 min at 90–100°C. The sterol acetates then are visualized as blue-green bands.

The mobile phase in system B viz. acetic acid–acetonitrile (25 : 75) considerably reduces the time of development. Because of the low R_F values obtained with this solvent mixture, a smaller degree of impregnation had to be applied. The drying period therefore was increased to 80 min. An amount of only 0.19 g of undecane then remains on the chromatoplate (viz. 0.04 g/g kieselgur). After this drying period the chromatoplate is developed during 1½–2 hrs with the acetic acid–acetonitrile (25 : 75) mixture.

Observing the above precautions distinct chromatograms were obtained, which show small semi-circular bands of approximately 2–4 mm width. Under non-ideal conditions the bands at the sides of the chromatoplate sometimes may be deformed. To obtain straight solvent fronts the adsorbent should be always scraped off the edges of the chromatoplate before chromatography.

Although the entire experimental procedure and especially the drying period was standardized closely, the reproducibility of the degree of impregnation is less than can be achieved in reversed-phase paperchromatography. Consequently, there is a greater variation in R_F values. The relative R_S values (s = cholesterol) in the systems A and B appeared to be fairly reproducible and are given in table 23. Analogous results were obtained in systems like tetradecane (bp 240–250°C)/acetic acid–water (70 : 30) and undecane/acetic acid–mono-chloroacetic acid–water (65 : 28 : 7). On account of the R_S values of the acetates of β-sitosterol, stigmasterol, and cholesterol we would expect a separation of the sterols present in mixtures of vegetable and animal fats. Actually,

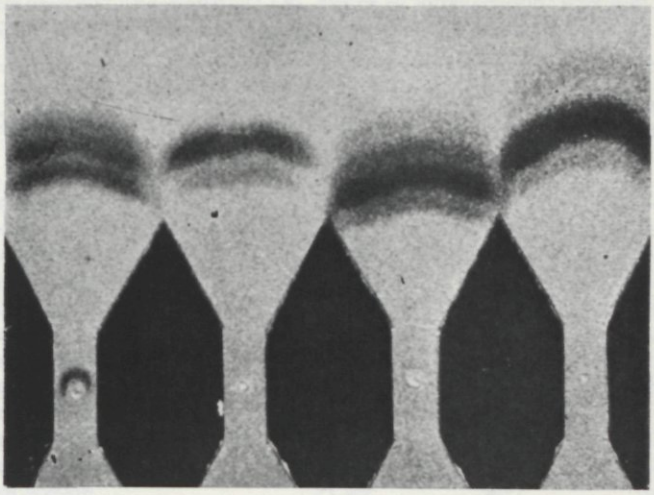


FIG. 32. Separation of sterol acetates by reversed-phase T.L.C. Adsorbent: kieselgur G (MERCK). System B: undecane/acetic acid–acetonitrile (25 : 75); time of run: 1½ h.

spot 1. 20 μg of a mixture containing the acetates of cholesterol, stigmasterol, and β-sitosterol.
spot 2. 80 μg of a mixture of acetates of cholesterol–dihydrocholesterol (8 : 2).
spot 3. 20 μg of the sterol acetates of hardened peanut oil.
spot 4. 80 μg of sterol acetates of hardened whale oil.

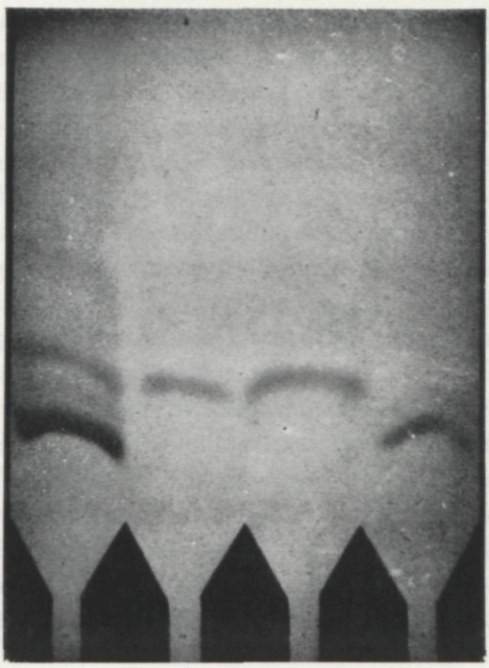


FIG. 34. Separation of sterol acetates using bromination on the chromatoplate. Adsorbent: kieselgur G; system: undecane/acetic acid–acetonitrile (25 : 75) + 0.5% bromine; time of run: 2 hrs; detection: heating; 50% antimony(III)chloride.

spot 1. 30 μg of coconut phytosterol acetates.
spot 2. cholesterol acetate.
spot 3. stigmasterol acetate.
spot 4. β-sitosterol acetate (impure preparation).

TABLE 23 R_S values of some sterol acetates in reversed-phase T.L.C.
Systems: (A) undecane/acetic acid–water (92 : 8), degree of impregnation: 0.09 g/g; (B) undecane/acetic acid–acetonitrile (25 : 75), degree of impregnation: 0.04 g/g kieselgur.

Compound	Abbr. formula	R_S values in system A	R_S values in system B
cholesterol acetate	FC_{27}	≡ 1.0	≡ 1.0
β-sitosterol acetate	FC_{29}	0.83	0.80
stigmasterol acetate	$FC_{29}F$	0.91	0.91
brassicasterol acetate	$FC_{28}F$	1.00	1.06
dihydrocholesterol acetate	C_{27}	0.89	0.84
ergosterol acetate	$2FC_{28}F$	1.19	1.35
7-dehydrocholesterol acetate	$2FC_{27}$	1.16	1.28
Δ7-cholestenol acetate	FC_{27}	—	0.99
3-dehydrocholesterol acetate (Δ3,5-cholestadiene-3β-ol acetate)	$2FC_{27}$	—	1.22
Δ3-cholestenol acetate	FC_{27}	—	1.00
lanosterol acetate	$FC_{30}F$	0.97	1.00
agnosterol acetate	$2FC_{30}F$	0.86	0.86
epi-cholesterol acetate	FC_{27}	1.16	1.19

amounts of 10 μg of the sterol acetates originated from such fat mixtures, are fractionated into three distinct small bands. The separations are for the greater part comparable to those obtained by reversed-phase paperchromatography (fig. 32, spot 1).

The R_F value of brassicasterol acetate is slightly higher than that of cholesterol acetate. Unfortunately, this difference is too small to enable a clear separation between both sterols to be made. All the applications of sterol analysis, which were discussed in the preceding chapters, can be also accomplished with rev-phase T.L.C. of the sterol acetates. Because of the considerable time reduction by this procedure, rev-phase T.L.C. is preferable in routine work of sterol analysis. Adventitiously, in this routine sterol analysis we usually have to prepare the sterol acetates. After two additional crystallizations these acetates are used for a melting point determination (phytosterol acetate test). Part of the portion of crude non-recrystallized sterol acetates is used directly for chromatographic analysis. Because of the very rapid development with the acetic acid–acetonitrile (25 : 75) solvent, *we prefer this system (viz. B) in routine sterol analysis.*

Although on the whole the data of table 23 correspond with the R_S values obtained in the paperchromatographic system studied – see table 14, chapter 3 – some peculiar differences are evident. The R_S value of the acetate of the triterpenoid alcohol lanosterol in rev-phase T.L.C. is nearly equal to that of cholesterol acetate, whereas the R_S value of this compound in paperchromatography only amounts to 0.68. The migration rate of agnosterol acetate in rev-phase T.L.C. is likewise higher than that in reversed-phase paperchromatography. Another striking difference is shown by the

migration rate of epi-cholesterol acetate. The R_S values of this compound in the systems A and B are higher than that of cholesterol acetate. On the other hand the R_S value in reversed-phase paperchromatography amounts to only 0.79. Since no keto-enol tautomerism can occur in Δ3-cholestenol *acetate*, the R_S value of this compound is equal to that of cholesterol acetate as contrasted with the behaviour of the "free Δ3-cholestenol" in paperchromatography.

Especially the undecane/acetic acid–acetonitrile (25 : 75) system enables a clear *separation of the acetates of cholesterol and dihydrocholesterol.* The advantage over the normal paperchromatographic technique consists in the detection procedure with phosphomolybdic acid, which for dihydrocholesterol is far more sensitive on chromatoplates (circa 5 μg) than on paper strips (circa 100 μg). By spotting 50–80 μg of sterol acetates we were able to detect even small amounts of dihydrocholesterol acetate (down to 5%) in an excess of cholesterol acetate (fig. 32, spot 2).

5.3.2 Separation of sterols

Using the same techniques we have attempted to separate the free sterols in stead of their acetates. Analysing the sterols in system A viz. undecane/acetic acid–water (92 : 8) too high R_F values were obtained, resulting in a bad separation of cholesterol–soybean phytosterols. By increasing the degree of impregnation to about 0.13 g undecane/g of kieselgur and by raising the water content of the mobile phase to 10%, the R_F value of cholesterol was decreased sufficiently. The drying period was again fixed at 60 min. Amounts of 2–5 μg of sterols are spotted on the chromatoplate. The R_S values of some sterols in this system C viz. undecane/acetic acid–water (90 : 10) are given in table 24. In addition to these acetic acid–water systems we have tested some acetic acid–acetonitrile solvent mixtures. Using the more rapid system B viz. undecane/acetic acid–acetonitrile (25 : 75) the migration rates of cholesterol, β-sitosterol etc. appeared to be very high. We therefore have modified this system by adding 10% of water to the acetic acid part of the mobile phase. Furthermore, the degree of impregnation was increased considerably by using a 12% solution of undecane in petroleum ether, to which an extra amount of 3% of tetradecane had been added (undecane–tetradecane–petroleum ether = 12 : 3 : 85). Finally the amount of stationary phase remaining on the chromatoplate was increased by a reduction of the drying period to only 30 min. The degree of impregnation then amounts to about 0.25 g/g of kieselgur. After having changed the experimental conditions in this way, the R_F value of cholesterol was decreased to 0.45, while simultaneously a much sharper fractionation of cholesterol and the soybean oil phytosterols was accomplished. The time of development required with this mobile phase was only about 2 hrs.

The R_S values of a great number of sterols, provitamins, and related compounds in this system D viz. undecane–tetradecane (80 : 20)/acetic acid–water–acetonitrile (22.5 : 2.5 : 75) are given in table 24. On the whole the sequence of these R_S values is similar to that in the reversed-phase paperchromatographic system. Sterol mixtures

TABLE 24 R_S values of some sterols and related compounds in reversed-phase T.L.C. Systems: (C) undecane/acetic acid–water (90 : 10), degree of impregnation: 0.13 g/g; (D) undecane–tetradecane (80 : 20)/acetic acid–water–acetonitrile (22.5 : 2.5 : 75), degree of impregnation: 0.25 g/g of kieselgur.

Compound	Abbr. formula	R_S values in system C	R_S values in system D*
cholesterol	FC_{27}	≡ 1.0	≡ 1.0
β-sitosterol	FC_{29}	0.86	0.81
stigmasterol	$FC_{29}F$	0.93	0.91
brassicasterol	$FC_{28}F$	1.02	1.05
dihydrocholesterol	C_{27}	0.90	0.86
coprostanol	C_{27}	—	0.33
ergosterol	$2FC_{28}F$	1.16	1.24
7-dehydrocholesterol	$2FC_{27}$	1.12	1.20
Δ7-cholestenol	FC_{27}	—	1.02
desmosterol	$FC_{27}F$	—	1.24
3-dehydrocholesterol (Δ3,5-cholestadiene-3β-ol)	$2FC_{27}$	—	0.99
"Δ3-cholestenol"	FC_{27}	—	0.63
α-spinasterol	$FC_{29}F$	—	0.95
cholestanone-3		—	0.61
epi-cholesterol	FC_{27}	0.90	0.80
lanosterol	$FC_{30}F$	0.84	0.80
24-dihydrolanosterol	FC_{30}	0.70	0.57
agnosterol	$2FC_{30}F$	0.76	0.68
24-dihydroagnosterol	$2FC_{30}$	0.75	0.67
zymosterol	$FC_{27}F$	—	1.25
vitamin D_2	$3FC_{28}F$	—	1.22
vitamin D_3	$3FC_{27}$	—	1.18
dihydrovitamin D_2	$3FC_{28}$	—	1.01
pyrocalciferol	$2FC_{28}F$	—	1.07
isopyrocalciferol	$2FC_{28}F$		1.28
lumisterol	$2FC_{28}F$	—	1.17
epi-lumisterol	$2FC_{28}F$	—	1.25
22-dihydroergosterol	$2FC_{28}$	—	1.11
5-dihydroergosterol	$FC_{28}F$	—	1.09
Δ4,6,8(14)-cholestatriene		—	1.28
cholestane		—	0.85
7-hydroxycholesterol		—	2.03

* R_P value of cholesterol approx. 0.45.

e.g. cholesterol–desmosterol; cholesterol–7-dehydrocholesterol; cholesterol–dihydrocholesterol are clearly separated, quite similar to their behaviour in paperchromatography and in rev-phase T.L.C. system B. The R_S values of lanosterol and epi-cholesterol in system D are considerably lower than that of cholesterol, as contrasted with the R_S values of their acetates in the system A and B. Using the terminology of BUSH (1960), discussed in paragraph 3.1.2, for these compounds the ΔR_{Mr} values caused by the conversion sterol to sterol acetate are quite different from that of the normal sterol types. A remarkable difference is also shown by 3-dehydrocholesterol, which

in system D has an R_S value of 0.99, whereas in system B and in the paperchromatographic system studied the R_S values approach that of 7-dehydrocholesterol.

The polycyclic alcohols of a commercial mixture of wool-fat alcohols are fractionated into a cholesterol, a lanosterol, and a dihydrolanosterol band, quite similar to their behaviour in reversed-phase paperchromatography (see fig. 33).

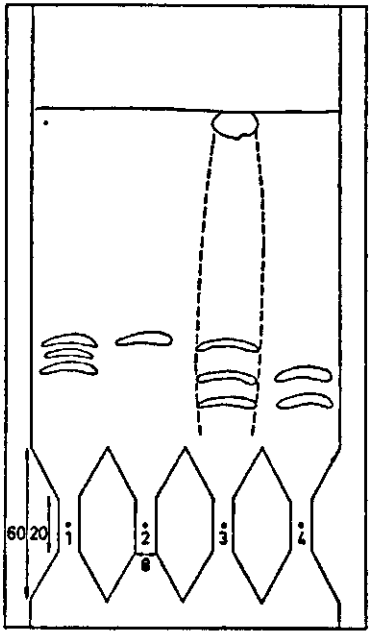


FIG. 33. Separation of sterols by reversed-phase T.L.C. System (D): undecane–tetradecane (80 : 20)/ acetic acid–water–acetonitrile (22.5 : 2.5 : 75).

spot 1. 20 μg of a cholesterol, stigmasterol, β-sitosterol mixture.
spot 2. 5 μg of 3-dehydrocholesterol.
spot 3. 150 μg of commercial wool-fat alcohols.
spot 4. 10 μg of a lanosterol–dihydrolanosterol mixture.

5.4 SOME APPLICATIONS

R_M values are calculated approximatively from R_S values of the sterols, using an average R_F value of cholesterol that has been determined in numerous experiments. When plotting these uncorrected R_M values of the sterols in system D versus the N_C values, the following correlation is found:

$$R_M = 0.086\ (N_C - 24) - 0.21$$

According to the system of chapter 3, some ΔR_M values have been calculated.

$\Delta R_M^{CH_3} = +0.09$
$\Delta R_M^{C=C} = -0.13$
$\Delta R_M^{C=C \rightarrow C=C-C=C-C=C} = +0.04$
$\Delta R_M^{\Delta 5 \rightarrow 7\ etc.} = -0.03$
$\Delta R_M^{3\beta\text{-OH}} = -0.01$
$\Delta R_M^{3\text{-one}} = +0.21$

TABLE 25 Colour reactions of some sterols in reversed-phase T.L.C.

Compound	Bismuth(III) chloride 33% in 96% ethanol Heat: 10–15 min at 90°C	*p*-Anis-aldehyde 1 g in 100 ml acetic acid + 2 ml sulfuric acid Heat: 10 min at 90°C	Thymol 20% in 96% ethanol Heat: 10 min at 80°C. Then 1N sulfuric acid Heat: 10 min at 90°C	Urea 50% in water Fluorescent bands under U.V. radiation of 365 nm	Antimony(III) chloride Saturated solution in chloroform Heat: 10 min at 90°C Then 1N sulfuric acid Heat: 10 min at 90°C	Salicyl-aldehyde Spray with the pure liquid Heat: 5 min at 80°C Then 1N sulfuric acid Heat: 10 min at 90°C	β-Naphtol 0.2% in 4N sulfuric acid Heat: 10 min at 90°C	Chloro-sulfonic acid 30% in acetic acid Heat: 10–15 min at 90°C	Acetic anhydride 30% in 50% sulfuric acid Heat: 10 min at 90°C	Resorcinol 20% in 96% ethanol Heat: 5 min at 80°C Then 1N sulfuric acid Heat 10 min at 90°C
cholesterol acetate	strong violet	strong blue-purple	strong violet	neg.	strong violet	strong violet	strong blue	strong violet	strong violet	faint blue
stigmasterol acetate	strong violet	strong blue-purple	strong violet	neg.	strong violet	strong violet	strong violet	strong violet	strong violet	faint blue
β-sitosterol acetate	strong violet	strong blue-purple	strong violet	neg.	strong violet	strong violet	strong purple	strong violet	strong violet	faint blue
third-band phytosterol acetates of coconut fat	grey-blue	strong blue-purple	faint purple-blue	neg.	strong purple	purple		purple-blue	purple-blue	faint blue
dihydrocholesterol acetate	neg.	neg.	white	neg.	white	white	white	white	white	neg.
ergosterol acetate	grey	grey-green	grey-brown	pos.	grey-blue	grey-blue	grey-blue	grey-brown	grey-green	faint green
7-dehydrocholesterol acetate	grey	grey-green	grey-brown	pos.	grey-blue	grey-blue	grey-blue	grey-brown	grey-green	purple
lanosterol acetate	yellow-brown	blue-purple	orange-brown	neg.	faint orange-brown	orange-brown	blue-purple	orange-brown	orange-purple	brown
Δ7-cholestenol	yellow-brown	violet	blue	neg.	grey-brown	purple	blue	grey	purple-blue	white
Δ7-ergostenol acetate	yellow-brown	violet	yellow-green	neg.	grey-brown	yellow-green	blue	yellow-green	yellow-brown	brown
5-dihydroergosterol acetate	yellow-brown	violet	grey-green	neg.	faint orange-brown	orange-brown	blue-purple	grey-brown	purple-brown	purple-brown
vitamin D_2	yellow-brown		purple-brown	weakly pos.	grey	grey-brown	grey	grey	grey-brown	orange-brown

In system D mixtures of cholesterol and soybean phytosterols are fractionated into three bands just like in reversed-phase paperchromatography. However, because of the somewhat clearer separations obtained in system B, and since in routine sterol analysis the acetates are always available, we prefer to use system B.

When analysing the sterol acetate mixtures of hardened whale oil and fish oils in this system (B), in addition to the cholesterol acetate band, two other distinct bands are shown in the same section of the chromatoplate (fig. 32, spot 4). The band under that of cholesterol acetate with an R_S value of 0.85 should be attributed to dihydrocholesterol acetate. The sterols isolated from other animal fats such as lard, tallow, butter fat etc. show only one band on the plate viz. that of cholesterol acetate. Similarly, phytosterol acetate mixtures isolated from hardened vegetable fats, analysed in system B, show a small band under that of β-sitosterol acetate. Most probably this band ($R_S = 0.75$) should be attributed to the presence of hydrogenated sterol acetates viz. β-, and γ-sitostanol acetate (see fig. 32, spot 3).

The detection of sterols on a chromatoplate can be accomplished with numerous colour reactions and we have tested a great variety of them. Some of these are suitable as non-specific reagents, all sterols then giving the same shade of colour a.o. protoporphyrin, silicotungstic acid, phosphotungstic acid, and the most sensitive one viz. phosphomolybdic acid. When spraying with some special reagents, sterols with different structures such as $\Delta 5$ and $\Delta 7$ sterols exhibit a great variety of hues. A set of colour reactions as comprehensive as possible, thus may be of use in structural analysis of unknown sterols or related compounds. Reagents that produce slightly differing colours with the several sterol types are a.o. cadmium chloride, zinc chloride, p. dimethylamino benzaldehyde–sulfuric acid, trichloroacetic acid, phenol–ammonium molybdate–perchloric acid, furfural–sulfuric acid. The most appropriate colour reactions, however, are listed in table 25 viz. bismuth(III)chloride, p-anisaldehyde, thymol, urea, antimony(III)chloride, salicylaldehyde, β-naphtol, chlorosulfonic acid, acetic anhydride, and resorcinol. Some of these colour reactions have not been described before, but were found to be very suitable for determining the structure of unknown sterols (see chapter 6).

With rev-phase T.L.C. of sterol acetates small amounts of animal fat in vegetable oils can be detected. However, the colour reagent phosphomolybdic acid has the disadvantage of visualizing even traces of third-band phytosterols, which are not perceptable in paperchromatography. Therefore, in routine sterol analysis preferably another colour reaction viz. that with bismuth(III)chloride is applied (see table 25). This reaction yields a strong violet colour with cholesterol acetate and only a faint blue-grey one with third band-phytosterol acetates from e.g. coconut fat. The colour reaction is carried out by spraying the plate with a 33% solution of bismuth(III) chloride in 96% ethanol. The plate is then heated during 10–15 min at 90°C till the bands are revealed by a faint orange-brown colour. Afterwards the colour of the cholesterol acetate band is intensified and changes in about 10–20 min to a violet hue.

At first the colour of the brassicasterol acetate band is quite similar to that of cholesterol acetate, but while the latter attains a violet colour, the former is *not* intensified and obtains a faint blue-grey hue.

When looking for admixture of animal fat to vegetable fats a violet coloured third band of the sterol acetate mixture by indicating cholesterol acetate definitely proves the presence of animal fat. The detection of animal fat admixture also holds in the presence of vegetable oils containing large percentages of third-band phytosterols such as coconut fat, rapeseed oil etc. This procedure of rev-phase T.L.C. (viz. system B and detection with bismuth(III)chloride) therefore is superior to the paperchromatographic analysis, described in the preceding chapters.

5.5 Separation of sterol acetate bromides

5.5.1 Bromine containing systems

Kaufmann *et al.* (1962b) have separated several critical pairs of fatty acids and of triglycerides by way of their bromo derivatives. By adding 0.5% of bromine to the mobile phase the bromination is carried out *on the chromatoplate* during the development process. We have applied this elegant procedure to the separation of the critical pair cholesterol-"$FC_{28}F$"-sterol. When spotting 30 μg of coconut sterol acetates on a chromatoplate impregnated with undecane (0.043 g/g) and eluting the plate with an acetic acid–acetonitrile (25 : 75) mixture containing 0.5% of bromine, the mixture of sterol acetates is separated into four distinct bands (fig. 34, spot 1). The first band with $R_S = 0.84$ is produced by β-sitosterol acetate (dibromide), the second band with $R_S = 0.95$ possibly by some C_{28}-phytosterol acetates (dibromides). Unfortunately, under these conditions stigmasterol acetate has the same R_F value as cholesterol acetate. Together they constitute another critical pair, forming the third band with $R_S = 1.0$. The fourth band with the highest R_S value viz. 1.44 has to be attributed most probably to the di-unsaturated "$FC_{28}F$"-sterol acetate (fig. 34). Phytosterol mixtures, containing 20–30% of third-band phytosterols according to the paperchromatographic analysis, all show this fourth "$FC_{28}F$"-sterol band in the bromine system. Furthermore, after separation in system B several bands of coconut third-band phytosterol acetates were scraped off the chromatoplate, extracted and then spotted on another plate, which was developed in the bromine system. The appearance of a major band with $R_S = 1.44$ ("fourth band") and only a minor with $R_S = 1.00$ on that chromatoplate evidences the identity of the sterols of this "fourth" band with the third-band phytosterols produced in system B and in paperchromatography. When comparing the behaviour of the third-band phytosterols and of stigmasterol in system B with that in the bromine system, the migration rates of these sterols in the latter system appear highly increased. On the other hand the migration rates of the monounsaturated sterols in both systems are almost equal. These phenomena suggest an

analogy in the unsaturation of third-band phytosterols and stigmasterol. This strongly affirmed the hypothetical di-unsaturated methyl-cholestadienol ($FC_{28}F$) structure of the third-band phytosterols of coconut fat, as suggested in chapter 4.

The bromination of a sterol acetate on the chromatoplate during the development process is carried out quantitatively. This statement is based on the following conclusive experiments. In the bromine system pure preparations of the various sterol acetates never produce more than one band on the chromatoplate. When analysing pure preparations of cholesterol acetate- and soybean phytosterol acetate dibromides in the bromine system and spraying with antimony(III)chloride, similar bands appear as those given by cholesterol- and soybean phytosterol acetate. Another affirmation of the nature of the bands shown in the bromine system is given by a specific reaction with cadmium chloride, which definitely established that the blue bands produced by antimony(III)chloride in fact are caused by the sterol acetate dibromides. The cadmium chloride reaction is quite specific for sterol *bromides*. It is carried out by spraying a spot of cholesterol acetate dibromide with a 50% solution of cadmium chloride ($2\frac{1}{2}$ H_2O) in 50% ethanol, heating at 90°C during 15 min and viewing the resulting bright fluorescence under U.V. radiation (365 nm). Cholesterol acetate as such does not produce this colour reaction. Amounts of 200 μg of the acetates and the acetate dibromides both of soybean phytosterol and of cholesterol were spotted on a chromatoplate. After development in the bromine system and spraying with the above cadmium chloride reagent, pairwise identical fluorescent bands were produced. These experiments demonstrate that in the mobile phase containing bromine the sterol acetates during development are brominated quantitatively yielding exclusively their *dibromo* compounds.

In the normal reversed-phase systems of paperchromatography and T.L.C. the pairs: cholesterol (FC_{27})–brassicasterol ($FC_{28}F$), and C_{28}-phytosterols (FC_{28})–stigmasterol ($FC_{29}F$) have equal R_F values. In the bromine system, however, brassicasterol acetate exhibits quite a higher migration rate than cholesterol acetate, thus enabling the separation of this critical pair of sterols. Similarly, the R_F value of stigmasterol acetate is higher than that of C_{28}-phytosterol acetates but unfortunately nearly equal to that of cholesterol acetate (fig. 34). Therefore, a new critical pair of the acetates of cholesterol and stigmasterol is constituted. Mixtures of cholesterol acetate and the soybean sterol acetates were analysed with this procedure, yielding three bands with a heavy third band. However, in this way the presence of cholesterol acetate in such mixtures cannot be detected unambiguously. In this system the sterol mixtures of vegetable oils comprising more than 20% of third-band phytosterols (as determined with paperchromatography, see chapter 4), which are suspected of containing cholesterol, can be investigated more in detail.

Only $\Delta 5$ mono-unsaturated and $\Delta 5$, side chain di-unsaturated, non-conjugated sterol acetates can be analysed in this system. The acetates of all $\Delta 7$-sterols, both mono-unsaturated as well as conjugated, like $\Delta 7$-cholestenol, α-spinasterol, 5-dihydroergo-

sterol, ergosterol, 7-dehydrocholesterol are apparently decomposed and do not produce any detectable band. The same phenomenon holds for aberrant compounds such as $\Delta 3$-cholestenol, zymosterol, lanosterol, agnosterol etc., all having double bonds in the nucleus in other positions than $\Delta 5$. This difference between $\Delta 5$ and $\Delta 7(8)$, $\Delta 8(9)$ etc. sterols can be applied in determining the structure of unknown sterols.

Several other solvent mixtures were tested as mobile phases in such bromine systems, but the acetic acid–acetonitrile (25 : 75) mixture enabled the best separations to be made.

5.5.2 Experimental procedure

Glass plates of 14 × 24 cm were coated with a MERCK's kieselgur G–water (1 : 2) mixture. After heating during ½ hr at 120°C the resulting 0.2 mm layer was impregnated with undecane (bp 190–220°C; of J. HALTERMANN, Hamburg). The chromatoplate is dipped carefully into a 10% solution of undecane in petroleum ether (bp 40–60°C) in a shallow tray. Care must be taken that the layer is not damaged. After impregnation the plate is held 1 min upside down and is then stored during 80 min at room temperature to allow the evaporation of the solvent. An amount of approximately 0.19 g of undecane is left on the plate (viz. 0.04 g/g of kieselgur). During this drying period hexagonal holes are removed from the layer with a brush, using an appropriate templet. The sterol acetate solution (30–40 mm^3 of a 0.1% ethereal solution) is spotted at the centre of the 8 mm wide "bridges". The undecane and the mobile phase have to be mutually saturated by shaking vigorously and leaving overnight. The chromatoplate is developed with the acetic acid–acetonitrile (25 : 75) mixture, to which 0.5% of bromine has been added *within* the chromatographic vessel just before the development. This mobile phase, containing bromine, has to be freshly prepared each time. The chromatographic vessel of 19 × 30 × 7 cm is supplied with filter paper at the sides to ensure complete saturation and is kept at a constant temperature of 20°C. When the solvent front has travelled 20 cm (in about 1½ hr) the development is discontinued and the plate is dried during 2 hrs in the air. Upon heating at 90°C in about 10 min most often blue coloured bands will appear. The band of brassicasterol acetate, however, is coloured only very faintly. The warm plate is then sprayed with 8 N sulfuric acid and finally with a 50% solution of antimony(III)chloride (in acetic acid). The colour of the bands is now intensified, especially that of brassicasterol acetate which attains a violet shade. The bands of the other sterol acetates are coloured bright blue. The colours are not stable but fade away after some hours.

5.5.3 Semi-quantitative evaluations

Generally, it is troublesome to adapt T.L.C. for quantitative determinations. In a rapid and simple but only semi-quantitative procedure the blue coloured bands of the sterol acetate dibromides are outlined with a sharp needle. The areas are determined by placing the chromatoplate upside down on a light screen and redrawing the outlines on transparent paper. The bands on this paper are cut out and weighed. By recalculating these weights to percentages of their total the respective percentages of the sterol acetate bromides on the chromatoplate are evaluated approximatively. However, the linear relationship between spot area and amount of sterol is only valid in case of small quantities (about 2–10 μg). With higher quantities the surface areas do not increase proportionally. Evidently, with the above procedure only semi-quantitative data can be obtained.

In this way the quantities of the bands 1, 2, 3, and 4 of a sample of refined coconut

fat were estimated as 26%, 13%, 31%, and 30%, whereas the corresponding band areas of peanut oil sterol acetates were calculated as 38%, 21%, 21%, and 20%. The percentage areas of the three bands of soybean oil phytosterol acetates in the bromine system were first band 39; second band 29, and third band 32.

Provisionally, we may assume the various bands in rev-phase T.L.C. and in paperchromatography to be produced by the following sterols.

T.L.C., bromine system			Rev-phase paperchromatography		
band	sterol	percentage	band	sterol	percentage
4	FC_{28}F-sterols	p	3	cholesterol/FC_{28}F-sterols	a
3	cholesterol/stigmasterol	q	2	stigmasterol/C_{28}-phytosterols	b
2	C_{28}-phytosterols	r	1	β-sitosterol	c
1	β-sitosterol	s			

The percentage of stigmasterol may be calculated as (b — r)% and that of cholesterol as (a — p)% or (q — b + r)%. When analysing mixtures of e.g. coconut sterol acetate with 10–50% of cholesterol acetate in the bromine system as well as in paperchromatography, we were able to evaluate the percentage of cholesterol acetate in these mixtures. In this way reasonably accurate results were obtained. However, in some cases the percentages of the first band of phytosterol mixtures in the bromine system appeared to be slightly lower than those obtained in paperchromatography ($s < c$). The same effect was observed with the respective "FC_{28}F-sterol" bands ($p < a$ for several pure vegetable oils). This discrepancy troubles the calculations and might indicate that some other unknown sterols are still present in these sterol mixtures.

6 BEHAVIOUR OF SOME ABERRANT PHYTOSTEROL TYPES IN CONNECTION WITH THEIR POSSIBLE STRUCTURE

6.1 Failures of the original phytosterol acetate test of Bömer

In the original phytosterol acetate test of Bömer an end-melting point exceeding 116–117°C, after two recrystallizations, proves the presence of vegetable fat. This effect is caused by the steep rise of the melting point diagram of cholesterol acetate–phytosterol acetate at low percentages of phytosterol acetate. However, we have found deviating diagrams obtained with some special vegetable fats, as was already mentioned in chapter 1. The diagram of the phytosterol acetates prepared from *safflower oil* shows an uniformly sloping curve (cf. fig. 12). Admixture of small to moderate amounts of safflower oil to butter, therefore, does *not* give rise to a sterol acetate melting point exceeding 116°C, even after four additional crystallizations (table 26). A melting point in excess of 116°C is only observed at a quantity of 50% of safflower oil (after *six* additional crystallizations).

In contrast to the failure of Bömer's phytosterol acetate test in these cases, the microscopic examination of sterols according to Den Herder did provide reliable results. In mixtures of cholesterol with 7–50% of phytosterols some swallow-tail crystals were observed, even in the sterol mixtures from butter fat with small amounts of safflower oil. Besides by this microscopic procedure, the presence of phytosterols obviously can be detected by chromatographic analysis.

6.1.1 Special behaviour of pumpkin phytosterols

Admixture of small amounts of pumpkin oil (Kürbiskernöl from Austria) also does not produce a sufficient rise in the sterol acetate melting point. Only when an amount of 40% of pumpkin oil was added and after four additional crystallizations we were able to detect the vegetable oil by the high sterol acetate melting point of 121.6°C (table 26). On the other hand microscopic observation of the sterols of butter fat–pumpkin oil mixtures (9 : 1, 8 : 2 etc.) always did reveal some swallow-tail crystals. The melting point of the pumpkin oil phytosterol acetates is very high viz. about 164°C and increases gradually upon recrystallization from ethanol to about 170°C. A second peculiarity is revealed by paperchromatographic analysis in the studied system (degree of impregnation 0.10 g/g). The paperchromatogram of the pumpkin oil sterol mixture shows a series of four distinct bands with R_S values of 0.85; 0.95; 1.05; and 1.15. The R_S value of the fourth band is indicative for a sterol structure

TABLE 26 Behaviour of some special vegetable oils in the phytosterol acetate test

	Mixtures of butter fat with	Melt. p. in °C after successive recrystallizations						
		0	1	2	3	4	5	6
A	20% of safflower oil	113.2	114.9	114.7	115.2	115.7	—	—
	50% of safflower oil	114.6	115.4	115.0	115.9	115.9	—	116.9
	80% of safflower oil	119.3	119.4	119.8	120.2	120.4	—	—
B	10% of pumpkin oil	105.2	107.2	107.4	107.1	107.4	—	—
	20% of pumpkin oil	102.0	103.5	104.5	104.6	104.7	—	—
	30% of pumpkin oil	99.0	105.2	110.2	110.2	110.4	—	—
	40% of pumpkin oil	99.0	106.5	110.0	114.0	121.6	122.2	—
C	10% of shea nut fat	110.9	112.2	122.6	113.4	113.2	113.5	—
	20% of shea nut fat	108.4	110.2	111.4	112.1	113.0	113.6	113.9
	40% of shea nut fat	103.3	105.6	106.8	109.0	110.5	110.7	112.6
	55% of shea nut fat	110.3	111.2	113.2	116.6	121.1	126.4	—

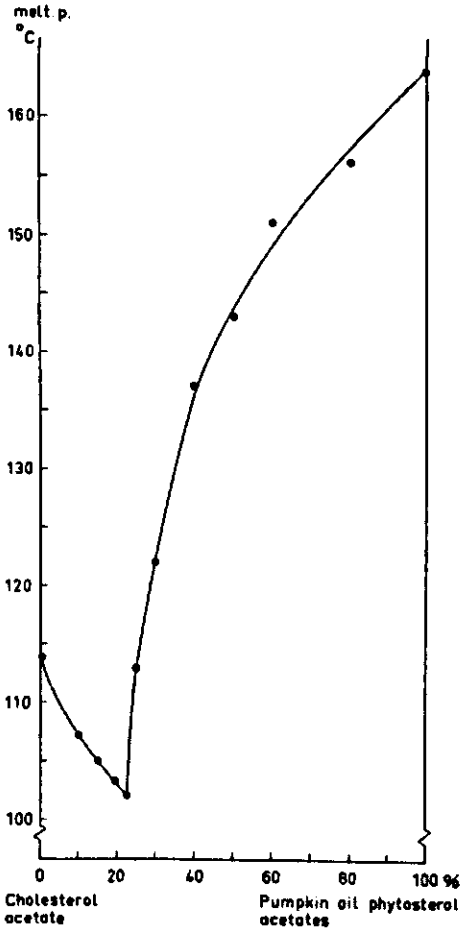


FIG. 35. Melting point diagram of cholesterol acetate with pumpkin oil phytosterol acetates.

TABLE 27 Sterol values of five samples of pumpkin oil

Pumpkin oil sample	Total sterols: percentages of the phytosterols in the chromatographic analysis* band 1	band 2	band 3	band 4	sterol content in %	melt. p.** sterol acetates in °C	Fre: percentages band 1	band 2	band 3
I	25	19	19	37	0.40	155.6	30	20	25
II	33	27	20	20	0.30	162.9	29	23	23
III	29	29	22	20	0.37	162.5	38	25	19
IV	27	27	27	19	0.22	163.4	33	27	20
V	27	33	16	24	0.39	164.8	23	28	25

* Determined by planimetry
** Determined after two recrystallizations

belonging to the $FC_{27}F$ — $2FC_{28}F$ critical pair. The occurrence of such a sterol in an edible vegetable oil in relatively high contents up to 0.09% has not yet been reported.

Therefore, the sterol values of several brands of pumpkin oil, commercially available in Austria, were investigated (table 27). The sterol composition of all these samples varies to some extent; their melting points are between 155.6° and 164.8°C and the quantities of fourth-band phytosterols between 19% and 37%. The exceptional behaviour in the phytosterol acetate test upon admixture of pumpkin oil was elucidated by determining its melting point diagram with cholesterol acetate. Whereas in a normal diagram a steep increase is shown at low percentages of phytosterol acetate, we found this special diagram displaying a distinct *decrease* in melting point in that region; at 22.5% of phytosterol acetate a pronounced *minimum* occurs (fig. 35). At higher percentages the melting point rises continuously. This melting point diagram illustrates the difficulties encountered in detecting admixture of pumpkin oil to butter by means of BÖMER's phytosterol acetate test. Because of the peculiar nature of this diagram, of the high acetate melting point, and of the chromatographic pattern as well, we suppose the structure of pumpkin oil sterols to be different from those of the normal phytosterols.

6.1.2 Phytosterols of shea nut fat and Mowrah fat

BERG *et al.* (1914a, 1914b) and OLIG (1914) did not succeed in determining admixture of shea nut fat and Mowrah fat to butter by the phytosterol acetate test. Both shea nut fat (or karité fat from *Butyrospermum Parkii Kotschy*) and Mowrah fat (or illipé

Free sterols			Bound sterols					
the phytosterols			percentages of the phytosterols					
band 4	sterol content in %	melt. p. sterol acetates in °C	band 1	band 2	band 3	band 4	sterol content in %	melt. p. sterol acetates in °C
25	0.26	159.4	30	25	15	30	0.14	161.4
25	0.21	168.0	33	20	27	20	0.09	157.4
18	0.23	164.4	29	24	24	23	0.14	157.0
20	0.14	158.4	28	28	22	22	0.08	160.8
24	0.25	162.5	29	22	25	24	0.14	158.0

butter from *Madhuca longifolia Macb.*) have high sterol acetate melting points viz. 169° and 173–175°C respectively. Admixture of small to moderate amounts of these fats to butter does not, however, give rise to a value of the sterol acetate melting point exceeding 116°C. In view of the accordance of these data with those of pumpkin oil, we suggested that the sterols from these fats should also have structures different from the normal Δ5-phytosterols. The behaviour upon admixture of 10, 20, 40, and 55% of shea nut fat to butter fat in the phytosterol acetate test was investigated. Just as in the case of pumpkin oil a sterol acetate melting point in excess of 116°C was only observed at considerable amounts of shea nut fat viz. of 55% (and after at least three crystallizations). The phytosterols of shea nut fat were isolated according to the procedure of BERG (1914b), by which the part of the unsaponifiable fraction insoluble in ethanol was removed before precipitating the sterols by digitonin. The melting point of the acetates of this sterol fraction was 146.5°C. Other characteristics are: total-sterol content 0.11%, according to BERG'S procedure; with $\alpha_{D,CHCl_3}$[6] = +20°; total unsaponifiable fraction 4.4% with α_D = +38°; sterol-free unsaponifiable fraction 4.2% with α_D = +36°. In the melting point diagram of these phytosterol acetates with cholesterol acetate a decrease nearly equal to that of pumpkin oil was observed at low percentages of phytosterol acetate. A pronounced minimum value was found at an amount of 30% of phytosterol acetate (fig. 36, curve A).

By the same procedure the phytosterols of Mowrah fat were investigated. The sterol acetate melting point is 157°C; the total-sterol content only 0.04%; total unsaponifi-

[6] All optical rotations are determined in chloroform solution.

able fraction: 1.31%; and the sterol-free unsaponifiable fraction: 1.26%. The shape of the diagram of these Mowrah fat phytosterol acetates with cholesterol acetate closely resembles those of pumpkin oil and shea nut fat and has a minimum at 32.5% of phytosterol acetates (fig. 36, curve B). In paperchromatographic analysis (0.15 g/g) the shea nut fat sterols were separated into two bands having R_S values of 0.79 and 0.91. The ratio of the areas of these bands was 64:36. The chromatogram of Mowrah fat phytosterols showed two bands with R_S values of respectively 0.77 and 0.90. The percentages of these phytosterol types were 55 and 45.

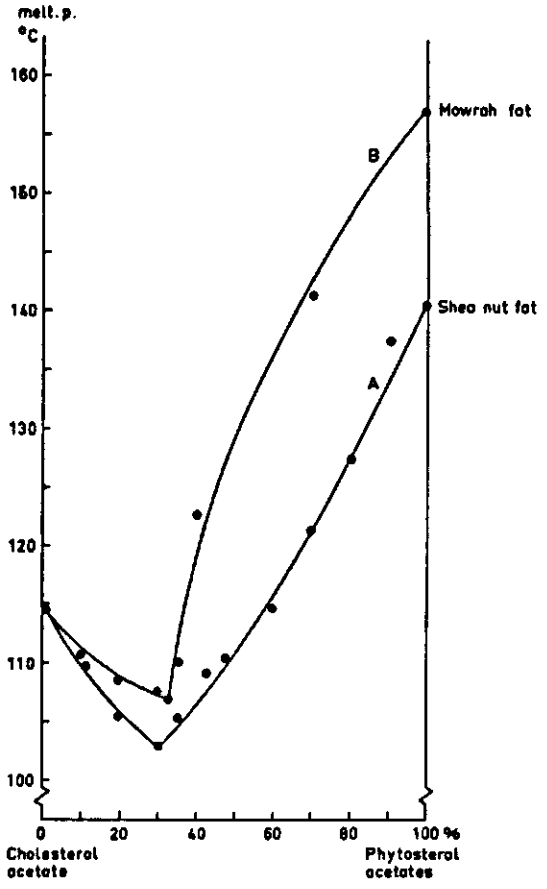


FIG. 36. Melting point diagram of cholesterol acetate with the phytosterol acetates prepared from shea nut fat (A) and Mowrah fat (B).

Furthermore, these phytosterol mixtures were analysed in all four systems of rev-phase T.L.C., described in chapter 5. In system B (undecane/acetic acid–acetonitrile (25 : 75)) the shea nut phytosterol acetates are fractionated into two bands with R_S values of 0.85 (60% of the total mixture) and 0.94 (40%). The Mowrah phytosterol acetates are fractionated into three bands with R_S values and percentages of 0.85(30);

0.96 (60); 1.05 (10) (see table 28). Both phytosterol acetate mixtures thus contain a compound with an R_S value of about 0.95.

TABLE 28 Separation of high-melting phytosterol acetate mixtures in rev-phase T.L.C.

I Rev-phase T.L.C., system B	Shea nut fat		Mowrah fat		Cucumber seed oil		Pumpkin seed oil (sample no V)	
	R_S	%	R_S	%	R_S	%	R_S	%
band 1	0.85	60	0.85	30	—	—	0.84	10
band 2	0.94	40	0.96	60	0.92	20	0.94	25
band 3	—	—	1.05	10	1.03	20	1.04	25
band 4	—	—	—	—	1.23	60	1.22	40
II T.L.C. bromine system								
band 1	0.86	100	0.90	60	—	—	—	—
band 2	—	—	1.00	<10	—	—	—	—
band 3	—	—	1.08	30	—	—	—	—

When analysed in the bromine system, the shea nut sterol acetates only show a single blue band with an R_S value of 0.86. However, there is no band of R_S value 0.94. In the bromine system only $\Delta 5$ mono-, and $\Delta 5,22$-di-unsaturated sterols produce distinct blue bands. Sterols having a nuclear double bond in another position e.g. 7(8), 8(9), or 9(11) are readily decomposed by bromine and their oxidation products ascend with the solvent front. The special component of shea nut sterols (parkeol) has been identified with $\Delta 9(11)$, 24(25)-lanostadienol. The R_S value of the isomeric lanosterol acetate ($\Delta 8(9)$, 24(25)) in this system is 0.97. Therefore, it appeared most likely that the "second band" of shea nut phytosterol acetates with $R_S = 0.94$ should be attributed to this parkeol. This statement was confirmed by applying various colour reactions. With some of the reagents mentioned in table 25 viz. antimony(III)chloride and bismuth(III)chloride the first and second band of the shea nut sterol acetates show different colours. Whereas the first band from these acetates has the same violet hue as that of β-sitosterol and cholesterol acetate, the second band has an orange-brown shade, quite similar to that of lanosterol acetate. Because of the $\Delta 9(11)$,24 unsaturation the second-band phytosterols of the shea nut sterol mixture are not revealed by a blue band in the bromine system at an R_S value of 1.00. The only band shown in this system is apparently due to $\Delta 5$-sterols, belonging to the first-band critical pair, most probably β-sitosterol. The presence of a quantity of about 60% of these normal $\Delta 5$-phytosterols in shea nut sterols is in accordance with the paperchromatographic data.

In the bromine system the Mowrah fat phytosterol acetates are fractionated into

three bands with R_S values of 0.90 (60% of the total mixture), 1.00 (10%), and 1.08 (30%). As compared with the percentages found in system B the content of the sterols belonging to the band with $R_S = 0.96$ is decreased from 60% to only 10% in the bromine system. Upon spraying with bismuth(III)chloride or antimony(III)chloride this band produces the same orange-brown shade as is shown by the second band of shea nut sterol acetates and by lanosterol acetate. Therefore, it may be assumed that this band also contains a triterpenoid alcohol, hitherto not described in the literature and probably related to the parkeol found in shea nut phytosterols.

Shea nut fat and Mowrah fat thus both contain a relatively large fraction of special tetracyclic triterpenoid alcohols having a nuclear double bond in the C-ring. Evidently, the presence of such triterpenoid "pseudosterols" in the sterol mixtures of these fats corresponds with a decrease in their melting point diagram, as is illustrated in the diagrams of shea nut and Mowrah phytosterol acetates with cholesterol acetate (fig. 36).

6.1.3 $\Delta 7$-sterol mixtures

The peculiar high melting point of the phytosterols from pumpkin seed (*Cucurbita Pepo*) viz. 159°C was observed already in 1910 by POWER *et al.* (1910a). Later on LENDLE (1938) has investigated this sterol mixture more thoroughly. Several other high-melting sterol mixtures, possibly of a comparable nature, were reported in the literature a.o.:

Cucurbitaceae

Cucurbita Pepo, melt.p.phytosterol 162–163°C; molecular weight (RAST) 408.4; C 82.77 H 11.69; $C_{28}H_{46}O \cdot \frac{1}{2}H_2O$; acetate melt.p.: 174–175°C (LENDLE 1938).

Cucurbita citrullus L., melt.p. 163–164°C; C 79.92 H 11.88; $C_{26}H_{44}O \cdot H_2O$; acetate melt.p. 170–173°C; $C_{26}H_{43}O \cdot C_2H_3O$ (FORTI 1890) and phytosterol melt.p. 163–164°C; C 82.6 H 11.3; $C_{20}H_{34}O$ (?) (POWER 1910b).

Citrullus colocynthus, melt.p. 158–160°C; $\alpha_D = +8.1°$; acetate melt.p. 167–170°C (POWER 1910c); and phytosterol melt.p. 164–165°C; $\alpha_D = -2°$; C 83.6 H 12.0; acetate melt.p. 184°C; C 81.7 H 11.2; molecular weight 428, identified with α-spinasterol (HAMILTON 1952).

Momordica cochinchinensis, two sterols (A) Bessisterol; melt.p. 175°C; $\alpha_D^{23} = -13.5°$; acetate melt.p. 185°C; $\alpha_D = -14.8°$, identified with α-spinasterol (KUWADA 1940a) and (B) melt.p. 163.5–167.5°C; $\alpha_D = +4.04°$; $C_{28}H_{46}O \cdot \frac{1}{2}H_2O$; acetate melt.p. 174.5–176.5°C, isomer of 5-dihydroergosterol(?) (KUWADA 1940b).

Bryonia dioica (roots) melt.p. 137°C; $\alpha_D = +0°$; $C_{27}H_{46}O$; acetate melt.p. 155–157°C (POWER 1911).

Ecballium elaterium (fruits) melt.p. 148°C; $\alpha_D = +3.2°$; $C_{27}H_{46}O$; acetate melt.p. 155–157°C (POWER 1909).
and also from e.g.:

Thea sinensis melt.p. 167–168°C; acetate melt.p. 181°C; molecular weight (RAST) 408; 5-dihydroergosterol (?) (TSUJIMURA 1932).

The melting points of *normal* Δ5-phytosterol mixtures, free from conjugated sterols, generally are higher than those of the corresponding acetates. However, the *above* phytosterol mixtures including those of pumpkin seed oil and cucumber seed oil have melting points significantly *lower* than those of their acetates. According to BERGMANN (1953) such data are characteristic of Δ7-sterols. This statement is affirmed by the small values of the optical rotation of $\pm 0°$. Such small rotations are specific for mono-unsaturated Δ7-sterols, the slightly more pronounced levo-rotations indicating an additional Δ22(23) double bond (BERGMANN 1952). BERGMANN (1953) stated that the presence of such Δ7-sterol types is quite typical for the fats of the *Cucurbitaceae* and of the *Sapotaceae* (including *Madhuca* and *Butyrospermum* species). We found some conclusive evidences for the first part of this statement. However, in the group of the *Sapotaceae* aberrant sterol types are found, some of these sterol mixtures containing triterpenoid "pseudosterol" types.

Besides the pumpkin oil sterols we have investigated still another high melting phytosterol mixture from the *Cucurbitaceae* group viz. the oil from cucumber seeds (*Cucumis sativus*). The phytosterol mixture of this oil has the following characteristics: sterol content 0.54%; melting point of the acetates: 170.1°C; $\alpha_D = +5.9°$; melting point of the sterols 148.6°C; $\alpha_D = +14.2°$. Paperchromatographic analysis showed three bands with R_S values and percentages of 0.94 (48); 1.12 (24); and 1.24 (28). Obviously, the third band belongs to the $FC_{27}F$–$2FC_{28}F$ critical pair. In rev-phase T.L.C. (system B) three bands with R_S values and percentages of 0.92 (20); 1.03 (20); and 1.23 (60) are shown. In the bromine system no bands are detected, thus excluding a Δ5-sterol structure for any component of this mixture (table 28). The melting points and optical rotations also point to a Δ7-sterol structure, the triterpenoid alcohols related to lanosterol having much higher dextro-rotation. Analogous data are found when analysing pumpkin oil sterols in such thin-layer chromatographic systems. The pumpkin sterols are supposed to have Δ7-sterol structures, as will be discussed more in detail in the next part.

We thus can assert that the occurrence both of triterpenoid alcohols and of certain Δ7-sterol types in edible oils and fats gives rise to quite abnormal melting point diagrams (fig. 35, 36). These diagrams are all typified by a distinct minimum value, as is shown by the phytosterol acetates prepared from shea nut fat, Mowrah fat, and pumpkin oil. This behaviour contrasts with the curvature shown by normal Δ5-phytosterol acetates.

Consequently, the fats that contain such aberrant phytosterols cannot be detected in

butter by means of the original phytosterol acetate test. The failure of detecting shea nut fat and Mowrah fat in butter with this procedure, therefore, is not caused by their low sterol contents, as was suggested at the time by BERG *et al.* (1914a).

The above statements were illustrated by determination of the melting point diagrams lanosterol acetate–cholesterol acetate and $\Delta 7$-ergostenol acetate–cholesterol acetate. In the lanosterol acetate–cholesterol acetate diagram a strong decrease in melting point is found at low percentages of lanosterol acetate. A very pronounced minimum with melting point of 86°C is observed at 44% of lanosterol acetate. Quite similar to the curvature of the pumpkin sterol diagram, the $\Delta 7$-ergostenol acetate–cholesterol acetate melting point diagram exhibits a minimum at about 20% of $\Delta 7$-ergostenol acetate (fig. 37).

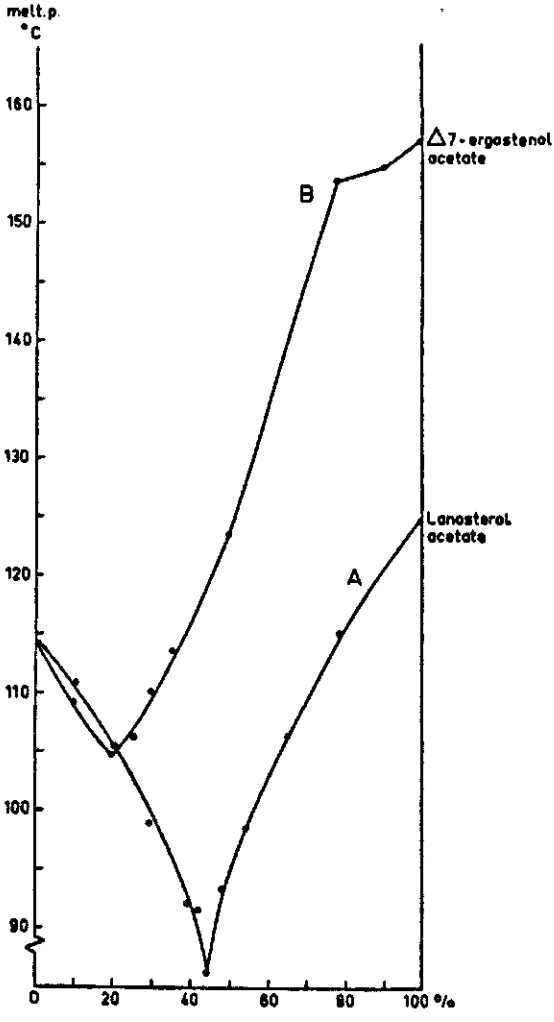


FIG. 37. Melting point diagrams of cholesterol acetate with lanosterol acetate (A) and $\Delta 7$-ergostenol acetate (B).

On the basis of the above phenomena, we have to conclude that a negative result in the phytosterol acetate test does *not* necessarily give a reliable indication as to the absence of vegetable fat in butter. The test should be completed by microscopic examination of the sterol mixture, according to the procedure of DEN HERDER, or preferably by a chromatographic analysis of the sterol mixture.

6.2 STRUCTURE OF THE PUMPKIN OIL PHYTOSTEROLS

As an example of the application of chromatographic methods as well as several other analytical techniques, the structure of pumpkin oil sterols was investigated more thoroughly. A complete structural analysis by all methods of organic chemistry including degradation procedures is beyond the scope of this investigation.

6.2.1 Characteristics of pumpkin oil

Crude pumpkin oil was prepared from pumpkin seeds in order to exclude the possibility of admixture of other vegetable oils. An amount of 10 kg of peeled pumpkin seed[7] was extracted with petroleum ether (bp 40–60°C). The dark brown coloured crude pumpkin oil (yield about 28 %) was investigated, determining the common fat chemical characteristics viz. refractive number 59.7; iodine value 122.5; density 0.9208; percentage of free fatty acids 0.46; peroxide number 32.6; R.M.W. value 0.90; POLENSKE value 0.28; critical temperature of dissolution (aniline) 13.5; saponification value 194.6; percentage of the unsaponifiable fraction 1.83–1.84 %, α_D of this fraction —9° and —10°; sterol-free unsaponifiable fraction 1.44 %, α_D of this fraction —7.5° and —8.5°.

FITELSON test on crude oil (with acetic anhydride): strong green colour. Colour reactions of TORTELLI-JAFFÉ positive, WELMANS weakly positive, BERTRAM weakly positive (on a refined sample).

Sterol values: total-sterol content 0.39–0.40 %; sterol acetate melting point 167.6°, after 5 crystallizations 170.5°C.

Molecular weight (RAST) of the pumpkin oil sterol mixture 386 and of the corresponding acetates 422 (theoretical values for a $C_{27}H_{44}O$ formula: 384 and 426).

6.2.2 Paperchromatography

In the studied system paraffin oil (0.10 g/g)/acetic acid–water (84 : 16) pumpkin phytosterols are fractionated into four distinct bands, belonging to the critical pairs of the first, second, third, and fourth band. Further on the sterols producing these bands are denoted as *cucurbitasterol* 1, 2, 3, and 4. The R_S and R_M values of these four

[7] The pumpkin seeds were obtained from Ölwerke PELZMANN, Leibnitz Österreich by courtesy of PROF. DR. L. SCHMIDT, Wien.

bands are respectively 0.85 (0.31); 0.95 (0.23); 1.05 (0.16); and 1.15 (0.09). The quantities of the four cucurbitasterols were evaluated as cucurbitasterol 1 : 27%; cucurbitasterol 2 : 26%; cucurbitasterol 3: 25%; cucurbitasterol 4: 22%, assuming that each band is produced by only one sterol. Cucurbitasterol 4 with an R_S value of 1.15 obviously belongs to the critical pair $2FC_{28}F$-$2FC_{27}$-$FC_{27}F$. However, the colour reactions mentioned in table 19 did not produce the colours indicative for conjugated sterol types. Excluding thus the conjugated $2FC_{27}$, $2FC_{28}F$ and higher unsaturated structures, cucurbitasterol 4 may have a di-unsaturated, non-conjugated $FC_{27}F$ structure or less probably a C_{27}-sterol skeleton with two non-conjugated, nuclear double bonds.

6.2.3 U.V. spectrophotometry

The pumpkin sterol acetate sample was recrystallized twice from iso-octane. The acetates were saponified and the U.V. spectrum of the sterol fraction, dissolved in chloroform, was measured with an Unicam SP 700 spectrophotometer. Only three small peaks at 272, 282, and 294 nm occur in the spectrum. The $E^{1\%}_{1\,cm}$ value at 282 nm of 0.98 indicates the presence of a small amount viz. about 0.35% of a conjugated provitamin D compound. If a considerable part of the sterol mixture would consist of 7-dehydrocholesterol ($2FC_{27}$) or related sterols, much higher extinction coefficients would be expected.

Therefore, neither the fourth band (cucurbitasterol 4) nor the other ones should be attributed to sterols having a system of 5,7 conjugated double bonds.

6.2.4 T.L.C. bromine system

When analysed in the T.L.C. system undecane/acetic acid–acetonitrile (25 : 75) with 0.5% bromine the pumpkin oil sterol acetates apparently are decomposed and no bands appear on the chromatoplate. This experiment proves unambiguously that the pumpkin sterol mixture does not contain any sterol of the non-conjugated $\Delta 5$-type. On account of this elegant procedure the conjugated $2FC_{27}$ and $2FC_{28}F$ structures for cucurbitasterol 4 thus are definitely excluded. This special method of analysis can be applied successfully in practice to detect admixture of common vegetable oils, containing $\Delta 5$-sterols, to pure pumpkin oil.

6.2.5 Colour reactions

Pumpkin phytosterols give a positive reaction in the TORTELLI-JAFFÉ colour reaction. This reaction is generally considered to be specific for sterols containing a ditertiary double bond in the nucleus or that are easily isomerized to such substances e.g. ergosterol, $\Delta 7$-ergostenol, 5-dihydroergosterol etc.

In the ROSENHEIM reaction (1 mg of sterol in 1 ml of 90% trichloroacetic acid) the pumpkin sterols show a distinct blue colour, whereas β-sitosterol and cholesterol produce only very faint violet to pink hues. Under the same circumstances $\Delta 7$-sterols

such as 5-dihydroergosterol and conjugated sterols like ergosterol show deep blue to blue-black colours.

In a colour reaction with anisaldehyde (KATZ 1960) pumpkin sterols as well as $\Delta 7$-sterols, 7-dehydrocholesterol, zymosterol etc. develop purple to brown-purple shades. The reaction was carried out by dissolving 1 mg of sterol in 1 ml of a mixture of anisaldehyde–acetic acid–sulfuric acid (1 : 100 : 2) and heating during 25 sec over a microflame.

With the reagent p-nitrobenzene diazonium chloride, indicative of conjugated sterol structures (FIESER 1930), pumpkin oil sterols only show a faint yellow colour.

The phytosterol mixtures of most common vegetable oils are slow-acting in the LIEBERMANN-BURCHARD colour reaction, similar to the behaviour of cholesterol, stigmasterol etc. However, the pumpkin sterols definitely belong to the fast-acting group of sterols. The maximum extinction coefficient of this sterol mixture is nearly as high as that of ergosterol (fig. 31 and 38).

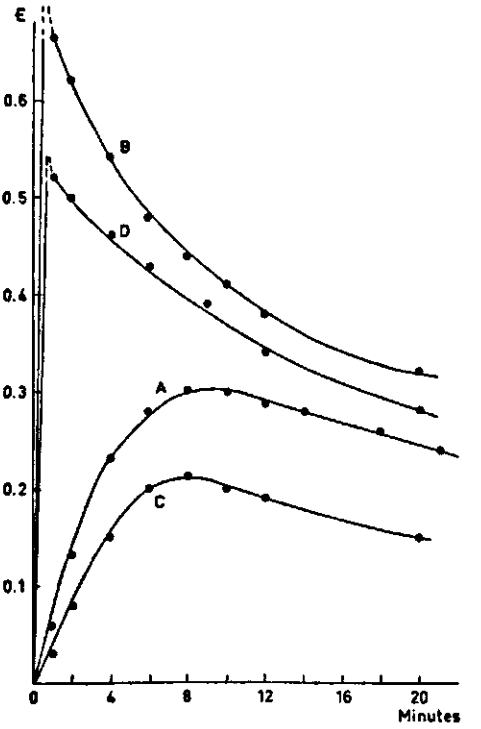


FIG. 38. Absorbances in the LIEBERMANN-BURCHARD reaction shown by cholesterol (A), ergosterol (B), and by the sterol mixtures from soybean oil (C) and pumpkin oil (D).

The presence of conjugated sterols such as ergosterol could be excluded on the basis of the U.V. spectrum and several colour reactions. In the group of the other sterol types only the $\Delta 7$-unsaturated sterols give rise to such high extinction coefficients. The $\Delta 8(14)$ and $\Delta 14(15)$ types have absorbances intermediate between those of the

slow-acting and of the fast-acting groups respectively (IDLER 1953b, TOYAMA 1956). In view of the above statements, we must conclude that the major parts of the cucurbitasterols have structures with an ethylenic linkage in the 7(8) position.
The high extinction coefficients in the LIEBERMANN-BURCHARD reaction can be applied in practice to the detection of small amounts of pumpkin oil in the common types of vegetable oils.

6.2.6 Melting point and optical rotation

The melting points of normal non-conjugated Δ5-sterols are generally higher than those of their acetates, whereas the reverse is characteristic of the group of Δ7-sterols. The melting points of the pumpkin sterols and their acetates viz. 159.0° and 170.5°C, therefore, also indicate a Δ7-sterol structure. The specific optical rotation of the pumpkin sterol mixture is +5.0°, of the acetates +1.0°. The molecular rotations thus are respectively +19.3 and +4.2. According to BERGMANN (1953) specific rotations of 0 to —25° suggest a 7(8) double bond, the more negative rotations in that region indicating an additional Δ22(23) double bond. In BARTON's (1945) system of additive molecular rotations of parts of the sterol molecule a small difference Δ_1 between the molecular rotations of the sterol acetates and the sterols of about –6 is indicative of a Δ7(8) ethylenic linkage. On the other hand tetracyclic triterpenoid "pseudosterols" have quite different Δ_1 values viz. exceeding +15. The rotations of pumpkin sterols closely resemble those of other compounds belonging to the Δ7-sterol group (table 29), the experimental value of Δ_1 of —15.1 being in reasonable accordance with BARTON's data.

TABLE 29 Comparison of pumpkin sterol and some other Δ7-sterols

	Sterol		Sterol acetate	
	melt.p.	α_D	melt.p.	α_D
Δ7-cholestenol	125°C	+5.7°	118°C	+1.5°
Δ7-ergostenol (fungisterol)	146	±0	160	—5
Δ7,22-ergostadiene-3β-ol (5-dihydroergosterol)	176	—20	180	—20
episterol (?)	151	—5	161	—3.5
Δ7-stigmastenol	146	+9	157	+8
Δ7,22-stigmastadiene-3β-ol (α-spinasterol)	167–169	—3	177–185	—5
β-spinasterol (Δ7,25(26)?)	149	+6	154	+5
pumpkin oil sterols	159	+2	170.5	±0

6.2.7 Infrared and N.M.R. spectra[8]

The infrared spectra of the pumpkin sterol mixture, their acetates and of some related $\Delta 7$-sterols were measured with an Unicam SP 100 infrared spectrophotometer. The spectrum of pumpkin sterols appeared to possess a band pattern nearly identical with that of $\Delta 7$-ergostenol and 5-dihydroergosterol. It shows absorption bands at 725, 776, 794, 828, 843, 868, *885*, 936, 969, 1015, 1040(1048), 1099, 1130, 1156, 1171, 1195, 1215, 1250, 1303, 1320, 1342, 1372(1380), 1445, *1643*, and 1663 cm^{-1} (fig. 39).

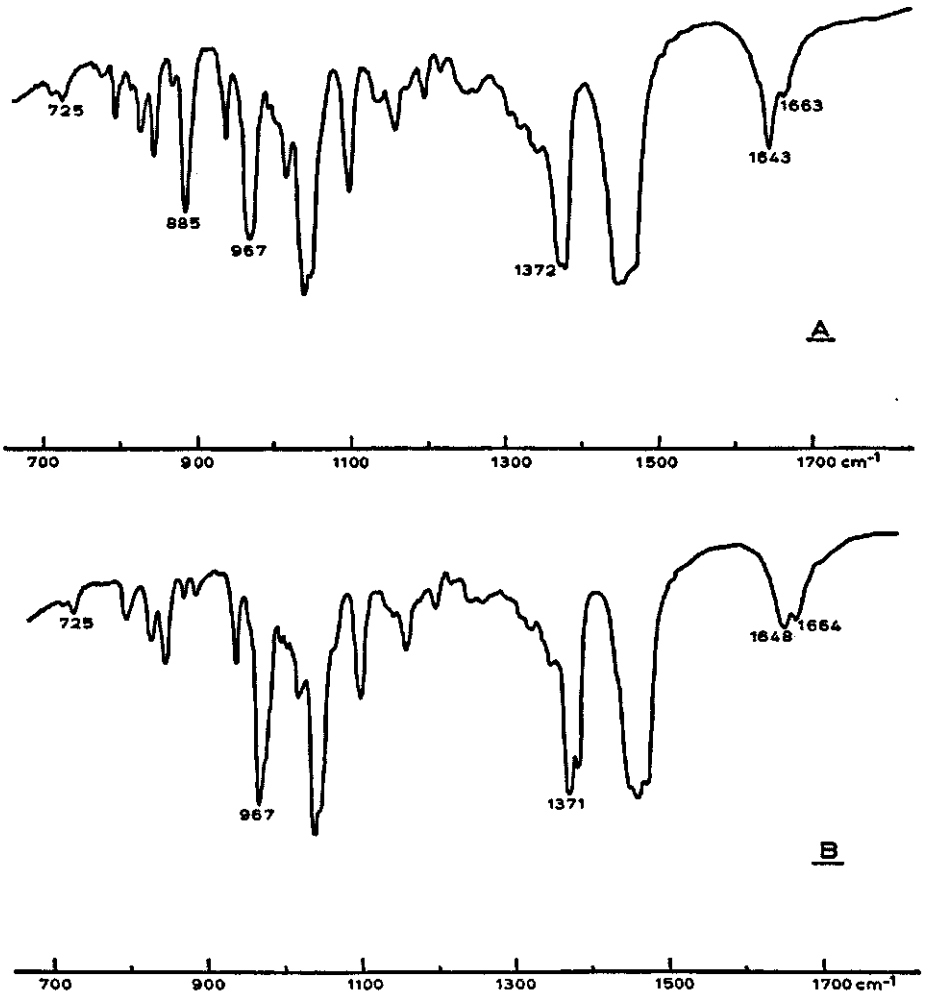


FIG. 39. Infrared spectra of pumpkin sterols (A) and 5-dihydroergosterol (B).

[8] I thank the scientists of the ORGANISCH CHEMISCH LABORATORIUM, Rijksuniversiteit at Leiden, Director PROF. DR. E. C. KOOYMAN, for their manifold help and advice.

According to IDLER *et al.* (1953a) $\Delta 7$-sterols such as $\Delta 7$-cholestenol, $\Delta 7$-ergostenol, and α-spinasterol show a common "$\Delta 7$-pattern" of bands at 729, 797, 830, 847, 937, 966, 976, 1020, 1053, and 1101 cm^{-1}, which is in accordance with our data. A very close resemblance was shown by many details of the spectrum of pumpkin sterols and that of 5-dihydroergosterol. The strong 969 peak may indicate that some cucurbitasterols have an additional $\Delta 22(23)$ unsaturation. These data confirm the hypothetical $\Delta 7$-sterol structure for all cucurbitasterols. A 24-b-configuration has to be attributed to the four C_{28} and C_{29}-cucurbitasterols, since the spectrum of $\Delta 7,22$-24a-stigmastadiene-3β-ol (chondrillasterol) is quite different from that of its 24-b-epimer α-spinasterol and other 24-b-sterols like 5-dihydroergosterol (cf. DOBRINER 1953). The most probable type of structure for the various cucurbitasterols, therefore, appeared to be that of 5-dihydroergosterol, its homologues or structural isomers.

However, some differences between the spectra of pumpkin sterols and other $\Delta 7$-sterols are noticed. The spectrum of the pumpkin sterols exhibits a very distinct band at 885 cm^{-1} and 1643 cm^{-1} and a minor band at 776 cm^{-1}, which form no part of the normal $\Delta 7$-pattern. The 885 cm^{-1} and 1643 cm^{-1} absorptions are characteristic of sterols and steroids with a terminal ethylenic linkage, as has been demonstrated by SONDHEIMER *et al.* (1957). Some special sterols having a terminal unsaturation are found in nature. The absorptions at 885 cm^{-1} and 1637 cm^{-1} shown by the sterol mixture isolated from chick embryo tissues were proved to be due to the presence of 25-dehydrocholesterol ($\Delta 5,25(26)$-cholestadiene-3β-ol) in this mixture (STOKES 1956).

Another possible situation for a terminal unsaturation in the skeleton of C_{28} and C_{29}-sterols is at the C_{24-28} linkage. Actually, the sterol mixtures isolated from certain molluscs and from queens of *Apis mellifica L.* show infrared spectra with quite similar absorptions at 885 cm^{-1} and 1637 cm^{-1}, due to the presence of 24-methylene cholesterol (viz. $\Delta 5,24(28)$-ergostadiene-3β-ol) in these mixtures (IDLER 1955, 1957, BARBIER 1959).

Analogously, the 885 cm^{-1} and 1645 cm^{-1} absorptions given by pumpkin sterols have to be attributed to a terminal linkage, $\Delta 25(26)$ or possibly $\Delta 24(28)$, in at least one or possibly two of the components of the cucurbitasterol mixture.

This evidence was confirmed by the nuclear magnetic resonance (N.M.R.) spectrum. Common $\Delta 7$-sterols like $\Delta 7$-cholestenol and $\Delta 7$-ergostenol show a peak at 5.2 p.p.m. (with trimethylsilane as zero), due to the C_7 proton (fig. 40,A). The C_6 and $C_{22,23}$ protons of stigmasterol respectively give rise to peaks at 5.35 and a doublet at 5.1–5.05 p.p.m. A solution of pumpkin sterols in deuterated chloroform shows a peak at 5.2 p.p.m. (C_7 proton) with a shoulder at 5.1 p.p.m. ($C_{22,23}$ protons), indicating that part of the $\Delta 7$-cucurbitasterols has an additional $\Delta 22(23)$ unsaturation. However, at 4.7 p.p.m. another marked peak is found (fig. 40, B). This evidences the presence of a component having a second double bond in a special position. Its content in the sterol mixture was estimated to be approximately 40%, which is in reasonable accordance with the content of fourth-band sterols, determined with rev-phase T.L.C. The 4.7

p.p.m. peak indicates a terminal unsaturation, analogous to the corresponding peak in the N.M.R. spectrum of e.g. methylene cyclobutane and methylene cyclohexane (BHACCA 1962).

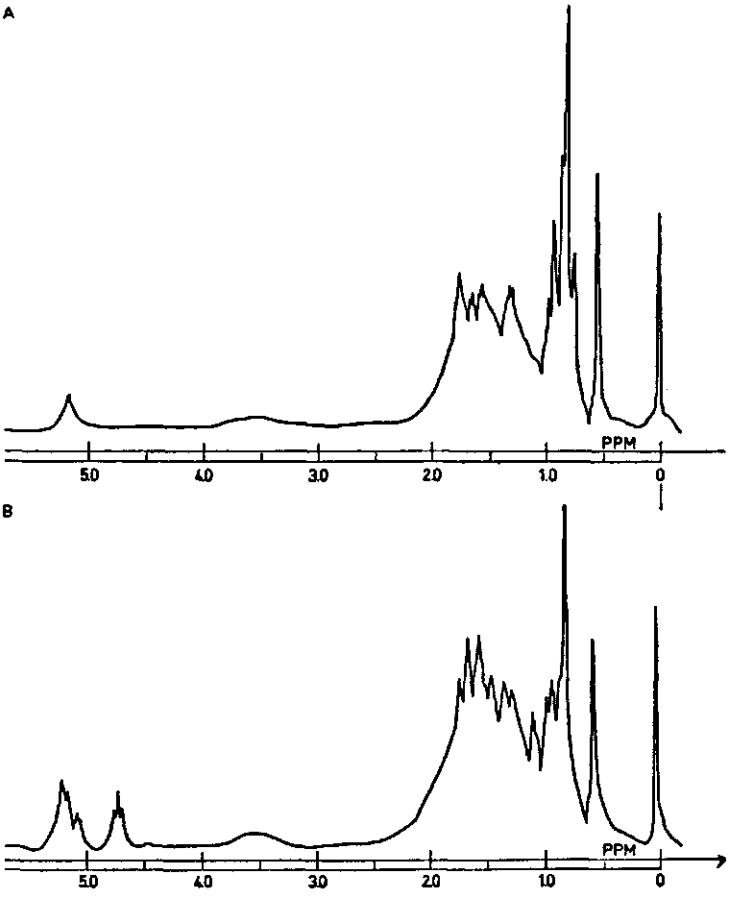


FIG. 40. Nuclear magnetic resonance (N.M.R.) spectra of Δ7-ergostenol (A) and pumpkin seed phytosterols (B).

The presence of a terminal ethylenic linkage was still further confirmed by the forming of formaldehyde upon oxidimetric degradation. Pumpkin oil sterol acetates were oxidised with periodic acid and potassium permanganate according to the procedure of BRICKER *et al.* (1949). The formaldehyde was separated from the reaction mixture by distillation. Its amount was determined by means of the colour reaction with chromotropic acid, the yield amounting to about 6% of the theoretical value[9].

On the basis of the above data it is likely that in addition to the Δ7-structure for all

[9] Under identical circumstances limonene yielded formaldehyde in 30% of the theoretical amount.

cucurbitasterols and a Δ22(23) unsaturation for some of them, at least one but possibly two of the other cucurbitasterols may have the second double bond in a terminal position, viz. the Δ25(26) or possibly the Δ24(28) position.

6.2.8 Thin-layer chromatography

Pumpkin oil sterols and their acetates were analysed with rev-phase T.L.C. in the systems C, D and A, B respectively. By spotting pure preparations of 5-dihydroergosterol and Δ7-ergostenol on the same chromatoplate an accurate comparison of the respective R_F values was obtained (table 30). In system B viz. undecane/acetic acid–acetonitrile (25 : 75) the pumpkin sterol acetates are fractionated into four bands, the respective amounts of the bands 1–4 being approximately 11, 31, 25, and 33% (fig. 41). When the pumpkin sterol acetates are recrystallized repeatedly from e.g. ethanol, the relative intensities of these four bands do not change appreciably. As compared with the percentages of the four bands determined with paperchromatography viz. 27, 26, 25, and 22%, it appears that part of the first-band sterols found in paperchromatography is shifted to the fourth band in rev-phase T.L.C.

TABLE 30 Comparison between characteristics of the four cucurbitasterols and some related Δ7-sterols in rev-phase T.L.C. (system B)

Acetates of	Abbr. formula	Δ at	R_S value	Antimony (III) chloride*	Trichloro-acetic acid	Salicyl-aldehyde*	Resor-cinol*
β-sitosterol	FC_{29}	5	0.80	violet	grey	violet	faint blue
cucurbitasterol 4	$FC_{27}F$?	7,25(26)?	1.22	purple-brown	blue	purple-brown	orange-brown
cucurbitasterol 3	$FC_{28}F$?	7,25(26)?	1.05	orange-brown	grey-brown	orange-brown	purple-brown
cucurbitasterol 2	$FC_{29}F$?	7,22 ?	0.94	orange-brown	grey-brown	orange-brown	purple-brown
cucurbitasterol 1	FC_{29} ?	7	0.84				
5-dihydroergosterol	$FC_{28}F$	7,22	1.06	orange-brown	grey-brown	orange-brown	purple-brown
Δ7-ergostenol	FC_{28}	7	0.97	grey-brown	yellow-brown	yellow-green	yellow-brown
α-spinasterol (second band from *Asterina* sterols)	$FC_{29}F$	7,22	0.95	faint orange-brown	grey-brown	orange-brown	
22-dihydroergosterol	$2FC_{28}$	5,7					
zymosterol	$FC_{27}F$	8(9),24(25)		orange-brown	grey-brown	orange-brown	

* The procedures for the colour reactions are mentioned in table 25.

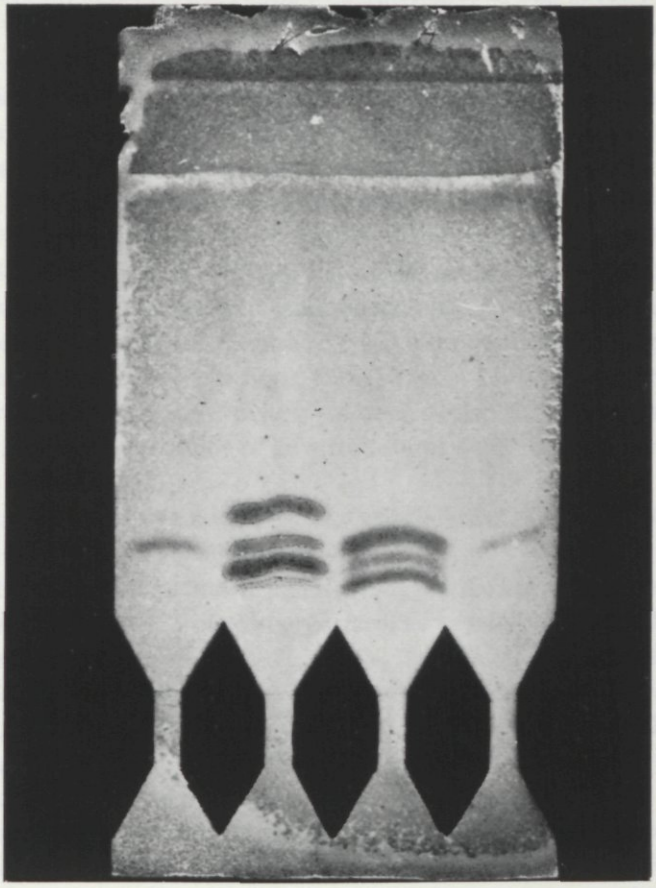


FIG. 41. Reversed-phase T.L.C. of cucurbitasterol acetates in the system: undecane/acetic acid–acetonitrile (25 : 75).

spot 1 and 4. 5-dihydroergosterol.
spot 2. pumpkin oil sterol mixture.
spot 3. sterol mixture from *Asterina Pectinifera.*

The R_S values of the cucurbitasterols and of some Δ7-sterols in system B are listed in table 30. On account of the results of paperchromatography and of rev-phase T.L.C. we may assume the presence of at least four cucurbitasterols in the pumpkin sterol mixture. The R_F values in the studied systems indicate that these four cucurbitasterols constitute a series of probably homologous Δ7-sterols, differing in length and unsaturation of the side chain. On the basis of the relationships between sterol structure and migration rate and of the above-mentioned characteristics we might tentatively assume the most probable structures for cucurbitasterol 1, 2, 3, and 4 to be respectively FC_{29}; $FC_{29}F$; $FC_{28}F$; and $FC_{27}F$. In the system B the migration rates of cucurbitasterol 3 and of 5-dihydroergosterol ($FC_{28}F$) are quite similar (fig. 41). This similarity was also found when applying the three other systems viz. A, C, and D. Furthermore, several chromatoplates developed in the system B were systematically sprayed with twenty colour reagents, some of which are given in table 25. With these reagents the various sterol types generally show diverse colours. Notwithstanding the variety of shades observed, those of the bands of cucurbitasterol 3 and of 5-dihydroergosterol matched exactly (table 30).

On the other hand further related sterols like Δ7-cholestenol, Δ7-ergostenol, lanosterol etc. show quite different shades.

On the basis of the above statements we therefore suggest the hypothesis that cucurbitasterol 3 may be identified with 5-dihydroergosterol or possibly with one of its isomers having a terminal unsaturation viz. Δ7,25(26)-, or Δ7,24(28)-ergostadiene-3β-ol. In view of the observed R_F values in the four studied systems, the above-mentioned colour reactions, and other characteristics, cucurbitasterol 2 may tentatively be identified with α-spinasterol ($FC_{29}F$), whereas there is some evidence suggesting the identity of cucurbitasterol 1 with Δ7-stigmastenol (FC_{29}).

The migration rates and colour reactions of cucurbitasterol 1 and 2 closely resemble those of the first two bands shown by a sterol mixture from *Asterina Pectinifera*[10] (fig. 41). TOYAMA *et al.* (1955, 1956) have identified the components of this mixture with Δ7-cholestenol, a Δ7,22-C_{28} sterol, α-spinasterol, and Δ7-stigmastenol. Upon chromatographic analysis in system B the first two sterols together form the third band, α-spinasterol forms the second, and Δ7-stigmastenol the first band (fig. 41). The resemblance in chromatographic behaviour between the bands 1 and 2 of this mixture with those of the pumpkin oil sterol mixture confirms the above hypothesis concerning the possible structure of cucurbitasterol 1 and 2.

With some specific spray reagents a.o. antimony(III)chloride and trichloroacetic acid–acetic acid (90 : 10) (in the latter reaction: 15 min heating at 80°C) cucurbitasterol 4 shows a colour shade differing from that of the other cucurbitasterols. Therefore, it appears probable that the second double bond of cucurbitasterol 4 (sterol type

[10] The sample of *Asterina Pectinifera* sterols was a most generous gift of PROF. Y. TOYAMA (Nagoya) Japan.

Δ7-$FC_{27}F$) is not at the normal 22(23) position. The infrared absorptions at 885 and 1643 cm^{-1} and the 4.7 p.p.m. peak in the N.M.R. spectrum most likely indicate the presence of a quantity of about 40% of a special sterol having an additional 25(26) ethylenic linkage. It appears probable that these phenomena might be correlated with the special behaviour of cucurbitasterol 4 (about 33% of the total mixture) in rev-phase T.L.C. In view of this circumstantial evidence, we suggest that cucurbitasterol 4 might be Δ7,25(26)-cholestadiene-3β-ol.

7 CALCULATIONS ON THE BASIS OF STEROL VALUES

7.1 IDENTIFICATION OF THE TYPE OF ADMIXED ANIMAL FAT

In a mixture of one type of vegetable fat with one single type of animal fat, the sterol values of the mixture are useful in evaluating the respective amounts of the components. Because of the variability of the composition of a fat and the influence of refining processes, the sterol values of that fat may vary to some extent. This natural variability implies that the results of such calculations are only of a semi-quantitative nature. Furthermore, the present imperfection in the determination of the cholesterol percentage also hampers the reliability of such estimations. The difficulties encountered in determining the cholesterol percentage, especially when considerable amounts of third-band phytosterols are present, have been discussed in chapter 4. It may be assumed that in the future still more reliable procedures for determining the cholesterol percentage will be available e.g. gas-liquid chromatography. This would further enhance the significance of the mathematical treatment now going to be discussed. In some cases these calculations can already furnish some evidence concerning the type of animal fat that is admixed in only small amounts to a vegetable fat. In these calculations which are essentially based on a balance of the cholesterol and phytosterol content of fat mixtures the following symbols are used:

x_0 = cholesterol content of the animal fat.
y_0 = phytosterol content of the vegetable fat.
a_0 = total-sterol content of the fat mixture.
c_0 = cholesterol content of the sterol mixture.
$100 - c_0$ = phytosterol content of the sterol mixture.
p = content of animal fat in the fat mixture.
$100 - p$ = content of vegetable fat in the fat mixture.

In addition to the sterol values the results of more classical methods of fat analysis have to be applied as well. When analysing mixtures of vegetable and animal fat usually the following characteristics are determined: R.M.W. and POLENSKE value (presence of coconut and palmkernel fat), arachidonic acid content, and the colour reactions of TORTELLI-JAFFÉ (specific for whale oil), BERTRAM (positive in the case of soybean oil and animal fats, except lard), and WELMANS (strong positive for whale oil and less pronounced for all vegetable oils).

The amount of cholesterol present in the fat mixture is evaluated in two different ways viz. from $p \,.\, x_0$ and $c_0 \,.\, a_0$ respectively. Thus:

$$p \,.\, x_0 = a_0 \,.\, c_0 \qquad [1]$$

The total quantity of sterols of the fat mixture is derived according to the equation:

$$p \,.\, x_0 + (100 - p) \,.\, y_0 = 100 \,.\, a_0 \qquad [2]$$

Of all the factors involved in this calculation the sterol values a_0 and c_0 are determined experimentally. In special cases the type of the vegetable fat is known by a true declaration or it is identified by other methods of fat analysis e.g. by specific colour reactions. The tables 2 and 20 then provide an approximate y_0 value. In a mixture of a single vegetable fat of known nature with an amount of animal fat (p), the latter is evaluated using the values of a_0, c_0, and y_0. From equations [1] and [2] we have:

$$(100 - p)y_0 = (100 - c_0)a_0 \qquad [3]$$

and hence

$$p = 100 - \frac{(100 - c_0) \,.\, a_0}{y_0} \qquad [4]$$

From the percentage of animal fat (p), derived from equation [4], the cholesterol content of that type of animal fat can be estimated according to:

$$x_0 = \frac{a_0 \,.\, c_0}{p} \quad \text{(from equation [1])} \qquad [5]$$

The cholesterol content of the admixed animal fat (x_0) can give some indication as to the origin of that fat.

If an x_0 value exceeding 0.20% is found and in addition the colour reactions on whale oil (viz. that of TORTELLI-JAFFÉ, BERTRAM, and WELMANS) are negative, we can decide upon the presence of (refined) inedible animal fat (ROOS 1956). The sterol contents of first quality pure animal fats such as lard and beef tallow are only 0.08–0.10%. The limit of 0.20% has been established by carefully taking into account the several influences on the sterol contents exerted by the origin of the fat, the refining processes etc. In fact fats with x_0 values exceeding 0.15% already have to be considered as questionable. The limit of 0.20% therefore has a substantial safety-margin (ROOS 1961).

Because of the large variety in sterol contents of (hardened) whale and fish oils drawing up a correct sterol balance is made more complicated by the presence of these fats. In the presence of marine animal fats, therefore, no evaluation of the amounts of other animal fats can be made. In practice the differentiation between animal fats of pure nature and refined inedible animal fats such as greases (a.o. White grease, Hog grease) and tallows e.g. Fancy tallow is of prime importance. This differentiation is even possible in the presence of an excess of vegetable fat. At present no other method of fat analysis can boast of comparable results.

Sometimes it is troublesome to identify all components of a vegetable fat mixture properly. However, in a special case the type of admixed *animal* fat may be known e.g. by declaration. Provided that no other animal fats are present, the percentage of this animal fat then can be estimated according to equation [1] viz. $p = c_0 \,.\, a_0/x_0$. In

this case a further investigation of the composition of the vegetable fat fraction is not necessary.

Admixture of a non-hydrogenated animal fat can be confirmed by the high value of the arachidonic acid content. This tetraenoic fatty acid with isolated double bonds occurs in relatively high percentages in fats of animal origin, whereas vegetable fats contain only very small amounts, not exceeding 0.04%. If besides a considerable amount of cholesterol no significant increase in arachidonic acid content is found, we may conclude to the presence of hydrogenated animal fats. The great difference in sterol content of (hydrogenated) Menhaden fish oil (a_0 = 0.4–2.0%) and (hydrogenated) whale oil (a_0 = 0.05–0.2%) should enable analogous calculations yielding indications as to the type of marine animal oil present in a mixture with vegetable fat. Some typical instances of these calculations in problems of fat analysis will be discussed in detail.

7.2 Analysis of a cottonseed oil sample

A sample of partly hydrogenated cottonseed oil gave the following characteristics: iodine value 48, no colour reactions of Bertram and Baudouin, and polybromide value negative. The colour reaction of Welmans showed a weakly positive result indicative of vegetable oils. Colour reaction of Halphen (for cottonseed oil) positive. Total-sterol content a_0 = 0.20% and sterol acetate melting point 124.7°C. After the addition of respectively 5% and 25% of cholesterol acetate melting points of 126.2° and 125.2°C were found. In the microscopic test of Roos-Van Dijk (chapter 1.6.2) some swallow-tail crystals were observed. These data point to the occurrence of cholesterol in this sterol mixture. No indications were obtained as to the presence of other vegetable fats and of marine animal fats. The amount of the third-band sterols was determined by planimetric evaluation of a chromatogram viz. 15%. According to table 20 cottonseed oil generally does not contain a detectable amount of third-band phytosterols. The third band therefore should be attributed exclusively to the presence of cholesterol: c_0 = *15%*. Table 2 indicates an average phytosterol content of cottonseed oil samples of about y_0 = 0.30%. In a tentative calculation we may suppose the animal fat to be constituted of pure lard or beef fat with x_0 = 0.08%. In that case we can draw up the following sterol balance:

$$\frac{p.0.08}{100} + \frac{(100-p)0.30}{100} = 0.20, \text{ resulting in } p = 45\%$$

The amount of 45% of lard or beef fat should contribute: 0.036% of cholesterol
The fraction of 55% of cottonseed oil should contain: 0.165% of phytosterol
Total-sterol content: 0.201%

and cholesterol percentage 36/201 . 100 = 18. This percentage agrees satisfactorily with the experimentally determined amount of third-band sterols (c_0 = 15%). This agreement confirmed the validity of the original assumption that the cholesterol would originate from pure lard or beef fat. On the other hand, if the presence of cholesterol should be attributed to an admixture of refined inedible animal fat e.g. White grease with x_0 = 0.3–0.5% another sterol balance should hold:

$$\frac{p.\,0.40}{100} + \frac{(100-p)\,0.30}{100} = 0.20$$

yielding no positive value of p. This possibility ,therefore, has to be excluded. The percentage of the admixed animal fat and its cholesterol content thus can be derived by equations [4] and [5].

$$p = 100 - \frac{(100-15)0.20}{0.30} = 43\% \text{ and } x_0 = \frac{15\,.\,0.20}{43} = 0.07\%$$

According to table 2 a calculated cholesterol content of 0.07% points to the presence of pure lard or beef fat.

7.3 Analysis of a palm oil sample

The analysis of a sample of refined hardened palm oil gave the following data: refractive number 47.9; iodine value 52; sterol content 0.08%; sterol acetate melting point 129.1°C; R.M.W. and Polenske values 1.2 and 0.8 (negative for coconut and palmkernel fat); colour reactions of Tortelli-Jaffé, Bertram, Halphen, and Baudouin negative; reaction of Welmans weakly positive (indicating vegetable fat); chromatographic sterol analysis 50% of third-band sterols; arachidonic acid content 0.03%.

Upon adding 20% of cholesterol acetate to the sterol mixture the melting point was decreased to 126°C. According to fig. 18 this indicates the presence of about 40% of cholesterol acetate in the mixture of palm oil phytosterol acetates. By chromatographic separation a third-band sterol content of 50% was estimated i.e. of the magnitude of the first-band phytosterols. According to the statements of chapter 4 (cf. 4.4.1. B.2 and 3), the occurrence of strong first and third bands together with only a small percentage of second-band phytosterols evidences the presence of cholesterol. Since hardened palm oil samples usually do not contain appreciable amounts of third-band phytosterols, we may assume a cholesterol content in the sterol mixture of about 45%. The arachidonic acid content of 0.03% also arouses suspicion as to the pure vegetable nature of the fat. The presence of any *marine* animal oil is precluded because of the negative colour reactions of Tortelli-Jaffé and Bertram. We were not able to detect the presence of any other type of vegetable fat. Because of the absence of marine

animal oils, the content of animal fat can be calculated according to equation [4], equation [5] giving some indication as to the nature of that fat. According to table 2 the phytosterol content of palm oil (y_0) is approximately 0.04–0.05%. Hence:

$$p = 100 - \frac{(100 - 45) \,.\, 0.08}{0.05} = 12\% \text{ and } x_0 = \frac{45 \,.\, 0.08}{12} = 0.3\%$$

The content of admixed animal fat thus is about 12% and the cholesterol content of that fat about 0.3%. The latter percentage proves that the animal fat is not a pure, edible body fat but consists of a (refined) inedible animal fat. We can now draw up the following sterol balance:

78% of palm oil with $y_0 = 0.05$ contributes:	0.039% of phytosterol
12% of inedible animal fat with $x_0 = 0.30\%$ contributes:	0.036% of cholesterol
Total-sterol content:	0.075%

and amount of cholesterol in the sterol mixture 36/75 . 100 = 48%. This corresponds with the experimental sterol values.

7.4 Analysis of a second palm oil sample

A third instance concerning the composition of a margarine fat, announced as 100% palm oil, was given originally by Den Herder. The way of reasoning is amplified by chromatographic data. Fat chemical characteristics: refractive number 47.2; iodine value 45; R.M.W. and Polenske values 3.2 and 2.1, indicating the presence of 13% of coconut fat; colour reactions of Tortelli-Jaffé, Bertram, and Welmans positive; total-sterol content 0.11%; sterol acetate melting point 130.3°C.

The total-sterol content is too high for pure palm oil (see chapter 1). Evaluating the colour intensity in the Bertram reaction in a semi-quantitative way, the presence of 10–25% of whale oil is indicated. We now have to consider whether the high sterol content can be explained by the above-mentioned amounts of coconut fat and whale oil. The sterol acetate mixture was mixed with 25% and 50% of cholesterol acetate, resulting in the melting points 128.0° and 122.0°C. The melting point diagram of fig. 18 indicates the presence of about 25% of cholesterol acetate in the palm oil sterol mixture. Since the nature of the animal fat was identified as whale oil, with an approximate cholesterol content of about 0.20%, the amount of that fat is circa $p = c_0 \,.\, a_0/x_0 \sim 14\%$. This percentage agrees with the above-mentioned rough estimate on the basis of the Bertram colour reaction. The presence of whale oil precludes the detection of other types of animal fat e.g. lard.

The phytosterol content of palm oil usually amounts at most to 0.05%. A mixture

of 73% of palm oil and 13% of coconut fat, therefore, would contribute only 0.036 + 0.013 = 0.049% of phytosterol. In addition to the amount of 14/100 . 0.2 = 0.028% of cholesterol some part of the phytosterol fraction still remained to be accounted for. This part had to originate in a third type of vegetable fat with a relatively high phytosterol content.

By paperchromatographic analysis of the sterol mixture a quantity of 33% of third-band sterols was established. According to the data of table 20 these amounts of coconut fat and palm oil can contribute only about 3% and 5% of third-band phytosterols respectively. Consequently, chromatographic analysis indicates a cholesterol percentage of about 25. The remaining quantity of phytosterols must be caused by a third, possibly hardened vegetable fat. This fat should have a rather high phytosterol content, should not contain a considerable amount of third-band phytosterols and should have a sterol acetate melting point of about 129.0–130.5°C. These data might point to a vegetable oil like soybean oil[11]. Substituting the data $p = 14$, $a_0 = 0.11$, and $c_0 = 25$ in the equation $(100 - p)\, y_0 = (100 - c_0) \,.\, a_0$, a value of $y_0 = 75 \,.\, 0.11/86 = 0.096\%$ results. Since the palm oil–coconut fat (73 : 13) mixture only accounts for about 0.05% of phytosterols, we must assume admixture of a vegetable oil like soybean oil. An amount of 15% of soybean oil might contribute the 0.03–0.05% of phytosterol, which still had to be accounted for. A possible[11] sterol balance is:

58% of palm oil with $a_0 = 0.05\%$:	0.029% of phytosterol
13% of coconut fat with $a_0 = 0.10\%$:	0.013% of phytosterol
14% of whale oil with $a_0 = 0.20\%$:	0.028% of cholesterol
15% of soybean oil with $a_0 = 0.30\%$:	0.045% of phytosterol
Total-sterol content:	0.115%

and cholesterol percentage: 28/115 . 100 = 24

7.5 Detection of cocoa extraction fat in pure cocoa butter

The last example deals with a possible application of the methods of sterol analysis to the detection of cocoa extraction fat in pure pressed cocoa butter. Usually, such an admixture is detected by the high amount of the unsaponifiabe fraction (Fincke 1962). Elaborate procedures for the detection of foreign fats in cocoa butter a.o. by colour reactions, by quantitative fractionation of the fatty acids, and by analysis of some specific glyceride fractions, are described in the literature (cf. Kaufmann 1958a, 1962c).

[11] In these calculations the types of fats employed in the manufacturing of the margarine should be taken into account.

Table 31 shows the respective contents of the total unsaponifiable fraction and of the sterol fraction, isolated from cocoa fats and from some other fats that may be used as adulterants. Furthermore, the optical rotation values of the sterols and of the sterol-free unsaponifiable fraction are listed.

TABLE 31 Typical guide values of cocoa butter and some other fats

	Contents in % of		Optical rotation of	
	unsaponifiable fraction	sterol fraction	sterols	sterol-free unsaponifiable fraction
cocoa butter	0.4–0.9	0.3–0.6		±0°
cocoa extraction fat	1.2–2.8	1.2–2.7	—35°	±0°
Tenkawang fat (from *Shorea stenoptera*)	0.5–0.7	0.25–0.32	—35°	—1.1°
Shea nut fat (from *Butyrospermum parkii Kotschy*)	3–7	0.09–0.14	+20°	+38°
Mowrah fat (from *Madhuca longifolia Macb.*)	1.1–2.3	0.03–0.05		+33/+35°
coconut fat	0.2–0.4	0.09–0.11		±0°
tallow	0.10–0.16	0.08–0.10		±0°

Recently, MEYER (1961) has stressed the possibility of applying the difference in phytosterol content of pure cocoa butters and cocoa extraction fats. In the investigation of the characteristics of pure pressed and extracted cocoa butters, we have determined the following sterol values of these fats viz. sterol acetate melting point, percentages of first-, and second-band phytosterols, and total-phytosterol content. The latter was determined by the LIEBERMANN-BURCHARD colour reaction, using an acetic anhydride–sulfuric acid (9 : 1) reagent. We have determined a calibration line giving the absorbance of the reaction mixture in dependance of the amount of β-sitosterol. From the calibration line the corresponding phytosterol contents of both fats were derived. These data correspond with those reported in the literature (KAUFMANN 1958a, MEYER 1961). Another, more reliable value for the phytosterol content was determined by the "precipitation in the soap" procedure of DEN HERDER using digitonin solution.

No significant differences were found in the sterol acetate melting points and chromatographic sterol patterns of pressed and extracted cocoa fats. When analysing *pressed* cocoa butters both phytosterol determinations agree within reasonable limits. In the case of cocoa *extraction* fat, however, the colorimetric estimation gives con-

TABLE 32 Detection of cocoa extraction fat in pure cocoa butter

	Melting point of sterol acetates in °C	Percentages of phytosterols of		
		first band	second band	third band
cocoa butter 1	129.8	56	44	0
cocoa butter 2	130.0	56	44	0
crude cocoa extraction fat 1	128.2	61	39	0
crude cocoa extraction fat 2	127.8	54	46	0
crude cocoa extraction fat 3	127.8	62	38	0
refined extraction fat	127.2	60	40	0
Shea nut fat	146.5	64	36	0
Tenkawang fat	126.0	73	27	0

siderably *higher* data than the more reliable gravimetric procedure (table 32). This difference indicates that, as compared with the pure cocoa butter, the extraction fat contains a higher quantity of components fast-acting in the LIEBERMANN-BURCHARD reaction. Since the sterol-free unsaponifiable fraction of the extraction fat showed an U.V. spectrum totally devoid of special absorption peaks in the 260–290 nm region, the fast-acting properties must be attributed exclusively to the sterol fraction. Consequently, we have determined the amount of poly-unsaturated sterols by measuring the U.V. spectra of the sterol digitonides, which procedure was introduced in chapter 4. The sterol digitonides are dissolved in the solvent mixture dimethylformamide–methanol (1 : 9) and the U.V. spectrum of this solution was determined.

The spectrum of the digitonides from extraction fat shows a four-band system characteristic of provitamins D with maxima at 261, 272, 282, and 294 nm. On the other hand the sterol digitonides of pressed cocoa butter have quite a normal spectrum, with only small absorbances at these wave-lengths. The $E_{1\,cm}^{1\%}$ values of the digitonides from extraction fats measured at 282 nm are about 3–4 times higher than those of the pressed fats (table 32). The variation in background absorption is eliminated by measuring the D_{282} values of both fats. This D_{282} value is defined as:

$$D_{282} = E_{282} - \tfrac{1}{2}(E_{276} + E_{288}) \ .$$

Whereas the pure cocoa butters have D_{282} values of about 0.003–0.006, the four extraction fats have much higher values viz. about 0.01–0.02. A still greater difference can be obtained upon calculating the extinction values (D_{282}^{10g}) to be attributed to the absorbance of the total amount of digitonides from 10 g of fat. These D_{282}^{10g} values are calculated by:

$$D_{282}^{10g} = 40\ x \text{ sterol content} \times D_{282} \ .$$

Phytosterol content in % by LIEBERMANN-BURCHARD reaction	Phytosterol content in % by digitonin precipitation	U.V. spectrophotometric values				
		$E^{1\%}_{1cm}$ at 276 nm	$E^{1\%}_{1cm}$ at 282 nm	$E^{1\%}_{1cm}$ at 288 nm	D_{282}	D^{10g}_{282}
0.29	0.26	0.0163	0.0167	0.0108	0.0032	0.033
0.30	0.28	0.0218	0.0228	0.0167	0.0036	0.040
0.73	0.60	0.0605	0.0666	0.0458	0.0135	0.32
1.05	0.64	0.0766	0.0850	0.0566	0.0184	0.47
0.73	0.59	0.0600	0.0651	0.0436	0.0133	0.31
0.61	0.49	0.0795	0.0880	0.06015	0.0181	0.34
—	0.08	0.0243	0.0237	0.0194	0.0018	0.019
—	0.30	0.0480	0.0478	0.0380	0.0048	0.025

The D^{10g}_{282} values of the two pressed cocoa butters are 0.03 and 0.04, whereas those of the extraction fats show the considerably higher values of 0.3–0.4 (other extraction fats varying between the extreme limits of about 0.2–0.5). The experimental data are listed in table 32.

By this procedure a reliable differentiation can be achieved between pure cocoa butter and extraction fats, the latter crude as well as refined. Although the sterol digitonides from Tenkawang fat (or Borneo tallow from *Shorea stenoptera*) and shea nut fat (or karité fat from *Butyrospermum parkii Kotschy*) have relatively high absorbances at 282 nm, their D^{10g}_{282} values are still lower than those of pure cocoa butter. These fats and also other common vegetable fats, therefore, do not interfere in this procedure. The presence of shea nut fat is revealed by its special characteristics a.o. by the high dextro-rotation of the sterol fraction and of the sterol-free unsaponifiable fraction (table 31).

This procedure clearly illustrates the various possibilities of applying sterol values to fat chemistry. The above calculations give the first practical application of a principle which requires and deserves further investigation in view of the natural variability of the various types of pressed cocoa butters and extraction fats.

In the above instances some applications of sterol analysis have been presented. In the analysis of complex fat mixtures one should also have recourse to other methods. In some special cases even one simple carefully selected characteristic value can furnish sufficient information. Sterol analysis has gained a place among our analytical procedures, which enable the chemist to accept the challenge offered by the complex nature of fat mixtures and the intricacies of interpretation caused by the large natural variability in composition.

SUMMARY

The occurrence of characteristic compounds (guide substances) in the unsaponifiable fraction of oils and fats can be applied to the analysis of fat mixtures. This type of fat analysis, denoted as guide (fat) analysis, makes use of characteristic values, based upon the properties of the sterols, tocopherols, and other minor components (sterol values, tocopherol values etc.).

Thus, of old adulteration of butter with vegetable oil has been detected by a rise in the sterol acetate melting point (i.e. the so-called phytosterol acetate test of BÖMER). However, in the analysis of mixtures of animal and vegetable fats, a chromatographic separation of cholesterol and the major phytosterols is desirable.

In *chapter 1* the application in actual practice of sterol values known from the literature and from unpublished results of DEN HERDER are discussed. In the following chapters the author's own investigations as to the chromatographic separation of sterols, especially of cholesterol and the phytosterols are described.

In *chapter 2* some experimental chromatographic separations are reviewed critically. A complete separation of cholesterol and the major phytosterols stigmasterol and β-sitosterol has been obtained in the paperchromatographic system: paraffin oil/acetic acid–water (84 : 16). The quantities of the sterols were evaluated quantitatively.

The fundamental principles of paperchromatography are discussed in *chapter 3*. Studying some physicochemical properties of the above-mentioned system, strong evidence was obtained as to the near-ideal nature of the partition process in this system. ΔR_M values for the introduction of several groups in the sterol molecule were calculated. The relation between structure and R_M values for a great number of sterols is given by: $R_M = 0.153\ (N_c - 24) - 0.05$ (where N_c = number of carbon atoms minus number of double bonds). This relationship may facilitate the structural analysis of as yet not identified sterols. As an example of such an application the structure of γ-sitosterol, according to the literature 24-a-ethyl cholesterol, is questioned.

Chapter 4 describes some problems of fat chemistry, which were solved by paperchromatography of the sterol mixtures. The sterol mixtures of several oils and fats were analysed thoroughly and their sterol values are compiled in table 20. Several edible vegetable oils contain a type of phytosterol, showing an R_F value quite similar to that of cholesterol. It was suggested that these phytosterols are either identical with brassicasterol or are isomers of this substance. The occurrence of these so-called third-band phytosterols hampers the reliable detection of cholesterol. Some general rules are given, according to which we still may conclude to the presence of cholesterol.

The total duration of the analysis is considerably decreased by the application of reversed-phase thin-layer chromatography, which is described in *chapter 5*. In the systems (A) undecane/acetic acid–water (92 : 8) and (B) undecane/acetic acid–acetoni-

trile (25 : 75) the sterol acetates were separated, the systems (C) undecane/acetic acid–water (90 : 10) and (D) undecane–tetradecane (80 : 20)/acetic acid–water–acetonitrile (22.5 : 2.5 : 75) being suitable for the separation of the free sterols.

The separation of sterols in these systems is analogous to that in the studied paper-chromatographic system. When 0.5% of bromine is added to the mobile phase of system B, di-unsaturated sterols move faster than the mono-unsaturated ones. Consequently, in this system cholesterol and the di-unsaturated third-band phytosterols were separated. Various sterol types can be differentiated by applying specific spray reagents. In the presence of third-band phytosterols cholesterol can be detected a.o. by means of the colour reaction with bismuth(III)chloride.

The aberrant behaviour of some special vegetable fats in the phytosterol acetate test of BÖMER is discussed in *chapter 6*. For most of the common vegetable oils the melting point diagram cholesterol acetate–phytosterol acetates has a maximum value. However, the phytosterol acetates of shea nut fat, Mowrah fat, cucumber seed oil, and pumpkin seed oil give rise to aberrant melting point diagrams with marked minimum values. Therefore, in this case the original phytosterol acetate test cannot give a reliable indication as to the presence of these vegetable fats.

The phytosterols of pumpkin seed oil ("Kürbiskernöl") are fractionated chromatographically into four components viz. the cucurbitasterols 1, 2, 3 and 4. Circumstantial evidence is given concerning the peculiar $\Delta 7$-sterol structures of these cucurbitasterols.

In *chapter 7* some examples are given of the application of sterol values to the analysis of fat mixtures. In special cases it is possible to calculate the amount of animal fat admixed to a vegetable fat. This procedure even enables a differentiation between an admixture of pure animal body fat and of the group of inedible animal fats such as White grease.

The percentage of provitamins D in the sterol mixtures is a further sterol value applicable to problems of fat analysis. An admixture of cocoa extraction fat to pure pressed cocoa butter can be detected by means of this provitamin D percentage.

SAMENVATTING

Van de aanwezigheid van enkele groepen van verbindingen zoals sterolen, tocoferolen en koolwaterstoffen in de onverzeepbare rest van oliën en vetten kan gebruik worden gemaakt bij de analyse van olie- en vetmengsels bijvoorbeeld voedingsvetten. In het bijzonder zal in dit proefschrift worden behandeld de analyse van vetmengsels met behulp van grootheden, behorend bij de uit het vet geïsoleerde sterolfractie. Deze grootheden zal ik verder aanduiden als „sterolgetallen".

Toevoeging van plantaardig vet aan dierlijk vet of omgekeerd kan o.a. worden aangetoond op grond van verschillen in de sterolgetallen van cholesterol en van de phytosterolmengsels.

Zo wordt reeds sinds lang een toevoeging van plantaardig vet aan boter aangetoond door een verhoging van het smeltpunt van het sterolacetaat, veroorzaakt door de aanwezigheid van phytosterolacetaat naast cholesterolacetaat (de z.g. phytosterolacetaatproef volgens BÖMER).

Bij de analyse van mengsels van dierlijke en plantaardige vetten is het nog beter cholesterol en de belangrijkste phytosterolen te scheiden d.m.v. chromatografie.

In *hoofdstuk 1* worden enkele praktische toepassingen van sterolgetallen: sterolgehalte en smeltpunt van het sterolacetaat behandeld, die bekend waren uit de literatuur en uit interne rapporten van DEN HERDER (RIJKSZUIVELSTATION).

In de volgende gedeelten van dit proefschrift worden eigen onderzoekingen beschreven betreffende de chromatografische analyse van sterolen en de toepassingen hiervan bij de analyse van mengsels van voedingsvetten.

Na een kritische beschouwing van de scheiding der sterolen m.b.v. papierchromatografie behandelt *hoofdstuk 2* de werkwijze waarmee een volledige scheiding van cholesterol en enkele phytosterolen wordt verkregen in het stelsel: vloeibare paraffine/azijnzuur–water (84 : 16). De gehalten van de verschillende aldus gescheiden sterolen werden kwantitatief bepaald.

De theoretische grondslagen van de papierchromatografie worden uiteengezet in *hoofdstuk 3*. Nadere bestudering van enkele eigenschappen van het paraffine/azijnzuur–water stelsel maakt aannemelijk, dat het chromatografische proces in dit stelsel als een voorbeeld van vrijwel ideale vloeistof-vloeistof verdelingschromatografie mag worden beschouwd. De R_M waarden van verscheidene groepen voorkomend in het sterolmolecuul werden berekend. Het verband tussen struktuur en R_M waarde blijkt voor een grote groep sterolen te worden benaderd door de betrekking:
$R_M = 0{,}153\ (N_c - 24) - 0{,}05$ (waarin N_c is aantal C-atomen van het molecuul verminderd met het aantal dubbele bindingen). Van deze relatie kan men gebruik maken bij onderzoekingen over de struktuur van nog niet geïdentificeerde sterolen. Zo moet bijvoorbeeld de aan γ-sitosterol toegeschreven struktuur 24-a-ethylcholesterol in twijfel worden getrokken.

Enkele toepassingen van deze papierchromatografische sterolanalyse op problemen

van de vetchemie worden besproken in *hoofdstuk 4*. Uit de sterolgetallen van verscheidene soorten eetbare oliën en vetten, bijeengebracht in tabel 20, bleek dat in vele oliën phytosterolen voorkomen met R_F waarden gelijk aan die van cholesterol. Het is gebleken dat deze derde-bandphytosterolen hetzij identiek zijn met brassicasterol of een hiermede isomere struktuur bezitten. De aanwezigheid van deze phytosterolen belemmert het aantonen van cholesterol in vetmengsels. Er werden echter enkele regels opgesteld die het mogelijk maken in vele gevallen toch de aanwezigheid van cholesterol vast te stellen.

Het bleek dat bij toepassing van dunnelaagchromatografie met omgekeerde fasen de analysetijd aanzienlijk wordt bekort. Deze techniek wordt besproken in *hoofdstuk 5*. Voor de analyse van sterolacetaten bleken de systemen (A) undecaan/azijnzuur–water (92 : 8) en (B) undecaan/azijnzuur–acetonitril (25 : 75), daarentegen voor de scheiding van de sterolen zelf de systemen (C) undecaan/azijnzuur–water (90 : 10) en (D) undecaan–tetradecaan (80 : 20)/azijnzuur–water–acetonitril (22,5 : 2,5 : 75) het best te voldoen. De scheidingen zijn nagenoeg identiek met die in het onderzochte papierchromatografische systeem. Door toevoeging van 0,5% broom aan de mobiele fase van systeem B verkrijgen sterolen met twee dubbele bindingen een grotere R_S waarde dan enkelvoudig onverzadigde sterolen. In dit „broomsysteem" worden cholesterol en de derde-bandphytosterolen geheel gescheiden. De verschillende steroltypen kunnen worden onderscheiden met behulp van specifieke kleurreagentia. Zo kan bijvoorbeeld cholesterolacetaat, ook bij aanwezigheid van derde-bandphytosterolen, worden aangetoond o.a. met bismuth(III)chloride.

In *hoofdstuk 6* wordt het afwijkende gedrag in de phytosterolacetaatproef van enkele bijzondere plantaardige vetten besproken. De phytosterolen uit de meeste plantaardige vetten geven een smeltpuntsdiagram cholesterolacetaat-phytosterolacetaat met een maximum. Diagrammen van phytosterolacetaten verkregen uit sheaboter, Mowrahvet, komkommerzaadolie en kalebaszaadolie vertonen echter duidelijke minima. De phytosterolacetaatproef geeft dan geen uitsluitsel omtrent de aanwezigheid van deze plantaardige vetten.

De sterolen van kalebaszaadolie ("Kürbiskernöl") werden chromatografisch gescheiden in vier componenten te weten de cucurbitasterolen 1, 2, 3 en 4. Voor deze cucurbitasterolen, alle met een $\Delta 7(8)$ dubbele binding, werden hypothetische struktuurformules opgesteld.

In *hoofdstuk 7* worden enkele voorbeelden besproken van de toepassing van sterolgetallen bij de analyse van vetmengsels. Bij toevoeging van dierlijk vet aan plantaardig vet kan men niet alleen de hoeveelheid van dit dierlijke vet ten naaste bij berekenen, maar soms kan men tevens aangeven of dit vet behoort tot de zuivere slachthuisvetten of tot de groep der technische dierlijke vetten zoals bijvoorbeeld White grease.

Het percentage aan provitaminen D in de sterolmengsels van bepaalde vetten kan eveneens als een sterolgetal worden beschouwd. Vermenging van zuivere geperste cacaoboter met zg. cacaoextractievet kan hiermede worden aangetoond.

REFERENCES

* These statements, experimental data and figures are based on our own experimental work.

Chapter 1

ALLAN, J. AND C. W. MOORE	1927	The phytosteryl acetate test and the phytosterol of sunflower seed oil. *J. Soc. Chem. Ind. (London) 46*, 433–434.
ANDERSON, R. J. AND R. L. SHRINER	1926	The phytosterols of corn oil. *J. Am. Chem. Soc. 48*, 2976–2986.
ANGLIN, C., J. H. MAHON, AND R. A. CHAPMAN	1955	Tocopherol content of canadian butterfat and its use in detecting adulteration. *J. Dairy Sci. 38*, 333–339.
AVIGAN, J. *et al.*	1960	The identification of desmosterol in serum and tissues of animals and man treated with MER-29. *J. Biol. Chem. 235*, 3123–3126.
BARTLET, J. C. AND D. G. CHAPMAN	1961	Detection of hydrogenated fats in butter fat by measurement of cis-trans conjugated unsaturation. *J. Agr. Food Chem. 9*, 50–53.
BAUER, K. H. UND R. NEU	1943	Zur Kenntniss des Kaffee-Öles III. *Fette, Seifen, Anstrichmittel 50*, 345–347.
BEHER, W. T., J. PARSONS, AND G. D. BAKER	1957	Comparative value of X-ray diffraction and infrared spectrophotometry in identifying certain sterols and their digitonides. *Anal. Chem. 29*, 1147–1151.
BERG, P. UND J. ANGERHAUSEN	1914a	Das Unverseifbare des Mowrahfettes und seine Bedeutung für den Nachweis von Mowrahfett in tierischen und pflanzlichen Speisefetten. *Z. Untersuch. Nahr. u. Genussm. 27*, 723–731.
—	1914b	Das Unverseifbare des Sheafettes und seine Beziehung zu dem Unverseifbaren des Mowrahfettes. *Z. Untersuch. Nahr. u. Genussm. 28*, 73–79.
—	1914c	Das optische Verhalten des sterinfreien Unverseifbaren der Fette. *Z. Untersuch. Nahr. u. Genussm. 28*, 145–150.
BERGMANN, W. AND E. M. LOW	1947	Contributions to the study of marine products XX. *J. Org. Chem. 12*, 67–75.
BERNSTEIN, S. AND E. S. WALLIS	1939	Studies in the sitosterol complex. The isolation of α_3-sitosterol. *J. Am. Chem. Soc. 61*, 1903–1904.
BERTRAM, S. H.	1937	Der Nachweis tierischer Fette und Öle insbesondere gehärterter Trane in Fettgemischen. *Seifen – Öle – Fette – Wachse 1*, 13–14.
BHALERAO, V. R. AND F. A. KUMMEROW	1956a	Modification of the refractive index method for the detection of foreign fats in dairy products. *J. Dairy Sci. 39*, 947–955.
—	1956b	A summary of methods for the detection of foreign fats in dairy products. *J. Dairy Sci. 39*, 956–963.
BIEFER, K. W. UND H. HADORN	1956	Nachweis von Sojabohnenöl auf Grund seines Gehaltes an δ-Tocopherol. *Mitt. Gebiete Lebensm. u Hyg. 47*, 445–455.

BLADON, P., H. B. HENBEST, AND G. W. WOOD	1952	Ultra-violet adsorption spectra of ethylenic centres. *J. Chem. Soc.* 2737–2744.
BLAIZOT, P. ET M. T. MELLIER	1956	Les tocophérols. *Oléagineux 11*, 539–543, 645–650.
BOCK, F.	1938	Über die bei einigen Avertebraten vorkommenden antirachitischen Provitaminen. *Z. physiol. Chem. 256*, 33–41.
BÖMER, A. UND K. WINTER	1901a	Beiträge zur Analyse der Fette VI. Über einige Ester des Cholesterins und Phytosterins. *Z. Untersuch. Nahr. u. Genussm. 4*, 865–888.
BÖMER, A.	1898	Beiträge zur Analyse der Fette I. Über Gewinnung und Krystallformen von Cholesterin und Phytosterin aus Fetten, *Z. Untersuch. Nahr. u. Genussm. 1*, 21–49.
—	1901b	Beiträge zur Analyse der Fette VII. Über den Nachweis von Pflanzenfetten in Thierfetten mittels der Phytosterinacetat-Probe. *Z. Untersuch. Nahr. u. Genussm. 4*, 1070–1095.
—	1902	Beiträge zur Analyse der Fette VIII. Über den Nachweis von Margarine in Butter mittels der Phytosterinacetat-Probe. *Z. Untersuch. Nahr. u Genussm. 5*, 1018–1035.
BONSTEDT, K.	1928	Über einige Sterine des Pflanzenreichs. *Z. physiol. Chem. 176*, 269–281.
BROWN, F.	1952	Separation of tocopherol mixtures occurring in natural products by paper chromatography. *Biochem. J. 51*, 237–239.
BROWN, H. H. *et al.*	1954	Rapid procedure for determination of free serum cholesterol. *Anal. Chem. 26*, 397–399.
CANADIAN FOOD AND DRUG REGULATIONS	1954	Section B 08006, July 14.
CANNON, J. H.	1955	Report on foreign fats in dairy products. Sterol acetate test. *J. Assoc. Offic. Agr. Chemists 38*, 338–347.
—	1956	Report on foreign fats in dairy products. Sterol acetate test. *J. Assoc. Offic. Agr. Chemists 39*, 359–365.
—	1957	Report on foreign fats in dairy products. Sterol acetate test. *J. Assoc. Offic. Agr. Chemists 40*, 517–522.
—	1958	Report on foreign fats in dairy products. Sterol acetate test. *J. Assoc. Offic. Agr. Chemists 41*, 268–271. see also:
OFFICIAL METHODS OF ANALYSIS	1960	9th Edition, Association of Official Agricultural Chemists, Washington pag. 371.
CAPELLA, P., G. DE ZOTTI, AND G. JACINI	1960	Chromatography on silicic acid of the unsaponifiable matter of fats. *J. Am. Oil Chemists' Soc. 37*, 564–567.
CODEX ALIMENTARIUS NEDERLAND	1914	no. 2 Spijsvetten en Kaas, pag. 3.
COOK, R. P.	1958	Cholesterol, first ed., Acad. Press. New York.
—	1961	Reactions of steroids with acetic anhydride and sulphuric acid. *Analyst 86*, 373–381.
COPIUS PEEREBOOM, J. W. EN J. B. ROOS	1960	Die Chromatographie von Sterinen und ihre Anwendung für den Nachweis tierischer und pflanzlicher Fette nebeneinander. *Fette, Seifen, Anstrichmittel 62*, 91–100.
COPIUS PEEREBOOM, J. W.	1961	Toepassing van de dunnelaagchromatografie bij de analyse van oliën en vetten. *Chem. Weekblad 57*, 625–630.

DELSAL, J. L.	1943	Remarques sur le dosage gravimétrique du cholestérol par la natigine. *Bull. soc. chim. biol. 25*, 361–363.
DEN HERDER, P. C. AND C. I. KRUISHEER	1953	The total cholesterol content of Dutch creamery butter. Proc. XIII Int. Dairy Congress, The Hague. 3, 1359–1361.
DEN HERDER, P. C.	1950	Système de recherches pour déceler la falsification du beurre par la margarine. VIII Congrès Intern. Ind. Agricoles, Bruxelles IV, 68–79.
—	1954	Toepassing van sterolen bij het vetonderzoek, unpublished results.
—	1955	Detection of adulteration of butter with foreign fats by examination of the sterols. *Neth. Milk Dairy J. 9*, 261–274.
DIRKX, I. P.	1961	Spectropolarimetrie. Een bijdrage tot de instrumentele ontwikkeling en stereochemische toepassing. Thesis, Amsterdam, V.R.B. Groningen. We gratefully thank Dr. I. P. Dirkx of measuring the optical rotation curves.
DREKTER, I. J., A. E. SOBEL, AND S. NATELSON	1936	Fractionation of cholesterol in blood by precipitation as pyridine cholesteryl sulfate and cholesterol digitonide. *J. Biol. Chem. 115*, 391–399.
ELSEVIER'S ENCYCLOPAEDIA OF ORGANIC CHEMISTRY	1952	Elsevier Publ. Co., Amsterdam. Vol. 14 (supplement).
FERNHOLZ, E. AND H. B. MC.PHILLABY	1941	Isolation of a new phytosterol: campesterol. *J. Am. Chem. Soc. 63*, 1155–1156.
FIESER, L. F. AND M. FIESER	1959	Steroids. Reinhold Publ. Corp., New York.
FRITZSCHE, M.	1913	Die Prüfung von tierischen Fetten auf Phytosterin. *Z. Untersuch. Nahr. u. Genussm. 26*, 644–648.
GATTORTA, G. E M. GISONDI	1961	Tentativi per la caratterizzazione dei grassi animali dai vegetali a mezzo della diffrazione dei raggi X nei constituenti dell' insaponificabile. *Riv. Ital. Sostanza Grasse 38*, 73–75.
GROSSFELD, J.	1938	Zur praktischen Untersuchung der Speisefette. *Z. Untersuch. Lebensm. 76*, 340–350.
GUEX, G.	1957	Die Umwandlung von Rinderfett in synthetische Butter (Bolzella). *Fleischwirtschaft 9*, 494–495.
HACKMANN, J. TH.	1950	Preparation of cholesterol from wool grease by means of addition products. *Rec. trav. chim. 69*, 433–438.
HADORN, H. UND R. JUNGKUNZ	1949	Nachweis und annährende Bestimmung des Olivenöles auf Grund seines Squalen-Gehaltes. *Mitt. Gebiete Lebensm. u Hyg. 40*, 61–95.
—	1950	Weitere Untersuchungen über die Squalenzahl. *Mitt. Gebiete Lebensm. u Hyg. 41*, 435–443.
—	1954a	Beitrag zum Nachweis von pflanzlichen Fetten und Ölen mittelst der Phytosterinacetatprobe. *Mitt. Gebiete Lebensm. u Hyg. 45*, 389–397.
—	1954b	Über den Gehalt am Unverseifbarem und Gesamtsterinen in Speisefetten und Speiseölen. *Mitt. Gebiete Lebensm. u Hyg. 45*, 397–401.
HANSSEN, E. G., W. STURM UND H. J. V. DRACHENFELS	1960	Untersuchungen an einem "synthetischen" Butterfett. *Z. Untersuch. Lebensm. 111*, 381–393.

HAWLEY, H.	1933	The phytosteryl acetate test as a routine method in the examination of butter fats with border-line R.M. values. *Analyst 58*, 529–531.
HERRAIZ, M. L. AND H. G. DE ALVAREZ HERRERO	1949	Tocopherol content of edible oils sold in the markets in the city of Buenos Aires. *Ann. N.Y. Acad. Sci. 32*, 306–308.
HESSE, O.	1878	Über Phytosterin und Cholesterin. *Ann. 192*, 175–179.
HOLDE, D.	1906	Über aktuelle Fragen der Fettchemie. *Z. angew. Chem. 19*, 1604–1610.
HORWITZ, W.	1954	Report on dairy products. *J. Assoc. Offic. Agr. Chemists 37*, 223–231.
HOTON, L.	1909	De l'importance des indices de réfraction pour la distinction des beurres purs d'avec les beurres falsifiés. *Ann. fals. et fraudes 2*, 8–26.
HUEBNER, V. R.	1961	The analysis of glycerides by high temperature gas-liquid partition chromatography. *J. Am. Oil Chemists' Soc. 38*, 628–631.
ICHIBA, A.	1936	On the phytosterol of wheat germ oil. *Sci. papers Inst. Phys. Chem. Research (Tokyo) 28*, 112–124.
IDLER, D. R. AND C. A. BAUMANN	1953	Skin sterols III. Sterol structure and the Liebermann-Burchard reaction. *J. Biol. Chem. 203*, 389–396.
I.U.P.A.C. MONOGRAPHS	1954	Standard methods for the analysis of oils and fats. Société d'Edition d'Enseignement supérieur, Paris. pag. 93.
JACINI, G. *et al.*	1960	Méthodes pour distinguer les huiles d'olive des huiles comportant un mélange. *Inf. Oléicoles intern. 9*, 81–99.
JAEGER, F. M.	1907	Enkele opmerkingen over de phytosterylacetaat-reaktie van Bömer, als middel ter bepaling van de vervalsching van dierlijke en plantaardige vetten. *Chem. Weekblad 4*, 1–10.
JANTZEN, E. UND W. GOHDES	1934	Über das Vorkommen von glucosidisch gebundenen Sterinen in Sojaöl. *Biochem. Z. 272*, 167–171.
KABARA, J. J., J. T. MCLAUGHLIN, AND C. A. RIEGEL	1961	Quantitative microdetermination of cholesterol using tomatine as precipitating agent. *Anal. Chem. 33*, 305–307.
KAUFMANN, H. P.	1941a	Über die Bedeutung der Begleitstoffe in natürlichen Fetten und ihr Schicksal bei der Raffination I. *Fette, Seifen, Anstrichmittel 48*, 53–59.
—	1941b	Untersuchung der Sterine in Fetten. *Fette, Seifen, Anstrichmittel 48*, 537–540.
KAUFMAN, H. P. *et al.*	1950	Über die Bedeutung der Begleitstoffe in natürlichen Fetten und ihr Schicksal bei der Raffination II. *Fette, Seifen, Anstrichmittel 52*, 35–38.
—	1958	Analyse der Fette und Fettprodukte Teil I. Springer-Verlag, Berlin. pag. 524.
—	1959	Die Papier-Chromatographie auf dem Fettgebiet XXX, XXXI, XXXV. *Fette, Seifen, Anstrichmittel 61*, 523–528; 631–636; 768–770.
—	1962	Die Dünnschicht-Chromatographie auf dem Fettgebiet VI, VII, VIII. *Fette, Seifen, Anstrichmittel 64*, 1–6; 81–85; 214–217.

KAUFMANN, H. P. UND Z. MAKUS	1960	Die Dünnschicht-Chromatographie auf dem Fettgebiet I. *Fette, Seifen, Anstrichmittel 62*, 1014–1020.
—	1961a	Die Papier-Chromatographie auf dem Fettgebiet XLIV. *Fette, Seifen, Anstrichmittel 63*, 125–132.
—	1961b	Die Dünnschicht-Chromatographie auf dem Fettgebiet II, III und IV. *Fette, Seifen, Anstrichmittel 63*, 235–238; 689–691; 807–811.
KLOSTERMANN, M.	1913	Zum Nachweis von Pflanzenfetten in tierischen Fetten. *Z. Untersuch. Nahr. u Genussm. 26*, 433–437.
KLOSTERMANN, M. UND H. OPITZ	1914a	Über das Cholesterin und seine Bestimmung in Fetten. *Z. Untersuch. Nahr. u Genussm. 27*, 713–723.
—	1914b	Über das Phytosterin und seine Bestimmung in Pflanzenfetten. *Z. Untersuch. Nahr. u Genussm. 28*, 138–145.
KOFLER, L. UND E. SCHAPER	1935	Über eine Mikro-Phytosterinacetatprobe. *Fettchem. Umschau 42*, 21–26.
KRUCKENBERG, W.	1948	Über eine Bestimmungsmethode von Cholesterin in Fetten. *Z. physiol. Chem. 283*, 68–70.
KRUISHEER, C. I.	1955	The total tocopherol (vitamin E) content of Dutch butter. *Neth. Milk Dairy J. 9*, 275–290.
KÜHN, B. UND J. WEWERINKE	1914	Über den Phytosterin-Nachweis in tierischen Fetten mit Abscheidung der Sterine durch Digitonin-Fällung. *Z. Untersuch. Nahr. u Genussm. 28*, 369–379.
KÜHN, B., F. BENGEN UND J. WEWERINKE	1915	Über den Phytosterin-Nachweis in tierischen Fetten nach der Bömer'schen Acetatprobe mit Abscheidung der Sterine durch Digitonin-Fällung. *Z. Untersuch. Nahr. u Genussm. 29*, 321–329.
KUZDZAL-SAVOIE, S. ET W. KUZDZAL	1960	La recherche du lait de vache ajouté au lait de chèvre. Application au cas du fromage. *Lait 40*, 393–407.
LADA, A.	1954	Volatile acids from oxidation products of cholesterol and phytosterols. *J. Assoc. Offic. Agr. Chemists 37*, 551–552.
LANGE, W., R. G. FOLZENLOGEN, AND D. J. KOLP	1949	Colorless crystalline perchloric and hexafluorophosphoric acid salts of sterols. *J. Am. Chem. Soc. 71*, 1733–1737.
LANGE, W.	1950	Cholesterol, phytosterol, and tocopherol content of food products and animal tissues. *J. Am. Oil Chemists' Soc. 27*, 414–422.
LARSEN, C. D. AND H. P. MORRIS	1943	Thermal decomposition of lard. *J. Am. Chem. Soc. 65*, 2301–2303.
LEMBKE, A., W. KAUFMANN UND P. L. FARFALETTI-CASALI	1953	Spektralanalytische Messungen an Fetten. *Milchwissenschaft 8*, 118–120.
MAHON, J. H. AND R. A. CHAPMAN	1954	Detection of adulteration of butter with vegetable oils by means of the tocopherol content. *Anal. Chem. 26*, 1195–1198.
MAHON, J. H., C. ANGLIN, AND R. A. CHAPMAN	1955	Tocopherol content of the fat of dairy products as an index of adulteration. *J. Dairy Sci. 38*, 1089–1095.
MARCUSSON, J. UND H. SCHILLING	1913	Unterscheidung von Tier- und Pflanzenölen. *Chemiker Ztg. 37*, 1001–1002.
MARCUSSON, J. UND G. MEGENHEIM	1914	Die unverseifbaren Bestandteile natürlicher und gehärteter Fette. *Z. angew. Chem. 27*, 201–203.
MAZUR, Y., A. WEIZMANN, AND F. SONDHEIMER	1958	The carbon skeleton of the α-sitosterols. *Bull. Research Council Israel 7a*, 82–83.

McGugan, W. A.	1959	A chromatographic method of detecting foreign fats in butter-fat. Proc. XV Int. Dairy Congress, London. 3, 1534–1538.
Meyer, H.	1961	Ein Diskussionsbeitrag zur spektrophotometrischen Unterscheidung von Kakaopreszbutter und Kakaoextraktionsfett. *Mitt. Gebiete Lebensm. u Hyg. 52*, 419–421.
Michaels, G. D. *et al.*	1958	Technics for separation of plasma cholesterol esters for determination of iodine value and of cholesterol. *Proc. Soc. Exptl. biol. Med. 98*, 826–829.
Moore, P. R. and C. A. Baumann	1952	Skin sterols I. Colorimetric determination of cholesterol and other sterols in skin. *J. Biol. Chem. 195*, 615–621.
Morris, R. J., R. D. McPhee, and E. L. Randall	1952	Ultraviolet spectrophotometry in detection of food product substitutes. *Anal. Chem. 24*, 1396–1399.
Nazir, D. J. and N. G. Magar	1959	Tocopherol content of indian butter and its use in detection adulteration of butterfat. *Indian J. Dairy Sci. 12*, 125–132.
Nederlandse Norm N.E.N. 1046	1961	Onderzoekingsmethoden plantaardige en dierlijke oliën en vetten, 's-Gravenhage. pag. 71.
Olig, A.	1914	Der Bömer'sche Phytosterinnachweis nach dem abgeänderten Marcusson-Schilling'schen Digitonin-ausfällungsverfahren. *Z. Untersuch. Nahr. u Genussm. 28*, 129–138.
Philippe, E. und M. Henzi	1944	Über den Nachweis von Olivenöl in Speiseöl. *Mitt. Gebiete Lebensm. u Hyg. 35*, 94–111.
Pollak, O. J. and B. Wadler	1952	Rapid turbidimetric assay of cholesterol. *J. Lab. Clin. Med. 39*, 791–794.
Privett, O. S. and M. L. Blank	1961	A new method for the analysis of component mono-, di-, and triglycerides. *J. Lipid Res. 2*, 37–43.
Quaife, M. L., R. P. Geyer, and H. R. Bolliger	1959	Rapid paper chromatographic microassay of free and ester cholesterol of blood. *Anal. Chem. 31*, 950–955.
Riffart, H. und H. Keller	1934	Colorimetrische Bestimmung mit dem Stufenphotometer von Zeiss bei der Untersuchung von Lebensmitteln. *Z. Untersuch. Lebensm. 68*, 113–138.
Roos, J. B.	1956	Die Qualitätsbeurteilung von Schweineschmalz im Hinblick auf den Nachweis von raffiniertem "White Grease". *Fette, Seifen, Anstrichmittel 58*, 118–121.
Roos, J. B. en D. M. van Dijk	1961a	Unpublished results.
Roos, J. B.	1958	Unpublished results.
—	1961b	Unpublished results.
Salkowski, E.	1887	Beiträge zu den Untersuchungsmethoden des Leberthrans und der Pflanzenöle. *Z. anal. Chem. 26*, 557–582.
Schoenheimer, R. und H. Dam	1933	Über die Spaltbarkeit und Löslichkeit von Sterindigitoniden. *Z. physiol. Chem. 215*, 59–63.
Schoenheimer, R. and W. M. Sperry	1934	A micromethod for the determination of free and combined cholesterol. *J. Biol. Chem. 106*, 745–760.

Schmidt-Thomé, J. und H. Augustin	1942	Über eine titrimetrische Mikrobestimmung des Cholesterins mit Hilfe der Blutkörperchenhämolyse und ihre Anwendung auf Serum. *Z. physiol. Chem. 275*, 190–207.
Schoorl, N.	1909	Commentaar op de codex alimentarius no. 2. Spijsvetten en kaas. Noordhoff, Groningen.
Schramme, A.	1939	Zur Bestimmung der Sterine. *Fette, Seifen, Anstrichmittel 46*, 443–444.
Shipe, W. F.	1954	Cited by V. R. Bhalerao and F. A. Kummerov. *J. Dairy Sci. 39*, (1956) 956–963.
Sperry, W. M. and M. Webb	1950	A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem. 187*, 97–106.
Sprinkmeyer, H. und A. Diedrichs	1914	Zum Nachweis von pflanzlichen Fetten in tierischen Fetten. *Z. Untersuch. Nahr. u Genussm. 28*, 236–244.
Staub, H.	1947	Titrimetrische, klinische Cholesterinbestimmung II. *Schweiz. med. Wochenschr.* 77, 60–62; Cited in *Z. Untersuch. Lebensm. 89*, (1949) 201.
Steuart, D. W.	1923	Unsaponifiable matter and sterols of edible fats. *Analyst 48*, 155–160.
Swaving, A. J.	1939	l'Indice Reichert-Meissl peut-il nous fixer sur la purité d'un beurre? *Lait 19*, 462–468.
Täufel, K. und W. Preisz	1929	Über die Umesterung von Neutralfetten mit Buttersäure. *Z. Untersuch. Lebensm. 58*, 425–433.
Täufel, K., M. Achtzehn und Ch. Franzke	1958	Über die Umesterung niederer Fettsäuren in natürliche Fette und Öle. *Nahrung 2*, 178–192.
Tanret, M. C.	1890	Sur un nouveau principe immédiat de l'ergot de seigle: l'ergostérine. *Ann. chim. phys.* [6] *20*, 289–297.
Thornton, M. H. *et al.*	1940	Sterol glucosides from expressed soybean oil. *J. Am. Chem. Soc. 62*, 2006–2008.
Thuesen, W.	1943	Über eine fällungstitrimetrische Mikromethode zur schnellen Bestimmung des Cholesterins. *Acta Physiol. Scand. 6*, 307–318.
—	1953	The cholesterol content of marine animal oils, commercially produced in Norway. *Norwegian Whaling Gaz. 42*, no. 1, 25–29.
Trappe, W.	1942	Eine einfache Methode zur getrennten, quantitativen Bestimmung von freiem und verestertem Cholesterin im Blutserum ohne Digitoninfällung und Verseifung. *Z. physiol. Chem. 273*, 177–190.
Vahouny, G. V. *et al.*	1960a	Rapid quantitative determination of total and free cholesterol with anthrone reagent. *Anal. Biochem. 1*, 371–381.
—	1960b	Determination of 3-β-hydroxy sterols with anthrone reagent. *Arch. Bioch. Biophys. 86*, 210–214.
Van der Wal, R. J.	1960	Calculation of the distribution of the saturated and unsaturated acyl groups in fats from pancreatic lipase hydrolysis data. *J. Am. Oil Chemists' Soc. 37*, 18–20.
Van Rijn, J. J. L.	1899	Onderzoekingen over de samenstelling van de Nederlandsche boter, Leeuwarden.
Vitagliano, M. e A. d'Ambrosio	1957a	Ricerche sull' agguinta di grassi di altra origine all' olio di oliva. *Olearia 11*, 169–184.
Vitagliano, M. e E. Turri	1958	La misura dei constituenti minori negli oli di oliva italiani. *Olearia 12*, 145–156.

VITAGLIANO, M.	1961	Sopra il punto di fusione dell' aceto sterolico. *Olearia 15*, 5–9.
WAGHORNE, D. AND C. D. BALL	1952	Semimicromethod for the determination of plant sterols. *Anal. Chem. 24*, 560–564.
WALLIS, E. S. AND E. FERNHOLZ	1936	α-sitosterol. *J. Am. Chem. Soc. 58*, 2446–2448.
WEBSTER, W. W., C. W. NICHOLS, AND I. L. CHAIKOFF	1959	Determination of 3-β-OH sterols in serum and tissues by the anthrone reagent. *Arch. Bioch. Biophys. 82*, 195–202.
WEIGENSBERG, B. I. AND G. C. MCMILLAN	1959	Ultraviolet spectrophotometric method for the determination of cholesterol. *Am. J. Clin. Path. 31*, 16–25.
WERNER, P.	1911	Beiträge zum Nachweis von Beimischungen tierischen Fettes zu Pflanzenfett mit Hilfe von Cholesterin und Phytosterin. Thesis, Berlin. cited in *Z. Untersuch. Nahr. u Genussm. 21*, (1911) 691.
WINDAUS, A. UND A. HAUTH	1906b	Über Stigmasterin, ein neues Phytosterin aus Calabar-Bohnen. *Ber. 39*, 4378–4384.
WINDAUS, A. UND A. WELSCH	1909b	Über die Phytosterine des Rüböles. *Ber. 42*, 612–616.
WINDAUS, A.	1906a	Über ein Verfahren zur Trennung tierischem und pflanzlichem Cholesterin. *Chemiker Ztg. 30*, 1011–1012.
—	1909a	Über die Entgiftung der Saponine durch Cholesterin. *Ber. 42*, 238–246.
—	1910	Über die quantitative Bestimmung des Cholesterins und der Cholesterinester in einigen normalen und pathologischen Nieren. *Z. physiol. Chem. 65*, 110–117.
WINDHAM, E.	1957	Report on foreign fats in dairy products. Tocopherol test. *J. Assoc. Offic. Agr. Chemists 40*, 522–531.
WITTKA, F.	1960	Die Krise in der Olivenöl-Industrie Italiens. *Seifen – Öle – Fette – Wachse 86*, 110–112.
WOLFF, G. ET J. P. WOLFF	1953	Méthodes d'analyse et de contrôle industriel des matières grasses. Dunod, Paris. pag. 109.
WYCOFF, H. D. AND J. PARSONS	1957	Chromatographic microassay for cholesterol and cholesterol esters. *Science 125*, 347–348.
ZWIKKER, J. J. L.	1917	De afzondering der sterinen uit vetten. *Pharm. Weekblad 54*, 101–102.
ZETZSCHE, F.	1898	Beiträge zur Phytosterinprobe. *Pharm. Zentralhalle 39*, 877–883.

BARBIER, M. UND O. SCHINDLER	1959	Isolierung von 24-Methylencholesterin aus Königinnen und Arbeiterinnen der Honigbiene (Apis mellifica L.). *Helv. Chim. Acta 42*, 1998–2005.
BARTON, D. H. R.	1945	The application of the method of molecular rotation differences to steroids, parts I and V. *J. Chem. Soc.* 813–818 and 1354–1355.
BATE-SMITH, E. C. AND R. G. WESTALL	1950	Chromatographic behaviour and chemical structure I. *Biochim. et Biophys. Acta 4*, 427–439.
BAUER, K. H. UND H. MOLL	1939	Zur Kenntnis der unverseifbaren Bestandteile des Sheafettes. *Fette, Seifen, Anstrichmittel 46*, 560–563.
BEERTHUIS, R. K. EN J. H. RECOURT	1960a	Sterol analysis by gaschromatography. *Nature 186*, 372–374.
—	1960b	Sterol analysis by gaschromatography. *Colloques Int. Centre Nat. Rech. Scient. no XCIX*, Marseilles.
BENSON, A. A. *et al.*	1950	The path of carbon in photosynthesis V. Paper chromatography. *J. Am. Chem. Soc. 72*, 1710–1719.
BERG, P. UND J. ANGERHAUSEN	1914a	Das Unverseifbare des Mowrahfettes und seine Bedeutung für den Nachweis von Mowrahfett in tierischen und pflanzlichen Speisefetten. *Z. Untersuch. Nahr. u Genussm. 27*, 723–731.
—	1914b	Das Unverseifbare des Sheafettes und seine Beziehung zu dem Unverseifbaren des Mowrahfettes. *Z. Untersuch. Nahr. u Genussm. 28*, 73–79.
BERGMANN, W. AND E. M. LOW	1947	Contributions to the study of marine products XX. *J. Org. Chem. 12*, 67–75.
BERGMANN, W.	1940	The splitting of digitonides. *J. Biol. Chem. 132*, 471–472.
—	1952	Sterols. *Progress in the chemistry of fats and other lipids. 1*, 18–69.
—	1953	The plant sterols. *Annual review of plant physiology 4*, 383–426.
BHACCA, N. S., L. F. JOHNSON, AND J. N. SHOOLERY	1962	N.M.R. Spectra catalog. Varian Associates.
BOCK, F. UND T. WETTER	1938	Über die bei einigen Avertebraten vorkommenden antirachitischen Provitamine. *Z. physiol. Chem. 256*, 33–41.
BOER, A. G. *et al.*	1936	A naturally occurring chicken provitamin D. *Koninkl. Ned. Akad. Wetenschap. Proc. 39*, 622–632.
BONSTEDT, K.	1928	Über einige Sterine des Pflanzenreichs. *Z. physiol. Chem. 176*, 269–281.
BRICKER, C. E. AND K. H. ROBERTS	1949	Determination of end unsaturation in organic compounds. *Anal. Chem. 21*, 1331–1334.
BROCKMANN, H. UND F. VOLPERS	1947	Ein neues Verfahren zur Trennung farbloser Stoffe. *Ber. 80*, 77–82.
BUSH, I. E.	1960	The biosynthesis and secretion of adrenocortical steroids. *Biochem. Soc. Symposia (Cambridge) 18*, 1-23.
—	1961	The chromatography of Steroids. Pergamon Press, Oxford.
CONSDEN, R., A. H. GORDON, AND A. J. P. MARTIN	1944	Qualitative analysis of proteins: a partition chromatographic method using paper. *Biochem. J. 38*, 224–232.
COOK, R. P.	1961	Reactions of steroids with acetic anhydride and sulphuric acid. *Analyst 86*, 373–381.

COPIUS PEEREBOOM, J. W. EN J. B. ROOS	1960a	Die Chromatographie von Sterinen und ihre Anwendung für den Nachweis tierischer und pflanzlicher Fette nebeneinander. *Fette, Seifen, Anstrichmittel 62*, 91–100.
COPIUS PEEREBOOM, J. W.	1960b	The analysis of plasticizers by microadsorption chromatography. *J. Chromatog. 4*, 323–328.
COPIUS PEEREBOOM, J. W., J. B. ROOS EN H. W. BEEKES	1961	The paperchromatography of some sterols, provitamins and triterpenoid alcohols. *J. Chromatog. 5*, 500–514.
COPIUS PEEREBOOM, J. W. EN H. W. BEEKES	1962	Separation of some sterols and sterol acetates by thin-layer chromatography. *J. Chromatog. 9*, 316–320.
CORMIER, M. ET P. JOUAN	1957	Séparation des lipides par chromatographie sur papier imprégné d'acide silicique. *Bull. soc. chim. biol. 39*, 1321–1327.
DANIEL, D., E. LEDERER ET L. VELLUZ	1945	Sur les constituants de la graisse de laine. *Bull. soc. chim. biol. 27*, 218–225.
DAVIS, R. B., J. M. MCMAHON, AND G. KALNITSKY	1952	Separation of D vitamins from other sterols by paper chromatography and the quantitative determination of 7-dehydrocholesterol. *J. Am. Chem. Soc. 74*, 4483–4484.
DE ZOTTI, G., P. CAPELLA E G. JACINI	1959	Papier-chromatographie der Sterine. *Fette, Seifen, Anstrichmittel 61*, 1114–1119.
DIRSCHERL, W. UND H. NAHM	1943	Die Seitenkette des β- und γ-Sitosterins. *Ann. 555*, 57–69.
DOBRINER, K., E. R. KATZENELLENBOGEN, AND R. NORMAN JONES	1953	Infrared absorption spectra of steroids. Interscience, New York.
DONIA, R. A., A. C. OTT, AND N. DRAKE	1957	Determination of stigmasterol in soysterols by radioactive isotope dilution. *Anal. Chem. 29*, 464–467.
ELSEVIER'S ENCYCLOPAEDIA OF ORGANIC CHEMISTRY	1952	Elsevier's Publ. Comp. Amsterdam. vol. 14 (supplement).
EISNER, J. *et al.*	1962	Gas chromatography of unsaponifiable matter I. Butter and margarine sterols. *J. Assoc. Offic. Agr. Chemists 45*, 337–342.
FIESER, L. F. AND W. P. CAMPBELL	1930	Concerning dehydroabietic acid and the structure of Pine resin acids. *J. Am. Chem. Soc. 60*, 159–170.
FINCKE, A. UND I. MÖSSNER	1962	Untersuchungen zur Reinheitsprüfung von Kakaobutter und Schokoladenfetten. *Süsswaren 6*, 724–727.
FORTI, C.	1890	Gegenwart von Cholesterin und eines löslichen Kohlehydrats in den Samen der Melonen. *Cited in Chem. Zentr. 61*, 581–582.
FRANC, J. UND J. JOKL	1956	Über die Beziehung zwischen Dipolmoment und Rf-Wert homologer Reihen. *Collection Czech. Chem. Commun. 21*, 1161–1169.
FRANCO, P. E G. PIO	1962	Determinazione di composti steroidici mediante gascromatografia con rivelatore a jonizzazione di fiamma. *Riv. Ital. Sostanza Grasse 39*, 302–307.
GALINOVSKY, F. UND O. VOGEL	1948	Chromatographische Trennung epimerer Sterin-alkohole. *Monatsh. Chem. 79*, 325–326.
GRACIAN, J. Y J. MARTEL	1959	Chromatografia en papel de esteroles. *Grasas y aceitas 10*, 7–11.

Hais, I. M. und K. Macek	1958	Handbuch der Papierchromatographie I. G. Fischer Verlag, Jena. pag. 64, 345 etc.
Hamilton, B. and W. O. Kermack	1952	The isolation of α-spinasterol from colocynth. *J. Chem. Soc.* 5051–5052.
Hasselstrom, T.	1949	Pine tree resenes in the biogenesis of the resin acids. *Papertrade J. 128*, 17–21.
Hunter, R. F., A. D. Scott, and I. R. Edisbury	1942	Palm oil carotenoids. *Biochem. J. 36*, 697–702.
Idler, D. R. and C. A. Baumann	1952	Isolation of $\Delta 7$-cholestenol. *J. Biol. Chem. 195*, 623–628.
—	1953b	Sterol structure and the Liebermann-Burchard reaction. *J. Biol. Chem. 203*, 389–396.
Idler, D. R. *et al.*	1953a	Separation and tentative identification of two new sterols from oats. *J. Am. Chem. Soc. 75*, 1712–1715.
Idler, D. R. and U. H. M. Fagerlund	1955	Marine sterols I. Isolation of 24-methylene cholesterol. *J. Am. Chem. Soc. 77*, 41412–41413.
—	1957	Marine sterols III. *J. Am. Chem. Soc. 79*, 988–991.
Izmailov, N. A. and M. S. Schraiber	1938	A drop-chromatographic method of analysis and its utilization in pharmacy. Farmatsiya 3, 1–7. Cited in *Chem. Abstr. 34* (1940) 855[8].
Janecke, H. und I. Maass-Goebels	1960	Beobachtungen bei Vitaminbestimmungen. *Z. anal. Chem. 178*, 161–172.
Johnson, J. L., M. F. Grostic, and A. O. Jensen	1957	Determination of stigmasterol in soybean sterol mixtures by infrared method. *Anal. Chem. 29*, 468–470.
Kabasakalian, P. and A. Basch	1960	Paper chromatography of steroids. *Anal. Chem. 32* 458–461.
Kaiser, E. and B. M. Kagan	1956	Separation of vitamin A alcohol and vitamin A esters by paper chromatography. *Arch. Bioch. Biophys. 63*, 118–121.
Katz, S.	1960	The location and tentative identification of steroids on paper chromatograms by means of a system of color tests. *Arch. Bioch. Biophys.* 91, 54–60.
Kaufmann, H. P. und W. H. Nitsch	1954	Die Papier-Chromatographie auf dem Fettgebiet XVI. *Fette, Seifen, Anstrichmittel 56*, 154–158.
Kaufmann, H. P. *et al.*	1958a	Analyse der Fette und Fettprodukte. Teil II. Springer-Verlag, Berlin.
Kaufmann, H. P. und E. Mohr	1958b	Die Papier-Chromatographie auf dem Fettgebiet XXIV. *Fette, Seifen, Anstrichmittel 60*, 165–177.
Kaufmann, H. P. und Z. Makus	1960	Die Papier-Chromatographie auf dem Fettgebiet XL. *Fette, Seifen, Anstrichmittel 62*, 153–160.
Kaufmann, H. P., Z. Makus und B. Das	1961	Dünnschicht-Chromatographie auf dem Fettgebiet IV. *Fette, Seifen, Anstrichmittel 63*, 807–810.
Kaufmann, H. P., Z. Makus und T. H. V. Khoe	1962a	Dünnschicht-Chromatographie auf dem Fettgebiet VI. *Fette, Seifen, Anstrichmittel 64*, 1–5.
Kaufmann, H. P. und B. Das	1962b	Dünnschicht-Chromatographie auf dem Fettgebiet VIII. *Fette, Seifen, Anstrichmittel 64*, 214–217.
Kaufmann, H. P., H. Wessels und B. Das	1962c	Über die Reinheitsprüfung der Kakaobutter. *Fette, Seifen, Anstrichmittel 64*, 723–729.
Kirchner, J. G., J. M. Miller, and G. I. Keller	1951	Separation and identification of some terpenes by a new chromatographic technique. *Anal. Chem. 23*, 420–425.

KISS, P. UND T. SZELL	1956	Paperchromatographic separation of certain steroids on filter paper with aluminium soap impregnated. *Naturwiss. 43*, 448–449.
KODICEK, E. AND D. ASHBY	1954	Paper chromatography of vitamin D and other sterols. *Proc. Biochem. J. 57*, XIII.
KRITCHEVSKY, D. AND M. R. KIRK	1952	Paper chromatography of steroids. *J. Am. Chem. Soc. 74*, 4484–4486.
KUČERA, J., Z. PROCHAZKA UND K. VERES	1957	Die Papier-Chromatographie neutraler Steroidverbindungen. *Collection Czech. Chem. Commun. 22*, 1185–1191.
KŬKSIS, A. AND J. M. R. BEVERIDGE	1960a	The paperchromatographic fractionation of plant steryl esters. *Can. J. Biochem. and Physiol. 38*, 95–107.
—	1960b	Composition of molecular distillates of corn oil: isolation and identification of sterol esters. *J. Lipid Res. 1*, 311–320.
—	1960c	Preparation of certain physical properties of some plant steryl esters. *J. Org. Chem. 25*, 1209–1219.
KUWADA, S. UND S. YOSHIKI	1940a	Über die Identität des Bessisterins mit Spinasterin. *J. Pharm. Soc. Japan 60*, 161–162.
—	1940b	Ein Sterin aus den Samen von Momordica cochinchinensis. *J. Pharm. Soc. Japan 60*, 232–233.
LADENBURG, K., E. FERNHOLZ, AND E. S. WALLIS	1938	The separation of sterols by the chromatographic adsorption method. *J. Org. Chem. 3*, 294–299.
LATA, C. F. AND C. S. VESTLING	1952	Extraction and determination of cholesterol in adrenal tissue. *Anal. Chem. 24*, 208–209.
LEDERER, E. *et al.*	1946	Sur les constituants de l'ambre gris II. *Helv. Chim. Acta 29*, 1354–1365.
LENDLE, A.	1938	Über die Inhaltsstoffe der Kürbissamen. *Arch. Pharm. 276*, 45–53.
LIFSCHÜTZ, I.	1935	Zur Analyse des Cholesterins. *Biochem. Z. 282*, 441–443.
LINKS, J.	1955	De carotenoiden, steroiden en vetzuren van Polytoma uvella. Thesis, Leiden, Excelsior 's-Gravenhage.
MACEK, K. AND Z. J. VEJDELEK	1955	Application of paperchromatography to structural problems in the veratrum alkaloid group. *Nature 176*, 1174–1175.
MCMAHON, J. M., R. B. DAVIS, AND G. KALNITSKY	1950	Identification of sterols on filter paper and their separation by paper chromatography. *Proc. Soc. Exptl. Biol. Med. 75*, 799–801.
MANGOLD, H. K.	1961	Thin-layer chromatography of lipids. *J. Am. Oil Chemists' Soc. 38*, 708–727.
MARCUSSON, J. UND H. SCHILLING	1913	Unterscheidung von Tier- und Pflanzenölen. *Chemiker Ztg. 37*, 1001–1002.
MARTIN, A. J. P. AND R. L. M. SYNGE	1941	A new form of chromatogram employing two liquid phases. *Biochem. J. 35*, 1358–1386.
MARTIN, A. J. P.	1948	Some theoretical aspects of partition chromatography. *Biochem. Soc. Symposia* (Cambridge) *3*, 4–20.
MARTIN, R. P. AND I. E. BUSH	1955	Application of a reversed-phase system for separation of the less polar steroids on paper. *Federation Proc. 14*, 252–253.
MARTIN, R. P.	1957	Reversed-phase paperchromatography and detection of steroids of the cholesterol class. *Arch. Bioch. Biophys. 25*, 408–417.

MATTHIAS, W.	1954	Serienuntersuchungen mit Hilfe einer neuen Form der Streifenpapierchromatographie. *Naturwiss. 41*, 17–18.
MAZUR, Y. AND A. WEISZMANN	1958	The carbon skeleton of the α-sitosterols. *Bull. Research Counc. Israel 7A*, 82–83.
MEINHARD, J. E. AND N. F. HALL	1949	Surface chromatography. *Anal. Chem. 21*, 185–188.
MELLIER, M. T.	1951	Les stérols de l'huile de palme. *Oléagineux 6*, 20–24.
MEYER, H.	1961	Ein Diskussionsbeitrag zur spektrophotometrischen Unterscheidung von Kakaopreszbutter und Kakaoextractionsfett. *Mitt. Gebiete Lebensm. u Hyg. 52*, 419–421.
MICHALEC, C. AND J. STRASEK	1960	A new paper chromatographic technique for the separation of cholesterol and cholesteryl esters. *J. Chromatog. 4*, 254–257.
MULVANY, P. K. *et al.*	1951	A chromatographic study of sulfite waste liquor sugars. *J. Am. Chem. Soc. 73*, 1255–1257.
NEHER, R. UND A. WETTSTEIN	1952	Papier Chromatographie von schwach polaren Steroiden. *Helv. Chim. Acta 35*, 276–283.
NEHER, R.	1958	Chromatographie von Sterinen, Steroiden und verwandten Verbindungen. Elsevier's Publ. Comp., Amsterdam.
OLIG, A.	1914	Der Bömer'sche Phytosterinen-nachweis nach den abgeänderten Marcusson-Schilling'schen Digitonin-ausfällungsverfahren. *Z. Untersuch. Nahr. u Genussm. 28*, 129–138.
OPPENAUER, R. V.	1935	Synthesis of dehydroandrosterone by the composition of γ-sitosterol from soybeans. *Nature 135*, 1039.
PARDEE, A. B.	1951	Calculations on paperchromatography of peptides. *J. Biol. Chem. 190*, 757–762.
POWER, F. B. AND C. W. MOORE	1909	The constituents of the fruit of Ecballium Elaterium. *J. Chem. Soc. 95*, 1985–1993.
POWER, F. B. AND A. H. SALWAY	1910a	Chemical examination of pumpkin seed. *J. Am. Chem. Soc. 32*, 346–360.
—	1910b	Chemical examination of watermelon seed. *J. Am. Chem. Soc. 32*, 360–374.
POWER, F. B.	1910c	The constituents of colocynth. *J. Chem. Soc. 97*, 99–110.
POWER, F. B. AND C. W. MOORE	1911	The constituents of bryony root. *J. Chem. Soc. 99*, 937–946.
QUAIFE, M. L., R. P. GEYER, AND H. R. BOLLIGER	1959	Rapid paper chromatographic microassay of free and ester cholesterol of blood. *Anal. Chem. 31*, 950–955.
RECOURT, J. H.	1962	Personal communication.
REICHL, E. R.	1956	Papierchromatographische Strukturanalyse. *Mikrochim. Acta*, 955–965.
REITSEMA, R. H.	1954	Characterization of essential oils by chromatography. *Anal. Chem. 26*, 960–963.
RIDDEL, C. AND R. P. COOK	1955	Cholesterol metabolism. *Biochem. J. 61*, 657–670.
RIEMERSMA, J. C.	1957	Unpublished results.
RIEMERSMA, J. C. EN W. STOUTJESDIJK	1958	Über die Papierchromatographie von Sterinen aus Tier und Pflanzenfetten. *Mitt. Gebiete Lebensm. u Hyg. 49*, 115–119.

RITTER, F. AND J. HARTEL	1958	Qualitative, quantitative and preparative chromatography of steroids on fully acetylated paper. *J. Chromatog. 1*, 461–469.
ROOS, J. B.	1956	Die Qualitätsbeurteilung von Schweineschmalz im Hinblick auf den Nachweis von raffiniertem "White Grease". *Fette, Seifen, Anstrichmittel 65*, 118–121.
—	1961	Unpublished results.
ROUSER, G. *et al.*	1961	Paper chromatography of lipids: methods, applications, and interpretations. *J. Am. Oil Chemists' Soc. 38*, 565–581.
SANDQVIST, H. UND E. BENGTSSON	1931	Zur Frage der Bruttoformel des Sitosterins. *Ber. 64*, 2167–2172.
SCHAUER, H. K. UND R. BURLISCH	1955	Über die Beziehungen zwischen Rf-Wert und chemische Konstitution organischer Verbindungen in der Papierchromatographie. *Z. Naturforsch. 10b*, 683–693.
—	1958	Über die Beziehungen zwischen der chemischen Konstitution organischer Verbindungen und ihrem Rf-Wert in der Papierchromatographie II. *Z. Naturforsch. 13b*, 327–335.
SCHOENHEIMER, R. UND H. DAM	1933	Über die Spaltbarkeit und Löslichkeit von Sterindigitoniden. *Z. physiol. Chem. 215*, 59–63.
SHULL, G. M., J. L. SARDINAS, AND R. C. NUBEL	1952	Paper chromatography of steroid compounds. *Arch. Bioch. Biophys. 37*, 186–198.
SMITH, L. L.	1954	Autoxidation of cholesterol during purification via the dibromide. *J. Am. Chem. Soc. 76*, 3232–3233.
SOCZEWINSKI, E. AND C. A. WACHTMEISTER	1962	The relation between the composition of certain ternary two-phase solvent systems and R_M values. *J. Chromatog. 7*, 311–319.
SONDHEIMER, F. AND R. MECHOULAM	1957	Synthesis of steroidal methylene compounds by the Wittig reaction. *J. Am. Chem. Soc. 79*, 5029–5033.
SPITERI, J.	1954	Chromatographie de partage des acides gras. *Bull. soc. chim. biol. 36*, 1355–1362.
STOKES, W. H., W. A. FISH, AND F. C. HICKEY	1955	Chromatographic separation and quantitative estimation of iodine-131-labeled derivates of sterols etc. *Anal. Chem. 27*, 1895–1898.
—	1956	Isolation and chemical nature of two companion sterols. *J. Biol. Chem. 220*, 415–430.
STOKES, W. H.	1957	The separation of the compounds of the lanosterol group present in isocholesterol by chromatography of the p-iodobenzoates-I^{131} and benzoates. *Arch. Bioch. Biophys. 67*, 272–279.
SULSER, H. UND O. HÖGL	1957	Die Unterscheidung von Tier und Pflanzenfetten. *Mitt. Gebiete Lebensm. u Hyg. 48*, 248–258.
SWELL, L. *et al.*	1956	The absorption of plant sterols and their effect on serum and liver sterol levels. *J. Nutrition 58*, 385–398
TOYAMA, Y. AND T. TAKAGI	1955	Reinvestigation of hitodesterol and its identity with α-spinasterol. *J. Chem. Soc. Japan 28*, 469–473.
—	1956	Mono-unsaturated sterol components of the starfish, Asterina Pectinifera. *J. Chem. Soc. Japan 29*, 317–319.
TSCHESCHE, R. UND G. SNATZKE	1960	Über Digitanol-Glykoside V. *Ann. 636*, 105–111.
TSCHESCHE, R., F. LAMPERT UND G. SNATZKE	1961	Über Triterpene VII. Dünnschicht- und Ionenaustauscherpapierchromatographie von Triterpenoiden. *J. Chromatog. 5*, 217–224.

Tsujimura, H.	1932	Carotin and dihydroergosterol in green tea. *Sci. Papers Inst. Phys. Chem. Research (Tokyo) 18*, 13–21.
Tunmann, P.	1956	Über die Natur des Sterins aus Potentilla anserina. *Archiv. Pharm. 289*, 704–714.
Van Duin, H.	1960	Bijdrage tot de vloeistof-vloeistof verdelingschromatographie der alifaten. Thesis Amsterdam, Epe, Hooiberg.
Vitagliano, M. e A. d'Ambrosio	1957a	Richerche sull'aggiunta di grassi di altra origine all' olio di oliva. *Olearia 11*, 169–184.
Vitagliano, M.	1957b	La ricerca dell'aggiunta di oli di origini animale nell' olio di oliva effettuata con tecnica cromatografica sulle rispettive sterine. *Publ. Univ. Cattolica S. Cuore 53*, 60–67.
—	1961	Sopra il punto di fusione dell'acetato sterolico. *Olearia 15*, 5–9.
Von Christiani, A. und V. Eck	1944	Trennung von Koprosterin und Cholestanol mit Hilfe der chromatographischen Adsorptionsanalyse. *Z. physiol. Chem. 280*, 127–129.
Williams, T. I.	1947	Introduction to chromatography. Blackie and Son, Glasgow. pag. 36.
Wallis, E. S. and P. N. Chakravorty	1937	The nature of the sterols in cottonseed oil. *J. Org. Chem. 2*, 335–340.
Windaus, A. und O. Stange	1936	Über das Provitamin des Eiersterins. *Z. physiol. Chem. 244*, 218–220.
Winterstein, A. und G. Stein	1933	Fraktionierung und Reindarstellung organischer Substanzen nach dem Prinzip der chromatographischen Adsorptionsanalyse. *Z. physiol. Chem. 220*, 247–463.
Zimmermann, G. und G. Bräse	1956	Zur Papierchromatographie des Cholesterins und seiner Ester. *Pharmazie 11*, 715–716.

TABLE 6 Data concerning the paperchromatographic separation of some sterols, vitamins, and provitamins D

R_F values in the paperchromatographic systems

sterol	SHULL 1952	KRITCHEVSKY 1952		DAVIS 1952		VITAGLIANO 1957*a*, 1957*b*	KISS 1956	MCMAHON 1950	NEHER 1952	RIDDEL 1955	KODICEK 1954			SWELL 1956	KAISER 1956	GRACIAN 1959	TUNMANN 1956	SULSER 1957	DE ZOTTI 1959	KUKSIS 1960*b*	Own experiments	
	1	2	3	4	5	6	7	8	9[b]	10	11	12	13	14	15	16	17[b,d]	18	19	20[b]	21[g]	22[g]
cholesterol	0.77	0.52	0.56	0.49	0.73	0.56	0.34	0.0	≡1.0	≡1.0	0.29	0.49	0.50	0.33		0.74		0.42	0.35		0.43	0.34
cholesterol acetate							0.13														0.10	0.11
β-sitosterol	0.80	0.54	0.65	0.43	0.65	0.47–0.58				1.0	0.00	0.51	0.42	0.00		0.64	0.33	0.34	0.26	0.32	0.31	0.24
phytosterols from vegetable oils						0.33–0.41[a]								0.00			0.62[e]	0.42[e]	0.33[a,b]–0.40	0.68[f]	0.33/0.38/0.43[e]	0.25/0.30/0.34[e]
γ-sitosterol																	0.50			0.50	0.29	0.29
stigmasterol	0.80	0.53	0.52						0.57	1.0				0.00				0.38			0.37	0.30
7-dehydrocholesterol		0.94	0.88	0.48	0.67			0.9		0.7	0.39	0.57	0.56	0.43							0.51	0.43
dihydrocholesterol		0.56	0.63							1.1											0.33	0.25
coprostanol										1.3											0.09	0.07
ergosterol	0.60	0.95	0.84	0.43	0.68		0.52	0.0	0.48	0.6	0.00	0.56	0.00				0.89	0.89	0.42		0.56	0.43
zymosterol											0.41	0.61	0.48								0.60	0.47
fucosterol																			0.39[b]			
vitamin D_3				0.76	0.91						0.46	0.68	0.68								0.22	0.40
vitamin D_2				0.80	0.91						0.44	0.66	0.69								0.50	0.42
lumisterol											0.27	0.59	0.52								0.49	0.37
suprasterol II											0.36	0.52	0.57									
tachysterol (?)											0.37	0.61	0.58									
vitamin A alcohol											0.93–0.98				~0.9							
vitamin A acetate															~0.5							
α-tocopherol											0.15	0.58									0.16	0.12

The solvent mixtures used for the paperchromatographic fractionation of sterols, mentioned in this table, are the following:

1. Aluminium oxide/hexane–ether (3 : 1)
2. Quilon/ethanol–water (80 : 20)
3. Quilon/methanol
4. Quilon/methanol–water–ethylene glycol monomethylether (62 : 19 : 19)
5. Quilon/methanol–water (95 : 5)
6. Quilon/ethanol–water (80 : 20)
7. Aluminium stearate–palmitate/methanol–carbon tetrachloride–water (72 : 20 : 8)
8. Water/phenol–methanol–water (13.5 : 30 : 56.5)
9, 10. Phenyl cellosolve/heptane
11. Paraffin/ethylene glycol monomethylether–n-propanol–methanol–water (35 : 10 : 30 : 25)
12. Paraffin/n-propanol–methanol–water (15 : 82 : 3)
13. Paraffin/methanol–water (95 : 5)
14. Paraffin/ethylene glycol monomethylether–n-propanol–methanol–water (35 : 10 : 30 : 25)
15. Water/isopropanol–water (1 : 1)
16. Silicone and paraffin oil/ethanol–isopropanol–cyclohexane (60 : 30 : 10)
17. Paraffin/acetic acid or ethanol
18. Paraffin/acetic acid–water (84 : 16) (circular technique)
19. Petroleum (bp 220–240°C)/pyridine–water (85 : 15)
20. Paraffin/propionic acid–water (88 : 12)

Our own experiments in:

21. Paraffin/acetic acid–water (84 : 16), degree of impregnation: 0.09–0.10 g/g paper
22. Paraffin/acetic acid–water (84 : 16), degree of impregnation: 0.15–0.16 g/g paper

[a] Determined for olive oil phytosterols
[b] Measured roughly from the chromatograms published by these authors
[c] Determined for coconut fat phytosterols
[d] Analysed as sterol acetates
[e] Unknown phytosterol acetate from *Potentilla anserina*
[f] Third-band phytosterol from corn oil sterol ester hydrolysate, denoted as "α-sitosterol"
[g] The R_F values of no. 21 and 22 are corrected via their R_S values

TABLE 20 Sterol values and percentage of unsaturated fatty acids of some samples of common vegetable and animal oils and fats

Fats	Total-sterol fraction					Free-sterol fraction					Bound-sterol fraction					Percentage of unsaturated fatty acids		
	percentage of the phytosterols of			sterol content in %	melting point of the sterol acetates in °C	percentage of the phytosterols of			sterol content in %	melting point of the sterol acetates in °C	percentage of the phytosterols of			sterol content in %	melting point of the sterol acetates in °C	linoleic acid	linolenic acid	arachidonic acid
	band 1	band 2	band 3			band 1	band 2	band 3			band 1	band 2	band 3					
VEGETABLE FATS																		
soybean oil, refined 1	50	50	0	0.34	128.6	48	52	0	0.21	131.8	86	14	0	0.13	121.4	12.1	2.3	0.03
soybean oil, refined 2	59	41	0	0.34	128.8	45	55	0	0.22	131.9	100	0	0	0.12	121.4	19.0	4.3	0.03
soybean oil, crude	50	50	0	0.41	129.2											33.0	6.2	0.06
soybean oil, refined	50	50	0	0.37	130.4	43	57	0	0.25	132.0						24.4	6.1	0.10
soybean oil, bleached	55	45	0	0.39	131.8	50	50	0	0.29	132.8	100	0	0	0.10	122.5	14.8	3.1	0.000
soybean oil, refined				0.37	130.4				0.27	132.0				0.10	118.4	19.1	3.6	0.000
soybean oil, hardened 1	60	40	0													10.2	2.4	0.01
soybean oil, hardened 2	66	34	0	0.30	130.2													
soybean oil, hardened 3	50	50	0	0.30	130.7													
soybean oil, hardened 4	60	40	0	0.33	129.8													
soybean oil, hardened 5	50	50	0	0.32	130.4													
soybean oil, hardened 6	57	40	3	0.17	126.3	60	31	9	0.10	130.8	75	25	0	0.06	118.8			
rapeseed oil, refined	60	30	10															
rapeseed oil, refined	38	37	25	0.62	133.8	42	33	25	0.26	138.0	40	40	20	0.36	135.6	2.3	7.3	0.000
rapeseed oil, bleached	70	20	10	0.63	135.2	63	25	12	0.26	137.8	60	30	10	0.36	135.3	9.9	8.1	0.000
rapeseed oil, crude	47	28	25	0.68	137.0	50	25	25	0.29	137.3	60	40	0	0.39	133.9	15.4	9.8	0.16
rapeseed oil, hardened	50	40	10	0.56														
peanut oil, crude	70	20	10	0.20	123.4	69	17	14	0.15	127.3	67	17	16	0.05		17.3	0.06	0.000
peanut oil, refined	60	20	20	0.22	128.4	70	15	15	0.13	127.4	83	12	5	0.09	126.4	9.2	0.15	0.000
peanut oil, refined	47	28	25	0.28	128.6	45	45	10	0.17	131.2	86	14	0	0.11	130.6	14.0		0.002
peanut oil, hardened 1	63	31	6	0.18	127.8					120.2	50	33	17	0.15	129.4			
peanut oil, hardened 2	70	17	13	0.05	124.8	86	14	0	0.01	122.1	82	18	0	0.04	129.1			
peanut oil, hardened 3	76	15	9	0.06	127.8	89	11	0	0.03	128.6	82	18	0	0.02				
maize oil, refined 1	67	33	0	0.89	128.8	62	38	0	0.28	128.0	50	38	12	0.61	128.8	24.6	0.51	0.000
maize oil, refined 2	80	20	0	0.82	128.8	67	33	0	0.24	129.7	80	20	0	0.57	129.6	27.4	0.43	0.002
maize oil, refined 3	80	20	0	0.81	129.2	60	40	0	0.20	128.2	67	33	0	0.61	130.2	13.0	0.31	0.000
maize oil, bleached	60	34	6	0.92	129.6	57	43	0	0.30	129.4	60	40	0	0.63	130.4	29.5	0.43	0.000
maize oil, crude	60	40	0													51.7	0.49	0.04
palm oil (Congo, crude)	62	31	7	0.06	128.6	72	23	5	0.05	131.5	70	21	9	0.01	125.8	10.7	0.22	0.015
palm oil (Sumatra, crude)	63	37	0	0.06	127.8	74	26	0	0.05	131.2	70	30	0	0.01	128.4	9.7	0.20	0.01
palm oil, refined	50	50	0	0.07	128.0													0.01
palm oil, refined	60	36	4	0.05	129.9													
palm oil, refined	60	33	7	0.03	129.2													0.01
palm oil, refined	67	33	0	0.06	129.6													
palm oil, refined	56	37	7	0.04	128.0													
palm oil, hardened	55	33	12	0.04	129.5	45	33	22	0.03	130.1	50	30	20	0.01				
palm oil, hardened	56	36	8	0.07	130.8													
palm oil, hardened	55	41	4	0.06	128.8													
palmkernel oil, refined	50	50	0	0.05	125.4	50	50	0	0.03	126.4						7.6	0.48	0.000
palmkernel oil, refined	66	28	6	0.11	126.0													
olive oil, spanish 1	100	0	0	0.13	118.2				0.00		100	0	0	0.12	122.0			
olive oil, spanish 2						100	0	0	0.06	121.2	100	0	0	0.05	116.4			
olive oil, spanish 3	100	0	0	0.10	119.6	100	0	0	0.05	122.6	100	0	0	0.04	119.2			
olive oil, algerian	85	0	15	0.09	121.4	55	0	45	0.07	122.4						7.6	0.48	0.001
olive oil, french	90	0	10													10.5	0.48	0.000
olive oil, israelitish	100	0	0	0.12	117.4	100	0	0	0.05	120.4				0.07		6.7	0.61	0.000
cottonseed oil	100	0	0	0.37	124.8	100	0	0	0.25	124.6	100	0	0	0.12	126.8	25.4	0.20	0.01
cottonseed oil, hardened 1	100	0	0	0.26	122.2	86	14	0	0.21	124.6	82	18	0	0.05				
cottonseed oil, hardened 2	100	0	0	0.26	124.8	92	8	0		123.9	91	9	0		120.0			
safflower oil, crude	66	34	0	0.35	123.0	63	25	12	0.24	121.8	54	36	10	0.11	125.2			
safflower oil, refined	70	30	0	0.27	120.7	66	26	8	0.22	116.8	64	22	14	0.05	118.6	34.0	0.05	0.000
safflower oil, crude	70	30	0	0.31	120.8	62	30	8	0.21	121.0	73	19	8	0.09	122.4	50.4	0.20	0.000
coconut fat	45	29	26	0.09	128.8	50	30	20	0.06	129.4	46	23	31	0.03	126.2	1.6	0.70	0.000
coconut fat, strongly refined	51	16	33	0.07	127.2	48	29	23	0.04	126.4	52	27	21	0.02	123.4			
coffee oil	65	35	0	0.82	130.5	34	66	0	0.38	132.9	64	36	0	0.45	129.6			
wheatgerm oil	75	25	0	1.8														
grapeseed oil (old sample)	60	40	0	0.39	114.6											50.0	0.22	0.03
tabaccoseed oil (old sample)	50	30	20	0.70	126.4													
almond oil, refined 1	54	0	46	0.15														
almond oil, refined 2	89	0	11	0.16	123.4	83	0	17	0.12	123.8	86	0	14	0.03	122.0			
avocado oil, crude	85	0	15	0.42	119.4	87	0	13	0.10	122.3	89	0	11	0.33	115.2			
avocado oil, refined	100	0	0	0.34	121.3	83	0	17	0.17	123.4	87	0	13	0.18	114.8			
castor oil, crude				0.21	130.6													
castor oil, refined	38	37	25	0.20	130.0	50	25	25	0.15	129.1	50	33	17	0.05	126.2			
castor oil, crude				0.27	130.6													
tall oil, crude	100	0	0	4.1	122.0	89	11	0	0.08	122.2	85	15	0	1.82(?)	123.4	58.5	0.46	0.05
sunflower oil	70	30	0	0.44	119.4	66	34	0	0.19	127.4	100	0	0	0.25	120.8	21.0	0.20	0.000
sesame oil	50	37	13	0.51	128.1	60	30	10	0.22	128.0	50	30	20	0.29	129.8			
cocoa butter 1	50	50	0	0.24	130.2	50	50	0	0.17	130.4	67	33	0	0.07	128.0			
cocoa butter 2	56	44	0	0.26	129.8				0.21	128.4								
EDIBLE ANIMAL BODY FATS																		
lard 1	0	0	100	0.09	115.0	0	0	100	0.08	115.3								
lard 2	0	0	100	0.08	114.0	0	0	100	0.06	114.2	0	0	100	0.02	114.4			
beef fat 1	0	0	100	0.08	115.0	0	0	100	0.06	114.7	0	0	100	0.02				
beef fat 2	0	0	100	0.11	115.0	0	0	100	0.10	113.9	0	0	100	0.02	115.4			
whale oil (crude)	0	0	100	0.23	114.5	0	0	100	0.22	115.0	0	0	100	0.02	114.6			
whale oil (neutralized, bleached)	0	0	100	0.21	114.5	0	0	100	0.19	114.7	0	0	100	0.02	114.7			
whale oil (refined, hardened)	8	0	92	0.20	116.0	4	0	96	0.14	116.0	7	0	93	0.06	115.2			
fish oil (neutralized, bleached)	0	0	92*	0.58	114.7	0	0	89*	0.52	115.1	0	0	79*	0.06	114.6			
fish oil (refined)	0	0	81*	0.56	115.3	0	0	80*	0.47	115.1	0	0	83*	0.08	115.3			
fish oil (refined, hardened)	0	0	100	0.42	114.1	0	0	100	0.33	114.8	0	0	100	0.08	114.7			
INEDIBLE ANIMAL FATS																		
white grease 1	0	0	100	0.30	115.2	0	0	100	0.19	115.2	0	0	100	0.11	113.9			
white grease 2	0	0	100	0.31	115.3	0	0	100	0.22	114.6	0	0	100	0.09	113.0			
white grease 3	0	0	100	0.26	115.1	0	0	100	0.23	115.0	0	0	100	0.03	113.8			
grease	0	0	100	0.27	116.8	0	0	100	0.17	116.0	0	0	100	0.07	116.5			
fancy tallow 1	0	0	100	0.25	116.2	0	0	100	0.21	115.8	0	0	100	0.04	114.4			

* With fourth bands of about 8–21%.