THE ANALYSIS OF MIXTURES OF ANIMAL AND VEGETABLE FATS

II. THE PAPER CHROMATOGRAPHY OF SOME STEROLS, PROVITAMINS, VITAMINS AND PENTACYCLIC TRITERPENOID ALCOHOLS*

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INTRODUCTION

Unlike the paper chromatographic separation of the more polar steroids, the chromatography of highly fat-soluble sterols, vitamins, provitamins and pentacyclic triterpenoid alcohols has not been the subject of many comprehensive studies.

Because of the poor solubility of these compounds in water and polar solvents, the "normal" paper chromatographic methods find little application here. The literature only deals with separations of cholesterol and 7-dehydrocholesterol[1] and of vitamin A and vitamin A acetate[2] in similar "normal" systems.

Impregnation of the paper beforehand is necessary to achieve good chromatographic separations. For this purpose the following agents may be used: phenyl cellosolve[4–6], salts of carboxylic acids[7], aluminium oxide[3] and Quilon (a chromium stearato complex); see Table I. The last-mentioned compound, a water-repellent impregnating agent, has been applied for the separation of some naturally occurring sterols[9–11].

The solvent mixtures used for the paper chromatographic fractionation of sterols, mentioned in Table I, are the following:

(1) Quilon/ethanol–water (8:2)[9].
(2) Quilon/methanol[9].
(3) Quilon/methanol–water–ethylene glycol monomethyl ether (65:20:20)[10].
(4) Quilon/methanol–water (95:5)[10].
(5) Quilon/ethanol–water (8:2)[11].
(6) Sodium stearate, -palmitate/methanol–carbon tetrachloride–water (18:5:2)[7].
(7) Aluminium oxide/hexane–ether (3:1)[3].
(8) Water/phenol–methanol–water (13.5:30:56.5)[1].
(9) Water/isopropanol–water (1:1)[2].
(10) Phenyl cellosolve/heptane[6].
(11) Phenyl cellosolve/heptane[5].
(12) Silicone grease/acetonitril–water (6:4)[8].

* For Part I of this series, see ref.[22].
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TABLE I

DATA CONCERNING THE PAPER CHROMATOGRAPHIC SEPARATION OF SOME STEROLS, VITAMINS AND PROVITAMINS

Compound	R_F in paper chromatographic system																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Cholesterol	0.52	0.56	0.49	0.73	0.56	0.30	0.77	0.0		0.03	≡0.1		0.29	0.49	0.50	0.35	0.42
Cholesterol acetate						0.11											
β-Sitosterol	0.54	0.65	0.43	0.65	0.47–0.58		0.80				1.0		0.00	0.51	0.42	0.26	0.38 / 0.33
Phytosterol from rapeseed- or olive-oil					0.33–0.41						1.0					0.33 / 0.40	0.42
Stigmasterol	0.53	0.52					0.80			0.30	1.0		0.00				0.38
7-Dehydrocholesterol	0.94	0.88	0.48	0.67				0.9			0.7		0.39	0.57	0.56		
β-Cholestanol	0.56	0.63									1.1						
Ergosterol	0.95	0.84	0.43	0.68		0.47	0.60	0.0		0.37	0.6		0.00	0.56	0.00		0.89
Zymosterol													0.41	0.61	0.48		
Vitamin D_3			0.76	0.91									0.46	0.68	0.68		
Vitamin D_2			0.80	0.91									0.44	0.66	0.69		
Lumisterol													0.27	0.59	0.52		
Suprasterol II													0.36	0.52	0.57		
Tachysterol													0.00	0.00	0.00		
													0.37	0.61	0.58		
													0.98	0.98	0.98		
Vitamin A									about 0.9				0.03 / 0.08				
Vitamin A acetate									about 0.5			0.32					
α-Tocopherol												0.07	0.15	0.58			

(13) Paraffin/ethylene glycol monoethyl ether–*n*-propanol–methanol–water (35:10:30:25)[16].
(14) Paraffin/*n*-propanol–methanol–water (15:82:3)[16].
(15) Paraffin/methanol–water (95:5)[16].
(16) Petroleum b.p. 220–240°/pyridine–water (85:15)[12].
(17) Paraffin/acetic acid–water (84:16)[13–15].

The best separations of these highly fat-soluble sterols and vitamins have been obtained by reversed-phase chromatography, applying as stationary phase: silicone grease[8], petroleums fractions[12] or paraffinum liquidum[13,16].

In the analysis of fats and oils, a good method for the detection of small amounts of animal or vegetable fats in their mixtures is required[22]. Therefore, a paper chromatographic method for the identification of cholesterol, which occurs in animal fats, was badly needed. It is, however, not easy to obtain a complete paper chromatographic separation of cholesterol from the closely related phytosterols occurring in vegetable oils (mainly β-sitosterol and stigmasterol). Of all the systems mentioned in Table I, system No. 17: paraffin/acetic acid–water (84:16) gave the best separation of cholesterol from these phytosterols. The paper chromatographic separations of several sterols, vitamins and provitamins, that are possible with this system were more closely studied. Besides this, an attempt was made to establish more general rules for classifying and "explaining" the paper chromatographic data obtained from the literature and from our own experiments.

PAPER CHROMATOGRAPHIC DATA

Table II presents the R_F values, relative R_S values (S = cholesterol) and the corresponding R_M values of some sterols, fat-soluble vitamins, provitamins and pentacyclic triterpenoid alcohols in the above-mentioned system. The formulae of some of these sterols are given in Fig. 1.

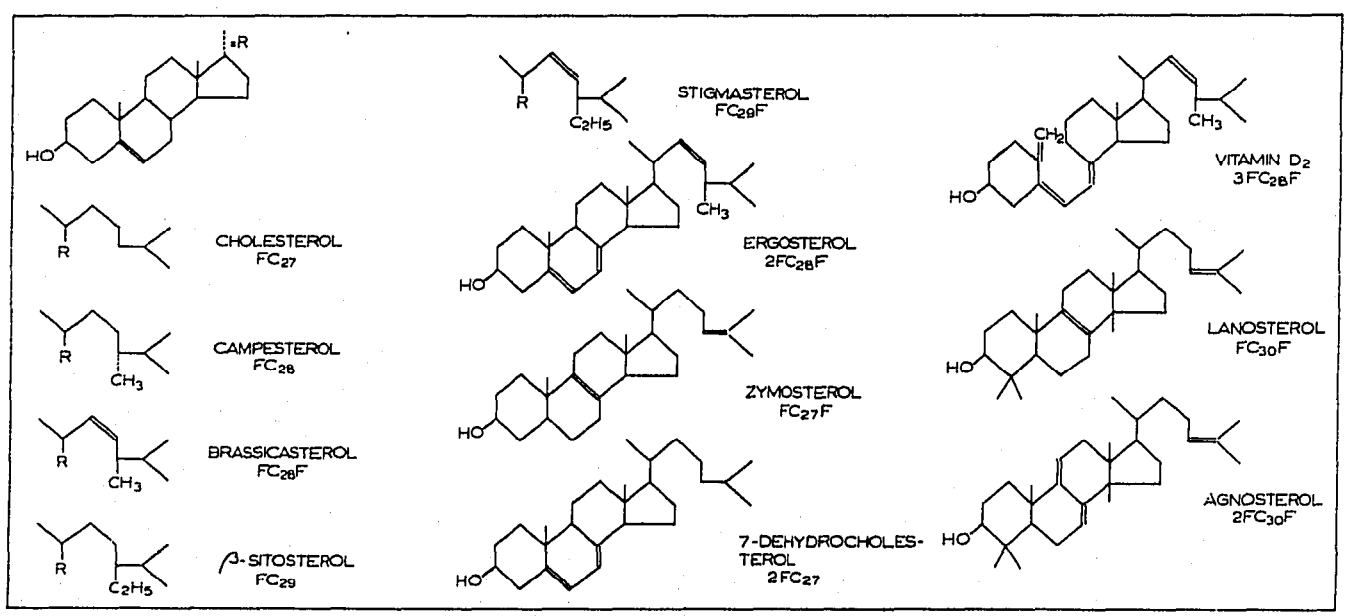


Fig. 1. The formulae of some sterols, mentioned in Table II.

Paper chromatographic reversed phase systems generally present a pure liquid–liquid partition chromatography. The relation between the R_F values or, even better the R_M values, and the number of carbon atoms of substances belonging to a homologous series should be more or less linear. This nearly linear relation is observed, for instance, when separating higher fatty acids in the system undecane/95 % acetic

TABLE II

THE R_F, R_S* (S = CHOLESTEROL) AND R_M** VALUES OF SOME STEROLS, VITAMINS, PROVITAMINS AND PENTACYCLIC TRITERPENOID ALCOHOLS IN THE SYSTEM PARAFFIN/ACETIC ACID–WATER (84 : 16)

Paper: Schleicher-Schüll 2043 b mgL.; temp. 22–24°.

Compound	*Formula*	*Abbr. form.***	R_F *value*	R_S* *value*	R_M** *value*	R'_M† *calcd.*
Cholesterol	$C_{27}H_{46}O$	FC27	0.33	1.0	0.31	0.28
4-Cholestenol	$C_{27}H_{46}O$	FC27	0.33	1.0	0.31	0.28
7-Dehydrocholesterol	$C_{27}H_{44}O$	2FC27	0.40	1.22	0.18	0.20
Dihydrocholesterol ((β-cholestanol))	$C_{27}H_{48}O$	C27	0.27	0.81	0.43	0.39
Coprostanol	$C_{27}H_{48}O$	C27	0.07	0.22	1.13	—
Epicholesterol	$C_{27}H_{46}O$	FC27	0.26	0.79	0.45	—
			(0.11	0.34	0.91)	
			(0.09	0.30	1.005)	
β-Sitosterol	$C_{29}H_{50}O$	FC29	0.25	0.75	0.48	0.52
Campesterol††	$C_{28}H_{48}O$	FC28	0.29	0.87	0.39	0.40
Stigmasterol	$C_{29}H_{48}O$	FC29F	0.27	0.83	0.43	0.41
Brassicasterol†† ((and chalinasterol))	$C_{28}H_{46}O$	FC28F	0.33	1.0	0.31	0.29
γ-Sitosterol	$C_{28}H_{48}O$	FC28?	0.29	0.88	0.39	0.40
22-Dihydroergosterol	$C_{28}H_{46}O$	2FC28	0.34	1.04	0.29	0.32
Zymosterol	$C_{27}H_{44}O$	FC27F	0.43	1.31	0.12	0.17
Ergosterol	$C_{28}H_{44}O$	2FC28F	0.42	1.26	0.14	0.21
Vitamin D_2	$C_{28}H_{44}O$	3FC28F	0.38	1.15	0.21	0.26
Vitamin D_3	$C_{27}H_{44}O$	3FC27	0.36	1.10	0.25	0.25
Dihydrovitamin D_2	$C_{28}H_{46}O$	3FC28	0.29	0.87	0.39	0.37
Pyrocalciferol	$C_{28}H_{44}O$	2FC28F	0.31	0.94	0.35	—
Isopyrocalciferol	$C_{28}H_{44}O$	2FC28F	0.38	1.15	0.21	—
Lumisterol	$C_{28}H_{44}O$	2FC28F	0.33	1.00	0.31	—
Epilumisterol	$C_{28}H_{44}O$	2FC28F	0.37	1.12	0.23	—
Lanosterol	$C_{30}H_{50}O$	FC30F	0.22	0.66	0.55	0.53
Dihydrolanosterol	$C_{30}H_{52}O$	FC30	0.15	0.44	0.75	0.64
Agnosterol	$C_{30}H_{48}O$	2FC30F	0.19	0.59	0.63	0.45
Dihydroagnosterol	$C_{30}H_{50}O$	2FC30	0.18	0.54	0.66	0.56
Cholesterol acetate	$C_{29}H_{48}O_2$	—	0.09	0.25	1.00	—
β-Sitosterol acetate	$C_{31}H_{52}O_2$	—	0.06	0.17	1.20	—
Stigmasterol acetate	$C_{31}H_{50}O_2$	—	0.08	0.24	1.06	—
Ergosterol acetate	$C_{30}H_{46}O_2$	—	0.10	0.29	0.95	—
Cholesterol butyrate	$C_{31}H_{52}O_2$	—	0.04	0.12	1.38	—
7-Hydroxycholesterol	$C_{27}H_{46}O_2$	—	0.74	2.23	−0.46	—
Vitamin A	$C_{20}H_{30}O$	—	0.30	0.91	0.37	—
Vitamin A acetate	$C_{22}H_{32}O_2$	—	0.01	0.03	1.99	—
dl-α-Tocopherol	$C_{29}H_{50}O_2$	—	0.13	0.40	0.83	—

* The R_S value is calculated from R_F sterol/R_F cholesterol with R_S cholesterol = 1.0.

** $R_M = \log(1/R_F - 1)$.

*** The abbreviated formula FC2SF means a sterol structure with 28 carbon 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.

† These theoretical R'_M values are calculated from R_{M_0} and ΔR_M values.

†† These sterols were not available as pure preparations.

acid[18] and when separating cholesterol esters in the system paraffin/acetic acid–chloroform–paraffin oil (65:25:10)[19].

Analogously, an almost linear relation is obtained when the R_F values of some sterols are plotted against their number of carbon atoms (see Fig. 2).

DISCUSSION

The appearance of critical pairs

The introduction of a double bond produces an increase in the polarity of the molecule (and an increase in R_F value), which is approximately equal to that caused by a decrease in length of the carbon chain by one $-CH_3$ group. Therefore stigmasterol (FC29F), campesterol (FC28) and dihydrocholesterol (C27) have approximately equal R_F values.

Similar to the paper chromatographic separation of higher fatty acids[18], all sterols are arranged in critical pairs (mimic substances according to BUSH[23]). Brassicasterol (FC28F), cholesterol (FC27) and 7-dehydrocholesterol (2FC27), ergosterol (2FC28F), and zymosterol (FC27F) also belong to two different critical pairs.

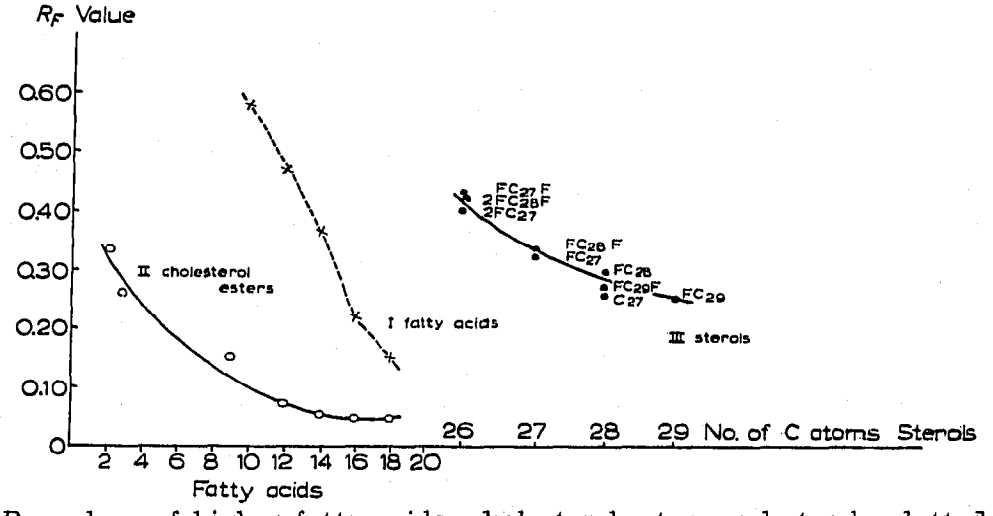


Fig. 2. The R_F values of higher fatty acids, cholesterol esters and sterols plotted against their number of carbon atoms. The higher fatty acids are separated in the system undecane/95% acetic acid[18] (curve I), the cholesterol esters in the system paraffin/acetic acid–chloroform–paraffin oil (65:25:10)[19] (curve II), and the sterols in the system paraffin/84% acetic acid (curve III).

The above-mentioned sterols are separated into four zones on a paper chromatogram, made according to the technique of MATTHIAS[32]. The lowest zone contains β-sitosterol, the second zone campesterol and stigmasterol, the third zone cholesterol and brassicasterol, and the fourth zone 7-dehydrocholesterol, ergosterol and zymosterol (see Fig. 5).

Each critical pair can be characterized by a paper chromatographic value, *pc.V.*, calculated as follows: *pc.V.* = number of carbon atoms minus number of double bonds[21]. In Table III all the sterols with the same *pc.V.* number are arranged horizontally, thus producing the various critical pairs.

The sterols belonging to the same zone may still present a slight difference in R_F

TABLE III

CRITICAL PAIRS OF NON-CONJUGATED STEROLS, CLASSIFIED BY THEIR *pc.V.* NUMBER

pc.V. = n — M (n is the number of carbon atoms and M the number of double bonds in the molecule).

pc.V.	$M = 0$	$M = 1$	$M = 2$	$M = 3$	Zone	R_F values	R_M values
25			Zymosterol, FC27F 7-Dehydrocholesterol, 2FC27	Ergosterol, 2FC28F	4	0.39–0.44	0.10–0.19
26		Cholesterol, FC27	Brassicasterol, FC28F		3	0.32–0.35	0.27–0.32
27	Dihydrocholesterol, C27	Campesterol, FC28	α_1-Sitosterol, FC29F Stigmasterol, FC29F		2	0.26 0.30	0.37–0.45
28	Dihydrocampesterol, C28	β-Sitosterol, FC29	α_2-Sitosterol, FC30F Lanosterol, FC30F		1	0.21 0.26	0.45–0.58
29	Dihydrositosterol, C29	Dihydrolanosterol, FC30				0.14–0.19	0.63–0.79
30	Tetrahydrolanosterol, C30 Tetrahydro-α_2-sitosterol, C30					0.06–0.10	0.95–1.20

value. This difference is too small to permit a separation under normal conditions. We succeeded, however, in separating a mixture of zymosterol and ergosterol, using the circular technique described by SULSER[13].

A complete separation of cholesterol from the most important naturally occurring phytosterols (β-sitosterol and stigmasterol), which is necessary for the analysis of mixtures of animal and vegetable fats[22] and a separation of vitamin A and vitamin A esters can be obtained in the studied system.

The pentacyclic triterpenoid alcohols occurring in wool fat, lanosterol (FC30F) and dihydrolanosterol (FC30), can also be separated. It is, however, impossible to separate agnosterol (2FC30F) and dihydroagnosterol (2FC30), which both have another double bond. A complete separation of vitamin D_2 (3FC28F) and dihydrovitamin D_2 (3FC28), which both have three conjugated double bonds but differ by one non-conjugated double bond, is also impossible. Conjugated double bonds seem to have a strong influence on the R_F value of a sterol molecule.

Calculation of ΔR_M values

The introduction of a non-conjugated double bond into the molecule generally causes a decrease in R_M value. ($R_{M2} - R_{M1} = \Delta R_M^{C=C} = \ln \Delta\mu^{C=C}/RT$). The $\Delta R_M{}^{C=C}$ value varies between −0.05 and −0.20, with a mean value of −0.11 (see Table IV A and Fig. 4). This value agrees with the difference in the R_M value caused by the introduction of an extra $-CH_3$ group ($\Delta R_M^{CH_2} = +0.12$).

TABLE IV

THE EFFECT OF THE INTRODUCTION OF ANOTHER DOUBLE BOND ON THE R_M VALUE

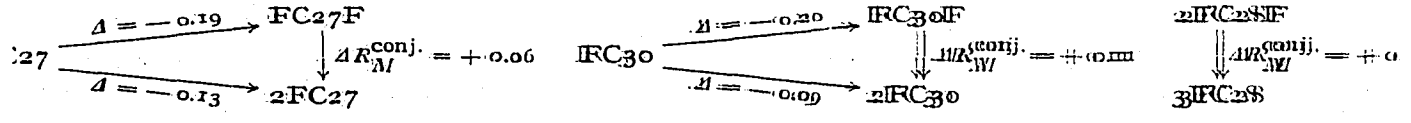
A. The molecule contains no double bond or only one			$\Delta R_M{}^{C=C}$
Dihydrocholesterol (C27)	→	cholesterol (RC27)	−0.12
Cholesterol (FC27)	→	zymosterol (RC27F)	−0.09
Campesterol (FC28)	→	brassicasterol (RC28F)	−0.08
β-Sitosterol (FC29)	→	stigmasterol (RC29F)	−0.05
Cholesterol (FC27)	→	7-dehydrocholesterol (2RC27)	−0.13
Dihydrolanosterol (FC30)	→	lanosterol (RC30F)	−0.20
Dihydrolanosterol (FC30)	→	dihydroagnosterol (2RC30)	−0.09

B. The molecule already contains two double bonds			$\Delta R_M{}^{C=C-C=C-C=C}$
1. Ergosterol (2FC28F)	→	vitamin D_2 (3RC28F)	+0.07
2. 7-Dehydrocholesterol (2FC27)	→	vitamin D_3 (3RC27)	+0.07
3. Lanosterol (FC30F)	→	agnosterol (2RC30F)	+0.08
4. Dihydroagnosterol (2FC30)	→	agnosterol (2RC30F)	−0.03
5. 22-Dihydroergosterol (2FC28)	→	dihydrovitamin D_2 (3RC28)	+0.08

If the molecule contains two (conjugated) double bonds, the introduction of a third one causes only a slight decrease in R_M value (see Table IV B, No. 4) or in most cases when the third double bond is conjugated, even an increase in R_M value.

By irradiation of 7-dehydrocholesterol and ergosterol with U.V. light, vitamins D_3 and D_2, respectively, are formed. When ring B of the cholesterol nucleus opens, producing a system of three conjugated double bonds, an increase in R_M value of about +0.07 is obtained.

In a critical pair, the sterols with several conjugated double bonds generally have higher R_M values than sterols with fewer conjugated double bonds or without any. The R_M values of dihydroagnosterol (2FC30), 7-dehydrocholesterol (2FC27) and dihydrovitamin D_2 (3FC28) are higher than those of lanosterol (FC30F), zymosterol (FC27F) and ergosterol (2FC28F) respectively.



From these examples a mean value of +0.10 caused by the presence of several conjugated double bonds can be calculated.

The agreement between the values: 0.07, 0.10, due to conjugation, and the $\Delta R_M^{CH_3}$ and $\Delta R_M^{C=C}$ values causes the existence of critical pairs. Introduction of a double bond as well as shortening of the carbon chain by removing one $-CH_2$ group results in the sterol being shifted one step to the left in the graph of Fig. 2 (*i.e.* to higher R_F values).

Introduction of another (conjugated) double bond when the molecule already contains two of them, will shift the R_F value one step to the right (*i.e.* to lower R_F values).

The same effect is produced when separated double bonds are "rearranged" into a system with (more) conjugated double bonds. If, for instance, cholesterol (FC27) is first converted into 7-dehydrocholesterol (2FC27) and this into vitamin D_3 (3FC27), the R_F values of cholesterol and vitamin D_3 will be very similar.

Consequently these sterols for the greater part follow the rule of MARTIN regarding the thermodynamic meaning and additivity of the R_M values. The validity of this rule in the field of the more polar adrenocortical steroids has been shown by BUSH[23].

The R_F value of a sterol molecule with a steric configuration related to 3β-cholestanol is predominantly determined by the total number of carbon atoms and the number of non-conjugated and conjugated double bonds in the molecule.

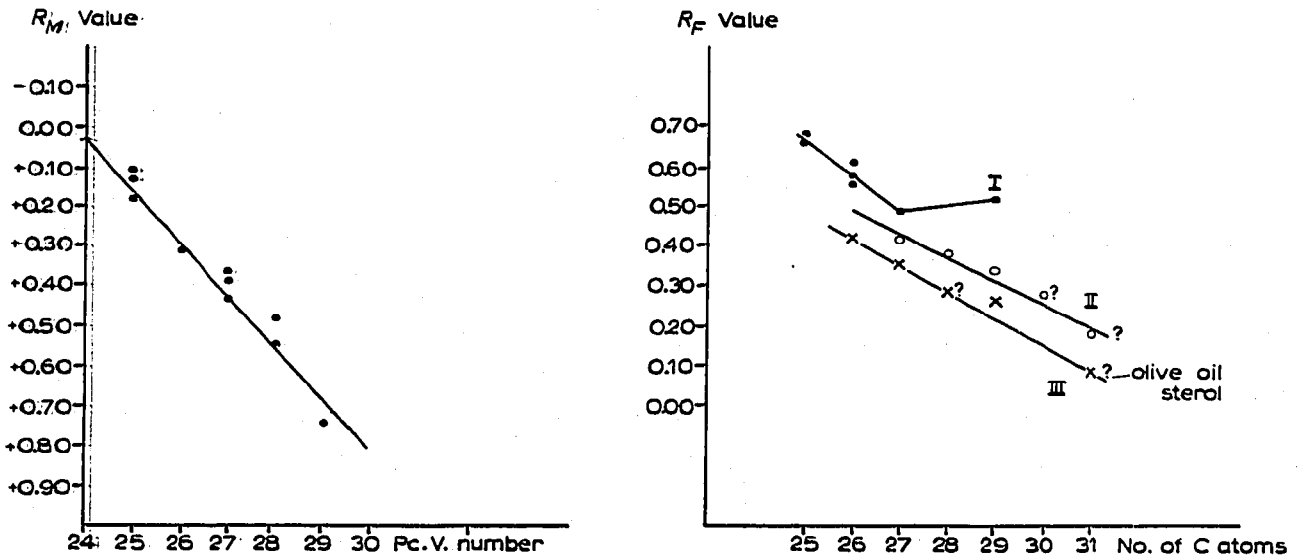


Fig. 3. (a) The R_M values of lanosterol and dihydrolanosterol fit into the linear relation obtained with the other sterols. (b) The linear relation of the R_F values in the systems described by KODICEK[16] (I), SULSER AND HÖGL[13] (II), and DE ZOTTI[12] (III).

The position of the double bonds in the molecule has no influence. Thus cholesterol (Δ^5) and Δ^7-cholestenol have the same R_F values. The place of attachment of the alkyl groups in the molecule has also no influence. Therefore the R_M values of the related pentacyclic triterpenoid alcohols lanosterol (FC30F), dihydrolanosterol (FC30), agnosterol (2FC30F) and dihydroagnosterol (2FC30), which all have two methyl groups at C-4, for the greater part agree with the above-mentioned rules.

Fig. 3a illustrates that the R_M values of the first two non-conjugated alcohols fit into a straight line with the R_M values of the other sterols.

From the graph of Fig. 3a and from the above-mentioned rules, the following ΔR_M values, characteristic for the studied system, can be calculated:

$$R_{M(0)} = +0.03 \text{ (C}_{27}\text{ number} = 27)$$
$$\Delta R_M^{CH_3} = +0.12$$
$$\Delta R_M^{C=C} = -0.11$$

When two conjugated double bonds are formed:

$$\Delta R_M^{C=C-C=C} = -0.11 + \text{exaltation value A of } +0.03 = -0.08$$

Introduction of another double bond, when the molecule already contains two of them, gives:

$$\Delta R_M^{C=C-C=C-C=C} = -0.11 + \text{exaltation value B of } +0.19 = +0.08$$

The theoretical R'_M values, mentioned in Table III, are calculated from R_{M_0} and ΔR_M values, according to the formulae:

$$R_{M2} - R_{M1} = \Delta R_M^{x} = \ln \Delta \mu^{x}/RT$$
$$R'_M\text{sterol} = R_{M(0)} + m \ln \Delta \mu^{(CH_3)}/RT + nn \ln \Delta \mu^{C=C}/RT + \text{exaltation values}$$

An example of such a calculation gives: R'_M dihydrowitanium $D_{2,3}$, $3FC_{28} = 0.03 + 4 \times 0.12 - 3 \times 0.11 + 0.19 = 0.37$ (experimental value 0.39).

When the number of double bonds in a sterol molecule is plotted against the R_M values, sigmoid curves are obtained. This is illustrated in Fig. 4.

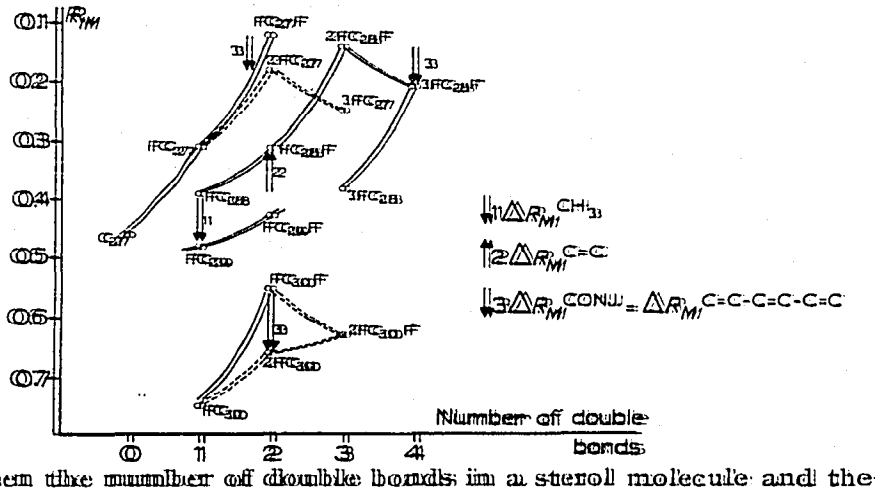


Fig. 4. Relation between the number of double bonds in a sterol molecule and the R_M value. The various ΔR_M values are presented.

APPLICATIONS

By applying these rules regarding the effect of various groups in the sterol molecule on the R_F value, important data concerning not yet identified sterol structures may be obtained.

KABASAKALIAN[24] recently calculated the ΔR_M values of several substituents in the pregnane nucleus. Using these values it is possible to calculate a theoretical R_F value of a steroid of known structure. Good agreement between calculated and experimental values has been achieved. Analogously, the structure of not yet identified steroids or naturally occurring sterols[25] can be determined by using these previously calculated ΔR_M values. Other paper chromatographic systems are useful for this purpose as well. The "R_F–carbon number" graphs of systems Nos. 14, 16 and 17, mentioned in Table I, also show a nearly linear relation (see Fig. 3b).

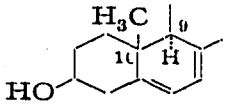
In system 14, the R_F values of the vitamins D_2 and D_3 also fit into the linear relation[16], unlike their behaviour in the system studied by us. The R_F value of β-sitosterol in system 14 is higher than might be expected from the linear relation. In the corresponding systems 13 and 15 of Table I, the deviating R_F values for β-sitosterol and ergosterol (R_F = 0.0) indicate non-ideal liquid–liquid partition chromatography.

In the analogous system: petroleum/pyridine–water (85:15)[12] a similar linear relation can be found (Fig. 3b). Only the R_F value of "fucosterol" obtained by DE ZOTTI[12] does not fit into this relation. This sterol, which has a structure isomeric with that of stigmasterol ($C_{29}H_{48}O$ with Δ^5 and Δ^{28}) should also present an R_F value corresponding with that of stigmasterol. According to curve III of Fig. 3b we should expect an R_F value of 0.37. The R_F value of about 0.41, obtained by DE ZOTTI for "fucosterol", points more in the direction of a sterol belonging to the critical pair: zymosterol–ergosterol.

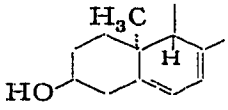
The R_F values of some unidentified sterols from peanut and whale oil (0.28 and 0.18 respectively) reported by SULSER AND HÖGL[13] fit into the linear relation shown by the other sterols (curve II of Fig. 3b).

Separation of some sterols and steroids that have different steric configurations

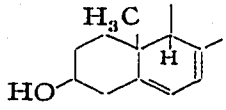
Some provitamins such as lumisterol, isopyrocalciferol and pyrocalciferol are isomers of ergosterol. Because of their different steric configurations, they have, however, different R_S values.



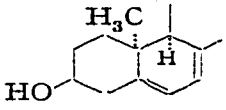
Ergosterol



Lumisterol



Isopyrocalciferol



Pyrocalciferol

Configuration of the molecule: 3β-OH, juncture A/B: *trans*

and	10β,9α	10α,9β	10β,9β	10α,9α
R_S value:	1.26	1.00	1.15	0.94

These data show that the R_S values of provitamins belonging to the (allo) series of β-cholestanol and possessing β-configuration at carbon atom 10 are always higher than these of the corresponding provitamins with α configuration at carbon atom 10. The separation of the provitamins with different configurations at carbon atom 10 *i.e.* ergosterol (10β,9α) and lumisterol (10α,9β), or isopyrocalciferol (10β,9β) and pyrocalciferol (10α,9α), is possible under favourable conditions. A separation of provita-

mins with the same configuration at C-10 cannot be obtained. The series of increasing R_S values is as follows:

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

$$\Delta R_M\ 9\alpha,10\alpha \longrightarrow 9\alpha,10\beta = -0.21$$

$$\Delta R_M\ 9\beta,10\alpha \longrightarrow 9\beta,10\beta = -0.10$$

The lumisterol preparation that we had at our disposal showed also a second zone ($R_S = 1.72$), only visible by a greenish colour in U.V. light (365 mμ), and of unknown origin.

If the steric configuration of a sterol molecule is different from the (allo) series of β-cholestanol with respect to the configuration at C-5 at the juncture of rings A and B, the R_S values obtained deviate considerably. The R_S value of cholestanol (3β, equatorial OH group, juncture A/B: *trans* or 3β,5α) is considerably higher than that of coprostanol (3β, axial OH, A/B: *cis* or 3β,5β). Thus we can derive a difference in polarity of the molecule given by: 3β,5α > 3β,5β. ΔR_M 3β,5α ⟶ 3β,5β = +0.70.

This corresponds with data in the literature[26] concerning C_{19} and C_{21} steroids, which show the polarity sequence: 3β,5α ≥ 3α,5β > 3β,5β ≥ 3α,5α.

In general, steroids with an equatorial OH group show a higher polarisy in partition chromatography than those with an axial OH group[26]. Thus choletterol (3β equatorial) likewise has a higher R_S value than epicholesterol (3α axial). ΔR_M 3β ⟶ 3α = ± 0.14.

EXPERIMENTAL

Several hexagonal holes are cut out of a sheet of Schleicher and Schüll No. 2043b mgl paper of 20 × 50 cm, according to MATTHIAS[32] (see Fig. 5). The direction of the paper fibres should be parallel to the direction in which the mobile phase moves.

The paper is immersed three times in a 10% solution of medicinal paraffinum liquidum in petroleum ether (b.p. 60–80°). After drying in the air the degree of impregnation is about 0.15 g/g paper. The stationary and mobile phases are mutually saturated. In the centre of the 1 cm wide bridges 15 μg sterol mixture is spotted. After 16 h accommodation, the chromatogram is developed with the mobile phase: acetic acid–water (84:16) for 40–48 h, by the ascending method. The temperature should be 22–24°; the length of the run 25–30 cm. After drying the chromatogram some sterols are visible in U.V. light (365 mμ) as fluorescent spots (see Table V). After drying for 2 h in the air and 1 h at 80°, the chromatogram is sprayed with a 10% ethanolic solution of phosphomolybdic acid (Merck) and then heated for about 1–4 min at 80°.

Blue-green spots develop on a quickly darkening light green background.

Dihydrocholesterol and other saturated sterols, if present in quantities of 100 μg, become visible as yellow spots. By spraying with a mixture of ether–concentrated sulphuric acid (2:1) and heating 5–10 min at 80° all saturated sterols become visible as blue-green spots[27].

The R_F values mentioned in Table I were calculated from a great number of

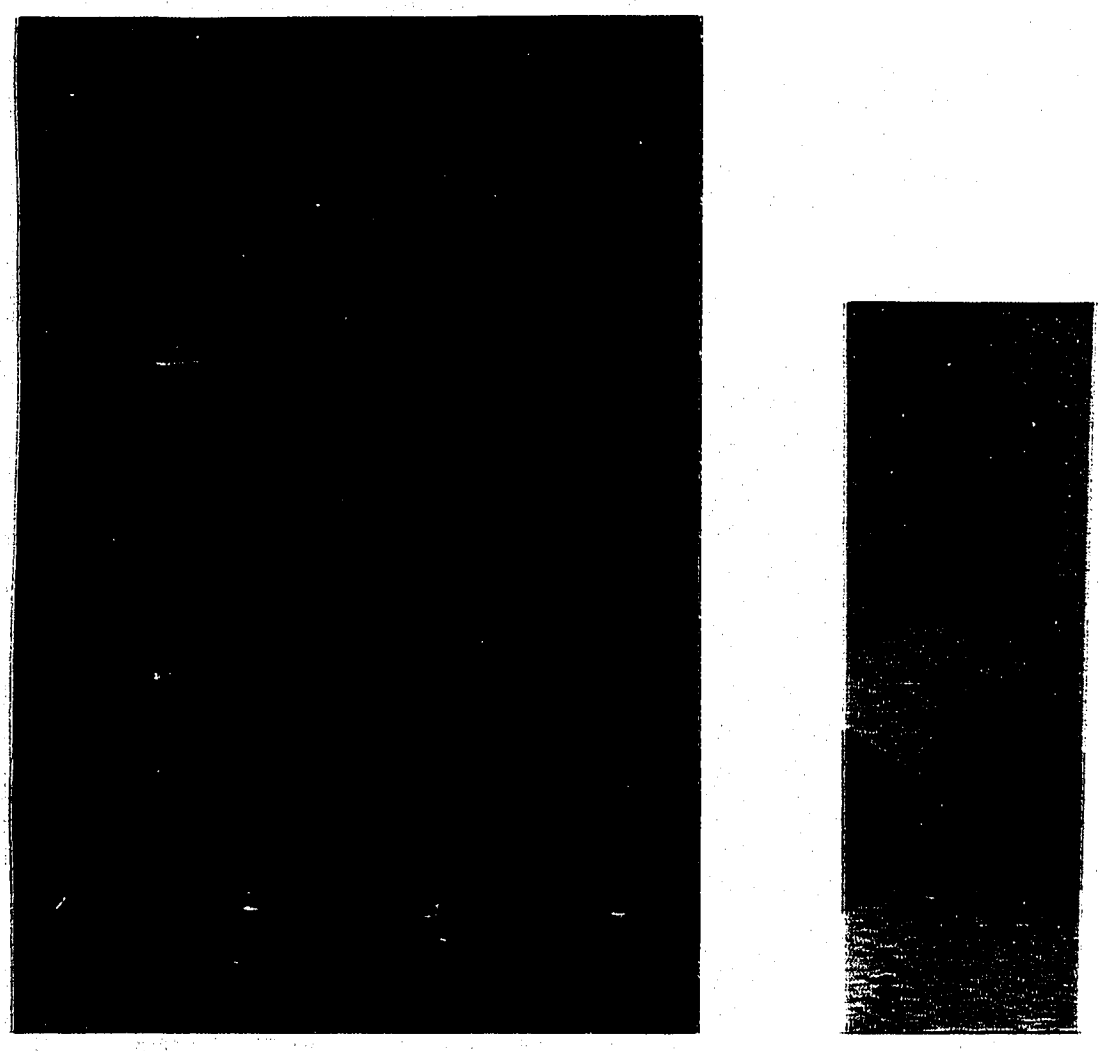


Fig. 5. Separation of some sterols in the system: paraffin/acetic acid–water ((8:1:16)). Spot 1: technical wool-fat alcohols, showing cholesterol, lanosterol and dihydrolanosterol. Spot 2: a mixture of ergosterol, cholesterol, stigmasterol, β-sitosterol, dihydrolanosterol and cholesteryl acetate. Spot 3: lumisterol. Spot 4: vitamin D_2. Spot 5: 15 μg of a mixture of cholesterol and phytosterols from soja oil ((2:8)).

determinations. The precision of the R_F values is approximately ± 0.01. The R_M values were calculated with the formula: $R_M = \log((1/R_F) - 1)$.

Near the front some spots produced by oxidation products appear, e.g. 7-hydroxy-cholesterol.

Accompanying sterols in quantities of about 10–30% are demonstrated by spotting 100–400 μg sterol mixture on a sheet of Whatman No. 3 paper, cut in a similar way with bridges of 0.5 cm. Besides the principal spots some weakly coloured zones can be demonstrated.

Colour reactions

Besides the above-mentioned very sensitive detection with phosphomolybdic acid, more specific colour reactions are available for the identification of sterols belonging to the same critical pair.

TABLE V

COLOUR REACTIONS SHOWN BY SOME STEROLS

	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*
Compound	*U.V. light 365 mμ*	*$SbCl_3$*	*Sb_2Cl_3*	*$BiCl_3$*	*$CaCl_2$ in U.V. light*	*Trichloroacetic acid*	*Phosphotungstic acid*	*Silicotungstic acid*	*Dimethyl-p-phenylenediamine*	*Millon's reagent*	*Urea in U.V. light 365 mμ*	*$NaIO_4$-$KMnO_4$*
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	—	—	—(365mμ) +(256mμ)	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 (365mμ) +(256mμ)	yellow
7-Dehydrocholesterol	+	purple-brown	brown-blue	faint purple-brown	+	green-purple	purple-brown	purple-brown	—	yellow	+	yellow
Vitamin D_2	+	brown	brown-blue	grey-brown	+	—	brown	brown	blue	faint yellow	—	yellow
Lumisterol	+	faint brown	purple	—	+	purple	purple-brown	purple-brown	blue	yellow	+	yellow

In the literature the following colour reactions are mentioned: with antimony (III) chloride[6], antimony (V) chloride[11,27,28], phosphotungstic acid[29], $SnCl_2$ with benzoyl chloride[6] and silicotungstic acid[30,31]. We have found some other colour reactions, *e.g.* with trichloroacetic acid, $CaCl_2$, $BiCl_3$, $NaIO_4$–$KMnO_4$, urea and dimethyl-*p*-phenylenediamine–*m*-tolylenediamine (1:1) also very useful.

The colour reactions are carried out on spots of 100 µg sterol on Whatman No. 3 paper. These more specific reactions, mentioned in Table V, can be used for the identification of not yet identified, naturally occurring sterols[25].

Execution of the colour reactions mentioned in Table V. The dried chromatogram is sprayed with:

II. A solution of antimony (III) chloride (50% in ethanol). Heat 5–10 min at 70°. The colours produced in daylight (and in U.V. light of 365 mµ) are noted.

III. A solution of antimony (V) chloride (20% in $CHCl_3$).

IV. A solution of bismuth (III) chloride (33% in ethanol). Heat some seconds at 60°. Colours are produced in daylight and U.V. light.

V. A solution of $CaCl_2$ (50% in ethanol–water) and heated 10–15 min at 80–90°. In U.V. light of 365 mµ fluorescent spots are visible.

VI. Moisten the chromatogram with trichloroacetic acid, dissolved in two drops of water.

VII. A solution of phosphotungstic acid (15% in ethanol). Heat some minutes at 60°.

VIII. A solution of silicotungstic acid (25% in ethanol). Heat some minutes at 60°.

IX. A mixture of dimethyl-*p*-phenylenediamine and *m*-tolylenediamine, 1:1 (1% in water).

X. Millon's reagent (one part of mercury dissolved in two parts of concentrated nitric acid). Heat 2–4 min at 40–50°; spray again and heat.

XI. A solution of urea in water (50%). Heat 10–50 min at 80°. In U.V. light of 365 mµ and 256 mµ, fluorescent spots appear.

XII. A solution of $NaIO_4$ (1% in water). Spray after 5 min with 1% $KMnO_4$.

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SUMMARY

The determination of R_F values of several sterols, fat-soluble vitamins, provitamins and pentacyclic triterpenoid alcohols in the system paraffin/acetic acid–water (84:16) is discussed.

There is a nearly linear relation between the R_M (R_F) values and the number of carbon atoms.

Some rules are given regarding the effect on the R_F value of the introduction of one or more double bonds into the molecule.

The ΔR_M values, due to the introduction of a CH_3 group, or of non-conjugated or conjugated double bonds into the molecule are calculated. Sterols with nearly the same R_F values are arranged in so-called critical pairs. Rules concerning these critical pairs are discussed.

In the system mentioned above the separation of the following sterols has been achieved: cholesterol–stigmasterol, β-cholestanol–coprostanol, cholesterol–epicholesterol, lanosterol–dihydrolanesterol and ergosterol–lumisterol, etc.

The system can be used for the identification of not yet identified sterols, isolated from natural sources.

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