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Study on the Chemical Nature of Sterols contained in Bulgarian Sunflower Oil

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The free sterols, the sterol esters and the sterol glycosides of the crude sunflower oil as well as those of the technical lecithin, the pitch and the deodorizer distillate of the latter oil were isolated by preparative TLC. The nature of sterols contained in the isolated sterol derivatives was elucidated by GLC and combined GLC-MS.

Major sterols of all examined sterol fractions are sitosterol, campesterol and stigmasterol, the amount of sitosterol being prevalent. Unknown sterol with a molecular weight of 428 is present in sterols of the crude oil and the deodorizer distillate. Sterols of the deodorizer distillate contain an unknown sterol with a molecular weight of 430. Stigmasterol is present in the sterol fraction of the deodorizer distillate in high amounts. It was established that Δ^7-sterols of the crude oil occur only in esterified form.

The hydrolysis of the sterol derivatives in acid medium leads to dehydration products known as steroid dienes and disteroid ethers. Hydrolysis without dehydration was achieved by enzyme preparations.

Some sterols of the crude oil were esterified with the same higher fatty acids contained in the glycerides of the sunflower oil.

Recently we found that Bulgarian sunflower oil contains free sterols, sterol esters and sterol glycosides [1, 2]. The quantity of free sterols in the crude oil is predominant, the content of sterol glycosides and esterified sterol glycosides being very low. In the sunflower phosphatides (technical lecithin) sterol glycosides are prevalent, whereas sterol esters occur in very low quantities. The pitch contains only esterified sterols, while in the deodorizer distillate sterols occur only in a free state.

In this paper we present the results from our investigations on the nature of individual sterols present as free sterols, sterol esters and sterol glycosides of the crude sunflower oil and the refinement byproducts, i.e. technical lecithin, pitch and deodorizer distillate.

Such data are not available in the literature, although a great number of publications on the sterol composition of the so called unsaponifiables, i.e. the mixture of free sterols and those obtained by saponification of sterol esters of sunflower oil, are known [5—9]. The sterol glycosides of the technical lecithin [3] and the unsaponifiables of the deodorizer distillate [4] were the only refinement byproducts of the latter oil characterized in their sterol composition.

Materials and methods

In our studies on the crude oil, the technical lecithin and the pitch we used industrially obtained products. The deodorizer distillate was prepared by laboratory deodorizing of hydrated oil [2].

The free sterols were isolated by preparative TLC on Silica gel G using petroleum ether:diethyl ether (1:1) system.

The sterol esters were isolated by preparative TLC on Silica gel G using petroleum ether:diethyl ether (75:5) system. The hydrolysis was carried out in alkaline medium by the method of NORDBY et al. [10]. The free sterols were isolated by preparative TLC using petroleum ether:ethyl ether (1:1) system.

The sterol glycosides and the esterified sterol glycosides were isolated by preparative TLC using chloroform:methanol (94:10) system. The acid hydrolysis was caried out by the method of GAVER et al. [11]. The product obtained was separated by TLC using petroleum ether:diethyl ether (1:1) system into two spots marked A (at the front) and C (Rf —0,50). Substance A was separated by preparative TLC using petroleum ether system into substances B (at the front) and E (Rf —0,20).

Enzyme hydrolysis of a mixture of sterol glycosides and esterified sterol glycosides dispersed in acetate buffer (pH 5,5) was carried out with equivalent amount Luizym (Fa. Luitpold-Werk, Chemisch-Pharmazeutische Fabrik, München.) at 37 °C for 72 h. The free sterols were isolated by preparative TLC using petroleum ether:ethyl ether (1:1) system.

The nature of the individual sterols contained in the fractions was elucidated chiefly by GLC, MS and combined GLC-MS analysis.

The GLC analysis of sterol fractions were performed with Pye Unicam gas chromatograph. Operating conditions: Glass column packed with Gas Chrom P and coated with 3% OV-17. Temperature 265 °C, carrier gas at 60 ml/min.

The MS and combined GLC-MS analysis of sterol fractions was performed with LKB 2091 gas chromatograph — mass spectrometer. The chromatograph was fitted with a glass column, packed with Chromosorb W and coated with SE-30. Operating conditions: column 235 °C, molecular separator and ion source 200 °C, ionizing voltage 70 eV.

The nature of some products obtained by acid hydrolysis of sterol glycosides and esterfied sterol glycosides was elucidated by their U. V. and I.R. spectra compared with those of authentic samples of cholesterol, sitosterol and stigmasterol.

The study of the acyl components of sterol esters was carried out with PYE Unicam gas chromatograph, fitted with a 1,5 m glass column, 4 mm ID, packed with chromosorb W and coated with 14% PEGS. Operating conditions: column 170 °C, carrier gas at 40 ml/min.

Results and Discussion

Since free sterols are present only in the crude oil, the technical lecithin and the deodorizer distillate, these sterols were isolated from the latter products only. Sterol esters only occur in the crude oil, the pitch and, in insignificant amounts, in the technical lecithin and they were isolated from the first two products only. Analogously, sterol glycosides and esterified sterol glycosides were obtained only from the technical lecithin.

The nature of sterols present in the examined materials was partially elucidated during the chromatographic separation. TLC of sterols obtained from the sterol glycosides and the esterified sterol glycosides by enzyme hydrolysis showed besides the major spot typical for sterols, two other spots corresponding in chromatographic behaviour to 4-monomethyl- and 4,4-dimethyl sterols. The same spots were observed in the TLC of sterols obtained from sterol esters of the crude oil. This chromatogram did not show spots for Δ^7-sterols probably due to the low content of the latter. Such a procedure using higher amount of oil afforded a clearly visible TLC spot for Δ^7-sterols. Further separation of the latter by TLC-method was not achieved by us [6].

The GLC, MS and combined GLC-MS analysis of the sterol fractions led to the following results. All examined substances contain sitosterol, stigmasterol and campesterol, the amount of sitosterol being prevalent.

The position of the double bond in Δ^5-sterols favours dehydration, since with the cleavage of the OH group at C-3 a system of conjugated double bonds arises. This structural condition was not present in another position of the double bond, which was used to differentiate Δ^5- from Δ^7-sterols. It was established that in the mass spectrum of the Δ^7-sterols examined by us, the ratio $\frac{M^{\cdot+}}{M - H_2O]^{\cdot+}}$ reaches 40, whereas in Δ^5-sterols it is from 1 to 2. Taking into consideration the fact that the ration $\frac{M^{\cdot+}}{M - H_2O]^{\cdot+}}$ in the mass spectrum of some sterol mixtures exceeds two, it can be concluded that the latter contain Δ^7-sterols.

An intensive peak at m/e 314 was observed in the mass spectrum of some of the examined sterol mixtures. This peak indicates content of C_{29}-dienol with a second double bond in the side chain at C_{24}-C_{28} or at C_{24}-C_{25} [12].

A mixture of C_{29}-dienols, among which a low amount of stigmasterol (less intensive peak at m/e 300) and higher amounts of other sterols with unknown position of the double bond in the side chain were observed in the mass spectrum of sterols obtained from sterol esters of the crude oil.

The mass spectrum of the sterol fraction of the deodorizer distillate showed presence of small quantities of dihydrositosterol, dihydrocampesterol and dehydrocampesterol (molecular weight 416, 402 and 398).

Besides this, substances with the molecular weights 428 and 430 were present in the mass spectra of some sterol fractions. The MS analysis has not elucidated the structure of these substances, which will be an object of a separate investigation. Further in this paper the above substances will be marked as substance with a molecular weight of 428 and substance with a molecular weight of 430.

The data referring to the composition of the examined sterol fractions are shown in Table 1.

Acid hydrolysis of sterol glycosides [12] afforded a product whose TLC showed two spots (A and C) giving the typical sterol colouring. We showed spot C to be a sterol (or a mixture of sterols). Substance A was separated using a non-polar eluent into two spots marked as B and E.

In the TLC substance B is present at the front and gives pink colouring with sulphuric acid (50%) at room temperature. The U.V. spectrum of the product shows absorption maximum at 228, 235 and 243 nm. Its I.R. spectrum did not show presence of any oxygen-containing groups. These data give us reason to assume that product B is a hydrocarbon.

Substance E is more polar than substance B in chromatographic behaviour. Its I.R. spectrum showed strong absorption at 1095 cm^{-1}, which is typical for the –C–O– bond. The lack of absorption in the 3600 and 1700 cm^{-1} region refuted the possibility of substance E to contain hydroxyl and carbonyl groups, which led to the assumption that an ether bond is present.

Similar treatment of a number of individual sterols, such as cholesterol, sitosterol and stigmasterol afforded apolar steroids. Their U.V. and I.R. spectra corresponded to cholesta-3,5-diene, sitosta-3,5-diene and stigmasta-3,5-diene, respectively and were

Table 1
Composition of the examined sterol fractions

Sample	Sterols	Per cent distribution in the mixture
I. Sterols of the crude oil		
1. Free	sitosterol	50
	campesterol	13
	stigmasterol	25
	substance with m.w.* 428	12
2. Esterified	sitosterol	47
	campesterol	6
	C_{29}-dienols combined with low amount of stigmasterol	10
	Δ^7-stigmasterol	4
	substance with m.w. 428	33
II. Sterols of the lecithin		
3. Free	sitosterol	61
	campesterol	19
	stigmasterol	14
	substance with m.w. 428	6
4. Sterol glycosides (after enzymatic hydrolysis)	sitosterol	83
	campesterol	7
	stigmasterol	10
III. Sterols of the pitch		
5. Esterified	sitosterol	74
	campesterol	7
	stigmasterol combined with other C_{29}-dienols	19
IV. Sterols of the deodorizer distillate		
6. Free	sitosterol	52
	campesterol	3
	stigmasterol combined with other C_{29}-dienols	28
	substance with m.w. 428	10
	substance with m.w. 430	7

* m.w. = molecular weight

very similar to those of substance B. Bisteroid ethers were obtained in very low quantities from the three individual sterols and our attempts failed to isolate them.

The TLC of the native starting materials as well as of the isolated sterol glycosides and esterified sterol glycosides did not give spots corresponding to substances B and E, which shows that the latter are formed during the acid hydrolysis of sterol glycosides. The lack of free hydroxyl groups both in substances B and E shows that the latter are products of secondary denaturating processes. In 1951 RAOUL et al. [13] have carried out dehydration of cholesterol with conc. sulphuric acid with the view to obtaining a product with antirachitic activity and they have obtained a mixture consisting of cholesta-2,4-diene, cholesta-3,5-diene, dicholesteryl ether and disteroid hydrocarbon. Later BRIESCORN et al. [14] assumed that the colouring in the reaction of LIEBER-

MANN-BURCHARD (conc. sulphuric acid) is due also to a steroid diene obtained under the dehydrating action of the acid.

In 1950 KAUFMANN et al. [15] found that the bleaching earth causes dehydration of sterols of the vegetable oils. The result was confirmed by a number of investigations [16–19], which showed that the main product of denaturation of sterols obtained by bleaching of vegetable oils are $\Delta^{3,5}$-steroid hydrocarbons. The absorption maximum was observed at 228, 235 and 243 nm in their U.V. spectrum. KAUFMANN et al. [18] and HOMBERG [19] have shown that the bleaching of vegetable oils results also in disteroid ethers. The I.R. spectrum of the latter revealed strong absorption at 1095 cm^{-1}.

On the basic of these results it can be concluded that under the conditions of the acid hydrolysis, denaturation of the same type proceeds, which results in the formation of apolar steroids and disteroid ethers.

The described denaturation of sterols may prove to be an essential shortcoming of the acid hydrolysis as a method of investigation of the sterol derivatives, particularly if the latter completely undergo dehydration. We established that similar process also occurs during other routine variants of the acid hydrolysis, i.e. the method of THORNTON et al. [20] and POWER et al. [21].

The same dehydration was observed when we attempted to carry out hydrolysis under mild conditions (in presence of Amberlite 15, ROHM and HAAS Co) or ethanolysis in absolute ethanol with p-toluene-sulphonic acid [22].

We accomplished the hydrolysis of sterol glycosides without dehydration using β-glucosidase (Luizym or Zease T). Hydrolysis of sterol glycosides and esterified sterol glycosides isolated from technical lecithin was carried out with Luizym at room temperature and no products of dehydration were observed.

The attempts to hydrolyse the sterol glycosides with α-amylase were unsuccessful. This gives us reason to assume that the glycoside linkage in the sterol glycosides is of β-type.

The percent composition of some more important fatty acids contained as acid components of the sterol esters in the crude oil is shown in the following:

$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
18,2%	2,2%	6,8%	27,6%	45,2%

Zusammenfassung

TS. MILKOVA, N. MAREKOV, S. POPOV, N. WULFSON und I. BOGDANOVA: Untersuchungen über die chemische Natur der Sterine in bulgarischem Sonnenblumenöl

Die freien Sterine, die Sterinester und Steringlykoside im rohen Sonnenblumenöl sowie im technischen Lecithin, im Fettsäurenpech und im Desodorationsdestillat dieses Öls werden durch Dünnschichtchromatographie isoliert. Die chemische Natur der in den einzelnen Sterinderivaten vorhandenen Sterine wird durch GLC und GLC-MS bestimmt. Die Hauptsterine aller untersuchten Sterinfraktionen sind Sitosterin, Campesterin und Stigmasterin; die überwiegende Menge stellt Sitosterin dar. Ein nichtidentifiziertes Sterin vom Molekulargewicht 428 ist in den Sterinen des rohen Öls und des Desodorationsdestillates vorhanden. Die Sterine des Desodorationsdestillates enthalten außerdem ein nichtidentifiziertes Sterin vom Molekulargewicht 430. Bemerkenswert ist der hohe Gehalt an Stigmasterin in der Sterinfraktion des Desodorationsdestillates. Die Δ^7-Sterine des rohen Öls sind nur in veresterter Form festgestellt worden.

Die Hydrolyse der Sterinderivate in saurem Medium führt zur Bildung von Dehydratationsprodukten, die als Steroid-Diene und Bisteroid-Äther identifiziert worden sind. Hydrolyse ohne Dehydra-

tation wird durch die Anwendung von Enzympräparaten erzielt. Ein Teil der Sterine im rohen Öl ist mit denselben höheren Fettsäuren verestert, die auch in den Glyceriden des Sonnenblumenöls vorhanden sind.

Резюме

Ц. Милкова, Н. Мареков, С. Попов, Н. Вульфсон, И. Богданова: Исследования химмической природы стеролов, содержающихся в болгарском подсолнечном масле

Свободные стеролы, эфирно- и гликозидно связанные стеролы сырого подсолнечного масла, технического лецитина, пека и дезодорационного дистиллята этого масла изолированы при помощи препаративной ТСХ. Химическая природа стеролов, входящих в составе каждого отдельного вида изолированных стероловых производных установлена при помощи ГЖХ и комб. ГЖХ-МС.

Главные стеролы всех исследованных стероловых фракции-ситостерол, кампестерол и стигмастерол, причем количество ситостерола преобладает. Неидентифицированный стерол м. в. 428 содержится в стеролах сырого масла и дезодорационного дистиллята. Стеролы дезодорационного дистиллята содержат и неидентифицированный стерол м. в. 430. Нужно отметить высокое содержание стигмастерола в строеловой фракции дезодорационного дистиллята. Найдено, что Δ^7-стеролы сырого масла находятся только в виде сложных эфиров.

При гидролизе стероловых производных в кислой среде образуются продукты дегидратации, которые идентифицированны как стероидные диены и бистероидные эфиры. Для избежания дегидратации проведена гидролиза в присутствии энзимов.

Часть стеролов сырого масла этерифицированы с теми высшими жирными кислотами, которые участвуют в составе глицеридов подсолнечного масла.

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