Identification of New Steroidal Hydrocarbons in Refined Oils and the Role of Hydroxy Sterols as Possible Precursors

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The dehydration of sterols during the refining process of vegetable oils results in the formation of steroidal hydrocarbons (sterenes or steradienes) with two double bonds in the ring system. Other steroidal hydrocarbons whose structures were in agreement with the presence of three double bonds in the ring system were detected in the sterene fractions of refined vegetable oils. The 5α-, 7α-, and 7β-hydroxy derivatives of cholesterol and phytosterols have been dehydrated in *n*-butanol/H_3PO_4 to form steroidal hydrocarbons with three double bonds at the 2, 4, and 6 positions in the ring system. These hydrocarbons had the same relative retention time and mass spectra as those present in the sterene fractions of refined oils. The dehydration of the hydroxy sterols dissolved in extra virgin olive oil and in the presence of 1% bleaching earths at 80 °C for 1 h results in the formation of the same steroidal hydrocarbons found in the refined oils.

Keywords: *Steroidal hydrocarbons; sterenes; steratrienes; hydroxy sterols; refined oils; gas chromatography/mass spectrometry; bleaching*

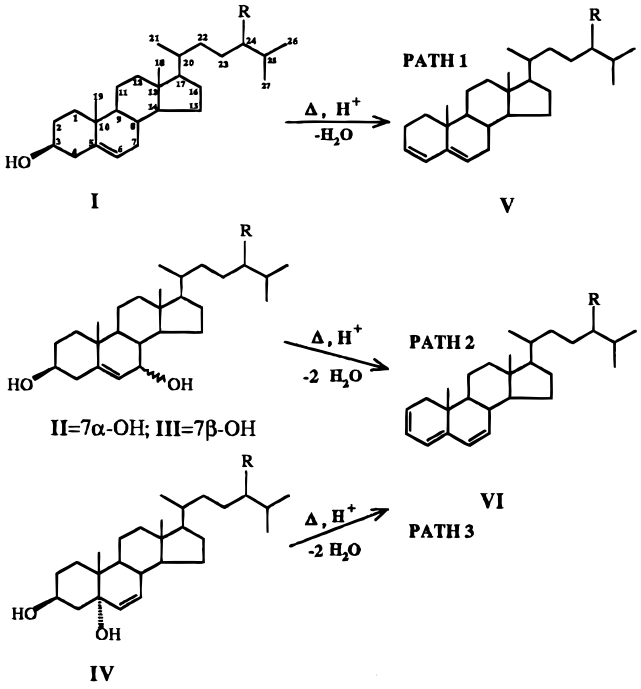
INTRODUCTION

The identification and quantitation of minor components in edible oils, naturally present or produced as a consequence of refining, are utilized both for characterization purposes as well for the detection of frauds. With a few exceptions, among which is extra virgin olive oil, edible oils undergo, prior to sale, more or less intense processes such as neutralization, bleaching, and deodorization.

The bleaching with acidic earths and deodorization at high temperature cause the sterols to undergo transformations such as isomerization with a shift of the double bond (Biederman et al., 1996) and degradations such as dehydration with the formation of steroidal hydrocarbons (sterenes or steradienes) as shown in Scheme 1, path 1. By dehydration, each sterol gives rise to three sterene isomers with the two double bonds at the 3,5-, 2,4-, and 2,5-positions. In Scheme 1, only the 3,5-diene derivative has been reported, since it is the isomer more abundant. (Niewiadomski et al., 1966; Kaufmann et al., 1970; Grob et al., 1994; Mennie et al., 1994; Cert et al., 1994, 1998). The determination of sterenes can detect the addition of refined oil to extra virgin olive oil as low as to the 1% level. This analysis is now a European Union Official analytical method for the control of extra virgin olive oil (Regulation EC/656/95; Lanzon et al., 1989; Grob et al., 1995). Crews et al. (1997) utilized the analysis of sterenes to detect the presence of refined vegetable fats in chocolate.

The GC-MS analyses of the sterenes fraction isolated from refined oils revealed other compounds whose mass spectra had a molecular ion two mass unit less than those of the sterenes derived from the dehydration of β-sitosterol (**Ib**), stigmasterol (**Id**), and campesterol (**Ic**) (Grob et al., 1994; Mennie et al., 1994; Bortolomeazzi et al., 1996).

Scheme 1



R = H: **Ia, IIa, IIIa, IVa, Va, VIa**
R = C_2H_5: **Ib, IIb, IIIb, IVb, Vb, VIb**
R = CH_3: **Ic, IIc, IIIc, IVc, Vc, VIc**
R = C_2H_5 and C_{22}-C_{23} double bond: **Id, IId, IIId, IVd, Vd, VId**
R = CH_3 and C_{22}-C_{23} double bond: **Ie, IIe, IIIe, IVe, Ve, VIe**

Two mass units less is indicative of the presence of another double bond in the molecule; moreover, the occurrence of the ions at *m*/*z* 253 and 211, respectively due to loss of the side chain and of the chain plus ring D, typical of sterolic structures, supports the presence of the double bond in the ring system, Scheme 2.

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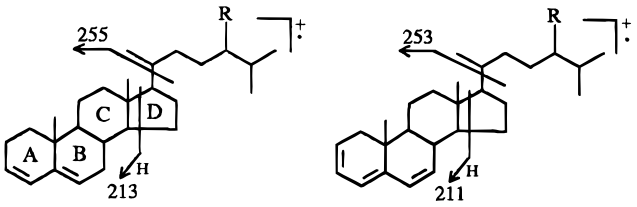
10.1021/jf9912147 CCC: $19.00
Published on Web 03/29/2000

Scheme 2



The formation of these products can be rationalized by the loss of two molecules of water from the hydroxy derivative of a sterol as reported in Scheme 1, paths 2 and 3.

Grob et al. (1994) tentatively identified the product formed from β-sitosterol as the 24-ethyl-cholest-2,4,6-triene claiming that oxidation is involved in its formation. Smith (1981) reported the formation of colest-2,4,6-triene (**VIa**) among the degradation products of 7-hydroperoxy and 7-hydroxy derivatives of cholesterol. The formation of steroidal hydrocarbons and ketosteroids from 7-hydroxy derivatives of β-sitosterol (**IIb**, **IIIb**) treated with bleaching earths was also reported long ago by Niewiadomski et al. (1964) and by Kaufman et al. (1970). Bortolomeazzi et al. (1996) demonstrated the correlation between the oxidation products of β-sitosterol, campesterol, and stigmasterol and the formation of compounds with three double bonds in the ring system.

In this work, the 5α-, 7α-, and 7β-hydroxy derivatives of the principal sterols were subjected to the action of activated bleaching earths in order to confirm the hydroxy sterols as possible precursors as well as to identify the steroidal hydrocarbons formed.

MATERIALS AND METHODS

Materials and Reagents. All solvents were of analytical grade. Cholest-3,5-diene (**Va**) was purchased from Sigma. Silica gel 60, 70−230 mesh ASTM, and the thin-layer chromatography (TLC) silica gel plates, 0.25 mm thickness, were provided by Merck (Bracco, Milan, Italy). The silica gel was equilibrated with 2% (w/w) of water before use.

The bleaching earth "Tonsil Supreme NFF" was obtained from an oil refining industry.

The melting points were determined on a Gallen Kamp MFB-595 (England) melting apparatus.

Gas Chromatography (GC) and GC-Mass Spectrometry (GC-MS). A Carlo Erba Mega 5160 gas chromatograph equipped with a split−splitless injector and a flame ionization detector was used. The fused silica column was an SPB5, 30 m × 0.32 mm i.d., 0.25 μm film thickness (Supelco, Bellefonte, PA). The column temperature was 280 °C isotherm and the detector and injector 300 °C. The carrier gas (helium) flow rate was 1.3 mL/min, and the split ratio was 1:40 (v/v).

For the GC-MS analysis, a Varian 3400 gas chromatograph coupled to a Varian Saturn ion trap detector was used. The fused silica column was a DB-5 (J&W, Folsom, CA), 30 m × 0.25 mm i.d., 0.25 μm film thickness, operating at 280 °C. The injection was in split mode (split ratio 1:40) with helium as the carrier gas at a flow rate of 1 mL/min. The injector, transfer line, and ion trap temperatures were, respectively, 300, 300, and 170 °C. The electron impact (70 eV) spectra were recorded at 1 s/scan with a filament emission current of 10 μA.

Synthesis of the Hydroxy Derivatives of Sterols. Synthesis of the 5α-, 7α-, and 7β-hydroxy derivatives of cholesterol and phytosterols was carried out as reported by Bortolomeazzi et al. (1999). Due to the presence of ca. 8% campesterol in the β-sitosterol standard, the corresponding hydroxy derivatives of campesterol itself were formed. The same thing happened in the case of stigmasterol, which contained, as impurities, 1.5% brassicasterol, 2.6% campesterol, and 4.8% β-sitosterol. This also made it possible to study the dehydration products of the hydroxy derivatives of campesterol and brassicasterol.

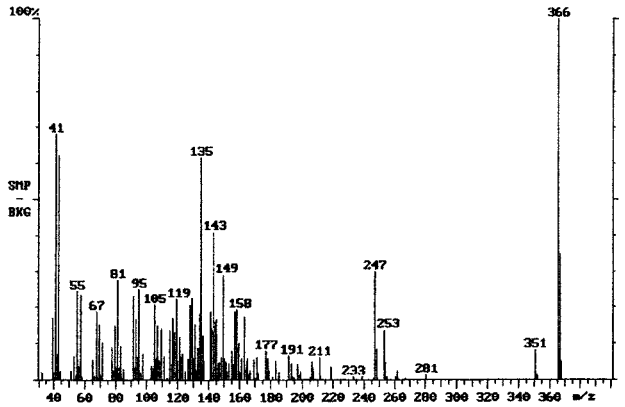


Figure 1. Mass spectrum of cholest-2,4,6-triene.

Table 1. Mass Spectrometric Data of the Dehydration Products of the Hydroxy Derivatives of Cholesterol and Phytosterols

	m/z				
ion	**VIa**	**VIb**	**VIc**	**VId**	**VIe**
M	366 (100)	394 (100)	380 (100)	392 (100)	378 (90)
M − CH_3	351 (8)	379 (5)	365 (8)	377 (4)	363 (5)
M − C_3H_7				349 (3)	335 (3)
M −C_9H_{11}	247 (30)	275 (25)	261 (27)	273 (5)	259 (6)
M − R[a]	253 (13)	253 (13)	253 (14)	253 (15)	253 (19)
M − (R + 2H)				251 (8)	251 (10)
M − (R + C_3H_6)	211 (6)	211 (6)	211 (8)	211 (7)	211 (9)

[a] R = side chain.

Synthesis of Cholest-2,4,6-triene. Concentrated phosphoric acid (0.5 mL) was added to a refluxing solution of 150 mg of 7α-OH-cholesterol (**IIa**) in 10 mL of *n*-butanol. The mixture was then refluxed for 1 h. After the mixture was cooled to room temperature, the volume was reduced under reduced pressure with a rotavapor, treated with water, and extracted with *n*-hexane. The hexane extract was washed several times with water, dried over anhydrous sodium sulfate, and filtered. Removal of the solvent under reduced pressure gave 143 mg of a yellow syrup which slowly solidified. The residue dissolved in *n*-hexane was loaded into a silica chromatography column (20 cm length × 0.75 cm i.d.) and eluted with *n*-hexane. The first fraction, 83 mg, immediate blue color with 80% H_2SO_4 (Smith et al., 1973), gave 29 mg of pure cholest-2,4,6-triene (**VI a**) after crystallization from aqueous acetone: mp 72.5−73.5 °C [lit. 71.5−72.5 °C (Selter and McMichael, 1967), 71−72 °C (Schmutz et al., 1951), 72−76 °C (Smith et al., 1973)]. Calcd for $C_{27}H_{42}$: C, 88.45; H, 11.55. Found: C, 88.04; H, 11.63. $\lambda_{max}^{cyclohexane}$ = 307 mμ (ϵ = 15 687) with shoulders at 321 (ϵ = 10 121) and 295 mμ (ϵ = 13 977) [lit. $\lambda_{max}^{cyclohexane}$ 307 mμ (ϵ = 15 400), shoulders at 321 (ϵ = 10 080) and 295 mμ (Selter and McMichael, 1967); $\lambda_{max}^{cyclohexane}$ 307.5 (ϵ = 12 600), 322.5 (ϵ = 8020), and 296.5 mμ (ϵ = 11 900), (Smith et al., 1973); $\lambda_{max}^{cyclohexane}$ 307 mμ (ϵ = 15 200) (Schmutz et al., 1951)]. The mass spectrum of **VIa** is reported in Figure 1, while the fragment ions, *m/z*, and relative abundances are reported inTable 1. Besides the formation of cholest-2,4,6-triene, the GC-MS analysis revealed the presence of two other isomers, probably with a different position of the double bonds, with a relative retention time with respect to **VIa** of 1.09 and 1.15 and in the amount of 1.9% and 0.3%, respectively.

Synthesis of the 2,4,6-Trienes from the Hydroxy Derivatives of Phytosterols. The 2,4,6-trienes (**VIb**, **VIc**, **VId**, **VIe**) were synthesized by dehydration of the 7α-OH derivatives of the corresponding sterols in the same experimental conditions used for the preparation of **VIa**.

Bleaching with Acidic Earths. About 5.00 mg of the hydroxy derivative of a sterol and 1 mL of a cholest-3,5-diene (internal standard, I.S.) solution (1.0 mg/mL) were pooled in a 10 mL round-bottom flask. After evaporation of the solvent under reduced pressure at room temperature, about 5.00 g of extra virgin olive oil was weighted in the same flask and the mixture was vigorously stirred by a magnetic bar until complete dissolution of the two compounds in the oil. After 50 mg (1% w/w) of acidic earths was added, the flask was connected to a water pump. The mixture was then warmed to 80 °C by a water bath, and the decoloration was carried out for 1 h at this temperature, under vacuum and vigorous stirring. After the mixture was cooled, it was immediately filtered on a Büchner to remove the earths.

Isolation of the Steroidal Hydrocarbons from the Bleached Oil. The bleached oil, 1 g, was loaded onto a silica gel column (1.5 cm × 15 cm) with two 1 mL portions of *n*-hexane. The chromatography was followed by spotting the eluate on a TLC silica plate sprayed with 80% sulfuric acid and warming with a hair dryer, the internal standard cholest-3,5-diene gave a red color, while the triene derivatives gave a blue color. The first fraction, about 30 mL, containing alkanes, was discarded; the second 40 mL fraction, containing the steroidal hydrocarbons, was reduced to dryness. The residue, dissolved in *n*-hexane, was transferred to a conical vial, and the volume was adjusted to about 0.5 mL prior to GC and GC-MS.

RESULTS AND DISCUSSION

Identification of the dehydration products of the hydroxy derivatives of phytosterols was carried out by comparing their UV absorption and mass spectral data with those of cholest-2,4,6-triene (**VIa**).

The products obtained from the dehydration, in acidified alcoholic medium, of **IIb** and **IId** had the following UV absorption characteristics: $\lambda_{max}^{cyclohexane} = 307$ mμ ($\epsilon = 15\,515$) with shoulders at 321 ($\epsilon = 9826$) and 296 mμ ($\epsilon = 14\,044$) and $\lambda_{max}^{cyclohexane} = 307$ mμ ($\epsilon = 15\,098$) with shoulders at 321 ($\epsilon = 9252$) and 296 mμ ($\epsilon = 14\,075$), respectively.

Both λ_{max} as well as ϵ are in agreement with the values of **VIa**, which means a structure with three double bonds at the 2,4,6-positions. The data are, moreover, in agreement with λ_{max} theoretically calculated on the basis of the number of double bonds and their position in the rings of the steroidal skeleton (Fieser and Fieser, 1959), therefore, excluding other possible structures, for example, that with the double bonds at the 3,5,7-positions.

As reported under Materials and Methods, 8% of **IIc** was present as an impurity together with **IIb**. GC-MS analysis of the dehydration product of **IIb** also revealed, in fact, the presence of the dehydration product of **IIc**, which was not able to be distinguished by UV absorption. The two dehydration compounds, **VIb** and **VIc**, have, in fact, pratically the same UV spectrum. Similarly, GC-MS analysis of the dehydration product of **IId** also revealed the presence of dehydration products of **IIb**, **IIc,** and **IIe**. The fragments, *m*/*z* values, and relative abundances of all the dehydration products **VIb**, **VIc**, **VId**, and **VIe** are given in Table 1.

The mass spectrum of **VIa** was characterized by the molecular ion (*m*/*z* 366) as the base peak. The ions at *m*/*z* 253 and 211 originated from the loss of the side chain and the side chain plus ring D, respectively, as shown in Scheme 2, while the ion at *m*/*z* 247 derived from the loss of rings A and B by fission of the bonds 7−8 and 9−10 (Mennie et al., 1994; Dumazer et al., 1986). The mass spectrum of **VIa** and those of the dehydration products of the hydroxy phytosterols **VIb**, **VIc**, **VId,** and **VIe** show pratically the same fragmentation patterns and similar relative ions abundances with the molecular ion at *m*/*z* 366, 394, 380, 392, and 378, respectively. The differences of 28, 14, 26, and 12 amu reflect the differences in the structure of the side chain (Scheme 1).

Some differences were present in the spectra of **VId** and **VIe**, due to the presence of a double bond in the side chain of these compounds. The ion at *m*/*z* 251 in the mass spectra of **VId** and **VIe** originates from the loss of the side chain together with two hydrogen atoms from the ring system. This cleavage is, in fact, characteristic of the presence of a double bond in the side chain (Wyllie et al., 1968; Dumazer et al., 1986). Moreover, the ions at *m*/*z* 349 and 335 present in the spectra of **VId** and **VIe,** respectively, were derived from the loss of an isopropyl (C_{25}, C_{27}) group at the end of the side chain, characteristic of the presence of a Δ^{22} double bond in the side chain of the steroidal structure (Dumazer et al., 1986; Pizzoferrato et al., 1993).

On the basis of the UV absorption and mass spectral data, the following structures have been assigned to the dehydration products of the 7α-OH phytosterols: from **IIb**, 24-ethyl-cholest-2,4,6-triene (*β*-sitotriene) (**VIb**); from **IIc**, 24-methyl-cholest-2,4,6-triene (campestatriene) (**VIc**); from **IId**, 24-ethyl-cholest-2,4,6,22-tetraene (stigmatetraene) (**VId**); from **IIe**, 24-methyl-cholest-2,4,6,22-tetraene (brassicatetraene) (**VIe**). The name in parentheses is given so that one remembers the original sterol from which the steroidal hydrocarbon with two new double bonds derives. In the same way that sterenes or steradienes are the trivial names used for the dehydration products of sterols, the name steratrienes could be used for the dehydration products of hydroxy sterols.

Also, in the case of the dehydration of **IIb** and **IId**, besides the 2,4,6-triene derivatives, the other two isomers were formed in amounts similar to those formed in the dehydration of **IIa**. In the case of the dehydration of **IIc** and **IIe**, the low amount of these compounds did not allow the detection of the triene isomers.

To verify the formation of the same dehydration products also in conditions similar to those utilized during the bleaching of oils, all three hydroxy derivatives of each sterol, dissolved in extra virgin olive oil, have been decolored in the presence of bleaching earths.

The 7α-OH as well the 5α-OH and 7*β*-OH derivatives of the same sterol dehydrated to form the same dehydration product, which was in agreement with the product obtained by dehydration of the 7α-OH isomer in alcholic acidic medium.

β-Sitotriene (**VIb**), campestatriene (**VIc**), and stigmatetraene (**VId**) have the same GC retention time and mass spectra as the compounds previously revealed in the sterene fractions of commercial sunflower, maize, soya bean, and peanut oils (Bortolomeazzi et al., 1996). Brassicatetraene (**VIe**) has, instead, a mass spectrum that is the same as a compound found by Mennie et al. (1994) in rapeseed oil, where the brassicasterol is a relatively abundant sterol. This confirms the presence of these compounds in refined oils and the possible role as a precursor of the hydroxy derivatives of sterols. The relative retention times of **VIa**, **VIb**, **VIc**, **VId**, and **VIe** with respect to the I.S. are 0.96, 1.36, 1.16, 1.23, and 1.04, respectively. To verify whether similar results were obtained also by the dehydration of the hydroperoxides, a mixture of 7-hydroperoxy derivatives of

Table 2. Dehydration Percentage of the Hydroxy Derivatives of Cholesterol and Phytosterols

hydroxy sterol	% dehydration[a]	standard deviation
IIa	21.4	0.96
IIb	22.5	1.48
IIc	21.9	1.41
IId	22.0	1.27
IIIa	17.8	1.53
IIIb	18.2	1.38
IIIc	16.2	1.28
IIId	17.7	0.89
IVa	18.4	0.46
IVb	20.8	1.61
IVc	19.7	1.77
IVd	20.9	0.81

[a] Mean of three repetitions.

cholesterol was bleached in the same conditions. In this case only about 1.2% of dehydration product **VIa** was formed, probably because of the degradation of the hydroperoxides to other products.

The extra virgin olive oil, used as the matrix, was bleached in the same conditions and without the addition of hydroxy sterols in order to verify the eventual formation of dienes and trienes which would have interfered with the quantitative analysis. Under our relatively mild conditions of bleaching, the sterols did not dehydrate; moreover, there was no formation of trienes, probably because hydroxy sterols or other possible precursors were absent or present only in a very low amount.

The percentage of dehydration, on a molar basis, of the hydroxy sterols (**IIa**, **IIb**, **IIc**, **IId**; **IIIa**, **IIIb**, **IIIc**, **IIId**; **IVa**, **IVb**, **IVc**, **IVd**) was determined by quantitative analysis of the corresponding dehydration products using cholest-3,5-diene as an I.S. and considering the relative response factor between the I.S. and the steratrienes to the FID detector to be equal to 1. The results are reported in Table 2. The dehydration percentages are similar for the same hydroxy derivative of the vegetable sterols and of cholesterol, whereas considering the different isomers, the 7α-OH and 5α-OH derivatives have similar average dehydration percentages which are a little higher than that of the 7β-OH isomers.

In the bleaching conditions used, dehydration of the hydroxy sterols was about 20%, whereas there was no dehydration of the sterols present in the extra virgin olive oil used as matrix. This is an indication of the higher dehydration rate of the hydroxy sterols with respect the sterols, independent of the fact that the dehydration depends on many factors such as the kind of oil, the bleaching earths, and the time and temperature of the process as outlined by Mariani et al. (1992) and Grob et al. (1995).

During the bleaching of the hydroxy derivatives of phytosterols, there was the unexpected formation, besides that of the trienes, also of 3,5- and 2,4-dienes such as those obtained by dehydration of the corresponding sterols. The total amount of 3,5- and 2,4-dienes formed was about one-tenth of the corresponding 2,4,6-trienes. The dienes corresponding to **Ie** were not detected due to the low amount of the hydroxy derivatives of brassicasterol. The origin of these dienes from the corresponding sterols was however excluded in this case on the basis of the following considerations: (a) in the hydroxy derivatives of the sterols there were no impurities of the original sterol; (b) the decoloration of the extra virgin olive oil, used as the matrix, which contained 1067 ppm of **Ib** and 12 ppm of **Id**, did not give rise to any diene compounds as previously reported (an eventual dehydration of these sterols should have been independent of the particular hydroxy sterol added); (c) a bleaching of **IId** in lard, which is free of vegetable sterols, gave the same results as bleaching in olive oil.

The formation of small amounts of 3,5- and 2,4-dienes seems to derive, in this case, from a reduction of the corresponding hydroxy sterol or of the triene derivative, probably due to the catalyzing action of the acidic earths. This can explain the formation of only the dienes of **Ib** and **Ic** in the bleaching of the hydroxy derivatives of β-sitosterol and the formation of only the dienes of **Id** in the bleaching of the hydroxy derivatives of stigmasterol. Because there was no formation of dienes in the synthesis of the trienes in *n*-butanol/H_3PO_4, the presence of the bleaching earths seems to be a determinant factor.

The hydroxy derivatives of vegetable sterols can act as precursors, by dehydration, of the steroidal hydrocarbons with three double bonds in the ring system found in refined vegetable oils. Each steroidal hydrocarbon has the structure of the corresponding original sterol with the double bonds located at the 2,4,6-positions. The rate of dehydration of the hydroxy sterols is higher than that of the corresponding sterols.

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Received for review November 9, 1999. Revised manuscript received February 11, 2000. Accepted February 11, 2000.

JF9912147