Formation of $\Delta^{3,5}$-Diene and 3-Chloro Δ^5-Ene Analogues of Sterols in Protein Hydrolysates

Jan Velíšek,* Jiří Davídek, and Vladislav Kubelka

Unsaponifiable material isolated from commercial soybean meal hydrolysate and the corresponding waste product humins was separated by thin-layer and column chromatography on silica gel, and the isolated hydrocarbons were evaluated by gas chromatography on packed columns and capillary gas chromatography–mass spectrometry. A total of 14 hydrocarbons, mainly $\Delta^{3,5}$-dienes and 3-chloro Δ^5-enes derived from sterols occurring in the raw material lipids, were identified and described for the first time as protein hydrolysate constituents. Quantitatively, in humins, $\Delta^{3,5}$-dienes, the predominant compounds, were found in concentrations about 60 mg·kg^{-1} and the levels of 3-chloro Δ^5-enes were 7 mg·kg^{-1}. The content of these compounds in hydrolysate was approximately $^1/_{1000}$ of that found in humins.

Sterols occur widely in animals as well as in plants. Their role in living organisms is increasingly studied. The major sterol in foodstuffs of animal origin is cholesterol (Δ^5-cholesten-3β-ol) and those of vegetable-derived foodstuffs are sitosterol ((24*R*)-24-ethyl-Δ^5-cholesten-3β-ol) and stigmasterol ((24*R*)-24-ethyl-$\Delta^{5,22}$-cholestadien-3β-ol), which are often accompanied by campesterol ((24*R*)-24-methyl-Δ^5-cholesten-3β-ol and some minor constituents.

Several papers on the chemical reactions of sterols during autoxidation and industrial treatment of edible fats and oils, reviewed by Seher (1976), showed that sterols may undergo, to a certain extent, dehydration reaction giving the corresponding unsaturated hydrocarbons. For example, the passage of anhydrous butter fat over Fuller's earth caused formation of $\Delta^{3,5}$-cholestadiene from cholesterol. Hydrogenation further progressively transformed cholestadiene into cholestenes and cholestane. These compounds were employed as indicators of the presence of hydrogenated butter fat in normal butter fat (Roderbourgh and Kuzdzal-Savoie, 1979; Niiya et al., 1980). Nonpolar steroid hydrocarbons were also formed by the action of bleaching earth onto pure cholesterol, stigmasterol, sitosterol, and brassicasterol in the presence of hexane. $\Delta^{3,5}$-Cholestadiene as a product of dehydration of cholesterol has been identified (Kaufmann and Hamza, 1970). Sterol hydrocarbons were also formed, partly removed due to deodorizing, partly transformed to other sterols during the hardening and hydrogenation. All edible oils and margarines thus contained not only steroid hydrocarbons (from 0.013 to 0.023% by weight) but also steroidal products of autoxidation and hydrogenation (Niewiadomski et al., 1965; Niewiadomski, 1975).

$\Delta^{3,5}$-Diene has been also found as one of the major transformation products of sitosterol formed by its treatment in pure state with atmospheric oxygen (Yanishlieva et al., 1980). The same compound has been found as a component of tall oil rosin (Lehtinen et al., 1965).

$\Delta^{3,5}$-Cholestadiene has been also detected as a degradation product of cholesteryl heptafluorobutyrate (Poole and Morgan, 1974) and as a byproduct derived from cholesterol during methanolysis and the subsequent gas–liquid chromatography (Kawamura and Taketomi, 1973; Lea and Jones, 1980). Pyrrolysis of cholesterol in the liquid phase at 180–250 °C in the presence of aluminosilicates gave several products such as Δ^5-cholestene, $\Delta^{3,5}$-cholestadiene, and $\Delta^{1,3,5}$-cholestatriene (Pustil'nikova et al., 1982).

$\Delta^{3,5,7}$-Trienes derived from campesterol and stigmasterol have been identified in the sterol fraction in some vegetable oils (Jovanovic and Bastic, 1979).

Soybean meal and flour, wheat gluten, and keratin (the principal raw materials currently employed for the industrial production of chemical protein hydrolysates in many countries) contain residual lipids such as triacylglycerols, phospholipds, etc., amounting about 1–2% of the raw material weight. Several papers have been directed at examining the fate of these lipids during the hydrolytic process and at evaluating the chemical composition of their degradation products. New chlorine-containing compounds derived from triacylglycerols, i.e. glycerol chlorohydrins and their esters with higher fatty acids, have been identified as chemical constituents of protein hydrolysates (Velíšek et al., 1978, 1980; Davídek et al., 1985).

The present study is a part of the research on chemical composition of protein hydrolysates. It is directed on the main constituents of the unsaponifiable matter of the raw material lipids, i.e. sterols, their main reactions encountered during the production of hydrolysates, and their distribution between the edible hydrolysates and the waste product humins.

EXPERIMENTAL SECTION

Materials. Soybean meal hydrolysate and humins were obtained from Tukový průmysl, k.p., Vitana Byšice, Czechoslovakia.

Methods. Soybean meal hydrolysate (1 kg) was placed in a separatory funnel and extracted once with 200 mL and twice with 100 mL of diethyl ether. The combined extracts were washed with 20 mL of saturated sodium chloride and three times with 20 mL of water and dried over anhydrous sodium sulfate.

Humins (200 g) were extracted in a mortar. The solvent, its amount, and procedure were the same as above.

Unsaponifiable material from soybean meal hydrolysate and humin lipids was obtained according to the IUPAC standard method (1973). An aliquot of the obtained unsaponifiables (0.25 g) was fractionated on a 400 × 10 mm (i.d.) glass column packed with Kieselgel (0.2–0.5 mm, Merck). The fraction eluted with 100 mL of hexane was dried over anhydrous sodium sulfate, evaporated to dryness, dissolved in 1 mL of hexane, and rechromatographed under the same conditions. The obtained eluate was dried again, concentrated to about 1 mL, and used for gas chromatographic–mass spectrometric analysis. Another aliquot of the unsaponifiable matter was separated by thin-layer chromatography according to the IUPAC procedure (1973). The obtained fractions of hydrocarbons,

Department of Food Chemistry and Analysis (J.V., J.D.) and Laboratory of Mass Spectrometry (V.K.), Institute of Chemical Technology, Suchbátarova 1905, 166 28 Prague 6, Czechoslovakia.

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Table I. Hydrocarbons Identified in Nonpolar Unsaponifiable Material Isolated from Humins

peak no.[a]	compd	derived from	mass spectral data, m/e	retentn index, I_x
1	diene	cholesterol	368 M^+	
2	diene	cholesterol	368 M^+	
3	$\Delta^{3,5}$-cholestadiene	cholesterol	368 M^+ (100), 353 (27), 260 (25), 255 (22), 247 (36), 213 (16)	2880
4	triene	cholesterol	366 M^+	
5	24-methyl-$\Delta^{3,5}$-cholestadiene	campesterol	382 M^+ (100), 367 (31), 274 (22), 255 (30), 261 (34), 213 (18)	2950
6	24-ethyl-$\Delta^{3,5,22}$-cholestatriene	stigmasterol	394 M^+ (100), 379 (8), 255 (64), 273 (5), 213 (17), 351 (15), 281 (9), 253 (17), 228 (11)	2990
7	diene	sitosterol	396 M^+	
8	24-ethyl-$\Delta^{3,5}$-cholestadiene	sitosterol	396 M^+ (100), 381 (22), 288 (20), 255 (22), 275 (21), 213 (15)	3040
9	3-chloro-Δ^5-cholestene	cholesterol	see Table II	3080
10	chloro diene	cholesterol	402 M^+, 387 (M − 15), 366 (M − HCl), 351 (M − 15 − HCl), 289 (M − side chain), 253 (M − side chain − HCl)	
11	3-chloro-24-methyl-Δ^5-cholestene	campesterol	see Table II	3170
12	3-chloro-24-ethyl-$\Delta^{5,22}$-cholestadiene	stigmasterol	see Table III	3200
13	chloro triene	sitosterol	428 M^+, 413 (M − 15), 392 (M − HCl), 377 (M − 15 − HCl)	
14	3-chloro-24-ethyl-Δ^5-cholestadiene	sitosterol	see Table II	3300

[a] The peak numbers correspond to the numbers in Figure 1.

tocopherols, triterpenic alcohols, and sterols were separated and quantitatively evaluated by gas–liquid chromatography with cholesteryl isovalerate (3-methylbutanoate) as an internal standard.

Instruments. Gas–liquid chromatographic separations were performed on a Hewlett-Packard Model 5880A instrument equipped with a flame ionization and electron capture detector and an 2400 × 2 mm glass column packed with methylpolysiloxane OV-101 stationary phase (3% w/w) on a Chromaton N-AW-DMCS (0.125–0.16 mm; Lachema, Brno, Czechoslovakia) solid support. Column temperature was programmed from 100 to 240 °C at a rate of 5 °C·min^{-1}; injector and detectors were held at 250 °C. Nitrogen carrier gas flow rate was 30 mL·min^{-1}; injection volume was 5 μL.

The mass spectrometer, Jeol D-3000, was coupled to a gas chromatograph equipped with an OV-101 fused silica capillary column (length 20 m, 0.2 mm i.d., film thickness 0.2 μm) and used for gas chromatographic–mass spectrometric measurements. The conditions were as follows: column and injector temperature, see above; carrier gas flow rate, 2.5 mL·min^{-1} (helium); temperature of ion source and all connection parts, 200 °C; electron energy, 70 eV; cathodic current, 0.8 mV; injection volume, 0.1 μL.

RESULTS AND DISCUSSION

The unsaponifiable material amounted to approximately 4% of lipids isolated from hydrolysates, 15% of lipids isolated from humins, and 1.7% of the original soybean meal oil lipids. A substantial part of the unsaponifiable material of lipids isolated from hydrolysates and humins was sterols and hydrocarbons. The main sterols in humins were sitosterol and cholesterol (from keratin); the main constituent of the sterol fraction of soybean hydrolysate was sitosterol. Campesterol, stigmasterol, brassicasterol, Δ^5-avenasterol, and other unidentified compounds were present in low quantities (Davídek et al., 1985).

The main hydrocarbons of soybean meal oil unsaponifiables were aliphatic hydrocarbons paraffins, diterpene squalene etc., hydrolysates, and humins contained as the main components hydrocarbons derived from sterols (Davídek et al., 1985).

The concentrate of nonpolar unsaponifiable material obtained from humins by column chromatography on silica gel was investigated by gas–liquid chromatography and capillary gas chromatography–mass spectrometry (Figure 1). The compounds identified by mass spectrometry are listed in Table I. In total, 14 volatile hydrocarbon compounds, derived from sterols, were found; six compounds were tentatively identified. Compounds 5, 6, 8, 11, 12, and 14 were also found analyzing soybean meal hydrolysate unsaponifiables. Their mass spectra were consistent with those obtained for humins. All of the identified compounds were described for the first time from protein hydrolysates and the corresponding humins constituents. Mass spectral data of 3-chloro-Δ^5-cholestenes derived from cholesterol, campesterol, and sitosterol (identified in humins) are summarized in Table II, and the mass spectral data of 3-chloro-24-ethyl-$\Delta^{5,22}$-cholestadiene (derived from stigmasterol), in Table III. The mass spectra were characterized by the presence of their molecular ions and their expepcted major fragment ions. The most abundant ions above m/e 210 are presented. The retention indices and the mass spectra of the identified compounds were consistent with those obtained under the same instrument

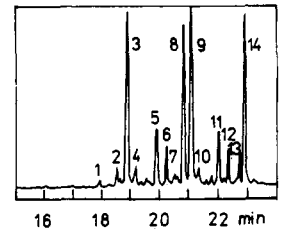

Figure 1. Gas chromatographic separation of nonpolar unsaponifiable volatiles isolated from humins. The peak numbers correspond to the numbers in Table I.

Table II. Mass Spectra (m/e) of 3-Chloro-Δ^5-cholestenes Isolated from Humins

	fragmentation	3-chloro-Δ^5-cholestene	3-chloro-24-methyl-Δ^5-cholestene	3-chloro-24-ethyl-Δ^5-cholestene
M	molecular ion	404 (100)	418 (100)	432 (100)
a	M − 15 (CH_3)	389 (44)	403 (39)	417 (36)
b	M − HCl	368 (53)	382 (59)	396 (68)
c	M − 15 − HCl	353 (20)	367 (19)	381 (19)
d	M − HCl − 67 (C_5H_7)	301 (22)	3125 (22)	229 (22)
e	M − side chain	291 (52)	291 (50)	291 (52)
f	M − HCl − 93 (C_7H_9)	275 (42)	289 (33)	303 (32)
g	M − side chain − 27 (C_2H_3)	264 (15)	264 (15)	264 (17)
h	M − side chain − HCl	255 (17)	255 (20)	255 (22)
i	M − side chain − 42 (C_3H_6)	249 (69)	249 (71)	249 (73)
j	M − HCl − 121 (C_9H_{13})	247 (33)	261 (27)	275 (26)
k	M − side chain − HCl − 42	213 (22)	213 (22)	213 (24)

Table III. Mass Spectrum (m/e) of 3-Chloro-24-ethyl-$\Delta^{5,22}$-cholestadiene Isolated from Humins

	fragmentation	3-chloro-24-ethyl-$\Delta^{5,22}$-cholestadiene
M	molecular ion	430 (44; base peak 55)
a	M − 15 (CH_3)	415 (5)
b	M − HCl	394 (64)
c	M − 15 − HCl	379 (9)
e	M − side chain	291 (50)
h	M − side chain − HCl	255 (46)
i	M − side chain − 42 (C_3H_6)	249 (14)
k	M − side chain − HCl − 42	213 (12)
l	M − 43 (C_{25}–C_{27})	387 (26)
m	M − 13 − HCl	351 (11)
n	M − 15 − (C_{23}–C_{27})	318 (36)
o	M − side chain − 2 H	289 (37)
p	M − (C_{23}–C_{27} + 1 H)	332 (11)
q	M − 15 − (C_{23}–C_{27} + 1 H)	317 (16)

conditions for authentic synthesized compounds (Daughenbaugh and Allison, 1929).

Diene and trienes derived from cholesterol, sitosterol, campesterol, and stigmasterol have been already known to occur in oils and fats, while chlorine-containing analogues of sterols were identified in foodstuffs for the first time. No attempt has been made, however, to examine their possible physiological effects.

The average levels of $\Delta^{3,5}$-dienes and chlorine-containing hydrocarbons found in the analyzed humins (mg/kg, fresh weight) were as follows: $\Delta^{3,5}$-cholestadiene, 29.1; 24-methyl-$\Delta^{3,5}$-cholestadiene, 4.2; 24-ethyl-$\Delta^{3,5,22}$-cholestatriene, 1.2; 24-ethyl-$\Delta^{3,5}$-cholestadiene, 29.5; 3-chloro-Δ^5-cholestene, 2.1; 3-chloro-24-methyl-Δ^5-cholestene, 1.2; 3-chloro-24-ethyl-$\Delta^{5,22}$-cholestadiene, 1.0; 3-chloro-24-ethyl-Δ^5-cholestene, 2.4. Amounts of the same compounds (with the exception of cholesterol originating from keratin) in commercial soybean meal hydrolysate were, however, about 1000 times lower than those found in humins.

The proportions between $\Delta^{3,5}$-dienes and chlorine-containing hydrocarbons in Figure 1 and the naturally occurring $\Delta^{3,5}$-dienes and chlorine-containing hydrocarbons are a little different, as the latter compounds were concentrated by chromatography on silica gel column prior to the mass spectrometric analysis.

3-Chloro-Δ^5-cholestene (3-cholesteryl chloride) is known to be the reaction product of cholesterol in trichloroacetic acid–hydrochloric acid mixtures (Kurasawa et al., 1976, 1978). Yield of reaction products obtained from the reaction of cholesterol with hydrochloric acid has been shown to be low in some solvents (e.g., diisopropyl ether), and substitution products such as 3-chloro-Δ^5-cholestene are the dominant, while in other solvents (e.g., ethanol) yields of elimination products predominate. Hydrochloric acid in 2-chloroethanol is unexpectedly reactive with cholesterol (Patel and Peal, 1964). The formation of $\Delta^{3,5}$-cholestadiene, by acid-catalyzed dehydration of cholesterol, has been shown to proceed by a unimolecular elimination process. It was further shown that 3β-hydroxy-Δ^5-steroids in general undergo dehydration on treatment with ethanolic hydrochloric acid to form steroidal 3,5-dienes. The absence of the 3α epimers from all the reaction products (including substitution products such as chloride and ether) showed that no bimolecular substitution occurred, moreover, and that the formed 3-chloro-Δ^5-cholestene slowly reacts in the presence of hydrochloric acid and is solvolyzed to cholesterol (Peal, 1957).

The same or similar reactions also occur in 20% (6 M) hydrochloric acid, which is employed for the production

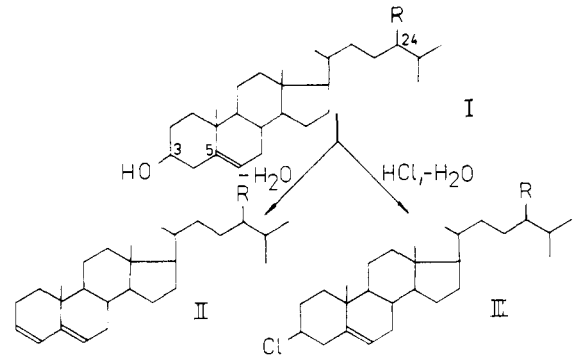

Figure 2. Reaction of Δ^5-cholesten-3β-ols with hydrochloric acid: I, Δ^5-cholesten-3β-ol; II, $\Delta^{3,5}$-cholestadiene; III, 3-chloro-Δ^5-cholestene.

of protein hydrolysates used as food seasonings (Figure 2), and products such as ethers and oligomers of hydrocarbons can be expected (Niiya et al., 1980; Patel and Peal, 1964). The same products formed from sterols, i.e. unsaturated and chlorine-containing hydrocarbons, could be also obtained from other structurally related compounds occurring in soybean meal, i.e. triterpenic alcohols, sapogenins (sapogenols), etc.

Registry No. II (R = H), 747-90-0; II (R = Me), 102491-95-2; 22-ene-II (R = Et), 102491-96-3; II (R = Et), 4970-37-0; III (R = H), 96345-96-9; III (R = Me), 102491-97-4; 22-ene-III (R = Et), 102491-98-5; III (R = Et), 102491-99-6.

LITERATURE CITED

Davídek, J.; Velíšek, J.; Pánek, J. *Proc. Eur. Conf. Food Chem.* **1985**, *2*, 47.
Daughenbaugh, P. J.; Allison, J. B. *J. Am. Chem. Soc.* **1929**, *51*, 3665.
IUPAC *Standard Methods for the Analysis of Oils, Fats and Soaps*, 5th ed.; Butterworths: London, 1973; p 1E.
Jovanovic, J. A.; Bastic, M. *Glas. Hem. Drust. Beograd* **1979**, *44*, 313; *Chem. Abstr.* **1979**, *91*, 156254a.
Kaufmann, H. P.; Hamza, Y. *Fette, Seifen, Anstrichm.* **1970**, *72*, 432.
Kawamura, N.; Taketomi, T. *Jpn. J. Exp. Med.* **1973**, *43*, 157.
Kurasawa, Y.; Takeda, A.; Ueda, T. *Chem. Pharm. Bull.* **1976**, *24*, 859.
Kurasawa, Y.; Takeda, A.; Ueda, T. *Steroids* **1978**, *31*, 163.
Lea, E. J. A.; Jones, S. P. *J. Chromatogr.* **1980**, *221*, 367.
Lehtinen, T.; Elomaa, E.; Alhojarvi, J.; Bruun, H. H. *Suom. Kemistil.* **1965**, *38*, 127.
Niewiadomski, N. *Nahrung* **1975**, *19*, 525.
Niewiadomski, N.; Sawicki, J. Zesz. *Probl. Postepow Nauk Roln.* **1965**, *53*, 175.
Niiya, T.; Goto, Y.; Ono, Y.; Ueda, Y. *Chem. Pharm. Bull.* **1980**, *28*, 1747.
Patel, M. S.; Peal, W. J. *Tetrahedron* **1964**, *20*, 2499.
Peal, W. J. *Chem. Ind. (London)* **1957**, 1451.
Poole, C. F.; Morgan, E. D. *J. Chromatogr.* **1974**, *90*, 380.
Pustil'nikova, S. D.; Abryutina, N. N.; Kayvkova, G. P.; Lubenko, V. G.; Petrov, A. A. *Geokhimiya* **1982**, 144; *Chem. Abstr.* **1982**, *97*, 6634*j*.
Roderbourgh, H.; Kuzdzal-Savoie, S. *J. Am. Oil Chem. Soc.* **1979**, *56*, 485.
Seher, A. In *Lipids*; Paoletti, R., Jacini, G., Porcellati, G., Eds.; Raven: New York, 1976; Vol. 2, p 293.
Velíšek, J.; Davídek, J.; Hajšlová, J.; Kubelka, V.; Janíček, G.; Mánková, B. *Z. Lebensm.—Unters.—Forsch.* **1978**, *167*, 241.
Velíšek, J.; Davídek, J.; Kubelka, V.; Janíček, G.; Svobodová, Z.; Šimicová, A. *J. Agric. Food Chem.* **1980**, *28*, 1142.
Yanishlieva, N.; Schiller, H.; Marinova, E. *Riv. Ital. Sostanze Grasse* **1980**, *57*, 572.

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