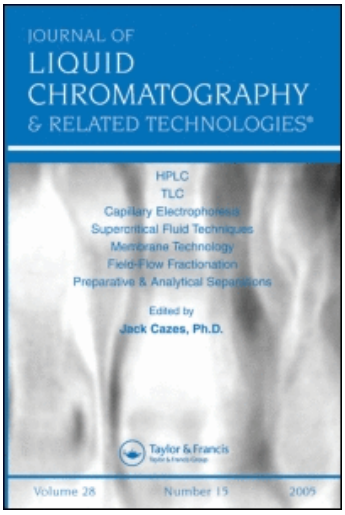

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ANALYSIS OF OXYSTEROLS BY LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatography of oxysterols of current biochemical and biomedical interest is reviewed.

INTRODUCTION

The facile oxidation of cholesterol in biological systems by active oxygen species generates a complex farago of oxysterols, the simple oxidation products of cholesterol and related sterols, that exhibit divers biological activities of biomedical interest.[1-8] The oxysterols of human tissues and foods have similar chemical and physical properties, requiring good resolution coupled with adequate independent means of identification.[1,9-11] Additionally, sensitivity is an issue, as oxysterols are present in human blood at nanomolar levels only and in foods at the parts-per-million levels.[12]

Although high performance liquid chromatography (HPLC) was applied early to resolution of sterols (including oxidized cholecalciferol derivatives), application to oxysterols began only

in 1977, with resolution of isomeric cholesterol 5,6-epoxide 3ß-benzoates,[13] (25RS)-cholest-5-ene-3ß,26-diol 3ß,26-diacetates,[14,15] and several C_{27}-ketosteroids[16] on silica adsorption columns. Our 1979 contribution[17] demonstrated applicability of both adsorption and reverse phase columns to esterified and unesterified oxysterols as well, and the topic has received continuous attention since that time. Both analytical and preparative applications now abound, and the present review careers through selected examples.

DISCUSSION

The oxysterols of interest are those encountered regularly in human tissues and in foods, chiefly the common B-ring cholesterol autoxidation products: epimeric cholesterol 7-hydroperoxides (7α-OOH, 7ß-OOH) and cholest-5-ene-3ß,7-diols (7α-OH, 7ß-OH) and 3ß-hydroxycholest-5-en-7-one (7-ketone) (and its dehydration product cholesta-3,5-dien-7-one), isomeric 5,6α-epoxy-5α-cholestan-3ß-ol (5α,6α-epoxide) and 5,6ß-epoxy-5ß-cholestan-3ß-ol (5ß,6ß-epoxide), and their common hydration product 5α-cholestane-3ß,5,6ß-triol (triol). Tissue oxysterols (20S)-cholest-5-ene-3ß,20-diol (20-OH), (24S)-cholest-5-ene-3ß,24-diol (24-OH), cholest-5-ene-3ß,25-diol (25-OH), and cholest-5-ene-3ß,26-diols (26-OH) need likewise be included, as also should the ketones cholest-4-en-3-one, 7α-hydroxycholest-4-en-3-one, cholesta-4,6-dien-3-one, and cholest-4-ene-3,6-dione *inter alia*. To these common oxysterols must be added the unique oxysterols formed by electronically excited (singlet) dioxygen 3ß-hydroxy-5α-cholest-6-ene-5-hydroperoxide (5α-OOH), 3ß-hydroxycholest-4-ene-6ß-hydroperoxide (6ß-OOH), and 5α-cholest-6-ene-3ß,5-diol (5α-OH) and by ozone, together with other oxysterols of natural and synthetic origins of current interest.

The complete pattern of oxysterols present in a sample may be useful in assessing the past oxidation history of the sample,[18,19] but resolution of all these oxysterols with one HPLC system is unrealistic. Generally a limited set of oxysterols is of concern, and silica adsorption columns and reverse phase systems provide adequate means for most analyses. Many commercially available

adsorption columns such as μPorasil (Waters), Zorbax SIL (DuPont), LiChrosorb Si 60 (E. Merck), Ultrasphere SIL (Beckman, Altex), and the like, with isocratic irrigation with the binary solvent hexane (Hx)/2-propanol (iPrOH) is by far the most frequently used, but other binary mixtures such as C_6H_6/ethyl acetate (EtOAc), Hx/$CHCl_3$, Hx/butanol, and CH_2Cl_2/EtOAc and ternary CH_2Cl_2/Hx/EtOAc and quaternary Hx/EtOAc/iPrOH/acetone[20] mixtures have been used. Reverse phase columns such as μBondapak C_{18} (Waters), Ultrasphere-ODS (Beckman), Supelcosil-LC18 (Supelco), Ultrasil-ODS (Altex), Microsorb C_{18} (Rainin), and Zorbax C_8 (DuPont) with binary solvents acetonitrile (MeCN)/iPrOH, MeCN/methanol (MeOH), MeCN/H_2O, MeOH, and MeOH/H_2O, ethanol/H_2O, iPrOH/H_2O, MeOH/iPrOH, and ternary solvent MeOH/iPrOH/H_2O all find use. The 3-5 μm particles size packings provide superior resolution with shorter columns than do the older 10 μm size materials. Stepwise and linear gradients are used,[17,21-23] but programmed curvilinear gradients have not been exploited.

For quantitation the issues of linearity of detector response and sensitivity arise. Oxysterols are detected suitably by their ultraviolet light end-absorption (205-214 nm) and by differential refractometry, flame ionization, and mass spectrometry and for derivatives by chemiluminescence, electrochemical, and fluorimetric detection. The 5,6-epoxides having much diminished end-absorption must be detected with less sensitive refractometry, but those oxysterols with strong chromophores (such as 235-240 nm for α,ß-enones, 284 nm for α,ß;γ,δ-dienones) may be individually detected by such means. The increasing use of photodiode array detectors capable of evaluation of a range of ultraviolet light absorption bands permits specific and sensitive detection via ultraviolet light absorption properties.

Improved sensitivity of detection may be had by esterification of oxysterols as 3ß-benzoates with increased ultraviolet light absorptivity[24] or as fluorescent 3ß-anthroates[25] and by a variety of post-column reactions, including cholesterol oxidase dehydrogenation of Δ^5-3ß-oxysterols to corresponding Δ^4-3-ketones with increased ultraviolet light absorption.[26]

TABLE 1
Chromatographic Properties of A- and B-Ring Oxidized Oxysterols

Oxysterol[a]	Retention Time, min.[b]				
	I	II	III	IV	V
7α-OOH	7.82	59.6	31.4	12.2	7.36
7ß-OOH	7.28	49.6	27.2	13.3	-
5α-OOH	5.35	31.6	19.8	13.9	8.75
7α-OH	9.4	108.8	59.5	13.9	8.27
7ß-OH	16.3	102.0	55.2	14.4	-
5α-OH	8.51	29.6	19.0	17.4	11.1
7-Ketone	5.93	37.6	22.6	16.2	10.1
$\Delta^{3,5}$-7-Ketone	0.13	-	-	44.6	-
Δ^{4}-3-Ketone	0.44	-	-	53.3	-
Δ^{5}-3-Ketone	0.12	-	-	59.0	-
$\Delta^{4,6}$-3-Ketone	0.44	-	-	43.5	-
5α,6α-Epoxide	4.4	-	-	29.5	-
5ß,6ß-Epoxide	16.4	-	-	27.2	-
3ß,5α,6ß-Triol	-	-	-	11.9	-

a Systematic names of oxysterols as defined in the text.
b Systems defined: I, 10 μm μPorasil, Hx/iPrOH (24:1); II, 3μm Zorbax SIL, Hx/iPrOH (100:1.4); III, 3 μm Zorbax SIL, Hx/iPrOH (50:1); IV, μBondapak C_{18}, MeCN/H_2O (9:1); VI, 5 μm Ultrasphere-ODS, MeCN/H_2O (9:1).

The present review emphasizes resolution of oxysterols for component identification and quantitation, but applications for isolations are also of great value. Resolution of all the common oxysterols yet poses a problem, as most systems fail in some manner or other, but the extent to which the technique has been established is illustrated by review of HPLC applications to ten items of continuing interest.

1. Common Oxysterol Types

Resolution of the commonly encountered oxysterols oxidized in the A- and B-rings is had with both adsorption and reverse phase

systems, and some of our retention data for these oxysterols is presented in Table 1.[17,27-30] As mentioned, the complete oxysterol composition of a sample may disclose the nature of the oxidative processes to which the sample has been exposed,[17,18] but this goal is seldom attained.

The order of elution of seven commonly encountered B-ring oxidized sterols is quite variable, depending on the specific silica adsorption column and proportions of binary HX/iPrOH used. In a comparative study the elution order on 10 μm μPorasil is 7-ketone > 5α-OH > (5α-OOH/7ß-OOH) > 7α-OOH > 7ß-OH > 7α-OH; that on 5 μm Ultrasphere SIL is 5α-OH > (7-ketone/5α-OOH) > 7ß-OOH > 7α-OOH > (7α/7ß-OH); that on 3 μm Zorbax SIL is (5α-OH/5α-OOH) > 7-ketone > 7ß-OOH > 7α-OOH > 7ß-OH > 7α-OH.[28]

The failure of some systems to provide resolution of the three isomeric hydroperoxides from one another and from other common oxysterols is overcome by using a 5 μm microbore Ultrasphere Si column and Hx/iPrOH (10:2.3), with the order of elution: 20-OH > 24-OH > 25-OH > 26-OH > 5α-OOH > 5α-OH > 7-ketone > 7ß-OOH > 7α-OOH > (7α/7ß-OH).[27] Selection among these columns should provide proper analysis of samples containing these components (the isomeric 5,6-epoxides, 3ß,5α,6ß-triol, etc. not included here).

2. Monohydroxylated Cholesterol Derivatives

Some of our retention data for a series of synthetic and metabolite monohydroxylated cholesterol derivatives on silica columns is presented in Table 2.[9] The mobilities reveal a trend, those of oxysterols hydroxylated in the side-chain and D-ring being uniformly less polar than of those substituted in the A/B ring system. Thus, derivatives hydroxylated in the 1-, 4-, 7-, and 19-positions are substantially more polar than are those substituted in the 16-26 positions. The nearer the second hydroxyl group is to the 3ß-hydroxyl the more polar the oxysterol.[9,31] This relationship is not apparent in reverse phase systems.[32]

The order of elution of side-chain oxysterols (20S)-3ß,20-, 3ß,24-, 3ß,25-, and 3ß,26-diols and the synthetic 3ß,23-diols is

TABLE 2
Chromatographic Properties of Monohydroxylated Cholesterols

Cholest-5-ene-3ß,x-diol	Retention Time, min.[a]	
	I	VI
Cholest-5-ene-1α,3ß-diol	137.9	24.8
Cholest-5-ene-1ß,3ß-diol	181.3	30.9
Cholest-5-ene-3ß,4α-diol	-	15.0
Cholest-5-ene-3ß,4ß-diol	-	18.9
Cholest-5-ene-3ß,7α-diol	139.7	24.9
Cholest-5-ene-3ß,7ß-diol	118.0	21.0
Cholest-5-ene-3ß,12α-diol	29.4	18.2
Cholest-5-ene-3ß,16ß-diol	18.5	9.5
Cholest-5-ene-3ß,17α-diol	16.6	9.2
Cholest-5-ene-3ß,19-diol	30.5	54.0
(20S)-Cholest-5-ene-3ß,20-diol	17.4	11.2
Cholest-5-ene-3ß,21-diol	-	10.8
(22R)-Cholest-5-ene-3ß,22-diol	23.5	-
(22S)-Cholest-5-ene-3ß,22-diol	23.5	-
(23R)-Cholest-5-ene-3ß,23-diol	18.0	11.6
(23S)-Cholest-5-ene-3ß,23-diol	18.0	11.6
(24R)-Cholest-5-ene-3ß,24-diol	19.6	9.5
(24S)-Cholest-5-ene-3ß,24-diol	19.6	9.5
Cholest-5-ene-3ß,25-diol	26.4	10.7
(25R)-Cholest-5-ene-3ß,26-diol	33.2	12.0

a Systems defined: I, μPorasil, Hx/iPrOH (24:1); VI, μPorasil, Hx/iPrOH (9:1).

uniformly the same as the arbitrary carbon atom numbering system used,[17,27,28] thus suggesting that the polarity of the side-chain hydroxyl substituents increases with increased distance from the steroid nucleus (the primary 21-hydroxyl and 3ß,22-diols excepted). On reverse phase systems a varied order of elution is observed.

Nuclear substituted epimeric pairs are resolved, but effects of conformation on mobility may not be uniform. The equatorial 4α- and 7ß-hydroxy derivatives are less polar than their axial epimers, whereas the axial 1α-hydroxy derivative is eluted before the equatorial 1ß-epimer. Side-chain substituted stereoisomers are not

resolved in these adsorption systems, but resolution of some on reverse phase systems and on chiral adsorption columns has been described. The epimeric 24-hydroxylated (24R)- and (24S)-saringosterols (stigmasta-5,28-diene-3ß,24-diols) from giant kelp are resolved on an Utrasphere-ODS reverse phase column with the solvent methanol/water (97:3).[33] Even more impressive is the resolution of the epimeric (24R)- and (24S)-cholest-5-ene-3ß,24-diols on a 5 μm Bakerbond DNBPG chiral column with Hx/iPrOH (97.5:2.5)[34] and on a reverse phase 5 μm C_{18} Resolve (Waters) column with MeOH/H_2O (22:3),[35] the (24S)-epimer of 24ß$_F$ configuration eluting ahead of the (24R)-3ß,24-diol (24α_F configuration). Furthermore, the isomeric (25R)- and (25S)-cholest-5-ene-3ß,26-diols previously resolved only as 3ß,26-diacetates[14,15] or 3ß,26-dibenzoates[36] may be resolved without derivitization using TSKgel ODS-120T with MeOH/H_2O (93:7).[37]

Although successful analysis of the simpler oxysterols is readily had, analysis of more highly oxidized very polar cholesterol autoxidation products has not been pursued despite the utility of reverse phase systems for such work. Using μBondapak C_{18} columns and MeCN/H_2O many such as yet unidentified cholesterol oxidation products have been resolved and the steroid acids 3ß-hydroxyandrost-5-ene-17ß-carboxylic acid, 3ß-hydroxy-22,23-bisnorchol-5-enic acid, and 3ß-hydroxychol-5-enic acid identified.[38]

3. Sterol Hydroperoxides and Peroxides

Sterol hydroperoxides as initial products of cholesterol oxidation are resolved in the same systems as the secondary nonperoxidic oxysterols,[9,17,27,28,30] but their inclusion in oxysterols analyses is infrequent. The introduction of electrochemical[39] and chemiluminescence[40-42] detectors may improve matters.

Interest continues to increase in the presence of cholesterol ester hydroperoxides in human blood at low (3.4 nM) levels, and several different systems have been devised for such analyses. These oxysterols appear to be oxidized in the unsaturated ester moiety and not the sterol nucleus or side-chain, but reverse phase systems with binary solvents MeCN/iPrOH (1:1),[43] MeOH/tert-butanol (1:1), or

(19:1), and the ternary system MeCN/tert-butanol/H_2O (11:6:1) with chemiluminescence detection[40-42] have been described.

Semi-preparative HPLC isolation of sterol peroxides is exemplified in the recent recovery of isomeric cholesterol 20,25-dihydroperoxides from air-aged cholesterol with 5 μm Microsorb C_{18} column and MeCN/H_2O (63:37), the (20S)-20,25-dihydroperoxide eluting before the (20R)-20,25-dihydroperoxide.[44] Similar semi-preparative reverse phase Partisil M9 10/50 ODS-2 (Whatman), μBondapak C_{18}, and Ultrasphere-ODS columns with MeOH/H_2O as solvent have been useful in isolation of sterol 5α,8α-epidioxides (formed from stera-5,7-dien-3ß-ols) from plant and marine creatures.[45-50]

4. Ozone and Singlet Oxygen Products

Cholesterol is oxidized by singlet molecular oxygen to sterol hydroperoxides different from those formed by autoxidation, but all have the same properties (Table 1) and require careful attention to their analysis. The major 5α-hydroperoxide and minor 6ß-hydroperoxide are resolved from one another and from the cholesterol 7α- and 7ß-hydroperoxides on a reverse phase column with ternary solvent MeOH/H_2O/MeCN (45:4:2) as are also their corresponding reduced alcohol derivatives.[39] Resolution of the 5α-hydroperoxide from other frequently encountered oxysterols may pose a problem, but a recently devised system 5 μm microbore Ultrasphere Si column and Hx/iPrOH (10:2.3) achieves this objective.[28]

The cholesterol ozonides pose yet other problems, as their stability is such that it is uncertain whether any can be detected in tissues or foods exposed to ozone. Our HPLC retention data for acknowledged ozonide derivatives of cholesterol are presented in Table 3.[18,19,51,52]

Preparative oxysterol HPLC is well illustrated in our recovery of cholesterol ozonization products from complex reaction product mixtures. In these cases the 10 μm μPorasil columns irrigated with C_6H_6/EtOAc (25:3) or better 5 μm IBM silica column with C_6H_6/EtOAc (50:3) resolved the major ozonization products. Our work with the oligomeric cholesterol ozonides provides an heuristic example of the

TABLE 3
Cholesterol Ozonization Derivatives

Oxysterol[a]	Retention Time, min.[b]		
	I	VII	VIII
Seco 6-aldehyde	17.2	-	-
Hydroxy-epidioxide	28.0	-	-
Methoxy-epidioxide	19.3	-	-
Ethoxy-epidioxide	15.0	-	-
5α,6α-Epoxide	13.0	-	-
5ß,6ß-Epoxide	14.0	-	-
α-Ozonide	-	19.4	-
ß-Ozonide	-	20.8	-
Dimer α-ozonide	-	9.5	16.1
Dimer ß-ozonide	-	9.5	16.6
Trimer ozonide	-	8.4	14.8
Tetramer ozonide	-	8.1	13.9
Pentamer ozonide	-	7.9	13.4
Dimer Δ^5-sterol	-	8.8	15.6
Dimer 5ß,6ß-epoxide	-	10.0	18.2

a For systematic nomenclature of the several ozone products, see references 51,52.
b Systems defined: I, μPorasil, Hx/iPrOH (24:1); VII, μPorasil, C_6H_6/EtOAc (25:3); VIII, IBM silica column, C_6H_6/EtOAc (50:3).

application of HPLC analytical and semi-preparative columns for resolution and recovery of individual oxysterols from a complex mixture of congeners otherwise not possible to fractionate.[52]

5. Food and Tissue Oxysterols

Food analysis for oxysterols has more often been conducted by gas chromatography in conjunction with mass spectrometry, but HPLC analysis began in 1980 with μPorasil columns and Hx/iPrOH,[53] with many subsequent applications.[54-57] Reverse phase systems μBondapak C_{18} with MeCN/H_2O (9:1)[58] and with a ternary mixture of MeCN/iPrOH/H_2O[59]

have also been reported. Because of the complexity of food matrices and low oxysterols levels (parts-per-million range[12]), preliminary concentration of oxysterols is generally necessary, and preparative chromatography with silica columns and ethyl acetate has been used for such purpose.[60]

Similar considerations hold for oxysterols analysis in blood and tissues, even in synthetic liposomes,[61] where preliminary concentration with preparative cartridges and semi-preparative columns of μPorasil (with Hx/iPrOH) and μBondapak C_{18} (with MeCN and MeCN/iPrOH), including gradient elution, serve well.[62,63] The low nanomolar concentration of the common oxysterols in human blood[12] poses considerable burden on analyses, however conducted.

6. Other Oxysterols

Other oxysterols implicated in sterol metabolism are the 24,25-epoxides of lanosterol and desmosterol (24,25-epoxy-5α-lanost-8-en-3ß-ol and 24,25-epoxycholest-5-en-3ß-ol). These are resolved with μPorasil columns with Hx/iPrOH,[35] Zorbax ODS with MeOH,[64] and Resolve columns with MeOH/H_2O (9:1).[65] The (24R)- and (24S)-isomers of each 24,25-epoxide are resolved on 5 μm Ultrasphere-Si with Hx/iPrOH (98.8:1.2),[66] and on Chiralcel-OD (J. T. Baker) with Hx/iPrOH (49:1),[67] Resolve with MeOH/H_2O (9:1), and Vydac 201T54 reverse phase columns with MeOH.[64] Yet other C_{30}-oxysterols 3ß-hydroxy-5α-lanost-7-en-32-al, 3ß-hydroxy-5α-lanost-8-en-32-al, and 5α-lanost-8-ene-3ß,32-diol, are also resolved in these systems.[35,68]

7. Oxysterol Esters

The reverse phase system μBondapak C_{18} with Hx/iPrOH resolves oxysterol fatty acyl esters (Table 4),[62,69] as does also Ultrasphere Si with cyclohexane/EtOAc (19:1).[70] Retention times increase as the fatty acyl chain increases in length but decrease upon introduction of olefinic bonds.

Oxysterol acetates are resolved on a 5 μm Spherisorb column with Hx/EtOAc (19:1) and on 5 μm Spherisorb ODS-II reverse phase column with MeOH, the order of elution of side-chain monohydroxy-

TABLE 4
Retention Data for Oxysterol Esters

Oxysterol Ester	Retention Time, Min.[a]		
	IX	X	XI
3ß-Hydroxycholest-5-en-7-one			
3ß-myristate	-	15.0	-
3ß-palmitate	-	19.4	-
3ß-stearate	-	25.6	-
Cholest-5-ene-3ß,25-diol			
3ß-myristate	-	18.8	-
3ß-palmitate	-	24.6	52.7
3ß-stearate	-	32.4	74.8
3ß-oleate	-	-	49.1
3ß-linoleate	-	-	33.0
3ß-linolenate	-	-	27.0
3ß,25-dipalmitate	37.8	-	-
3ß,25-distearate	69.4	-	-
3ß,25-dioleate	33.4	-	-
Cholest-5-ene-3ß,26-diol			
3ß-palmitate	-	-	52.3
26-palmitate	-	-	48.7
3ß-stearate	-	-	75.2
26-stearate	-	-	69.6
3ß,26-dipalmitate	23.0	-	-
3ß,26-distearate	30.8	-	-
3ß,26-dioleate	18.6	-	-

a μBondapak C_{18} and MeCN/iPrOH in the proportions: IX, (1:1); X, (4:1); XI, (9:1).

cholesterol diacetates (22 > 24 > 26) and 3ß-monoacetates (20 > 25) being the same as found for the unesterified oxysterols. Moreover, the 3ß,24-diacetates of the isomeric (24R)- and (24S)-cholest-5-ene-3ß,24-diols were resolved with a 3 μm Spherosorb ODS-II column with MeOH/iPrOH (4:1).[71]

8. Oxysterol Natural Products

Other less commonly encountered oxysterols have been detected in divers tissues, including cholest-4-en-3-one from adrenal tissue

using Nucleosil C_{18} (Macherey Nagel & Co.) with MeOH/CH_2Cl_2/H_2O (48:1:1)[72] and several oxysterols from sponges, including 24-alkyl-Δ^5-7-ketones and alcohols with μPorasil,[73] $\Delta^{5,8}$-7-ketones using 7 μm LiChrosorb Si 60 with Hx/EtOAc (1:9) and 3 μm Micropak ODS-2 with MeOH/H_2O (23:2),[74] 3ß,5α,6ß-triols with 7 μm Hibar RP-18 reverse phase columns with Me/H_2O (17:3) and (4:1),[75] and 24-ketones with Partisil ODS-2 irrigated with MeOH.[49,76]

9. Oxysterol Metabolites

Regarding another sort of oxysterol 3ß-hydroxy-5α-cholest-8(14)-en-15-one, its isolation from rat skin using 5 μm Spherisorb ODS-II with MeOH has been described. In this case, the 3ß-trimethylsilyl ether derivative was also chromatographed successfully on Spherisorb with Hx/EtOAc (4:1).[77]

Applications to oxysterol metabolism involve similar procedures, with reverse phase columns, ternary solvent mixtures, and gradient operations for 15-keto metabolites,[78-80] both reverse phase (with MeOH/H_2O) and silica adsorption (with Hx/iPrOH) systems for bile acid precursors 7α-hydroxycholest-4-en-3-one and cholesta-4,6-dien-3-one.[81-84]

10. Cholesterol 7α-Hydroxylase Assay

For assay of mitochondrial cholesterol 7α-hydroxylase (EC 1.14.13.17) resolution and measurement of cholest-5-ene-3ß,7α-diol formed from cholesterol is necessary, and silica adsorption columns with Hx/iPrOH (41:9) have been used.[85-88] Increased sensitivity is obtained by oxidation of the 3ß,7α-diol to 7α-hydroxycholest-4-en-3-one by cholesterol oxidase, followed by HPLC separations in the same system irrigated with Hx/iPrOH (41:9),[86] μPorasil column with Hx/iPrOH (19:1),[87] or with a C_{18} reverse phase column 10 μm Ultrasil-ODS developed with MeCN/MeOH (7:3)[88]

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