COMPOSITION OF BILE ACIDS IN *BOS TAURUS*, *SUIDAE SUS* AND *GALLUS GALLUS*

By

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COMPOSITION OF BILE ACIDS IN BOS TAURUS,

SUIDAE SUS AND GALLUS GALLUS

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CHAPTER I

INTRODUCTION

Chemical investigations of bile acids have been carried on since the early 19th century. Studies of bile acids in the biles of various animals have been extensive in more recent years, and there has been a great amount of work published on the bile of *Bos taurus* (ox), *Suidae sus* (hog) and *Gallus gallus* (chicken) biles. However, most of these investigations were limited to the isolation and determination of different individual bile acids from the biles of these animals. Work on the complete analysis of the bile acids occurring in the bile of these three animals has not been reported at this time.

An investigation of the constituents of the bile of various animals can furnish useful results related to bile acid metabolism. A knowledge of the structures of the various bile acids produced will aid in outlining the metabolic pathways in the formation of bile acids. The search for new bile acids and for companion substances may also aid in establishing the physiological relationship of the bile acids to other products of the animal body. With the aid of gas-liquid chromatography, thin-layer chromatography and the combination of gas-liquid chromatography with mass spectrometry as new techniques for the analysis of bile acids, the possibilities of new advances in the study of the occurrence and metabolism of these compounds have been greatly increased. This work reports the results from a study on the composition of bile

acids in the biles of the ox, hog and chicken using these chromatographic techniques.

CHAPTER II

REVIEW OF LITERATURE

The early literature on bile acids has been reviewed by Sobotka (1). During the late 19th century and the early 20th century, a great deal of work on the bile acids was centered on colorimetric determinations. Pettenkofer (2) found that samples of bile acids formed a purple color when heated with glucose or starch in concentrated sulfuric acid, whereas a brown color indicated the absence of bile acid. Numerous modifications have appeared since, attempting to increase the specificity and sensitivity of the Pettenkofer reaction. Other color reactions for bile acids were also developed during the same period. Enderlin (4) discovered that a blue color was formed when bile acids were heated with less concentrated sulfuric acid or phosphoric acid. Nakagawa (3) produced red colorations by heating bile acids with phosphoric acid and furfural. Mylius (5) found a reaction of cholic acid and iodine under a certain condition formed a blue "iodocholic acid" product. Hammersten (6) based his blue colored reaction on dehydration by strong mineral acids using concentrated hydrochloric acid. The fluorescnece of bile acids was first reported by Tashiro (7). He observed differences in the fluorescence spectra of the products obtained from the Pettenkofer reactions of cholic acid and other bile acids. Minibeck (8) developed a quantitative fluorescence method for the determination of bile acids. In this method a sulfuric acid-acetic acid reagent was used to dissolve

the bile acid sample, and the characteristic green fluorescence developed was measured.

Bile acids form ultraviolet absorbing compounds when dissolved in strong sulfuric acids. These ultraviolet absorbing compounds can be used for the determination of bile acids. Reinhard (9) was the first to study the human bile by its ultraviolet absorption curve. Jenke (10) found that factors such as the concentration of sulfuric acid, temperature and the time of heating, etc., could change the ultraviolet spectra of bile acids. It is necessary to purify the bile acid samples before they can be used for determination. Kier (11) showed that the conjugated and the corresponding free bile acids have the same absorption spectra in concentrated sulfuric acid. Wysocki (12) used sulfuric acid-acetic acid (9:1) as a reagent, and obtained bile acid spectra similar to those produced by using concentrated sulfuric acid.

The use of aluminum oxide columns is now a standard procedure and has been used extensively for the separation of steroids. Wieland _et al_. (13) used alcohol as the solvent to separate some cholenic acids on an alumina column. Haslewood and Wootten (14) also used alumina column chromatography for the separation of bile acids in their comparative studies of bile acids from various animal species. However, they found that this method would give satisfactory resolution for unconjugated bile acids only. Arima (15) described a method for the quantitative analysis of both free and conjugated di- and trihydroxycholanic acids. Furuebisu (16) employed alumina chromatography to separate the monohydroxy-, dihyroxy- and trihydroxy bile acids in the bile of rabbit, chicken, ox, pig and glove fish.

Silicic acid columns have also been used by authors such as Wotton (17), Hirsch and Ahrens (18) and Goldsmith et al. (19) etc. to separate

the fecal bile acids from sterols, triglycerides and fatty acids.

The reversed-phase partition chromatography was developed by Howard and Martin (20) for the separation of fatty acids. Sjövall and Bergström (21) adapted this method to separate bile acids. The separation was effected on a column of Hyflo-Supercel made hydrophobic by storage in a desiccator over dimethyldichlorosilane for one to two weeks. Phase systems with aqueous methanol as the moving phase and chloroform containing a small amount of heptane as the stationary phase were found to be most suitable for the separation of free bile acids. Norman (22) developed the phase systems for the separation of conjugated bile acids so that the bile acids can be separated in the form in which they occur in the bile.

Lambiotte (23) described the method of ion-exchange column chromatography as a means of purifying bile acids in biological extracts. Recently, Gordon _et al._ (24) reported the separation of conjugated bile acids by ion-exchange chromatography. This method permitted a complete resolution of glycine and taurine conjugates. In addition, there was an effective though not complete separation of the tri- and dihydroxycholanic acid derivatives within each conjugated group. Gordon's group applied this method to the gall bladder biles of dog, ox and man, and obtained satisfactory fractionation.

Bile acids have also been separated by paper electrophoresis. The differences in acidity between taurine-conjugated, glycine-conjugated and free bile acids make the separation by this method feasible. Dessi (25) was the first to use paper electrophoresis to analyze biles from different animals. He separated the bile acids of the bile from bilirubin and cholesterol. Briggs _et al._ (26) separated the bile salts mix-

ture into the groups, unconjugated bile acids, glycine conjugates and taurine conjugates. Biserta et al. (27) described the separation of deoxycholic acids, cholic acids, glycocholic acids, taurocholic acids and taurochenodeoxycholic acids by paper electrophoresis.

Counter-current distribution as a method to separate bile acids was investigated by Ahren and Craig (28). Wiggins and Wotton (29) used this method to separate glycine- and tauring-conjugated bile acids.

Paper chromatographic separation of bile acids was also studied by Kritchevsky et al. (30). A basic solvent system using ammonium hydroxide as stationary phase was reported to give satisfactory and reproducible separations of the bile acids. Sjövall (31) at the same time, developed an acidic solvent systems for the paper chromatography of bile acids in which acetic acid was used as the stationary phase. Cerri et al. (32) used the solvent system consisting of n-butanol saturated with water and alkalinized with a trace of ammonia to separate the methyl esters of the bile acids. Ritter (33) demonstrated the chromatography of bile acids on acetylated filter papers, while Szendy (34) used filter papers impregnated with propylene glycol for the same purpose. Watanabe (35) developed a solvent system which could separate free bile acids and glycine conjugates without showing tailing.

Several spray reagents have been used for the detection of bile acids on paper chromatograms, e.g. Kritchevsky (30) used 15% phosphomolybdic acid in ethanol. Carey and Bloch (36) used saturated antimony trichloride in chloroform. Most of these reagents give different colors with different bile acids.

Ganshirt et al. (37) showed the separation and determination of bile acids by thin layer chromatography. Hofman (38) has developed two

different solvent systems for the separation of free and conjugated bile acids. Eneroth (39) investigated fifteen solvent systems suitable for thin layer chromatographic separation of bile acids. It was found, in general, that acidic solvents (containing acetic acid) gave the best separations. However, in analyzing a completely unknown mixture of bile acids, the neutral systems had the advantage of permitting a rough identification of bile acids present. Derivatives of bile acids can also be separated by thin layer chromatography. Solvent systems which are particularily good for the separation of bile acid methyl esters were described by Sodhi and Wood (40).

Color detection offers a good identification of bile acids supplementary to the identification by R_F values in thin layer chromatography. Kritchevsky et al. (41) used a spray reagent consisting of 0.5 ml. anisaldehyde, 50 ml. glacial acetic acids and 1 ml. concentrated sulfuric acid to detect bile acids on a thin layer chromatogram, and found the reagent gave specific color for each free bile acid under ultraviolet and visible lights. Anthony and Beher (42) stressed the color detection of bile acids, and investigated four different sprays for free and conjugated bile acids. They found that the conjugated bile acids gave colors similar to their corresponding free bile acids.

VandenHeuvel, Sweetly and Horning (43) first introduced the technique of separation of bile acids by gas-liquid chromatography in 1960. The bile acids were converted to their methyl esters by reaction with diazomethane, and analyzed on a SE-30 column. Well defined peaks with no evidence of decomposition were obtained for all compounds analyzed. Blomstrand (44) used the same technique to analyze the human bile acids, and found that cholic, chenodeoxycholic and deoxycholic acids were pre-

sent. Lewis (45) obtained good separations of trimethylsilyl ethers of bile acid methyl esters on a 1% QF-1 column. Kuksis and Gordon (46) separated acetates of bile acid methyl esters on a SE-30 column. Briggs and Lipsky (47) described the preparation of partial trimethylsilyl ethers of bile acid methyl esters, and their analysis by gas chromatography. The partial trimethylsilyl ethers were found to be valuable in the gas chromatographic analysis of bile acids since they provided high resolution of structurally similar compounds as well as information concerning both the number and the position of bile acid hydroxyl groups. Supina (48) investigated the silane treatment of solid supports for gas chromatography. Better resolution of bile acid derivatives were obtained by using the siliconized supports.

Bergström et al. (49) employed mass spectrometry for the identification of bile acids. They found that the mass spectra of the methyl esters of bile acids showed characteristic features useful in analysis. The mass spectra obtained from acetylated methyl esters of monohydroxy, dihydroxy and trihydroxy cholanic acids were published by the same investigators in a later report (50). Recently, Eneroth (51) used the gas chromatography-mass spectrometry combination instrument to analyze fecal bile acids.

Some of the bile acids present in the biles of ox, hog and chicken have been isolated and identified by different authors. Table I summarizes the bile acids found in these animals. This table is a modification of Haslewood's table in his survey of distribution of bile salts in various animals (52).

TABLE I

BILE ACIDS IDENTIFIED IN OX, HOG AND CHICKEN BILES

Animal	Bile Acid Isolated From Bile	References
Ox (*Bos taurus*)	Cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid;	Fiser and Fiser (53)
	3α-hydroxy-12-ketocholanic acid;	Wieland and Kishi (54)
	3α, 12α-dihydroxy-7-ketocholanic acid, 7α, 12α-dihydroxy-3-ketocholanic acid; 3α-hydroxy-7, 12-diketocholanic acid	Haslewood (55)
Hog (*Suidae sus*)	Lithocholic acid;	Shimizu (56)
	Chenodeoxycholic acid;	Ido and Sakurai (57)
	α-hyodeoxycholic acid;	Windaus and Bohne (58)
	β-hyodeoxycholic acid;	Kimura (59)
	3α-hydroxy-6-ketocholanic acid;	Fernholz (60)
	Hyocholic acid	Haslewood (61)
Chicken (*Gallus gallus*)	Chenodeoxycholic acid;	Yonemura (62)
	Isolithocholic acid;	Hosizima *et al.* (63)
	Cholic acid;	Yamasaki (64)
	3α-hydroxy-7-ketocholanic acid;	Wiggins (65)
	3α, 7α, 12α, 23-tetrahydroxy-5α-cholanic acid	Yamasaki (66)

CHAPTER III

MATERIALS AND METHODS

Extraction and Purification of Bile Acids from Crude Bile

The general procedure outlined by Sjovall (67) was followed; however, the specific procedure used is described. Crude bile was obtained from the gall bladder of *Bos taurus* (ox), *Suidae sus* (hog), and *Gallus gallus* (chicken), and was dissolved in twenty volumes of absolute ethanol. After boiling 30 min., the solution was colled, then filtered to remove the precipitate which was discarded. The filtrate was evaporated to dryness on a rotary evaporator and dissolved in 50% ethanol. An appropriate volume of 15% sodium hydroxide was added to the ethanol solution to make the concentration of ethanol : sodium hydroxide = 1:1. The solution was hydrolyzed in a nickel crucible at 115°C and 15 psi for 10 hours. Following hydrolysis, the solution was acidified with 1 N hydrochloric acid and extracted three times with ten volumes of anhydrous ethyl ether. The ether extracts were combined and washed with samll portions of water until a neutral PH was obtained; and each of the water portions was reextracted with ethyl ether. The combined neutral ether extracts were evaporated to dryness, and the residue was subjected to a three step counter-current extraction between equal volumes of 70% ethanol and petroleum ether to remove cholesterol and fatty acids. The free bile acids were recovered from the ethanol phases by evaporating the solvent on a rotary evaporator. The samples were stored at -15°C until used.

Preparation of Methyl Esters (ME)

Free bile acid standards, 3α-hydroxycholanic acid (lithocholic acid), 3α, 12α-dihydroxycholanic acid (deoxycholic acid), 3α, 7α-dihydroxycholanic acid (chenodeoxycholic acid), 3α, 6α-dihydroxycholanic acid (α-hyodeoxycholic acid), 3β, 6α-dihydroxycholanic acid (β-hyodeoxycholic acid), 3α, 7β-dihydroxycholanic acid (ursodeoxycholic acid), 3α, 7α, 12α-trihydroxycholanic acid (cholic acid), 3α, 6α, 7α-trihydroxycholanic acid (hyocholic acid) (above compounds were purchased from Calbiochem, Los Angeles); 3α-hydroxy-7-ketocholanic acid, 3α-hydroxy-12-ketocholanic acid, 3α, 12α-dihydroxy-7-ketocholanic acid (above compounds were obtained from Dr. Jan Sjövall as a gift); and the free bile acids extracted from animal biles were weighed, and dissolved in ether: methanol (9:1) solution. A suitable amount of freshly prepared diazomethane in ether was added to the solution to completely methylate the bile acids. Methyl esters of bile acids thus prepared were used directly for chromatography.

Thin Layer Chromatography (TLC)

Thin layer plates of 0.5 mm in thickness were prepared according to Stahl (68). Glass plates of 20 cm x 20 cm were coated with a suspension of 58 ml of distilled water and 30 g of Silica Gel G (Brinkmann and Co.) using a commercial apparatus (Brinkmann and Co.). The chromatoplates were allowed to air dry, then activated in a oven at 110°-120°C for three hours before use.

Samples of methyl esters of bile acid standards and mixtures of unknown bile acids extracted from animal biles were dissolved in ethanol, and applied on the plates through sharpened micropipettes (10-20 μg in

3-4 µl). During this procedure, the chromatoplates were warmed by heat from a electrical hair dryer which helped in the evaporation of the solvent. The glass plates were cooled to room temperature and placed in the chamber previously saturated with the developing solvent. When the solvent front was about 17 cm from the origin, the plates were taken out of the chamber and dried in an oven at 110°C. The plates were then sprayed with concentrated sulfuric acid and heated in an oven at 110°C. R_F values and the R_D values (ratio between the absolute mobility of a compound and the absolute mobility of standard methyl deoxycholate) were calculated for each sample. The unknown bile acids in the animal bile samples were identified by their R_F and R_D values with the standard bile acids. The solvent systems used for the thin layer chromatography of the methyl esters of bile acids were:

Solvent I; ethyl acetate: acetic acid (96:4)

Solvent II; chloroform: methanol (90:10).

All solvents were redistilled before use and were measured exactly by pipetting since it was found that small changes in composition of the solvent system interfered with reproducibility.

Gas-Liquid Chromatography (GLC)

The columns used for gas-liquid chromatography were packed according to the technique developed by Horning _et al_. (69). Gas Chrom Q (silanized), 100-120 mesh purchased from Applied Science Laboratories, Philadelphia, was used as the supporting material for the liquid phase. The stationary liquid phases, QF-1 and SE-30, were also purchased from Applied Science Laboratories. One hundred ml of 3% solution of the stationary phase in toluene were added to 20 g of Gas Chrom Q. Vacuum

was applied, and after 30 minutes the excess solution was filtered off. The packing material was spread on a filter paper to air dry, then dried in the oven at 80°C. The columns used were ¼ in x 10 ft coiled glass designed to fit the mass spectrometer. The columns were carefully washed and silanized before packing. The coated supports were packed into the column under light tapping, e.g. with a glass rod with rubber policeman, and with the aid of a water aspirator connected to the other end of the column. The packed columns (3% QF-1 and 3% SE-30) were conditioned at 300°C for 72 hours without gas flowing through the column and then for another day with a slow flow of helium.

A modified Barber Colman Model 5000 gas chromatograph with a hydrogen flame detector was used. The modification consisted of an oven and injection port of the same dimensions as used on the mass spectrometer-gas chromatograph (70, 71). The column temperature during chromatography was 250°C for the 3% SE-30 column, and 290°C for the 3% QF-1 column. The flash heater and the detector temperature were kept at 300°C. The helium gas flow rate was maintained at 55 ml/min. Samples of methyl esters of bile acids dissolved in ether and methanol (9:1) were injected directly onto the gas chromatograph with a micro syring (Hamilton Co., Inc., Whittier, Cal.). Sample sizes of 50-100 µg of the mixture of unknown bile acids, and 20-50 µg on individual bile acid standards were used. Methyl esters of bile acids from the bile of animals were identified by their retention times as compared with the standard ME of bile acids. For quantitative determination of the unknown methyl esters peak areas were measured using a planimeter[1] to measure the area underneath the curves. Areas of the unknown methyl esters were compared to areas

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[1]Lasico Planimeter Model 700

from known amounts of standard methyl esters.

Gas Chromatography-Mass Spectrometry (GC-MS)

The prototype of the LKB 9000 combination gas chromatograph-mass spectrometer (GC-MS) instrument (70,71) was used. The 3% SE-30 column used for the gas-liquid chromatography was used. The column temperature was 230°C. The helium flow rate was 50 ml/min. The molecule separator was kept at 260°C, and the ion source and flash heater both at 280°C. The ionizing current was 20 μ amp., the ionization voltage was 70 eV, the electron multiplier voltage was 1.7 kV, and the accelerating voltage was 3.5 kV.

Samples analyzed were methyl esters of unknown bile acid mixtures from animal biles and individual bile acid standards. Mass spectra were obtained as the peaks appeared on the TIC[2] recorder serving as the monitor for the GLC unit. Background mass spectra derived mainly from the column liquid phase were always recorded and subtracted from the sample spectra. Mass spectra were computer-plotted from tabular intensity data. A Cal Comp 565 Plotter driven by an IBM 1620 computer required 3 min. of plotting time and 6 min. of computer time using a Fortran II-D program.

Unknown bile acid methyl esters in the animal biles were identified by their molecular ion, their fragmentation patterns and their GLC retention times.

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[2]TIC: Total Ion Current (proportional to total no. of ion formed and consequently to amount of compound).

CHAPTER IV

RESULTS AND DISCUSSION

Thin layer chromatographic (TLC) analyses of ox, hog and chicken bile acid methyl esters together with the mixture of methyl esters of standard bile acids in solvent systems, chloroform: methanol (90:10) and ethyl acetate: glacial acetic acid (96:4), were shown in Figure 1. The R_F and R_D values of each methyl ester of bile acid occurring in the animal biles identified by using these two solvent systems were shown in Tables II and III. Methyl esters of 3α-hydroxycholanic acid; 3α-hydroxy-12-ketocholanic acid; 3-keto-7α, 12α-dihydroxycholanic acid; 3α, 7α-dihydroxycholanic acid; 3β, 6α-dihydroxycholanic acid; 3α, 7α, 12-diketocholanic acid; 3α, 12α-dihydroxy-7-ketocholanic acid; 3α, 6α-dihydroxycholanic acid and 3α, 7α, 12α-trihydroxycholanic acid were found in the ox bile. Methyl esters of 3α-hydroxycholanic acid; 3α-hydroxy-6-ketocholanic acid; 3α, 7α-dihydroxycholanic acid; 3β, 6α-dihydroxycholanic acid; 3α, 6β-dihydroxycholanic acid; 3α, 6α-dihydroxycholanic acid and 3α, 7α, 6α-trihydroxycholanic acid were found in the hog bile. Methyl esters of 3α-hydroxycholanic acid; 3α-hydroxy-7-ketocholanic acid; 3α, 7α-dihydroxycholanic acid; 3β, 6α-dihydroxycholanic acid; 3α, 6α-dihydroxycholanic acid; 3α, 6α-dihydroxycholanic acid; 3α, 7α, 12α-trihydroxycholanic acid and one unknown bile acid with very low mobility were found in the chicken bile. All bile acid methyl esters except methyl 3-keto-7α, 12α-dihydroxycholanate, methyl 3α-hydroxy-7, 12-diketocholanate,

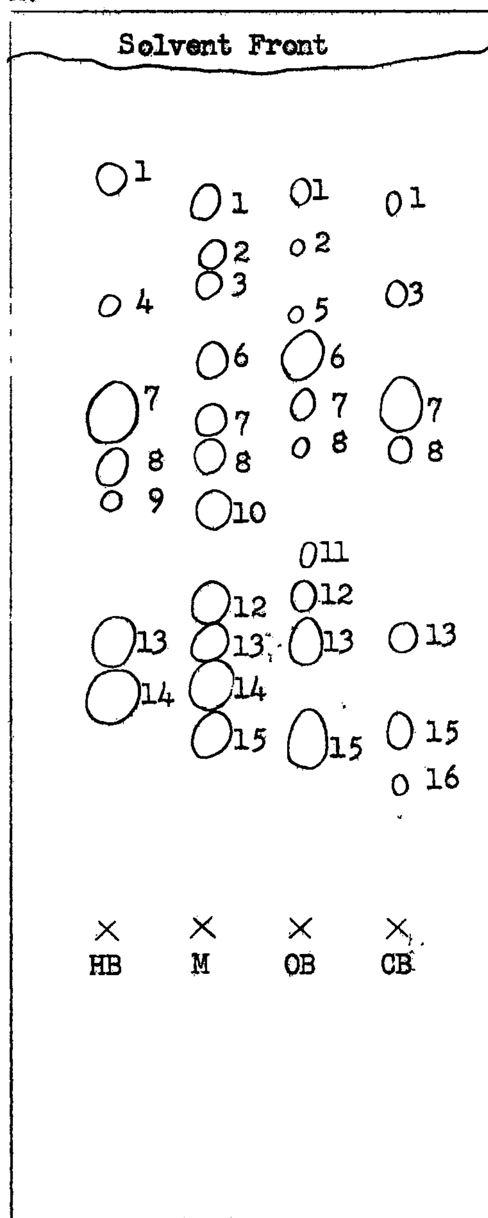
Figure 1. TLC Analyses of Bile Acids as Methyl Esters in Ox, Hog and Chicken Biles, and Methyl Esters of Standard Bile Acids.

A. In solvent system, ethyl acetate: acetic acid (96:4).

B. In solvent system, chloroform: methanol (90:10).

Abbreviations: Ox bile: OB, hog bile: HB, chicken bile: CB, mixture of methyl esters of standard bile acids. M.

Numbers, 1-16, representing methyl esters of bile acids are as indicated in Tables II and III.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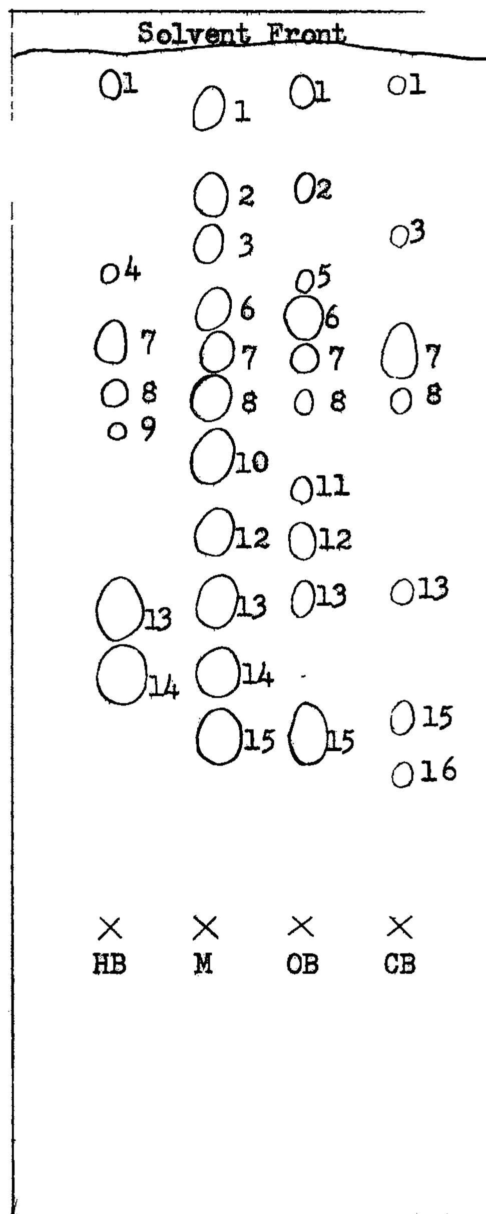
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TABLE II

THE R_F^1 AND R_D^2 VALUES OF BILE ACIDS AS METHYL ESTERS IN OX, HOG AND CHICKEN BILES IDENTIFIED BY TLC IN SOLVENT SYSTEM, ETHYL ACETATE : ACETIC ACID (96:4)

No.	Methyl Ester of Bile Acid	Authenic		In Ox Bile		In Hog Bile		In Chicken Bile	
		R_F	R_D	R_F	R_D	R_F	R_D	R_F	R_D
1.	3αOH	0.84	1.25	o.85	1.29	0.87	0.32	0.80	1.22
2.	3αOH, 12=0	0.78	1.18	0.79	1.20	-	-	-	-
3.	3αOH, 7=0	0.74	1.13	-	-	-	-	0.74	1.12
4.	3αOH, 6=0	-	-	-	-	0.72	1.09	-	-
5.	3=0, 7αOH, 12αOH	-	-	0.71	1.08	-	-	-	-
6.	3αOH, 12αOH	0.65	1.00	0.67	1.00	-	-	-	-
7.	3αOH, 7αOH	0.59	0.89	0.60	0.90	0.59	0.90	0.60	0.92
8.	3βOH, 6αOH	0.54	0.83	0.55	0.84	0.54	0.81	0.55	0.84
9.	3αOH, 6βOH	-	-	-	-	0.51	0.77	-	-
10.	3αOH, 7βOH	0.48	0.73	-	-	-	-	-	-
11.	3αOH, 7=0, 12=0	-	-	0.43	0.65	-	-	-	-
12.	3αOH, 7=0, 12αOH	0.37	0.56	0.38	0.58	-	-	-	-
13.	3αOH, 6αOH	0.32	0.49	0.33	0.50	0.33	0.50	0.34	0.52
14.	3αOH, 7αOH, 6αOH	0.28	0.42	-	-	0.27	0.41	-	-
15.	3αOH, 7αOH, 12αOH	0.22	0.33	0.22	0.33	-	-	0.22	0.34
16.	3αOH, 6αOH, 12αOH, 23αOH, 5α	-	-	-	-	-	-	0.17	0.25

$$^1R_F = \frac{\text{Distance travelled by methyl ester spot}}{\text{Distance travelled by the solvent front}}$$

$$^2R_D = \frac{\text{Distance travelled by methyl ester spot}}{\text{Distance travelled by methyl deoxycholate spot}}$$

TABLE III

THE R_F^1 AND R_D^2 VALUES OF BILE ACIDS AS METHYL ESTERS IN OX, HOG AND CHICKEN BILES IDENTIFIED BY TLC IN SOLVENT SYSTEM, CHLOROFORM : METHANOL (90:10)

No.	Methyl Ester of Bile Acid	Authenic		In Ox Bile		In Hog Bile		In Chicken Bile	
		R_F	R_D	R_F	R_D	R_F	R_D	R_F	R_D
1.	3αOH	0.94	1.32	0.95	1.34	0.96	1.35	0.96	1.35
2.	3αOH, 12=0	0.84	1.18	0.84	1.18	-	-	-	-
3.	3αOH, 7=0	0.78	1.09	-	-	-	-	0.79	1.10
4.	3αOH, 6=0	-	-	-	-	0.75	1.06	-	-
5.	3=0, 7αOH, 12αOH	-	-	0.74	1.04	-	-	-	-
6.	3αOH, 12αOH	0.71	1.00	0.70	1.00	-	-	-	-
7.	3αOH, 7αOH	0.66	0.92	0.65	0.91	0.66	0.93	0.66	0.93
8.	3βOH, 6αOH	0.61	0.85	0.60	0.84	0.61	0.85	0.60	0.84
9.	3αOH, 6βOH	-	-	-	-	0.57	0.80	-	-
10.	3αOH, 7βOH	0.54	0.75	-	-	-	-	-	-
11.	3αOH, 7=0, 12=0	-	-	0.50	0.70	-	-	-	-
12.	3αOH, 7=0, 12αOH	0.45	0.63	0.44	0.62	-	-	-	-
13.	3αOH, 6αOH	0.39	0.53	0.38	0.53	0.37	0.51	0.38	0.53
14.	3αOH, 7αOH, 6αOH	0.29	0.41	-	-	0.29	0.40	-	-
15.	3αOH, 7αOH, 12αOH	0.22	0.30	0.23	0.31	-	-	0.24	0.33
16.	3αOH, 6αOH, 12αOH 23αOH, 5α	-	-	-	-	-	-	0.17	0.24

$^1R_F = \frac{\text{Distance travelled by methyl ester spot}}{\text{Distance travelled by the solvent front}}$

$^2R_D = \frac{\text{Distance travelled by methyl ester spot}}{\text{Distance travelled by methyl deoxycholate spot}}$

in the ox bile, methyl 3α, 6β-dihydroxycholanate, methyl 3α-hydroxy-6-ketocholanate in the hog bile and methyl 3α-hydroxy-7-ketocholanate in the chicken bile were identified by their R_F and R_D values comparing with the known standards run on the same chromatogram. The above mentioned bile acid methyl esters were identified by comparing R_D values with those reported by others (38,39). The unknown bile acid methyl ester found in chicken bile was believed to be methyl 3α, 6α, 12α, 23-tetrahydroxy-5α-cholanate due to its low mobility on the thin layer chromatogram. This bile acid has been reported by Wiggins (65) to appear in chicken bile, but the R_F and R_D values of its methyl ester in TLC are not known. Therefore, this finding was not confirmed. All bile acids but 3α, 6α-dihydroxy- and 3β, 6α-dihydroxycholanic acids, found in ox, hog and chicken biles have been reported previously (52). The 3α, 6α-dihydroxy- and 3β, 6α-dihydroxycholanic acids have been reported to be found in hog bile only, not in chicken or ox bile.

During the course of investigation of the TLC analysis of bile acids, many solvent systems which had been reported to give good separations for bile acids were tried. Most of these solvent systems gave good separations for the standard bile acids; however, poor resolution was obtained when they were used to analyze the naturally occurring bile acids in animal biles. It was also found that methyl esters of bile acids can be separated much easier than the bile acids themselves. The bile acids formed long tailing when separated by TLC, whereas methyl esters yielded uniform round spots without any tailing. The purity of solvents and accuracy in measurement of their volume was found to affect the resolution. Better separations occurred when freshly distilled and accurately measured solvents were used.

The gas liquid chromatographic (GLC) analyses of ox, hog, chicken bile acid methyl esters and the mixture of standard bile methyl esters on the 3% SE-30 column and the 3% QF-1 column are shown in Figures 2 and 3 respectively. The R_D values and the amount in per cent of each identified bile acid methyl ester for the two columns were shown in Tables IV and V. The percentage of each bile acid methyl ester in the total bile acid methyl ester mixture was calculated by comparing its specific peak area on the recorder response with that of a known standard bile acid methyl esters. These areas were measured by a planimeter.

Similar methyl esters of bile acids were found in the ox, hog and chicken biles by the GLC technique as the TLC analyses, except that there was no unknown methyl ester found in the chicken bile. The methyl 3-keto-7α, 12α-dihydroxycholanate, methyl 3α-hydroxy-7, 12-diketocholanate in ox bile; the methyl 3α, 6β-dihydroxycholanate, Me 3α-hydroxy-6-ketocholanate in hog bile and the methyl 3α-hydroxy-7-ketocholanate in the chicken bile were also identified by comparisons of their R_D values with those reported by others (72). Methyl esters of 3α, 6α-dihydroxycholanate and 3β, 6α-dihydroxycholanate were also found in the GLC analyses in ox and chicken biles, besides the hog bile. This finding agreed with the result of TLC though these two bile acids had not been reported to occur in these two species before.

The 3% QF-1 column gave better separation for bile acid methyl esters than the 3% SE-30 column. The SE-30 column is a rather non-selective column which can not separate stereo-isomers such as methyl 3α, 7α-dihydroxycholanate and methyl 3α, 7β-dihydroxycholanate; but it did give good separation of the positional isomers such as methyl 3α, 7α-dihydroxycholanate and methyl 3α, 6α-dihydroxycholanate. Therefore, the

methyl 3β, 6α-dihydroxycholanate and methyl 3α, 6β-dihydroxycholanates which are stereoisomers of methyl 3α, 6α-dihydroxycholanate were not shown in the gas chromatograms of SE-30 column (Fig. 2). The QF-1 column in the other hand gave separations to both of the stereo-and positional isomers, and hence all the methyl esters of bile acids analyzed were shown in the chromatogram of this stationary liquid phase (Fig. 3).

Both of the SE-30 and QF-1 columns showed some degree of irreversible adsorption of bile acid methyl esters to the supports, and there were losses of compounds on the columns. The losses were indicated by the decrease of specific peak areas for the compounds. The extend of column adsorption is far greater on the QF-1 column than the SE-30 column. The loss of compound on the column increased with the increase in number of hydroxyl groups in the compound. In Fig. 3B which shows the GC analysis of ox bile methyl esters on QF-1 column, the specific peak area for the methyl 3α, 7α, 12α-trihydroxycholanate was much smaller than the peak area for methyl 3α, 12α-dihydroxycholanate, and yet it represented more amount of the compound in weight than the other. Ox bile was found to contain 54% of 3α, 7α, 12α-trihydroxycholanic acid and only 17% of the 3α, 12α-dihydroxycholanic acid.

Results of gas chromatography-mass spectrometric (GC-MS) studies of the ox, hog and chicken bile acids are shown in Figures 4, 5, 6, 7 and 8. Mass spectra of bile acid methyl esters from the animal biles are shown together and compared with the spectrum of a standard bile acid methyl ester in each of these figures. The similarity in mass spectra between the standard and unknown provides further evidence for the occurrence of a specific bile acid in the animal bile. Mass spectra were not obtained from all the bile acid methyl esters of the animal

biles identified by GLC or TLC. Only the mass spectra of methyl 3α-hydroxycholanate from the ox, hog and chicken biles (Fig. 4); methyl 3α, 12α-dihydroxycholanate from the ox bile (Fig. 6); methyl 3α, 7α-dihydroxycholanate from ox, hog and chicken biles (Fig. 7); methyl 3α, 6α-dihydroxycholanate from ox, hog and chicken biles (Fig. 5); and methyl 3α, 7α, 12α-trihydroxycholanate from ox and chicken biles (Fig. 8) were obtained. A good mass spectrum of the methyl esters of bile acids which occurred in very small amounts in the animal biles was not obtained although they were detected by GC or TLC. The mass spectra of methyl 3α, 6α-dihydroxycholanate (Fig. 5) obtained from the ox and chicken biles provided further evidence of its presence in the bile of the two species. However, no mass spectrum was obtained from the methyl 3β, 6α-dihydroxycholanate due to its trace amount in the biles, and its presence in the ox and chicken bile was not further confirmed.

The molecular peaks for the bile acids with ring hydroxyl groups was found to be very small, since the hydroxyl groups are easily eliminated as H_2O. The side chain was also readily split off and a peak corresponding to the ionized and unsaturated nucleus occurred. Methyl esters of monohydroxycholanic acids such as 3α-hydroxycholanic acid (Fig. 4) has a nuclear peak occurred at m/e 257. For dihydroxycholanic acids, such as 3α, 12α-dihydroxycholanic acid (Fig. 6), 3α, 7α-dihydroxycholanic acid (Fig. 7) and 3α, 6α-dihydroxycholanic acid (Fig. 5), the nuclear peak occurred at m/e 255. For a trihydroxycholanic acid such as 3α, 7α, 12α-trihydroxycholanic acid (Fig. 8) the nuclear peak occurred at m/e 253. Therefore, for a saturated bile acid the nuclear peak indicates the number of hydroxyl groups present in the sterol ring system and hence serves as an aid in detecting the positional isomers.

Other fragment ion peaks useful in interpretation of the spectra occur due to the loss of one or more hydroxyl groups of the side chain, ring A, ring D or combination of these. Stereo-isomers such as methyl 3α, 7α-dihydroxycholanate (Fig. 7) and methyl 3α, 7β-dihydroxycholanate (Fig. 9) which differ only in regard to the orientation of the substituent at C_7 gave very similar spectra whereas the positional isomer, methyl 3α, 12α-dihydroxycholanate (Fig. 6) gave a quite different spectrum.

The results of analyses of ox, hog and chicken biles by thin layer chromatography, gas liquid chromatography and gas chromatography-mass spectrometry, and the methyl esters of bile acids found in each animal bile are finally summarized in Table V. The notation '+' refers to a positive identification of the specific bile acid methyl ester in the animal biles.

Figure 2. GLC Analyses of Methyl Esters of Bile Acids on 3% SE-30 Column at 250°C.

A. Mixture of methyl esters of standard bile acids.

B. Methyl esters of bile acids in ox bile.

C. Methyl esters of bile acids in hog bile.

D. Methyl esters of bile acids in chicken bile.

Numbers, 1-16, representing methyl esters of bile acids are as indicated in Tables IV and V.

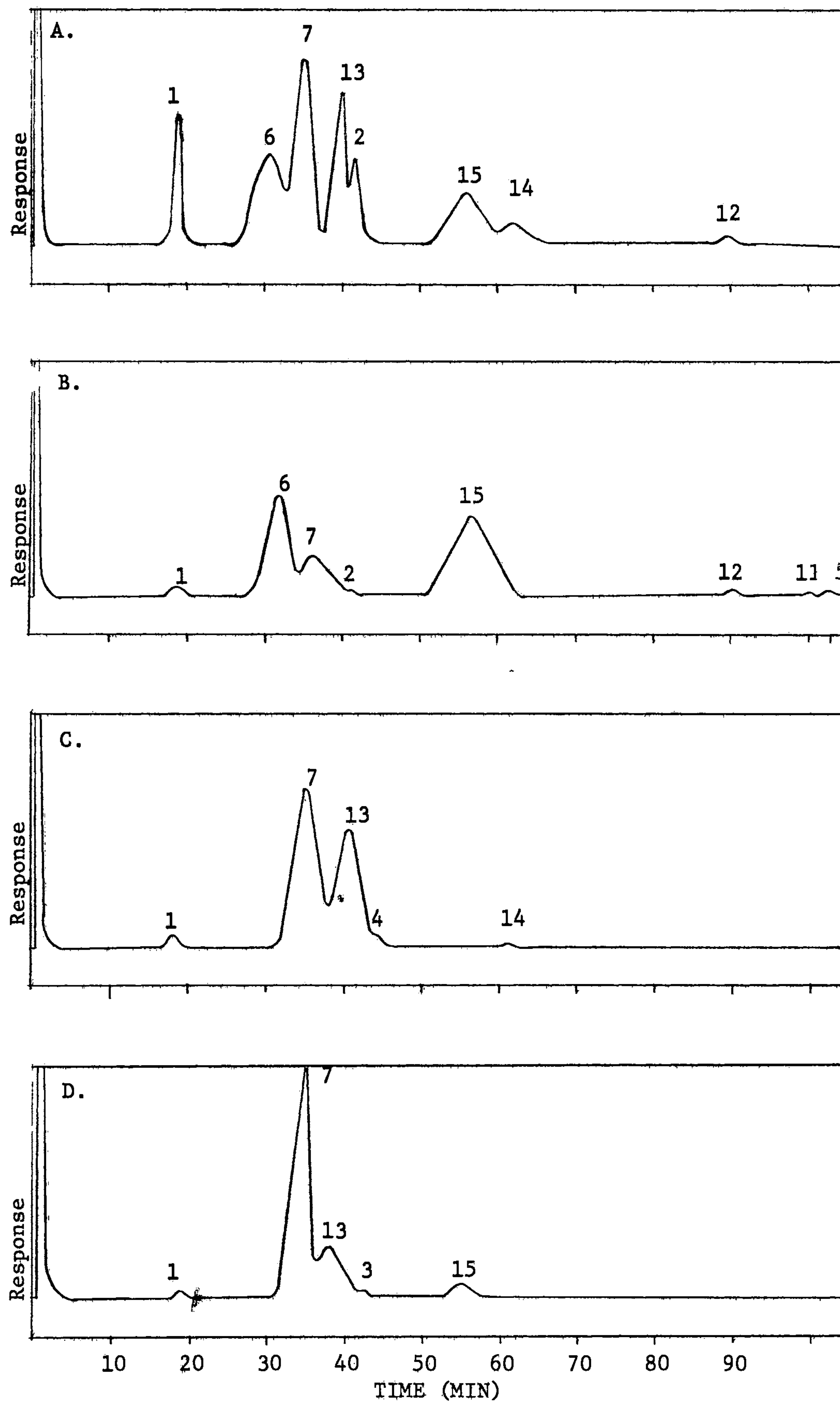

A.
Response
1
6
7
13
2
15
14
12
B.
Response
1
6
7
2
15
12
11
5
C.
Response
1
7
13
4
14
D.
Response
1
7
13
3
15
10
20
30
40
50
60
70
80
90
TIME (MIN)

Figure 3. GLC Analyses of Methyl Esters of Bile Acids on 3% QF-1 Column at 290°C.

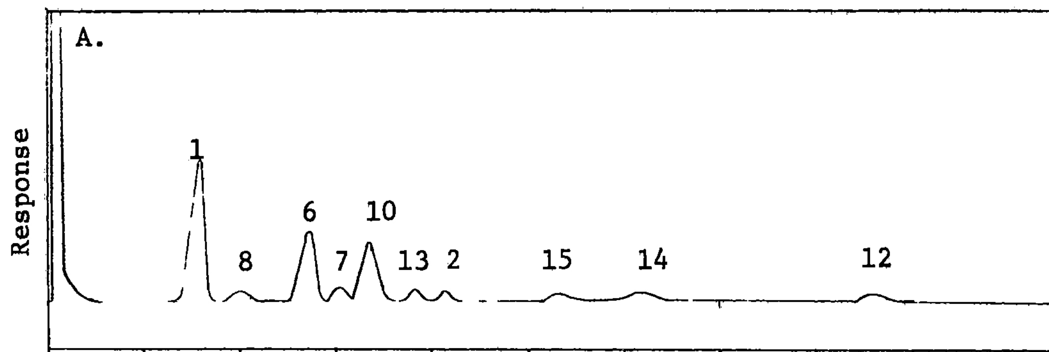
A. Mixture of methyl esters of standard bile acids.

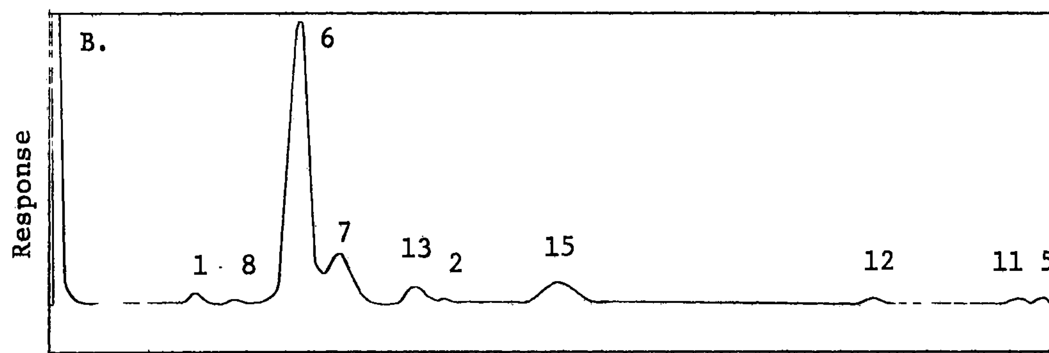
B. Methyl esters of bile acids in ox bile.

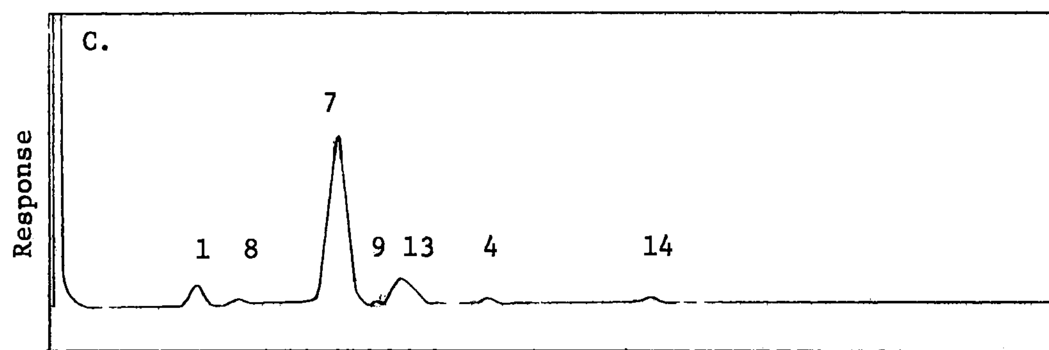
C. Methyl esters of bile acids in hog bile.

D. Methyl esters of bile acids in chicken bile.

Numbers, 1-16, representing methyl esters of bile acids are as indicated in Tables IV and V.


A.
Response
1
8
6
7
10
13
2
15
14
12


B.
Response
6
1
8
7
13
2
15
12
11
5


C.
Response
7
1
8
9
13
4
14

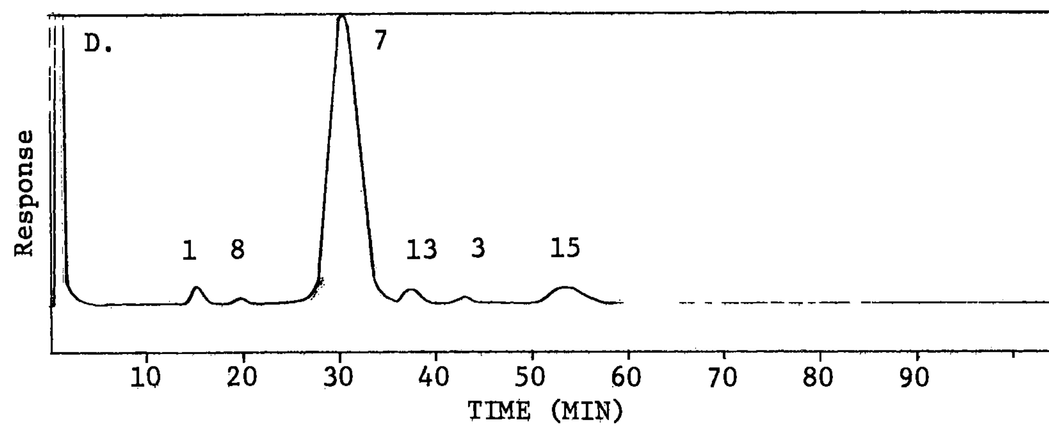

D.
Response
7
1
8
13
3
15
10
20
30
40
50
60
70
80
90
TIME (MIN)

TABLE IV

THE R_D^1 VALUES AND AMOUNTS OF BILE ACIDS AS METHYL ESTERS IN OX, HOG AND CHICKEN BILES IDENTIFIED BY GLCON 3% SE-30 COLUMN AT 250°C

No.	Methyl Ester of Bile Acid	Authentic	In Ox Bile		In Hog Bile		In Chicken Bile	
		R_D	R_D	% In Total Me of Bile Acids	R_D	% In Total Me of Bile Acids	R_D	% In Total Me of Bile Acids
1.	3αOH	0.63	0.62	0.35	0.63	0.51	0.62	0.18
2.	3αOH, 12=0	1.43	1.41	trace	-	-	-	-
3.	3αOH, 7=0	-	-	-	-	-	1.38	trace
4.	3αOH, 6=0	-	-	-	1.46	trace	-	-
5.	3=0, 7αOH, 12αOH	-	4.10	trace	-	-	-	-
6.	3αOH, 12αOH	1.00	1.00	8.97	-	-	-	-
7.	3αOH, 7αOH	1.14	1.10	12.05	1.12	71.0	1.12	85
8.	3βOH, 6αOH	-	-	-	-	-	-	-
9.	3αOH, 6βOH	-	-	-	-	-	-	-
10.	3αOH, 7βOH	1.21	-	-	-	-	-	-
11.	3αOH, 7=0, 12=0	-	3.90	trace	-	-	-	-
12.	3αOH, 7=0, 12αOH	3.07	3.10	trace	-	-	-	-
13.	3αOH, 6αOH	1.29	-	-	1.30	28.0	1.29	12.2
14.	3αOH, 7αOH, 6αOH	2.13	-	-	2.15	trace	-	-
15.	3αOH, 7αOH, 12αOH	1.85	1.80	77.77	-	-	1.81	1.6
16.	3αOH, 6αOH, 12αOH, 23αOH, 5α	-	-	-	-	-	-	-

$$^1R_D = \frac{\text{Actual retention time of the methyl ester of bile acid}}{\text{Actual retention time of methyl deoxycholate}}$$

TABLE V

THE R_D^1 VALUES AND AMOUNTS OF BILE ACIDS AS METHYL ESTERS IN OX, HOG AND CHICKEN BILES IDENTIFIED BY GLC ON 3% QF-1 COLUMN AT 290°C

No.	Methyl Ester of Bile Acid	Authentic	In Ox Bile		In Hog Bile		In Chicken Bile	
		R_D	R_D	% In Total Me of Bile Acids	R_D	% In Total Me of Bile Acids	R_D	% In Total Me of Bile Acids
1.	3αOH	0.57	0.56	0.43	0.56	0.90	0.56	0.18
2.	3αOH, 12=0	1.51	1.50	trace	-	-	-	-
3.	3αOH, 7=0	-	-	-	-	-	1.57	trace
4.	3αOH, 6=0	-	-	-	1.63	trace	-	-
5.	3=0, 7αOH, 12αOH	-	4.30	trace	-	-	-	-
6.	3αOH, 12αOH	1.00	1.00	17	-	-	-	-
7.	3αOH, 7αOH	1.11	1.11	18	1.11	86	1.12	77
8.	3βOH, 6αOH	0.73	0.70	trace	0.71	trace	0.71	trace
9.	3αOH, 6βOH	-	-	-	1.31	trace	-	-
10.	3αOH, 7βOH	1.22	-	-	-	-	-	-
11.	3αOH, 7=0, 12=0	-	4.15	trace	-	-	-	-
12.	3αOH, 7=0, 12αOH	3.30	3.32	trace	-	-	-	-
13.	3αOH, 6αOH	1.38	1.38	7	1.38	13	1.37	0.42
14.	3αOH, 7αOH, 6αOH	2.31	-	-	2.32	trace	-	-
15.	3αOH, 7αOH, 12αOH	1.93	1.92	54.3	-	-	1.93	23
16.	3αOH, 6αOH, 12αOH 23αOH, 5α	-	-	-	-	-	-	-

$$^1R_D = \frac{\text{Actual retention time of methyl ester of bile acid}}{\text{Actual retention time of methyl deoxycholate}}$$

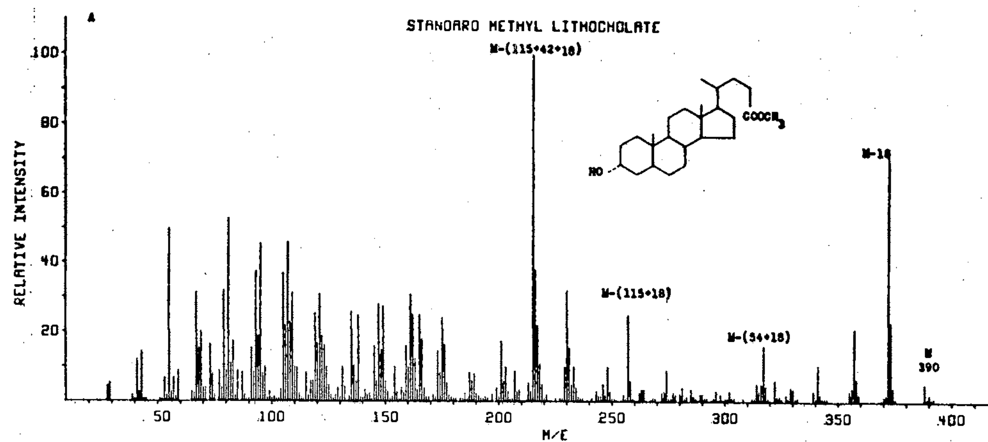
Figure 4. Mass Spectra of Methyl Lithocholate (Methyl 3α-hydroxycholanate).

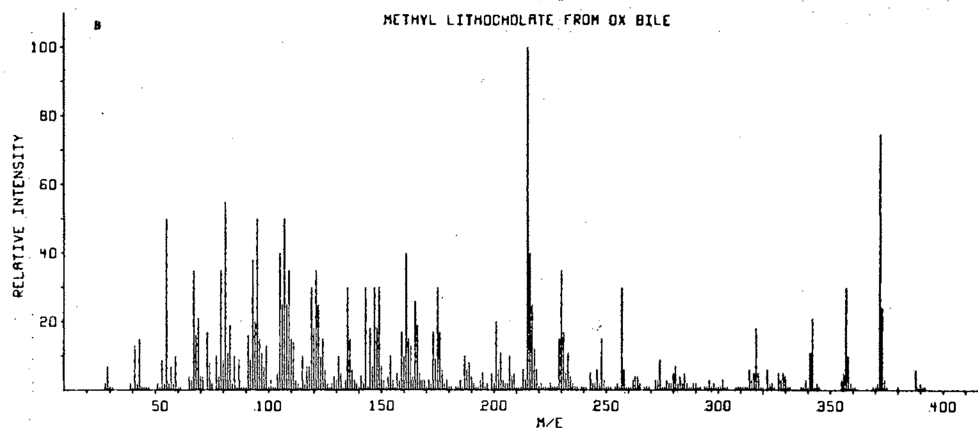
A. Standard methyl lithocholate.

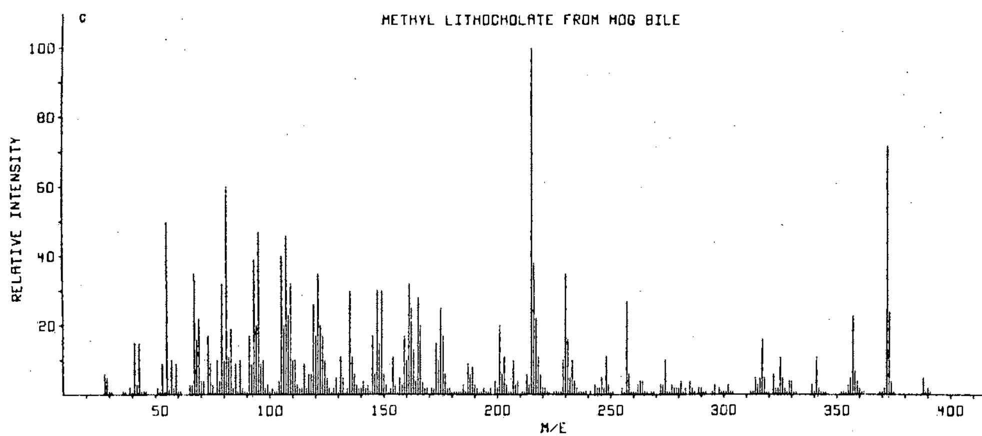
B. Methyl lithocholate from ox bile.

C. Methyl lithocholate from hog bile.

D. Methyl lithocholate from chicken bile.


A
STANDARD METHYL LITHOCHOLATE
M-(115+42+18)
COOCH3
HO
M-18
M-(115+18)
M-(34+18)
M
390
RELATIVE INTENSITY
M/E


B
METHYL LITHOCHOLATE FROM OX BILE
RELATIVE INTENSITY
M/E


C
METHYL LITHOCHOLATE FROM HOG BILE
RELATIVE INTENSITY
M/E

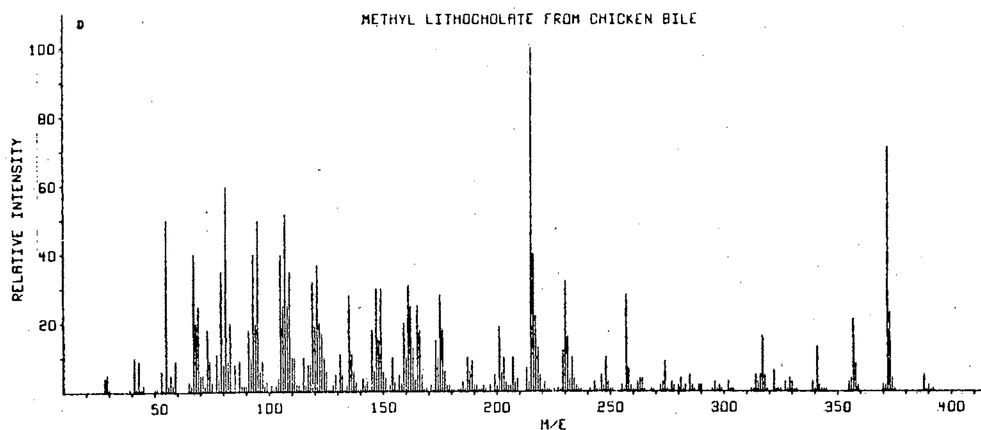

D
METHYL LITHOCHOLATE FROM CHICKEN BILE
RELATIVE INTENSITY
M/E

Figure 5. Mass Spectra of Methyl α-Hyodeoxycholate (Methyl 3α, 6α-Dihydroxycholanate).

A. Standard methyl α-hyodeoxycholate.

B. Methyl α-hyodeoxycholate from ox bile.

C. Methyl α-hyodeoxycholate from hog bile.

D. Methyl α-hyodeoxycholate from chicken bile.

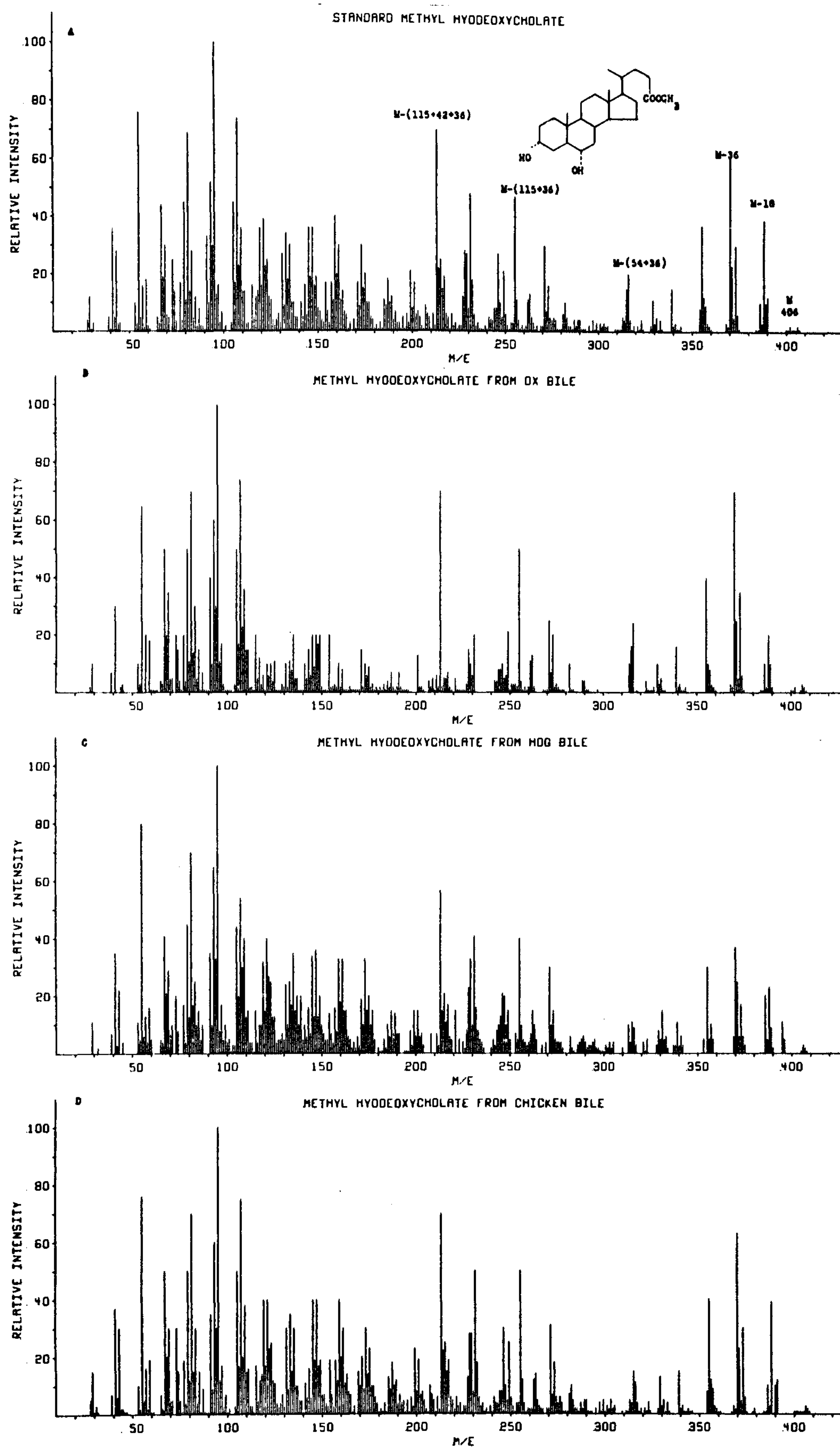
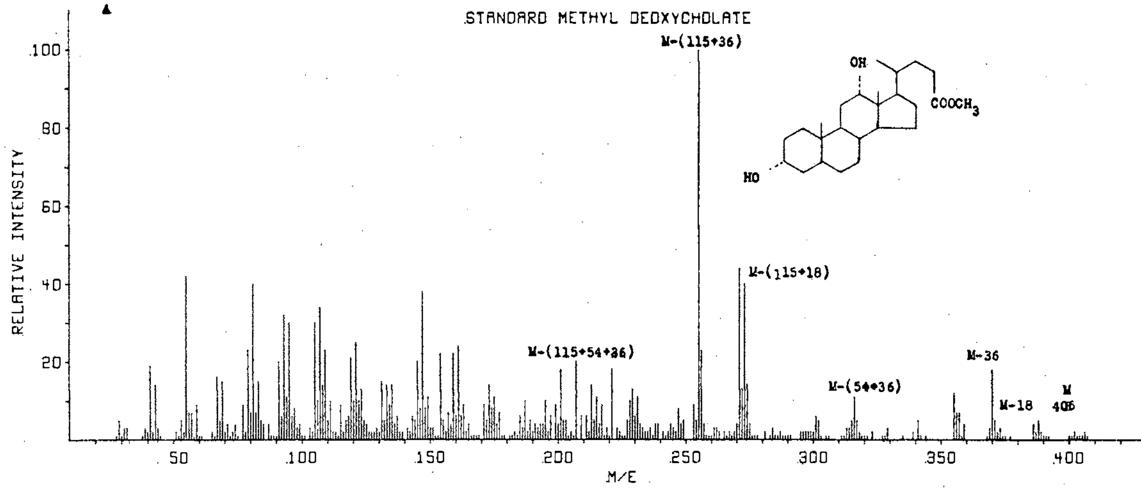

A
STANDARD METHYL HYODEOXYCHOLATE
RELATIVE INTENSITY
M/E
M-(115+42+36)
M-(115+36)
M-(54+36)
M-36
M-18
M
406
COOCH3
HO
OH
B
METHYL HYODEOXYCHOLATE FROM OX BILE
C
METHYL HYODEOXYCHOLATE FROM HOG BILE
D
METHYL HYODEOXYCHOLATE FROM CHICKEN BILE

Figure 6. Mass Spectra of Methyl Deoxycholate (Methyl 3α, 12α-Dihydroxycholanate).

A. Standard methyl deoxycholate.

B. Methyl deoxycholate from ox bile.


A
STANDARD METHYL DEOXYCHOLATE
M-(115+36)
M-(115+18)
M-(115+54+36)
M-(54+36)
M-36
M-18
M
406
OH
COOCH3
HO
RELATIVE INTENSITY
M/E

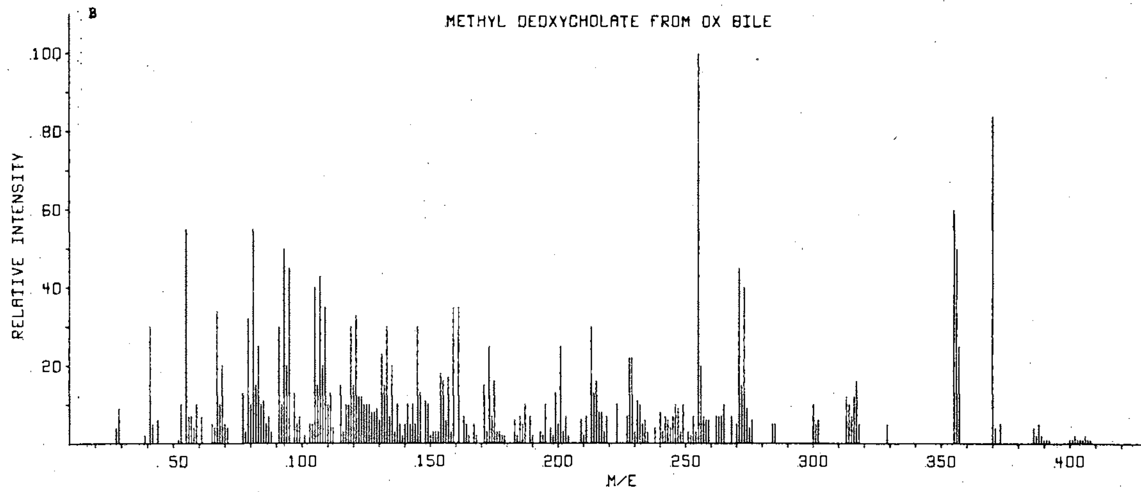

B
METHYL DEOXYCHOLATE FROM OX BILE
RELATIVE INTENSITY
M/E

Figure 7. Mass Spectra of Methyl Chenodeoxycholate (Methyl 3α, 7α-Dihydroxycholanate).

A. Standard methyl chenodeoxycholate.

B. Methyl chenodeoxycholate from ox bile.

C. Methyl chenodeoxycholate from hog bile.

D. Methyl chenodeoxycholate from chicken bile.

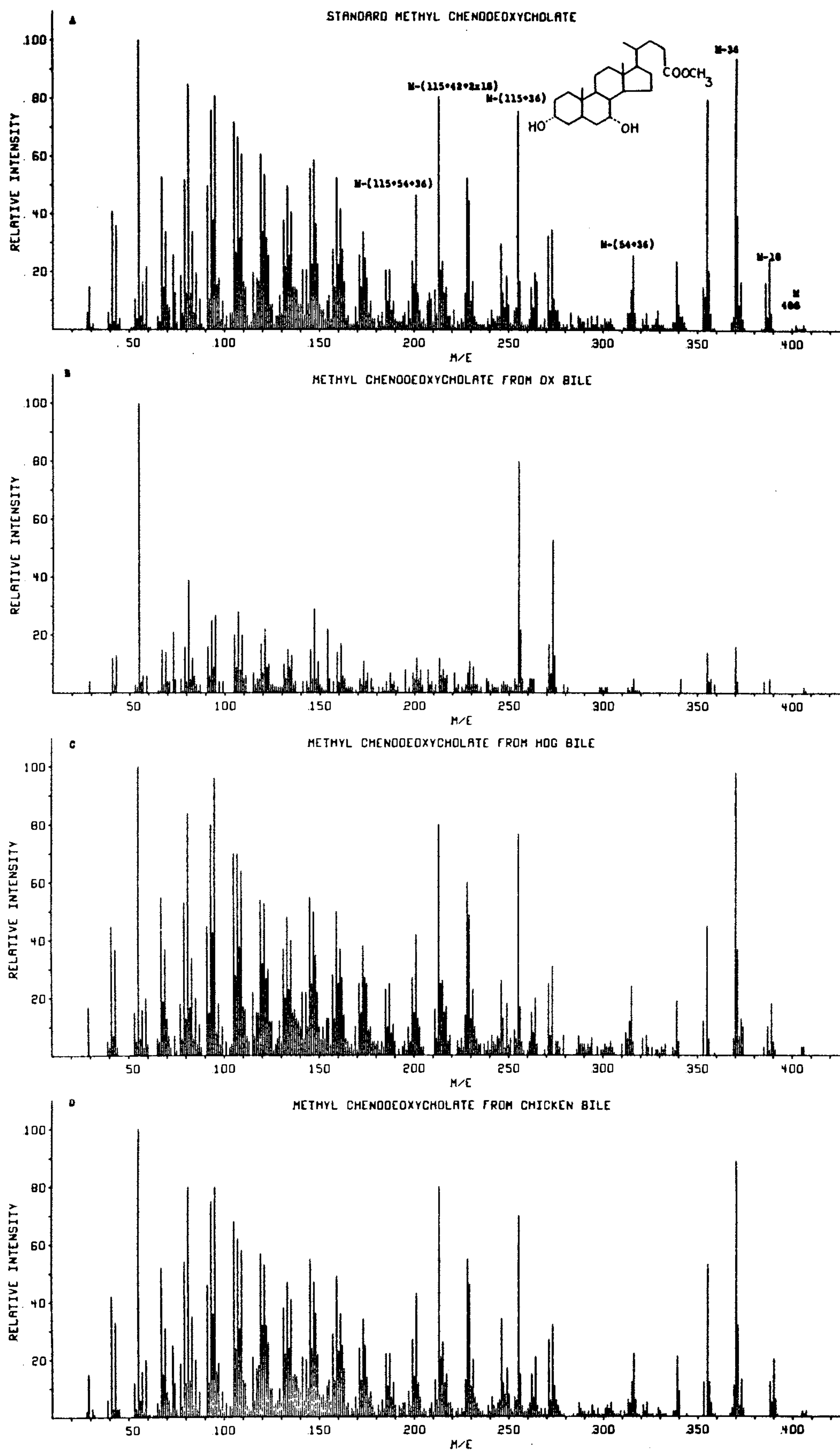
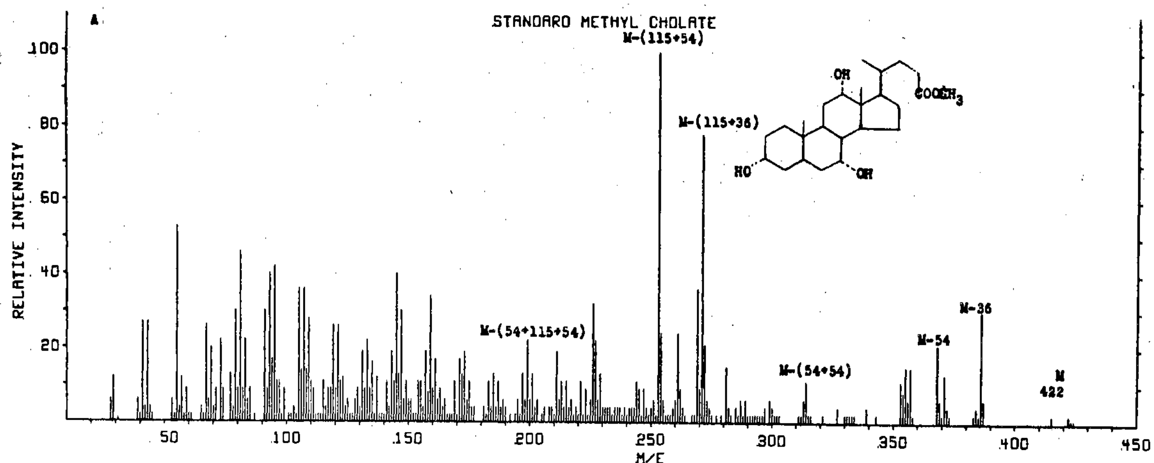

A
STANDARD METHYL CHENODEOXYCHOLATE
M-(115+42+2x18)
M-(115+36)
M-(115+54+36)
M-(54+36)
M-36
M-18
M
406
COOCH3
HO
OH
B
METHYL CHENODEOXYCHOLATE FROM OX BILE
C
METHYL CHENODEOXYCHOLATE FROM HOG BILE
D
METHYL CHENODEOXYCHOLATE FROM CHICKEN BILE
RELATIVE INTENSITY
M/E
20
40
60
80
100
50
150
200
250
300
350
400

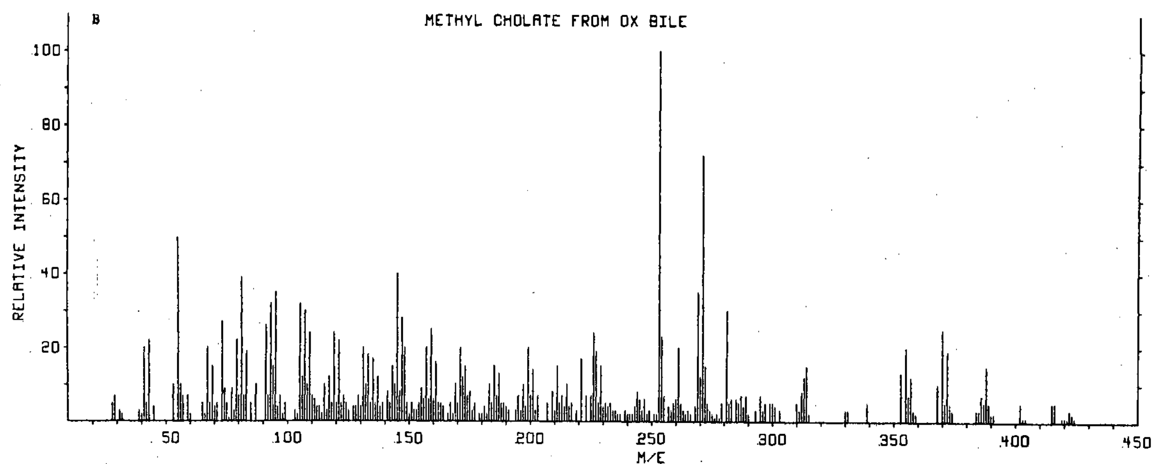
Figure 8. Mass Spectra of Methyl Cholate (Methyl 3α, 7α, 12α-Trihydroxycholanate).

A. Standard methyl cholate.

B. Methyl cholate from ox bile.

C. Methyl cholate from chicken bile.


A
STANDARD METHYL CHOLATE
M-(115+54)
M-(115+36)
M-(54+115+54)
M-(54+54)
M-54
M-36
M
422
OH
COOCH3
HO
OH
RELATIVE INTENSITY
M/E


B
METHYL CHOLATE FROM OX BILE
RELATIVE INTENSITY
M/E

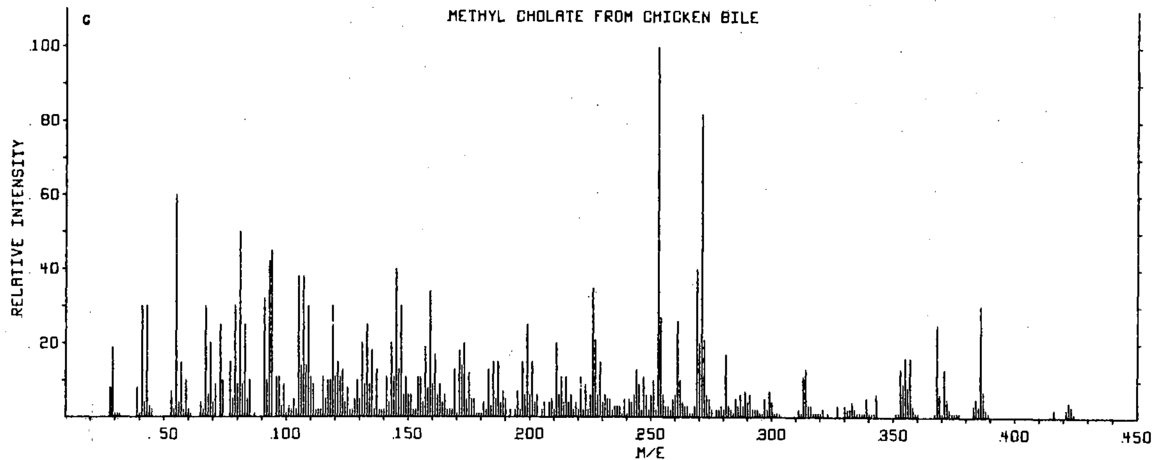

C
METHYL CHOLATE FROM CHICKEN BILE
RELATIVE INTENSITY
M/E

Figure 9, Mass Spectrum of Standard Methyl Ursodeoxycholate (Methyl 3α, 7β-Dihydroxycholanate).

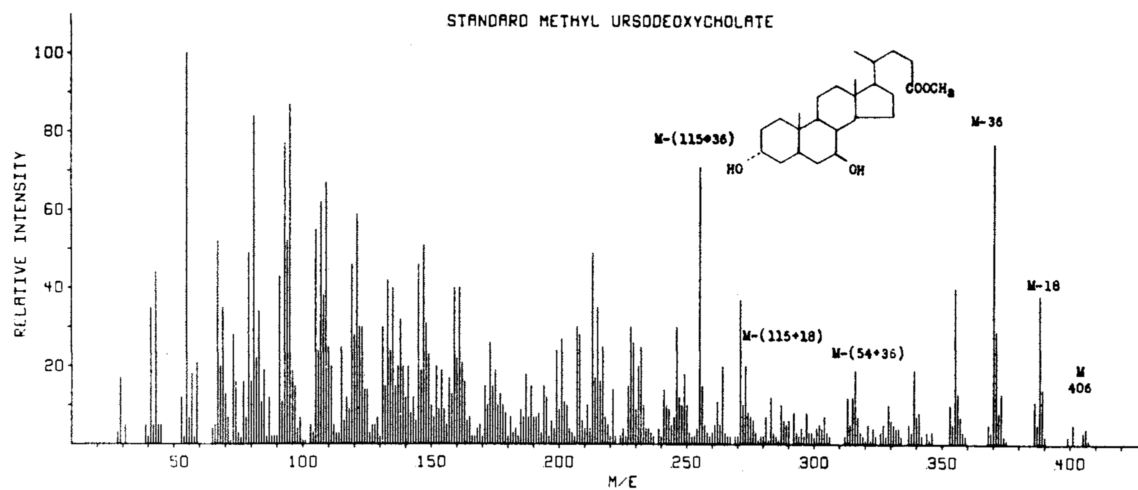

STANDARD METHYL URSODEOXYCHOLATE
RELATIVE INTENSITY
M/E
COOCH3
HO
OH
M-(115+36)
M-36
M-18
M-(115+18)
M-(54+36)
M
406

TABLE VI

BILE ACIDS AS METHYL ESTERS IN OX, HOG AND CHICKEN BILES IDENTIFIED BY TLC, GLC AND GC-MS

No.	Methyl Esters of Bile Acid	Ox Bile			Hog Bile			Chicken Bile		
		TLC	GLC	GC-MS	TLC	GLC	GC-MS	TLC	GLC	GC-MS
1.	3αOH	+[1]	+	+	+	+	+	+	+	+
2.	3αOH, 12=0	+	+							
3.	3αOH, 7=0							+	+	
4.	3αOH, 6=0				+	+				
5.	3=0, 7αOH, 12αOH	+	+							
6.	3αOH, 12αOH	+	+	+						
7.	3αOH, 7αOH	+	+	+	+	+	+	+	+	+
8.	3βOH, 6αOH	+	+		+	+		+	+	
9.	3αOH, 6βOH				+	+				
10.	3αOH, 7βOH									
11.	3αOH, 7=0, 12=0	+	+							
12.	3αOH, 7=0, 12αOH	+	+							
13.	3αOH, 6αOH	+	+	+	+	+	+	+	+	+
14.	3αOH, 7αOH, 6αOH				+	+				
15.	3αOH, 7αOH, 12αOH	+	+	+				+	+	+
16.	3αOH, 6αOH, 12αOH, 23αOH, 5α							+		

[1]+ refers to a positive identification

CHAPTER V

SUMMARY

Bile acids of ox, hog and chicken biles were extracted with ethanol, and hydrolyzed with 15% sodium hydroxide in a nickel crucible at 115°C and 15 psi for 10 hours.

The hydrolyzed bile acids were separated and identified as their methyl ester derivatives. Thin layer and gas-liquid chromatography showed the occurrence of lithocholic acid (0.5%), 3α-hydroxy-12-ketocholanic acid (trace), 3-keto-7α, 12α-dihydroxycholanic acid (trace), deoxycholic acid (17%), chenodeoxycholic acid (18%), β-hyodeoxycholic acid (trace), 3α-hydroxy-7, 12-diketocholanic acid (trace), 3α, 12α-dihydroxy-7-ketocholanic acid (trace), α-hyodeoxycholic acid (7%) and cholic acid (54%) in ox bile; lithocholic acid (0.9%), 3α-hydroxy-6-ketocholanic acid (trace), chenodeoxycholic acid (86%), β-hyodeoxycholic acid (trace), 3α-6β-dihydroxycholanic acid (trace), α-hyodeoxycholic acid (13%) and 3α, 6α, 7α-trihydroxycholanic acid (trace) in hog bile; and lithocholic acid (0.2%), 3α-hydroxy-7-ketocholanic acid (trace), chenodeoxycholic acid (77%), β-hyodeoxycholic acid (trace), α-hydeoxycholic acid (0.4%) and cholic acid (23%). Mass Spectrometric studies confirmed the presence of lithocholic acid, deoxycholic acid chenodeoxycholic acid, α-hyodeoxycholic acid, and cholic acid in ox bile; lithocholic acid, chenodeoxycholic acid and α-hyodeoxycholic acid in hog bile; and lithocholic acid, chenodeoxycholic acid, α-hyodeoxycholic acid and cho-

lic acid in chicken bile.

The finding of α- and β-hyodeoxycholic acids in the chicken and ox biles has not been reported previously. However, the occurrence of β-hyodeoxycholic acid in these two species was not confirmed by mass spectrometry, although it was shown by thin-layer chromatography and gas-liquid chromatography. A good mass spectrum of this bile acid was not obtained due to the small amount in the two animals.

Part of the material from this thesis is being published in Lipids (73).

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