*Journal of Chromatography*, 347 (1985) 155–162
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 048

# BILE ACIDS

# LXXVI. ANALYSES OF BILE ACIDS AND STEROLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A MICROBORE COLUMN

PING H. PATRICK and WILLIAM H. ELLIOTT*
*Edward A. Doisy Department of Biochemistry, St. Louis University, School of Medicine, St. Louis, MO 63104 (U.S.A.)*
(Received June 26th, 1985)

## SUMMARY

The mobilities of several free and conjugated 5β-bile acids, cholesterol and analogues, and α,β-unsaturated sterols and steroidal acids have been investigated with a microbore reversed-phase high-performance liquid chromatographic column (50 cm × 1 mm I.D., 12% $C_{18}$) with appropriate solvent mixtures at flow-rates of 50–100 μl/min and a UV monitor set at 193, 198, 212, or 243 nm. With a solvent mixture of 2-propanol–10 m*M* phosphate buffer, pH 7.0 (160:340) bile acids or their conjugates were separated in a manner similar to those by μBondapak columns (10% $C_{18}$). The lower detection limit of the conjugates was 20 pmoles with the UV detector set at 193 nm, whereas the lower limit for α,β-unsaturated keto sterols or steroidal acids was 5 pmoles at 243 nm. The detection limit for cholesterol with the UV monitor at 198 nm was 10 pmoles. Contributions of substituent groups of sterols to their time of elution (capacity factor) were calculated for several substituted 4-cholesten-3-ones.

## INTRODUCTION

Micro high-performance liquid chromatography (HPLC) has now received considerable attention[1–7]. Microcolumns (0.5–2.0 mm I.D.) are capable of higher chromatographic efficiency and considerably reduced consumption of the mobile phase[8–10]. Because of the small diameter of microcolumns, special modifications are required for the HPLC instrument[11–13]. The volume of injector and the detector cell must be reduced, and the pump must deliver with accuracy a flow-rate as low as 10 μl/min. This report provides results of analyses of free and conjugated bile acids*, unsaturated sterols, and steroidal acids with a microbore column (50 cm × 1 mm,

* Common bile acids are derivatives of 5β-cholan-24-oic acid with hydroxyl substituents; their trivial names and orientations of the hydroxyl groups are: cholic acid (C), 3α,7α,12α-$(OH)_3$; chenodeoxycholic acid (CDC), 3α,7α-$(OH)_2$; ursodeoxycholic acid (UDC), 3α,7β-$(OH)_2$; deoxycholic acid (DC), 3α,12α-$(OH)_2$; lithocholic acid (LC), 3α-OH.

0021-9673/85/$03.30 

I.D.) and a modified Waters HPLC system. A preliminary account of some of these results has appeared[14].

## EXPERIMENTAL

A Waters Model 6000A pump was coupled with a Waters electronic flow splitter (Series 1-83-08013) to achieve flow-rates of 10 to 90 μl/min. A Waters Model U6K injector was fitted with a 10-μl injection loop, which was attached to the inlet of a stainless-steel microbore column (HR SM-50-$C_{18}$, C-M Laboratories, Nutley, NJ, U.S.A., 50 cm × 1 mm I.D. × 1.59 mm O.D., 10-μm particle size, 12% carbon). The outlet was connected to a UV detector (Schoeffel-Kratos Model 770), which was modified as follows: (a) the 8-μl cell was replaced with a 0.5-μl cell, (b) two resistors in the Signal Electronics Section (R60 and R67) were changed to 1 kΩ, 1/8 W, 1% metal film for faster response times, (c) the heat exchanger was removed, and (d) a 15-cm section of 0.23-mm I.D. stainless-steel tubing was connected to the exit port of the detector cell to reduce the formation of air bubbles. A Waters Data Module 730 system was used for recording the chromatograms.

Samples for HPLC were prepared in methanol; 1 μl of sample was injected into the system. The column was washed with methanol–2-propanol–water (1:1:1) continuously at a rate of 10 μl/min when not in use and was regenerated with 100% methanol before analyses. All organic solvents were Fisher HPLC grade. Distilled water was deionized and purified with a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Mobile phase was filtered through a disc of filter paper (47 mm diameter, 0.45 μm porosity, Schleicher and Schuell, Keene, NH, U.S.A.) and degassed before use[15].

Purified bile acids were available from stock[16]; conjugated bile acids were obtained from Calbiochem (La Jolla, CA, U.S.A.). Purified samples of cholesterol, campesterol, and β-sitosterol were at hand[17]; stigmasterol was obtained from Sigma (St. Louis, MO, U.S.A.). 6β-Hydroxy-4-cholesten-3-one, 5-cholesten-7-on-3β-ol acetate, 5,22-cholestadien-24-ethyl-7-on-3β-ol acetate, 4,6,22-cholestatrien-3-one, and 4,22-cholestadien-3-one were products of Steraloids (Wilton, NH, U.S.A.). 4-Cholesten-24-methyl-3-one, 4,22-cholestadien-24-ethyl-3-one, and 4-cholesten-24-ethyl-3-one were prepared from campesterol, stigmasterol, and β-sitosterol, respectively, by known procedures. 7α,12α,25-Trihydroxy-4-cholesten-3-one, 7α,25-dihydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, 7α-hydroxy-4-cholesten-3-one, 4-cholesten-3-one, 3-oxo-7α,12α-dihydroxy-4-cholenate, 3-oxo-7α,12α-dihydroxy-4-cholene-24-carboxylate, 3-oxo-7α-hydroxy-4-cholenate, and 3-oxo-7α-hydroxy-4-cholene-24-carboxylate were prepared for other purposes[18].

Cholic (C), chenodeoxycholic (CDC), deoxycholic (DC) and lithocholic (LC) acids were chromatographed with a solvent mixture of 2-propanol–10 m*M* phosphate buffer, pH 7.0 (160:340) at a flow-rate of 50 or 80 μl/min with the UV monitor set at 193 nm, range 0.01 or 0.02[15]. Glycine (G) and taurine (T) conjugates were analyzed in the same manner. The UV detector was set at 212 nm for samples of cholesterol and analogues, and at 243 nm for α,β-unsaturated sterols and steroidal acids. Methanol or a mixture of 94% methanol–methylene chloride (300:12) was used for the free sterols at a flow-rate of 90 μl/min. α,β-Unsaturated sterols were eluted with acetonitrile–methanol–water (30:30:10) at a flow-rate of 90 μl/min. Unsaturated acids

were eluted with the solvent system 2-propanol–10 m*M* phosphate buffer, pH 7.0 (130:370) at a flow-rate of 50 μl/min.

## RESULTS AND DISCUSSION

Although these studies were undertaken to evaluate the performance of a microbore column and to ascertain the lower limits of detection of bile acids (free and conjugated) and a series of unsaturated sterol derivatives, additional useful information can be derived from these results. From Table IA it is clear that separations of four common bile acids were achieved with a 50 cm microbore column (1 mm I.D.) packed with $C_{18}$ silica (12% carbon loading, 10-μm particles), much as they were with a μBondapak column (300 × 3.9 mm I.D.) with 10% carbon loading[19]. The capacity factors (*k'*) of these compounds differ for the two columns, but the relative capacity factors (*rk'*)[16] are similar.

With the same solvent system (2-propanol–10 m*M* phosphate buffer, pH 7.0 (160:340) for glycine and taurine conjugates of these bile acids, neither column was able to separate GC from TC, or GDC from TDC (Table IB). The lower carbon content of the μBondapak column afforded a separation of GCDC and TCDC, but the 12% carbon packing of the microbore column was unable to effect this separation under these conditions. Separations of conjugated bile acids by HPLC are achieved with comparable columns and solvent systems, but at lower pH[19]. A separation of ten well defined peaks of the glycine and taurine conjugates of C, CDC, DC, UDC, and LC was recently reported[20] using Nova radial compression cartridges ($C_{18}$, 5-

TABLE I

CAPACITY FACTORS (*k'*) AND RELATIVE CAPACITY FACTORS (*rk'*) OF FREE BILE ACIDS (A) AND CONJUGATED BILE ACIDS (B)

Microbore column: 50 cm × 1 mm I.D., $C_{18}$, 10-μm particle size 12% carbon loading; detector, Schoeffel UV at 193 nm, 0.5 μl cell; mobile phase, 2-propanol–10 m*M* phosphate, pH 7.0 (160:340); flow-rate, 80 μl/min at 1900 p.s.i. μBondapak column: 30 cm × 3.9 mm I.D., $C_{18}$, 10-μm particle size, 10% carbon loading; detector, Waters 401 differential refractometer, 10-μl cell; mobile phase, 2-propanol–10 m*M* phosphate, pH 7.0 (160:340); flow-rate, 1 ml/min at 1200 p.s.i.

| | *Microbore* | | *μBondapack* | |
|---|---|---|---|---|
| | *k'* | *rk'* | *k'* | *rk'* |
| *A. Free bile acids* | | | | |
| C | 0.70 | 0.34 | 1.15 | 0.35 |
| CDC | 1.70 | 0.83 | 2.77 | 0.80 |
| DC | 2.05 | 1.00 | 3.25 | 1.00 |
| LC | 4.71 | 2.30 | 7.10 | 2.15 |
| *B. Conjugated bile acids* | | | | |
| GC | 0.72 | 0.38 | 1.06 | 0.37 |
| GCDC | 1.58 | 0.84 | 2.13 | 0.73 |
| GDC | 2.02 | 1.07 | 2.88 | 0.99 |
| TC | 0.73 | 0.39 | 1.04 | 0.36 |
| TCDC | 1.51 | 0.80 | 2.47 | 0.86 |
| TDC | 1.89 | 1.00 | 2.90 | 1.00 |

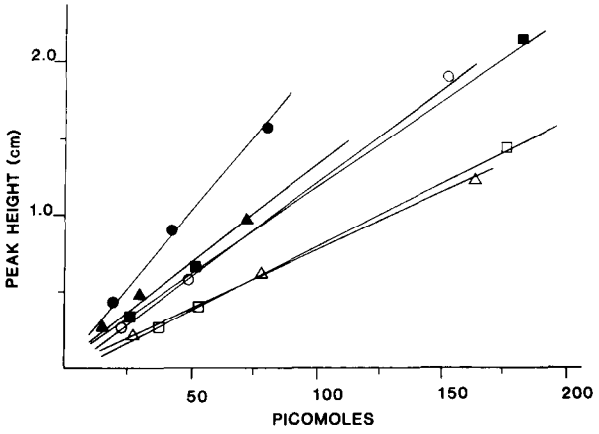


Fig. 1. Quantitation of conjugated bile acids. Conditions: A microbore column (50 cm × 1 mm I.D. packed with $C_{18}$ coated silica, 12% carbon, 10-$\mu$m particles) was used with a solvent system of 2-propanol–10 m$M$ phosphate buffer, pH 7.0 (160:340) at a flow-rate of 80 $\mu$l/min with a Schoeffel UV monitor (0.5 $\mu$l cell) set at 193 nm. ● = GC; ○ = TC; ▲ = GCDC; △ = TCDC; ■ = GDC; □ = TDC.

$\mu$m particles, Waters) and a solvent system of acetonitrile–2-propanol–50 m$M$ phosphate buffer, pH 3.0 (90:110:320). The conjugates of lithocholate were not included in the present study because of their prolonged elution time[15,16]. The lower limit of detection (signal-to-noise ratio, 2:1) of these conjugates with the microbore column was found to be about 20 pmoles (Fig. 1), 100 times smaller than previously reported[19].

In order to ascertain the effectiveness of this microbore column for separation of free sterols, cholesterol and several of its homologues were studied (Table II) in two solvent systems: (A) 100% methanol, and (B) 94% methanol–methylene chloride (300:12). Solvent system B retained the four sterols longer and provided a poorer separation than did solvent A. The lower limit of detection of cholesterol with System A and the UV detector at 212 nm (range 0.01) was 40 ng or about 100 pmole. With the UV detector set at 198 nm (range 0.01), the lower limit of detection was 4 ng or about 10 pmoles. Relative capacity factors (Table II) can be compared to $\alpha_c$ values reported by DiBussolo and Ncs[21] using a $\mu$Bondapak $C_{18}$ column (300 × 3.9 mm I.D., 10% C loading, Waters), acetonitrile as solvent, and a UV detector set at 208 nm. The $\alpha_c$ values are the ratios of $k'$ of the test sterol to the $k'$ of cholesterol, and

TABLE II

CAPACITY FACTORS FOR CHOLESTEROL AND HOMOLOGUES

Conditions: (A) 100% methanol; flow-rate 100 $\mu$l/min at 600 p.s.i.; UV detector 212 nm. (B) 94% Methanol–methylene chloride (300:12); flow-rate 90 $\mu$l/min at 700 p.s.i.; UV detector 212 nm.

| | $k'_A$ | $rk'_A$ | $k'_B$ | $rk'_B$ |
|---|---|---|---|---|
| Cholesterol | 1.82 | 1.00 | 9.16 | 1.00 |
| Campesterol | 2.26 | 1.24 | 9.80 | 1.07 |
| $\beta$-Sitosterol | 2.53 | 1.39 | 11.35 | 1.24 |
| Stigmasterol | 2.09 | 1.15 | 10.29 | 1.12 |

are therefore identical in meaning to $rk'$ (ref. 15). Despite the differences in solvents and carbon loading between the microbore column (12%) and the μBondapak column, the $rk'_A$ and $\alpha_c$ values for β-sitosterol (1.39 and 1.36) and stigmasterol (1.15 and 1.18) agree well; the disparity in the values for campesterol (1.24 and 1.16) may be related to purity of the sample. Similarly, DiBussolo and Nes calculated the contribution of a particular functional group to the retention time as $\sigma$, the ratio of $k'$ values of the sterol with the feature to the sterol without the feature. For the double bond at C-22, they reported $\sigma = 0.87$ (the ratio of $k'$ values of stigmasterol to β-sitosterol); the ratio from $k'_A$ values in Table II is 0.83. For the ratio of $k'$ values of β-sitosterol to campesterol their data provide $\sigma = 1.17$; from Table II the value is 1.12. As expected, these data confirm that homologues of cholesterol with $C_9$ or $C_{10}$ side chains are eluted later than the $C_{27}$ sterol[16]. With 100% methanol stigmasterol, the $\Delta^{22}$-$C_{24}$-ethyl sterol, was eluted before campesterol, the $C_{24}$-methyl sterol. With acetonitrile and the μBondapak column this was not the case ($k'$ values of 1.18 and 1.16, respectively)[21].

A series of α,β-unsaturated sterols was investigated (Table III) using the microbore column with the solvent system acetonitrile–methanol–water (30:30:10) at a flow-rate of 90 μl/min and the UV detector set at 243 nm. Under these conditions (UV detector at range 0.01), the lower limit of detection of 7α-hydroxy-4-cholesten-3-one was 2 ng or 5 pmoles. The solvent system is not an ideal system; it was used primarily to afford elution of the 13 sterols, although this required 137 min for elution of the least polar substance, 4-cholesten-24-ethyl-3-one. Fig. 2 shows a separation of the first 10 α,β-unsaturated sterols. Two of the sterols are 3β-acetoxy derivatives with the α,β-unsaturated moiety present as a 5-en-7-one; all others are derivatives of 4-cholesten-3-one. The latter materials were eluted in a predictable manner: the most polar sterol (the 7α,12α,25-trihydroxy derivative) was followed by the two dihydroxy analogues (7α,25- and 7α,12α-diols), the monohydroxy derivatives (the 7α- and 6β-ols), the unsaturated $\Delta^{4,6,22}$-triene, and $\Delta^{4,22}$-diene, the parent 4-

TABLE III

CAPACITY FACTORS OF SOME UNSATURATED STEROLS

Conditions: acetonitrile–methanol–water (30:30:10); flow-rate 90 μl/min at 500 p.s.i.; UV detector at 243 nm.

| *Compound* | $k'$ | $rk'$ |
|---|---|---|
| 7α,12α,25-Trihydroxy-4-cholesten-3-one | 0.81 | 0.14 |
| 7α,25-Dihydroxy-4-cholesten-3-one | 1.42 | 0.24 |
| 7α,12α-Dihydroxy-4-cholesten-3-one | 2.47 | 0.42 |
| 7α-Hydroxy-4-cholesten-3-one | 5.91 | 1.00 |
| 6β-Hydroxy-4-cholesten-3-one | 11.08 | 1.87 |
| 5-Cholesten-7-on-3β-ol acetate | 12.71 | 2.15 |
| 5,22-Cholestadien-24β-ethyl-7-on-3β-ol acetate | 15.84 | 2.68 |
| 4,6,22-Cholestatrien-3-one | 21.93 | 3.71 |
| 4,22-Cholestadien-3-one | 25.10 | 4.25 |
| 4-Cholesten-3-one | 26.69 | 4.52 |
| 4-Cholesten-24-methyl-3-one | 31.00 | 5.25 |
| 4,22-Cholestadien-24-ethyl-3-one | 35.20 | 5.96 |
| 4-Cholesten-24-ethyl-3-one | 37.80 | 6.40 |

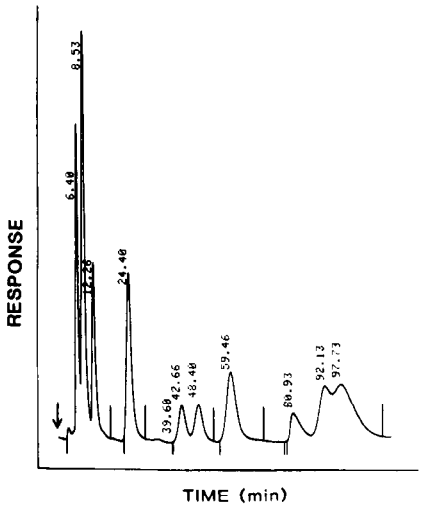


Fig. 2. Separation of α,β-unsaturated sterols and acetates. The column was that described in Fig. 1; the solvent system was acetonitrile–methanol–water (30:30:10) at a flow-rate of 90 μl/min with the UV detector set at 243 nm. The compounds identified by time of elution are: 7α,12α,25-trihydroxy-4-cholesten-3-one, 6.40; 7α,25-dihydroxy-4-cholesten-3-one, 8.53; 7α,12α-dihydroxy-4-cholesten-3-one, 12.26; 7α-hydroxy-4-cholesten-3-one, 24.40; impurity, 39.60; 6β-hydroxy-4-cholesten-3-one, 42.66; 3β-acetoxy-5-cholesten-7-one, 48.40; 3β-acetoxy-5,22-cholestadien-24-ethyl-7-one, 59.46; 4,6,22-cholestatrien-3-one, 80.93; 4,22-cholestadien-3-one, 92.13; 4-cholesten-3-one, 97.73.

cholesten-3-one and its 24-alkylated homologues (Table III). The sterol (4,22-cholestadien-24-ethyl-3-one) derived from stigmasterol was eluted before 4-cholesten-24-ethyl-3-one (derived from β-sitosterol) as was the case with these analogues of cholesterol (Table II). The $\sigma$ value calculated from data in Table III was 0.93, the contribution due to the C-22 double bond.

If the *rk'* of 4-cholesten-3-one is arbitrarily set to unity, the α values[21] for the three analogues eluted later are: 4-cholesten-24-methyl-3-one (1.16), 4,22-cholestadien-24-ethyl-3-one (1.32), and 4-cholesten-24-ethyl-3-one (1.42). Upon comparison of these data with the published values for the analogous 3β-hydroxy-5-ene sterols[21], the first of these values (1.16) agrees exactly with the published value, and the second and third differ by 0.14 and 0.06, respectively. The latter two peaks were distorted (broad base, lengthy tailing, and a wide rounded peak), probably resulting from a failure of the integrator to locate the peak properly. The method, however, shows potential for use with any standard if sufficient analogues of that standard are at hand.

The elution of 6β-hydroxy-4-cholesten-3-one after 7α-hydroxy-4-cholesten-3-one may appear unusual. From an extensive study of a number of bile acids[16] the *rk'* values for the 3α,6β-diols of the 5β- (0.24) and 5α-series (0.33) were found to be smaller than *rk'* values of the 3α,7α-diols of the 5β- and 5α-series (0.80 and 0.82, respectively). The axial 6β-hydroxyl group projects upward into the β-face of either the 5β- or 5α-series, somewhat like the equatorial 7β-hydroxyl of ursodeoxycholate (*rk'* 0.34), which is more polar than cholic acid in HPLC. However, in a study of the activity of hepatic microsomal 12α-steroid hydroxylase where 3α,7α-dihydroxy-5α-cholestane was almost as active as 7α-hydroxy-4-cholesten-3-one, we noted the co-

planarity of these two ring systems[22] and demonstrated that the two sterols were coplanar. Thus, it appears more reasonable to compare the mobility of 6β-hydroxy-4-cholesten-3-one with the corresponding 7α-hydroxy-3-oxo-4-cholenic acid (*rk'* 0.27)[16] rather than with the saturated 3α,7α-dihydroxy-5β- or 5α-cholanates.

Since only the 7α- and 6β-monohydroxy $C_{27}$ α,β-unsaturated sterols were available, it was not feasible to calculate the contribution of individual functional groups to the mobility of the parent sterol nucleus in order to predict mobility in HPLC, as has been done with the bile acids[16,19]. For calculation of *rk'* of the 13 sterols in Table III, 7α-hydroxy-4-cholesten-3-one was chosen arbitrarily as the standard in order to reduce the values of those compounds eluted later. A less polar system should be used for HPLC of 4-cholesten-3-one, and the $\Delta^6$- and $\Delta^{6,22}$-analogues and their 24-methyl and 24-ethyl homologues. The two 3β-acetoxy-$\Delta^5$-7-ones should be studied similarly.

The $C_{24}$ and $C_{25}$ acid analogues of the above five α,β-unsaturated sterols were studied with a solvent system of 2-propanol–10 mM phosphate buffer, pH 7.0 (130/370) a flow-rate of 50 μl/min with the UV detector set at 243 nm (Table IV). The *rk'* values are based on the *k'* of 7α-hydroxy-3-oxo-4-cholenic acid; note that the components of the solvent system are those used for the bile acids (Table I), but the mixture is more polar. Again, the dihydroxy acids were eluted prior to the monohydroxy acids, and the $C_{24}$ derivatives preceded the $C_{25}$ analogues in elution. The sigma values for the contribution of the 12α-hydroxyl group calculated according to DiBussolo and Nes[21] are 0.23 (A/C) and 0.18 (B/D), and for the 7α-hydroxyl group (C/E) is 0.37. The minimum detectable quantity of 7α-hydroxy-3-oxo-4-cholenic acid was found to be 5 pmoles, in agreement with the α,β-unsaturated sterols.

TABLE IV

CAPACITY FACTORS OF UNSATURATED STEROIDAL ACIDS

Conditions: 2-propanol–10 m*M* phosphate buffer, pH 7.0 (130:370); flow-rate 50 μl/min at 1500 p.s.i.; UV detector at 243 nm.

| *Compound* | | *k'* | *rk'* |
|---|---|---|---|
| A | 3-Oxo-7α,12α-dihydroxy-4-cholenate | 0.31 | 0.23 |
| B | 3-Oxo-7α,12α-dihydroxy-4-cholene-24-carboxylate | 0.52 | 0.39 |
| C | 3-Oxo-7α-hydroxy-4-cholenate | 1.33 | 1.00 |
| D | 3-Oxo-7α-hydroxy-4-cholene-24-carboxylate | 2.93 | 2.20 |
| E | 3-Oxo-4-cholenate | 3.60 | 2.71 |

These studies support the reported effectiveness of a microbore column for HPLC separations in this instance of small quantities of bile acids, free or conjugated, cholesterol and homologues, α,β-unsaturated sterols, and steroidal acids, and provide supporting data for contributions of functional groups to the mobilities of certain sterols. Under the conditions used, the minimum detectable quantities with a UV detector are: conjugated bile acids, 20 pmoles, 3-oxo-$\Delta^4$-derivatives, 5 pmoles, and cholesterol, 10 pmoles.

ACKNOWLEDGEMENTS

These studies were supported by U.S.P.H.S. Grant HL-07878. We gratefully acknowledge the advise of Mr. Vincent Caniglione, Kratos, for details of modification of the UV detector and installation of the micro cell, the electronic assistance of Curtis Dismore and Ronald Paulick, and the assistance of Drs. S. V. Hiremath, M. B. Mattammal and M. J. Joyce in the synthesis of the series of hydroxylated analogues of 7α-hydroxy-4-cholesten-3-one.

REFERENCES

1 B. A. Stuart, *Anal. Chem.*, 56 (1984) 1031A.
2 V. L. McGuffin, *LC, Liq. Chromatogr. HPLC Mag.*, 2 (1984) 282.
2 R. F. Meyer, R. J. Gaydosh and R. A. Hartwick, *LC, Liq. Chromatogr. HPLC Mag.*, 1 (1983) 298.
4 F. J. Yang, *J. Chromatogr.*, 236 (1983) 265.
5 R. P. W. Scott, P. Kucera and M. Munroe, *J. Chromatogr.*, 186 (1979) 475.
6 T. Takeuchi, S. Saito and D. Ishii, *J. Chromatogr.*, 258 (1983) 125.
7 T. Takeuchi and D. Ishii, *J. Chromatogr.*, 253 (1983) 41.
8 R. Majors, *LC, Liq. Chromatogr. HPLC Mag.*, 1 (1983) 146.
9 M. Novotny, *Anal. Chem.*, 53 (1981) 1293A.
10 R. Majors, *LC, Liq. Chromatogr. HPLC Mag.*, 1 (1983) 80.
11 R. Majors, *LC, Liq. Chromatogr. HPLC Mag.*, 1 (1983) 464.
12 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 169 (1979) 51.
13 C. E. Reese and R. P. W. Scott, *J. Chromatogr. Sci.*, 18 (1980) 479.
14 P. Patrick and W. H. Elliott, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 43 (1984) 1903.
15 R. Shaw, J. A. Smith and W. H. Elliott, *Anal. Biochem.*, 86 (1978) 450.
16 R. Shaw, M. Rivetna and W. H. Elliott, *J. Chromatogr.*, 202 (1980) 347.
17 P. M. Hyde and W. H. Elliott, *J. Chromatogr.*, 67 (1972) 170.
18 M. J. Joyce, S. V. Hiremath, M. B. Mattammal and W. H. Elliott, *Steroids* 44, (1984) 95.
19 W. H. Elliott and R. Shaw, in M. P. Kautsky (Editor), *Steroid Analysis by HPLC*, Marcel Dekker, New York, 1981, pp. 1–40.
20 W. H. Elliott and P. H. Patrick, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 44 (1985) 659.
21 J. M. DiBussolo and W. R. Nes, *J. Chromatogr. Sci.*, 20 (1982) 193.
22 M. M. Mui and W. H. Elliott, *Biochemistry*, 14 (1975) 2712.