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SEPARATION AND QUANTITATION OF FATTY ACIDS, STEROLS AND BILE ACIDS IN FECES BY GAS CHROMATOGRAPHY AS THE BUTYL ESTER—ACETATE DERIVATIVES

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SUMMARY

A system allowing the separation and quantitation of individual species of fecal fatty acids, sterols and bile acids in a single chromatographic step is described. The system is based on the butylation of carboxyl groups and acetylation of free hydroxyls of the compounds in fecal lipid extracts, followed by their resolution by temperature-programmed gas chromatography. As the butyl ester-acetate derivatives, fatty acids, sterols and bile acids elute separately and with no overlap on a variety of chromatographic columns, obviating the need for prior separation of each class by thin-layer or column chromatography. All common bile acids, a wide variety of sterols and keto-steroids, as well as saturated and unsaturated fatty acids may be routinely resolved. Quantitation is facilitated by the addition of the internal standards, heptadecanoic acid and nor-deoxycholic acid to each sample. With an automatic sample injector, the rapid assessment of a wide range of potential risk factors for colorectal cancer may be carried out in large numbers of samples.

INTRODUCTION

A possible role of bile acids, sterols and fatty acids in the genesis of large bowel cancer has created great interest in analyses of these classes of compounds in human fecal samples and intestinal content. High levels of fecal bile acids correlate with a high incidence of colon cancer [1,2] and they have been shown to be toxic to the colonic epithelium in animals [3,4]. Reduced bacterial degradation of cholesterol has been suggested to be associated with increased incidence of the disease [5]. Plant sterols, on the other hand, may confer some protective effect on the colonic epithelium [6]. Fecal fatty acid levels are of interest because of their direct toxicity [7] but also as monitors of dietary fat intake or fecal fat content [8]. Clearly, a single method for the quantitation of these potential risk factors in feces or colonic content would be advantageous.

Determination of these classes of compounds typically involves three separate

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analyses. Fatty acids are usually assessed as their methyl esters following prior separation of other compounds [9]. Sterols have been determined by gas chromatography (GC) after removal of the more polar lipids by selective extraction, thin-layer chromatography (TLC) or column liquid chromatography [10,11]. Bile acids have been determined in biological material by GC as the methyl esters [11,12], chromate-oxidized methyl esters [13], methyl ester trimethylsilyl ethers [12,14,15], methyl ester trifluoroacetates (TFA) [12,16], methyl ester acetates [17] and hexafluoroisopropyl–TFA esters [18]. Generally, fecal lipid classes have been separated from each other prior to chromatographic analysis. Methods utilizing selective extraction [14], TLC [17] and ion-exchange chromatography [11,12,15] have been described.

Many of the derivatization reagents used for bile acids also react with sterols and fatty acids to produce material suitable for gas–liquid chromatography but conditions allowing the simultaneous resolution of these "contaminating" compounds have generally not been explored. The simultaneous derivatization and chromatographic resolution of disparate classes of lipid have been used to good advantage in the analysis of plasma lipids [19] and in the analysis of total fecal fatty acids [20] and suggested the feasibility of a similar system for the analysis of fecal total lipids. It was the goal of this research, therefore, to investigate conditions allowing the simultaneous derivatization, separation and quantitation of fatty acids, sterols and bile acids in human fecal samples. We report that useful separations of individual species of lipid may be achieved with packed or capillary GC columns when the compounds are chromatographed as the butyl ester–acetates.

EXPERIMENTAL

Chemicals

All lipid and bile acid standards were purchased from Sigma (St. Louis, MO, U.S.A.) with the exception of nor-deoxycholic acid, which was prepared by Research Plus (Bayonne, NJ, U.S.A.) in greater than 99% purity, coprostanol from Supelco (Oakville, Canada), and cholesterol and stigmasterol from Fluka (Hauppauge, NY, U.S.A.).

Magnesium sulphate and potassium carbonate were purchased from Fisher (Toronto, Canada) and 99% pure dimethylamino pyridine was from Aldrich (Montreal, Canada). All other solvents and reagents were from local suppliers. [24-^{14}C]Deoxycholic acid was purchased from the Radiochemical Centre (Amersham, U.K.).

Preparation of fecal lipid extracts

The method used is a modification of that of De Wael et al. [21]. To 100 mg of lyophilized human feces was added 1 ml of methanol containing 1 mg each of heptadecanoic acid and nor-deoxycholic acid, followed by 2 ml of 2.5 *M* sodium hydroxide. Nitrogen was blown over the sample, the tube was sealed and the mixture heated at 120°C for 2 h. After cooling the tube, the sample was acidified to pH 1 with concentrated hydrochloric acid and the lipids were extracted with

three 6-ml volumes of trichloromethane–methanol (2:1). If the extracts were to be stored for long periods, they were passed through a pasteur pipette containing ca. 1 g of a mixture of magnesium sulphate–potassium carbonate (1:1) to remove water and neutralize any residual acid. Otherwise, they were dried under a nitrogen stream at 40°C prior to derivatization.

In some cases, fecal supernatants were also analysed. These were obtained by centrifuging fresh feces for 120 min at 15 000 *g* at room temperature. A 2-ml sample of the supernate was taken for analysis and treated as the freeze-dried feces with the exception that only 0.2 mg of each internal standard was used instead of 1.0 mg.

Preparation of butyl ester–acetate derivatives

Butylation was carried out essentially as described by Marai and Kuksis [22]. A 1-ml volume of a solution of 6% sulphuric acid in *n*-butanol was added to the lipid extract and the sample was sealed under a nitrogen atmosphere and heated at 40°C for 1.5 h. After cooling, 5 ml of trichloromethane were added and the organic layer was washed four times with 1 ml of distilled water. The extract was then passed through a pasteur pipette containing magnesium sulphate–potassium carbonate and evaporated under nitrogen at 40°C.

The dry butyl ester mixture was acetylated in 1 ml of trichloromethane containing 3 mg of dimethylaminopyridine and 0.5 ml of acetic anhydride. The tube was sealed under nitrogen and heated at 40°C overnight. The butyl ester–acetates were extracted by adding 5 ml of trichloromethane to the cooled sample. The organic phase was backwashed with two 1.0-ml volumes of water and passed through a pasteur pipette containing magnesium sulphate. The dried extract was evaporated under nitrogen and taken up in 2 ml of trichloromethane. Typically, 1 μl was injected into the gas chromatograph.

Gas chromatography

Capillary chromatography was carried out on a Hewlett-Packard 5890 chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) fitted with a split/splitless injector and flame ionization detector. When fitted with either a 15-m DB-5+ column [polymethyl(5% phenyl)siloxane, 0.32 mm I.D., 0.25 μm film thickness, Chromatographic Specialties, Brockville, Ontario, Canada] or a 15-m DB-1701 fused-silica column [polymethyl(50% phenyl)siloxane, 0.25 mm I.D., 0.25 μm film thickness, Chromatographic Specialties], the carrier gas was helium at a head pressure of 275 kPa. The detector make-up gas was also helium. The injector temperature was 300°C and injections were made with the oven at 50°C. The purge vent was off for the first 1 min after injection. The oven was then temperature-programmed to 200°C at 50°C/min. The rate was then reduced to 4°C/min to 285°C, then to 2°C/min between 285 and 320°C. Integration of peak areas was performed by a Hewlett-Packard 3392A integrating recorder. No correction was required for column bleed with either column.

Separations obtained with DB-1701 megabore columns (15 m$\times$0.5 mm I.D., Chromatographic Specialties) were achieved with a Varian 3700 gas chromatograph (Varian Assoc., Sunnyvale, CA, U.S.A.). The instrument possessed a packed

column injector which had been modified to accept megabore columns using a commercially available adaptor (Chromatographic Specialties). A helium flow-rate of 15 ml/min was used with no make-up gas. The column oven was temperature-programmed from 190 to 280°C at 3°C/min after a dwell time of 10 min at the initial temperature.

Packed column profiles were also obtained using a Varian 3700 gas chromatograph. Columns were 2 m×2 mm I.D. nickel packed with 3% SP-2401 (trifluoropropylsiloxane, Supelco). The carrier gas was nitrogen at a flow-rate of ca. 30 ml/min. The injector temperature was 280°C and the samples were injected with the oven temperature at 164°C. The oven was then programmed at 2°C/min to 274°C and held at that temperature for 20 min. Peak areas were determined using a Hewlett-Packard 3390A integrating recorder.

Peak-area correction factors [$F(W)$] were determined using data obtained from mixtures containing known amounts of standard compounds. The factors were calculated as described by Kuksis et al. [19] using the following equation:

$$F(W) = (W_X/W_{I.S})(A_{I.S.}/A_X)$$

W_X and $W_{I.S.}$ are the weights of component X and the internal standard, respectively; A_X and $A_{I.S}$ are the peak areas for component X and the internal standard, respectively.

Gas chromatography–electron-impact mass spectrometry (GC–MS) was carried out on a Hewlett-Packard Model 5985A instrument, equipped with the 15-m DB-1701 fused-silica column described above. The column oven was temperature-programmed between 200 and 280°C at 5°C/min.

RESULTS

Separation of fatty acids, sterols and bile acids

To enhance the resolution between the acidic and non-acidic sterols, we explored a variety of alcohols for the esterification process. Chromatograms of a mixture of fatty acids, sterols and bile acids as the methyl, ethyl and butyl ester–acetates obtained using a DB-5+ non-polar capillary and a more polar packed SP-2401 column are shown in Figs. 1 and 2. On both columns, the fatty acids were well resolved from the steroids and showed a similar resolution based on carbon number and unsaturation. The best resolution of oleate and stearate occurred with the butyl esters on both columns. Neither system provided a useful separation of linoleate from oleate, but in both cases arachidonic was well resolved from other fatty acids. The relative retention times for the fatty acyl species are given in Table I.

On the non-polar system, only the butyl ester–acetates gave complete separation of the bile acids (peaks 7–11, Fig. 1) from the sterols (peaks 5 and 6, Fig. 1). With the polar system, shown in Fig. 2, adequate separation of the two classes was achieved with the methyl ester–acetates, although several non-acidic steroids, such as sitosterol, lanosterol, and the keto-steroids, elute in the bile acid region using this derivative. As seen in Table I, this overlap did not occur with the butyl ester–acetates on either column.

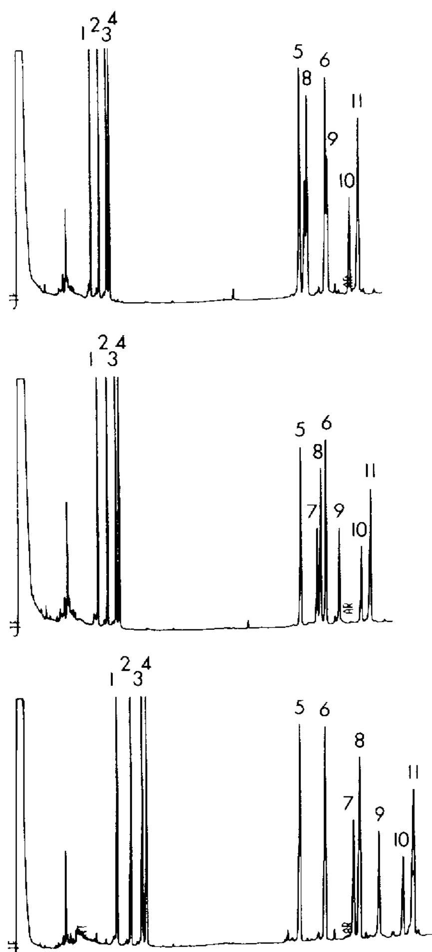

Fig. 1. Resolution of fatty acids, sterols and bile acids by capillary GC as the methyl, ethyl and butyl ester–acetates. A mixture containing equal weights of each component was derivatized as outlined in Experimental using methanol (upper panel), ethanol (middle panel) or *n*-butanol (lower panel) as the esterifying alcohol. The mixture was chromatographed on a 15-m DB-5 fused-silica column as described, using a gas chromatograph fitted with a split/splitless injector and a flame ionization detector. Peaks: 1 = palmitic acid; 2 = heptadecanoic acid; 3 = oleic acid; 4 = stearic acid; 5 = cholesterol; 6 = stigmasterol; 7 = nor-deoxycholic acid; 8 = lithocholic acid; 9 = deoxycholic acid; 10 = cholic acid; 11 = ursodeoxycholic acid.

The resolution of the bile acid species themselves was adequate for many purposes on the non-polar capillary column. All common acids, including deoxycholate, chenodeoxycholate, and ursodeoxycholate, were separated from each other, regardless of the esterifying alcohol. The overall order of elution was more predictable with SP-2401, however, with the monohydroxy, dihydroxy, and trihydroxy acids eluting in sequence. As shown in Table I and the upper panel of Fig. 3, a similar elution order was brought about by both wide- and narrow-bore DB-1701 columns. This was an advantage in the identification of unknown com-

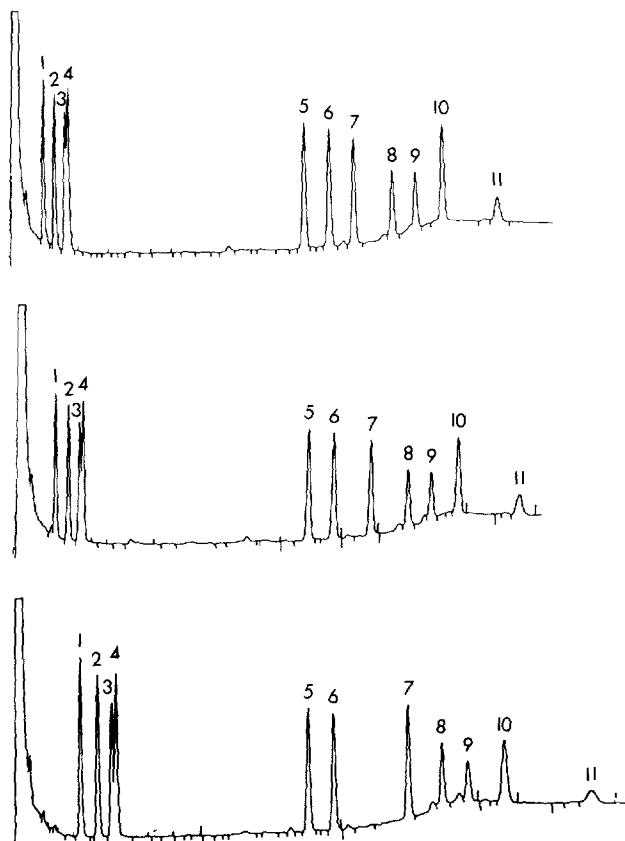

Fig. 2. Resolution of fatty acids, sterols and bile acids by packed column GC as the methyl, ethyl and butyl ester–acetates. A mixture containing the compounds shown in Fig. 1 was derivatized using methanol (upper panel), ethanol (middle panel) or *n*-butanol (lower panel) as the esterifying alcohol. Chromatography was carried out on a 2-m packed column of SP-2401 using a gas chromatograph fitted with a flame ionization detector and an on-column injector. Peaks: 1 = palmitic acid; 2 = heptadecanoic acid; 3 = oleic acid; 4 = stearic acid; 5 = cholesterol; 6 = stigmasterol; 7 = lithocholic acid; 8 = nor-deoxycholic acid; 9 = deoxycholic acid; 10 = ursodeoxycholic acid; 11 = cholic acid.

pounds such as may occur in fecal samples. Nor-deoxycholate was found to be a useful internal standard in all of the chromatographic systems tested, eluting before lithocholate on DB-5 and between lithocholate and deoxycholate on SP-2401 and DB-1701. The complete analysis time of the butyl ester–acetates was ca. 40 min with the capillary systems and ca. 70 min on the packed column.

Recovery and quantitation of compounds

To determine the effect of the butylation and acetylation conditions on the structural integrity of compounds of interest, the lipids listed in Table I were derivatized and chromatographed individually on DB-1701 columns. Using the derivatization conditions described in Experimental, degradation of fatty acids, neutral steroids, and bile acids was negligible. Small amounts of bile acid dehydration products (ca. 5% of the total amount) were formed when the butylation

TABLE I

RELATIVE RETENTION TIMES OF FATTY ACIDS, STEROLS AND BILE ACIDS AS THE BUTYL ESTER-ACETATES

Butyl ester-acetates were chromatographed as described in Experimental. The C.V. in the relative retention times was less than 0.5% for fatty acids, less than 1.3% for the sterols and less than 1.4% for the bile acids on all columns. Baseline separation of compounds was achieved with a difference of 0.02 in the relative retention time with the capillary columns and 0.10 with the packed column.

Compound	Relative retention time			
	DB-5*	DB-1701		SP-2401§
		Capillary**	Megabore***	
Palmitic	0.89	0.89	0.81	0.80
Heptadecanoic	1.00	1.00	1.00	1.00
Stearic	1.13	1.12	1.20	1.23
Oleic	1.09	1.10	1.16	1.17
Linoleic	1.10	1.11		1.24
Nonadecanoic	1.26			1.53
Arachidonic	1.33			1.52
5α-Cholestane	1.79			
Cholesterol	2.36	2.35	3.06	3.60
Campesterol	2.45		3.27	3.95
Sitosterol	2.57		3.43	4.16
Stigmasterol	2.56	2.54	3.27	3.91
Lanosterol	2.65			4.12
5α-Cholestanol	2.38			3.60
Coprostanol	2.28	2.26	2.94	3.53
5α-Cholestan-3-one	2.23			4.04
4-Cholesten-3-one	2.33	2.51	3.32	4.35
5-Cholesten-3-one	2.36			4.32
Nor-deoxycholic	2.79	3.03	3.90	5.27
Lithocholic	2.83	2.96	3.84	4.84
Deoxycholic	2.98	3.29	4.26	5.61
Chenodeoxycholic	3.09	3.50	4.55	5.97
Ursodeoxycholic	3.24	3.70	4.85	6.08
Cholic	3.17	3.86	5.00	7.17

*Capillary, 15 m×0.25 mm I.D.
**15 m×0.25 mm I.D.
***15 m×0.5 mm I.D.
§Packed column, 2 m×2 mm I.D.

reaction was carried out at 70°C for 3 h rather than at 40°C as recommended. The dehydration product of deoxycholic acid eluted in the monohydroxy region as expected. The monounsaturated nature of the compound was confirmed by MS.

Completeness of butylation at 40°C was confirmed by TLC using deoxycholic acid as a model compound. Butylation was essentially complete within 30 min at

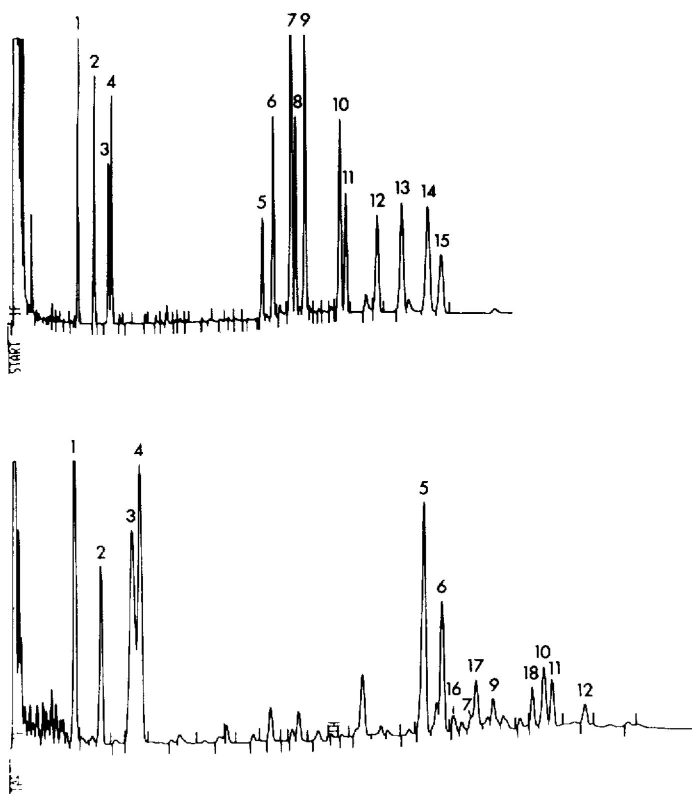

Fig. 3. Resolution of human fecal fatty acids, sterols and bile acids as the butyl ester–acetates on a DB-1701 megabore column. The chromatograms show the resolution obtained with a mixture of standard fatty acids, sterols and bile acids (upper panel) and with human fecal lipids (lower panel) when using a moderately polar, bonded liquid phase. The mixture of standards was derivatized and chromatographed on a 15-m DB-1701 fused-silica megabore column, installed in a Varian 3700 gas chromatograph with flame ionization detectors as described in Experimental. The column was temperature-programmed from 200 to 300°C at 5°C/min. Lyophilized human feces (100 mg) were analysed using DB-1701 as outlined in Experimental. Peak: 1 = palmitic acid; 2 = heptadecanoic acid; 3 = oleic plus linoleic acid; 4 = stearic acid; 5 = coprostanol; 6 = cholesterol; 7 = campesterol; 8 = 4-cholesten-3-one; 9 = sitosterol; 10 = lithocholic acid; 11 = nor-deoxycholic acid; 12 = deoxycholic acid; 13 = chenodeoxycholic acid; 14 = ursodeoxycholic acid; 15 = cholic acid; 16 = 5β-campestanol; 17 = 5β-sitostanol; 18 = unknown.

this temperature. Acetylation of deoxycholic acid under basic conditions was essentially complete after 4 h at room temperature but it was convenient to leave the reaction to proceed overnight. Deoxycholate was used as a model in this case because of the known difficulty in acetylating the hydroxyl at the 12-α position [23]. Extensive degradation of unsaturated fatty acids occurred when acetylation was carried out using the perchloric acid-catalysed method commonly used for bile acids [17].

To further examine the losses incurred during the derivatization process, a standard mixture such as that shown in Figs. 1 and 2 was labelled with [^{14}C] deoxycholate. This species was chosen because it is one of the most difficult

TABLE II

PEAK-AREA CORRECTION FACTORS OF FATTY ACIDS, STEROLS AND BILE ACIDS AS THE BUTYL ESTER-ACETATES

Compound	Peak-area correction factor*					
	DB-5 Capillary		SP-2401 Packed		DB-1701 Megabore	
Palmitic	0.96 ± 0.02	1.82 ± 0.13	0.98 ± 0.02	1.51 ± 0.22	1.04 ± 0.04	2.18 ± 0.20
Heptadecanoic	1.00	1.89 ± 0.13	1.00	1.57 ± 0.22	1.00	2.10 ± 0.13
Stearic	0.98 ± 0.01	1.86 ± 0.10	0.74 ± 0.02	1.29 ± 0.08	0.77 ± 0.02	1.61 ± 0.09
Oleic	0.91 ± 0.03	1.72 ± 0.13	0.71 ± 0.04	1.20 ± 0.07	0.47 ± 0.02	0.99 ± 0.02
Cholesterol	0.89 ± 0.05	1.69 ± 0.03	0.98 ± 0.03	1.53 ± 0.23	0.84 ± 0.03	1.76 ± 0.05
Coprostanol					0.92 ± 0.03	1.92 ± 0.07
Stigmasterol	0.83 ± 0.06	1.57 ± 0.05	0.95 ± 0.04	1.49 ± 0.21	0.76 ± 0.03	1.59 ± 0.04
Cholestan-3-one					0.71 ± 0.01	1.50 ± 0.07
Nor-deoxycholic	0.53 ± 0.03	1.00	0.56 ± 0.13	1.00	0.48 ± 0.03	1.00
Lithocholic	0.85 ± 0.07	1.62 ± 0.06	0.91 ± 0.06	1.48 ± 0.19	0.73 ± 0.03	1.53 ± 0.07
Deoxycholic	0.60 ± 0.06	1.13 ± 0.04	0.54 ± 0.13	0.87 ± 0.08	0.51 ± 0.02	1.06 ± 0.05
Chenodeoxycholic					0.77 ± 0.04	1.55 ± 0.04
Ursodeoxycholic	0.83 ± 0.07	1.57 ± 0.07	0.83 ± 0.05	1.29 ± 0.17	0.73 ± 0.06	1.51 ± 0.06
Cholic	0.38 ± 0.03	0.71 ± 0.03	0.29 ± 0.05	0.61 ± 0.05	0.35 ± 0.03	0.72 ± 0.02

*Peak area correction factors were calculated as described in Experimental. The factors are expressed relative to the internal standards, heptadecanoic and nor-deoxycholic acids. The columns used were as described in Table I.

to extract and acetylate. The sample was then butylated and acetylated and all fractions retained for scintillation counting. We found that 97% of the label remained in the sample after butylation and water washing of the extract. However, 9% of the total label was lost during passage of the extract through the magnesium sulphate–potassium carbonate column. Less than 1% was lost during subsequent acetylation and water washing, but an additional 9% was lost during the passage through magnesium sulphate. The overall recovery, therefore, was 78% of the original radioactivity, with the major losses occurring during the drying and neutralization of the extracts with magnesium sulphate and potassium carbonate.

The losses described above point out the need for the inclusion of internal standards that are closely similar in structure to the compounds of interest. We have chosen to use two standards, heptadecanoic and nor-deoxycholic acids, because of the widely different structures of the compounds of interest. Peak-area correction factors for a representative group of compounds chromatographed on DB-5, DB-1701 and SP-2401 are given in Table II. It is seen that the recoveries of all compounds are generally high, with the possible exception of cholic acid. While most compounds show a recovery of 60–100% of that of the fatty acyl standard on each of the columns, the recovery of cholic acid was typically between 30 and 40%.

To determine which internal standard gave the most reproducible quantitation

of the various classes of compounds, we compared the coefficient of variation [C.V. = (S.D./mean) ×100] of the peak-area correction factors obtained with heptadecanoate and nor-deoxycholate when chromatographed on the DB-1701 megabore column. Standard mixtures such as that shown in the upper panel of Fig. 3 and containing the two internal standards were used for this analysis. The C.V. of the response of fatty acids was 4.1% against heptadecanoate and 5.8% against nor-deoxycholate. For the sterols the variability was 3.1% against the fatty acid standard and 3.4% against the bile acid. The variability in the bile acid response factors was 6.3% against heptadecanoate and 3.8% against nor-deoxycholate. Thus the reproducibility of the quantitation of the fatty acids is higher when expressed relative to the peak area of heptadecanoic acid, while the bile acids are more reliably quantitated against the bile acid standard. The sterols may be quantified against either standard.

A representative profile of lipids prepared from lyophilized human feces and chromatographed on a DB-1701 megabore column is shown in the lower panel of Fig. 3. The identity of peaks indicated in the legend to the figure was confirmed by GC–MS. As with the standard mixtures, the molecular species of most fatty acids, sterols and bile acids were resolved from each other. Butyl linoleate was shown by GC–MS to be present in this sample, overlapping with butyl oleate (peak 3) in this system. The peaks eluting between butyl stearate (peak 4) and coprostanyl acetate (peak 5) were not identified, but their retention times and

TABLE III

REPRODUCIBILITY OF QUANTITATION OF FECAL LIPIDS

Values represent the means ± S.D. of analyses performed on five separate samples of the freeze-dried, human fecal sample used in Fig. 3. Chromatography and sample handling was as described in the legend to Fig. 3 and in Experimental.

Compound	Amount (mg/g of feces)	Coefficient of variability (%)
Palmitic	18.7 ±0.3	1.6
Stearic	14.3 ±1.0	7.0
Oleic, linoleic	24.1 ±3.0	12.4
Cholesterol	5.6 ±0.3	5.4
Coprostanol	11.7 ±1.3	11.1
Campesterol	0.18±0.06	33.3
5β-Campestanol	1.00±0.06	6.0
Sitosterol	3.4 ±0.2	2.9
5β-Sitostanol	2.9 ±0.4	13.0
Lithocholic	3.1 ±0.2	6.5
Deoxycholic	4.9 ±0.4	8.2
Total fatty acid	56.7 ±2.5	4.4
Total sterol	24.8 ±1.8	7.3
Total bile acid	8.3 ±0.9	10.8

MS characteristics were consistent with those of hydroxylated fatty acids. Peak 18 was not identified.

To illustrate the reproducibility of the quantitation of the compounds in this chromatogram, five separate samples of the freeze-dried stool were worked up and chromatographed. The results are shown in Table III. Generally, the coefficient of variability of individual values was less than 13% from the mean. The large uncertainty in the campesterol value is the result of the small size of the peak and its incomplete resolution from sitostanol. The variability of peak areas seen with replicate injections of the same sample was comparable with that shown for separate aliquots, indicating that integrator error is a large component of the reported variability. Coefficients of variability in fecal bile acid quantitations have been reported to be 3.4–5.8% using enzymic methods [21] and 3.3–16.2% using methyl ester, trimethylsilyl ether derivatives and GC [15]. Values of 2.0–3.6% have been reported for the quantitation of sterols in feces using a method based on GC of the sterol trimethylsilyl ethers [10].

DISCUSSION

Methods for the extraction of fecal lipids have been developed by many laboratories [24] and, while the subsequent work-up of the components differs widely, the initial treatment is generally based on a solvent extraction of fresh or lyophilized material. As we were not concerned with retention of intact higher glycerides, we opted for the simplest method, which is based on an alkaline saponification followed by extraction of the acidified hydrolysate [21]. However, the procedures described here may be applied equally well to total extracts derived by other means.

The use of butanol as the esterifying alcohol ensured the complete separation of sterols and keto-steroids from bile acids on a wide variety of columns. This allowed peaks to be identified with much more certainty, and without their prior separation by TLC or column chromatography. As the butyl ester–acetates are readily amenable to GC–MS, structural confirmations are possible using this technique. Using the esterification conditions described, any alcohol giving rise to the appropriate chromatographic separations can be substituted for *n*-butanol and yield quantitative results. For some analyses, as seen in Fig. 2, esterification with methanol or ethanol provided useful separations of sterols and bile acids on the SP-2401 column. With fecal samples, however, several late-running sterols overlap with the bile acid region when using these esters. The extra resolution imparted by the propyl or butyl esters is, therefore, a great advantage. This choice of the higher-molecular-weight alcohols is associated with a small decrease in the chromatographic recovery of cholic acid with some columns, but the use of short megabore columns renders this difference insignificant.

During the course of these investigations, several columns and derivatization combinations were assessed that did not prove suitable for total lipid profiling. Esterification of fecal extracts with hexafluorisopropanol as described by Imai et al. [18] did not bring about useful separations of acidic and non-acidic steroids on DB-5, SP-2401 or DB-1701 columns. We had a similar lack of success with trimethyl silylation or dimethyl *tert.*-butyl silylation of the total lipid extracts.

Chromatography of the butylated and acetylated extracts on a packed FFAP (substituted terephthalic acid) column increased the resolution between the fatty acyl species, but a low upper temperature limit did not allow the elution of the more polar steroids.

For routine analysis of fecal extracts, we have found 10–15 m DB-1701 megabore columns to be the most suitable. The reasons for the superiority of the megabore in our view are five-fold. First, the orderly elution of the sterols, followed by the mono-, di-, and trihydroxy bile acids is important in the identification of compounds in complex biological mixtures. Second, is the large sample handling capacity offered by these columns. Third, megabore columns are readily adaptable for use with an automatic injector using direct on-column injection. The latter is important in the chromatography of compounds of widely differing molecular weights [19]. Fourth, the bonded DB-1701 phase is long lasting and requires little conditioning prior to use. The minimal column bleed at high temperatures allows increased sensitivity over the SP-2401 packed column alternative. Fifth, the megabore system allows the entire chromatogram to be obtained in ca. 40 min, compared with ca. 70 min with packed columns.

Conditions for extraction and derivatization were investigated for their ability to degrade compounds of interest, and although these were minimized, there are deficiencies of which we are aware. Unsaturated fatty acids or ketones were not degraded during the acidic butanolysis, but almost complete loss of unsaturated fatty acids occurred when acetylations were carried out under acidic conditions such as those used earlier for the preparation of methyl ester acetates of bile acids [17]. The basic conditions of Hofle and Steglich [23] were therefore adopted. Methods based on the use of acetyl chloride [20,25] were not investigated.

Some degradation of keto bile acids is known to occur during saponification with sodium hydroxide [14,26]. The saponification also brings about a loss of information on the source of the fatty acids, that is, whether they are derived from triacylglycerol, phospholipids or sterol esters. Similarly, information concerning the conjugation or sulphation of the bile acids is not given by the present technique. There is, furthermore, room for improvement in the resolution of the fatty acyl species. Linoleic acid elutes between oleic and stearic acids on the DB-5 capillary column, but this information is lost on the packed SP-2401 and 15-m megabore systems. The saturates and unsaturates are, however, separated from each other and additional information can be derived from re-chromatography on a more polar column, aided by the stability of the butyl ester–acetate derivative.

Given these deficiencies, these systems provide information concerning the structure and quantity of three biologically important classes of compounds, fatty acids, sterols, and bile acids, in a single chromatographic step. This represents to our knowledge, the first attempt at the development of a chromatographic total lipid profile for intestinal content and feces. The present system was characterized using about two dozen standard compounds that we know represent the major lipids present in human stool. We recognize that there are many more trace components typically present that we have not dealt with in this report but the systems can be expanded to accommodate these if the appropriate standard compounds are available. Similarly, components in hydrolysates prepared by

methods other than that described may be resolved using the systems reported. This may occur, for example, in cases where enzymic hydrolysis of glycerides or conjugated bile acids is preferable to alkaline hydrolysis.

Information provided by the lipid profiling system may be used to assess several hypotheses or risk factors relating to large bowel cancer. For example, the fecal ratios of lithocholic to deoxycholic acids [27] and secondary to primary bile acids [28] have been suggested to be associated with elevated risk of bowel cancer. The ratios of plant sterols to total sterols [6] and degraded to non-degraded cholesterol [5], as well as the presence of coprostanol [29] or keto-steroids [30] may also indicate risk of the disease. These compounds are readily identifiable in the chromatographic systems described here. The information has the potential to allow the estimation of risk of bowel cancer in large numbers of samples of readily available material. We are currently applying this technique in studies of the effect of diet on fecal risk factors, and the levels of these factors in patients with colonic polyps and bowel cancer.

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