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ARTICLES

Simultaneous Characterization of Bile Acid, Sterols, and Determination of Acylglycerides in Feces from Soluble Cellulose-Fed Hamsters Using HPLC with Evaporative Light-Scattering Detection and APCI–MS

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The rapid rise in obesity-related diseases has increased interest in oral and dietary agents that disrupt fat metabolism, resulting in the excretion of dietary lipids in the feces. In this study, a rapid and convenient liquid chromatography method to comprehensively analyze fecal lipids in a single injection was developed. An evaporative light-scattering detector (ELSD) for routine analysis or atmosphere pressure chemical ionization tandem mass spectrometry [(+)APCI–MS/MS] for structural confirmation and peak purity was used. The method was applied to characterize lipid components of feces from hamsters fed high-fat diets with either 5% microcrystalline cellulose or 5% hydroxypropyl methylcellulose (HPMC) fibers, to test the effect of HPMC on lipid metabolism. HPMC is a nonfermentable, soluble cellulose fiber. The fecal lipid components identified using this method includes two secondary bile acids, deoxycholic acid, lithocholic acid, and neutral sterols including cholesterol, coprostanol, stigmastanol, and sitosterol. The profile of fecal lipid components was compared between two groups. It was found that the bile acid excretion was increased 2-fold in HPMC-fed hamsters. More interestingly, diacylglycerides and triacylglycerides were detected in feces from hamsters on HPMC-included high-fat diets. We believe that this is the first report of excretion of acylglycerides following neutral soluble fiber feeding.

KEYWORDS: Fecal fat; bile acids; sterols; acylglycerides; soluble dietary fiber; hydroxypropyl methylcellulose; HPMC; hamster; high-fat diet; obesity

INTRODUCTION

Overnutrition and insufficient physical activity at the end of the 20th century have resulted in an overweight and obese population in most developed countries. As a result, public health agencies and medical associations recommend weight reduction and decreased intake of dietary fat. In recent years, some success at reducing fat and cholesterol bioavailability has been achieved by the use of oral lipase inhibitors (*1*), plant sterols (*2, 3*), and soluble fibers (*4*) that reduce cholesterol absorption. Use of these gastrointestinally active biological agents inevitably results in the characteristic profile of excreted lipid.

It is known that dietary fibers decrease caloric density but increase sterol and bile acid excretion (*4*). For example, it has been demonstrated that one of the soluble dietary fibers (SDFs), psyllium, increases conversion of cholesterol into bile acids to replace bile acids excreted into feces (*4*). The properties of SDF have been known for over 50 years; however, there has not yet been a definitive explanation of the mechanism of the hypocholesterolemic effect of soluble fibers. In addition to psyllium, other water-soluble viscous polysaccharides, such as cereal β-glucans (*5*), guar gum, pectin (*6*), and hydroxypropyl methylcellulose (HPMC) (*7, 8*), are also known to reduce blood cholesterol (*9*). HPMC is a highly purified, well-characterized,

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nonfermentable viscous polysaccharide, which has previously been shown to not be degraded in rats (*10*).

Diets as well as pharmaceutical inhibitors of lipid digestive enzymes can change lipid metabolism, resulting in the altered profiles of fecal lipid components. Specifically, the appearance of triacylglycerides in feces indicates impaired fat absorption by an inadequate bile-acid pool (*11*) or lipolytic activity (*12*) and the type and composition of the dietary fat (*13*). A lipase activity decrease has also been reported in diets containing cereals, wheat bran, and wheat germ (*14*), soybean (*15*), chitosan (*16*), and teasaponin (*17*). Tetrahydrolipstatin is a potent inhibitor of gastric, pancreatic, and carboxylester lipase (*18*), which has proven effective for the treatment of human obesity (*19*).

The major impediment to a convenient and reliable analysis of bile acids has been the difficulties as aresult of their similar yet complex chemical structures, low ultraviolet (UV) and visible light absorbance, low volatility, and low concentration in complex biomatrices. Gas chromatography (GC) in combination with mass spectrometry (MS) has been used for many years and is probably the method of choice for a comprehensive analysis, but this instrumental methodology requires laborious sample derivatization steps before analysis (*20, 21*) and, unlike high-performance liquid chromatography (HPLC), is limited to a single lipid class. HPLC in conjunction with mass spectrometry (LC–MS) has proven to be one of the most powerful and convenient tools, particularly for the characterization of chemical constituents in biological matrices, including the complex profile of secondary bile acids in feces resulting from colonic bacterial metabolism (*22*). Cholesterol and phytosterols in human serum and phytosterols in plants are also typically analyzed by GC and radioisotope methods (*23, 24*) and more recently by HPLC with an electrospray mass spectrometer (ESI–MS). However, the analysis of sterols by ESI–MS is technically challenging because these hydrophobic molecules are not easily ionized through conventional electrospray methods. Generally, atmosphere pressure chemical ionization (APCI) has been found to be more efficient than ESI for the sterol analysis in different samples and matrices (*25, 26*). Triacylglycerides (TAG) from plant oils have also been analyzed by LC–APCI–MS (*27*) because of their importance to nutrition. However, the studies on fecal TAG have only been reported under the conditions with fat malabsorption (*28, 29*). Fecal TAG from dietary sources is usually low or nonexistent in normal humans and animals. To our knowledge, no studies have simultaneously analyzed fecal bile acids, neutral sterols, and acylglycerides, including TAG, diglycerides (DAG), and monoglycerides (MAG).

This study describes a HPLC analytical method for fecal components, including bile acids, neutral sterols, and other lipid compounds, which uses evaporative light-scattering detection (ELSD) or APCI–MS detection. The use of ELSD not only provides the ability to detect nonvolatile compounds but also allows for the use of gradient elution because the baseline remains stable during evaporation of the chromatographic mobile phase. The limit of detection (LOD) of bile acids and neural sterols by ELSD has been demonstrated to be in the nanograms per injection range (*30*).

The utility of this method is demonstrated in the analysis of a study of the effect of a soluble dietary fiber, HPMC, on excreted lipids in feces in Golden Syrian hamsters. This animal model was chosen because of its similarity in sterol and bile acid metabolism to humans (*31, 32*) and is widely used in studies of dietary lipid metabolism.

We believe that this method is highly suitable for routine screening of fecal bile acids and lipid profiles and to determine the effects of diet and changes in lipid absorption and the effects of diet on the profile of fecal components.

MATERIALS AND METHODS

Chemicals. All reference standards were purchased from Steraloids, Inc. (Newport, CT). Deoxycholic acid (DCA) and lithocholic acid (LCA) were used as bile acid standards, and coprostanol, cholesterol, sitosterol, and stigmastanol were used as sterol standards. Hyodeoxycholic acid and 5α-cholestane were used as internal standards. HPLC-grade methanol, isopropyl alcohol, acetonitrile, hexane, ethyl acetate, and water were obtained from Fisher (Somerville, NJ). Sodium hydroxide and hydrochloric acid used for hydrolysis were also purchased from Fisher. HPMC supplied by The Dow Chemical Company (Midland, MI) was a food-grade sample, as defined by the U.S. Pharmacopoeia (USP 27 NF22 S1 for Hypromellose), with viscosity of 100 000 cP. The viscosity refers to a 2% solution, as determined by the USP procedure for cellulose derivatives under the viscosity method.

Animals and Diets. Male Syrian Golden hamsters (*Mesocricetus auratus*) were obtained from Charles River Laboratories (Kingston Colony, Laboratory Area K62, Wilmington, MA). The animals were housed individually in wire-bottom cages in an environmentally controlled room maintained at 20–22 °C and 60% relative humidity. Upon arrival, the animals were fed a ground commercial chow (Purina Rodent Laboratory Chow) and were acclimated to a 12 h alternating light–dark cycle. After 1 week, the hamsters were randomized ($n = 10$) to test diets, which were divided into control and experimental test diets. The test diets contained 5% dietary fiber, 20% casein, 10% butterfat, 8% corn oil, 2% fish oil, choline bitartrate, methionine, vitamin mix, mineral mix, and corn starch (Dyets, Inc., Bethlehem, PA). The control diet contained 5% (w/w) of "inactive fiber" microcrystalline cellulose (MCC) with 20% high fat content (referred to as HF-MCC), whereas the experimental diet contained 5% of the modified cellulose dietary fiber (HPMC) with the same 20% content of fat (referred to as HF-HPMC). Diet feed consumption was measured twice weekly, and the animals were weighed weekly.

Sample Collections and Extraction Processes. The feces were collected during the last 3 days of the study, weighed, and frozen. To avoid the difference caused by intragroup animal–animal variability, the freeze-dried feces from three different hamsters fed the same diet were pooled and weighed to determine the dry weight. The lyophilized feces were ground to produce a homogeneous mixture and stored at −20 °C before the analysis.

A sample of dry feces (0.1 g) was transferred to a sample vial, to which the internal standards were added: 100 μL of hyodeoxycholic acid and 5α-cholestane (final amount to be 400 and 800 μg, respectively), spiked in a mixture of methanol and isopropyl alcohol (1:1). The sample vial was loaded on a Dionex ASE 200 automatic extractor (Dionex Corp., Sunnyvale, CA). Extraction was performed using a 20 mL mixture of hexane and isopropyl alcohol (3:2, v/v, 2% acetic acid) at 2178 psi and 60 °C for 30 min. After extraction, the solvent was evaporated under N_2 stream, the extract was reconstituted in 400 μL of isopropyl alcohol then filtered using a 0.45 mm polytetrafluoroethylene (PTFE) syringe membrane filter, and 40 μL was injected into HPLC.

LC–ELSD Analysis. A reversed-phase HPLC 1100 series system (Agilent Technologies, Palo Alto, CA) equipped with a Luna C18 column (5 μm, 250 × 4.6 mm; Phenomenex, Torrance, CA) were used. Separation was performed using a gradient of two mobile phases: (A) methanol/acetonitrile/water (53:23:24, v/v/v) and (B) isopropyl alcohol (100%). To both phases an appropriate amount of crystalline ammonium acetate was added and mixed to form a 30 mM solution. Solvent A was acidified by the addition of 24 mL of glacial acetic acid in 1 L. A linear gradient from A to B at a flow rate of 0.5 mL/min was performed follows: time of 0–30 min, 8–36% B; time of 30–40 min, 36–50% B; time of 40–100 min, 50–56% B; time of 100–105 min, 56–70% B; time of 105–145 min, 70–88% B; and time of 145–170 min, 88–95.5% B. In all experiments, the columns were re-equilibrated between

injections with the equivalent of 10 mL of the initial mobile phase. The LC effluent was monitored by a ELSD (PL-ELS 1000, Varian, Inc., Amherst, MA) with a nitrogen gas flow of 1.3 L/min and desolvation and evaporation temperatures set to 100 and 120 °C, respectively. Peaks detected by ELSD were further identified with LC–(+)APCI–MS.

LC–(+)APCI–MS Analysis. The same feces were analyzed on a LC system (Surveyor system including a LC pump, autosampler, PDA, Thermo Finnigan, San Jose, CA) using a Luna C18 column (3 μm, 150 × 2.0 mm) that was more compatible with the mass spectrometer. The outlet of the PDA detector of the liquid chromatograph was connected to a Finnigan LCQ quadrupole ion-trap mass spectrometer (Thermo Finnigan, Inc., Waltham, MA) through an APCI source in positive-ion mode. The flow rate was changed to 0.1 mL/min in the LC condition, but the same gradient was applied. The optimized operating parameters of the APCI–MS interface were as follows: vaporization temperature, 400 °C; capillary temperature, 280 °C; capillary voltage, 10 V; corona discharge, 5 μA; sheath N_2 gas flow, 80 arbitrary (instrument) units; aux gas flow, 0 arbitrary units; tube lens offset, 10 V; and ion collection time, 200 ms. The MS experiment was performed first in full scanning mode over a mass range of *m*/*z* 150–2000, followed by the selective ion monitoring for those corresponding to the masses of all possible lipid compounds, including bile acids, neutral sterols, and acylglycerides. Peaks showing *m*/*z* values relevant to bile acids and neutral sterols were further investigated using atmosphere pressure chemical ionization–tandem mass specterometry (LC–APCI–MS/MS). For the MS/MS experiment, parent ions were detected by select ion monitoring (SIM) and fragments generated from parent ions were scanned at the range of 100–400 *m*/*z* for bile acids and neutral sterols. The collision energy was applied as numbers between 35 and 42 [unit, NCE (%)] depending upon the compounds analyzed. No MS/MS experiment were performed for acyglycerides. Data were collected and processed using Excalibur software (version 1.3, Thermo Finnigan, San Jose, CA).

Quantitation of Bile Acids and Sterols by LC–ELSD. Peaks observed by ELSD were identified using APCI–MS by determining the mass to charge ratio (*m*/*z*) in the positive mode. The recovery of bile acids and sterols in feces was determined by a comparison with the recovery of internal standards: hyodeoxycholic acid and 5α-cholestane. Once bile acids and sterols in feces samples were identified by MS, the calibration curves of compounds in hamster feces were generated according to peak areas acquired by ELSD and used to quantitate these compounds in feces samples.

RESULTS AND DISCUSSION

In contrast to gas–liquid chromatographic methods that either require the derivatization of the carboxylic acid and hydroxyl groups prior to their analysis or other methods that require the separation of a single lipid class, such as bile acids or neutral sterols, from biological matrices, the method described here uses a simple solvent blend to simultaneously extract lipid classes: bile acids, neutral sterols, free fatty acids (FFAs), MAG, DAG, and TAG. A mixture of hexane and isopropyl alcohol (3:2) with 2% acetic acid at high temperature and pressure was chosen as the extraction solvent because it has been known to be equivalent to the Folch solvent (*33*), chloroform and methanol mixture (2:1), but presents fewer waste problems (*34*). The extract can be used directly for the analysis of bile acids and lipid in feces, because bile acids in hamster feces are present mainly (75–80%) in the unconjugated form (*20*); therefore, the customary hydrolysis step can be eliminated. However, deoxycholic acid is polymerized in human and hamster feces (*35*), and alkaline hydrolysis is necessary for determination in the feces.

The extraction conditions (temperature, pressure, and time) were chosen to one that results in the highest recovery of internal standards. The recoveries of internal standards were 75.2 ± 1.24 and 70.9 ± 3.43% for hyodeoxycholic acid and 5α-cholestane, respectively (n = 3 for each). These internal standards were chosen because of the similarity in structure to bile acids and neutral sterols, respectively (**Figure 1**). The elution gradient required a dramatic change in polarity, careful consideration of parameters, such as the presence of acidifying additives, and the use of volatile organic modifiers. The use of ammonium acetate to acidify the mobile phase was adapted from bile acid separations described by Torchia et al. (*36*). The addition of ammonium acetate and glacial acetic acid provides the suppression of ionization of carboxylic group ionization in the bile and free fatty acids, improving the peak shape. Gradient blending of isopropanol (solvent B) into the methanol/acetonitrile/water ternary mixture (solvent A) was needed to increase the elution strength for the more hydrophobic lipids, such as DAG and TAG.

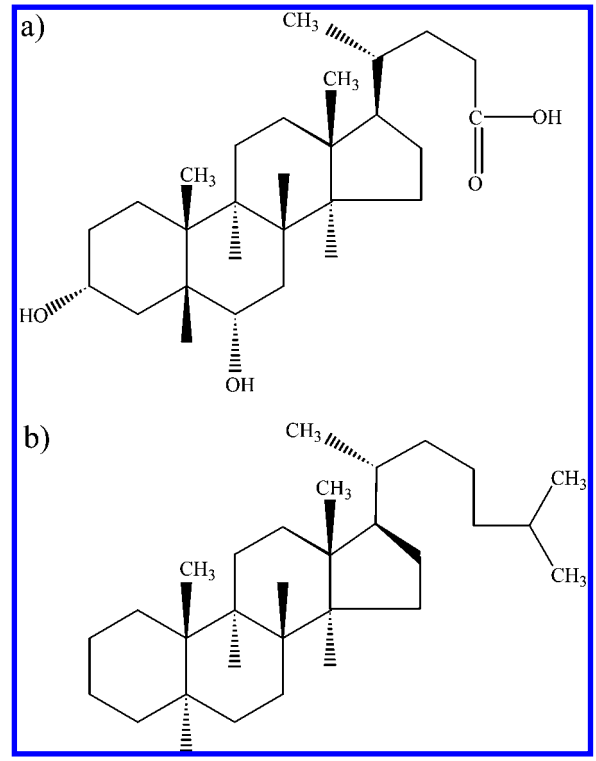

Figure 1. Structure of (a) hyodeoxycholic acid and (b) 5α-cholestane used as internal standards.

Figure 2 illustrates the LC–ELSD chromatogram of a standard mixture of several bile acids, FFA, MAG, neutral sterols, DAG, and TAG, separated as described in the Materials and Methods. The FFA, MAG, DAG, and TAG standards used for this separation were selected to represent the major fatty acids of the dietary fat in the hamster diet, a mixture of corn oil and butter fat (1:3). The retention times of all compounds were highly reproducible between different runs and between long periods of column inactivity.

This method was employed to separate and quantify individual members of all lipid classes in hamster feces. **Figure 3** shows the chromatogram of fecal lipids from hamsters on the HPMC-included high-fat diet (HF-HPMC) as an example of the profile of hamster fecal lipids. An absence of background effects and a similar resolution to the standard injection (**Figure 2**) with baseline separation were achieved with samples from the animals. LC does not have the resolution of GLC, and it was possible that the major peaks detected by LC contained more than one lipid component. Peaks detected by ELSD were further analyzed by LC–(+)APCI–MS/MS for the confirmation of identity and peak purity. APCI is the most frequently used ionization technique for sterols and TAG analysis because of

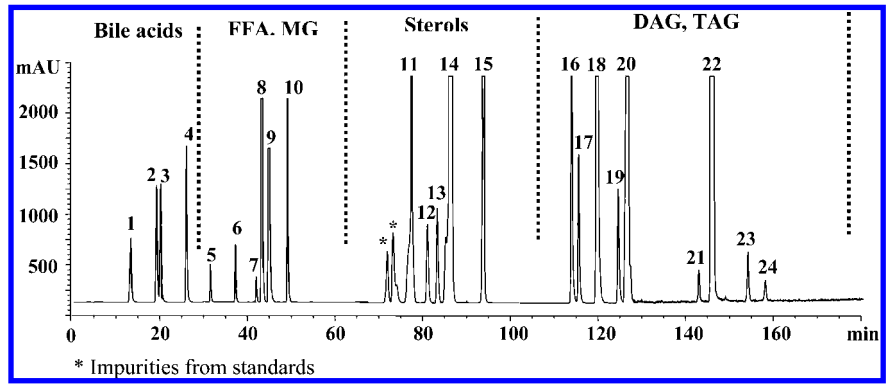

Figure 2. LC–ELSD chromatogram of bile acids, FFAs, MAG, sterols, DAG, and TAG standard mixture using the solvent system described in the Materials and Methods: (1) hyodeoxycholic acid, (2) chenodeoxycholic acid (CDCA), (3) deoxycholic acid (DCA), (4) lithocholic acid (LCA), (5) monolinolenin (18:3), (6) monolinolein (18:2), (7) monoolein (18:1), (8) FFA mixture (16:0, 18:0, 18:1, 18:2, and 18:3), (9) monopalmitin (16:0), (10) monostearin (18:0), (11) coprostanol, (12) cholesterol, (13) cholestanone, (14) stigmastanol, (15) sitosterol, (16) dilinolein, (17) diolein, (18) dipalmitin, (19) 1-palmitoyl, 3-stearol glycerol, (20) trilinolenin, (21) 1,2-dipalmitoyl, 3-oleyl glyceride, (22) tripalmitin, (23) palmitoyl-oleoyl-stearin, and (24) 1,3-dioleic, 2-stearic glyceride.

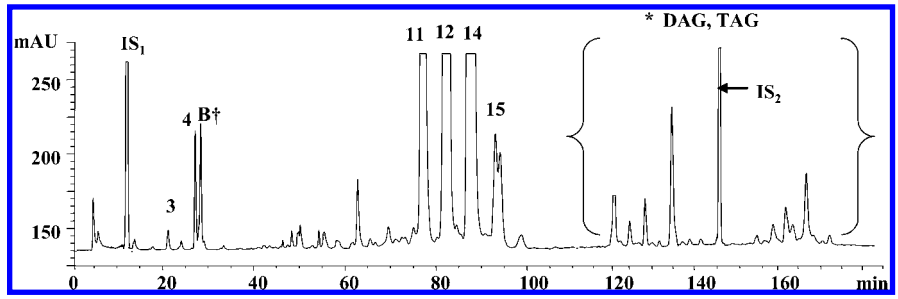

Figure 3. LC–ELSD chromatogram of the feces collected from HF-HPMC hamsters: (IS_1) hyodeoxycholic acid, (3) deoxycholic acid, (4) lithocholic acid, ($B^{\dagger}$) lithocholic acid isomer, (11) coprostanol, (12) cholesterol, (14) stigmastanol, (15) sitosterol, and (IS_2) 5α-cholestane. (∗) The results of DAG and TAG identification by APCI/MS are illustrated in **Figure 6**.

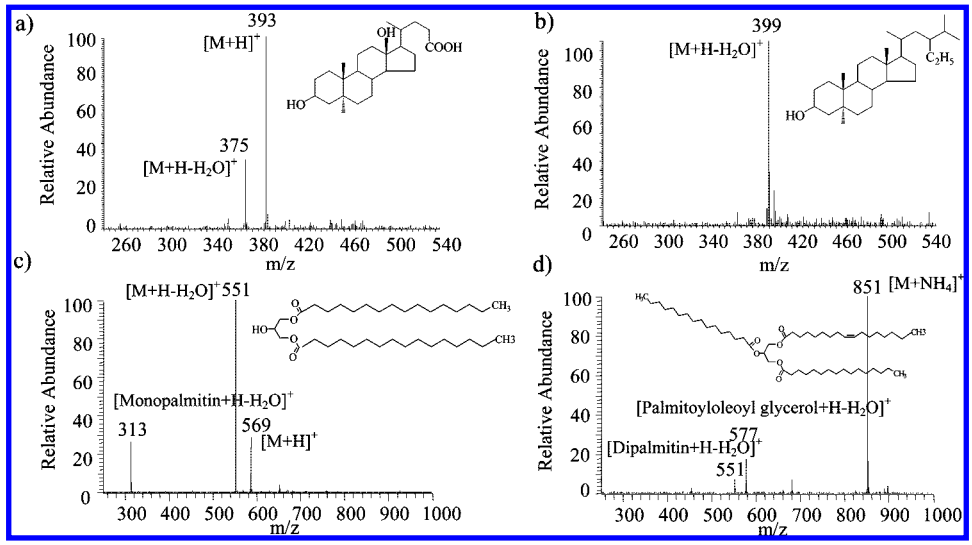

Figure 4. LC–(+)APCI–MS spectra of (a) deoxycholic acid, (b) stigmastanol, (c) dipalmitin, and (d) dipalmitoyl oleoyl glycerol found in feces.

the ease of coupling to nonaqueous mobile-phase systems used for the LC separation and high ionization efficiency for nonpolar molecules (*27*, *37*).

Peak identification was performed by acquiring total ion chromatograms (TICs) of fecal extracts over a mass range of *m*/*z* 150–2000 with three scans summed. The *m*/*z* value for all peaks in the total ion count mode (TIC) was determined to identify all possible bile acids and lipids in feces. Peaks showing *m*/*z* values corresponding to bile acids, neutral sterols, DAG, and TAG were further investigated using atmospheric pressure chemical ionization mass spectrometry [LC–(+)APCI–MS] in selective ion monitoring (SIM); using the same system, the mass spectrum was first acquired for the standards and compared to the mass spectrum obtained from the fecal samples. These mass spectra of bile acids, sterols, DAG, and TAG show the relative abundance of relevant ions. For deoxycholic acid (**Figure 4a**), APCI yielded two major ions, a base peak of $[M + H]^+$ *m*/*z* 393 and a second major ion with a neutral loss of water at $[M + H - H_2O]^+$ *m*/*z* 375. For neutral sterols, such as stigmastanol, APCI gave only one major ion formed with a neutral loss of water *m*/*z* 399. No other ions were formed (**Figure 4b**). Similar results were observed for other bile acids and sterols. DAG

Table 1. Characteristic Ions of Representative Fecal Components Observed in (+)APCI–MS Full Scanning

chemical name	chemical formula	theoretical mass	observed ions[a] *m/z*
deoxycholic acid	$C_{24}H_{40}O_4$	392.6	393 ($[M + H]^+$), 375 ($[M + H - H_2O]^+$)
lithocholic acid	$C_{24}H_{40}O_3$	376.6	377 ($[M + H]^+$), 359 ($[M + H - H_2O]^+$)
coprostanol	$C_{27}H_{48}O$	388.6	371 ($[M + H - H_2O]^+$)
cholesterol	$C_{27}H_{46}O$	386.6	369 ($[M + H - H_2O]^+$)
stigmastanol	$C_{29}H_{52}O$	416.7	399 ($[M + H - H_2O]^+$)
sitosterol	$C_{29}H_{50}O$	414.7	397 ($[M + H - H_2O]^+$)
dipalmitin	$C_{35}H_{68}O_5$	568.9	569 ($[M + H]^+$), 551 ($[M + H - H_2O]^+$) 313 ($[M + H - RCOOH - H_2O]^+$)
1,2-dipalmitoyl-3-oleoyl glycerol	$C_{53}H_{100}O_6$	833.4	851 ($[M + NH_4]^+$) 577 ($[M + H - {}^*RCOOH - H_2O]^+$ 551 ($[M + H - {}^\dagger R'COOH - H_2O]^+$

[a] *R, palmitic acid; †R′, oleic acid.

produced mainly protonated ions as well as the ions from the neutral loss of water. For instance, the full scan mass spectrum of dipalmitin showed a base peak of the ion $[M + H - H_2O]^+$ *m/z* 551 and a second major ion of $[M + H]^+$ *m/z* 569 (**Figure 4c**). In addition, the weak signal of monopalmitin was observed, because the ion form derived from a neural loss of water of monopalmitin at [monopalmitin + H – H_2O]$^+$ *m/z* 313. In the case of TAG, the most abundant ion formed was the ammonium adduct. 1,2-Dipalmitoyl-3-oleoyl glycerol, used as one example of TAG, showed an ammonium adduct $[M + NH_4]^+$ *m/z* 851 as a base peak. Additional ions relevant to its corresponding diacylglycerides were also observed, which are the neutral water loss ion of both dipalmitin and palmitoyloleoyl glycerol at *m/z* 551 and 577, respectively (**Figure 4d**). These characteristic ions of representative fecal compounds, such as bile acids, sterols, diglycerides, and triglycerides, are summarized in **Table 1**. APCI tends to create some fragments in MS experiments, whereas ESI produces no fragments. This weak fragmentation in APCI was used to advantage in the present study, in particular, for the identification of DAG and TAG because it provided more information on their structure. Therefore, the information provided by the APCI mass spectra combined with the reproducible retention time from LC separation made it possible to identify DAG and TAG in feces without further MS/MS experiments.

The structure of bile acids and sterols was confirmed by MS/MS. The *m/z* of protonated ions corresponding to bile acids and neutral sterols were selected for the precursor molecular ion, and daughter ions were scanned within the ranges covering possible fragments. The parent ion–daughter ion transitions of bile acids and sterols gave rise to the characteristic fragmentation pattern that agrees with those reported from previous studies (*38*, *39*), such as the retro-Diels–Alder reaction and neutral molecule elimination (**Figure 5**). In particular, the MS/MS spectra of bile acid ions are dominated by $[M + H - nH_2O]^+$ ions, where *n* is the number of ring hydroxyl groups present in the bile acid. Deoxycholic acid (3α,12α-dihydroxycholanic acid) (**Figure 5a**) can be identified by the MS/MS spectrum that shows the presence of the ion $[M + H - 2H_2O]^+$ *m/z* 357 and ions from further successive water loss $[M + H - 3H_2O]^+$ *m/z* 339 and $[M + H - 4H_2O]^+$ *m/z* 321. On the other hand, lithocholic acid (3α-hydroxycholanic acid) only shows the presence of two ions, $[M + H - H_2O]^+$ *m/z* 359 and $[M + H - 2H_2O]^+$ *m/z* 341 (not shown). This MS/MS experiment allowed us to determine bile acids and lipid compounds in feces by comparing their mass spectra with those obtained from standards of each compound. All fecal samples injected onto the LC system produced typical chromatograms, such as shown in **Figure 3**.

The major bile acids found in feces from HF-HPMC-fed hamsters were deoxycholic acid and lithocholic acid (**Figure 3**). This agrees with previous studies on fecal bile acid analysis in hamsters fed various dietary fibers (*20*, *35*). A peak (B^T in **Figure 3**) that eluted adjacent to lithocholic acid had an ion formed at *m/z* 377, which is the same as lithocholic acid and the same fragmentation pattern in collision-induced dissociation (CID) mass spectra (data not shown). This indicated that it was a stereoisomer of lithocholic acid (3α-hydroxyl-5β-cholanic acid). Daggy et al. (*20*) reported the presence of 3β-hydroxyl-5α-cholanic acid, a stereoisomer of lithocholic acid, in hamster feces as a minor component as analyzed by GC–MS.

Four principal neutral sterols excreted in HF-HPMC-fed hamster feces were identified. They were cholesterol and its bacterial metabolite coprostanol and stigmastanol and sitosterol derived from the dietary corn oil (**Figure 3**). The sterol profiles in feces were investigated because cholesterol can originate from the diet or from hepatic synthesis, and excretion of cholesterol-derived metabolites can be affected by environmental factors, such as diets or disease status. On the other hand, the excretion of plant sterols is less variable because it originates from the dietary fat fed to the hamsters (*40*).

The identification of DAG and TAG was also successfully achieved by employing LC–ELSD and LC–(+)APCI–MS. MAG and FFA eluted before neutral sterols (**Figure 2**). Other compounds present in feces tended to coelute with MAG and FFA, making the mass spectrum complex and difficult to interpret. Therefore, only DAG and TAG were analyzed in the feces from hamsters fed the HF-HPMC diet. There were no detectable amounts of DAG or TAG (data not shown) found in feces from HF-MCC-fed hamsters. As shown in **Figures 3** and **6**, DAG and TAG eluted according to the equivalent carbon number (ECN). The separation of TAG by ECN has been well-described in several studies (*27*, *41*). **Figure 6** shows TIC of DAG and TAG in the feces obtained by APCI. This figure illustrates that all DAG and TAG with different ECN were well-resolved and eluted in the order of increasing ECN, except for DAG with ECN 24 and 26. The DAG and TAG identified by APCI/MS are summarized in **Table 2**, with *m/z* values of the most abundant ion and their corresponding ECN. Close observation of the mass spectrum obtained from each peak proved the presence of their corresponding fragments, such as MAG and DAG, as well as their base peak. The complete separation of TAG with the same ECN is possible using the HPLC method described in some of studies (*27*) and could be applied to our APCI/MS system. This profile of DAG and TAG is similar to that of the composition of the dietary fat fed to hamsters, which consisted of palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3).

The quantitation of bile acids and sterols was developed to measure the effect of HPMC on the excretion of bile acids and lipid compounds in feces. The feces from HF-HPMC- and HF-MCC-fed hamsters were compared by LC–ELSD and LC–(+)APCI–MS/MS . Quantitation was made using ELSD chromatograms because ELSD chromatograms showed baseline separation with no matrix effect. Given the fact that ELSD has a disadvantage because the relationship between eluted mass and peak area is not linear, quantitative analysis requires careful multipoint calibration curves for each compound that is to be determined. The calibration curves generated for bile acids and neutral sterols were logarithmically proportional to the range of the concentra-

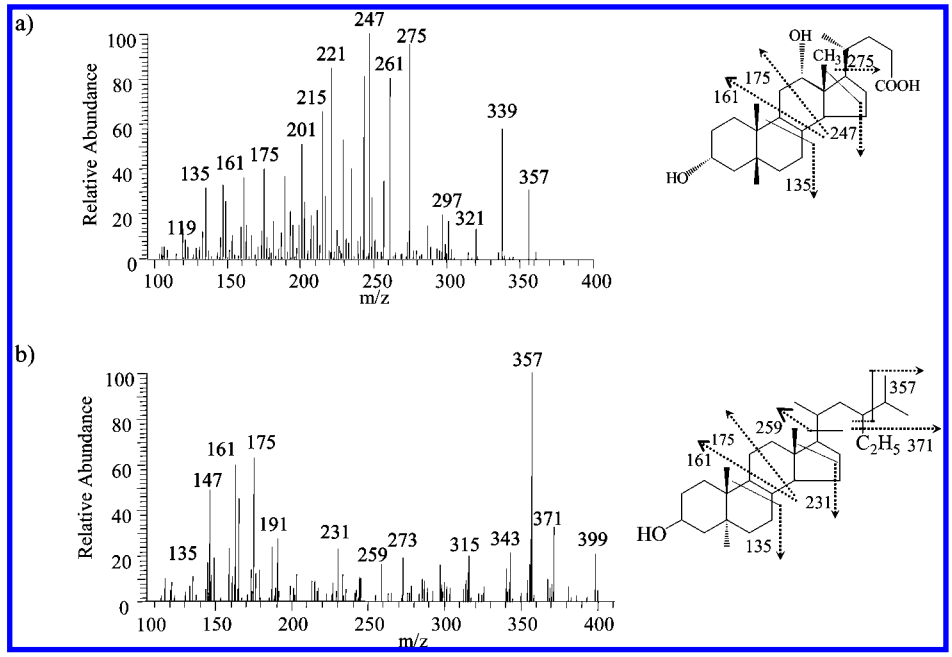

Figure 5. LC–(+)APCI–MS/MS spectra of (a) deoxycholic acid and (b) stigmastanol.

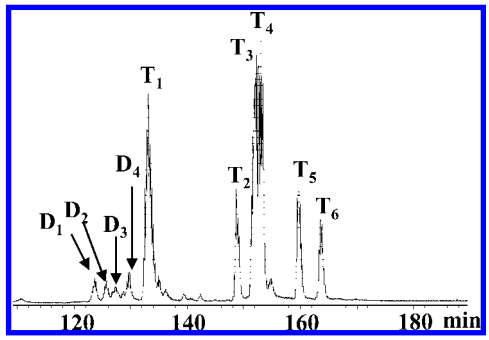

Figure 6. LC–(+)APCI–MS total ion chromatogram of DAG and TAG in feces collected from HF-HPMC-fed hamster: D_1–D_4 (DAG) and T_1–T_6 (TAG).

Table 2. Identification of DAG and TAG in Feces from HF-HPMC-Fed Hamsters[a]

	$[M + H - H_2O]^+$				$[M + NH_4]^+$		
peak	(*m*/*z*)	DAG	ECN	peak	(*m*/*z*)	TAG	ECN
D_1	595	LnLn	24	T_1	896	LLL	42
	597	LLn	26				
D_2	599	LL	28	T_2	898	OLL	44
	599	OLn			872	OPL	
	547	PoPo					
D_3	575	OPo	30	T_3	900	OLO, SLnO	46
	601	OL			874	SLnP, OLP	
	549	PPo			848	OPoO, PLP,	
	575	LP				POPo	
D_4	603	OO	32	T_4	902	OOO, SLO	48
	577	OP			876	SLnS, SLP	
	551	PP			850	OOP,POP	
					824	SMO, PPP	
				T_5	904	SLS, OSO	50
					878	SOP	
					852	SPP	
				T_6	906	SOS	52
					880	SPS	

[a] Abbreviations: M, myristic acid; P, palmitic acid; Po, palmitoleic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Ln, linolenic acid.

tion, with correlation coefficients (r^2) greater than 0.95, and the relative standard deviation (RSD) was lower than 6% ($n = 3$) for all measurements (**Table 3**). The detection limit ranging between 0.1 and 1 μM was achieved for compounds analyzed, including bile acids and neutral sterols. This is similar to those reported from the analysis of sterols and bile acids in biological fluids by LC–(+)APCI–MS/MS (*30*).

Table 3. LOD, Linearity, and RSD of the LC–ELSD Method

compound	LOD (μM)	linearity	RSD
deoxycholic acid	1	0.9962	2.13
lithocholic acid	0.5	0.9957	3.62
cholesterol	0.5	0.9699	2.56
coprostanol	0.2	0.9566	2.90
sitostanol	0.5	0.9999	5.51
stigmastanol	0.1	0.9542	4.99

Figure 7 shows the results of quantitation of major secondary bile acids as well as neutral sterols found in hamster feces. There was some difference in the excretion of secondary bile acids between the two diets. The amounts of deoxycholic acid and lithocholic acid were increased about 2 and 1.8 times, respectively, in feces of hamsters fed 5% HPMC ($p < 0.01$) compared to control hamsters. Bile acids in feces are the metabolites of colonic bacteria that may provide information of the population of bacteria. Increased amounts of deoxycholic acid have been reported in patients with colon cancer as compared to controls (*42*). Benson et al. (*35*) reported that the presence of polydeoxycholic acid in hamster and human stools may result from the reduction of toxicity of deoxycholic acid by colonic microorganisms. The results from neutral sterol quantitation showed that the major sterol component was coprostanol, the main bacterial transformation product of cholesterol, generated alternatively through a direct reduction of cholesterol (*43*) or indirectly via cholestenone and coprostanone; the latter was shown by Ren et al. (*44*). There was the significant increase in the excretion of coprostanol in HF-HPMC-fed hamsters ($p < 0.05$) by 15%, but no changes in the excretion of stigmastanol and sitosterol as expected. The total cholesterol-derived metabolites, including cholesterol and coprostanol, were also significantly increased in HF-HPMC-fed hamsters by 12%. Plant sterols are also known to have beneficial physiological functions of reducing cholesterol absorption by displacing cholesterol in the intestine because of their greater affinity for intestinal

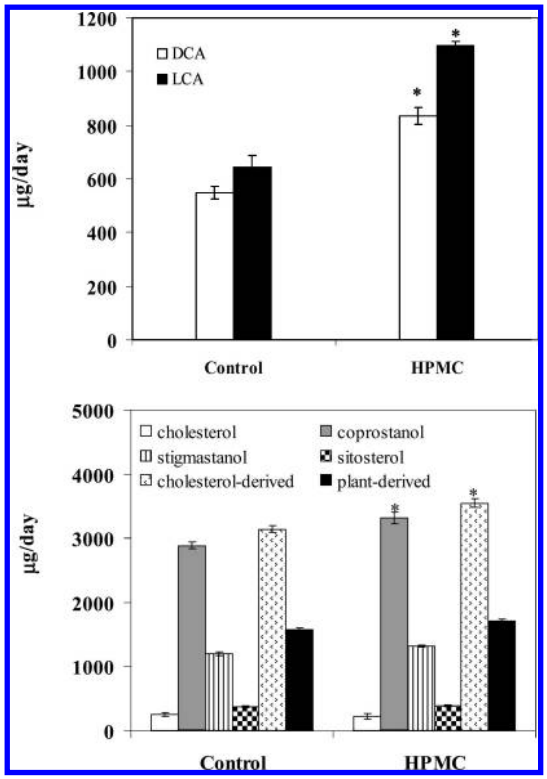

Figure 7. Calculated (a) secondary bile acids, deoxycholic acid, and lithocholic acid [mean ± standard error of the mean (SEM)] and (b) neutral sterols (mean ± SEM) in feces of HF-MCC- and HF-HPMC-fed hamsters. Cholesterol-derived sterols was obtained by summing the excreted cholesterol and coprostanol, and plant-derived sterols was obtained by summing the excreted stigmastanol and sitosterol. An asterisk indicates a significant difference, $p < 0.05$.

micelles (*45*, *46*). The total neutral sterols derived from plant sterols, including stigmastanol and sitosterol, were not significantly affected by the HF-HPMC diet. This analysis of fecal bile acids and sterols clearly shows that HPMC-soluble fiber facilitates the excretion of bile acids as well as cholesterol-derived metabolites in the feces of hamsters.

In summary, we have developed a highly simplified and convenient method for the analysis of total fecal lipid (bile acids, neutral sterols, FFA, MAG, DAG, and TAG) and showed its utility in the analysis of fecal lipids of hamsters fed the soluble, cholesterol-lowering fiber, HPMC. In this method, extensive sample preparation, such as extraction of bile acids from lyophilized feces, removal of neutral sterols, and chemical derivatization of acid and hydroxyl groups, was avoided. This makes the assay easier to perform and minimizes the production of artifacts that may interfere with the analysis of compounds in interests.

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