An optimized method for measuring fatty acids and cholesterol in stable isotope-labeled cells

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Abstract Stable isotope labeling has become an important methodology for determining lipid metabolic parameters of normal and neoplastic cells. Conventional methods for fatty acid and cholesterol analysis have one or more issues that limit their utility for in vitro stable isotope-labeling studies. To address this, we developed a method optimized for measuring both fatty acids and cholesterol from small numbers of stable isotope-labeled cultured cells. We demonstrate quantitative derivatization and extraction of fatty acids from a wide range of lipid classes using this approach. Importantly, cholesterol is also recovered, albeit at a modestly lower yield, affording the opportunity to quantitate both cholesterol and fatty acids from the same sample. Although we find that background contamination can interfere with quantitation of certain fatty acids in low amounts of starting material, our data indicate that this optimized method can be used to accurately measure mass isotopomer distributions for cholesterol and many fatty acids isolated from small numbers of cultured cells. Application of this method will facilitate acquisition of lipid parameters required for quantifying flux and provide a better understanding of how lipid metabolism influences cellular function.—Argus, J. P., A. K. Yu, E. S. Wang, K. J. Williams, and S. J. Bensinger. An optimized method for measuring fatty acids and cholesterol in stable isotope-labeled cells. J. Lipid Res. 2017. 58: 460–468.

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Fatty acids and cholesterol perform essential structural, energetic, and signaling roles in all animal cells (1). Renewed interest in understanding how cholesterol and fatty acid homeostasis is dynamically modulated in normal cellular states and during pathophysiologic processes (e.g., oncogenic signaling in cancer) has led to increased demand for methodologies that can accurately interrogate these lipid classes (2–4). One approach that has gained significant favor is the use of stable isotope labeling to quantify how cellular requirements for cholesterol and fatty acid pool sizes are achieved in normal and neoplastic cells (5). This methodology allows for defining the origin of cellular lipid (e.g., synthesized versus imported from extracellular sources) and determining the carbon source(s) contributing to the acetyl-CoA pool used for the synthesis of lipids (e.g., glucose versus amino acids) (6, 7).

Analysis of stable isotope-labeled fatty acids or cholesterol is commonly performed using GC/MS due to its low cost and relative simplicity (8–10). In this approach, cellular fatty acids or cholesterol are converted to nonpolar derivatives [e.g., fatty acid methyl esters (FAMEs) or trimethylsilyl ether (TMSE) cholesterol] before injection to increase volatility and improve chromatography. However, conventional methods are not ideal for analyzing total fatty acids and cholesterol from small amounts of stable isotope-labeled cells for one or more of the following reasons. First, many published methods have been developed for analyzing specific classes of lipids and, as a consequence, may not accurately quantitate total fatty acid or cholesterol content (9, 11, 12). Second, protocols are sometimes not validated for the small amounts of material commonly used in stable isotope-labeling experiments; thus, application of these methods can result in issues with background signal and/or destruction of analytes (e.g., cholesterol or unsaturated fatty acids) (13, 14). Third, stable isotope labeling often requires a large amount of sample to be injected onto the column for accurate determination of the mass isotopomer distribution (MID) of rare molecular ions (7, 15). However, many published methods, which have been optimized for nonlabeled samples,
cannot inject enough material onto the column to visualize these MIDs (16–18). Finally, few methods allow for efficient measurement of both fatty acids and cholesterol from the same sample (19).

To address these challenges, we developed a method optimized for analysis of total fatty acids and cholesterol from small numbers of cultured stable isotope-labeled cells. In this approach, cells are derivatized in situ (without initial lipid extraction) using a short acid-catalyzed methanolation reaction. FAMEs and cholesterol are extracted and concentrated. FAMEs are subsequently analyzed by a rapid GC/MS program. The sample is then dried, further derivatized using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA):trimethylchlorosilane (TMCS) (99:1) and pyridine, and reanalyzed by GC/MS to quantify TMSE cholesterol. In validation experiments, we demonstrate that long chain fatty acid (LCFA; 14–18 carbons) and very long chain fatty acid (VLCFA; 20–24 carbons) yields are near 100% for representatives from all major animal lipid classes (FFA, TG, cholesteryl ester (CE), phosphoglycerolipid, phosphophingolipid, and glycosphingolipid), while maintaining cholesterol yields at approximately 80–90%. Our data indicate that background fatty acid and cholesterol contamination can be a limiting factor when small amounts of starting material are used, depending on the analytes of interest. Nevertheless, we found that application of this method can generate robust MIDs for cholesterol and many fatty acids from a relatively wide range of cell numbers (0.4–2.5 × 10^6 H1299 lung cancer cells), representing approximately 20–125 μg of total fatty acid and cholesterol. Thus, we anticipate that utilization of this method will facilitate the application of stable isotope labeling to model lipid metabolic parameters in normal and disease states.

MATERIALS AND METHODS

Reagents

Optima grade methanol, Optima grade water, Optima grade n-hexane, and ACS plus grade hydrochloric acid were purchased from Fisher. Chromosolv Plus grade toluene, ≥99.0% butylated hydroxytoluene (BHT), ACS grade sodium carbonate, 99:1 BSTFA:TMCS, and anhydrous 99.8% pyridine were purchased from Sigma-Aldrich. The 99+% acetyl chloride was purchased from Alfa Aesar. FAMEs, FFAs, glycerol trinonadecanoate, and cholesteryl heicicosanoate were purchased from NuChek Prep. The 1,2-ditricosanoyl-sn-glycero-3-phosphocholine, N-lignoceroyl-D-erythro-sphingosylphosphorylcholine, and D-galactosylβ-L-1′-Nervonoyl-D-erythro-sphingosine were purchased from Avanti Polar Lipids. Cholesterol, stigmastanol, stigmasterol, and cholesterol-d₆ were purchased from Sigma-Aldrich. H1299 (also referred to as NCi-H1299) cells were purchased from ATCC. 13C₆-glucose was purchased from Cambridge Isotope Laboratories. FBS was purchased from Omega Scientific. All other cell culture reagents were purchased from Gibco.

Internal standards, lipid class standards mix, and standard curves

FAMEs and sterols (17:1 n-9, 19:0, 19:1 n-12, 23:0, 23:1 n-9, cholesterol-d₆, stigmastanol, and stigmasterol) were used as internal standards in one or more experiments, as described in the text. The mass of internal standards used per tube was varied based on the nature of the experiment; for experiments with 0.4–2.5 × 10^6 H1299 cells, 1–3 μg of each relevant internal standard were used. Internal standards were generally added in 10 μl of toluene. The lipid class standards mix contained equal masses of the six lipids listed in Table 1. Lipid (150 μg) was added to each sample in 50 μl of toluene. The standard curve for the lipid class standards mix was GLC-14A (methyl ester version; NuChek Prep) supplemented with 23:0, 24:0, and 24:1 n-9 methyl esters in a 62.5:12.5:12.5:12.5 mass ratio. The standard curve used to test for nonhydrolytic FAME loss and to quantify fatty acids and cholesterol in H1299 cells was designed to roughly mimic the content of animal cells. It consisted of GLC-96 (methyl ester version; NuChek Prep), 18:1 n-7 methyl ester, and cholesterol in an 83.2:7.0:9.2 mass ratio.

Cell culture

Unlabeled H1299 cells were cultured at 37°C (5% CO₂) in RPMI supplemented with antibiotic and 10% heat-inactivated FBS. Cells were trypsinized and counted in RPMI supplemented with antibiotic and 2.5% FBS using trypan blue exclusion and a Nexcelom Cellometer K2. Cells were then aliquoted into glass tubes containing 1.5–3.5 ml of PBS (used to dilute out FBS) and pelleted. The supernatant was aspirated until 50–100 μl remained in the tube. H1299 cells were labeled at 37°C (5% CO₂) using glucose-free DMEM supplemented with 25 mM 13C₆-glucose, antibiotic, and 5% FBS. Labeled medium was refreshed daily and cells were maintained in a subconfluent state by splitting as necessary. After ≥5 divisions in labeled medium (to ensure metabolic and isotopic steady state), cells were collected with trypsin and 2 ml of PBS directly into glass tubes and pelleted. The supernatant was aspirated until 50–100 μl remained in the tube. All cell pellets were held at −20°C until derivatization.

Free and esterified fatty acid method derivatization

Derivatization was as described previously (13) with minor modifications. Briefly, 200 μl of tolouene, 1.5 ml of methanol, and 300 μl of methanolic HCl (8.0% w/v) were added, in that order,
to samples in glass tubes. Methanolic HCl was prepared by adding 1.9 ml concentrated aqueous HCl to 8.1 ml methanol. Tubes were capped, shaken, and incubated at 100°C for 1.5 h in a closed fume hood or at 45°C for 16 h on the benchtop.

**Amidified fatty acid method derivatization**

Derivatization was as described previously (9, 20) with minor modifications. Briefly, 2 ml of methanolic HCl (5:1 v/v) were added to samples in glass tubes. Methanolic HCl was prepared by adding 4 ml concentrated aqueous HCl to 20 ml methanol. Tubes were capped, shaken, and incubated at 80°C for 5 h in a closed fume hood or at 50°C for 24 h on the benchtop.

**Current method derivatization**

Two milliliters of current method master mix were added to samples in glass tubes. The current method master mix contained 9% acetyl chloride, 18% tolune, and 73% methanol by volume. The master mix was made by slowly adding acetyl chloride to pre-mixed tolune and methanol on ice to avoid splashing. Reaction mixes were supplemented to contain approximately 100 µl of “sample” water unless otherwise stated. Tubes were capped, shaken, and incubated at 100°C for 2 h or overnight in a closed fume hood. Tubes were shaken again after 1 h for the 2 h incubation, and after several hours for the overnight incubations.

**Neutralization, extraction, and concentration**

After incubation, tubes were cooled to room temperature. To neutralize, 2.5 ml of 0.31 M [free and esterified fatty acid (FEFA) method], 0.99 M [amidified fatty acid (AFA) method], or 0.66 M (current method) aqueous sodium carbonate was added to yield a final aqueous layer of approximately 0.1 M sodium carbonate. After adding 2 ml of n-hexane, the tubes were shaken and centrifuged at 2,000 g for 5 min. The organic layer was extracted to a new tube, and the 2 ml n-hexane extraction was repeated. The combined organic layers were evaporated to dryness using the EZ-2 Elite Speedvac (“Low BP Mix” program, 50 min to final stage, 0 min final stage, lamp off). The dried lipids were redissolved in a small amount of toluene appropriate for the sample (75 µl recommended for 0.4–2.5 × 10⁶ stable isotope-labeled CE-1299 cells) and transferred to robovials containing 300 µl glass inserts for GC/MS analysis.

**GC/MS analysis**

For FAMEs, an Agilent 7890A/5975C GC/MS equipped with a 27.75 m, 0.25 mm ID, 25 µm film, DB-Wax column (Agilent) was used. Sample (0.5–2 µl) was injected in splitless mode into an inlet held at 275°C. The oven program was as follows: 95°C for 1 min, followed by 40°C/min to 115°C for 0 min, 30°C/min to 190°C for 2 min, 4°C/min to 218°C for 3 min, 4°C/min to 250°C for 7.8 min (31.8 min total). Helium was the carrier gas, and the column flow rate was 1 ml/min for 23.75 min followed by 1.7 ml/min for the remainder of the run. The MS was run in EI mode (70 eV). The transfer line, EI source, and quadrupole were maintained at 250, 230, and 150°C, respectively. For experiments without 13C labeling, M+0 molecular ions were collected in SIM mode (m/z 454.4 for TMSE cholesteryl, m/z 464.4 for TMSE cholesteryl-d₆, m/z 488.5 for TMSE stigmastanol, m/z 486.5 for TMSE stigmastanol). For experiments with 13C labeling, TMSE cholesteral molecular ions were collected in SIM from M-2 to M+27, while M+0 molecular ions (listed previously) were collected for internal standards. Chromatographic and spectral analyses were performed using ChemStation and MassHunter (Agilent).

**RESULTS**

**Difficulties in completely derivatizing fatty acids from representatives of all major animal lipid classes**

In cells, the majority of fatty acids are either esterified or amidified into complex lipids (1). A variety of methods have been developed to derivatize cellular fatty acids into FAMEs (9, 11). Historically, one strategy involves extraction of lipids from the biological source material followed by base-catalyzed hydrolysis (saponification) and extraction of the resultant FFAs. These FFAs are then methylated in a second reaction and extracted for subsequent analysis (12, 21). However, we chose to pursue in situ acid-catalyzed methanolyis because it only requires a single derivatization reaction followed by a single extraction step (8). Multiple in situ acid-catalyzed methanolyis methods have been developed for derivatization of FFAs (8, 13, 22), but it remains unclear whether these methods can efficiently derivatize AFAs. To begin testing this, we applied a commonly used FEFA method to a lipid class standards mix containing equal masses of standards representing the major fatty acid-containing lipid classes in animal cells (1, 9), spanning a range of polarities and fatty acid linkages (Table 1). One hundred and fifty micrograms of the lipid class standards mix were derivatized using 2 ml of 0.39 M methanolic HCl containing 10% toluene at 45°C for 16 h (13). One hundred microliters of water were added to mimic the water present in typical biological samples. As expected, FFAs [FA, TG, CE, and phosphatidylcholine (PC)] were efficiently derivatized (absolute yield of 89–98%; Table 2). However, yields from AFAs [SM and galactocerebroside (CRB)] were far lower (9–20%; Table 2). Increasing the temperature and decreasing the time of the reaction improved yield (49–62%; Table 2), but still failed to fully...
derivatize SM and CRB. These data support the notion that methods optimized to derivatize FEFAs may not be able to fully derivatize fatty acids from amidified lipids, likely because amide bonds are more resistant to methanolysis than ester bonds (9, 16).

We next tested two variants of a representative method specifically developed for derivatizing AFAs by reacting 150 μg of lipid class standards mix with 2 M methanolic HCl at 50°C for 24 h or 80°C for 5 h (9, 20). One hundred microliters of water were added to mimic the water content commonly present in biological sample preparations. Both variants of this AFA method resulted in sufficient yields (≥88%) from polar lipids (FFA, PC, SM, CRB; Table 2). However, both of the AFA methods had significantly decreased yields (4–47%) from nonpolar lipids (TG, CE; Table 2), likely due to their insolubility in a water-methanol mix (13). Taken together, these results indicate that methods developed for specific lipid classes may not be able to completely derivatize fatty acids from representatives of other major animal lipid classes. As a result, using these methods could result in an incomplete assessment of total cellular fatty acid content.

**Development of a method for derivatization of fatty acids from representatives of all major animal lipid classes**

One previously published method used an in situ acid-catalyzed methanolysis approach that completely derivatized fatty acids from CE, PC, and SM (16). In this method, 1.3 M HCl and 100°C incubation were used to fully derivatize the AFAs in SMs. Neutral CEs were brought into solution by including benzene in the reaction mix and by using acetyl chloride as an anhydrous source of acid (in place of aqueous HCl). This method increased the range of complex lipids derivatized in a single reaction; however, there are several aspects of this derivatization that do not make it ideal. Benzene is a regulated carcinogen, and acetyl chloride (due to its reactivity) was added dropwise on a per sample basis, greatly increasing labor time. Furthermore, other important lipid classes (TG, FFA, glycosphingolipids) were not directly tested. To address this, we modified the derivatization by replacing benzene with noncarcinogenic toluene, and added the reagents in a single master mix (2 ml of 9:18:73 acetyl chloride:toluene:methanol, v/v/v). Reaction time was extended from 1 to 2 h at 100°C to ensure complete derivatization of AFAs. To test whether these changes allowed for the derivatization of fatty acids from representatives of all major animal lipid classes, 150 μg of our lipid class standards mix were reacted in the presence of 100 μl of water. We found that FFA, TG, CE, PC, SM, and CRB were all efficiently derivatized at 95–101% of theoretical yield (Table 2). Extending the reaction to 21 h did not change the results, further demonstrating the completion of the reaction at 2 h (Table 2).

**Optimizing extraction and GC/MS analysis for limiting amounts of stable isotope-labeled FAMEs**

One intrinsic challenge with stable isotope labeling of lipids is that it requires intensive analysis of the molecular ion mass isotopomers to gain useful information about synthesis (6, 7). For fatty acids, the molecular ion mass isotopomers are rare (<2% of all ions created), particularly for PUFA (9). Furthermore, the high cost of stable isotope-enriched metabolites limits the amount of starting material that can be feasibly generated from in vitro labeling experiments. As a result, many published methods, which are optimized for large amounts of unlabeled starting material, do not produce enough signal to be used for analysis of stable isotope-labeled lipids. To address this, we improved extraction efficiency and concentrated the final samples by drying under vacuum. After derivatization, samples were neutralized with 2.5 ml of 0.66 M aqueous Na2CO3 and extracted twice with 2 ml of n-hexane. We observed extraction efficiencies of greater than or equal to 95% for FAMEs derivatized from the lipid class standards mix described above. The combined organic layers were then dried under vacuum and subsequently redissolved in as low as 75 μl of toluene before GC/MS analysis. As a result of our extraction, drying, and redissolving steps, we were able to significantly increase the final sample concentration.

Published FAME GC/MS programs can also be problematic when applied to stable isotope-labeled samples. Programs often call for the GC/MS inlet to be run in “split” mode, which is simple, rapid, and yields sharp peaks because only a small fraction (1–10%) of the injected sample is loaded onto the column (9, 11, 16). This may be sufficient for large amounts of unlabeled starting material, but usually will not result in adequate signal for analysis of FAMEs from small numbers of stable isotope-labeled cells. Furthermore, run times can be an issue (sometimes exceeding 90 min) because of challenges in chromatographically separating FAME isomers and the slow elution of cholesterol (16, 19). To overcome signal deficiencies, we used a “splitless” injection.

### TABLE 2. Current method derivatizes representatives of all major classes of animal lipids containing fatty acids

| Lipid Class | FEFA Method (Percent Yield) | AFA Method (Percent Yield) | Current Method (Percent Yield) |
|-------------|-----------------------------|-----------------------------|--------------------------------|
|             | 45°C, 16 h                  | 100°C, 1.5 h                | 50°C, 24 h                     | 80°C, 5 h | 100°C, 2 h | 100°C, 21 h |
| FFA         | 98 ± 1                      | 99 ± 0                      | 95 ± 1                         | 95 ± 1     | 96 ± 1     | 95 ± 1     |
| TG          | 89 ± 1                      | 96 ± 2                      | 4 ± 1                          | 35 ± 4     | 98 ± 1     | 96 ± 0     |
| CE          | 96 ± 1                      | 96 ± 4                      | 14 ± 3                         | 47 ± 2     | 101 ± 1    | 99 ± 1     |
| PC          | 92 ± 0                      | 95 ± 1                      | 92 ± 0                         | 92 ± 1     | 95 ± 1     | 93 ± 0     |
| SM          | 20 ± 0                      | 62 ± 8                      | 94 ± 2                         | 98 ± 3     | 98 ± 4     | 98 ± 3     |
| CRB         | 9 ± 0                       | 49 ± 7                      | 88 ± 1                         | 90 ± 1     | 96 ± 1     | 94 ± 1     |

One hundred and fifty micrograms of lipid class standards mix were added to each tube and reacted as described in the Materials and Methods. Samples were normalized to a 23:1 n-9 methyl ester internal standard added before extraction. Yield was determined by comparing samples to a FAME external standard curve. Values reported are the mean ± SD (n = 3).
paired with solvent focusing to load the entire injected sample onto the column while still resolving FAME isomers. An extended final bake out of the column is avoided by allowing sterols to elute in empty or noncritical areas of subsequent chromatograms, decreasing run time to 32 min. Taken together, these results show that our method has been optimized for rapid analysis of limiting amounts of stable isotope-labeled FAMEs by increasing signal (up to 150-fold) and reducing run time (up to 3-fold) compared with conventional methods (11, 16, 19).

Minimal losses of FAMEs due to hydrolysis, oxidation, or evaporation

Excess water can interfere with methyl ester derivatization by both driving nonpolar lipids out of solution and hydrolyzing methyl esters to free acids (15). Conversely, insufficient water has been reported to hinder sphingolipid derivatization (16). Because we designed the current method to work in situ, we sought to determine the water tolerance of the reaction. To that end, we derivatized the lipid class standards mix described above using the new procedure in the presence of 0–200 µl of water, approximating the range of residual water commonly found in pellets of cultured cells. Yields for FAMEs from all lipid classes were 92–101% when 50–200 µl of water were present, indicating that this amount of water is well-tolerated in the reaction. In agreement with previous results (16), we also observed that a completely anhydrous reaction modestly decreased yield from sphingolipids (approximately 85% of theoretical yield), but not other lipid classes. Additionally, increasing the water content in the reaction mixture resulted in a dose-dependent decrease in FAME yields, however this decrease was less than 5% and affected all fatty acids analyzed equally.

Evaporative loss of low molecular weight FAMEs during drying and destruction of PUFAs under harsh acid-catalyzed methanolysis conditions has been reported (9, 14, 16, 23). To address these issues, we analyzed 20–125 µg of a FAME/cholesterol mix designed to roughly mimic the content of animal cells (see the Materials and Methods). Samples were analyzed by GC/MS directly (external standard curve) or after being subjected to the current derivatization reaction (internal standard curve). Water (50, 100, or 200 µl) was added before derivatization to mimic sample water. A 19:0 methyl ester internal standard added before derivatization was used to correct for minor losses due to hydrolysis. When yield was determined using the ratio of internal to external standard curve slopes, we observed losses of less than or equal to 5% for all fatty acids measured, with the exception of 7% for 14:0 with 200 µl of water present. We found that performing derivatization under argon and in the presence of the antioxidant BHT (100 mg/l) did not result in significant improvement of the yield of PUFAs, likely because loss in the reaction was already low (<5%). Thus antioxidants and inert gas can be included, but they do not appear to be necessary to preserve PUFAs in the current method with the amounts of lipid used. In sum, these data demonstrate that FAME losses due to hydrolysis, evaporation, and oxidation are minimal when 50–200 µl of water is present and lipid mass exceeds 20 µg.

Complete derivatization of LCFAs and VLCFAs from all major animal lipid classes in cellular matrix

Cellular matrix can interfere with acid-catalyzed methanolysis by neutralizing acid or protecting complex lipids. Thus, we tested whether this method could completely derivatize fatty acids in the presence of cellular matrix. To that end, the yield from our lipid class standards mix was determined when spiked into matrix from 2.5 × 10⁶ H1299 lung cancer cells. We observed that yields were nearly identical in the presence or absence of cellular matrix (Table 3). We also considered the possibility that endogenous lipids from the cellular matrix might behave differently than exogenously added lipid. To test for this, we reacted cellular matrix from 2.5 × 10⁶ H1299 cells for 2 h or overnight (15 h). Incubation for both 2 and 15 h resulted in nearly identical yields of endogenous fatty acids (<5% difference), suggesting that, similar to the lipid class standards, endogenous lipids are fully derivatized in the first 2 h of the reaction. We also observed that the method is linear for ≥17 fatty acids over a range of cancer cell numbers (0.4–2.5 × 10⁶ H1299), with R² values greater than 0.99 (Table 4).

To further demonstrate the importance of fully derivatizing fatty acids from all major classes of lipid in animals, we assessed fatty acid content from cultured cells (H1299) using the current method and a commonly used FEFA method (13). The two methods yielded similar results for LCFAs, but the current method resulted in an increase of 1.5- to 3-fold for 22:0, 24:0, and 24:1 n-9 (Fig. 1). This is likely due to significant enrichment of saturated and mono-unsaturated VLCFAs in sphingolipids (24, 25), which can be poorly derivatized by FEFA methods (Table 2). Taken together, these results demonstrate that the current method fully derivatizes LCFAs and VLCFAs from all major animal lipid classes in the presence of cellular matrix, and that failure to completely derivatize fatty acids from these different classes can result in significant underestimation of the fatty acid content of cultured cells.

Efficient quantitation of cholesterol from the same sample

It is difficult to analyze both fatty acids and cholesterol using a single derivatization and GC column (9). To address this, we developed an integrated approach where we...

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**TABLE 3.** Complete derivatization of lipid class standards in cellular matrix

| Lipid Class | Without Cellular Matrix (Percent Yield) | With Cellular Matrix (Percent Yield) |
|-------------|----------------------------------------|-------------------------------------|
| FFA         | 97 ± 1                                  | 96 ± 1                              |
| TG          | 99 ± 1                                  | 97 ± 1                              |
| CE          | 103 ± 1                                 | 103 ± 1                             |
| PC          | 96 ± 1                                  | 97 ± 1                              |
| SM          | 100 ± 4                                 | 103 ± 6                             |
| CRB         | 98 ± 1                                  | 98 ± 1                              |

One hundred and fifty micrograms of lipid class standards mix were added to tubes containing 0 or 2.5 × 10⁶ H1299 cells. All tubes were analyzed using the current method. Samples were normalized to a 19:1 n-12 methyl ester internal standard added after dry down. Yield was determined by comparing samples to a FAME external standard curve. The contribution of cellular fatty acids was corrected for. Values reported are the mean ± SD (n = 3).
could analyze cholesterol content after FAME analysis. Samples were dried and rederivatized in 1:1 (99:1 BSTFA:TMCS):pyridine to produce TMSE sterols and a new rapid GC/MS program was run on a ZB-MR1 column (<10 min per sample, see the Materials and Methods). Fatty acids are also trimethylsilated, but they do not interfere with TMSE sterol quantitation because they elute at much lower temperatures. One potential concern was that a harsh acid methanolysis reaction could result in the destruction of cholesterol (13, 19, 26). To determine the yield of cholesterol in our method, we processed three concentrations of deuterated cholesterol (cholesterol-d6) alone or in the presence of cellular matrix (2.5 × 10⁶ H1299 cells). We found that the yield of cholesterol-d6 was 77–80% in the absence of cellular matrix and 86–90% in the presence of cellular matrix (Table 5). Though we saw modest destruction of cholesterol in this method, in unlabeled samples it could be accounted for by using cholesterol-d6 as an internal standard to control for any differences in destruction between samples (Table 4). In sum, these data demonstrate that this method can be used to quantify fatty acids and cholesterol from the same sample.

**Successful application of method to small numbers of stable isotope-labeled cells**

To determine whether the current method was capable of measuring MIDs from small numbers of stable isotope-labeled cells, H1299 cells were brought to metabolic and isotopic steady state in glucose-free DMEM supplemented with 25 mM 13C₆-glucose and 5% FBS. In applying the current method to these samples, LCFA data was collected from a 0.5 μl injection, while data from rarer VLCFAs were collected using a 2 μl injection. Dilution tests indicated that consistent MIDs can be collected for cholesterol and many fatty acid species down to the equivalent of 0.4 × 10⁶ H1299 cells (approximately 20 μg of total cellular fatty acid and cholesterol). The 14 fatty acid species included nonessential LCFAs (14:0, 16:0, 16:1 n-7, 18:0, 18:1 n-9, 18:1 n-7), PUFAs (18:2 n-6, 20:3 n-6, 20:4 n-6, 22:4 n-6), and nonessential VLCFAs (20:0, 22:0, 24:0, 24:1 n-9) (Fig. 2 and data not shown).

**Background contamination can be an issue for fatty acid and cholesterol analysis from small numbers of cells.** Fatty acids are present even in the purest solvents and the cleanest glassware, and can cause inaccurate quantitation. Cholesterol contamination is less common than fatty acid contamination, but it can also be an issue when serum is used in collection media. Furthermore, in stable isotope-labeled samples, unlabeled background contamination can distort MIDs. Thus, we assessed fatty acid and cholesterol background using the y-intercept of the H1299 linearity test (Table 4, modified to include only points from 0 to

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**Table 4. Fatty acid and cholesterol response is linear with cell number**

| Fatty Acid | Slope (μg/10⁶ Cells) | y-Intercept (μg) | R² |
|------------|----------------------|-----------------|----|
| 14:0       | 0.70                 | 0.02            | 1.00|
| 16:0       | 9.27                 | 0.06            | 1.00|
| 16:1 n-7   | 2.06                 | 0.04            | 1.00|
| 18:0       | 4.65                 | 0.06            | 1.00|
| 18:1 n-9   | 12.55                | −0.06           | 1.00|
| 18:1 n-7   | 3.71                 | 0.00            | 1.00|
| 18:2 n-6   | 1.24                 | 0.00            | 1.00|
| 20:0       | 0.08                 | 0.00            | 0.999|
| 20:1 n-9   | 0.35                 | 0.00            | 0.999|
| 20:2 n-6   | 0.08                 | 0.00            | 0.999|
| 20:3 n-6   | 0.49                 | 0.01            | 1.000|
| 20:4 n-6   | 0.01                 | 0.00            | 0.999|
| 22:0       | 0.14                 | 0.00            | 0.999|
| 22:4 n-6   | 0.18                 | 0.01            | 0.999|
| 22:6 n-3   | 1.30                 | 0.02            | 1.000|
| 24:0       | 0.67                 | 0.02            | 1.000|
| 24:1 n-9   | 0.57                 | 0.01            | 1.000|
| Cholesterol| 9.70                 | 0.22            | 0.998|

H1299 cells (0–2.5 × 10⁶) were added to tubes and analyzed using the current method. Linear regression was performed after analyte responses were normalized to 19:0 methyl ester (fatty acids) or cholesterol-d6 (cholesterol) internal standards (added before derivatization) and fit to an internal standard curve. Cellular samples were performed in technical triplicate.
The current method provides an approach for quantifying LCFAs, VLCFAs, and cholesterol from limited amounts of stable isotope-labeled cells.

**DISCUSSION**

There has been reinvigorated interest in how lipid metabolism affects the fate and function of cells. Because of this, stable isotope labeling of lipids, which can give unique insights into metabolic flux, is rapidly becoming an important tool for assessing changes in lipid homeostasis. As such, development of new techniques that facilitate the isolation and analysis of lipids is an important objective.

Herein, we describe a method that can be used to analyze both fatty acids and cholesterol from small numbers of stable isotope-labeled cultured cells. To our knowledge, this is the first method optimized to quantify cholesterol as well as LCFAs and VLCFAs from all major animal lipid classes. Importantly, the current method also addresses the critical challenge in analysis of stable isotope-labeled lipids, obtaining sufficient molecular ion mass isotopomer signal from limited starting material and low abundance analytes.

We acknowledge that quantitation of thermally labile lipids (e.g., eicosanoids), endogenous lipid species that may also be generated by ex vivo oxidation (e.g., oxysterols), or highly volatile fatty acids (e.g., short or medium chain FAs) may require alternate methodology (10, 23, 27). Nevertheless, we anticipate that this new method will be useful to a wide variety of scientists investigating the impact of fatty acid and cholesterol homeostasis on cell biology.

Due to the high cost of stable isotope-labeled metabolites, in vitro stable isotope labeling experiments are usually done on the smallest scale feasible. As a result, we validated our method from 0.4–2.5 × 10^6 H1299 cells (approximately 20–125 μg total fatty acids and cholesterol), which represent one 50% confluent well in a 6-well plate to a fully confluent T-25 flask. This is within the range of many in vitro stable isotope-labeling experiments (22, 28–30).

![Fig. 2. FAME MIDs collected from stable isotope-labeled cells using the current method. H1299 cells were brought to isotopic steady state in DMEM containing 100% 13C6-glucose and 5% FBS. Cells were collected at 85% confluency from 60 mm plates in triplicate and were analyzed using the current method. A: 22:4 n-6. B: 24:1 n-9. Data are presented as the mean ± SD (n = 3).](image-url)
and to include appropriate controls to visualize background and limit of detection for each analyte. For example, we have found that in low numbers of cultured cells, background contamination significantly interferes with quantitation of 22:1 n-9. As a result, accurately measuring that fatty acid would require increased sample material.

Fatty acids can be incorporated into a wide variety of complex lipid species. Thus, fully derivatizing fatty acids from all the major classes of complex lipids in animals is critical for accurately quantifying fatty acid pool sizes. For example, our data indicate that poor derivatization of sphingolipids can result in significant errors in quantifying the amount of VLC saturates and monounsaturates in cultured cancer cells (Fig. 1). This issue would likely be amplified in cells with higher concentrations of sphingolipids, such as neurons, glia, or cancer cells of neural origin (31). Additionally, we expect that the current method can facilitate accurate quantification of fatty acid pool sizes in cells containing large amounts of neutral lipids, such as hepatocytes and adipocytes (32, 33). Finally, we hypothesize that this new method will be particularly useful in ensuring accurate quantification of MIDs of fatty acids. Metabolic channeling has been shown to be important in the fluxes of metabolites, including selective incorporation of fatty acid species into particular complex lipids (34). Underrepresenting the labeled fatty acids from one or more classes of lipid as a result of poor extraction or derivatization could interfere with the accuracy of mass isotope labeling studies designed to determine a cell’s reliance on synthesis or import in achieving fatty acid homeostasis.

Cholesterol can be subject to degradation under harsh derivatization procedures (9, 13) and, in our methodology, we did observe modest destruction (Table 5). In unlabeled samples, this issue is remedied by the use of cholesterol-d₄ as an internal standard (Table 4). In stable isotope-labeled samples, cholesterol-d₄ cannot be used because it cannot be fully distinguished from ¹³C-labeled sample cholesterol chromatographically or by mass using our instrumentation. Thus, we used stigmasteranol as the internal standard when analyzing ¹³C-labeled samples. We found that stigmasteranol was resistant to degradation in the current reaction, likely due to its lack of double bonds (Table 5). One potential consequence of using stigmasteranol as an internal standard in the current method is that absolute cellular cholesterol content may be overestimated by up to 15% if a matrix-free internal standard curve is applied. However, we anticipate that relative error in cholesterol content between cellular samples will be significantly reduced as cellular matrix concentrations become closer. Thus, we strongly recommend using similar amounts of cellular matrix to maintain accuracy when assessing cholesterol in isotopic labeling studies. Alternatively, one could employ milder methods developed specifically for cholesterol analysis if small changes in cholesterol parameters are expected or very accurate absolute quantitation is required (35). Finally, when using the current method to observe MIDs of cholesterol, we strongly recommend culturing cells in an amount of ¹³C-labeled substrate that results in modest labeling of the cellular acetyl-CoA pool (no more than 35% ¹³C). This results in a more accurate molecular ion MID by limiting potential interference by the MID of the largest fragment ion, M-15 (36). Use of modest labeling also reduces the possibility of kinetic isotope effects selectively stabilizing ¹³C-labeled cholesterol in the derivatization reaction (37).

In conclusion, we believe that the broad range of input sample material that this method can accommodate, combined with heightened efficiencies of lipid derivatization and sample usage, make this new approach highly amenable to a variety of biologic applications, and will facilitate studies interrogating lipid homeostasis.

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