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Determination of stable isotopic enrichment and concentration of glycerol in plasma via gas chromatography–mass spectrometry for the estimation of lipolysis in vivo

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Abstract

Measuring glycerol's rate of appearance into the plasma compartment provides an excellent estimation of whole-body lipolysis. The glycerol rate of appearance can be calculated by estimating the plasma dilution of continuously infused stable or radioactive isotopes of glycerol. Previously, determination of glycerol stable isotopic enrichment has required either chemical ionization gas chromatography–mass spectrometry (GC–MS) or electron impact ionization GC–MS in which a fragment containing only a portion of the glycerol molecule was measured. The present method uses *tert*.-butyldimethylsilyl (tBDMS) derivatization and electron impact ionization to measure a fragment including the entire glycerol molecule. The method determines concentration and enrichment of plasma glycerol in a simple, precise, and cost-efficient manner, providing a basis from which lipid homeostasis can be assessed.

Keywords: Stable isotopic enrichment; Glycerol

1. Introduction

Non-esterified free fatty acids released during lipolysis can immediately undergo intracellular reesterification and reincorporation into triglycerides. However, because peripheral tissues lack the enzyme glycerol kinase to phosphorylate glycerol intracellularly, the glycerol released during lipolysis must exit the cell, enter the circulatory system, and be transported to the liver for reutilization. Therefore, the dilution of glycerol isotopes provides an ideal measure of glycerol rate of appearance resulting in an estimate of whole-body lipolysis in vivo.

The oldest methods of measuring glycerol rate of appearance used tritium- or carbon-14-labeled glycerol. However, these methods are problematic with regard to safety and methodological considerations. This caused investigators to turn to stable isotope methodology for estimation of lipolysis, particularly in clinical situations. However, a majority of the previously described methods for measuring stable isotopic tracers of glycerol required gas chromatography–mass spectrometry (GC–MS) equipment with a chemical ionization (CI) source [1–3], a feature that many table-top mass spectrometers do not possess.

In 1992, an alternative method for measuring d_5-

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glycerol was described using electron impact (EI) GC–MS and tris-trimethylsilyl (TMS) derivatization [4]. However, the most suitable fragment for detection with glycerol-TMS derivatives contains only two of three carbons and only three of five hydrogens from the glycerol molecule, as one carbon and two hydrogens are lost with the neutral fragment. This poses several disadvantages. First, the contribution of background measurements to total enrichment is increased and sensitivity is reduced when the fragment containing the glycerol molecule is lost. For d_5-glycerol, the fragmentation pattern in which two hydrogens are not included results in m+3 enrichment being measured versus m+5 if the fragment contained the entire glycerol molecule. For uniformly labeled ^{13}C-glycerol, this fragmentation results in M+2 enrichment being measured versus m+3 if the fragment contained the entire glycerol molecule. In either case, glycerol enrichment would be measured above a greater background when the fragment containing one carbon and two hydrogen atoms from the glycerol is lost, thereby reducing sensitivity. Second, by losing one carbon on the glycerol isotope, the use of ^{13}C-labeled glycerol is limited. For example, it would have to be assumed that glycerol labeled in either the one or three positions would lose one-half of its enrichment when the TMS-fragment is lost. Furthermore, analysis of secondary labeling of glycerol from the infusion of uniformly labeled [^{13}C]glucose using this method would be inconclusive. Finally, although there are no known examples of this with glycerol in vivo, any selective loss of label due to metabolism or hydrogen exchange would not be detected with the TMS-derivatization method. Thus, it is important to develop a method that would measure the intact glycerol molecule within its fragmentation pattern.

The use of *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) to form *tert.*-butyldimethylsilyl (tBDMS) derivatives appeared as early as 1975 [5]. In 1984, Schwenk et al. [6] reported the potential for GC–MS analysis of several physiological compounds found in plasma including glycerol, using tBDMS and an EI ionization source. However, these data were based on chemical standards and not on plasma samples. Furthermore, while other reports have documented the use of MTBSTFA as a derivative reagent for many compounds found in plasma [6–13], the potential utility of MTBSTFA to measure glycerol in human plasma has not been reported. The objective of this research was to develop a simple and reliable method to quantitate the enrichment of a stable isotopic tracer of glycerol in plasma through the use of EI ionization GC–MS, with the goal of applying the method to any form of glycerol stable isotopic tracer.

2. Experimental

2.1. Equipment

The GC–MS system (Hewlett-Packard, Palo Alto, CA, USA) consisted of a gas chromatograph (Model 5890), a mass selective detector (MSD; Model 5970B), and a computer system (9000 Serial Model 300). The gas chromatography system was equipped with an automatic sample injector (Model 7673A). Initially, the oven temperature was set at 100°C, and the injection port and transfer line temperatures were set at 270 and 280°C, respectively. The chromatographic capillary column was 25 m long with a 0.22-mm I.D. and a 0.11-μm film of cross-linked methyl silicone gum phase (HP-1, Hewlett-Packard, Avondale, PA, USA). A constant flow of research-grade helium (99.9999% pure) traversed the column at 1.1 ml/min. The mass spectrometry (MS) system was a quadrupole MSD with an EI ionization source. The MSD was placed in scan mode to determine mass spectra for isotopes of glycerol, but was operated in selected-ion monitoring mode (SIM) when tracer:tracee ratio measurements were required.

2.2. Reagent preparation

ACS reagent-grade glycerol (99.5% pure) was purchased from Sigma (St. Louis, MO, USA). [1,1,2,3,3-^{2}H$_5$]1,2,3-Propanetriol (d_5-glycerol) and [2-^{13}C]glycerol were obtained from Cambridge Isotope Laboratories (98% isotopic purity, Woburn, MA, USA). Perchloric Acid (PCA; ACS reagent grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pyridine (derivatization grade) and *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroaceta-

mide containing 1% *tert*.-butyldimethylchlorosilane (MTBSTFA+1% tBDMCS) were obtained from Regis (Morton Grove, IL, USA). The ion-exchange columns were prepared by adding 2.5 ml of AG-50W-X8 cation exchange resin in the hydrogen form (50:50, v/v; water–resin; Bio-Rad Laboratories, Hercules, CA, USA) to filtering columns, placing 2.5 ml of AG-1-X8 anion exchange resin (50:50, v/v, Bio-Rad) on top of the cation resin, and washing with 5×1 ml deionized water.

2.3. Preparation of standards, samples, and controls

Initially, both natural glycerol (500 μ*M*) and d_5-glycerol (100 μ*M*) stock standards were prepared in deionized water. Dilutions of the stock standards were used to determine elution times, mass spectra, and reproducibility. An internal standard (50 nmol of [2-^{13}C]glycerol) was added to each ml of the natural glycerol standards to establish a standard curve from which the concentration of glycerol in other samples could be calculated. Natural and d_5-glycerol standard curves were analyzed with each batch of unknowns. Standard solutions were stable for greater than 1 year when stored at −80°C and for greater than 6 months when stored at 4°C.

Blood was collected in vacuum tubes containing 15 mg dry disodium ethylenediaminetetraacetic acid (EDTA) per tube (Terumo Medical Corporation, Elkton, MD, USA). The blood was centrifuged at 4°C for 10 min at 2000 *g*, and the plasma was recovered and used for analysis. An internal standard (50 nmol of [2-^{13}C]glycerol per ml of plasma) was added to the plasma to determine the concentration of natural glycerol. Studies in our laboratory indicate plasma may be stored at −20°C for at least 1 year with no detectable change in glycerol isotopic enrichment.

Plasma samples to provide quality control (natural and enriched controls) were prepared from aliquots of pooled plasma. Enriched control plasma standards were prepared by combining 1 ml of the pooled plasma with five different quantities of the d_5-glycerol stock solution (0, 50, 100, 150, and 200 μl). Aliquots of the natural and enriched control plasma were analyzed daily to assess reproducibility.

2.4. Plasma glycerol analysis procedure

Prior to analysis, chemical standards, controls, and plasma samples were allowed to completely thaw. One ml of sample was added to a 12-ml plastic sample tube containing 1 ml of 4% PCA to precipitate any proteins. The tube was vortexed and centrifuged at 2000 *g* for 20 min at 4°C. The protein-free supernatant was poured directly onto an ion-exchange column prepared as described above and rinsed with four 1-ml aliquots of deionized water. Plasma components with positive and negative charges attached to the column, while the neutral plasma components, including glycerol, passed through the column. All of the eluate was collected in a 12-ml plastic sample tube and dried under a stream of dried compressed air at room temperature until approximately 200 μl of liquid remained (~15 h or overnight). At that point, the sample temperature was increased to 50°C while continuing the air stream until the sample was completely dried (~2 h). One hundred μl of pyridine and 100 μl of MTBSTFA were added to the dried residue, and the mixture was vortexed. The solution was allowed to remain at room temperature for 30 min allowing derivatization to reach completion. The derivatized sample was transferred into a plastic micro-injection autosampler vial (250 μl; Sun Division, Comar, Wilmington, NC, USA) and was sealed.

After a 2-μl splitless injection of the derivatized sample onto the column, the oven was held at the initial temperature of 100°C for 0.5 min, increased to 300°C at 25°C/min, and maintained at 300°C for 1.5 min. Thus, the total run time was equal to 10 min with glycerol and d_5-glycerol eluting at approximately 6.0 min. Using SIM, the major fragments monitored for the tBDMS derivatives of glycerol and d_5-glycerol were the [M-57] ion fragments, 377 *m*/*z* and 382 *m*/*z*, respectively. When d_5-glycerol was used as the tracer, the enrichment was quantitated in plasma as the ratio of d_5-glycerol:glycerol (ion abundance of 377/382 *m*/*z*).

Replicate injections of the chemical standards were made before and after each series of plasma samples to establish two standard curves: (1) the internal standard versus natural glycerol, and (2) 377 *m*/*z* versus 382 *m*/*z*. During a large series of plasma samples, the standards were injected every 6 h.

Enriched control plasma samples also were analyzed with each series of unknowns to establish intra- and inter-assay variation.

The recovery of the deproteinization and extraction procedures were evaluated by adding 0.1 μCi of [1,3-^{14}C]glycerol (NEN Research Products, Boston, MA, USA) to 10 ml of pooled plasma. One-ml aliquots of this solution were added to three scintillation vials. One-ml aliquots of the remaining solution were deproteinized and processed in the same manner as the experimental samples. After deproteinization, three of the aliquots were placed into scintillation vials. After the remaining four aliquots were passed over the ion-exchange column and dried, the dried residue was dissolved in one ml of water. These aliquots were added to scintillation vials, and 10 ml of scintillation solution (ICN Biomedicals, Irvine, CA, USA) was added to each of the scintillation vials. Radioactivity was measured by a liquid scintillation counter (LS 6000 Beckman Instruments, Fullerton, CA, USA).

2.5. Measurement of glycerol rate of appearance in vivo

Studies to determine the rate of appearance for glycerol were conducted in both humans ($n=3$) and canines ($n=3$). Both species were studied after an overnight fast, so they were post-absorptive. In addition, three humans were studied in the basal condition and after 60 min of a 60% maximum exercise on a recumbent bicycle. d_5-Glycerol was continuously infused into a peripheral vein at the rate of 0.12 μmol kg^{-1} min^{-1}. After a tracer equilibration period (>60 min), serial arterial blood samples were collected every 10 min for 30 min in canines. After a similar equilibration period, arterialized blood samples were collected every 10 min for 30 min under basal conditions in humans using the heated-hand method [14]. Only one arterialized blood sample was collected at the end of exercise in humans. The rate of appearance for glycerol (Ra_{Gly}) was calculated as in Eq. (1) by dividing the rate of tracer infusion (i) by the tracer:tracee enrichment (E) and subtracting the rate of tracer infusion from this quotient.

$$Ra_{Gly} = (i/E) - i \quad (1)$$

3. Results

The major derivative from glycerol using MTBSTFA contained three *tert.*-butyldimethylsilylated (tBDMS) groups (Fig. 1A,B). Other potential derivatives, such as mono- and di-tBDMS-glycerol, were not observed. The EI ionization mass spectrum for tBDMS-glycerol is shown in Fig. 1A and that for tBDMS-d_5-glycerol is presented in Fig. 1B. The fragmentation pattern of natural and d_5-glycerol in plasma samples and chemical standards were identical. The ion fragments most frequently used for monitoring isotopic enrichment of tBDMS derivatives correspond to those which have lost fragments of mass=15 (methyl group, [M-15]), mass=57 (*tert.*-butyl group, [M-57]), mass=71 (*tert.*-butyl-CH_2, [M-71]) and mass=145 (tBDMS-OCH_2, [M-145]). The most suitable fragments for SIM of glycerol were the [M-57], corresponding to 377, 378, and 382 m/z for tBDMS-glycerol, tBDMS-[^{13}C]glycerol and tBDMS-d_5-glycerol, respectively. Other suitable fragments for monitoring were not found since the smaller fragments lost one or more carbon and hydrogen atoms from glycerol.

Based upon recovery of radioactive glycerol added to pooled plasma controls, the calculated percent recovery of glycerol after deproteinization was 98.0% and that after extraction was 93.4%. Chemical standards and plasma samples presented similar chromatograms that contained baseline resolution of the base peak at 377 m/z, the internal standard peak at 378 m/z, and the tracer (if present) peak at 382 m/z (Figs. 2 and 3). All three peaks eluted at approximately 6.0 min. The elution time for natural and d_5-glycerol in plasma samples and chemical standards were identical.

The interference of other compounds or non-specific contaminants at 377, 378, and 382 m/z was not observed. Samples in which d_5-glycerol was not added had a non-detectable relative abundance at 382 m/z (m+5). Therefore, the natural background enrichment in such samples was negligible.

The isotopic enrichment curve of control plasma standards enriched with d_5-glycerol was linear over the range tested (0–15% enrichment). Regression analysis indicated strong agreement between theoretical and observed percentage enrichment values ($r^2=0.9993$). The observed percentage enrichment values

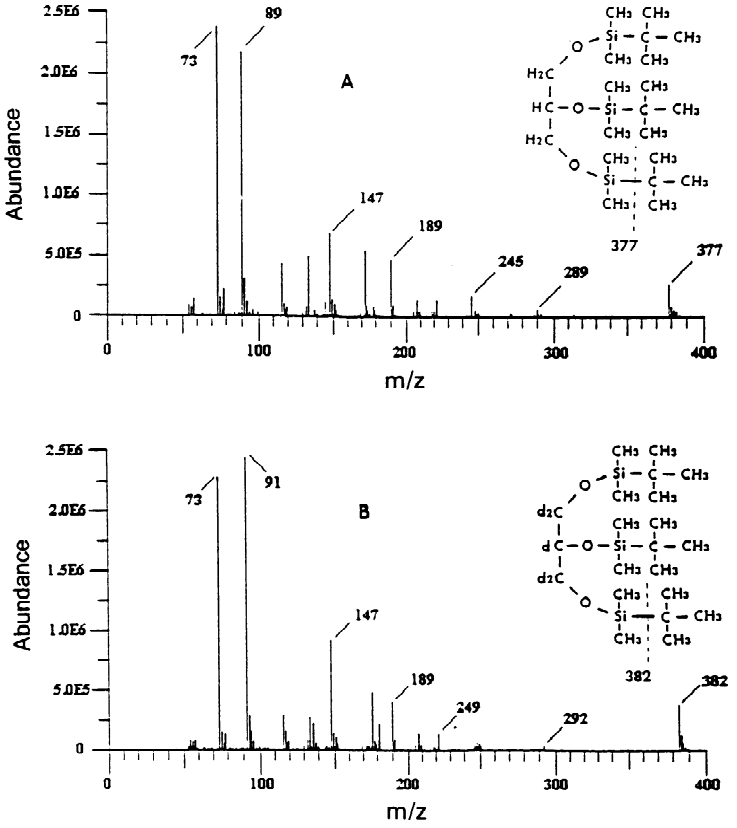

Fig. 1. EI ionization mass spectra and structure of (A) tBDMS natural glycerol derivative, and (B) tBDMS d_5-glycerol derivative.

from SIM were consistently stable for at least 48 h after the completion of derivatization. Ten replicate runs were made from each enriched control plasma sample to evaluate the reproducibility of the isotope ratio measurements. The intra-assay coefficient of variation calculated from these replicates was 1.06%. The inter-assay coefficient of variation of 20 assays performed on different days was 1.81%. Finally, the standard curve for natural glycerol concentration was linear over the range of 0–500 μ*M* with a correlation coefficient of 0.9977.

Whole-body estimates of basal lipolysis were made in humans and dogs by measuring glycerol rates of appearance (Ra) in vivo. Glycerol Ra (means±standard error of the mean) for overnight-fasted healthy dogs ($n=3$) and humans ($n=3$) were 2.39±0.26 and 1.73±0.16 μmol kg^{-1} min^{-1}, respectively. Plasma glycerol concentrations in these groups were 139±12 and 79±6 μ*M*, respectively. In vivo rates of whole-body lipolysis were also estimated in humans ($n=3$) after exercise. Glycerol Ra for three healthy overnight-fasted humans before and after a moderate bout of exercise were 1.59±0.18 and 4.25±0.43 μmol kg^{-1} min^{-1}, respectively. Plasma glycerol concentrations before and after exercise were 77±8 and 224±48 μ*M*, respectively.

4. Discussion

Measuring the rate of lipid breakdown is important to the understanding of lipid and energy homeostasis during various metabolic perturbations and disease states. After the intracellular catabolism of a triglyceride to three individual fatty acids and glycerol, fatty acids can be reesterified and reutilized within the same cell, but glycerol must be released into the circulation to be utilized. Therefore, the rate of

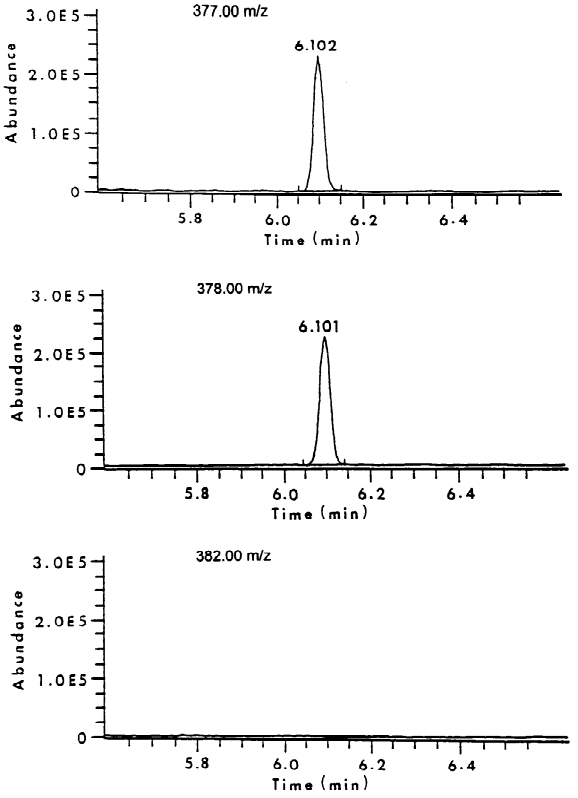

Fig. 2. The EI GC–MS selected ion chromatograph of glycerol from a pooled plasma control sample without d_5-glycerol added. No peaks were detected for ion 382.00 *m/z*.

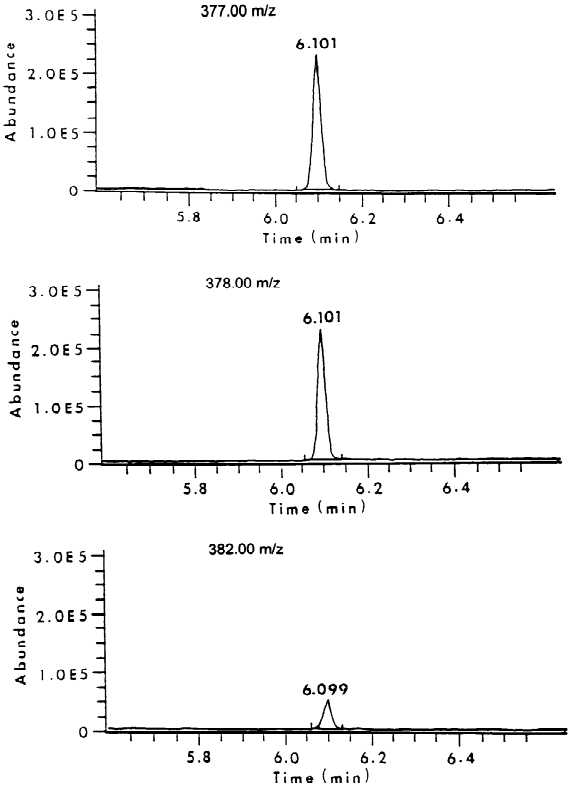

Fig. 3. The EI GC–MS selected ion chromatograph of glycerol from a pooled plasma control sample enriched with d_5-glycerol. A significant peak was detected for ion 382 *m/z*.

glycerol appearance is the most accurate reflection of lipolysis. Furthermore, the difference between the molar rates of glycerol appearance (multiplied by 3) and fatty acid appearance is an estimate of the rate of fatty acid reesterification. By combining whole-body measures of lipolysis, fatty acid reesterification and oxidation, and lipogenesis, whole-body lipid homeostasis can be established. However, the measure of glycerol flux is central to these measurements.

While an increase in plasma glycerol may be an indication of increased lipolysis, plasma glycerol concentration is the result of combined alterations in glycerol release from the periphery and uptake by the liver. Increased glycerol availability has been demonstrated to increase hepatic uptake of glycerol [15]. Therefore, increases in plasma glycerol concentration would tend to underestimate lipolysis. Rates of glycerol appearance for healthy canines after an overnight fast were nearly 40% greater than for healthy overnight-fasted humans. Although the data are too limited to conclude with certainty that the lipolytic rate in dogs is greater than in humans, it is interesting to note that the variability associated with these measurements is similar between the two species. The degree to which this variation may diminish with the control of variables such as gender, age, or body composition needs to be assessed. Based upon enzymatic data and plasma glycerol concentrations, it has been suggested that exercise evokes a significant increase in human lipolysis. This is consistent with our limited results in humans, as the glycerol rate of appearance was more than 2-fold greater at the conclusion of a 60-min bout of moderate-intensity exercise.

Several different approaches have been used to estimate glycerol turnover. The oldest method involves the measurement of the dilution of a radioac-

tive glycerol tracer [16,17]. However, this technique has several limitations. First, the exposure of human subjects and researchers to radiation poses a safety risk. In addition, the disposal of the radioactive waste is costly. Finally, glycerol-specific radioactivity measurements require separate measurements of radioactivity and cold glycerol concentration. The errors associated with each of these two measurements are combined when specific radioactivity is calculated, reducing the precision of this method. Conversely, while the mass of radioactive isotopes infused during in vivo studies is negligible, the mass of stable isotopic tracer infusions must be considered in the total glycerol rate of appearance.

Previously described methods to measure stable isotope tracers of glycerol have limitations. Many require the use of CI mass spectrometry, a feature that many table-top mass spectrometers do not possess. An alternative method utilizes EI ionization and derivatization with TMS. However, a major limitation of this method is that the fragment with the greatest relative intensity does not contain the entire glycerol moiety, and thereby, several of the labels are lost (two of the five deuteriums in d_5-glycerol and half of ^{13}C in [1-^{13}C]glycerol). This caveat has potential to be a significant limitation due to the increased background contribution to total enrichment, decreased sensitivity, the difficulty in using this method to assess secondary glycerol labeling, and the fact that any potential metabolic alterations to specific labels on the glycerol molecule may remain undetected. The present method uses tBDMS derivatization and EI fragmentation, thereby allowing measurement of the entire glycerol molecule.

A variety of techniques, including enzymatic [18], GC–flame ionization detection [19,20], and GC–MS [1,4,5] procedures, have been used to measure glycerol concentrations in physiological fluids. The method described herein allows measurement of both glycerol concentration and glycerol isotopic enrichment from the same sample. Furthermore, while we describe the use of this method with plasma, extrapolation to other biological fluids, such as cerebral spinal fluid or urine, would be appropriate.

In summary, a simple, precise, cost-efficient method has been described for the measurement of glycerol stable isotopic enrichment as well as for the determination of glycerol concentrations. This method offers several advantages over commonly used methods for determination of glycerol. The technique requires simple sample preparation and utilizes EI mass spectrometry to obtain measurement of the entire glycerol molecule. The use of standard non-specialized GC–MS equipment allows for near-continuous operation with low maintenance. In addition, the method uses well-established tBDMS chemistry, has a relatively short analysis time, and has a minimal cost. In conclusion, accurate and reproducible analysis of glycerol is permitted, providing a basis from which in vivo lipid homeostasis can be assessed.

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