Rapid measurement of plasma free fatty acid concentration and isotopic enrichment using LC/MS

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Abstract **Measurements of plasma free fatty acids (FFA) concentration and isotopic enrichment are commonly used to evaluate FFA metabolism. Until now, gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS) was the best method to measure isotopic enrichment in the methyl derivatives of ^{13}C-labeled fatty acids. Although IRMS is excellent for analyzing enrichment, it requires time-consuming derivatization steps and is not optimal for measuring FFA concentrations. We developed a new, rapid, and reliable method for simultaneous quantification of ^{13}C-labeled fatty acids in plasma using high-performance liquid chromatography-mass spectrometry (HPLC/MS). This method involves a very quick Dole extraction procedure and direct injection of the samples on the HPLC system. After chromatographic separation, the samples are directed to the mass spectrometer for electrospray ionization (ESI) and analysis in the negative mode using single ion monitoring. By employing equipment with two columns connected parallel to a mass spectrometer, we can double the throughput to the mass spectrometer, reducing the analysis time per sample to 5 min. Palmitate flux measured using this approach agreed well with the GC/C/IRMS method. This HPLC/MS method provides accurate and precise measures of FFA concentration and enrichment.—**Persson, X-M. T., A. U. Błachnio-Zabielska, and M. D. Jensen. **Rapid measurement of plasma free fatty acid concentration and isotopic enrichment using LC/MS. *J. Lipid Res.* 2010.** 51: **2761–2765.**

Supplementary key words mass spectrometry • liquid chromatography • palmitate • flux

Fatty acids are an important class of molecules for both energy metabolism (1, 2) and cell signaling (3, 4). The vast majority of fatty acids in humans are stored in a relatively inert form as adipose tissue triglyceride, being released into the circulation as free fatty acids (FFA) via lipolysis. Disturbances in FFA metabolism, including accelerated rates of FFA release under basal (5) and postprandial (6) conditions, are characteristic features of obesity. Plasma FFA concentrations are an imperfect indicator of FFA kinetics, as evidenced by the greater lipolysis rates in women than men at comparable FFA concentrations (7) and the divergence of FFA concentrations and flux during exercise (8, 9). Robust isotope dilution techniques have been developed to measure FFA kinetics, including radiotracer methods using high-performance liquid chromatography (HPLC) (10), gas chromatography-mass spectrometry (GC/MS) (11), and gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS) (12).

Each of these methods requires an isolation and derivatization step followed by a relatively time-consuming chromatography separation, potentially limiting sample throughput. To improve efficiency, we developed a new method for simultaneous measurement of concentration and stable isotopic enrichment of plasma FFA. Our method uses HPLC electrospray ionization (ESI) quadrupole mass spectrometry in the selected ion monitoring (SIM) mode. The method is simple and reliable for monitoring changes in plasma FFA concentration and enrichment. By using HPLC for the separation, we can avoid the derivatization step, allowing more rapid sample processing. In addition, by employing a tandem HPLC injection system, we are able to obtain sample data every 5 min. The results compare favorably with the GC/C/IRMS (12) approach for measuring palmitate flux using ultra-low tracer infusion rates.

MATERIALS AND METHODS

Supplies

Palmitic acid, sodium palmitate, sodium heptadecanoate, essentially fatty acid free albumin, potassium phosphate, potassium biphosphate, heptane, 2.0 M (trimethylsilyl)diazomethane solution, sulfuric acid, and ammonium acetate were purchased form Sigma-Aldrich Chemicals (St. Louis, MO). [$^{13}C_{16}$]palmitic acid was supplied by Cambridge Isotope Laboratories (Andover, MA). HPLC-grade acetonitrile, isooctane, isopropanol, methanol, and water were obtained from Burdick and Jackson Chemicals

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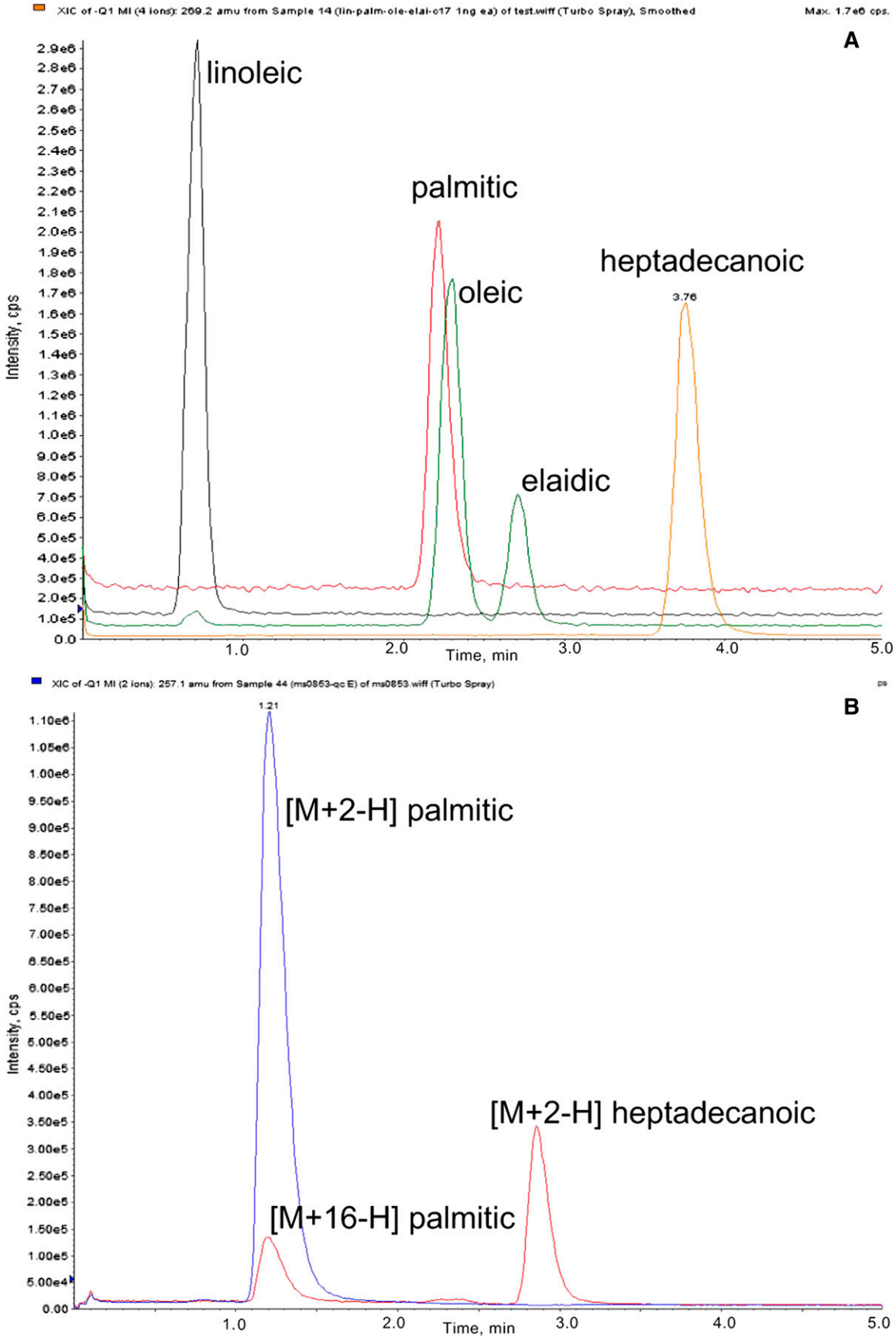

Fig. 1. A: Chromatograph separation of linoleic acid, palmitic acid, oleic acid, eladic acid, and heptadecanoic acid (internal standard) by LC/MS. The delay time set into the acquisition program after the injection is 2 min for this chromatogram. B: A typical plasma chromatogram from LC/MS system. Depicted are the M+2 peaks for palmitate and heptadecanoate and the M+16 peak for palmitate of a

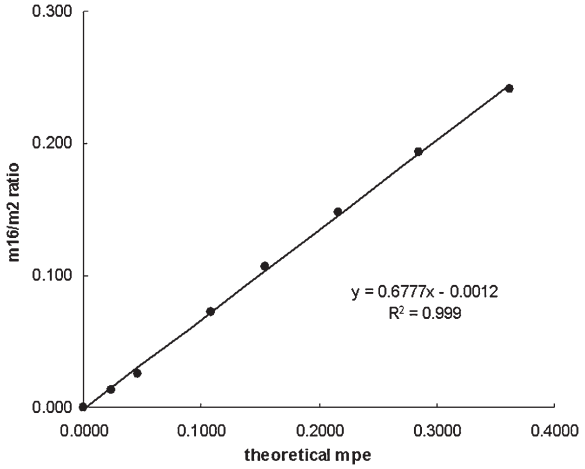

Fig. 2. $^{13}C_{16}$ palmitate enrichment curve obtained on LC/MS.

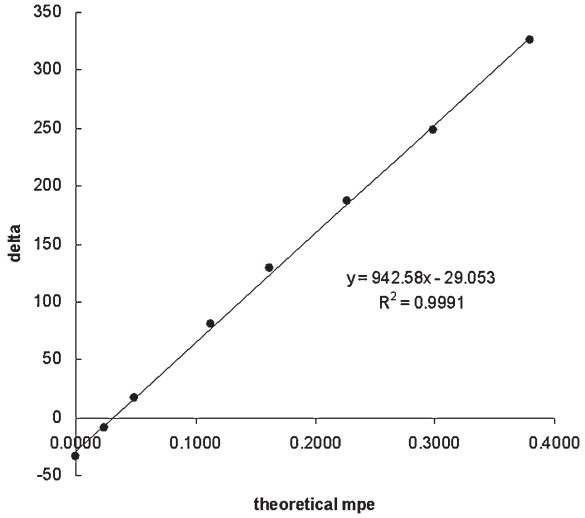

Fig. 3. $^{13}C_{16}$ palmitate enrichment curve obtained on GC/IRMS.

(Morristown, NJ). Four-milliliter screw-cap vials and 12 ml screw cap extraction tubes were supplied by Fisher Scientifics (Pittsburg, PA). All caps were lined with Teflon liners (Arthur H. Thomas, Philadelphia, PA). The GC column and LC column were purchased from Agilent Technologies (Santa Clara, CA) and Supelco (St. Louis, MO), respectively.

Stock solutions and standards

A mixed solution of 10 mM of potassium phosphate and diphosphate were prepared in water. The pH of the phosphate buffer was brought up to 7.6 using 10 N sodium hydroxide. Two percent albumin solution was made by slowing adding 2 g of albumin to 100 ml of 10 mM phosphate buffer. Stock palmitate concentration standard of 400 μM and heptadecanoate internal standard of 60 μg/ml were made in 2% albumin solution. A 1 N sulfuric acid solution was prepared in water. An eight-point (0.00%–0.40%) [$^{13}C_{16}$]palmitic enrichment curve was prepared in heptane. The curve was split into two separate aliquots for analysis in either LC/MS or GC/C/IRMS later.

In vivo experiments

Plasma samples were obtained from six lean and obese volunteers participating in two ongoing research studies of free fatty acid turnover in order to obtain data from individuals with a wide range of palmitate flux. These protocols were approved by Mayo Institution Review Board, and all participants provided signed, informed consent. On the day of the study, each subject was infused with [$^{13}C_{16}$]palmitate at rates of 2 or 6 nmol•kg^{-1}•min^{-1}. Blood samples were collected from a retrograde IV catheter using the heated hand vein approach and aliquotted into EDTA-containing tubes. Plasma was obtained by refrigerated centrifugation and stored at −80°C for analyses at a later date.

Procedures

On the day the samples were processed, a six-point palmitate concentration curve was constructed by taking 250 μl of the 400 μM stock solution and making dilutions with 10 mM phosphate buffer to yield 400, 200, 100, 50, 25. and 0 μM standards. Aliquots of 250 μl plasma were also placed into extraction tubes. A quantity of 50 μl of the heptadecanoate internal standard solution was spiked to each concentration standard and each plasma sample. The standards and plasma samples were extracted with freshly prepared Dole solution. The extracts were taken to dryness on a Speed Vac (Savant Instruments, Farmingdale, NY) and were analyzed first on the LC/MS system and then on GC/C/IRMS system as described below.

LC/MS

Concentration and isotopic enrichment of palmitic acid from extracted plasmas were simultaneously measured against an extracted concentration standard curve as well as an enrichment standard curve on the Applied Biosystem API5000 mass spectrometer-MS (Foster City, CA) coupled with a Cohesive TX2 liquid chromatography system-LC (Franklin, MA). This system provides two LCs connected in parallel to the mass spectrometer, allowing twice the number of samples to be analyzed by using tandem sample injection. The LCs were controlled by Aria software (Cohesive Technology). Palmitic and heptadecanoic acids were separated on the LCs using an Ascentis C18, 2.7 μm, 2.1 × 150 mm column using two buffers. Buffer A was 80% acetonitrile, 0.5 mM ammonium acetate; buffer B was 99% acetonitrile, 1% 0.5 mM ammonium acetate. The flow rate was 0.4 ml/min, and the gradient conditions were as follows: 0–8 min isocratic at 55% B, 8–8.5 min 55⇒95% B, 8.5–10 min isocratic at 95% B, 10–10.5 min 95⇒55% B, and 10.5–12 min isocratic at 55% B. One tenth of the volume of each concentration standard and each plasma sample were resuspended in 400 μl of buffer A prior to injecting 10 μl onto the LC/MS.

The chromographic separation of linoleic acid, palmitic acid, oleic acid, eladic acid, and heptadecanoic acid (internal standard) are depicted in **Fig. 1A**. Although palmitic and oleic acid peaks overlap, they are distinguishable by MS, whereas eladic acid, which is indistinguishable from oleic acid by MS, is easily separated by chromatography. Fig. 1B depicts the M+2 peaks for palmitate and heptadecanoate and the M+16 peak for palmitate of a plasma sample from a volunteer receiving a [U-^{13}C]palmitate infusion. Palmitate elutes at 6.2 min and heptadecanoate acid at 7.1 min; however, in Fig. 1B the retention times are shown as 1.2 and 2.9 min because of the 3 min delay set in the LC method before diverting the flow to the MS for acquisition. The MS was set to ac-

plasma sample from a volunteer receiving a [U-^{13}C]palmitate infusion. Palmitate elutes at 6.2 min and heptadecanoate acid at 7.1 min; however, the retention times are shown as 1.2 and 2.9 min because of the 3 min delay set in the LC method before diverting the flow to the MS for acquisition when focusing only on palmitate concentration and enrichment.

quire for only 5 min, allowing MS data acquisition from the second LC to begin while the first LC finishes its 12 min gradient.

The MS was equipped with a turbo ion spray interface with the heater set at 60°C, spray voltage at 5500 V, sheath gas at 50, sweep gas at 40, and transfer capillary at 270°C. Palmitate, [$^{13}C_{16}$]palmitate and heptadecanoate were selectively monitored at *m/z* 257, *m/z* 271 in negative mode. Palmitate was monitored as $[M+2-H]^-$ and $[M+16-H]^-$. Heptadecanoic was also monitor as $[M+2-H]^-$. Therefore *m/z* 271 was either [$^{13}C_{16}$]palmitate or heptadecanoate, depending on where it eluted in the LC gradient.

GC/C/IRMS

The second aliquot of the enrichment standards as well as the remaining plasma samples were methylated using (trimethylsilyl) diazomethane and methanol for analysis on the GC/C/IRMS as previously described (12). The enrichment of [$^{13}C_{16}$]palmitate, but not the concentration, was measured using this approach.

RESULTS

The enrichment standard curves are shown in **Figs. 2** and **3** for the LC/MS and GC/C/IRMS systems, respectively.

We measured $^{13}C_{16}$ palmitate enrichment in 80 different plasma samples belonging to six study subjects using both systems. **Fig. 4** shows the correlation between the two methods. As expected, the slope is not equal to 1.0 because the LC/MS measures the M+16 whereas the GC/C/IRMS measures total ^{13}C enrichment. However, the relationship between the tracer:tracee ratios obtained from the two instruments was such that the intercept was not different than 0, and slope was not different than 1.0 (data not shown).

Palmitate flux during 20 distinct steady-state intervals was calculated using the average palmitate enrichment from the two analytical approaches and the tracer infusion rates. For the LC/MS approach, we used the infusion rate of the [$^{13}C_{16}$]palmitate (M+16) (the tracer purity averaged 85.2%), whereas for the GC/C/IRMS we used the infusion rate of the [^{13}C]palmitate (tracer purity of 99% ^{13}C). Palmitate flux averaged 155 ± 71 and 147 ± 68 μmol/min (P = NS) for the LC/MS and GC/C/IRMS methods, respectively, and were well correlated (R^2 = 0.94, $P < 0.00001$). The slope of the relationship was not different than 1.0, and the intercept not different than 0. **Fig. 5** depicts the flux measures using the two approaches.

The palmitate concentration curve was linear from 0 to 400 μmol/l and was generated by monitoring M+2 species of both palmitate and heptadecanoate. A plasma quality control (QC) sample was analyzed with each set of samples to compare with the concentration value obtained from the HPLC-UV method that is routinely used in an adjacent laboratory (10). The QC palmitate concentration obtained from six different sets gave the value of 143 ± 1 μM, similar to the average value observed for the same QC using HPLC-UV of 145 μM.

DISCUSSION

A number of techniques are used to measure FFA concentration and isotopic enrichment. Most often FFA concentrations are measured using GC equipped with flame ionization detector (GC/FID) or coupled to mass spectrometer (13, 14). Gas chromatograph coupled to mass spectrometer (GS/MS) allows measurements of both concentration and isotopic enrichment (15, 16). Gas chromatography methods for FFA often employ a time-consuming lipid extraction procedure, such as solid phase extraction or liquid-liquid extraction, separation of FFA by TLC, and derivatization to fatty acid methyl esters for measurement by GS/MS (15, 17, 18). To date, the most sensitive method for measuring low levels of isotopic enrichment is by GC/C/IRMS (12, 14, 19). Although GC/C/IRMS is very accurate and precise in measuring very low enrichment samples, thus allowing low tracer infusion rates to be used, its narrow dynamic range compromises concentration measurement. Therefore, it is not the instrument of choice for quantifying concentration, especially in a protocol where lipolysis is either suppressed or increased.

This new method for simultaneously measuring plasma FFA concentration and fatty acid isotope enrichment by HPLC/MS involves extraction of FFA from plasma and direct injection of extract into the HPLC system, thus elimi-

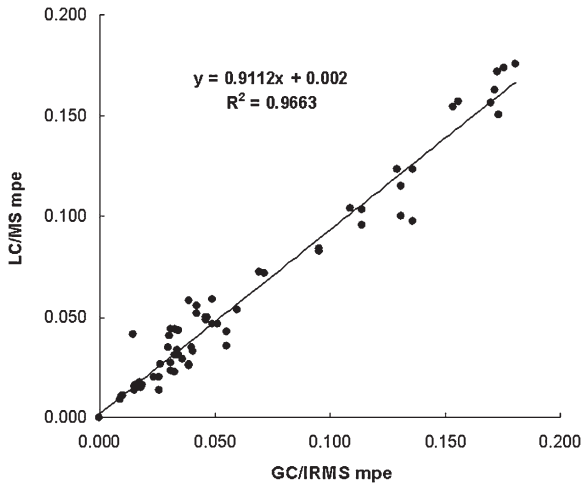

Fig. 4. Plasma mol% excess, LC/MS versus GC/IRMS.

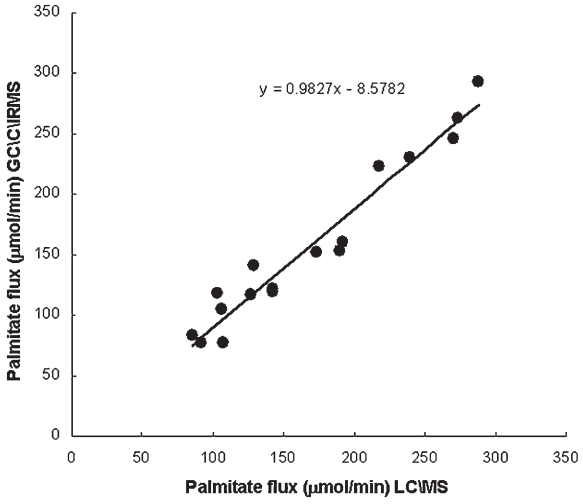

Fig. 5. Palmitate flux measured using LC/MS versus GC/IRMS.

nating extensive sample preparation. Because the method is specific for FFA without contribution from esterified fatty acids, no separation step using TLC or solid phase extraction is required. We believe a major advantage of this method is that the fatty acids do not require derivatization before analysis. After extraction, the samples can be dried, resuspended in solvent A used in HPLC gradient, and injected on the column. The HPLC system we employ uses two columns connected in parallel to a mass spectrometer, enabling sample throughput to double, essentially reducing the run time of plasma palmitate samples to 5 min. The reduced analysis time makes this method especially suitable for analysis of large numbers of samples.

After chromatographic separation, the samples are directed to a mass spectrometer. The concentration and enrichment are measured using SIM in negative electrospray ion mode. To optimize ionizing conditions, we used palmitic acid (C16:0) as the standard fatty acid. We were able to simultaneously measure palmitate concentration and enrichment in the low enrichment range by LC/MS. By using the M+2 as the base peak for measuring M+16 abundance, we were able to accurately assess palmitate enrichment at low levels typically best measured by GC/C/IRMS. This LC/MS method for measuring isotopic enrichment compared extremely well with the GC/C/IRMS approach to measure palmitate flux.

We found only one publication describing the use of HPLC/MS to measure fatty acid enrichment (20). The authors describe an LC/MS in negative mode using SIM approach to measure deuterium-labeled fatty acids. They were able to trace the presence of an orally administered deuterium-labeled fatty acid in rat plasma lipids using this approach.

In summary, the method we describe allows the measurement of palmitate concentration and flux using an ultra-low dose [U-^{13}C]palmitate tracer and LC/MS. The technique enables the accurate measurement of FFA with a substantial reduction in both sample processing and analytical run time. We believe that this approach will improve the efficiency of laboratory analysis of plasma samples from FFA flux studies.

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