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Total Serum Fatty Acid Analysis by GC-MS: Assay Validation and Serum Sample Stability

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Abstract

Analysis of n3 fatty acids in serum samples has clinical applications in supplementation trials, but the analysis can be challenging due to low levels, stability issues and intra-individual variation. This study presents the single laboratory validation of a gas chromatographic-mass spectral (GC-MS) assay for analysis of fatty acid methyl esters (FAME) using sensitive single ion monitoring and provides data on fatty acid stability under different sample handling conditions. Recovery of total fatty acids from serum with Folch extraction was optimized and parallelism tests with spiked samples indicated that the serum matrix did not interfere with mass spectral quantitation. Precision and accuracy of the assay at the lowest limit of quantitation and at low, medium and high levels met with accepted guidelines for single laboratory validation. Several storage conditions that can be encountered with clinical samples also were evaluated for impact on fatty acid levels in serum. Serum from blood that was stored refrigerated for 3 days yielded similar results as serum that was prepared and frozen at -80°C immediately. Serum storage at room temperature for 3–24 hours and serum subjected to one freeze/thaw cycle had minimal effects on fatty acid levels. The intra-individual variability in pregnant women was reasonably small, with significant correlation coefficients ranging from 0.35 to 0.76 for blood drawn between 12–20 weeks versus 34–36 weeks of gestation. These results indicate that GC-MS with single ion monitoring is valid for the analysis of total fatty acids in clinical samples, even when blood processing cannot be performed in a timely manner.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

The authors declare no conflicts of interest with the work reported.

Keywords

Fatty acids; fatty acid methyl esters; GC-MS; fish oils; pregnancy; blood levels

1. INTRODUCTION

The omega 3 fatty acids are thought to have beneficial effects in multiple health domains, but dietary intakes in western populations unfortunately are often low [1]. This has increased interest in fish oil supplementation for maintenance of optimal health. The main side effects of fish oil supplementation that have been identified are a fish after-taste, belching and diarrhea, and these can interfere with adherence to recommended dosing [2, 3]. This makes monitoring of serum fatty acids important in clinical studies of the health effects of fish oil supplementation. Total serum fatty acids, rather than phospholipid fatty acids, are well suited for this purpose and appear to be relatively more sensitive to small dietary changes [4, 5].

Analysis of fatty acid methyl esters (FAME) extracted from serum phospholipids is a well-established procedure. Extraction of phospholipids, however, adds significant cost and analysis time to the assay. For studies in which determination of n3 (omega 3) fatty acids is the primary aim, several laboratories have shown that fatty acid analysis in whole blood is sufficient and compares very well with phospholipid and red blood cells fatty acids [6, 7]. For clinical studies, however, serum is often prepared since many other markers of health risk are assayed in serum. We therefore sought to evaluate reliability and accuracy of fatty acid analysis in serum, with a focus on long chain polyunsaturated fatty acids since fish oil supplementation is currently being investigated in many studies related to human health.

Although fatty acid analyses have been fairly well established using gas chromatography (GC) with flame ionization detectors, there are fewer reports of GC with mass spectral detection for analysis of fatty acids, and these have been limited in scope. Gas chromatographic-mass spectral (GC-MS) assays of fatty acid methyl esters (FAME) are sensitive, especially when single ion monitoring is used [8, 9]. This is important since in some clinical applications, the amount of sample available may be limited. The GC-MS method also provides structural confirmation of the analyte that is useful in samples that may have interfering compounds. For eicosapentanoic acid (EPA), Abu and Oluwatowoju used GC with single ion monitoring and reported excellent intra- and inter-assay precision for 0.5–50 µg/ml EPA with a CV less than 3% [10]. In addition, the “omega 3 index” in blood (sum of EPA and DHA as a percent of total fatty acids in red blood cells) was the same when measured in the fasting and non-fasting state, indicating that an overnight fast is not required for determination of omega 3 status [10]. Results for other fatty acids were not reported in that study.

Here we report on the accuracy and precision of a GC-MS method for profiling fatty acids in serum using Association of Analytical Communities (AOAC) International guidelines [11]. This method was then used to evaluate the recovery of fatty acids from serum and the stability of fatty acids in serum that was processed using several types of conditions that

might be encountered with clinical samples. Finally, we show intra-individual variation in fatty acids in pregnant women to aid in the planning of subsequent clinical trials.

2. MATERIALS AND METHODS

2.1. Chemicals and Standards

Fatty acids were purchased in the triglyceride form from NuChek Prep (Elysian, MN), and they were stored at -35°C until used. MethPrep II was obtained from Alltech Inc. (Deerfield, IL). Solvents were B&J Brand HPLC-grade quality (Honeywell Inc., Morristown, NJ). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Standards for calibration were prepared in hexane:chloroform (1:1) and combined into a single fatty acid mixture. The fatty acids are named using the standard convention which is number of carbons followed by number of double bonds after the colon and number of carbon atoms from the last double bond (termed omega bond in the older literature). The final concentrations of each triglyceride fatty acid in the standard fatty acid mixture was: tridodecanoin (laurin, 12:0) 0.05 mg/ml, tritetradecanoin (myristin, 14:0) 0.25 mg/ml, trihexadecanoin (palmitin, 16:0) 2 mg/ml, trihexadecenoin (palmitolein, 16:1, n9) 1 mg/ml, trioctadecanoin (stearin, 18:0) 0.5 mg/ml, trioctadecenoin (petroselinin, 18:1 n9) 2 mg/ml, trioctadecadienoin (linolein, 18:2 n6) 2 mg/ml, trioctadecatrienoin (alpha-linolenin, 18:3 n3) 0.2 mg/ml, triicosenoin (20:1) 0.2 mg/ml, trihomogamma linolein (20:3 n6) 0.2 mg/ml, triicosatetraenoin (arachidonin, 20:4 n6) 1 mg/ml, triicosapentaenoin (20:5 n3) 0.25 mg/ml and tridocosahexaenoin (22:6 n3) 0.25 mg/ml. The internal standard, triheptadecanoin (17:0), was prepared in a concentration of 1 mg/ml in hexane. Standard curves were prepared by pipetting 10 μL of internal standard into each autosampler vial and either 0, 2.5, 5, 10, 25 or 50 μL of the standard fatty acid mixture. Standards were allowed to evaporate at room temperature prior to derivatization.

2.2. Blood Samples

Peripheral blood for the method validation studies was obtained from healthy volunteers. Human serum for gauging reproducibility of batches was frozen in aliquots from a single pool of blood samples drawn into red-top tubes. For evaluating intra-individual variability in fatty acids from pregnant women, blood samples were obtained from a clinical trial of fish oil supplementation to prevent depression in pregnancy. This trial has been described previously [12]. Only the women assigned to the placebo arm were included in this analysis ($n=41$), and samples were analyzed before randomization assignment was made available. The placebo pills contained soybean oil and a small amount of fish oil resulting in an intake of 7.8 mg EPA and 5.8 mg DHA each day, if the prescribed dose was taken in full. Each subject had a blood draw at study entry, which was between 12 and 20 weeks of pregnancy and a second blood draw at 34–36 weeks of pregnancy. The blood was drawn in the morning and subjects were asked to eat no more than a light low-fat breakfast such as dry toast and juice before the blood draw. All procedures with human subjects were reviewed and approved by the University of Michigan Institutional Review Board (HUM000004684 and AME00030604).

2.3. Fatty Acid Extraction

Serum was thawed at room temperature for 30 minutes and extracted using the Folch method (chloroform:methanol, 2:1, v/v) [13]. For 100 μ L aliquots of serum, 10 μ L of internal standard and 1 ml of Folch reagent were added. Recovery was evaluated using 1 or 2 minutes of vortexing, and one or two extractions. All recovery experiments were done spiking serum with 10, 25 and 50 μ L of the standard fatty acid mixture. After vortexing samples with Folch reagent, layers were separated using centrifugation for 5 minutes at 2400 g. The chloroform layer was removed taking care to exclude the interface containing precipitated proteins, and it was dried in a Speed-vac with heating at 37 °C.

2.4. Derivatization

Formation of fatty acid methyl esters by transesterification can be accomplished by a variety of reagents. Advantages of MethPrep II include that it is a one-step reaction, it is done at room temperature, it requires no extraction before analysis and it is a faster reaction than using sodium methoxide reagents. Initial experiments determined that derivatization with MethPrep II used in a mixture of hexane and chloroform yielded superior peak shape for longer-chain fatty acids including eicosapentanoic acid (EPA) and docsahecanoic acid (DHA), both of which elute late in the chromatogram (data not shown). The Meth-Prep II reagent (methanolic m-trifluoromethylphenyltrimethylammonium hydroxide) was used at room temperature, which is desirable since n-3 fatty acids are not highly stable to heating.

Dried lipid samples or standards were derivatized by adding 90 μ L of chloroform:hexane (1:1, Folch extractant) and 10 μ L Meth-Prep II. Samples were capped, vortexed briefly and allowed to react at room temperature for 20 minutes prior to transferring to autosampler vials. The derivatized samples were stable for up to a week at room temperature.

2.5. GC-MS

A SupelcoSP2330 column, 30m X 0.32mm X 0.2 μ m film thickness, was used (Cat. No. 24073, Sigma-Aldrich, St. Louis, MO). Gas chromatography was done with a HP 7673 GC and a HP 7673 autosampler. Helium was the carrier gas with a column head pressure of 10 psi. Total flow rate at the split vent was 50 ml/min, the flow rate through the column was 2.5 ml/min and the septum purge was 2.5 mL/min. The injector was set at 220°C using the splitless injection mode, and 1 μ L injections were made. The temperature gradient started with a 70°C initial temperature, a linear increase to 170°C at 11°C/min, a slower linear increase to 175°C at 0.8°C/min to separate closely-eluting fatty acids, followed by an increase to reach 220°C at 20°C/min, and a final 2.5 minute hold. The total run time was 20.1 minutes.

The detector was a HP 5971 mass spectral detector operated in the single ion monitoring mode at 250°C. The ions monitored are shown in Table 1. When more than one ion was monitored simultaneously, the dwell time was 50 ms. The saturated fatty acid 20:0 elutes before 20:1 but was typically very low in serum, often required manual peak integration, and therefore was not quantified.

2.6. Lowest Limit of Detection (LOD) and Lowest Limit of Quantification (LOQ)

The LOD was calculated as the lowest concentration that can be detected and the LOQ was defined as lowest concentration that can be determined with acceptable precision and accuracy using guidelines from the Association of Analytical Communities (AOAC) International [11]. Using data from 10 blank samples, the LOD was calculated as an amount of fatty acid that yields a peak three times the standard deviation (SD) of the blank divided by the slope. The LOQ was calculated as the blank SD times ten divided by the slope. This was then confirmed using standards prepared at the LOD and LOQ levels.

2.7. Precision and Accuracy

These procedures were designed to meet AOAC guidelines for a single laboratory validation (SLV). Standard curves were constructed using a range that would encompass a wide range of the expected levels in serum for each fatty acid. The precision and accuracy were determined at the LOQ using five independent samples. Intra- and inter-day precision and accuracy was determined at low-range (3-fold the LOQ), and the medium range and high range (25 or 50 μ L of the standard fatty acid mixture, respectively). For the low, medium and high spiked serum samples, five samples per level were prepared and analyzed on three separate days. The inter-day precision and accuracy is shown in Table 2 as the average CV across three days.

2.8. Parallelism

It is possible that components of serum can alter response of the mass spectral detector in a non-linear way. It was therefore important to evaluate the performance of standard curves prepared with and without addition of serum. Standard curves were constructed with and without a serum spike (Fig. 1). The resulting curves would be expected to be parallel if the serum was not affecting assay performance. Parallelism was assessed by means of linear regression models in which ratio was the outcome and concentration, serum indicator (added serum or not), and concentration*serum indicator interaction were used as independent variables. Statistical significance of the interaction term would be indicative of lack of parallelism. All statistical analyses were done in SPSS software (PASW Statistics, version 18.0, release 18.0.2).

2.9. Stability

Clinical blood samples, especially from umbilical cord blood, often are drawn at times when the laboratory is closed. This makes evaluation of fatty acid stability important. We evaluated the effects of storage of whole blood and serum to simulate short-term clinical handling scenarios that might be encountered. Blood was drawn into red-top tubes containing no anti-coagulant. One tube was placed in the refrigerator for 72 hours prior to preparing serum. The other tubes were allowed to clot at room temperature in the dark for 30 minutes followed by preparation of serum by centrifugation. Serum aliquots were stored as follows: 1) at room temperature and light for 12 hours, 2) at room temperature and light for 3 hours, and 3) frozen immediately after the 30 minute clot time at room temperature in the dark. In addition, since clinical samples are often limited in volume, we performed the assay

on serum that was frozen immediately and then thawed and re-frozen (re-frozen at least overnight) one or two times. Frozen aliquots were stored at -80°C before analysis.

3. RESULTS AND DISCUSSION

3.1. Mass spectral Detection Parameters

Initial studies were done with the mass spectral detector operated in full scan mode to determine optimal ions for detecting each fatty acid. The ions chosen were of mass/charge ratios greater than 100 that gave a high intensity. Mass spectra of two fatty acid methyl esters are shown in Fig. (2) as an example. For closely eluting fatty acids, 2–3 ions were monitored simultaneously, one for each fatty acid, since small changes in retention times during analysis might obscure capturing peaks in their entirety if the switch in detection was too close to the peak (Table 1). In this case, ions of sufficient intensity were chosen that were as similar in mass to charge ratios as possible to minimize cycling time of the mass spectrometer.

3.2. Fatty Acid Extraction

Extractions of serum done with one minute of vortexing with Folch extractant resulted in recovery of fatty acids that ranged 55–78%. Using two minutes of vortexing, recovery was 75–93%. Recovery could be improved further using two sequential extractions, with average recovery yield improving from 84% to 96%, but this did not affect the relative fatty acid percentages. Since results for fatty acid analysis are generally given as percent of fatty acids, rather than concentration in serum, all subsequent studies were done using one extraction and two minutes of vortexing.

3.3. Lowest Limit of Detection (LOD) and Lowest Limit of Quantification (LOQ)

The LOD and LOQ were determined for each fatty acid using standards. These data are shown in Table 2. It is important to note that these limits were determined using mass spectral voltage settings that were optimized for analysis of human serum. Lower limits could be reached if MS voltages were increased, which might be needed for other type of samples that are more limited in volume. The AOAC guidelines call for a coefficient of variation (CV) for precision and accuracy that does not exceed 15%, except for the LOQ which can have a CV of 20%. These limits were met (Table 2).

3.4. Precision and Accuracy

The precision and accuracy at the LOQ was well within AOAC guidelines, with a CV below 10% for each fatty acid and an average CV of 6.4%. Accuracy was between 8.3 and 19.9% at the LOQ, which meets with AOAC guidelines, and the average CV was 14%. Intra- and inter-day precision and accuracy was also determined at low-range (3-fold the LOQ), medium range and high range expected in serum. Both the intra- and inter-day precision and accuracy, as shown in Table 2, was acceptable with most values being below 10% and all values being below 15%. Precision ranged 4.3–6.1%, on average, for all fatty acids for the intra-day tests and 5.6–9.3% for the inter-day tests. Accuracy was slightly better with a range of 3.5–9.0%, on average, for intra-day tests and 2.4–4.6% for inter-day tests.

3.5. Parallelism

Standard curves were prepared by spiking serum with fatty acids to evaluate detector response over a wide range of fatty acid concentrations. Examples of the results are shown in Fig. (1). Statistical analyses indicated that the detector response did not differ by whether or not serum was spiked into the standard fatty acid mixture with p-values >0.10 in each case.

3.6. Stability of Fatty Acids

Once serum samples are frozen, they appear to be quite stable. Storage of serum at -80°C for up to 10 years reportedly did not significantly affect fatty acid profiles in serum [14]. Before serum can be frozen, however, processing is needed. Published data on fatty acid stability using longer time points has indicated that room temperature storage of blood as a dried spot on paper resulted in significant degradation after 1–2 months but that refrigerated blood on paper cards treated with butylated hydroxytoluene was stable for 1–3 weeks [15, 16]. Zheng reported that fatty acids in blood were stable for 16 hours at room temperature and for three freeze-thaw cycles, but the inter-day precision of their method for 22:6 was 21% [17].

In this report, we evaluated several storage conditions that might be expected to occur in the clinical handling of blood samples. We were especially interested in stability of long chain polyunsaturated fatty acids. Fish oil supplementation is currently the subject of research for beneficial effects in many disease states, making validation of this assay important.

Serum fatty acids appeared to be stable to various processing conditions. Serum could be thawed and refrozen once without large differences as compared with serum that was frozen at -80°C immediately after clotting. The percentage of 18:2, n6, was significantly decreased and the percentages of 12:0, 14:0, 16:0 and 16:1 were slightly increased by one freeze/thaw cycle (Table 3). Two freeze/thaw cycles did, however, result in significant changes, and this is not recommended. When whole blood was refrigerated for 72 hours, the fatty acid profile was quite similar to blood that had been thawed and refrozen once. Serum that was allowed to sit at room temperature for 3–24 hours also had a few fatty acids that were significantly different versus serum that was frozen right away, but 18:2, n6, was not affected.

Importantly, the fish oil fatty acids 20:5 and 22:6, which are likely the least stable of all the fatty acids analyzed, did not decrease under any of these conditions (Table 3). The decrease in 18:2, n6 with thawed and refrozen serum and whole blood refrigerated 3 days is a potential problem for analysis of fatty acids. For evaluation of the health benefits of n3 fatty acids, however, a score based on the percentage of n3 fatty acids in highly unsaturated fatty acids fatty acids of 20 carbon length or longer has been suggested to be useful [18]. This score calculates the relative amount of long chain omega 3 fatty acids relative to the sum of all fatty acids of 20 carbon length or longer and would not be affected by changes in 18:2.

3.7. Intra-individual Variability in Pregnant Women

Intra-individual variability in fatty acid levels was good despite the fact that the subjects were pregnant and considerable fetal growth would have occurred between the two blood draws. The correlation coefficients for levels on the first draw with levels on the second blood draw were all statistically significant and greater than 0.35 (Table 4). This indicates that a single blood draw would be a reasonable representation of fatty acid levels for an individual even during pregnancy.

The soybean oil placebo capsules contained a small amount of fish oil fatty acids, but levels of 20:5 or 22:6 did not increase between the two blood draws as determined by paired t-tests ($p > 0.89$ in each case, Table 4). Levels of arachidonic acid (20:4 n6) did go down slightly but significantly over from the first to the second blood draw (from 8.3 to 6.5% of fatty acids, $p < 0.001$), and this could be due to accretion of arachidonic acid by the growing fetus. Other significant changes in fatty acids by the paired t-test were decreased 18:0 (from 9.5 to 8.4%) and 20:3 (from 2.2 to 1.7%) and increased 18:1 (from 22.2 to 24.4%).

A longitudinal study of changes in red blood cell fatty acids during pregnancy indicated that many fatty acids increased from 12.5 to 26.1 weeks of pregnancy (namely 16:0, 16:1, 24:1, 18:2, 18:3, 20:3 and 22:6) but changes after 26 weeks were small [19]. Another study of red blood cells fatty acids also found the biggest changes earlier in pregnancy, namely from 20 to 30 weeks versus from 30 to 37 weeks, but the nature of the changes were different with no change in 22:6 and decreases in 20:4 and 20:5 [20]. The initial blood sample in our study was collected over a fairly wide range (12–26 weeks of pregnancy), making it difficult to determine exactly when the changes at 34–36 weeks occurred. Another study evaluated plasma phospholipids during pregnancy and found that 20:5 and 22:6 increased from <19 to 38 weeks of pregnancy, which we did not observe [21]. The differences between studies could be due to differences in the populations sampled, the blood sampling intervals during pregnancy, and in the blood fraction analyzed. In a study of blood samples from pregnant women at 16, 22 and 32 weeks of gestation, the pattern of changes in n6 polyunsaturated fatty acids (PUFA), was different in erythrocytes and plasma phospholipids [22]. With our approach using total serum fatty acids, there were no significant changes in fish oil fatty acids (20:5 and 22:6) over the two blood draws which will facilitate analysis of fish oil supplementation effects in pregnancy. In addition, to being important for brain development in the fetus, these n3 fatty acids could have an important role in preventing mood disorders during pregnancy [12].

CONCLUSION

In summary, this study showed that GC-MS with single ion monitoring is a reliable and accurate method for quantitation of FAME that meets AOAC standards. This method, using GC-MS parameters appropriate for analysis of human serum, had acceptable performance characteristics for quantitation of all major fatty acids. Degradation due to variations in processing before freezing serum samples are predicted to be minimal, and intra-individual reproducibility of fatty acid levels was reasonable in pregnant subjects. This assay therefore should be well suited for clinical studies of fish oil supplementation.

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ABBREVIATIONS

AOAC	Association of Analytical Communities
CV	coefficient of variation
DHA	docosahexanoic acid
EPA	eicosapentanoic acid
FAME	fatty acid methyl esters
GC-MS	gas chromatography –mass spectroscopy
HUFA	highly unsaturated fatty acids
LOD	limits of detection
LOQ	limits of quantitation
PUFA	polyunsaturated fatty acids
SD	standard deviation

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Fig. (1).

Parallelism of detector response for 22:6, n3 and 20:4, n6. Standard fatty acid samples at six levels were analyzed with and without a serum spike.

Fig. (2).

Mass spectra of fatty acid methyl esters found in human serum from a chromatogram using full scan mass spectral detection. In **A**, arachidonic acid (20:4, n6) is shown for which the fragment m/z 203 was selected for monitoring in the quantitative assay, and in **B**, linoleic acid (18:2, n6) is shown for which the molecular ion of m/z 294 was selected for monitoring. The y-axis is abundance and the x-axis is mass to charge ratio (m/z).

Table 1
GC-MS Parameters for Detection of Fatty Acid Methyl Esters Prepared from Human Serum Using Single Ion Monitoring.

Fatty Acid	Retention Time (min)	Molecular Ion (m/z)	Ion (m/z) Monitored	Structure of Ion Monitored	Voltage Increment ^a
12:0	5.78	214	183	M-CH ₃ O ⁺	506
14:0	7.38	242	199	M-C ₃ H ₇ ⁺	0
16:0 ^b	8.91	270	199	M-C ₅ H ₁₁ ⁺	0
16:1 ^b	9.23	268	194	M-CH ₃ OOOC(CH ₂) ₂ ⁺	0
17:0 ^b	9.64	284	185	M-C ₇ H ₁₅ ⁺	0
18:0 ^c	10.47	298	298	M ⁺	0
18:1 ^c	10.83	296	296	M ⁺	0
18:2 ^c	11.57	294	294	M ⁺	0
18:3 ^d	12.58	292	292	M ⁺	835
20:1 ^d	13.09	324	292	M-CH ₃ OH ⁺	835
20:3 ^e	15.04	320	222	M-C ₇ H ₁₄ ⁺	459
20:4 ^e	15.67	318	203	M-CH ₃ OOOC(CH ₂) ₄ ⁺	459
20:5	16.69	316	201	M-CH ₃ OOOC(CH ₂) ₄ ⁺	588
22:6	18.41	342	199	M-CH ₃ OOOC(CH ₂) ₆ ⁺	365

^aOptimal detection of some ions required a voltage that was higher than what is set during the automatic tune procedure. The voltage shown in the table is the increase in voltage (EMV) above the auto-tune set point. The dwell time was 75 ms for all fatty acids except 18:0, 18:1 and 18:2 where it was 50 ms.

^{b,c,d,e} Ions for closely-eluting fatty acids with the same superscript were monitored simultaneously and selected ions extracted for quantitation.

Table 2

Limits of Detection (LOD), Limits of Quantitation (LOQ), Intra- and Inter-Assay Precision and Accuracy (given as the coefficient of Variation) for Fatty Acid Quantitation by GC-MS Using Mass Spectral Electron Multiplier Voltages Optimized for Analysis of Human Serum.

Validation Parameter	Fatty Acid											
	12:0	14:0	16:0	16:1	18:0	18:1	18:2(n6)	18:3(n3)	20:1	20:3(n6)	20:4(n6)	22:6(n3)
LOQ (µg/ml)	3	13	100	5	25	100	100	10	10	10	5	13
LOQ Precision	7.1	6.8	6.9	6.3	7.1	5.3	7.2	4.9	5.5	5.9	6.4	6.5
LOQ Accuracy	11.2	17.9	19.2	19.9	17.5	16.5	17.0	9.6	13.4	8.3	13.9	8.9
Intra-assay												
Precision, low range	3.6	2.9	5.2	3.3	7.5	4.5	9.0	13.9	3.3	6.3	7.4	8.1
Accuracy, low range	1.0	2.3	3.1	2.4	4.5	5.4	7.9	3.1	2.3	0.1	4.9	6.0
Precision, mid-range	3.6	2.6	4.7	2.5	5.5	7.9	2.0	3.9	3.8	5.0	4.1	7.0
Accuracy, mid-range	8.3	9.1	8.8	7.0	12.7	10.5	13.4	0.3	7.8	12.0	10.5	12.7
Precision, high-range	2.7	4.4	6.2	3.3	5.9	6.9	7.4	8.0	3.6	5.2	5.7	5.3
Accuracy, high-range	3.0	5.2	7.1	5.3	11.8	11.4	5.3	6.2	2.1	2.2	5.6	5.2
Inter-assay												
Precision, low-range	5.5	5.0	6.0	5.1	10.5	8.9	7.8	10.8	4.4	7.8	7.5	9.2
Accuracy, low- range	1.5	2.4	4.5	0.5	1.1	0.1	7.2	1.7	0.4	1.8	5.2	4.0
Precision, mid-range	10.5	9.2	6.4	7.7	9.5	8.2	10.7	6.3	9.4	12.9	8.9	13.7
Accuracy, mid- range	0.8	2.8	6.6	2.2	8.6	7.4	7.7	2.5	1.1	1.9	5.1	2.9
Precision, high-range	4.4	3.6	6.7	2.6	5.4	9.2	6.5	9.3	3.1	5.5	4.6	7.1
Accuracy, high- range	0.9	4.4	9.0	5.8	11.0	9.9	6.8	0.6	1.3	0.1	4.6	0.01

Table 3

Effect of Storage Conditions on Fatty Acid Levels. Data Shown is Mean and SD for Six Replicates.

Fatty Acid	Storage Conditions Before Freezing at -80°C				
	No storage ^d	3 hr. room temp. ^b	24 hr. room temp. ^b	Frozen, Thawed ^c	Refrigerated 72hr ^d
12:0	0.68, 0.04	0.71, 0.01	0.73, 0.01*	0.73, 0.01*	0.73, 0.01*
14:0	1.06, 0.05	1.11, 0.01*	1.11, 0.01*	1.12, 0.02*	1.13, 0.03*
16:0	25.79, 1.03	24.84, 0.04	25.23, 0.45	27.25, 0.50*	27.12, 0.99*
16:1	2.02, 0.08	2.05, 0.04	2.05, 0.03	2.13, 0.06*	2.14, 0.07*
18:0	7.51, 0.57	7.23, 0.10	6.89, 0.07*	7.25, 0.12	7.22, 0.07
18:1	20.37, 0.82	21.06, 0.48	20.42, 0.29	19.43, 0.47	19.52, 0.94
18:2	19.58, 0.99	19.74, 0.51	20.05, 0.49	18.08, 0.29*	18.36, 0.85*
18:3	0.90, 0.03	0.96, 0.02*	0.93, 0.01*	0.90, 0.02	0.92, 0.03
20:1	0.21, 0.01	0.22, 0.00	0.20, 0.00	0.21, 0.00	0.21, 0.00
20:3	1.59, 0.09	1.58, 0.02	1.56, 0.07	1.64, 0.12	1.68, 0.07
20:4	13.57, 0.71	13.40, 0.28	13.96, 0.30	14.21, 0.37	13.94, 0.65
20:5	0.65, 0.05	0.67, 0.01	0.66, 0.02	0.69, 0.03	0.70, 0.02*
22:6	6.09, 0.32	6.43, 0.14*	6.20, 0.14	6.36, 0.11	6.34, 0.21

^a Serum samples were prepared from blood and frozen at -80°C immediately after preparation (first column).

^b Serum samples were stored at room temperature for 3 or 24 hours before freezing.

^c Serum was frozen immediately after preparation, thawed, and refrozen before analysis.

^d Whole blood was refrigerated for 72 hours in a red-top tube before preparation of serum and freezing.

* Values within a row that differ significantly ($P < 0.05$) from the "frozen immediately" sample by ANOVA with Tukey post-hoc tests are starred.

Table 4

Spearman Correlations and Paired T-Tests of Fatty Acid Levels Drawn from Women at 12–26 and 34–36 Weeks of pregnancy.

Fatty Acid	Correlation Coefficient	P-Value for Correlation	P-Value for Paired T-test
Saturated fatty acids	0.748	<0.001	0.289
Monounsaturated fatty acids	0.713	<0.001	0.002
n3 PUFA ^a	0.564	<0.001	0.883
n6 PUFA	0.504	<0.001	0.024
n3 HUFA ^b	0.663	<0.001	0.001
12:0	0.354	0.023	0.871
14:0	0.355	0.023	0.410
16:0	0.597	<0.001	0.325
16:1, n7	0.671	<0.001	0.947
18:0	0.698	<0.001	0.004
18:1, n9	0.723	<0.001	0.002
18:2, n6	0.764	<0.001	0.356
18:3, n3	0.345	0.027	0.814
20:1, n9	0.670	<0.001	0.497
20:3, n6	0.478	0.002	<0.001
20:4, n6	0.658	<0.001	<0.001
20:5, n3	0.613	<0.001	0.891
22:6, n3	0.554	<0.001	0.958

^aPUFA is the sum of all polyunsaturated fatty acids.

^bn3 HUFA is the proportion of 20:5, n3, and 22:6, n3, relative to the sum total of all fatty acids of 20 carbon length or longer.