HHS Public Access

Author manuscript

Anal Bioanal Chem. Author manuscript; available in PMC 2019 March 01.

Published in final edited form as:

Anal Bioanal Chem. 2018 March ; 410(9): 2321–2329. doi:10.1007/s00216-017-0841-5.

Standard Reference Material 2378 Fatty Acids in Frozen Human Serum. Certification of a Clinical SRM based on Endogenous Supplementation of Polyunsaturated Fatty Acids

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Abstract

Dietary fatty acids can be both beneficial and detrimental to human health depending on the degree and type of saturation. Healthcare providers and research scientists monitor the fatty acid content of human plasma and serum as an indicator of health status and diet. In addition, both the Centers for Disease Control & Prevention (CDC) and the National Institutes of Health – Office of Dietary Supplements are interested in circulating fatty acids (FAs) because they may be predictive of coronary heart disease. The National Institute of Standards and Technology (NIST) provides a wide variety of reference materials (RMs) and Standard Reference Materials® (SRM®s) including blood, serum, plasma, and urine with values assigned for analytes of clinical interest. NIST SRM 2378 Fatty Acids in Frozen Human Serum was introduced in 2015 to help validate methods used for the analysis of FAs in serum, and consists of three different pools of serum acquired from (1) healthy donors who had taken fish oil dietary supplements (at least 1000 mg per day) for at least one month (level 1 material), (2) healthy donors who had taken flaxseed oil dietary supplements (at least 1000 mg per day) for at least one month (level 2 material), and (3) healthy donors eating “normal” diets who had not taken dietary supplements containing fish or plant oils (level 3

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COMPLIANCE with ETHICAL STANDARDS.

This work represents no known conflicts of interest with respect to the authors or their institutions. The contractor who collected, processed, and packaged SRM 2378 was required to submit documentation to NIST of their relationship with an appropriate Institutional Review Board (IRB) registered with the US Department of Health and Human Services’ Office of Human Research Protections and located within the community in which the human subject research was conducted. The contractor also had to submit documentation for NIST institutional review and approval. The donors to SRM 2378 signed a form acknowledging that they had read information describing what their blood would be used for, that they had received answers to any questions, and that they gave consent for their blood to be pooled and used in the preparation of a serum SRM.

material). The use of dietary supplements by donors provided SRMs with natural endogenous ranges of FAs at concentrations observed in human populations.

Results from analyses using two methods at NIST, including one involving a novel microwave-assisted acid hydrolysis procedure, and one at the CDC are presented here. These results and their respective uncertainties were combined to yield certified values with expanded uncertainties for 12 FAs and reference values with expanded uncertainties for an additional 18 FAs.

INTRODUCTION

Natural-matrix Standard Reference Materials (SRMs) can be used to validate analytical methods and for quality assurance in the analysis of test samples. Because the molecular form of an analyte of interest may be impacted by metabolic processes, reference materials containing naturally occurring (rather than spiked) analytes are preferred. To support the demand for clinical reference materials (RMs) that better represent native samples, NIST has refined its focus on procuring materials that require minimal post-collection processing to meet target values observed in populations. Obtaining matrix materials from specific donor groups has reduced the need for exogenous spiking and dilution to obtain target values in many materials including NIST SRM 2378 Fatty Acids in Frozen Human Serum [1].

SRM 2378 was issued in February 2016 (expiration 30 September 2025) and addresses a need expressed by the Centers for Disease Control and Prevention (CDC) for human serum materials with endogenous levels of fatty acids (FAs), including those associated with fish oil and flaxseed oil supplementation, observed in the general population. Specific FAs have been implicated in cardiovascular health [2-4], fetal development and health [5], and eye disease [6], and may serve as markers for Alzheimer's disease [7]. Thus, SRM 2378 will be of importance to the health care community by providing better accuracy in the measurements of FAs in human populations. Results from analyses using two methods at NIST, including one involving a novel microwave-assisted acid hydrolysis procedure, and one at the CDC are presented below. These results and their respective uncertainties were combined to yield certified values with expanded uncertainties for 12 FAs and reference values with expanded uncertainties for an additional 18 FAs.

EXPERIMENTAL

Preparation of the SRM and selection of samples for analysis

Serum for this SRM was collected from three groups of individuals: (1) three healthy donors who took 1000 mg/day of fish oil supplements for a minimum of one month prior to collection (level 1 material); (2) three healthy donors who took 1000 mg/day of flaxseed oil supplements for a minimum of one month prior to collection (level 2 material); and (3) three healthy donors who did not take either fish or flaxseed oil supplements for one month prior to collection (level 3 material). Flaxseed oil supplementation usually results in elevated serum levels of α-linolenic acid (C18:3n3) [8, 9], while fish oil supplementation typically elevates serum levels of eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acid (C22:6n3, DHA) [8, 9]. All serum was collected and processed according to CLSI C37-A guidelines [10]. Details of the two analyses of FAs by NIST, one of the two methods

involving a novel microwave-assisted acid hydrolysis, and by CDC researchers follows, resulting in certified mass fraction values for 12 FAs and reference mass fraction values for 18 FAs.

Fatty acid measurements after KOH (in methanol) saponification and acidic methanol esterification with gas chromatography with flame ionization detection (GC-FID) (NIST-1 method)

Materials—High performance liquid chromatographic (HPLC) or better grade solvents were used throughout this work. SRM 2377 Fatty Acid Methyl Esters in 2,2,4-Trimethylpentane was used to prepare six calibration solutions in iso-octane [containing butylated hydroxytoluene (BHT) at a concentration of 1 g/L as an anti-oxidant] spanning the concentration ranges of the individual FAs in the sera. In addition, four calibration solutions were gravimetrically prepared in chloroform (also containing 1 g/L BHT) from 24 neat FAs obtained from NuChek Prep, Inc. (Elysian, MN). Palmitic-d_{31} acid was obtained from Cambridge Isotope Laboratories (Andover, MA), and heneicosanoic acid was obtained from Nu-Check Prep, Inc. (Elysian, MN). These were used to prepare an internal standard solution in iso-octane containing 1 g/L BHT. NIST SRM 1950 Metabolites in Frozen Human Plasma was used as a quality control material for these analyses. This material was collected and pooled from 50 men and 50 women, of ages between 40 and 50 years old, who had fasted overnight and overall whose racial distribution was similar to that of the U. S. population.

Sample Preparation—Sample preparation involved spiking triplicate serum aliquots with the internal standards described above, saponifying the samples for 10 min at room temperature using 2 mL of 0.4 mol/L potassium hydroxide in methanol (KOH in MeOH), extracting the FAs three times using 2 mL hexane, combining the extracts, and esterifying using 2 mL 5% (volume fraction) sulfuric acid in methanol (H_2SO_4 in MeOH) for 30 min at 80 °C. The resulting fatty acid methyl esters (FAMEs) were then extracted three times with 2 mL hexane and the combined hexane phases were concentrated to approximately 0.3 mL under a nitrogen stream for subsequent gas chromatography-flame ionization detection (GC-FID) analysis. The calibration solutions and blank sample were processed in the same manner as the serum samples and the quality control material (SRM 1950).

Instrumental method—The GC-FID analyses were performed using a 0.25 mm × 100 m SP2560 (nonbonded; biscyanopropyl polysiloxane) fused silica capillary column (Supelco, Bellefonte, PA), 0.25 μm film thickness. The column was held isothermally at 100 °C for 10 min and then temperature programmed at 1 °C/min to 240 °C. The injection port and the flame ionization detector were both maintained at 240 °C. Injections of 1 μL were performed in the split mode (30:1 ratio) with helium as the carrier gas at a constant flow rate of 0.5 mL/min. Quantitation was based on average response factors calculated from peak areas from the independently prepared calibration solutions relative to the internal standards.

NIST-1 method was used to assess the homogeneity of the three levels of SRM 2378. Ten individual vials of the three levels of SRM 2378 were selected using a random stratified sampling method.

Fatty acid measurements after microwave-assisted acid hydrolysis with pentafluorobenzyl esterification and GC/MS with negative chemical ionization (NIST-2 method)

Materials—Neat fatty acids were obtained from Nu-Chek Prep Laboratories (Elysian, MN), Sigma-Aldrich (St. Louis, MO) and Indofine Chemical Co. (Hillsborough Township, NJ), and four stable isotope labeled FAs, including myristic acid-d_{27}, palmitic acid-d_{31}, stearic acid-d_{35}, and arachidic acid-d_{39} were purchased from Cambridge Isotope Laboratories (Andover, MA). The anti-oxidant BHT was added to the calibration and labeled FA solutions at a concentration of 1 mg/g. Acetonitrile was mixed with the 6 mol/L HCl in a 90:10 volume fraction to be used as the hydrolyzing solution (prepared immediately prior to microwave-assisted hydrolysis). Hexane (Sigma, 98.5 %, HPLC Grade) was used to extract the FAs from the hydrolyzed serum, and a 10 % solution of pentafluorobenzyl bromide (Supelco) in acetonitrile was prepared for derivatization in the presence of triethylamine (Sigma-Aldrich, ≥ 99 %) of the FAs to their pentafluorobenzyl esters.

Microwave-assisted digestions—The microwave-assisted acid hydrolysis of the serum samples is a novel approach for these matrices and analytes, and for the preliminary application of this new method, previous published procedures [11-13] were used as guides for solvent/acid concentrations, as well as for the temperature and duration of the hydrolyses. A microwave digestion system (Mars 5, CEM Corporation, Matthews, NC) was used for the microwave-assisted hydrolyses of the serum, quality control material (SRM 1950), calibrants, and blanks. Four aliquots of approximately 100 μL each from three randomly chosen vials of the three levels of SRM 2378 (duplicate aliquots taken from one of the vials for the three levels) were weighed into Teflon microwave vessels, followed by known masses of the labeled FA solution. Four aliquots of SRM 1950, calibration solutions, and blanks were also transferred to Teflon microwave vessels to be processed in the same manner as the samples. To each microwave vessel, 3.5 mL of acetonitrile/6 mol/L HCl (90:10, volume fraction) were added, and the vessel was capped tightly and manually mixed by briefly shaking. The vials were then uncapped and allowed to digest at room temperature for 1 h prior to re-capping and processing in the microwave for 45 min at 100 °C (± 5 °C). This was done to minimize the build-up of pressure during the microwave-assisted hydrolysis. Hydrolyzed samples were transferred to 15 mL capped glass centrifuge tubes after which two separate 2.5 mL volumes of hexane were used with 1 min vortex mixing to extract the FAs from the hydrolyzed samples, and these combined extracts were concentrated under a nitrogen stream to dryness. The pentafluorobenzyl esters of the FAs were then generated as described in [11-13], and as below for the method used by the CDC.

Instrumental method—The measurement of the pentafluorobenzyl esters of the FAs was achieved using gas chromatography/mass spectrometry (GC/MS) with negative chemical ionization (NCI) using methane as the collision gas [13]. Given the three orders of magnitude range in the concentrations of the individual FAs measured in the serum and quality control samples (SRM 1950), both scan [mass/charge (*m/z*) 40 to *m/z* 550] and selected ion runs were performed, with on-column sample injection volumes of 0.2 μL to 1.0 μL and dilutions of both calibrants and samples for measurement of FAs at the highest concentrations. A 30 m × 0.25 mm ZB-5ms column (0.25 μm phase, 5 % phenylmethyl-substituted polysiloxane, Phenomenex, Torrance, CA) was used at a constant flow of 1.5

mL/min He. The mass spectrometer's source, quadrupole, and interface temperatures were set at 200 °C, 150 °C, and 280 °C, respectively. The pentafluorobenzyl FA esters readily capture low energy electrons, subsequently losing the pentafluorobenzyl as a neutral radical, leaving the corresponding carboxylate anion $[M-1]^-$ for the FA. Selected ion monitoring was employed for the [M-1]- ions for the corresponding FAs, including isotopically labeled standards, during sample and calibrant runs. Peaks areas of analytes and labeled standards were used to calculate response factors that were in-turn used to determine mass fractions (μg/g) of FAs in SRM 1950 (control) and SRM 2378.

Fatty acid measurements after acid/base hydrolysis with pentafluorobenzyl esterification and gas chromatography-mass spectrometry (GC/MS) with negative chemical ionization (CDC method)

Materials—Neat FAs were obtained from Nu-Chek Prep Laboratories (Elysian, MN) and sixteen stable isotope labeled FAs were purchased from Cayman Chemical (Ann Arbor, MI) or IsoSciences (King of Prussia, PA), and were used as internal standards for this method. These labeled FAs included capric-d_3, lauric-d_3, myristic-d_{27}, pentadecanoic-d_3, palmitoleic-d_{14}, palmitic-d_{31}, margaric-d_3, stearic-d_{35}, 13C-oleic, 13C-linoleic, alpha-linolenic-d_{14}, arachidic-d_{39}, eicosapentaenoic-d_5, docosanoic-d_4, docosahexaenoic-d_5, and tetracosanoic-d_4. High performance liquid chromatography (HPLC) grade solvents were used throughout this method.

Sample Preparation—Acid and base hydrolyses of the serum samples and the quality control material (SRM 1950) were performed in triplicate by the procedure described in [14]. Briefly, 100 μL aliquots of serum, 4 levels of quality control materials, including SRM 1950, calibrants, and blanks were spiked with a solution containing the internal standards and hydrolyzed sequentially with acetonitrile/6 mol/L HCl (90:10, volume fraction) and methanol/10 mol/L NaOH (90:10, volume fraction) each for 45 min at 100 °C. The preparations were then acidified with HCl, extracted with hexane in triplicate, concentrated to dryness, and derivatized for 15 min with 10 μL trimethylamine and 50 μL of 10 % pentafluorobenzyl bromide in acetonitrile and dissolved in hexane.

Instrumental method—Similar to the NIST-2 method described above, GC/MS was employed with NCI to measure the FAs as described in [14]. Briefly, 1 μL split injections (typical split ratios of 100:1) were made on a 60 m × 0.25 mm (0.25 μm phase thickness) cyanopropyl-methylpolysiloxane phase (50-50) column operated at a constant carrier flow of 2 mL/min helium. Methane (collision gas) was operated at 40 % of total flow with the ion source and quadrupoles set at 170 °C and 150 °C, respectively. As for method NIST-2, selected ion monitoring was employed for [M-1]- ions for the corresponding 48 FAs and labeled FAs during sample and calibrant runs. Peak areas of the individual FAs and labeled standards were used to generate linear and quadratic calibration curves used to quantify the FAs.

RESULTS AND DISCUSSION

Results for the quality control material SRM 1950 Metabolites in Frozen Human Serum measured using the three methods are shown in Table 1. Good agreement was observed among the three methods for most of the FAs except for palmitoleic acid, *cis*-vaccenic acid, and arachidonic acid. Palmitoleic acid and *cis*-vaccenic acid were measured at significantly higher concentrations by the NIST-2 method than by the NIST-1 and CDC methods. A low bias was observed for the NIST-2 arachidonic acid result compared the NIST-1 and CDC results. Table 2 summarizes some of the FA mass fraction results for the three levels of SRM 2378 and the three analytical procedures described above (NIST-1, NIST-2, and CDC), with good agreement observed among methods (see Electronic Supplementary Material Fig. S1 – S3 for typical GC-FID and GC/MS chromatograms). Phytanic acid was measured by the NIST-2 method and was included in this group of health-related FAs because at higher concentrations, phytanic acid along with pristanic acid, can indicate metabolic disorders [12, 15]. Significant differences in mass fractions of specific FAs were observed among the levels representing different supplementations (flaxseed oil and fish oil) or no supplementation (see Figure 1 for graphical representation). For example, the concentration of α-linolenic acid, a major component of flaxseed oil [3], was observed at about two times the concentration of the level 3 material (no supplementation) in both the level 2 (flaxseed oil supplementation) and level 1 materials (fish oil supplementation). The concentrations of gamma-linolenic acid, typically enriched in vegetable oil, were 30 % and 50 % higher in the level 2 material than observed in the level 1 and 3 materials, respectively, suggesting that the contributors to the level 2 material may have consumed significant quantities of other vegetable oils in addition to the flaxseed oil supplements. In addition, the level 1 material (fish oil supplemented) was enriched with 4 times more eicosapentaenoic acid (EPA) and two times more docosohexaenoic acid (DHA) than observed in the level 2 and 3 materials. Docosapentaenoic acid (DPA), considered to be a metabolic intermediate between EPA and DHA [16] was measured in the level 1 material at 1.4 and 2 times the concentrations observed in the level 2 and level 3 materials, respectively. In summary, the enrichments in specific FAs observed in the level 1 and level 2 materials of SRM 2378, with respect to the level 3 material (no supplementation), are consistent with donors who used fish oil and flaxseed oil, respectively.

The mass fractions of five FAs (α-linolenic, γ-linolenic, EPA, DPA, and DHA) in the level 3 material (no supplementation, see Table 2) were similar to the ranges observed in the serum of healthy adults in [17]. The levels of EPA and DHA were similar to those reported in older adults taking a placebo in a randomized intervention trial [6]. Similarly, the range of concentrations of EPA and DHA in the supplemented population in [6] are of similar concentrations to those observed for these two FAs in the level 1 materials of SRM 2378 (fish oil supplements). The results of these comparisons suggest that the three materials composing SRM 2378 are suitable for supporting population studies targeting FAs in serum.

Certified and reference mass fraction and molar concentrations for 12 and 18 FAs are shown in Tables 3 and 4, respectively. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account. A NIST reference value is a noncertified value that is the

best estimate of the true value based on available data; however, the value does not meet the NIST criteria for certification and is provided with associated uncertainties that may reflect only measurement reproducibility, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods [18].

The certified values are calculated as the mean of method means determined using results from at least two and sometimes three methods. The uncertainty provided with each value is an expanded uncertainty about the mean. The expanded uncertainty $U_{95\%}$ is calculated as $U_{95\%} = ku_c$, where u_c incorporates the observed difference between the results from the methods and their respective uncertainties, consistently with the ISO/JCGM Guide and with its Supplement 1, and k is a coverage factor, where $k = 2.0$, corresponding to approximately 95 % confidence [19-23]. Reference mass fractions and molar concentrations shown in Table 4 include expanded uncertainties that were determined in the same manner as those for the certified values, using data from one to three methods.

A recent paper [24] describes the use of SRM 2378 in 2012 and 2015 as unknown and quality control materials in quality assurance program interlaboratory comparisons of individual FAs in serum and plasma. The variability in the results of these interlaboratory comparisons led the authors to conclude that additional exercises were necessary. Performance in exercises such as these can often be improved when laboratories use a reference material like SRM 2378 to evaluate their analytical methods for biases.

CONCLUSIONS

The certification of SRM 2378 Fatty Acids in Frozen Human Serum by three methods yielding mass fractions of fatty acids is described above. This SRM is intended primarily for validation of methods for determining fatty acids in human serum and similar materials. The FA profiles of the three serum materials making up this SRM show the impact of endogenous supplementation on the relative levels of specific FAs of nutritional interests, thereby demonstrating the utility of supplementation to achieve ranges of specific analytes observed in populations. Whether targeting specific analyte levels or developing materials that reflect chemical characteristics of certain populations, serum and other biological matrices can thus be obtained from donors who possess specific endogenous levels. This approach eliminates the need for dilution and fortification, and results in a reference material that contains relevant levels of specific analytes observed in populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Partial financial support for the development and certification of SRM 2378 Fatty Acids in Frozen Human Serum was provided by the National Institutes of Health, Office of Dietary Supplements (NIH-ODS), with guidance from Joseph M. Betz (NIH-ODS) and Stephen A. Wise (NIST). Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment are the best available for the purpose. The findings and conclusions of the article are those

of the authors and do not necessarily represent the official views or positions of the CDC/Agency for Toxic Substance and Disease Registry or the Department of Health and Human Services, or NIST.

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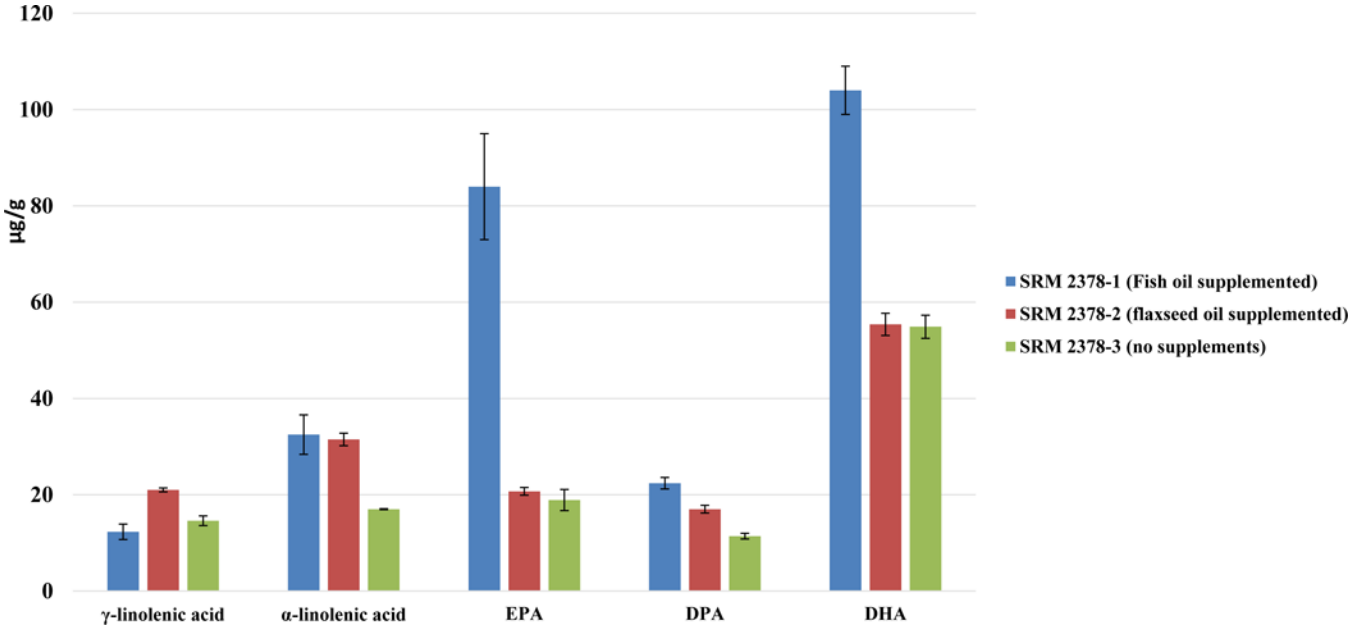

Figure 1.

Concentrations of Selected Fatty Acids in SRM 2378 (Error bars represent expanded uncertainties, $U_{95\%}$)

Table 1

Comparison of Results of Selected Fatty Acids in SRM 1950 (μg/g) Measured using GC-FID and GC/MS (NCI)

Lipid Name	Chemical Name (Common Name, g/mol)	Certified[a]	Reference[b]	NIST-1[c]	NIST-2[d]	CDC[e]
C14:0	Tetradecanoic acid (Myristic acid, 228.38)		17.9 ± 3.8	26.2 ± 0.3	25.4 ± 2.2	25.1 ± 0.6
C16:0	Hexadecanoic acid (Palmitic acid, 256.43)	594 ± 19		624 ± 14	650 ± 31	646 ± 18
C16:1 n-7	(Z)-9-Hexadecenoic Acid (Palmitoleic acid, 254.43)	53.5 ± 6.4		56.1 ± 0.8	252 ± 13	69.1 ± 1.5
	2,6,10,14-Tetramethylpentadecanoic acid (Pristanic acid, 298.50)				< 0.02	
	(7R,11R)-3,7,11,15-Tetramethyl-hexadecanoic acid (Phytanic acid, 312.53)				0.25 ± 0.03	
C18:0	Octadecanoic acid (Stearic acid, 284.48)	179 ± 12		174 ± 8	211 ± 29.4	170 ± 5
C18:3n6	(Z,Z,Z)-6,9,12-Octadecatrienoic acid (*gamma*-Linolenic acid, 278.48)		10.9 ± 2.3	13.4 ± 0.1	15.5 ± 0.69	14.8 ± 0.2
C18:2n6	(Z,Z)-9,12-Octadecadienoic acid (Linoleic acid, 280.48)	780 ± 39		879 ± 9	774 ± 14	842 ± 18
C18:1n9	(Z)-9-Octadecenoic Acid (Oleic acid, 282.48)	447 ± 43		451 ± 12	535 ± 39	527 ± 11
C18:1n7	Z)-11-Octadecenoic acid (*cis*-Vaccenic acid, 282.48)		37.7 ± 0.9	35.6 ± 0.9	99 ± 14	36.2 ± 0.9
C20:0	Eicosanoic acid (Arachidic acid, 312.54)		5.5 ± 0.2	5.57 ± 0.05	6.16 ± 0.4	5.55 ± 0.15
C20:4n6	(Z,Z,Z,Z)-5,8,11,14-Eicosatetraenoic acid (Arachidonic acid, 304.52)		293 ± 54	274 ± 13	201 ± 3.3	224 ± 5
C20:5n3	(*Z,Z,Z,Z,Z*)-5,8,11,14,17-Eicosapentaenoic acid (EPA, 302.52)		11.4 ± 0.1	12.3 ± 0.5	11.4 ± 0.2	12.5 ± 0.6
C20:1n9	(Z)-11-Eicosenoic acid (Gondoic acid, 310.54)		3.5 ± 0.1	3.44 ± 0.19	5.35 ± 0.26	3.77 ± 0.05
C22:0	Docosanoic acid (Behenic acid, 340.59)	15.9 ± 1.5		12.3 ± 0.4	19.1 ± 0.58	17.4 ± 0.4
C22:6n3	(*Z,Z,Z,Z,Z,Z*)-4,7,10,13,16,19-Docosohexaenoic acid (DHA, 328.57)		37.9 ± 6.8	38.1 ± 0.9	32.5 ± 1.17	36.5 ± 1
C22:5n3	(*Z,Z,Z,Z,Z*)-7,10,13,16,19-Docosapentaenoic acid (DPA, 330.57)		12.5 ± 0.2	12.5 ± 0.3	13.3 ± 0.25	13.7 ± 0.5
C22:1n9	(Z)-13-Docosenoic acid (Erucic acid, 338.59)		1.1 ± 0.4	1.79 ± 0.08	1.01 ± 0.11	1.45 ± 0.14
C24:0	Tetracosanoic acid (Lignoceric acid, 368.64)		16.8 ± 0.9	15.7 ± 0.3	18.2 ± 0.9	16.4 ± 0.4
C24:1n9	(Z)-15-Tetracosenoic acid (Nervonic acid, 366.63)		25.6 ± 1.2	17.1 ± 0.3	33.2 ± 0.49	25.6 ± 0.7

[a] Certified values and expanded uncertainties, $U_{95\%}$, from Certificate of Analysis.

[b] Reference values and expanded uncertainties, $U_{95\%}$, from Certificate of Analysis.

[c] KOH in methanol hydrolysis, acidic methanol derivatization, GC-FID quantification.

[d] Microwave-assisted acid hydrolysis, pentafluorobenzyl bromide derivatization, GC/MS (NCI).

[e] Acid/base hydrolysis, pentafluorobenzyl bromide derivatization, GC/MS (NCI).

Table 2

Comparison of Results[a] of Selected Fatty Acids in the Three Levels of SRM 2378 (μg/g) Measured by GC-FID[b] and GC/MS (NCI)[c,d]

		SRM 2378-1 – Fish Oil Supplementation		
Lipid Name	**Chemical Name (Common Name)**	**NIST-1[b]**	**NIST-2[c]**	**CDC[d]**
C18:3n3	(Z,Z,Z)-9,12,15-Octadecatrienoic acid (*alpha*-Linolenic acid)	35 ± 2		32.2 ± 0.1
C18:3n6	(Z,Z,Z)-6,9,12-Octadecatrienoic acid (*gamma*-Linolenic acid)	13.8 ± 0.7	12.1 ± 0.3	11.6 ± 0.1
C20:5n3	(*Z,Z,Z,Z,Z*)-5,8,11,14,17-Eicosapentaenoic acid (EPA)	94 ± 3	77 ± 5	85 ± 2
C22:5n3	(*Z,Z,Z,Z,Z*)-7,10,13,16,19-Docosapentaenoic acid (DPA)	21.2 ± 1.4	23.4 ± 0.9	24.1 ± 0.1
C22:6n3	(*Z,Z,Z,Z,Z*)-4,7,10,13,16,19-Docosohexaenoic acid (DHA)	109 ± 5	100 ± 5	109 ± 2
	(7R,11R)-3,7,11,15-Tetramethyl-hexadecanoic acid (Phytanic acid)		0.55 ± 0.01	
		SRM 2378-2 – Flaxseed Oil Supplementation		
		NIST-1[b]	NIST-2[c]	CDC[d]
C18:3n3	(Z,Z,Z)-9,12,15-Octadecatrienoic acid (*alpha*-Linolenic acid)	30.8 ± 1.0		33.5 ± 0.4
C18:3n6	(Z,Z,Z)-6,9,12-Octadecatrienoic acid (*gamma*-Linolenic acid)	20.7 ± 1.4	21.4 ± 0.8	21.6 ± 0.2
C20:5n3	(*Z,Z,Z,Z,Z*)-5,8,11,14,17-Eicosapentaenoic acid (EPA)	21.5 ± 0.9	19.4 ± 0.8	22.1 ± 0.2
C22:5n3	(*Z,Z,Z,Z,Z*)-7,10,13,16,19-Docosapentaenoic acid (DPA)	17.7 ± 0.6	16.5 ± 0.5	17.5 ± 0.4
C22:6n3	(*Z,Z,Z,Z,Z*)-4,7,10,13,16,19-Docosohexaenoic acid (DHA)	57 ± 2	52 ± 2	59.8 ± 0.2
	(7R,11R)-3,7,11,15-Tetramethyl-hexadecanoic acid (Phytanic acid)		0.31 ± 0.01	
		SRM 2378-3 – No Supplementation		
		NIST-1[b]	NIST-2[c]	CDC[d]
C18:3n3	(Z,Z,Z)-9,12,15-Octadecatrienoic acid (*alpha*-Linolenic acid)	17.0 ± 0.5		17.6 ± 0.2
C18:3n6	(Z,Z,Z)-6,9,12-Octadecatrienoic acid (*gamma*-Linolenic acid)	15.6 ± 0.6	14.2 ± 0.2	14.5 ± 0.2
C20:5n3	(*Z,Z,Z,Z,Z*)-5,8,11,14,17-Eicosapentaenoic acid (EPA)	20.9 ± 0.6	18.4 ± 1.4	19.0 ± 0.3
C22:5n3	(*Z,Z,Z,Z,Z*)-7,10,13,16,19-Docosapentaenoic acid (DPA)	10.9 ± 0.4	11.4 ± 0.5	12.2 ± 0.4
C22:6n3	(*Z,Z,Z,Z,Z*)-4,7,10,13,16,19-Docosohexaenoic acid (DHA)	53.5 ± 1.7	54.1 ± 1.2	59.0 ± 0.7
	(7R,11R)-3,7,11,15-Tetramethyl-hexadecanoic acid (Phytanic acid)		0.49 ±0.03	

[a] Mean ± 1 standard deviation.

[b] KOH in methanol hydrolysis, acidic methanol derivatization, GC-FID quantification, n = 3.

[c] Microwave-assisted acid hydrolysis, pentafluorobenzyl bromide derivatization, GC/MS (NCI), n = 4.

[d] Acid/base hydrolysis, pentafluorobenzyl bromide derivatization, GC/MS (NCI), n = 3.

Table 3

Certified Values for Fatty Acids in SRM 2378

		2378-1		2378-2		2378-3	
Lipid Name	**Chemical Name (Common Name, g/mol)**	**Mass Fraction (μg/g)**	**Conc.[a] (μmol/L)**	**Mass Fraction (μg/g)**	**Conc.[a] (μmol/L)**	**Mass Fraction (μg/g)**	**Conc.[a] (μmol/L)**
C14:0	Tetradecanoic acid (Myristic acid, 228.38)[b]	44.6 ± 1.5	200 ± 7	33.8 ± 1.2	151 ± 5	34.6 ± 0.8	155 ± 4
C16:0	Hexadecanoic acid (Palmitic acid, 256.43)[b]	833 ± 87	3320 ± 350	715 ± 111	2850 ± 440	642 ± 114	2560 ± 455
C16:1 n-7	(Z)-9-Hexadecenoic Acid (Palmitoleic acid, 254.43)[c]	53.4 ± 3.8	214 ± 15	69.1 ± 6.9	278 ± 28	45.7 ± 3.2	184 ± 13
C18:0	Octadecanoic acid (Stearic acid, 284.48)[b]	221 ± 25	795 ± 90	231 ± 14	830 ± 50	194 ± 21	696 ± 75
C18:1 n-9	(Z)-9-Octadecenoic Acid (Oleic acid, 282.48)[b]	604 ± 66	2190 ± 240	738 ± 52	2670 ± 189	569 ± 66	2060 ± 240
C18:2 n-6	(Z,Z)-9,12-Octadecadienoic acid (Linoleic acid, 280.48)[c]	1030 ± 180	3740 ± 640	1220 ± 12	4460 ± 45	913 ± 6	3330 ± 20
C18:3 n-3	(Z,Z,Z)-9,12,15-Octadecatrienoic acid[c] (*alpha*-Linolenic acid, 278.48)	32.5 ± 4.1	119 ± 15	31.5 ± 1.3	116 ± 5	17.0 ± 0.1	62.4 ± 0.5
C18:3 n-6	(Z,Z,Z)-6,9,12-Octadecatrienoic acid[b] (*gamma*-Linolenic acid, 278.48)	12.3 ± 1.6	45.1 ± 6.0	21.0 ± 0.4	77.1 ± 1.4	14.6 ± 1.0	53.6 ± 3.6
C20:4 n-6	(Z,Z,Z,Z)-5,8,11,14-Eicosatetraenoic acid[b] (Arachidonic acid, 304.52)	196 ± 20	659 ± 69	235 ± 26	790 ± 86	228 ± 14	765 ± 46
C20:5 n-3	(Z,Z,Z,Z,Z)-5,8,11,14,17-Eicosapentaenoic acid (EPA, 302.52)[b]	84 ± 11	284 ± 37	20.7 ± 0.8	70.1 ± 2.6	18.9 ± 2.2	63.8 ± 7.6
C22:5 n-3	(Z,Z,Z,Z,Z)-7,10,13,16,19-Docosapentaenoic acid (DPA, 330.57)[b]	22.4 ± 1.2	69.4 ± 3.6	17.0 ± 0.8	52.6 ± 2.4	11.4 ± 0.6	35.1 ± 1.9
C22:6 n-3	(Z,Z,Z,Z,Z)-4,7,10,13,16,19-Docosahexaenoic acid (DHA, 328.57)[b]	104 ± 5	323 ± 16	55.4 ± 2.3	173 ± 7	54.9 ± 2.4	171 ± 8

[a] Amount concentration values, mol/L and expanded uncertainties, calculated from mass fraction values using molar mass of the fatty acids and the densities of the sera.

[b] Means and expanded uncertainties, $U_{95\%}$, of NIST-1, NIST-2, and CDC method means ($k = 2.0$).

[c] Means and expanded uncertainties, $U_{95\%}$, of NIST-1 and CDC method means ($k = 2.0$).

Table 4

Reference Values for Fatty Acids in SRM 2378

		2378-1		2378-2		2378-3	
Lipid Name	**Chemical Name (Common Name, g/mol)**	**Mass Fraction (μg/g)**	**Conc.[a] (μmol/L)**	**Mass Fraction (μg/g)**	**Conc.[a] (μmol/L)**	**Mass Fraction (μg/g)**	**Conc.[a] (μmol/L)**
C10:0	Decanoic acid (Capric acid, 172.26)[b]	3.56 ± 0.04	21.1 ± 0.2	0.3 ± 0.1	1.9 ± 0.8	0.89 ± 0.15	5.3 ± 0.9
C14:1 n-5	(*Z*)-Tetradec-9-enoic acid (Myristoleic acid, 226.38)[c]	3.4 ± 1.5	16 ± 7	2.8 ± 1.5	13 ± 7	3.2 ± 1.7	14 ± 8
C15:0	Pentadecanoic acid (242.39)[b]	5.14 ± 0.16	21.7 ± 0.7	6.76 ± 0.16	28.5 ± 0.7	4.92 ± 0.12	20.7 ± 0.5
C17:0	Heptadecanoic acid (Margaric acid, 270.45)[b]	7.11 ± 0.47	26.9 ± 1.8	8.84 ± 0.21	33.4 ± 0.8	7.02 ± 0.37	26.5 ± 1.4
C18:1 n-7	(*Z*)-11-Octadecenoic acid (*cis*-Vaccenic acid, 282.48)[c]	41.3 ± 7.4	149 ± 27	35.6 ± 6.0	129 ± 22	32.0 ± 3.3	116 ± 12,
C18:4 n-3	(Z,Z,1Z,Z)-6,9,12,15-octadecatetra-enoic acid (Stearidonic acid, 276.40)[b]	2.20 ± 0.3	8.1 ± 1.1	1.11 ± 0.15	4.1 ± 0.5	0.77 ± 0.05	2.83 ± 0.17
C20:0	Eicosanoic acid (Arachidic acid, 312.54)[d]	7.6 ± 1.1	25.0 ± 3.6	8.7 ± 1.5	28.4 ± 4.8	7.9 ± 2.7	26.0 ± 9.0
C20:1 n-9	(Z)-11-Eicosenoic acid (Gondoic acid, 310.54)[c]	6.0 ± 1.0	19.7 ± 3.3	5.7 ± 1.0	18.9 ± 3.4	5.88 ± 0.43	19.4 ± 1.4
C20:3 n-9	(Z,Z,Z)-5,8,11-Eicosatrienoic acid (306.49)[b]	1.40 ± 0.09	4.67 ± 0.29	2.13 ± 0.07	7.10 ± 0.23	2.26 ± 0.03	7.54 ± 0.10
C22:0	Docosanoic acid (Behenic acid, 340.59)[c]	18.8 ± 4.3	57 ± 13	29 ± 9	86 ± 27	19.2 ± 4.5	58 ± 14
C22:1 n-9	(Z)-13-Docosenoic acid (Erucic acid, 338.59)[c]	1.7 ± 1.0	5 ± 3	1.7 ± 0.9	5 ± 3	1.9 ± 1.3	6 ± 4
C22:2 n-6	(Z,Z)-13,16-Docosadienoic acid (336.56)[a]	0.31 ± 0.02	0.93 ± 0.06	0.36 ± 0.03	1.09 ± 0.09	0.303 ± 0.003	0.92 ± 0.01
C22:5n-6	(Z,Z,Z,Z,Z)-4,7,10,13,16 Docosapentaenoic acid[b] (330.57)	2.71 ± 0.21	8.37 ± 0.64	5.20 ± 0.41	16.1 ± 1.3	5.16 ± 0.42	16.0 ± 1.3
C23:0	Tricosanoic acid (354.61)[b]	8.19 ± 0.19	23.6 ± 0.5	13.1 ± 0.5	37.9 ± 1.5	7.75 ± 0.07	22.3 ± 0.2
C24:0	Tetracosanoic acid (Lignoceric acid, 368.64)[d]	19 ± 5	54 ± 13	25 ± 9	70 ± 25	18 ± 6	49 ± 16
C24:1 n-9	(Z)-15-Tetracos-15-enoic acid (Nervonic acid, 366.63)[d]	32 ± 9	89 ± 26	30 ± 17	83 ± 49	22 ± 9	61 ± 25
C26:0	Hexacosanoic acid (Cerotic acid, 396.69)[b]	0.35 ± 0.05	0.89 ± 0.12	0.25 ± 0.04	0.63 ± 0.11	0.34 ± 0.09	0.86 ± 0.2
	(*7R,11R*)-3,7,11,15-Tetramethyl-hexadecanoic acid (phytanic acid, 312.53)[e]	0.55 ± 0.02	1.80 ± 0.06	0.33 ± 0.02	1.07 ± 0.06	0.48 ± 0.06	1.56 ± 0.18

[a] Amount concentration values, mol/L and expanded uncertainties, calculated from mass fraction values using molar mass of the fatty acids and the densities of the sera.

[b] Mean and expanded uncertainties, $U_{95\%}$, of CDC method ($k = 4.30$)

[c] Means and expanded uncertainties, $U_{95\%}$, of NIST-1and CDC method means ($k = 2.0$).

[d] Means and expanded uncertainties, $U_{95\%}$, of NIST-1, NIST-2, and CDC method means ($k = 2.0$).

[e] Mean and expanded uncertainties, $U_{95\%}$, of NIST-2 method ($k = 3.18$).