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Development of a Standard Reference Material for Metabolomics Research

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Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

Certain commercial equipment, instruments, or materials are identified in this paper 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 identified are necessarily the best available for the purpose. The findings and conclusions in this report are those of the authors and do not necessarily represent the official views or positions of the Centers for Disease Control and Prevention/Agency for Toxic Substances and Disease Registry or the Department of Health and Human Services.

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Abstract

The National Institute of Standards and Technology (NIST), in collaboration with the National Institutes of Health (NIH), has developed a Standard Reference Material (SRM) to support technology development in metabolomics research. SRM 1950 Metabolites in Human Plasma is intended to have metabolite concentrations that are representative of those found in adult human plasma. The plasma used in the preparation of SRM 1950 was collected from both male and female donors, and donor ethnicity targets were selected based upon the ethnic makeup of the U.S. population. Metabolomics research is diverse in terms of both instrumentation and scientific goals. This SRM was designed to apply broadly to the field, not toward specific applications. Therefore, concentrations of approximately 100 analytes, including amino acids, fatty acids, trace elements, vitamins, hormones, selenoproteins, clinical markers, and perfluorinated compounds (PFCs), were determined. Value assignment measurements were performed by NIST and the Centers for Disease Control and Prevention (CDC). SRM 1950 is the first reference material developed specifically for metabolomics research.

Graphical abstract



Metabolites are well-established indicators of human health, and measurement of specific metabolites has historically played a key role in disease diagnosis and risk assessment.¹ Fasting blood glucose levels are used to diagnose diabetes,^{2,3} and serum creatinine levels are monitored in assessment of kidney function.^{4,5} Individual metabolite markers tend to lack disease specificity, however, and results outside the normal range may only point to the need for further investigation rather than reflecting a clear cause and effect relationship.^{6,7} For example, elevated cholesterol levels are associated with a number of disorders, including hypothyroidism, diabetes, and kidney dysfunction. Diseases such as diabetes⁸ and Parkinson's disease^{9,10} tend to affect multiple biochemical processes in the body, and measurement of a single biomarker is often insufficient for definitive diagnosis or for classification of patients into disease subtypes. Therefore, focusing on one or even a few metabolites at a time has limited diagnostic or prognostic value and provides little insight into disease etiology.^{11,12}

Advances in technology have now made global profiling of hundreds or thousands of metabolites present in a given biological sample possible and thus have paved the way for

holistic investigations of biochemical pathways and the relationships between them.^{13,14} An examination of the complete collection of metabolites, known as the metabolome,^{15–17} may provide insight into disease mechanisms and identify markers that can pinpoint the early stages of disease when interventions are more likely to be effective.^{6,18} Similarly, metabolomics studies are also being employed to understand the mechanisms of drug-induced toxicity in the hopes of improving drug safety and efficacy.^{19–22}

Metabolomics studies can be either qualitative or quantitative in nature and either targeted (hypothesis-driven) or untargeted (global) in design.^{23,24} Given the complexity of the metabolome and the range of metabolite concentrations and polarities routinely encountered, no single analytical technique is able to provide a complete picture of the metabolites present in a specific sample. Hence, the metabolites observed are dependent to a certain extent upon the technique used as well as variables such as sampling and sample preparation protocols.^{25–27} Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the predominant analytical approaches in metabolomics research,¹⁴ although there have been applications of infrared and Raman spectroscopy as well as other techniques.^{6,15,26} The strengths and limitations of NMR and MS approaches for metabolomics have been summarized in a number of reviews.^{28–31}

Regardless of the analytical platform selected, metabolomics research relies upon the ability to compare two or more data sets and to identify particular patterns or features that differ between them. To achieve this goal and make meaningful comparisons of data, it must be possible to isolate “real” differences between samples and data sets from those that arise from analytical variability or experimental artifacts.^{32,33} Thus, identifying and controlling sources of intra- and interlaboratory variability have become crucial elements of metabolomics research.^{34,35}

Quality control (QC) materials are increasingly being employed in metabolomics as a means of assessing data quality and for identifying experimental artifacts.^{34,36} One approach to the implementation of QC materials involves combining aliquots from each of the study samples to prepare a QC pool that can be analyzed at the same time as the samples.³⁷ This technique can be particularly valuable for studies where samples are analyzed in different batches or if changes in instrument performance are suspected.³⁶ However, pooling of sample aliquots may not be feasible for long-term studies.³⁸ In addition, further confidence in data quality can be gained through the use of standards or QC materials that are independent of the samples being analyzed.

Standard Reference Material (SRM) 1950 Metabolites in Human Plasma was developed in collaboration with the National Institutes of Health (NIH) in response to a recognized need for a reference material to support metabolomics technology development. SRM 1950 consists of a human plasma pool collected from healthy adults. Concentrations of approximately 100 analytes have been determined in this material, including both metabolites and environmental contaminants. Isotope-dilution mass spectrometry approaches to analyte quantification were used whenever possible. This SRM is the first reference material developed specifically for metabolomics research.

EXPERIMENTAL SECTION

Standard Reference Material

SRM 1950 Metabolites in Human Plasma was prepared by Bioreclamation, Inc. (Hicksville, NY) according to specifications provided by NIST. The plasma pool was collected from 100 donors, with an equal number of men and women and from donors between 40 and 50 years of age. Donors were selected to be representative of the ethnic distribution of the U.S. population, based upon data from the U.S. Census in 2000. Donors were asked to fast overnight and to abstain from taking medications for 72 h prior to blood donation. A rapid glucose test was used to exclude individuals who did not comply with the fasting requirement. Individuals affected by overt disease or disorders and those having a body mass index (BMI) outside the 95th percentile were excluded.

Plasma was prepared from whole blood that was placed on ice immediately after collection, and lithium heparin was used as the anticoagulant. Each sample was centrifuged at 4 °C at $8000 \times g$ for 25 min. Samples were processed and frozen within 60 min from the time of collection. Units of plasma were thawed once and blended under nitrogen. The resulting plasma pool was dispensed in 1 mL aliquots into vials, and the vials were then stored at -80 °C prior to shipment to NIST. Approximately 20 000 vials of SRM 1950 were delivered to NIST, and a unit of SRM 1950 consists of 5 vials.

Safety Considerations

Each unit of human plasma used in the preparation of SRM 1950 was screened by Bioreclamation and found to be nonreactive for hepatitis B surface antigen (HBsAg), human immunodeficiency virus (HIV), hepatitis C virus (HCV), and human immunodeficiency virus 1 antigen (HIV-1Ag) by Food and Drug Administration (FDA)-licensed tests. However, because no test method can guarantee that these infectious agents are absent, appropriate safety precautions should be taken when handling this or any other potentially infectious human plasma or blood specimens.³⁹

Methods

Brief descriptions of the methods used in the value assignment of SRM 1950 are provided in the Certificate of Analysis⁴⁰ and are also included in the Supporting Information. Method details are also available online (<http://srm1950.nist.gov>). Some of the methods developed as part of the certification of SRM 1950 have been described in related publications.^{41–43} Certification measurements were performed at NIST and at the Centers for Disease Control and Prevention (CDC, Atlanta, GA). The methods used at NIST had, in most cases, been used previously for value assignment of other SRMs. The methods used by CDC had previously been validated for use in the National Health and Nutrition Examination Survey (NHANES). Analyte quantification was based upon isotope-dilution mass spectrometry (ID-MS) whenever possible. For analytes such as the carotenoids where ID-MS methods were not available, liquid chromatographic methods with UV/visible detection (LC-UV) or fluorescence detection (LC-FL) were employed at NIST and CDC. For some of the fatty acids and carotenoids, value assignment measurements were performed using a single method at the CDC.

RESULTS AND DISCUSSION

Validation of metabolites or profiles of metabolites as biomarkers will require the comparison of data sets from different laboratories and from different analytical platforms. Such comparisons are difficult at present, even if adequate experimental details (metadata) are provided with the results.^{1,22,44} The impact of certain preanalytical variables such as sample collection and storage can be minimized through the implementation of standard operating procedures (SOPs), but it is very difficult to adopt uniform approaches to reduce the many potential sources of variability in metabolomics, particularly when complex sample preparation and analysis schemes are required.^{32–34} As a result, experimental artifacts may be difficult to identify in the absence of appropriate measurement quality assurance materials.

Traditional approaches to detecting and minimizing analytical variability and ensuring measurement accuracy may not be easily adapted to metabolomics investigations. Internal standards, including stable isotope-labeled internal standards, can aid in correcting for variations in factors such as sample preparation, chromatographic retention, and ionization efficiency.²⁵ However, in metabolomics studies, selection of appropriate internal standards is complicated by the fact that the compounds of interest generally are not known in advance.³⁶ In addition, even when the target analytes are known, adding an internal standard for each compound when hundreds of analytes are being detected is not a viable option.

Reference materials are another mechanism for validating analytical methods and evaluating data quality. NIST SRMs have typically been developed with a particular application in mind. For example, SRM 967a Creatinine in Frozen Human Serum is directed toward laboratories performing measurements of creatinine in serum. Because metabolomics studies encompass such a wide range of interests including toxicology, nutrition, and drug development, as well as a number of different analytical platforms, it is nearly impossible to match the sample matrixes and metabolite profiles encountered by each end user of a potential reference material. An alternative approach is to develop a reference material that is as broadly applicable as possible. NIH and a panel of metabolomics investigators provided guidance to NIST in designing SRM 1950 Metabolites in Human Plasma and in selecting target analytes for value assignment.

SRM 1950 Metabolites in Human Plasma is intended to represent normal human plasma. The specifications for the preparation of the plasma pool were designed to minimize the influence of factors such as diet and medications on the metabolite profile.⁴⁵ As noted earlier, one of the goals of this work was to develop a reference material that would be suitable for applications that were difficult to predict in advance. Therefore, concentrations of a broad spectrum of metabolites were determined in SRM 1950, including amino acids, carotenoids, fatty acids, electrolytes, hormones, and vitamins. Additional measurements were performed for total protein, selenoproteins, and perfluorinated compounds (PFCs). PFCs are persistent environmental contaminants, and their concentrations in serum or plasma may be of interest for biomonitoring studies. Values were assigned for nearly 100 different species in SRM 1950, and this information is provided to users of the SRM in a Certificate of Analysis.⁴⁰ The assigned values are presented in the certificate as certified,

reference, or information values.⁴⁶ Both certified and reference values are given with their associated uncertainties, which were calculated in accordance with the International Organization for Standardization (ISO) Guide.⁴⁷

The methods used in the certification of SRM 1950 are not intended to be representative of analytical approaches typically used in metabolomics research. Candidate analytes of interest for value assignment in SRM 1950 were either known in advance or identified through preliminary screening of the material, and therefore method selection could be optimized. In many cases, metabolite concentrations were determined using methods previously employed at NIST for value assignment of other SRMs. These methods generally quantify only one or a small group of analytes at a time and are often based upon ID-MS approaches to analyte quantification. The following sections describe the value assignment process for several classes of metabolites in this reference material. Additional details can also be found in the Supporting Information.

Clinical Markers

Traditional clinical markers such as glucose, cholesterol, and triglycerides can be useful as surrogate markers for classifying or stratifying samples in metabolomics research.^{48,49} Glucose is one of the most abundant metabolites in serum or plasma, and glucose metabolism is also known to be altered in a number of disease states, including diabetes.⁸ Although electrolytes (calcium, magnesium, potassium, sodium) are not generally considered in metabolomics investigations, electrolyte imbalances are associated with a number of diseases. They are also some of the more abundant species in human serum or plasma.¹⁶ Steroid hormone profiles have been studied in relation to endocrine disorders and as potential cancer biomarkers.⁷

Table 1 summarizes the concentrations of the majority of the clinical markers that were value assigned in SRM 1950. For some analytes, mass concentrations presented in the table were calculated from mass fractions using the measured density of SRM 1950 (1.02086 g/mL). In general, each of these analytes was determined at NIST by a single method, as indicated in the table. Many of these methods are recognized as reference measurement procedures by the Joint Committee for Traceability in Laboratory Medicine (JCTLM). The GC-MS procedures for the clinical analytes involved derivatization of the analyte of interest after isolation from the plasma matrix. Labeled internal standards were added at the beginning of the sample preparation process and allowed to equilibrate with the sample for a predetermined period of time. A similar approach was utilized for the LC-MS and LC-MS/MS analyses, but derivatization was not required in these methods. Samples for ICP-MS analysis were subjected to acid digestion, and an appropriate isotope spike was employed for quantification. For the GC-MS, LC-MS, and LC-MS/MS analyses, the relative expanded uncertainties associated with these measurements are generally in the range of 1–3%, and relative expanded uncertainties <1% are common for the ICP-MS methods. This method performance is reflected in the expanded uncertainties shown with each certified or reference value in Table 1.

Amino Acids

Amino acids are the building blocks of proteins and also serve as important metabolic intermediates. Measurement of amino acids in biological fluids is routinely used in the diagnosis of metabolic disorders.⁵⁰ Thus, it is not surprising that metabolomics studies have frequently detected perturbations in amino acid concentrations that appear to be linked with particular disease states.^{51–53} Research has also indicated that amino acid metabolism is altered in diabetes, and certain branched chain amino acids may have utility as early indicators of diabetes risk.^{8,54} In some cases, changes in specific amino acid concentrations appear to have merit in classifying patients by disease subtype, as was reported for chronic obstructive pulmonary disease (COPD).⁵⁵

Amino acids were determined in SRM 1950 through a combination of data from four independent methods based upon liquid chromatography–tandem mass spectrometry (LC-MS/MS), gas chromatography–time-of-flight mass spectrometry (GC-TOF-MS), and two-dimensional GC coupled to time-of-flight mass spectrometry (GCxGC-TOF-MS).⁴¹ For the GC-TOF-MS analyses, either propyl chloroformate (PCF) or *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was used as the derivatizing agent. Only MTBSTFA was used as the derivatizing agent for the GCxGC-TOF-MS measurements. Isotopically labeled internal standards were employed for each of the amino acids and were added at the beginning of the sample analysis. In general, good agreement was observed among the results from the four methods. Details on the results from the individual methods are presented elsewhere.⁴¹ Coefficients of variation (CVs) for the LC-MS/MS method were typically in the range of 1–3%, reflecting the excellent precision commonly associated with ID-MS methods. The observed CVs for the GC-TOF-MS and GCxGC-TOF-MS analyses were generally higher (\approx 5–10%), perhaps because of the additional sample preparation steps that were required for these methods.

The concentrations of 18 amino acids were assigned based upon a combination of the GC-MS and LC-MS/MS methods, and the certified and reference values are summarized in Table 2. Arginine and ornithine could not be determined in SRM 1950 by the GC-MS methods because of thermal instability of the arginine derivative, leading to potential conversion to ornithine. Histidine could not be determined by GC-TOF-MS because of a large interference from glucose, but adding a second dimension to the separation (GCxGC) removed the interference. Hence, employing multiple independent methods reduced the likelihood of undetected measurement bias and provided greater confidence in the value assignment of amino acids in SRM 1950. The results obtained in this work were also comparable to a related literature report on analysis of SRM 1950 by NMR⁵⁶ and to information on amino acid concentrations found in the Human Metabolome Database.¹⁷ SRM 1950 is the first serum or plasma-based NIST SRM with values assigned for amino acids.

Fatty Acids

Dysregulation of fatty acid metabolism has been implicated in a number of diseases, including diabetes⁸ and cancer.⁵² Some drug therapies have been shown to cause widespread changes in lipid profiles,⁵⁷ and differences in fatty acid concentrations have also been

observed between groups having disparate levels of physical fitness.⁵⁸ Fatty acids were value assigned in SRM 1950 through a combination of GC methods with either flame ionization (FID) or mass spectrometric detection. A total of 26 fatty acids were determined in SRM 1950, including saturated, monounsaturated, and polyunsaturated fatty acids. The results are summarized in Table 3. Of these fatty acids, 14 were measured by a single method, either at NIST or at CDC. The two NIST methods (GC-FID and GC-MS) employed different sample preparation schemes and different chromatographic columns. The CDC method (GC-MS) represented a third approach and was independent from the NIST methodologies.

Figure 1 shows a comparison of results from the three methods for selected fatty acids. The certified or reference values derived from these results are also shown in the figure. In general, there was good agreement among the methods used, even for fatty acids present in SRM 1950 at low concentrations. Oleic acid, palmitic acid, and linoleic acid were the most abundant fatty acids measured in SRM 1950, and these results are consistent with U.S. population data from NHANES.⁵⁹ Omega-3 fatty acids are of interest because of their potential cardioprotective effects,⁴⁹ and several omega-3 fatty acids were measured in SRM 1950, as shown in Table 3. SRM 1950 represents the first serum or plasma-based NIST SRM with values assigned for fatty acids.

Vitamins and Carotenoids

Numerous studies have attempted to discern the relationship between diet and the risk of disease. Nutrients such as carotenoids and vitamin D may reduce the risk of cancer or other diseases in certain populations.^{60,61} However, identifying individuals more likely to respond to dietary interventions remains an elusive goal.⁶² Metabolomics studies may provide answers to some of the seemingly contradictory results that have been reported in nutrition research.⁶³

Vitamin A, vitamin E, and carotenoids were determined in SRM 1950 using a combination of LC-UV methods, as shown in Table 4. The NIST methods utilized two different types of chromatographic columns, and additional method details are provided in the Supporting Information. Whenever possible, the values in Table 4 represent a combination of the results obtained by NIST and CDC. In some cases, however, assigned values for the carotenoids were based solely upon measurements performed at CDC. Metabolites arising from two water-soluble vitamins (folate and vitamin B₆) and one additional fat-soluble vitamin (vitamin D) were also measured in SRM 1950, and these results are included in Table 4. The certified and reference values for these analytes were based primarily upon ID-MS methods, but an LC-FL method was included for analysis of pyridoxal 5'-phosphate, a vitamin B₆ metabolite.

CONCLUSIONS

SRM 1950 Metabolites in Human Plasma represents the first reference material developed specifically to support measurement quality assurance in metabolomics research. This SRM will complement existing approaches including the use of internal standards and QC materials that are already being employed in metabolomics analyses. In addition, SRM 1950

is intended to facilitate the development and validation of new metabolomics technology. Sufficient material was acquired to ensure approximately 10 years of availability for this SRM, and stability of the material is routinely monitored. Work to characterize the metabolite profile of SRM 1950 is continuing, and additional values will be added as they become available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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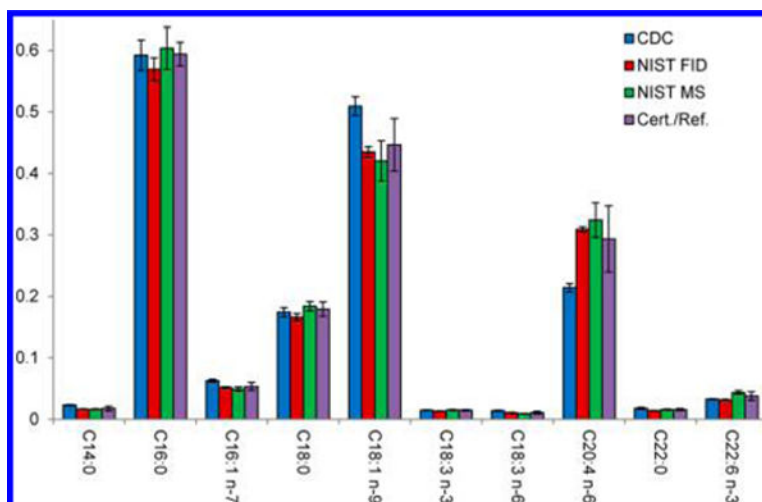


Figure 1. Comparison of results (mg/g) for selected fatty acids in SRM 1950. For the individual methods, the error bars represent one standard deviation. For the certified and reference values, the error bars represent the expanded uncertainty, U .

Table 1Concentrations of Clinical Markers in SRM 1950^a

analyte	mass concentration (mg/dL)	molar concentration (mmol/L)	method
bilirubin	0.344 ± 0.023		spectrophotometry
cholesterol	151.4 ± 3.3	3.917 ± 0.085	ID GC-MS
creatinine	0.6789 ± 0.0108	0.0600 ± 0.0009	ID LC-MS
glucose	82.16 ± 1.00	4.560 ± 0.056	ID GC-MS
total glycerides	99.0 ± 2.1	1.12 ± 0.02	ID GC-MS
calcium		1.936 ± 0.024	ID ICP-MS
magnesium		0.696 ± 0.004	ID ICP-MS
potassium		3.665 ± 0.025	ID ICP-MS
sodium		141.76 ± 0.31	gravimetry

analyte	mass concentration (mg/L)	molar concentration (μmol/L)	method
homocysteine	1.150 ± 0.026	8.50 ± 0.20	ID GC-MS

analyte	mass fraction (ng/g)	mass concentration (ng/mL)	method
cortisol	82.2 ± 1.7	83.9 ± 1.7	ID LC-MS/MS
progesterone	1.452 ± 0.037	1.482 ± 0.038	ID LC-MS/MS
testosterone	2.169 ± 0.046	2.214 ± 0.047	ID LC-MS/MS

^a Certified values (bold font) and reference values (normal font) are provided with their associated uncertainties.

Table 2Concentrations of Amino Acids in SRM 1950 Metabolites in Human Plasma^a

amino acid	mass fraction (mg/kg)	molar concentration ($\mu\text{mol/L}$)
alanine	26.2 ± 2.2	300 ± 26
arginine	13.89 ± 0.40	81.4 ± 2.3
cysteine	5.26 ± 0.81	44.3 ± 6.9
cystine	1.83 ± 0.08	7.8 ± 0.4
glutamic acid	9.7 ± 2.5	67 ± 18
glycine	18.0 ± 1.2	245 ± 16
histidine	11.04 ± 0.55	72.6 ± 3.6
isoleucine	7.13 ± 0.42	55.5 ± 3.4
leucine	12.90 ± 0.82	100.4 ± 6.3
lysine	20.0 ± 1.9	140 ± 14
methionine	3.26 ± 0.26	22.3 ± 1.8
ornithine	6.7 ± 0.4	52.1 ± 2.8
phenylalanine	8.2 ± 1.1	51 ± 7
proline	19.9 ± 1.1	177 ± 9
serine	9.87 ± 0.44	95.9 ± 4.3
threonine	13.94 ± 0.70	119.5 ± 6.1
tyrosine	10.17 ± 0.53	57.3 ± 3.0
valine	20.9 ± 1.2	182.2 ± 10.4

^aCertified values (bold font) and reference values (normal font) are provided with their associated uncertainties.

Table 3Concentrations of Fatty Acids in SRM 1950^a

lipid name	common name	mass fraction ($\mu\text{g/g}$)	molar concentration ($\mu\text{mol/L}$)
C12:0	lauric acid	1.86 ± 0.11	9.47 ± 0.57
C16:0	palmitic acid	594 ± 19	2364 ± 77
C 16:1 n-7	palmitoleic acid	53.5 ± 6.4	215 ± 26
C18:0	stearic acid	179 ± 12	644 ± 41
C18:3 n-3	α -linolenic acid	14.9 ± 1.0	54.6 ± 3.6
C18:1 n-9	oleic acid	447 ± 43	1614 ± 154
C18:2 n-6	linoleic acid	780 ± 39	2838 ± 143
C22:0	behenic acid	15.9 ± 1.5	47.8 ± 4.6
C14:0	myristic acid	17.9 ± 3.8	80.1 ± 17.0
C14:1	myristoleic acid	1.57 ± 0.03	7.1 ± 0.1
C15:0	pentadecanoic acid	1.08 ± 0.01	4.56 ± 0.04
C17:0	margaric acid	4.7 ± 0.2	17.6 ± 0.7
C18:3 n-6	γ -linolenic acid	10.9 ± 2.3	39.9 ± 8.5
C18:1 n-7	vaccenic acid	37.7 ± 0.9	136 ± 3
C20:0	arachidic acid	5.5 ± 0.2	18.0 ± 0.5
C20:1	gondolic acid	3.5 ± 0.1	11.5 ± 0.5
C20:2	(<i>Z,Z</i>)-11,14-eicosadienoic acid	5.7 ± 0.2	18.8 ± 0.6
C20:3 n-6	homo- γ -linolenic acid	41.8 ± 1.1	139 ± 4
C20:4 n-6	arachidonic acid	293 ± 54	984 ± 180
C20:5 n-3	EPA	11.4 ± 0.1	38.6 ± 0.5
C22:1	erucic acid	1.1 ± 0.4	3.4 ± 1.3
C22:4 n-6	(<i>Z,Z,Z,Z</i>)-7,10,13,16-docosatetraenoic acid	8.3 ± 0.2	25.5 ± 0.6
C22:5 n-3	DPA	12.5 ± 0.2	38.5 ± 0.7
C22:5 n-6	(<i>Z,Z,Z,Z,Z</i>)-4,7,10,13,16-docosapentaenoic acid	6.3 ± 0.1	19.5 ± 0.4
C22:6 n-3	DHA	37.9 ± 6.8	118 ± 21
C24:0	lignoceric acid	16.8 ± 0.9	46.6 ± 2.6
C24:1	nervonic acid	25.6 ± 1.2	71.3 ± 3.2

^a Certified values are presented in bold.

Table 4Concentrations of Vitamins and Carotenoids in SRM 1950^a

analyte	mass fraction (mg/kg)	mass concentration ($\mu\text{g/mL}$)	method(s)
retinol	0.396 \pm 0.034	0.404 \pm 0.035	LC-UV
retinyl palmitate	0.0067 \pm 0.0004	0.0069 \pm 0.0004	LC-UV
retinyl stearate	0.0022 \pm 0.0002	0.0023 \pm 0.0002	LC-UV
α -tocopherol	8.01 \pm 0.22	8.18 \pm 0.22	LC-UV
γ - + β -tocopherol	1.67 \pm 0.16	1.71 \pm 0.17	LC-UV
<i>trans</i> -lycopene	0.14 \pm 0.01	0.14 \pm 0.01	LC-UV
total lycopene	0.32 \pm 0.02	0.33 \pm 0.02	LC-UV
lutein	0.067 \pm 0.022	0.069 \pm 0.023	LC-UV
zeaxanthin	0.021 \pm 0.005	0.022 \pm 0.005	LC-UV
β -cryptoxanthin	0.038 \pm 0.003	0.039 \pm 0.003	LC-UV
total α -carotene	0.025 \pm 0.005	0.026 \pm 0.005	LC-UV
total β -carotene	0.077 \pm 0.004	0.079 \pm 0.004	LC-UV
<i>trans</i> - β -carotene	0.071 \pm 0.005	0.072 \pm 0.005	LC-UV
<i>cis</i> - β -carotene	0.0040 \pm 0.0003	0.0041 \pm 0.0003	LC-UV

analyte	mass fraction (ng/g)	mass concentration (ng/mL)	method(s)
25-hydroxyvitamin D ₂	0.51 \pm 0.17	0.52 \pm 0.17	ID LC-MS, ID LC-MS/MS
25-hydroxyvitamin D ₃	24.27 \pm 0.75	24.78 \pm 0.77	ID LC-MS, ID LC-MS/MS
5-methyltetrahydrofolate	12.11 \pm 0.31	12.36 \pm 0.32	ID LC-MS/MS
folic acid	1.48 \pm 0.44	1.51 \pm 0.45	ID LC-MS/MS
pyridoxal 5'-phosphate	8.02 \pm 0.45	8.19 \pm 0.46	ID LC-MS/MS, LC-FL

^a Certified values are given in bold.