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Development of a kelp powder (*Thallus laminariae*) Standard Reference Material

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Abstract

A Standard Reference Material (SRM) of seaweed, SRM 3232 Kelp Powder (*Thallus laminariae*) has been developed to support food and dietary supplement measurements in compliance with the Food Safety Modernization Act (FSMA) and the Dietary Supplement Health and Education Act of 1994 (DSHEA). The material was characterized for nutritional minerals, arsenic species, isomers of vitamin K_1, proximates, and toxic elements. Kelp is a rich source of vitamins and minerals, and it is an excellent source of dietary iodine. Kelp also contains a large amount of arsenic, which is toxic as inorganic species but much less so as organic species. To capture the dietary profile of kelp, certified values were issued for As, Ca, Cd, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Mo, Na, Pb, and Zn. Reference values for proximates were assigned. For the first time, a certified value for iodine, reference values for isomers of vitamin K_1, and reference values for arsenic species including arsenosugars were assigned in a seaweed. SRM 3232 fills a gap in Certified Reference Materials (CRMs) needed for quality assurance and method validation in the compositional measurements of kelp and similar seaweeds as food and as dietary supplements.

Keywords

Kelp; *Laminaria*; iodine; arsenosugar; vitamin K_1; SRM

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Introduction

Humans have been eating seaweeds for millennia. Early written accounts of seaweeds used as food can be traced back to 300 AD in China [1], 400 AD in Japan [2], and 600 AD in Ireland [1]. Seaweeds were served in 21 % of meals in Japan based on a 2001 research report of a typical Japanese diet [3]. The per capita consumption there nearly doubled from (5.2 ± 0.9, 1 standard deviation (SD)) g/d between 1949 and 1996 to approximately 10 g/d between 2010 and 2014 [1,4]. An estimated US$6 billion of seaweed was produced worldwide in 2003, of which US$5 billion worth was consumed as food products [2]. The increased demand for seaweeds in the last century surpassed the wild stocks of seaweed and more than 95% of the supplies are cultivated nowadays [1,2]. China is the largest producer of edible seaweeds, accounting for over 60% of the world's production, and *Laminaria japonica* is the largest cultivated crop in the region [2].

Although seaweeds have not been well known as food outside of Asian cuisine, the potential health benefits of seaweeds are being studied and exploited in dietary supplements. The consumption of seaweeds has been linked to lower incidences of cancer, hyperlipidemia, and coronary heart disease [5,6]. Seaweeds contain many novel polysaccharides that are indigestible by the human body and ferment in the large intestine [1,5], including laminarins, kelp's namesake storage glucans [7]. These undigested fermentable materials may beneficially affect the intestinal microbiota as prebiotics [1,5,8]. Alginic acid, a soluble fiber in seaweed, has been found to aid in weight loss [9]. In addition, seaweeds are a source of vitamins and mineral nutrients, especially iodine, which is essential for thyroid health [10].

Certified reference materials (CRM) for seaweeds were developed for consumer safety testing and environmental monitoring. Seaweeds are sentinels of environmental pollution because of their structural polysaccharides which sequester metals from the surrounding seawater [11,12,13]. In the aftermath of atmospheric nuclear weapons testing and accidents such as those in Sellafield and Chernobyl, National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 4359 Seaweed Radionuclide Standard and International Atomic Energy Agency (IAEA) CRM IAEA-446 Radionuclides in Baltic Sea Seaweed (*Fucus vesticulosus*) were issued in 2004 and 2013, respectively, for method validation in the measurements of natural and anthropogenic radionuclides in seaweeds and similar marine specimens. IAEA-140/TM Trace Elements and Methylmercury in Seaweed (*Fucus sp.*) was released in 1997 and included reference values for 24 elements and methylmercury. The reference values for most of the elements are listed in Table 1. The National Institute of Environmental Studies in Japan produced CRM No. 9 "Sargasso" (*Sargassum fulvellum*) that was certified for 16 elements. Reference values were provided for an additional 14 elements.

Around 2011, the National Metrology Institute of Japan (NMIJ) produced NMIJ CRM 7405-a Trace Elements and Arsenic Compounds in Seaweed (Hijiki) that was certified for 18 elements and arsenic acid (AsV) for method validation in food safety measurement [14]. (Hijiki (*Hizikia fusiforme*) is popular in Japanese diets; however, the Food Standards Agency in the UK advised consumers to avoid consumption of hijiki because it contains

high levels of toxic inorganic arsenic [15].) The National Institute of Metrology in China certified 3 elements in GBW08521, a CRM of laver that is also known as nori, gim, and zicai in different parts of the world. The Institute for Reference Materials and Measurements (IRMM) produced European Reference Material (ERM) CD 200, a bladderwrack (*Fucus vesiculosus*) specimen certified for 7 elements for environmental monitoring and food safety measurements.

All of the above CRMs are based on algae in the order of *Fucales* except for laver, which is in the order of *Bangiales*. Missing in the availability of seaweed CRMs is kelp in the order of *Laminariales* genus *Laminaria*, one of the most widely cultivated brown algae commodities for food, food ingredients, and dietary supplements [2]. The chemical composition and nutritional value in terms of elemental contents, vitamins, and other bioactive constituents varies widely among seaweed species [10]. Iodine and alginic acid contents in kelp are among the highest in edible seaweeds [10]. Laminarin that was found to possess regenerative properties for skin cells is abundant in kelp [16]. For accurate assessment of nutritional values and toxic impurities in kelp samples, a natural-matrix reference material that matches not only the matrix but also the analyte levels is needed for validation of measurement methods and for quality assurance of the measurement process.

In 2012, NIST and the National Institutes of Health Office of Dietary Supplements began a collaboration for the development of SRM 3232 Kelp Powder (*Thallus laminariae*) to fill the gap in the availability of edible seaweed matrix CRMs and the analytes certified therein. The resulting SRM 3232 became the first seaweed CRM assigned reference values for *cis*- and *trans*-vitamin K_1 and hydrophilic arsenic species, and the first seaweed CRM certified for the essential nutrient element iodine. SRM 3232 is intended to meet the measurement needs for compliance with the Food Safety Modernization Act (FSMA), Dietary Supplement Health and Education Act (DSHEA), and the current Good Manufacturing Practice (cGMP) to safeguard the consumption of seaweed commodities in *Laminaria* genus [17]. This paper describes the analytical approach to the measurement and value assignment of Al, As, Cd, Cl, Co, Cr, Cu, Fe, Hg, I, K, Mg, Mn, Mo, Na, P, Pb, Rb, Zn, *cis*- and *trans*-vitamin K_1, arsenic species, proximates, and calories in SRM 3232 through the use of either a primary method [18] or agreement of results among multiple validated methods in accordance with NIST requirements [19].

Experimental

Sample Collection and Preparation

The kelp used to produce SRM 3232 was harvested from the East China Sea when the kelp was approximately six months old. The pH of the water was between 7.8 and 8.2 and the quality of the water met grade 2 Chinese Seawater Quality Standard (GB 3097-1997) [20]. No other seaweed was grown near the kelp crop and no pesticide was applied during the life cycle of the kelp crop. The thallus was processed into powder that was passed through a 60-mesh sieve. The kelp powder was purchased from the manufacturer Sinochem Ningbo (Ningbo, China) by Modern Nutrition and Biotech (Ridgefield, CT, USA) and was received by NIST directly from the manufacturer in China. The kelp powder was homogenized by blending and was packaged into packets at High-Purity Standards (Charleston, SC). A

packet contains approximately 5 g of kelp powder sealed in a nitrogen-flushed plastic bag, which was sealed inside a nitrogen-flushed aluminized polyethylene bag with two packets of silica gel. To prevent mold growth during long-term storage, the packets of kelp powder were irradiated in a single lot at Neutron Products (Dickerson, MD) to an absorbed dose of 5.9 kGy to 7.6 kGy and then were packaged into units of SRM 3232 at NIST.

Correction to a Dry-Mass Basis

The results for the constituents in SRM 3232 are reported on a dry-mass basis, but the SRM was analyzed as received. Separate portions of the SRM were used to determine the moisture content for conversion of the analysis results from an as-received mass basis to a dry-mass basis. Approximately 1.0 g to 1.6 g of the SRM was used to fill the glass weighing vessel to approximately 1 cm in depth, and the content was dried to a constant mass. A constant mass was reached by drying over magnesium perchlorate in a desiccator at room temperature for 28 d or by drying in a forced-air oven at 80 °C for 3 h. The mean values of the two methods were combined to derive the unweighted mean and the expanded uncertainty at approximately 95 % confidence of the dry-mass fraction of 0.9368 g ± 0.0015 g dry-mass per gram of as-received mass. All measurement uncertainties are expanded uncertainties at approximately 95% confidence unless noted otherwise.

Determination of Elements

Value assignment of the mass fractions of the elements was based on NIST measurements using inductively coupled plasma optical emission spectrometry (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), isotope dilution ICP-MS (ID-ICP-MS), isotope dilution cold-vapor generation ICP-MS (ID-CV-ICP-MS), and instrumental neutron activation analysis (INAA). Veritas redistilled grade nitric acid from GFS Chemicals (Powell, OH, USA) was used in sample preparation for ICP-OES measurements. Optima grade nitric acid, Optima grade hydrofluoric acid, and Optima grade ammonium hydroxide from ThermoFisher Scientific (Waltham, MA, USA) were used in sample preparations for ICP-MS measurements. Puratronic grade ammonium carbonate from Alfa Aesar (Ward Hill, MA, USA) was used for buffer preparation. All other chemicals were reagent grade, and 18MΩ cm deionized water was used for dilution.

ICP-OES and ICP-MS methods—For the determination of Ca, Fe, Mg, Mn, P, K, Na, and Zn using PerkinElmer (Shelton, CT, USA) model Optima 3000 Dual View ICP-OES, 0.5 g test portions from ten packets of SRM 3232 were digested in a closed-vessel microwave system using nitric acid. In and Sc were used as internal standards. For ICP-MS determination of As, 0.5 g test portions from six packets of SRM 3232 were digested in a closed-vessel microwave system using nitric acid. Rb and Y were added and the sum of the intensities of ^{85}Rb and ^{89}Y was used as the internal standard for the determination of arsenic in high resolution mode (R≈10,000) by ThermoFisher Scientific model Element XR sector field ICP-MS. Se was used as the internal standard for the determination of arsenic by ThermoFisher Scientific model XseriesII quadrupole ICP-MS. The instrument was operated in collision cell mode using 7% H_2 in He as a collision gas. For the determination of iodine by ThermoFisher Scientific model X7 ICP-MS, 0.2 g test potions from eight packets of SRM 3232 were digested in a closed-vessel microwave system using ammonium hydroxide.

Rh was used as an internal standard. Quantification for all analyses was based on the method of single-point standard addition using the SRM 3100 series single-element standard solutions for calibration.

ID-CV-ICP-MS method—Test portions of 0.25 g from six packets were digested with an enriched ^{201}Hg isotope in a closed-vessel microwave system using a mixture of hydrochloric acid and nitric acid. The digests were stored in a refrigerator at 4 °C to allow degassing of excess nitrogen dioxide and carbon dioxide overnight. The following day, mercury isotopes were measured in time-resolved-analysis mode using cold vapor generation coupled with a ThermoFisher Scientific model X7 ICP-MS [21].

ID-ICP-MS methods—For the determination of Cr, Cu, and Mo, test portions of 0.5 g from six packets were digested with enriched ^{53}Cr, ^{65}Cu, and ^{97}Mo in a closed-vessel microwave system using a mixture of hydrofluoric acid and nitric acid. The analyte isotopes were measured at mass resolution of approximately 4000 using the Element XR sector-field ICP-MS. For the determination of Cd and Pb, test portions of 0.6 g were digested with enriched ^{111}Cd and ^{206}Pb in a closed-vessel microwave system using a mixture of hydrofluoric acid and nitric acid. The analyte isotopes were measured using a ThermoFisher Scientific XseriesII ICP-MS fitted with matrix-tolerant (Xt) cones. Lead was measured in standard mode, and Cd was measured in collision cell mode with kinetic energy discrimination using 8 % hydrogen in balance helium as the collision gas [22,23].

INAA methods—Test portions of 0.2 g from ten packets were made into 13 mm pellets, sealed in linear polyethylene bags, and irradiated at a neutron flux of $2.4 \cdot 10^{13}$ $cm^2 s^{-1}$ in the RT-2 channel of the NIST Center for Neutron Research [24]. For the determination of Al, Ca, Cl, I, K, Mg, Mn, and Na, the duration of the irradiation was 1 min. Gamma rays were counted for 5 min to 30 min after a 5 min to 3 h decay. For the determination of As, Co, Fe, Rb, and Zn, the duration of the irradiation was 6 h. The samples were rotated 180° after 3 h to ensure a uniform neutron flux for the irradiation. Gamma rays were counted for 2 h to 8 h after a 5 d to 1 month decay. The quantitative evaluation followed the NIST standard comparator method [25].

Determination of Arsenic Species

Value assignment of the mass fractions of arsenic species in SRM 3232 was based on measurements using liquid chromatography followed by off-line INAA determination (LC-INAA) and in-line ICP-MS determination (LC-ICP-MS) [27,28]. CRMs SPEC-AS3 and SPEC-AS5 from Spex CertiPrep (Metuchen, NJ, USA) were used as calibration standards for arsenite (AsIII) and AsV, respectively. High-purity monomethylarsonic acid (MMA) from Chem Service (West Chester, PA, USA), cacodylic acid (DMA) from Sigma-Aldrich (Milwaukee, WI, USA), trimethylarsine oxide (TMAO) from Wako Chemicals (Richmond, VA, USA), and a gift of As(328) (see Figure 1 for nomenclature) from John T. Creed (USEPA, Cincinnati, OH, USA) were characterized for use as calibration standards using a procedure described elsewhere [26]. Briefly, the total arsenic in the calibrant solution was determined by INAA using the same procedure for the determination of arsenic in LC fractions described below. Arsenic impurities in the calibrant solution were determined by

LC-ICP-MS. The mass fraction of the analyte arsenic species in the solution was calculated by subtracting the arsenic impurities from the total arsenic.

Extraction procedure—Test portions of 1 g from eight packets were transferred into 15 mL polypropylene test tubes, into which 10 mL of a solvent containing 50 % volume fraction of methanol in water were added. The contents were vortexed at 40 Hz for 1 min and then allowed to stand on the bench for 2 h at ambient temperature of 21 °C ± 1 °C. The contents were vortexed again at 40 Hz for 30 s and centrifuged at 3600 g_n for 30 min. The supernatant was used for speciation measurements by LC-INAA and LC-ICP-MS. The mass of the extracted arsenic species was calculated as the mass concentration of the species in the extract multiplied by the volume of the solvent.

LC-INAA method—From the above extraction procedure, a 0.9 g aliquot of the extract was diluted in a 2 mL vial by weighing into the vial 0.9 mL water and 0.2 mL internal standard. A 5 μg/g MMA solution and a 5 μg/g TMAO solution were used as the internal standards for separation by anion exchange and cation exchange, respectively. A PerkinElmer series 200 LC system consisted of a quaternary pump and an autosampler was used. A 50 μL aliquot of the extract containing the internal standard was injected onto a Macherey-Nagel (Dueren, Germany) Nucleosil 100-5 SA (250 mm × 4 mm, 5 μm), or a Hamilton (Reno, NV, USA) PRP X-100 (250 mm × 4.6 mm, 10 μm) column and eluted under isocratic conditions of 1 mL/min using 30 mmol/L pyridine at pH 3.0 or 20 mmol/L ammonium carbonate at pH 9.0, respectively. Arsenic species were quantitatively collected in the fractions of the eluent. The solvent of each fraction was evaporated at 75 °C under a stream of nitrogen. The residue was reconstituted in water and quantitatively transferred to a filter paper, which was dried under an infrared lamp and pressed into a pellet for measurement using the INAA procedure described above [27,28].

LC-ICP-MS method—From the above extraction procedure, a 0.9 g aliquot of the extract was diluted in a 2 mL vial by weighing into the vial 0.9 mL of water and 0.2 mL of 5 μg/g TMAO solution serving as an internal standard. A 0.5 g aliquot of the resulting solution was diluted to 2 g with water to form a measurement sample. A 1 g aliquot of the measurement sample solution was spiked with a 0.3 g standard containing 95 ng/g As(328), 30 ng/g DMA, and 30 ng/g AsV. An unspiked sample was prepared by mixing 1 g of the measurement sample with 0.3 g water. The spiked and the unspiked kelp extract samples were measured for AsIII, AsV, DMA, TMAO, and As(328) by cation exchange LC-ICP-MS using the Nucleosil 100-5 SA column coupled to a PerkinElmer DRCII ICP-MS via a PerkinElmer series 200 autosampler and quaternary pump. An injection of 50 μL sample was eluted at 1 mL/min in the gradient of mobile phases A and B each containing 2% methanol in water in addition to 0.1 mmol/L and 30 mmol/L (pH 3.0) pyridine, respectively. The gradient program was as follows: 0-4 min, isocratic at 100% A; 4-5 min, linear gradient from 100% A to 100% B; 5-20 min isocratic at 100% B; 20-25 min, equilibration at 100% A [27].

Determination of Vitamin K_1

Value assignment of total vitamin K_1 as a sum of *trans*-vitamin K_1 and *cis*-vitamin K_1 was based on measurements using an isotope dilution liquid chromatography atmospheric

pressure chemical ionization tandem mass spectrometry (ID-LC-APCI-MS/MS) method. The ratio of vitamin K_1 isomers in SRM 3232 was determined with the assumption that the instrument response was the same for *trans*- and *cis*- isomers. Phylloquinone (vitamin K_1) was obtained from ChromaDex (Irvine, CA, USA). Vitamin K_1-[$^2H_7$] was obtained from Isosciences (King of Prussia, PA). Three independently weighed stock solutions of vitamin K_1 and one solution of Vitamin K_1-[$^2H_7$] were prepared in ethanol. Three calibration solutions were prepared from each stock solution by combining the stock solution and labeled internal standard solution to obtain approximately equal amounts of the labeled and unlabeled forms of the analytes. For sample preparation, test portions of 2.5 g from 10 packets were transferred into 50 mL polyethylene centrifuge tubes containing an appropriate amount of vitamin K_1-[$^2H_7$] as an internal standard. Thirty milliliters of 30 mg/L butylated hydroxytoluene (BHT) in hexane was added as an antioxidant. The samples were sonicated in a water bath for 1 h, mixed on a rotary mixer at 1 Hz for 1 h, and centrifuged at 1400 g_n for 15 min, and the hexane was removed from the aqueous solution. Another 30 mL aliquot of hexane was added and the extraction process was repeated three more times. The hexane fractions from the four extraction steps were combined, and 1 g magnesium sulfate was added. The contents were mixed and centrifuged. The hexane phase was decanted into a drying vessel, dried under nitrogen, and the residue was reconstituted in 1.0 mL of 1:1 volume ratio of ethanol:ethyl acetate that contained 30 mg/L BHT. The separation of vitamin K_1 isomers was accomplished by eluting with a step gradient of methanol and water at 0.75 mL/min through a 150 mm × 3 mm Accucore (ThermoFisher Scientific) C30 reversed-phase column. Mobile phase A was 95:5 volume ratio of methanol: water. Mobile phase B was methanol. The gradient program was as follows: 0-20 min, isocratic at 100% A; 20-23 min, step gradient at 100% B; 23-27 min, step gradient for equilibration at 100% A. The injection volume was 10 μL. An Applied Biosystems API 5000 LC/MS/MS system (ThermoFisher Scientific) was operated in positive ion mode and multiple reaction monitoring (MRM) mode. The transitions at *m/z* 451.4 → *m/z* 187.3 and at *m/z* 458.4 → *m/z* 194.4 were monitored for vitamin K_1 and vitamin K_1-[$^2H_7$], respectively.

Determination of Proximates

Covance Laboratories (Madison, WI, USA) and The National Food Laboratory (Livermore, CA, USA) reported values for ash, protein, total fat, carbohydrates, moisture, and calories. Ash was determined using AOAC methods 923.03 Ash of Flour and 940.26 Ash of Fruits, and Fruit Products. Protein was determined using AOAC 992.15 Crude Protein in Meat and Meat Products Including Pet Foods method. Total Fat was determined using AOAC 922.06 Fat in Flour method. Carbohydrates were calculated per 100 g of the sample minus the sum of moisture, protein, total fat, and ash in the sample, wherein the moisture was measured using AOAC 920.151 Solids (Total) in Fruits and Fruit Products and AOAC 925.09 Solids (Total) and Moisture in Flour. Calories were calculated by summing the caloric equivalents of protein, carbohydrate, and fat according to the Atwater system using the average values of 4 Kcal/g for protein, 4 Kcal/g for carbohydrate, and 9 Kcal/g for fat.

Results and discussion

Mineral nutrients

The Institute of Medicine of the US National Academy of Sciences established Dietary Reference Intakes (DRIs) to guide health professionals in the United States and Canada in assessing and planning for the nutrient needs of individuals. Sixteen elements are listed as mineral nutrients with DRIs, which include Ca, Cl, Cr, Cu, F, Fe, I, K, Mg, Mn, Mo, Na, P, Se, sulfate, and Zn [29]. Seaweed in general is a good source of minerals because structural polysaccharides of seaweed sequester metals, but the mineral composition of seaweed varies with the endogenous factors and the surrounding seawater [13]. Kelp contains abundant alginic acid that is a sequester of alkali metals, while proteins in the seaweed tend to bind with Zn, Cr, and Fe forming metalloproteins [13]. That ion exchange is the main underlying mechanism of bioaccumulation of metals gives rise to positive correlation of mineral contents in the seaweed and the surrounding seawater [13]. This makes seaweed a useful tool for monitoring the marine environment [12]. SRM 3232 is value assigned for 13 of the 16 mineral elements having DRIs, 4 environmental pollutants, and 3 additional elements. Table 1 lists the elemental contents of the SRM together with the values assigned to the seaweed CRMs available worldwide. The certified and the reference values are listed in bold type and normal type, respectively. The certification was based on a primary method or a minimum of two independent NIST methods [19]. According to the definition of the Consultative Committee on Amount of Substance: Metrology in Chemistry and Biology (CCQM), a primary method of measurement is a method having the highest metrological qualities, whose operation can be completely described and understood, for which a complete uncertainty statement can be written down in terms of the International System of Units (SI), and whose results are, therefore, accepted without reference to a standard of the quantity being measured [18]. A primary ratio method, ID-ICP-MS was used to assign the certified values of Cd, Cr, Cu, Hg, Mo, and Pb [21–23].

Certification of total mass fraction of As, Ca, Fe, I, K, Mg, Mn, Na, and Zn in SRM 3232 was based on results from two or more independent methods. One of the methods was INAA, which required no sample digestion; the others were ICP-MS or ICP-OES that required mineralization of the kelp sample. The combination of measurement approaches was chosen to ensure orthogonality not only in measurement principles, but also in sample preparations prior to the measurement. Results obtained by multiple independent methods are provided in Table 2. The results of all elements were within 1SD of the method mean except for As and I, suggesting good agreement between methods. For the determination of As, two ICP-MS methods were used in addition to the INAA method. The kelp contains a large amount of Cl as shown in Table 1. Chlorine, which forms $^{40}Ar^{35}Cl^{+}$ and $^{38}Ar^{37}Cl^{+}$ in the argon plasma, interferes with the determination of monoisotopic ^{75}As by ICP-MS. Hydrogen was used as a collision gas to alleviate the $^{40}Ar^{35}Cl^{+}$ and $^{38}Ar^{37}Cl^{+}$ interferences, which produced results in agreement within 1SD of those by INAA. Alternatively, the $^{40}Ar^{35}Cl^{+}$ and $^{38}Ar^{37}Cl^{+}$ interferences can be resolved spectroscopically at mass resolutions of 7770 and 10,600, respectively. Measurements of arsenic in high-resolution mode ($R \approx 10{,}000$) of a sector-field ICP-MS yielded values outside 1SD but within 2SD intervals of results obtained using INAA and quadrupole ICP-MS. There was no technical reason for

rejection of sector-field ICP-MS results, so the results of the three methods were combined. The uncertainty of the combined mean was estimated using a bootstrap procedure based on a Gaussian random effects model for the between-method effects [30–32]. The expanded uncertainty is 3.4% of the certified value, which is slightly below the average relative expanded uncertainty (REU) of 4.2% for all elements certified by multiple independent methods listed in Table 1. The REU compares favorably to that of the seaweed CRMs listed in Table 1.

Iodine is an essential mineral required in the synthesis of thyroid hormones that regulate metabolism. Iodine intake must be carefully controlled to fall within the DRI range because both severe iodine deficiency and high iodine intake can harm health [29]. Seaweed is a rich source of dietary iodine [33]. Accurate accounting for iodine intake requires the measurement of major sources of iodine in the diet and dietary supplements such as seaweed; yet there is no certified value for iodine in seaweed CRMs that can be used for measurement validation. Iodine in SRM 3232 was determined by ICP-MS and INAA. ICP-MS is one of the most sensitive techniques for the determination of iodine [34]. Thanks to its mid-range atomic mass, iodine is not known to suffer isobaric interferences from plasma ions or solvent. Iodine is difficult to ionize in an argon plasma because of its high ionization potential, and its ionization is affected by the presence of carbon [35]. Therefore, the determination of iodine is susceptible to matrix effects [35,36]. Sample preparation and sample introduction for iodine measurement are also challenges for ICP-MS determination because iodine in oxidizing acid is susceptible to loss as I_2 and HI [37,38]. For this work, iodine in kelp was extracted in a closed-vessel microwave using 0.5% volume fraction ammonium hydroxide in water to prevent the loss of iodine due to volatilization. Calibration for the quantification was based on the method of standard addition to circumvent the matrix interferences. In comparison, INAA is described as the gold standard for the determination of iodine in biological samples [39,40]. The nondestructive measurement process ensures that INAA measurement of iodine is not affected by loss of the analyte due to volatility or incomplete extraction of the analyte in sample preparation. Although Na, Cl, and Br contributed to the background of measured iodine at 442.9 keV, the effect on the quantification of the element was negligible because of the high iodine content in the kelp. The replication relative standard deviation (RSD) of INAA implies that the sample inhomogeneity in terms of RSD was <2.1%. Therefore, the 6.5% replication RSD of the ICP-MS measurement was attributable to the ICP-MS measurement approach rather than the kelp material itself. The REU of iodine at 9.3% is the highest of all the certified values in SRM 3232; however, it is rather typical for certified mass fraction values of iodine in food matrices. A search of The European Virtual Institute for Speciation Analysis (EVISA) database using the terms ‘Iodine’ and ‘Certified’ in the Material category returned eleven food CRMs certified for iodine as shown in electronic supplementary Table S1 (DOI 10.18434/M3PM35). The relative expanded uncertainties of the certified value ranged from 8 % to 33% with the mean and median at 17% and 13%, respectively. The REU of iodine in SRM 3232 is below the mean and the medium REU of iodine in all food CRMs. The results suggest that certification of iodine in a food matrix remains a challenge. The certified value of iodine in SRM 3232 is fit for the purpose of method validation for iodine in kelp as food or dietary supplement.

Arsenic species—Arsenic is one of the ten chemicals of major public health concern of the World Health Organization (WHO), and it tops the Agency for Toxic Substances and Disease Registry's (ATSDR) Substance Priority List [41]. The toxicity of arsenic is determined by the speciation of the element. Only the most toxic inorganic arsenic species AsIII and AsV in food and drink are regulated [42]. Seaweeds contain large amount of arsenic as shown in Table 1, and kelp is no exception. Arsenic from a kelp dietary supplement was suggested to have caused toxicosis in a patient suffering from diarrhea, nausea, headache, vomiting, and weakness [43]. The case for arsenic intoxication was disputed in the subsequent correspondences because the speciation of arsenic in question was not presented in the publication, and an overdose of iodine in the kelp supplement could have caused the same symptoms [43]. It is evident that iodine intake must be controlled, and the information on the speciation of arsenic in the supplement is essential for toxicological assessment.

The efforts to meet the needs in the measurement of arsenic species in seaweeds were evident by the activities in ring trials and development of reference materials. In their 2005 publication, Raab and coworkers reviewed the results of an interlaboratory study for the measurement of seven seaweed specimens that showed identification of arsenic species to be a challenge [44]. In a more recent interlaboratory comparison of kelp measurement, participants reported results of AsIII, AsV, MMA, DMA, and arsenobetaine; however, none of the participants identified or quantified the most abundant arsenic species, i.e., arsenosugars [45]. Much of the difficulty in the identification and quantification of arsenosugars can be attributed to a lack of pure compounds for these species, because the retention time of the species used by many participants as the basis for identification was not sufficient for arsenosugars [44,46]. To advance the research of arsenic speciation of arsenosugars, Francesconi et al. developed an extract of algal (*Fucus serratus*) with reference values assigned for four arsenosugars As(328), As(482), As(392), and As(408) [47]. NMIJ CRM 7405-a is the first seaweed certified for AsV.

A common challenge in the determination of arsenic species in seaweed is that not all arsenic is extractable, and the extraction efficiency for a species is dependent on the solvent [44,48]. To prevent species conversion and to maximize the extraction efficiency of both the most toxic inorganic arsenic species and the most abundant arsenosugar species [49,50], a mild solvent consisting a mixture of methanol and water is frequently used in the determination of arsenic species in seaweeds [46,51,52]. LC-ICP-MS is used almost exclusively for the determination of arsenic species because of its sensitivity and selectivity; however, a lack of pure compounds for calibration of measurements hampers the accuracy in the LC-ICP-MS measurement of arsenosugars [28]. Gamma-ray spectroscopy of an element is not affected by the valence state of the analyte. Therefore, an inorganic arsenic primary standard can be used to calibrate INAA for the determination of organoarsenic, including arsenosugars, and the results of the measurement are traceable to the inorganic arsenic primary standard [28]. For this work, fractions of LC eluent containing isolated arsenic species were collected, transferred to filter papers that were pressed into pellets, and measured by INAA. Calibration for the INAA was accomplished using the characterized MMA and TMAO that served as the internal standards in the anion exchange and cation

exchange LC separation, respectively. Figure 2 shows the timing for collecting blank, DMA, As(482), As(392), and MMA fractions from the eluent of the anion exchange separation method for quantification by INAA using the MMA fraction as the calibrant. The eluent corresponding to As(328) retention time was not collected because an unknown component U1 cannot be excluded from the As(328) by the fraction collection approach. A pure fraction of As(328) was collected from the eluent of the cation exchange method as shown in Figure 3, which was quantified by INAA using the TMAO fraction as the calibrant [27]. Table 3 lists the results of DMA, As(328), As(482), and As(392) obtained by LC-INAA. LC-ICP-MS was used as the second method for the determination of AsV, MMA, and As(328) because the calibration standards for these species were available. The results of the measurement are listed in Table 3. DMA and As(328) were measured by LC-INAA and LC-ICP-MS. The results for these two analytes were in good agreement as indicated by the means of the two methods being within 1SD of each other. The replication uncertainty of LC-INAA was nearly an order of magnitude larger than that of LC-ICP-MS because the detection limit of the former was approximately an order of magnitude higher than the latter [28]. Like the inorganic arsenic species found in NMIJ CRM 7405-a hijiki, AsV was the only inorganic arsenic species detected in the kelp. Although the total arsenic mass fractions in SRM 3232 and CRM 7405-a are similar at (38.3 ± 1.3) mg/kg and (35.8±0.9) mg/kg, respectively, the contents of AsV differ by more than an order of magnitude at (0.247 ± 0.019) mg/kg and (10.1 ± 0.5) mg/kg, respectively. The large difference in the contents of arsenic species in the seaweed species reiterates the need for the species-specific reference material of frequently consumed seaweeds.

Vitamin K_1

Vitamin K functions as a coenzyme for blood coagulation and bone metabolism, and it is one of the essential vitamins with a DRI [29]. NIST issued certified values of total vitamin K_1 in SRM 3280 Multivitamin/Multielement Tablets and SRM 1849 Infant/Adult Nutritional Formula. An LC/MS method was used to determination the total vitamin K_1 in the two SRMs [53]. Vitamin K_1 exists in *cis*- and *trans*-forms, and the bioactivities of the two isomers are different with the former being practically inactive [54]. The isomers must be determined to accurately assess the nutritional value of a diet component. The C18 reversed-phase column used in the LC/MS analyses of SRMs 3280 and 1849, which are fortified with vitamin K_1, was not capable of separating the isomers of vitamin K_1. A method was developed to separate isomers of vitamin K_1 in SRM 3232 using a C30 column similar to that reported in the literature [55,56]. Baseline separation of the isomers was achieved under isocratic conditions in 100 % mobile phase A. To ensure stability of retention time and signal intensity, a step gradient of 100 % methanol was added to remove residues from the kelp matrix from the column. Figure 4 is a typical chromatogram of vitamin K_1 in samples of SRM 3232. A small shift in retention time was observed for the peaks in the labeled internal standard relative to those of vitamin K_1 because of the isotopic labeling. Most of the vitamin K_1 in SRM 3232 was found to be *trans*-isomer, which was consistent with the predominance of the isomer found in nature [57]. Table 3 lists the reference values assigned for vitamin K_1 and its isomers in SRM 3232. In comparison to the previous efforts that resulted in the certifications of fortified total vitamin K_1 in SRM 3280 and SRM 1849, SRM 3232 is the first SRM value-assigned for isomers of endogenous vitamin K_1 that would meet

the needs of more accurate assessment of nutritional values of vitamin K_1 in dietary supplements.

Proximates

Proximates are ash, moisture, proteins, fat, and carbohydrates, which provide a broad classification of food components [58]. The information about their content is used on labels for packaged food per FDA regulation under the Nutrition Labeling and Education Act of 1990 (NLEA) [59]. Table 4 lists the values of proximates in SRM 3232 provided by two collaborating laboratories. Except for total fat, the repeatability uncertainty within each lab was <2% RSD for all proximates. The repeatability RSD for fat was 3% and 20% for labs 1 and 2, respectively. Because of the low within-lab replication uncertainties, the between-lab bias was the primary source of uncertainty for the reference value of all proximates. Still, the relative expanded uncertainties are <7% for all proximates except fat, which is the minor component at (2.4±1.3) %.

Homogeneity

The homogeneity of analytes was assessed using graphical analysis and analysis of variance at a 5% significance level based on INAA, ID-ICP-MS, ID-CV-ICP-MS, and ICP-OES, LC-INAA, and ID-LC-MS/MS measurements using two to ten test portions per packet from six to ten packets. There was no detectable inhomogeneity for elements and arsenic species. For the values related to vitamin K_1, the uncertainty incorporates a component for possible inhomogeneity based on the standard deviation of replications. No homogeneity assessment measurements were made for proximates; however, results of proximates were treated as if they were distributed homogenously.

Conclusion

SRM 3232 Kelp Powder (*Thallus laminariae*) is the most characterized seaweed CRM in the order of Laminariales. The SRM fills the gap in reference values for vitamin K_1 and arsenosugars, and certified value for iodine in seaweed measurements. The SRM will be useful for method validation and quality assurance in measurements of nutritional and toxic contents of kelp in diet and dietary supplements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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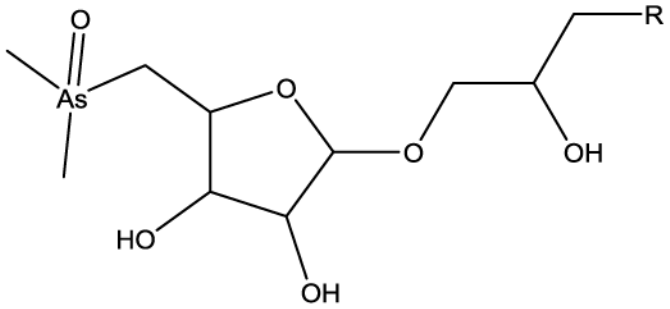

Arsenosugar As(328): R = OH
Arsenosugar As(392): R = SO_3H
Arsenosugar As(482): R = $OPO_2(OH)CH_2CH(OH)CH_2OH$
Arsenosugar As(408): R = OSO_3H

Figure 1.
Structural formula and nomenclature for arsenosugars.

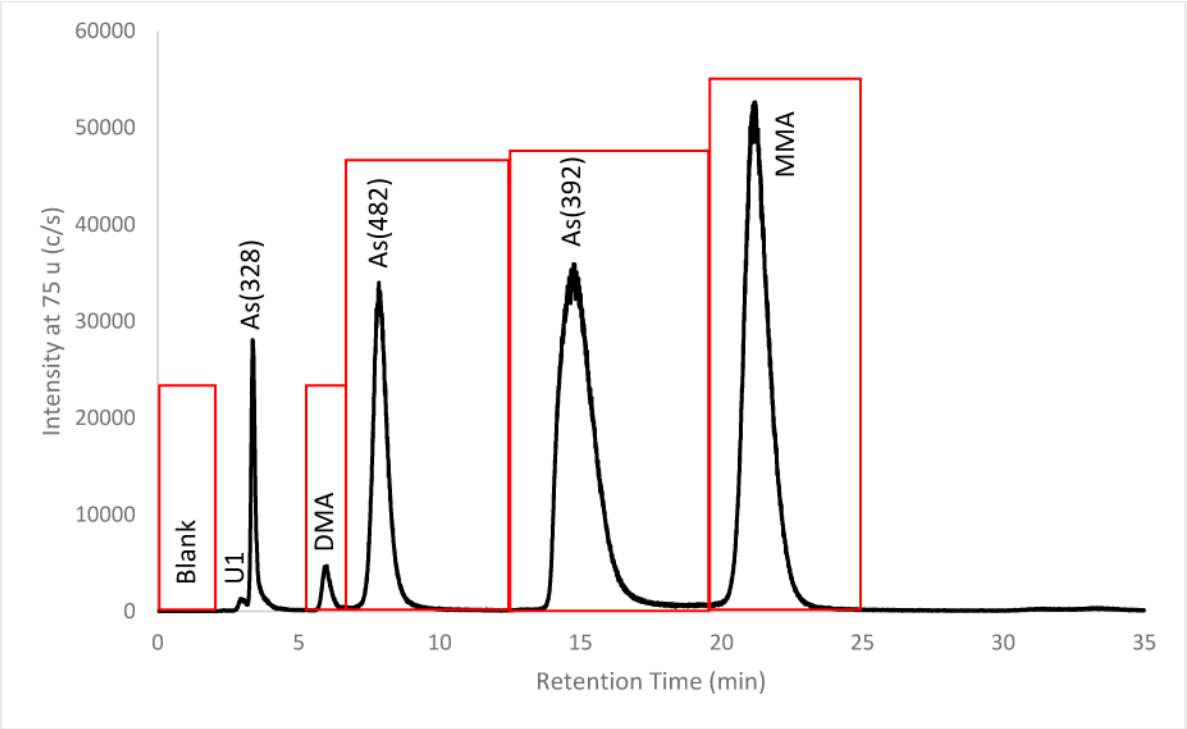

Figure 2.
Timing for collecting arsenic species from the eluent of the anion exchange method for INAA measurement.

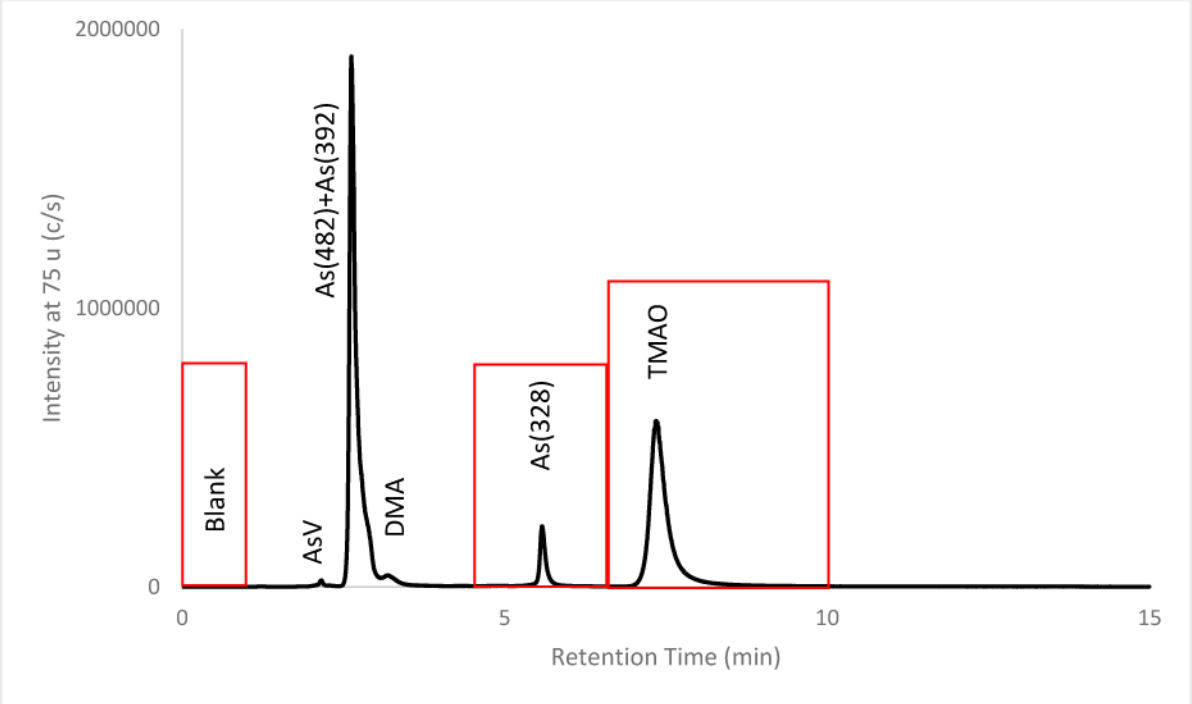

Figure 3.
Timing for collecting As(328) from the eluent of the cation exchange method for INAA measurement.

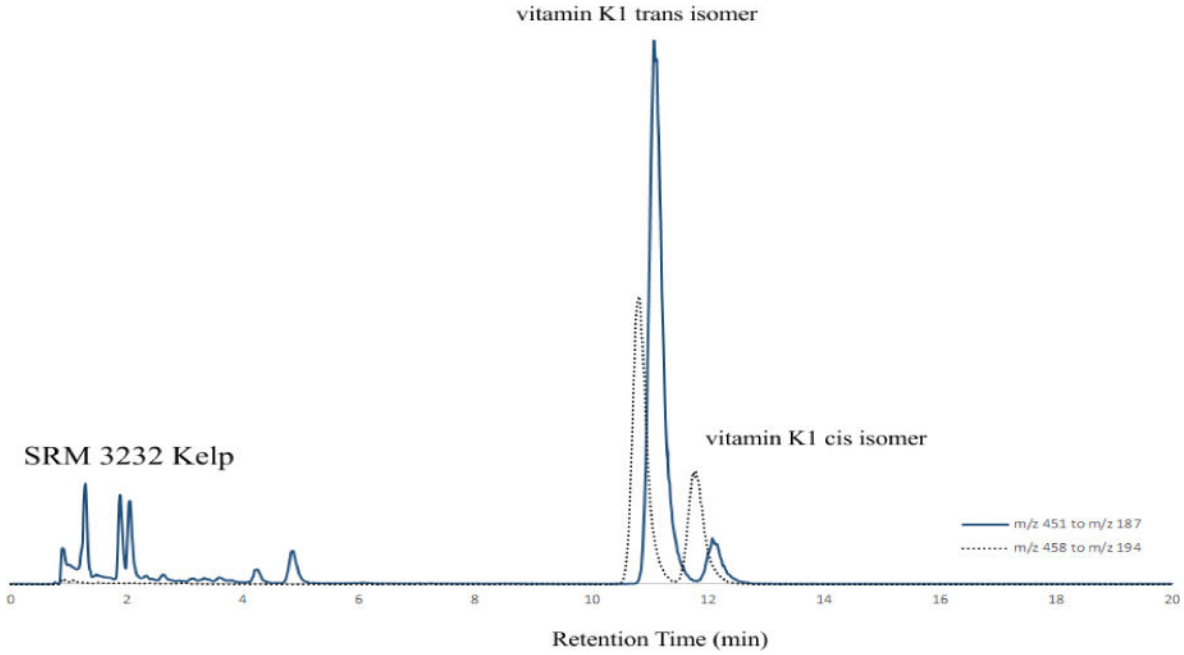

Figure 4.

A typical LC-MS/MS chromatogram of an SRM 3232 sample displaying the separation of the isomers. The solid line and the dotted line are the vitamin K_1 and the vitamin K_1-[$^2H_7$] labeled internal standard, respectively.

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Table 1

Comparison of certified (bold-type) and reference (normal-type) total mass fraction of elements in SRM 3232 to those in other seaweed certified reference materials in mg/kg. The uncertainties are expanded uncertainties at approximately 95% confidence.

Element	SRM 3232 (*Thallus laminariae*)	NIES CRM 9 (*Sargassum fulvellum*)	NMIJ CRM 7504a (*Hizikia fusiforme*)	IAEA140/TM (*Fucus sp.*)	ERM CD200 (*Fucus vesiculosus*)	GBW08521 (*Porphyra umbilicalis*)
As	**38.3±1.3**	**115±9**	**35.8±0.9**	**44.3±2.1**	**55±4**	**41±3**
Ca	**12260±680**	**13400±500**	**15200±300**	**12730±1760**		
Cd	**0.4259±0.0084**	**0.15±0.02**	**0.79±0.02**	**0.537±0.037**	**0.95±0.06**	**5.2±0.4**
Cr	**5.92±0.52**	0.2	**3.4±0.1**	**10.4±0.8**		
Cu	**3.875±0.087**	**4.9±0.2**	**1.55±0.07**	**5.05±0.28**	**1.71±0.18**	
I	**944±88**	520				
Fe	**672±13**	**187±6**	**311±11**	**1256±35**		
Pb	**1.032±0.039**	**1.35±0.05**	**0.43±0.03**	**2.19±0.28**	**0.51±0.06**	**0.81±0.03**
Mg	**6130±180**	**6500±300**	**6790±100**	**9070±880**		
Mn	**24.6±1.6**	**21.2±1**	**14.1±0.7**	**56.1±2.4**		
Hg	**0.1129±0.0032**	0.04		**0.038±0.006**	**0.0186±0.0016**	
Mo	**0.2441±0.0091**			**2.65±0.37**		
K	**76000±1100**	**61000±2000**	**47500±700**	**31100±2600**		
Na	**16330±380**	**17000±800**	**16200±200**	**32000±6600**		
Zn	**27.4±1.1**	**15.6±1.2**	**13.4±0.5**	**47.3±2**	**25.3±1.7**	
Al	1070±110	215	**147±7**	1184±265 *		
Cl	35600±1200	51000				
Co	0.307±0.011	**0.12±0.01**	**1.07±0.06**	**0.876±0.132**		
P	4551±51	2600	**1010±30**			
Rb	28.44±0.51	**24±2**		**16.4±2.3**		

* Information value.

Table 2

Certified values assigned by using multiple independent methods in mg/kg units. The uncertainties are expanded uncertainties at approximately 95% confidence

	Method	Mean	s	n	RSD%	Certified value ± U
As	INAA	38.98	0.89	27	2.3	38.3 ± 1.3
	ICP-MS	38.95	0.80	6	2.1	
	ICP-HRMS	36.39	0.94	6	2.6	
Ca	INAA	12000	650	23	5.4	12260 ± 680
	ICP-OES	12570	420	20	3.3	
I	INAA	899	19	23	2.1	944 ± 88
	ICP-MS	990	64	14	6.5	
Fe	INAA	665	30	27	4.5	672 ± 13
	ICP-OES	678	28	20	4.1	
Mg	INAA	6100	170	23	2.8	6130 ± 180
	ICP-OES	6150	280	20	4.6	
Mn	INAA	23.8	1.2	23	5.2	24.6 ± 1.6
	ICP-OES	25.4	1.7	20	6.8	
K	INAA	76440	700	23	0.9	76000 ± 1100
	ICP-OES	75600	2700	19	3.6	
Na	INAA	16520	170	23	1.0	16330 ± 380
	ICP-OES	16140	460	20	2.9	
Zn	INAA	26.9	1.2	27	4.3	27.4 ± 1.1
	ICP-OES	27.97	0.45	20	1.6	

Table 3

Mass fraction of arsenic species and Vitamin K_1 in SRM 3232 by method. The uncertainties are expanded uncertainties at approximately 95% confidence.

Analyte	Method	Average	s	n	Reference Value
DMA	LC-INAA	0.44	0.13	14	0.479 ± 0.077
	LC-ICP-MS	0.517	0.029	8	
As(328)	LC-INAA	1.18	0.26	16	1.20 ± 0.14
	LC-ICP-MS	1.216	0.021	8	
As(482)	LC-INAA	5.59	0.31	15	5.59 ± 0.51
As(392)	LC-INAA	14.06	1.03	14	14.06 ± 0.72
AsV	LC-ICP-MS	0.247	0.023	8	0.247 ± 0.019
Total Vitamin K_1	LC-MS/MS	0.431	0.013	20	0.431 ± 0.081
cis-Vitamin K_1	LC-MS/MS	0.0353	0.0012	20	0.0353 ± 0.0067
trans-Vitamin K_1	LC-MS/MS	0.396	0.012	20	0.396 ± 0.075

Table 4

Mass fraction value of proximates in SRM 3232 by lab. The uncertainties are expanded uncertainties at approximately 95% confidence.

	Lab ID	Mean	s (n=4)	Reference Value
Ash (%)	1	23.27	0.09	23.55±0.57
	2	23.84	0.08	
Carbohydrate (%)	1	57.2	0.1	56.7±1.0
	2	56.2	1.0	
Protein (%)	1	14.94	0.13	14.48±0.98
	2	13.98	0.09	
Total Fat (%)	1	1.71	0.05	2.4±1.3
	2	3.06	0.62	
Calories (kcal/100 g)	1	304.2	0.0	306.2±4.0
	2	308.3	2.2	