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ORIGINAL PAPER

Development of saw palmetto (*Serenoa repens*) fruit and extract standard reference materials

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Abstract As part of a collaboration with the National Institutes of Health's Office of Dietary Supplements and the Food and Drug Administration's Center for Drug Evaluation and Research, the National Institute of Standards and Technology has developed two standard reference materials (SRMs) representing different forms of saw palmetto (*Serenoa repens*), SRM 3250 *Serenoa repens* fruit and SRM 3251 *Serenoa repens* extract. Both of these SRMs have been characterized for their fatty acid and phytosterol content. The fatty acid concentration values are based on results from gas chromatography with flame ionization detection (GC-FID) and mass spectrometry (GC/MS) analysis while the sterol concentration values are based on results from GC-FID and liquid chromatography with mass spectrometry analysis. In addition, SRM 3250 has been characterized for lead content, and SRM 3251 has been characterized for the content of β-carotene and tocopherols. SRM 3250 (fruit) has certified concentration values for three phytosterols, 14 fatty acids as triglycerides, and lead along with reference concentration values for four fatty acids as triglycerides and 16 free fatty acids. SRM 3251 (extract) has certified concentration values for three phytosterols, 17 fatty acids as triglycerides, β-carotene, and γ-tocopherol along with reference concentration values for three fatty acids as triglycerides, 17 fatty acids as free fatty acids, β-carotene isomers, and δ-tocopherol and information values for two phytosterols. These SRMs will

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complement other reference materials currently available with concentrations for similar analytes and are part of a series of SRMs being developed for dietary supplements.

Keywords Saw palmetto · *Serenoa repens* · Fatty acids · Phytosterols · Certified reference material · Standard reference material

Introduction

Saw palmetto, *Serenoa repens*, is a palm plant that is common along the Atlantic and Gulf coasts of the USA. The plant produces large berries that are highly enriched in fatty acids, phytosterols, and flavonoids. The extract of the fruits has been used in numerous clinical studies to examine its effectiveness in treating benign prostatic hyperplasia [1–3]. Based on the positive results of these studies, saw palmetto extract is being marketed as a dietary supplement to treat enlarged prostates. Saw palmetto is available in many forms including capsules containing dried berry powder, tea, and gel capsules containing liquid extract. The labels on these products typically state that the materials contain 85% to 95% fatty acids and phytosterols.

The human body produces all of the fatty acids that it needs except for two essential fatty acids that are found in plant oils, linoleic acid and alpha-linolenic acid, and three essential fatty acids that are found in marine oils, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA). Generally fatty acids are bound to other molecules as triglycerides or phospholipids. Free fatty acids, not bound to other molecules, are also important for cellular processes. Saw palmetto products contain both free fatty acids and fatty acids as triglycerides, including linoleic acid and alpha-linolenic acid. Phytosterols or plant sterols are believed to have cholesterol-lowering properties and may act in cancer prevention. Phytosterols are often added to products targeted at lowering cholesterol levels.

As with all dietary supplements sold in the USA, saw palmetto products are regulated as foods, but it has been suggested that there should be more stringent regulation for dietary supplements [3]. Some potential side effects and drug interactions have been reported for saw palmetto products [4]. Several supplements, including saw palmetto, have been analyzed for metals and microbial contamination [5]. In that study, the supplements did not contain unacceptable concentrations of lead, mercury, cadmium, arsenic, uranium, chromium, vanadium, copper, zinc, molybdenum, palladium, tin, antimony, thallium, or tungsten but did show the presence of bacteria or fungi or both. In 2005, a review article examined the extent to which the supplements used in controlled clinical trials were characterized [6]. The conclusion of the review was that the supplements used in the published studies were inadequately characterized. Thus the results of the clinical trials are in question unless it is known if the products used in the studies were of sufficient or comparable quality.

To accelerate the development of appropriate analytical methods and validation of those analytical methods, the National Institute of Health's Office of Dietary Supplements and the Food and Drug Administration' s Center for Drug Evaluation and Research are collaborating with the National Institute of Standards and Technology (NIST) to develop standard reference materials (SRMs) for selected dietary supplements including saw palmetto. Typically, the SRM dietary supplement suites consist of plant material, extract, and finished product that represent different analytical challenges [7–9]. In the case of saw palmetto, two reference materials have been developed, SRM 3250 *Serenoa repens* fruit and SRM 3251 *Serenoa repens* extract. The fruit SRM 3250 has certified concentration values for three phytosterols, 14 fatty acids as triglycerides, and lead along with reference concentration values for four fatty acids as triglycerides and 16 free fatty acids. The extract SRM 3251 has certified concentration values for three phytosterols, 17 fatty acids as triglycerides, β-carotene, and γ-tocopherol along with reference concentration values for three fatty acids as triglycerides, 17 fatty acids as free fatty acids, β-carotene isomers, and δ-tocopherol and information values for two phytosterols.

Certified concentration values are values for which NIST has the highest confidence in their accuracy in that all known or suspected sources of bias have been investigated or taken into account [10]. Certified concentration values are generally determined using two or more independent methods at NIST, which are sometimes combined with data from collaborating laboratories. Reference concentration values are noncertified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification [10] and are 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. Reference concentration values are generally determined using one method at NIST or using only data from collaborating laboratories. Information concentration values are values that may be of interest to the SRM user; however, insufficient information is available to assess the uncertainty associated with the value and therefore no uncertainty is provided. The analytical methods used for the quantification of the fatty acids (gas chromatography with flame ionization detection [GC-FID] and GC/mass spectrometry [GC/MS]), phytosterols (GC-FID and liquid chromatography/MS [LC/MS]), β-carotene (two LC methods with absorbance detection), and tocopherols (LC with absorbance or fluorescence detection) are described in this paper along with the quantitative results.

Experimental

Materials

The material for production of SRM 3250 is ground *Serenoa repens* fruit that was packaged as received. The material was heat-sealed inside a nitrogen-flushed 4-mil polyethylene bag, which was then sealed inside a nitrogen-flushed aluminized plastic bag along with two packets of silica gel. Following packaging, SRM 3250 was irradiated at an absorbed dose of 3.6 kGy to 4.5 kGy. The material for production of SRM 3251 is a carbon dioxide liquid extract of saw palmetto berries and was ampouled as received. Two-milliliter amber ampoules were flushed with argon and filled with approximately 1 mL of extract.

Free fatty acids obtained from Nu-Chek (Elysian, MN) and from Sigma Aldrich (St. Louis, MO) were used to prepare calibration solutions in chloroform. The purities of the fatty acids used were assessed using GC-FID and differential scanning calorimetry (DSC). Stearic-d_{35} acid, arachidic-d_{39} acid, and myristic-d_{27} acid were obtained from Cambridge Isotope Laboratories, Inc (Andover, MA) and were used to prepare internal standard solutions in chloroform. For the fatty acid measurements, SRM 1846 infant formula was used as the control material for SRM 3250, and SRM 3276 carrot extract in oil was used as the control material for SRM 3251.

Sterols obtained from ChromaDex, Inc (Santa Ana, CA) and Sigma Aldrich were used to prepare calibration solutions in chloroform for GC-FID or in isopropanol for LC/MS. The purities of the sterols used were assessed using GC-FID, liquid chromatography with ultraviolet absorbance detection (LC/UV), and DSC. SRM 911a cholesterol was used to prepare internal standard solutions in chloroform for GC-FID or in ethanol for LC/MS. Since no suitable reference materials were found with certified concentrations for sterols, no control materials were used.

β-Carotene and the tocopherols were obtained from Sigma Aldrich and were used to prepare calibration solutions in ethanol. The concentration of each of these compounds was determined using Beer's law. Purity was assessed using LC. All solvents and reagents used were either HPLC-grade or ACS reagent grade.

For the analytes of interest, average response factors calculated from the independently prepared calibration solutions relative to the internal standards were used for quantification of each SRM.

Fatty acids as triglycerides

A graphical representation of the methods used to determine the fatty acids as triglycerides is shown in Fig. 1 and summarized below.

GC-FID analysis for fatty acids as triglycerides

For the measurement of fatty acids by GC-FID, duplicate portions of 0.5 g (exact mass known) from each of eight packets of SRM 3250, 0.5 g (exact mass known) of SRM 1846, or 1 mL (exact mass known) of each calibration solution were gravimetrically transferred into individual pressurized fluid extraction (PFE) cells containing hydromatrix (Isco, Lincoln, NE) and mixed with the hydromatrix. The remaining cell volume was filled with hydromatrix, and a known mass of the internal standard solution was added to the top of the extraction cell.

Hexane/acetone (4:1, v/v) was used as the extraction solvent. The cells were preheated at 125 °C for 1 min followed by the extraction heating at 125 °C for 6 min and a pressure of 10.4 MPa. There were four static cycles at 6 min each. These extraction conditions were repeated once for each cell (for a total of two extractions for each cell), and the resulting extracts were combined and concentrated to approximately 0.5 mL under nitrogen (N_2) gas. Each extract was then transferred to a capped culture tube. For SRM 3251, duplicate 0.02-g (exact mass known) portions from each of eight ampoules of the extract were gravimetrically transferred to individual capped culture tubes without a preliminary extraction step.

After adding 1.5 mL of 1.0 mol/L sodium hydroxide (NaOH in methanol), the concentrated PFE extract for SRM 3250 or the sample of SRM 3251 was heated in a dry bath at 100 °C for 5 min and cooled to room temperature. After adding 2.0 mL of 14% (mass fraction) boron trifluoride (BF_3 in methanol), the sample was heated in a dry bath at 100 °C for 30 min. The sample was then cooled to 40 °C, and 3 mL of 40 mg/L butylated hydroxytoluene (BHT) (in hexane) was added. Five milliliters of saturated NaCl(aq) was immediately added to the sample which was then allowed to cool to room temperature. The hexane/BHT layer was transferred to another culture tube, and the samples containing saturated NaCl(aq) were extracted twice with hexane/BHT. The hexane/BHT fractions were finally concentrated to approximately 2 mL under N_2. One blank was prepared by adding the internal standard solution to hydromatrix and processing as for the other samples.

GC-FID analysis was 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 120 °C for 3 min and then temperature programmed at 1.5 °C/min to 240 °C where it was held isothermally for 10 min. The injection port was maintained at 240 °C, and the FID was maintained at 240 °C. All injections were done in the split mode (1 μL) with helium as a carrier gas at a constant flow rate of 1 mL/min.

GC/MS analysis for fatty acids as triglycerides

For extraction of the ground fruit, duplicate 0.5-g (exact mass known) portions from each of eight packets of SRM

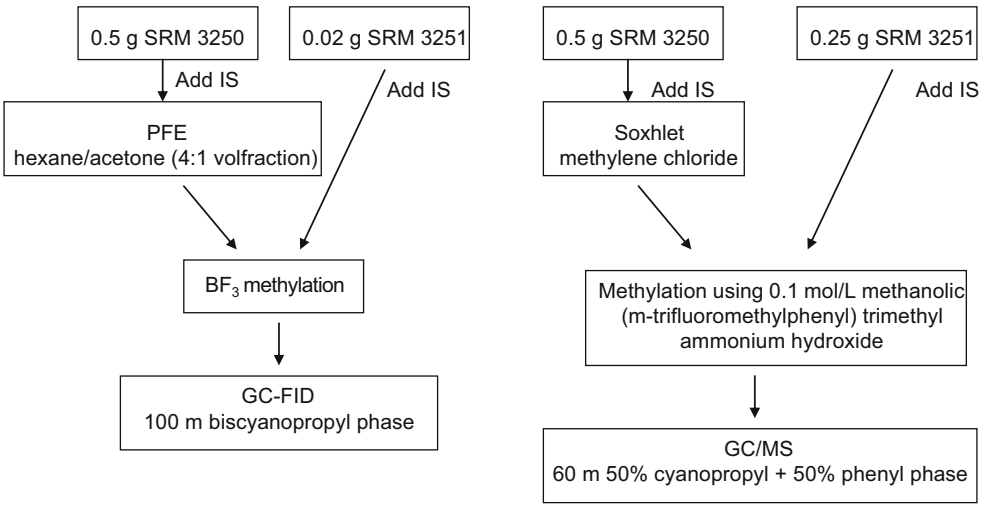

Fig. 1 Methods used for the analysis of fatty acids as triglycerides in SRM 3250 and SRM 3251. *IS* internal standard

3250, 0.5 g (exact mass known) of SRM 1846, or 1 mL (exact mass known) of a calibration solution were mixed with hydromatrix and transferred to individual glass extraction thimbles. Then, a known mass of the internal standard solution was added to the top of the extraction thimbles. The samples were then Soxhlet extracted for approximately 42 h using methylene chloride. Following extraction, the samples were concentrated with a solvent change to benzene. Approximately 0.25 mL of the sample was then mixed with approximately 0.25 mL (volumetric addition) of MethPrep II [0.1 mol/L methanolic (*m*-trifluoromethylphenyl)trimethylammonium hydroxide, Alltech Associates, Deerfield, IL]. The MethPrep II promotes the formation of the fatty acid methyl esters directly from the triglycerides in the extract. One blank was prepared by adding the internal standard solution to hydromatrix and processing as for the other samples.

For SRM 3251, duplicate 0.25-g (exact mass known) portions from each of eight ampoules of SRM 3251, 0.25 g (exact mass known) of SRM 3276, or 1 mL (exact mass known) of a calibration solution were gravimetrically transferred to individual autosampler vials using a gas-tight syringe. Then 0.5 mL (exact mass known) of the internal standard solution was added. Approximately 0.25 mL of the sample was then mixed with approximately 0.25 mL (volumetric addition) of MethPrep II as described above for SRM 3250. One blank was prepared by adding the internal standard solution to 0.5 mL of chloroform in an autosampler vial and processing as for the other samples.

The samples were analyzed using GC/MS with a 0.25 mm×60 m DB-23 (50% cyanopropyl +50% phenylpolysiloxane, mole fraction) fused silica capillary column (Agilent Technologies, Wilmington, DE), 0.25-μm film thickness. The column was held isothermally at 130 °C for 1 min, temperature programmed at 6.5 °C/min to 170 °C, and then temperature programmed at 2.75 °C/min to 215 °C where it was held isothermally for 25 min. The injection port was maintained at 250 °C, and the transfer line was maintained at 250 °C. All injections were done in the split mode (1 μL) with helium as a carrier gas at a constant flow rate of 1.2 mL/min. The MS was operated in the scan mode.

GC-FID analysis for free fatty acids

Only one method, GC-FID, was used for the analysis of the free fatty acids in SRM 3250 and SRM 3251. For SRM 3250, single 1.5-g portions from each of eight packets were combined with hydromatrix and a known mass of internal standard solution in individual Soxhlet extraction thimbles. The samples were Soxhlet extracted for 40 h using 4:1 hexane/acetone (v/v) as the extraction solvent. For SRM 3251, single 300-mg (exact mass known) portions from each of eight ampoules were combined with a known mass of internal standard solution in individual vials. Approximately 0.1 mL of each of the individual samples of SRM 3250 and SRM 3251 was then combined with 0.5 mL of MethPrep I [0.1 mol/L aqueous (*m*-trifluormethylphenyl) trimethylammonium hydroxide, Alltech Associates]. The MethPrep I promotes the formation of the fatty acid methyl esters from the free fatty acids in the extract. The samples were then analyzed by GC-FID using the same conditions as for the GC-FID analysis of the fatty acids as triglycerides summarized above.

Phytosterols

A graphical representation of the methods used to determine the phytosterols is shown in Fig. 2 and summarized below.

GC-FID analysis for phytosterols

The method used to determine the phytosterols by GC-FID was adapted from Sorenson and Sullivan [11]. For analysis,

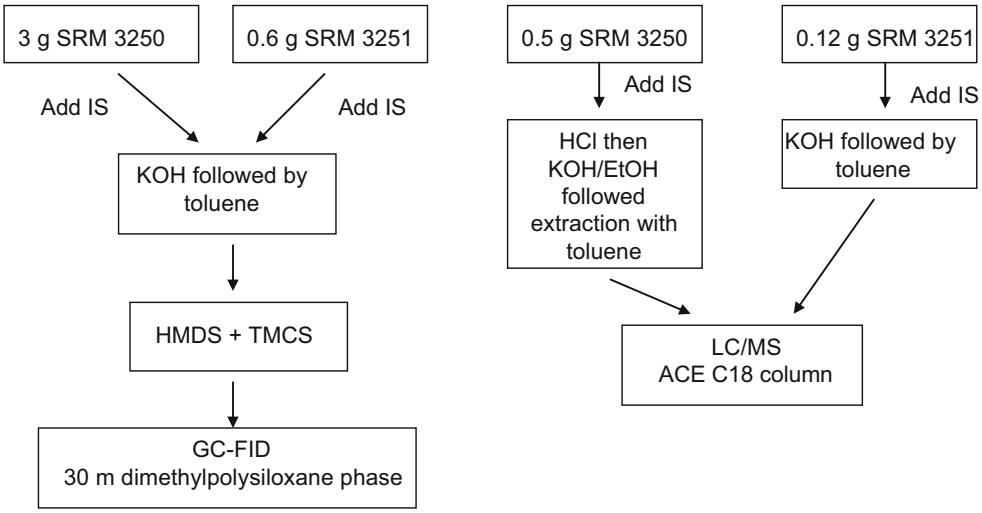

Fig. 2 Methods used for the analysis of phytosterols in SRM 3250 and SRM 3251. *IS* internal standard

single 3-g (exact mass known) portions from each of eight packets of SRM 3250, single 0.6-g (exact mass known) portions from eight ampoules of SRM 3251, 1 mL (exact mass known) of a calibration solution, or 1 mL of chloroform (as a blank) were added to individual round-bottomed flasks. Next, 0.5 mL (exact mass known) of the internal standard solution was added to each flask followed by 40 mL of ethanol and 8 mL of a 50% (mass fraction) potassium hydroxide (KOH) solution. The solutions were heated at reflux for approximately 90 min. At this point, 60 mL of ethanol was added through the top of the condenser. After cooling to room temperature, 100 mL toluene was added. Each toluene mixture was transferred to a separatory funnel, and 110 mL of a 1 mol/L KOH solution was added. After the layers separated, the aqueous layer was discarded. Next, 40 mL of a 0.5 mol/L KOH solution was added to the separatory funnel. The contents were swirled, the layers allowed to separate, and the aqueous layer was discarded. The toluene layer was then washed three times with water. The aqueous phase was discarded after each wash. The toluene layer was then poured from the top of the separatory funnel into a bottle containing approximately 5 g of sodium sulfate. The toluene solution was evaporated under N_2, and the concentrated toluene solution was then transferred to a centrifuge tube along with 0.4 mL hexamethyldisilane (HMDS) and 0.2 mL trimethylchlorosilane (TMCS). Following centrifugation, the upper layer (toluene) was transferred to an autosampler vial for analysis.

GC-FID analysis was performed using a 0.25 mm×30 m HP-1MS (100% [mole fraction] dimethylpolysiloxane) fused silica capillary column, 0.25-μm film thickness (Agilent Technologies). The column was held isothermally at 250 °C for 10 min and then temperature programmed at 4 °C/min to 280 °C where it was held isothermally for 22.5 min. The injection port was maintained at 280 °C, and the FID was maintained at 280 °C. All injections were done in the split mode (1 μL) with helium as a carrier gas at a constant flow rate of 1.2 mL/min.

Selected samples were also run on a GC×GC time of flight mass spectrometer (TOF-MS, Pegasus IV, Leco Corporation, St. Joseph, MI) using a 10 m×0.18 mm, 0.2-μm film RTX-5 column (5% phenyl methylpolysiloxane) coupled to a 1 m× 0.1 mm section of DB-17 (50% phenyl methylpolysiloxane) column. The separations were done in one dimension with the main oven held at 250 °C for 30 min, and the secondary oven held at 260 °C for 30 min. The injector and transfer line were held at 250 °C. All injections (1 μL) were in the split mode with helium as the carrier gas at a constant flow rate of 1 mL/min.

LC/MS analysis for phytosterols

The method used for these analyses is described in more detail in Bedner et al. [12]. Briefly, duplicate 0.5-g (exact mass known) portions from each of six packets of SRM 3250 were mixed with a known mass of the internal standard solution and heated with 6 mol/L hydrochloric acid at 100 °C for 30 min. The solution was cooled to room temperature followed by the addition of 4 g of KOH pellets. The solution was then heated at reflux for 45 min.

For SRM 3251 extract, duplicate 0.12-g (exact mass known) portions from eight ampoules were combined with a known mass of the internal standard solution. Next, 50% KOH in water was added, and the solution was heated at reflux for 80 min.

Toluene and 1 mol/L KOH solution were used to extract the sterols into the organic phase. This organic phase was then evaporated to dryness, and the residue was dissolved in isopropanol. LC/MS analysis was performed using an Ace C18 column (15 cm×3.0 mm, 3-μm particles). The mobile phase was 10% water/90% methanol. (LC mobile

phase conditions here and throughout this paper are reported as volume fractions.) The MS was operated in the atmospheric pressure chemical ionization mode in positive polarity with selected ion monitoring.

LC methods for β-carotene

β-Carotene was measured in SRM 3251 using two LC methods with absorbance detection. Duplicate (method 1) or single (method 2) 100-mg to 150-mg test portions from each of six ampoules were diluted in ethanol. Samples were mixed until the oil was visibly dissolved in the ethanol. For method 1, an isocratic mobile phase consisting of methanol/triethylamine/acetonitrile and a polymeric C_{18} column held at room temperature were used for determination of β-carotene. Absorbance was monitored at 452 nm. For method 2, an isocratic mobile phase consisting of 92% acetone/8% water containing 0.0125 mol/L ammonium acetate and a polymeric C_{30} column held at 10 °C were used for determination of β-carotene. Absorbance was monitored at 450 nm.

LC methods for tocopherols

Tocopherols were measured in SRM 3251 by using two LC methods with absorbance or fluorescence detection. Two 250-mg test portions (for fluorescence detection) or single 150-mg test portions (for absorbance detection) from each of six ampoules were diluted in ethanol containing tocol as an internal standard (fluorescence detection) or ethanol without an internal standard (absorbance detection). Samples were mixed until the oil was visibly dissolved in the ethanol and were analyzed by LC with fluorescence or absorbance detection. For LC with fluorescence detection, an isocratic mobile phase consisting of 97% methanol/3% water and a polymeric C_{30} column held at 25 °C were used for determination of δ- and γ-tocopherol. Excitation was at 298 nm; emission was monitored at 325 nm. For LC with absorbance detection, an isocratic mobile phase consisting of methanol/water and a C_{30} column were used for determination of γ-tocopherol at 5 °C. Absorbance detection was at 295 nm.

Toxic elements in SRM 3250

Two 0.5-g test portions from a single packet of SRM 3250 were screened for arsenic, cadmium, and lead using inductively coupled plasma with mass spectrometric detection (ICP-MS). Three 0.05-g test portions from a single packet were screened for mercury using a mercury analyzer. The material contains approximately 20 ng/g arsenic, 10 ng/g cadmium, and 3 mg/kg to 5 mg/kg lead; lead is not homogeneously distributed in this material. The material contains approximately 2 ng/g mercury.

Determination of moisture for SRM 3250

The moisture content of SRM 3250 was determined by (1) freeze-drying to constant mass over 7 days; (2) drying over magnesium perchlorate in a desiccator at room temperature for 38 days; and (3) drying for 4 h in a forced-air oven at 100 °C. Unweighted results obtained using all three techniques were averaged to determine a conversion factor of (0.9358±0.0097) gram dry mass per gram as-received mass, which was used to convert data from an as-received to a dry mass basis. A variability-in-moisture component is included in the uncertainties of the certified and reference values, reported on a dry mass basis.

Homogeneity assessment

The homogeneity of fatty acids and phytosterols in both SRMs was assessed at NIST by using the GC/MS and LC/MS methods, respectively, described above. The homogeneity of β-carotene and γ-tocopherol in SRM 3251 was assessed at NIST by using LC with absorbance detection, method 1 and LC with fluorescence detection, respectively. An analysis of variance did not show inhomogeneity for the test portions analyzed.

Results and discussion

Fatty acids

The certified and reference concentration values for the fatty acids as triglycerides in SRM 3250 *Serenoa repens* fruit and SRM 3251 *Serenoa repens* extract are summarized in Table 1, and the methods used are shown in Fig. 1. For the extraction of the fruit sample, several extraction procedures and conditions for each procedure were evaluated including PFE, Soxhlet extraction, and sonication. The two extraction procedures resulting in the highest mass fractions of extractable fatty acids from the fruit were PFE and Soxhlet extraction. In the case of the PFE, the choice of solvent, temperature of extraction, and pressure of extraction did not have a significant effect on the amount extracted so the conditions used were a solvent mixture of 4:1 (v/v) of hexane/acetone with a four static cycle extraction at 125 °C and 10.4 MPa. Each cell, however, was extracted a second time using the same conditions, and the extracts were combined to ensure efficient extraction. The solvent choice also did not have a significant effect on the extraction efficiency of the Soxhlet extraction, so methylene chloride was used. However, the duration of the extraction time was critical, and all of the extractions were allowed to continue for at least 40 h. If the extraction time was limited to 20 h, the concentrations of the fatty acids extracted were approxi-

Table 1 Certified (in bold) and reference concentration values (mass fraction as triglycerides) of fatty acids in SRM 3250 *Serenoa repens* fruit and SRM 3251 *Serenoa repens* extract

Fatty acid	SRM 3250[a,b] Mass fraction (%, dry mass)	SRM 3251[a,b] Mass fraction (%)
Hexanoic acid (C6:0)		2.25±0.03
Octanoic acid (C8:0) (caprylic acid)	**0.1072±0.0027**	**2.654±0.032**
Decanoic acid (C10:0) (capric acid)	**0.1175±0.0055**	**2.671±0.055**
Undecanoic acid (C11:0)		0.031±0.003
Dodecanoic acid (C12:0) (lauric acid)	**2.962±0.062**	**26.34±0.66**
Tridecanoic acid (C13:0)	0.0076±0.0014	**0.069±0.002**
Tetradecanoic acid (C14:0) (myristic acid)	**1.103±0.007**	**10.62±0.16**
Pentadecanoic acid (C15:0)	0.0047±0.0006	**0.0515±0.0018**
Hexadecanoic acid (C16:0) (palmitic acid)	**0.869±0.027**	**8.51±0.20**
(*Z*)-9-Hexadecenoic acid (C16:1 n-7) (palmitoleic acid)	**0.0158±0.0010**	0.271±0.004
Heptadecanoic acid (C17:0)	0.0061±0.0007	**0.0637±0.0024**
Octadecanoic acid (C18:0) (stearic acid)	**0.1791±0.0054**	**1.749±0.021**
(*Z*)-9-Octadecenoic acid (C18:1 n-9) (oleic acid)	**3.24±0.15**	**34.57±0.43**
(*Z*)-11-Octadecenoic acid (C18:1 n-7) (vaccenic acid)	**0.0547±0.0030**	**0.830±0.020**
(*Z*,*Z*)-9,12-Octadecadienoic acid (C18:2 n-6) (linoleic acid)	**0.824±0.055**	**5.990±0.093**
(*Z*,*Z*,*Z*)-9,12,15-Octadecatrienoic acid (C18:3 n-3) (linolenic acid)	**0.194±0.025**	**1.242±0.027**
Eicosanoic acid (C20:0) (arachidic acid)	**0.0097±0.0002**	**0.0932±0.0033**
(*Z*)-11-Eicosenoic acid (C20:1 n-9) (gondoic acid)	0.0173±0.0006	**0.1931±0.0031**
Docosanoic acid (C22:0) (behenic acid)	**0.0066±0.0002**	**0.0644±0.0016**
Tetracosanoic acid (C24:0) (lignoceric acid)	**0.0107±0.0003**	**0.0926±0.0028**

[a] Each certified (in bold) concentration value, expressed as a mass fraction, is an equally weighted mean of results provided by GC-FID and GC-MS. The uncertainty in the certified value, calculated according to the method described in the ISO Guide [13, 14], is expressed as an expanded uncertainty, *U*. The expanded uncertainty is calculated as $U=ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and drying components of uncertainty. The coverage factor (k) is determined from the Student's *t*-distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte

[b] Each reference concentration value, expressed as a mass fraction, is the mean of results provided using GC-FID and GC/MS, except for gondoic acid in SRM 3250 that is provided using only GC/MS results and hexanoic acid, undecanoic acid, and palmitoleic acid in SRM 3251 that are provided using only GC-FID results. The uncertainty in the reference value, calculated according to the method described in the ISO Guide [13, 14], is expressed as an expanded uncertainty, *U*. The expanded uncertainty is calculated as $U=ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of within-laboratory and drying components of uncertainty. The coverage factor (k) is determined from the Student's *t*-distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte

mately 10% lower than those after 40 h. Extending the extraction time longer than 40 h did not have an effect on the concentrations of the fatty acids. Once the triglycerides were extracted from the fruit sample, samples from both SRMs were treated the same. Samples were transesterified using either a commercial solution (Meth Prep II) or a boron trifluoride-catalyzed methylation and were analyzed using either GC-FID or GC/MS. A sample chromatogram from the GC-FID analysis of SRM 3251 is shown in Fig. 3.

The concentrations of the fatty acids as triglycerides (Table 1) range from 0.0047%±0.0006% for pentadecanoic acid to 3.25%±0.15% for oleic acid in SRM 3250 and from 0.0313%±0.0027% for undecanoic acid to 34.73%±0.43% for oleic acid in SRM 3251. The concentrations of the fatty acids as triglycerides in SRM 3251 range from 6 to 25 times higher in SRM 3251 compared with SRM 3250. The relative expanded uncertainties for the 14 fatty acids as triglycerides certified in SRM 3250 range from 0.6% (myristic acid) to 13% (linolenic acid), and the relative uncertainties for the 17 fatty acids as triglycerides certified in SRM 3251 range from 1.2% (caprylic acid, stearic acid, and oleic acid) to 4% (heptadecanoic acid). The difference in the concentration for linolenic acid in SRM 3250 determined using GC-FID (0.162%±0.010%) and GC/MS (0.202%±0.012%) was higher than for the other fatty acids resulting in the higher uncertainty for the certified concentration.

For the free fatty acids in SRM 3250 and SRM 3251, only one method of analysis was used resulting in the reference concentrations shown in Table 2 as mg/g with resulting relative uncertainties ranging from 0.5% to 14%. The concentrations of the free fatty acids in SRM 3250 range from 0.0121 mg/g±0.0009 mg/g on a dry mass basis (pentadecanoic acid) to 33.7 mg/g±1.9 mg/g on a dry mass basis (oleic acid), while the concentrations of the

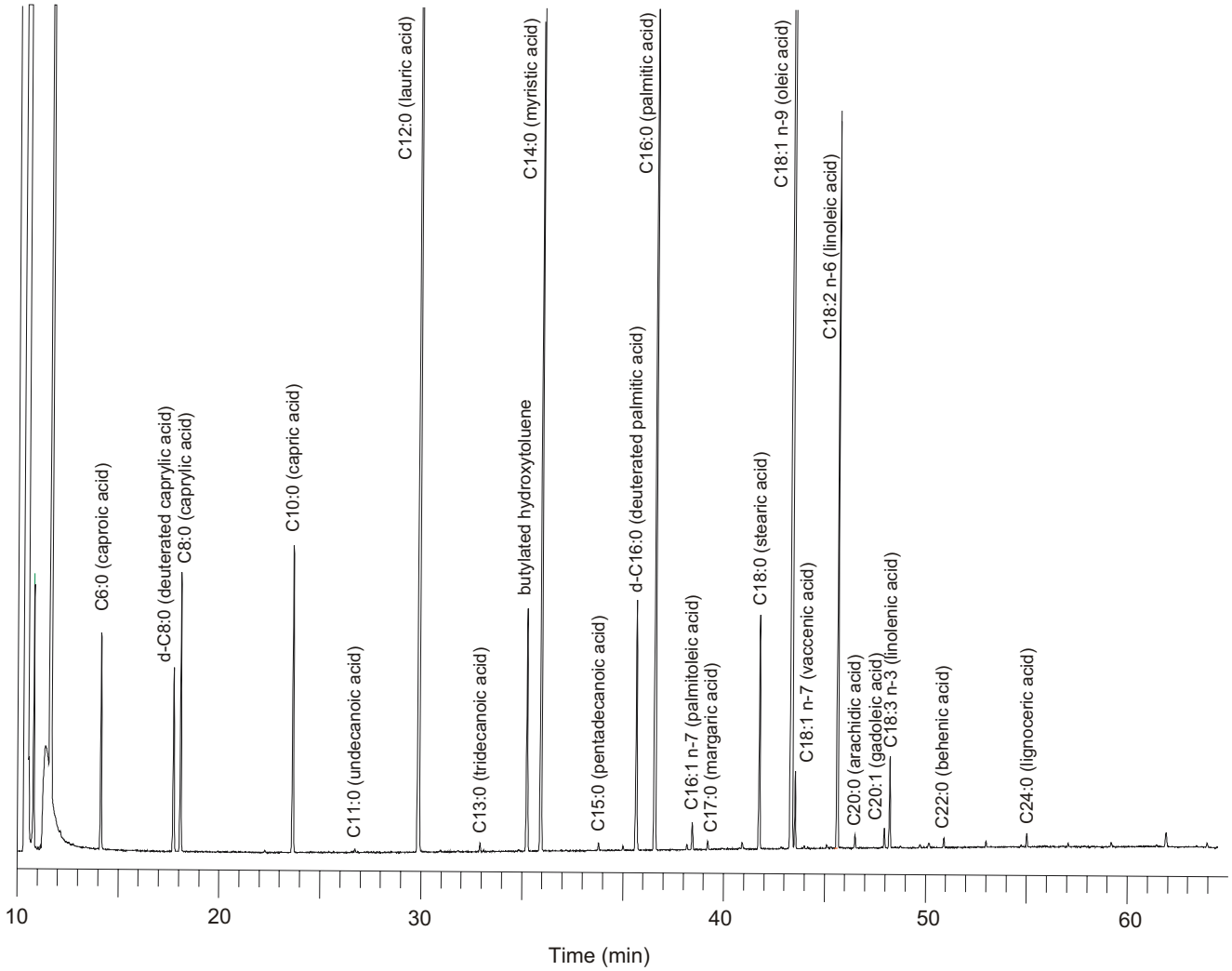

Fig. 3 Typical chromatogram for the measurement of fatty acids in SRM 3251 using GC-FID on a nonbonded biscyanopropyl polysiloxane column. The column was held isothermally at 100 °C for 4 min and then temperature programmed at 2.5 °C/min to 240 °C where it was held isothermally for 50 min. The injection port and FID were maintained at 240 °C. All injections were done in the split mode (1 μL) with helium as a carrier gas at a constant flow rate of 1 mL/min

free fatty acids in SRM 3251 range from 0.119 mg/g± 0.014 mg/g (pentadecanoic acid) to 221 mg/g±15 mg/g (oleic acid). The concentrations of the free fatty acids in SRM 3251 range from four to nine times higher than in SRM 3250.

In general, the concentration of the fatty acid as a triglyceride is higher than the concentration of the corresponding free fatty acid for both the fruit SRM 3250 and the extract SRM 3251 as shown in Fig. 4 for a selected number of fatty acids. This ratio is higher in the extract (SRM 3251) than in the fruit (SRM 3250). There is no information in the literature available on saw palmetto dietary supplements to support this observation. These SRMs will provide appropriate reference materials to make similar comparisons on dietary supplements.

As mentioned in the Introduction, linoleic and alpha-linolenic acids are essential fatty acids that are found primarily in plant oils. A number of SRMs have been characterized for these two fatty acids as triglycerides including fish oil and two fish tissues, as illustrated in Fig. 5. SRM 3251, the saw palmetto extract, has the second highest concentration of linoleic acid as the triglyceride compared with the other available SRMs, and the highest concentration of alpha-linolenic acid as the triglyceride compared with the other available SRMs. The concentration of linoleic and alpha-linoleic acid in the fruit SRM 3250 is approximately six times lower than in SRM 3251. Linoleic and alpha-linolenic acids are also lower in concentration as the free fatty acids in both SRM 3250 and SRM 3251 as compared with the fatty acids as triglycerides, as illustrated in Fig. 4.

Table 2 Reference concentration values (mass fraction) of free fatty acids in SRM 3250 *Serenoa repens* fruit and SRM 3251 *Serenoa repens* extract

Free fatty acid	SRM 3250[a] Mass fraction (mg/g, dry mass)	SRM 3251[a] Mass fraction (mg/g)
Octanoic acid (C8:0) (caprylic acid)	0.781±0.036	5.19±0.47
Dodecanoic acid (C12:0) (lauric acid)	7.21±0.036	51.8±0.3
Tridecanoic acid (C13:0)	0.0165±0.0011	0.151±0.015
Tetradecanoic acid (C14:0) (myristic acid)	5.96±0.21	46.1±3.6
Pentadecanoic acid (C15:0)	0.0121±0.0009	0.119±0.014
Hexadecanoic acid (C16:0) (palmitic acid)	8.72±0.45	58.6±4.4
(*Z*)-9-Hexadecenoic acid (C16:1 n-7) (palmitoleic acid)	0.216±0.014	2.04±0.26
Heptadecanoic acid (C17:0)	0.0926±0.0060	0.605±0.084
Octadecanoic acid (C18:0) (stearic acid)	2.023±0.094	7.96±0.68
(*Z*)-9-Octadecenoic acid (C18:1 n-9) (oleic acid)	33.7±1.9	221±15
(*Z*)-11-Octadecenoic acid (C18:1 n-7) (vaccenic acid)	0.789±0.053	4.48±0.42
(*Z*,*Z*)-9,12-Octadecadienoic acid (C18:2 n-6) (linoleic acid)	5.70±0.48	36.7±5.0
(*Z*,*Z*,*Z*)-9,12,15-Octadecatrienoic acid (C18:3 n-3) (linolenic acid)	1.351±0.050	6.53±0.27
Eicosanoic acid (C20:0) (arachidic acid)	0.1455±0.0076	0.573±0.051
Docosanoic acid (C22:0) (behenic acid)	0.0564±0.0050	0.212±0.020
Tetracosanoic acid (C24:0) (lignoceric acid)	0.0960±0.0033	0.463±0.052

[a] Each reference concentration value, expressed as a mass fraction, is the mean of results provided using GC-FID. The uncertainty in the reference value, calculated according to the method described in the ISO Guide [13, 14], is expressed as an expanded uncertainty, *U*. The expanded uncertainty is calculated as $U=ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of within-laboratory and drying components of uncertainty. The coverage factor (*k*) is determined from the Student's *t*-distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte

Phytosterols

The certified, reference, and information concentrations for the phytosterols in SRM 3250 and SRM 3251 are summarized in Table 3. Three phytosterols, campesterol, β-sitosterol, and stigmasterol, have certified concentration values in both SRMs. For SRM 3250, these certified concentration values are based on the results from two methods (GC-FID and LC/MS) performed at NIST; whereas for SRM 3251, the certified concentration values are based on the results from the GC-FID and LC/MS methods along with data from an AOAC collaborative study directed by W. Sorenson (Covance Laboratories, Madison, WI). The GC-FID method used at NIST is adapted from work done by Sorenson and coworkers [11] while the LC/MS method was developed for these SRM certification measurements and has been described in more detail elsewhere [12]. A sample LC/MS chromatogram is shown in Fig. 6. The agreement between the GC-FID and LC/MS data for SRM

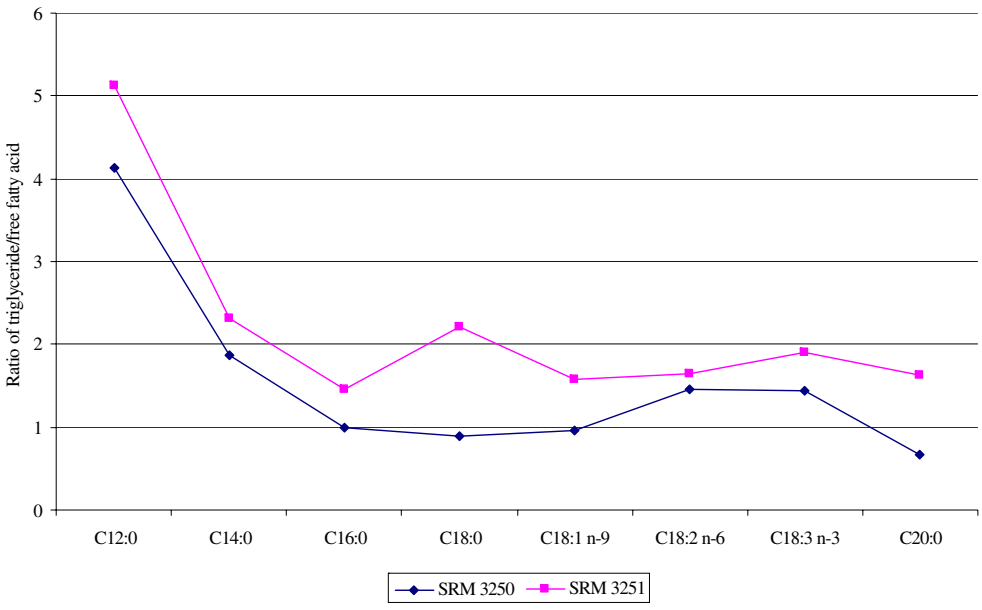

Fig. 4 Ratio of concentration of fatty acid as the triglyceride to that as the free fatty acid for SRM 3250 (*diamonds*) and SRM 3251 (*squares*). See Tables 1 and 2 for the individual concentrations and expanded uncertainties

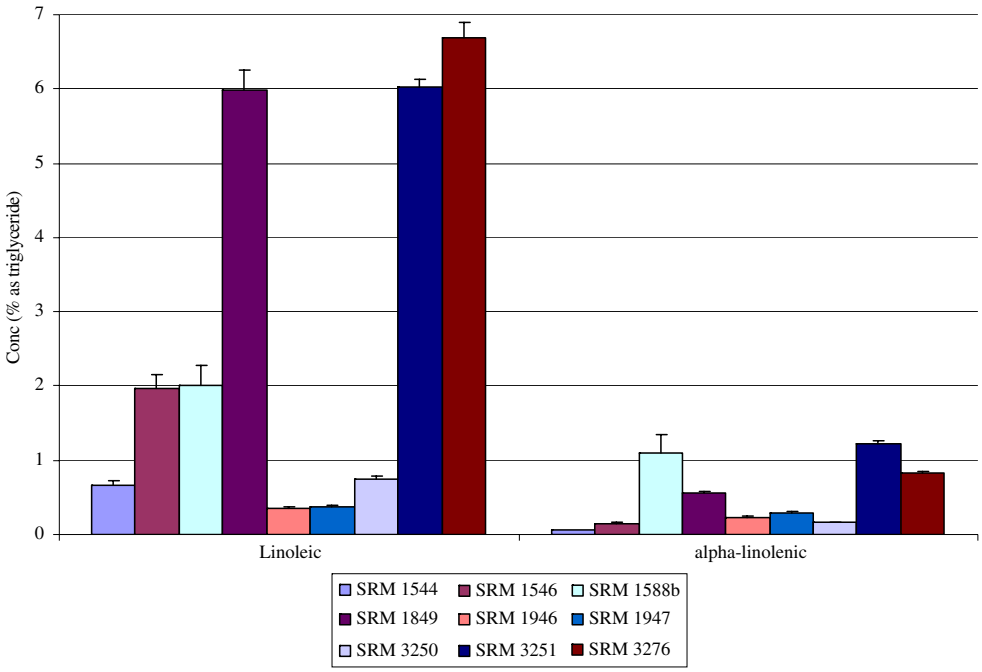

Fig. 5 Concentrations of essential fatty acids, linoleic and alpha-linolenic acids, in SRM 1544 fatty acids and cholesterol in frozen diet composite, SRM 1546 meat homogenate, SRM 1588b organics in cod liver oil, SRM 1849 infant/adult nutritional formula, SRM 1946 Lake Superior fish tissue, SRM 1947 Lake Michigan fish tissue, SRM 3250 *Serenoa repens* (fruit), SRM 3251 *Serenoa repens* (extract), and SRM 3276 carrot extract in oil. *Error bars* expanded uncertainties

3250 is very good for these three compounds resulting in uncertainties in the certified concentration values ranging from 2% for campesterol to 4% for stigmasterol. The uncertainties of the certified concentration values for the three phytosterols are higher in SRM 3251 ranging from 4% for β-sitosterol to 16% for stigmasterol. The concentrations of these three phytosterols in the extract (SRM 3251) are approximately four times higher than in the fruit (SRM 3250).

The reference concentration value for cycloartenol in SRM 3251 (Table 3) is based only on the data from the LC/MS analysis. The information concentration values for brassicasterol and lupeol in SRM 3251 are based only on the GC-FID data, but the identity of these phytosterols could not be confirmed by GC×GC/TOF-MS.

The only other certified reference material (CRM) available with certified values for phytosterols is BCR-633 anhydrous butter fat [15]. BCR-633 has certified concentrations for β-sitosterol (530 mg/kg±29 mg/kg) and stigmasterol (147 mg/kg±11 mg/kg). These concentrations fall in the range of those found in SRM 3250 and SRM 3251;

Table 3 Certified (in bold), reference, and information (no uncertainty) concentration values (mass fraction) of phytosterols in SRM 3250 *Serenoa repens* Fruit and SRM 3251 *Serenoa repens* Extract

Phytosterol	SRM 3250[a,b] Mass fraction (mg/g, dry mass)	SRM 3251[a,b] Mass fraction (mg/g)
Brassicasterol		0.056[c]
Campesterol	**0.1175±0.0025**	**0.533±0.031**
Cycloartenol		0.772±0.008
Lupeol		0.25[c]
β-Sitosterol	**0.454±0.018**	**1.666±0.064**
Stigmasterol	**0.0477±0.0020**	**0.247±0.040**

[a] Each certified (in bold) concentration value, expressed as a mass fraction, is an equally weighted mean of results provided by GC-FID and LC/MS. The uncertainty in the certified value, calculated according to the method described in the ISO Guide [13, 14], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U=ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and drying components of uncertainty. The coverage factor (k) is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte

[b] The reference concentration value, expressed as a mass fraction, is an equally weighted mean of results provided by LC/MS. The uncertainty in the reference value, calculated according to the method described in the ISO Guide [13, 14], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U=ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and drying components of uncertainty. The coverage factor (k) is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte

[c] Each information (no uncertainty) concentration value, expressed as a mass fraction, is a mean of results provided by GC-FID. The identities were not confirmed by GC×GC/TOF-MS. Insufficient information is available to provide an uncertainty on these values

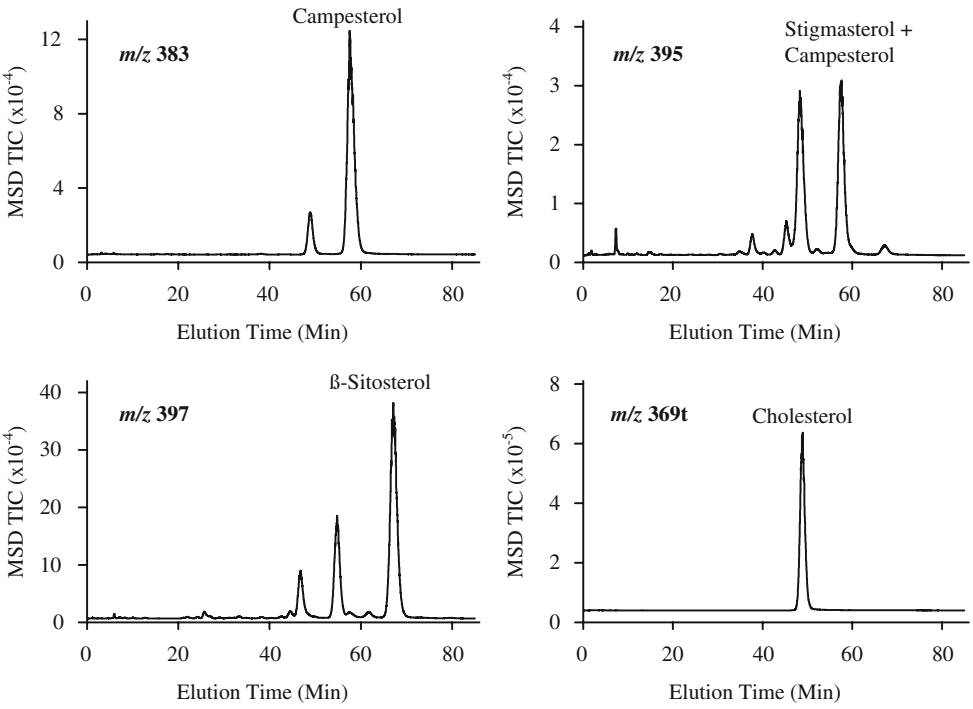

Fig. 6 Typical chromatograms for the measurement of phytosterols in SRM 3251 obtained by LC/MS with a 15-cm C_{18} column. The isocratic mobile phase consisted of 10% water, 90% methanol (v/v) at a flow rate of 0.8 mL/min. MS detection conditions were as follows: nebulizer pressure, 276 kPa (40 psi); fragmentor, 80 V; drying gas temperature, 350 °C; drying gas flow rate, 12 L/min; corona current, 8 μA; capillary voltage, 3,000 V; and vaporizer temperature, 400 °C

however, the matrices are very different, i.e., a plant fruit and fruit extract versus anhydrous animal fat. The two new SRMs and the available CRM will complement each other to assist analysts in developing and verifying phytosterol measurements in a variety of matrices.

β-Carotene and tocopherols

β-Carotene has been linked to cardiovascular benefits and cancer preventive effects while tocopherols are known for their antioxidative properties. β-Carotene, γ-tocopherol, and δ-tocopherol were quantified in SRM 3251 using LC methods described above and the resulting certified and reference concentration values are summarized in Table 4. The certified concentration values are for total β-carotene (46.8 μg/g±4.6 μg/g) and γ-tocopherol (280 μg/g±13 μg/g) with reference concentration values for *trans*-β-carotene, 9-*cis*-β-carotene, and δ-tocopherol. The other dietary supplement SRM currently available with certified and reference concentration values for these compounds is SRM 3276 carrot extract in oil [16]. SRM 3276 contains concentrations for total β-carotene (50.8 mg/g±3.6 mg/g), *trans*-β-carotene (30.7 mg/g±2.2 mg/g), 9-*cis*-β-carotene (11.4 mg/g± 1.6 mg/g), γ-tocopherol (443 mg/g±42 mg/g), and δ-

Table 4 Certified (in bold) and reference concentration values (mass fraction) of β-carotene and tocopherols in SRM 3251 *Serenoa repens* extract

	Mass fraction (μg/g)[a,b]
Total β-carotene	**46.8±4.6**
trans-β-Carotene	36.4±5.6
9-*cis*-β-Carotene	10.4±1.2
γ-Tocopherol	**280±13**
δ-Tocopherol	35.3±0.5

[a] Each certified concentration value, expressed as a mass fraction, is an equally weighted mean of results provided by two LC methods with absorbance and/or fluorescence detection. The uncertainty in the certified value, calculated according to the method described in the ISO Guide [13, 14], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U=ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory and within-laboratory components of uncertainty. The coverage factor (k) is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte

[b] Each reference concentration value, expressed as a mass fraction, is an equally weighted mean of results provided by two different LC methods with absorbance detection, except for δ-tocopherol that was obtained using results from one LC method with absorbance detection. The uncertainty in the reference value, calculated according to the method described in the ISO Guide [13, 14], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U=ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-method and within-method components of uncertainty. The coverage factor (k) is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte

tocopherol (372 mg/g±34 mg/g) that are between 840 times and 10,500 times higher than in SRM 3251. The largest difference is for δ-tocopherol. These two materials provide SRMs for both the lower concentration range (μg/g in SRM 3251) and higher concentration range (mg/g in SRM 3276) of β-carotene and tocopherols.

Conclusions

SRM 3250 *Serenoa repens* fruit and SRM 3251 *Serenoa repens* extract have been characterized for the concentrations of a wide range of fatty acids and phytosterols, and SRM 3251 has also been characterized for β-carotene, and tocopherols. These SRMs provide additional reference materials with relevant concentrations for fatty acids, including two essential fatty acids, and for the phytosterols for which only one other reference material has been characterized. For β-carotene and tocopherols, SRM 3251 is a lower concentration reference material compared with the currently available SRM 3276 carrot extract in oil.

Acknowledgment The authors thank the laboratories who participated in the AOAC collaborative study for phytosterols in SRM 3251.

Disclaimer 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.

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