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Preparation and Certification of Standard Reference Material 3278 Tocopherols in Edible Oils

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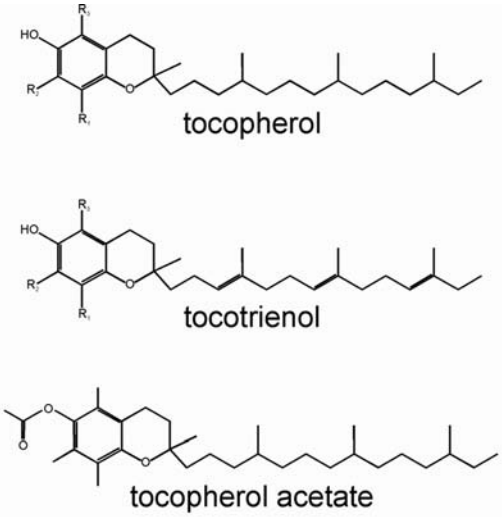
ABSTRACT: Standard Reference Material (SRM) 3278 Tocopherols in Edible Oils has been issued for use as a quality assurance tool in the measurement of tocopherols. Like other natural-matrix SRMs, this material can be used in method validation or in assignment of tocopherol values to in-house quality control materials. Because most edible oils contain one predominant tocopherol isoform, the SRM is a blend of sunflower, soy, canola, and safflower oils to provide roughly comparable chromatographic peak heights of the two main tocopherols, γ and α, with smaller amounts of δ and β. The four tocopherol isoforms were determined by three independent liquid chromatography methods with absorbance and fluorescence detection. Various chromatographic and detection modes are used for assignment of certified values because biases inherent to one method should not be present in the other, and the existence of bias can therefore be identified.

KEYWORDS: *Standard Reference Material, tocopherols, edible oils, quality assurance*

INTRODUCTION

Well-characterized certified reference materials are often essential to food and dietary supplement manufacturers in the development of new analytical methods or the evaluation of the performance of existing methods and for use as quality control materials. The National Institute of Standards and Technology (NIST) became involved in the development of food-matrix Standard Reference Materials (SRMs) after passage of the Infant Formula Act of 1980[1] and the Nutrition Labeling and Education Act of 1990.[2] Nutrient concentrations have been certified in a number of foods that differ in fat, carbohydrate, and protein concentrations and represent problems that may be encountered in the analysis of different types of foods. In a related effort, NIST has been working with the National Institutes of Health Office of Dietary Supplements (NIH-ODS) to develop SRMs in support of requirements of the Dietary Supplement Health and Education Act of 1994.[3] The accurate measurement of vitamin E is of interest to both the food and dietary supplement communities, and NIST has issued SRM 3278 Tocopherols in Edible Oils as a tool for its measurement. Vitamin E is a collective term for a number of structurally related fat-soluble analytes. Four tocopherol isoforms (α, β, γ, and δ) and four tocotrienol isoforms (α, β, γ, and δ) occur naturally in foods (Figure 1). Synthetic forms of α-tocopherol are sometimes added to processed foods as esters to prolong stability. Different bioactivities and bioavailabilites are associated with these isoforms, and each may offer unique health effects.[4] Some of the benefits attributed to vitamin E are from its actions as an antioxidant, an anticoagulant, and an essential regulator of metabolic processes.[5−7] It has been studied for the prevention of cardiovascular disease; however, the results from clinical studies are mixed at best.[8] In 2000 the Institute of Medicine recommended that α-tocopherol be considered as the only source of vitamin E for humans.[9] For this reason, α-tocopherol acetate and α-tocopherol succinate are often added to dietary supplements, foods, and cosmetics;[9] however, all of



	R_1	R_2	R_3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	CH_3	CH_3	H
δ	H	H	H

Figure 1. Structures of naturally occurring tocopherols and tocotrienols and the synthetic ester α-tocopherol acetate.

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Table 1. Certified and Reference Mass Fraction Values for Tocopherols in NIST SRMs[a]

	α-tocopherol (μg/g)	β-tocopherol (μg/g)	γ-tocopherol (μg/g)	δ-tocopherol (μg/g)
SRM 3251 *Serenoa repens* Extract			280 ± 13[b]	35.3 ± 0.5[c]
SRM 2387 Peanut Butter	108 ± 11	100 ± 19[b,d]	100 ± 19[b,d]	10 ± 3[b]
SRM 1849 Infant/Adult Nutritional Formula	369 ± 16	5.77 ± 0.79[b]	189 ± 13[b]	79.2 ± 2.4[b]
SRM 3280 Multivitamin/Multielement Tablets	21.4 ± 3.5[b]			
SRM 3284 Baking Chocolate	7.7 ± 1.7[c]		108.2 ± 1.9[c]	3.42 ± 0.47[c]
SRM 3276 Carrot Extract in Oil			443 ± 64[b]	373 ± 34[b]
SRM 2383 Baby Food Composite[e]	25.0 ± 3.3[b]		5.51 ± 0.93[b]	1.51 ± 0.43[b]

[a]The uncertainty in the certified value, calculated according to the method described in the ISO Guide, 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 Student's t distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte. [b]Certified value. [c]Reference value. [d]Combined γ/β-tocopherol value reported. [e]Currently out of stock; replacement material in preparation.

the natural tocopherol isoforms are found in high levels in plant oils, nuts and seeds, and green leafy vegetables.

To better understand the health effects of vitamin E isoforms, it is essential for laboratories to have the ability to qualitatively and quantitatively determine the levels of vitamin E isoforms in food and dietary supplement products. Whereas NIST has produced a number of SRMs that include certified values for tocopherols (Table 1), SRM 3278 was designed to contain the four tocopherol isoforms, with α-tocopherol and γ-tocopherol at similar response levels. This was achieved through the evaluation of a series of plant oils for the relative tocopherol levels and blending appropriate quantities of the selected oils.

The determination of tocopherols in foods has been the subject of a number of reviews.[5,10−13] Historically, normal-phase liquid chromatography (LC) separations have been used to resolve the four tocopherol isoforms in oils.[14−20] Although the methods are effective, there are problems with the reproducibility of retention times and long column equilibration times. Reversed-phase LC (RPLC) methods provide a good alternative to the normal-phase methods as the columns are more robust, solving the reproducibility and equilibration problems. Additionally, RPLC methods can separate certain tocopherol isoforms, retinol, and carotenoids in the same chromatographic run. However, β- and γ-tocopherol are usually unresolved with RPLC methods that use C_{18} columns, and the two isomers are often reported together.

Several authors have addressed the limitations of reversed-phase chromatographic methods for the separation and identification of tocopherol isoforms.[12,13,21] The majority of the work has focused on methods that use monomeric C_{18} columns for the separation. Improved separations of the four isoforms have been demonstrated with C_{30} columns with monomeric-type phases providing the best resolution of the β/γ-tocopherol pair.[22−25] Porous graphitic carbon stationary phases have been used for the analysis of vitamin E, and the separation of the four tocopherol isoforms and two tocopherol acetate entantionmers has been achieved.[21] In this paper, the development and certification of the SRM candidate material will be described.

MATERIALS AND METHODS

Source Materials and Processing. Seven different vegetable oils were purchased from grocery stores in Gaithersburg, MD. Each of the oils was screened for the relative amounts of the four isoforms; the oils and relative percentages of each tocopherol are listed in Table 2. The amounts of the tocopherols in the different oils were found to be consistent with literature reports; for example, γ-tocopherol was predominant in soybean oil, whereas α-tocopherol was predominant in sunflower oil.[15,26−30]

Table 2. Peak Area Ratios of Tocopherols in Oils Determined prior to Blending[a]

sample ID	oil type	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol
oil A	soybean	6.9	1.7	67.8	23.5
oil B	canola	23.6	1.2	73.6	1.6
oil C	corn	14.6	0.4	81.8	3.1
oil D	safflower	79.8	3.6	13.8	2.8
oil E	sunflower	81.2	4.6	12.6	1.6
oil F	safflower	90.3	2.5	6.2	1
oil G	olive	93.3	1.2	5.5	nd[b]

[a]Peak areas determined from the separation of the tocopherol isoforms on the Nomura Develosil C_{30} column with UV absorbance detection. [b]nd, not detected.

A 1:1 ratio of α-tocopherol/γ-tocopherol was targeted for the SRM. A 10% soybean oil (oil A), 10% canola oil (oil B), 10% safflower oil (oil F), and 70% sunflower oil (oil E) volume fraction blend was used to achieve the desired tocopherol ratios. A 500 mg/kg mass fraction of 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT) was added to the oil mixture as a competitive antioxidant to stabilize the vitamin E in the SRM. The mixture was manually shaken, immersed in an ultrasonic bath for several minutes, and then stirred on a magnetic stir plate for 2 h to ensure complete mixing of the oils and the BHT. The mixture was then ampuled under argon in 2 mL amber ampules; each ampule contains approximately 1.2 mL of the oil mixture. All of the preparation steps were performed in reduced light to avoid light-induced degradation of the tocopherols, and the final material was stored in the dark at room temperature.

Experimental Details. Assignment of the concentrations of tocopherols in SRM 3278 was based on a combination of measurements from different analytical methods at NIST: three independent LC methods were developed with absorbance and/or fluorescence detection. Four to five calibrants were gravimetrically prepared for each tocopherol (Chromadex, Irvine, CA, USA) for each method. The concentrations were determined spectrophotometrically, and the purity of each calibration material was assessed by LC with a monomeric C_{30} column and absorbance and fluorescence detection. Tocol (Tama Biochemical Co., Ltd., Tokyo, Japan) was used as an internal standard for all three methods. A fresh tocol solution was prepared for each sample set, and the same solution was used for calibrants and samples within each method. An internal standard approach to calibration was utilized for quantitation in which the individual calibrant solutions were closely bracketed around the sample concentrations. A response factor was calculated for each injection of each calibrant, and the average response factor was used for the determination of the tocopherols in the samples. Duplicate samples were prepared from each of 6 (method 3) to 10 (methods 1

and 2) ampules of the oil mixture. The samples were selected through a stratified random sampling scheme to ensure homogeneity throughout the packaging lot. In total, 26 ampules from 19 boxes were tested in duplicate.

For sample preparation, 50−300 mg test portions of the isopropanol−miscible oil blend were diluted by mass with 500−700 mg of internal standard solution in ethanol with 1% isopropanol (method 1) or isopropanol (methods 2 and 3). Samples were mixed until the oil was visibly dissolved in the solvent and then injected directly onto the LC columns.

LC with Fluorescence Detection, Method 1. A premixed isocratic mobile phase with 99:1 (volume fraction) methanol/water was used as the mobile phase with a monomeric C_{30} column (Nomura Develosil RP-C_{30}, 5 μm particle size, 250 mm × 4.6 mm; Phenomenex, Torrance, CA, USA). The flow rate was 1 mL/min, and the column temperature was held at 10 °C with a recirculating water bath and column jacket. A programmable detector with a xenon lamp was used to measure the fluorescence of the tocopherols and the internal standard at an excitation wavelength of 298 nm and an emission wavelength of 325 nm. A typical separation is shown in Figure 2A.

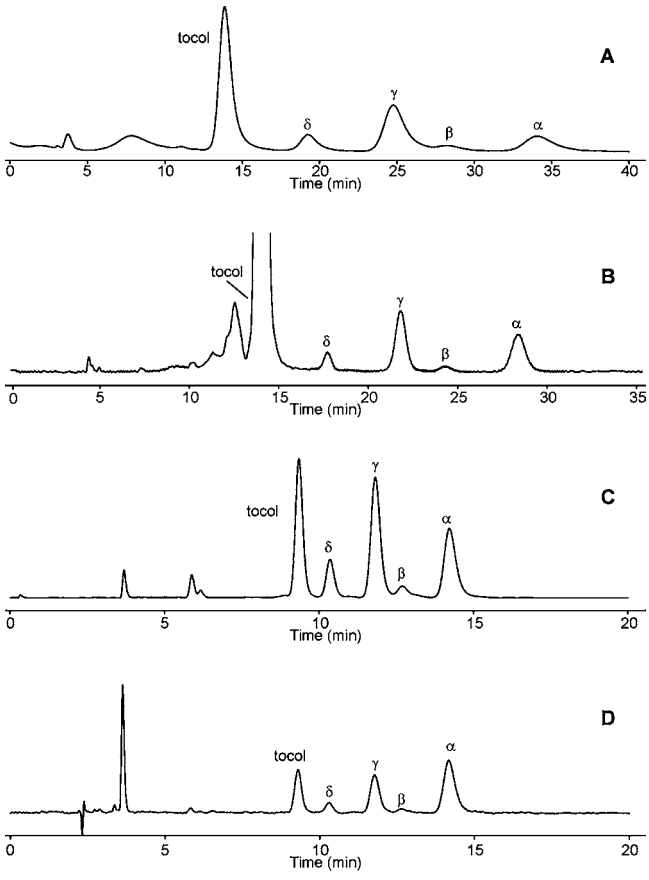

Figure 2. Typical chromatograms for the separation of tocopherols in SRM 3278: (A) monomeric C_{30} column used for separation at 5 °C with fluorescence detection; (B) polymeric C_{30} column used for separation at 5 °C with fluorescence detection; (C) π-naphthalene column used for separation at 30 °C with fluorescence detection; (D) π-naphthalene column used for separation at 30 °C with UV absorbance detection.

LC with Fluorescence Detection, Method 2. A premixed isocratic mobile phase with 95:5 (volume fraction) methanol/water was used as the mobile phase with a polymeric C_{30} column (YMC C_{30}, 5 μm particle size, 250 mm × 4.6 mm; Waters Corp., Milford, MA, USA). The flow rate was 1 mL/min, and the column temperature was held at 5 °C with a recirculating water bath and column jacket. A programmable detector with a xenon lamp was used to measure the fluorescence of the tocopherols and the internal standard at an excitation wavelength of 298 nm and an emission wavelength of 325 nm. A typical separation is provided in Figure 2B.

LC with Absorbance and Fluorescence Detection, Method 3. A premixed isocratic mobile phase with 90:10 (volume fraction) methanol/water was used as the mobile phase with a naphthalene bonded-phase column (Cosmosil π-NAP column, 250 mm × 4.6 mm, 5 μm particle size; Nacalai Tesque Co., Japan). The flow rate was 1.5 mL/min, and the column temperature was held at 30 °C with a recirculating water bath and column jacket. A variable-wavelength ultraviolet−visible detector with a deuterium lamp was used to measure the absorbance of the tocopherols and internal standard at 297 nm, and a programmable fluorescence detector with a xenon lamp was used to measure the response at an excitation wavelength of 298 nm and an emission wavelength of 325 nm. Typical separations are provided in Figure 2C,D.

RESULTS AND DISCUSSION

For the determination of tocopherols, SRM 3278 required no sample preparation other than dilution with a solvent that was miscible with the oil blend and compatible with the LC mobile phases; isopropanol was the best choice. Through careful selection of chromatographic parameters including the stationary phase, mobile phase, temperature, and detection technique, it was possible to achieve baseline separation of the γ/β-tocopherol pair with RPLC techniques. The independent techniques used for value assignment involved the use of columns with different selectivities (i.e., a polymeric C_{30}, a monomeric C_{30}, and a π-naphthalene column) and different methods of detection (absorbance and fluorescence).

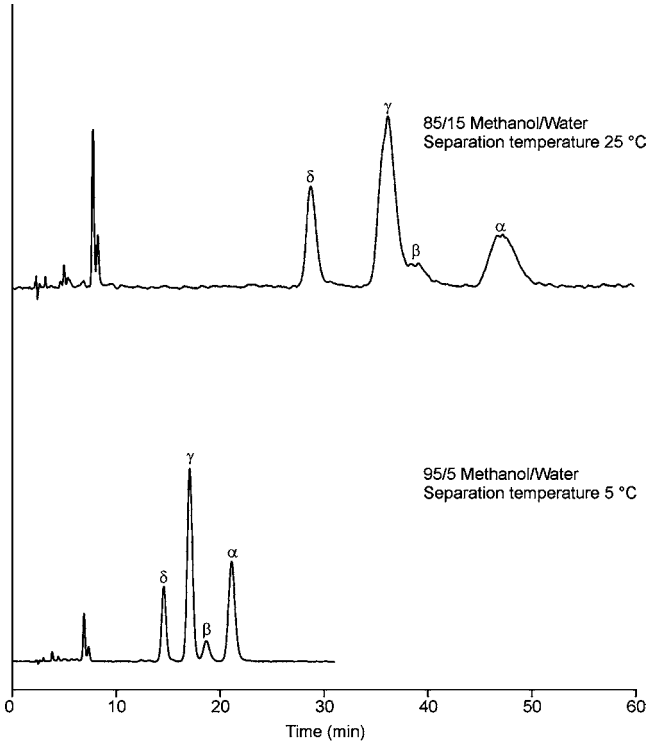

Figure 3. Effect of temperature and mobile phase composition on the resolution of tocopherol isoforms using a monomeric C_{30} column for separation.

Independence of the chromatographic methods of analysis is an important consideration in SRM certification because any coelution of constituents that may occur with one separation

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Table 3. Certified Values and Values Obtained from Individual Methods in Units of Micrograms per Gram

	certified value[a]	LC-fluorescence method 1 (monomeric C_{30} column)[b]	LC-fluorescence method 2 (polymeric C_{30} column)[b]	LC-fluorescence method 3 (π-naphthalene column)[b]	LC-UV absorbance method 3 (π-naphthalene column)[b]
α-tocopherol	290.1 ± 6.5	295.0 ± 8.7	287.7 ± 11.2	344.1 ± 11.8[c]	287.5 ± 6.9
β-tocopherol	11.38 ± 0.52	11.0 ± 1.0	11.9 ± 1.6[d]	11.5 ± 0.5	11.6 ± 0.6
γ-tocopherol	111.5 ± 5.8	131.3 ± 5.3[c]	106.8 ± 1.7	110.8 ± 1.5	116.8 ± 1.2
δ-tocopherol	28.8 ± 1.8	29.8 ± 1.5	28.2 ± 1.8	29.5 ± 0.3	27.7 ± 1.9

[a]The uncertainty in the certified value, calculated according to the method described in the ISO Guide, 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 Student's t distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte. [b]Average ± the standard deviation reported. [c]Values not used for certification due to suspected fluorescence quenching. [d]Values not used for certification due to increased relative standard deviation from integration uncertainty.

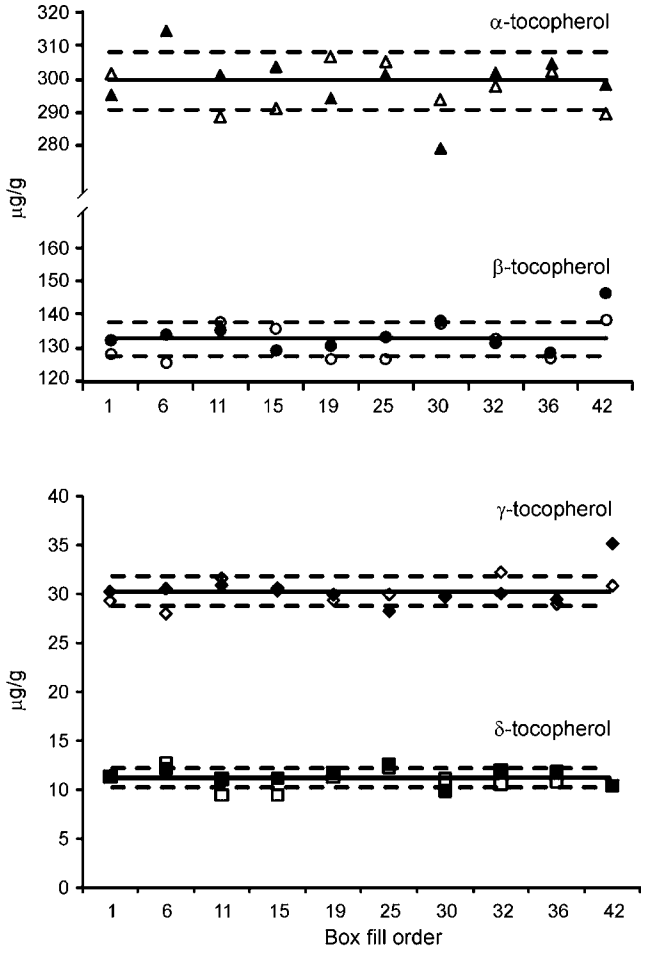

Figure 4. Evaluation of material homogeneity with respect to box-fill order. Duplicate measurements were made from each ampule. Solid symbols indicate the first measurement, and open symbols indicate the second measurement.

mechanism is unlikely to occur with a second separation mechanism. With two or more analytical methods, it is possible to evaluate sources of bias that may occur in the determination of analytes of interest. The two types of stationary phases used in the characterization of SRM 3278 separate compounds primarily on the basis of different molecular shapes and $\pi-\pi$ interactions. Nuclear magnetic resonance studies demonstrate that C_{30} phase ligands are more ordered at lower temperatures in a methanol/water mobile phase, causing shape recognition of the different tocopherol isoforms.[22] The π-naphthalene stationary phase exhibits little temperature dependence and may separate the tocopherols on the basis of $\pi-\pi$ interactions.

Temperature was an important factor in method optimization with C_{30} columns. Slight separation of γ- and β-tocopherol resulted for polymeric C_{30} columns operated at ambient temperature. Baseline resolution of the isomers was nearly achieved at 5 °C; however, reproducible integration of the β-tocopherol peak was difficult. As a result, the mean level of β-tocopherol was consistent with the other methods, but the standard deviation was significantly larger. Similar to the polymeric C_{30} column, the separation on the monomeric C_{30} column was improved at lower temperatures and with a higher percentage of methanol in the mobile phase (Figure 3). Through a systematic study of the effects of temperature and solvent composition, it was determined that the chromatographic selectivity was improved at lower temperatures; however, the retention and the band broadening increased. The addition of methanol allowed for optimized separation in a minimal period of time. The resulting chromatograms displayed well-resolved, symmetrical chromatographic peaks, and the integration was reproducible for all tocopherol isoforms. Unlike the C_{30} phases, the π-naphthalene column did not demonstrate selectivity changes with temperature, and as a result the column temperature was maintained at 30 °C to improve chromatographic speed and efficiency.

The use of the different methods of detection also increases method independence. Fluorescence detection is more sensitive and selective than absorbance detection, and it is less subject to interferences from coeluting constituents. Fluorescence detection is, however, susceptible to quenching effects that can result from changes in the solvent environment; absorbance detection is less sensitive to solvent-induced changes. Differences that result from different methods of detection are indicative of biases.

The results from the individual methods and certified values are reported in Table 3. When the data were combined and compared, it was observed that the results for γ-tocopherol from method 1 and α-tocopherol from method 3 were higher than the results from the other methods. Tocopherol response factors were compared with and without sparging of the mobile phases with helium, and it was found that the response factor for α-tocopherol in method 1 and for γ-tocopherol in method 3 changed slightly with the sparging; other tocopherols in methods 1 and 3 were unaffected by sparging. These data indicate fluorescence quenching may have occurred for specific

analytes. As there were at least three measurements that contributed to the value assignment of each of the tocopherols, the suspect results were not used in the final certification of SRM 3278.

Figure 4 shows the results for tocopherols measured in 10 samples prepared in duplicate. Samples were selected using a stratified random sampling scheme to evaluate homogeneity across the production lot. From Figure 4 it is evident that measurement error is randomly scattered and relatively small; no indications of sample inhomogeneity or analyte degradation are apparent.

SRM 3278 Tocopherols in Edible Oils provides a valuable method development tool and quality control material for food and dietary supplement laboratories determining vitamin E. Previous SRMs based on individual oils are most applicable as control materials for samples that have similar tocopherol profiles. The blend of the four oils in SRM 3278 makes it appropriate for use as a control material for most edible oils as well as foods containing mixed tocopherols.

AUTHOR INFORMATION

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 authors declare no competing financial interest.

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