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Determination of Total Cholesterol in Coconut Oil: A New NIST Cholesterol Standard Reference Material

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A new Standard Reference Material (SRM) consisting of coconut oil with various nutrients added has been developed at the National Institute of Standards and Technology in response to the needs of the food measurement community. SRM 1563 consists of ampules of a coconut oil with added cholesterol and selected fat-soluble vitamins and ampules of the natural coconut oil. We have measured cholesterol in this material by a modification of the definitive method based on isotope dilution mass spectrometry coupled with gas chromatography, originally developed for the measurement of cholesterol in serum. The cholesterol concentration, as total cholesterol, in the fortified oil was determined to be 64.2 ± 0.6 mg/100 g of oil. This value with its precision complies with the request of the food nutrient measurement community for a standard with an uncertainty within ±5% of the certified value at 95% confidence limits. The natural oil was found to contain 0.344 ± 0.014 mg/100 g of oil.

Understanding the relationship between food nutrient levels and human health requires the accurate determination of nutrient levels by laboratories making such measurements. Food matrix reference materials with accurate nutrient compositions can be useful as a means to standardize nutrient level measurements and thus make nutrient levels measured by different laboratories comparable. A workshop held at the National Institute of Standards and Technology recommended cholesterol in

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a stable, partially hydrogenated vegetable oil as a priority in the development of a series of food-related Standard Reference Materials (SRMs) (NBS Special Publication 635).

Isotope dilution combined with gas chromatography–mass spectrometry (IDMS) is the analytical method of choice for highly accurate and precise measurements of cholesterol in a complex matrix because of its specificity and high precision. A method for the measurement of cholesterol in human serum (Cohen et al., 1980) has been described, which has been extensively investigated for possible sources of inaccuracy (none were found) and gives results of high precision. In this paper, we show the results of adapting the method to measure cholesterol in a food matrix. The work reported here was performed as part of the certification of SRM 1563, which consists of a coconut oil to which no cholesterol has been added and a coconut oil to which cholesterol has been added.

In the IDMS method, isotopically labeled cholesterol is added in an amount about equal to the approximately known quantity of total cholesterol (unesterified + esterified cholesterol) in the sample. The sample is treated with base to saponify the cholesteryl esters, and the mixture of labeled and unlabeled cholesterol is extracted and converted to trimethylsilyl ethers for GC/MS analysis. Once the isotopically labeled cholesterol has been added and equilibrated, quantitative recovery of cholesterol is no longer critical, since it is the *ratio* of unlabeled to labeled cholesterol, not the absolute amount, that is measured and used for quantitation. In the GC/MS analysis, the intensity ratio for a specific fragment ion of unlabeled and of labeled cholesterol is determined according to a prescribed measurement protocol. Samples are bracketed with standard calibration mixtures composed of unlabeled and labeled cholesterol whose ratios are close to that of the sample. Duplicate measurements are performed on two different days. The total cholesterol concentration in the sample is calculated from the quantity of oil, the quantity of labeled cholesterol added, and the weight ratio calculated from the measurements. A substance could interfere with the measurement and not be detected only if (a) it had exactly the same retention time on the various GC columns used; (b) it gave an ion at every mass used for measurement; (c) its ratio of intensities of every ion used for measurement was exactly the same as that of cholesterol.

MATERIALS AND METHODS

Preparation of SRM 1563. Coconut oil with and without added cholesterol was prepared (Certificate of Analysis). For the oil fortified with cholesterol, NIST SRM 911a cholesterol with a certified purity of 99.8% was dissolved in UV-grade toluene and the resultant mixture added with thorough mixing to the coconut oil. Cholesterol was added to the oil by weight, corrected for the purity of the cholesterol and for the amount of added toluene. The calculated value is 63.57 mg/100 g of oil (Certificate of Analysis). Other analytes of interest to the food community (retinyl acetate, tocopheryl acetate, vitamin D_2) and an antioxidant (2,6-di-*tert*-butyl-4-methylphenol) were added. The total toluene content of the oil was 1.88% (by weight). Vials of coconut oil for analysis were chosen according to a stratified random sampling scheme. All other materials used have been previously described (Cohen et al., 1980).

Sample Preparation. Cholesteryl esters in coconut oil were hydrolyzed by the AOAC method (1984) for the determination of cholesterol in vegetable fats and oils, except that hexane was used for extraction in place of ether and a water-cooled condenser was used in place of an air-cooled condenser.

The coconut oil was melted by immersing the vial in warm water. About 1.5 g of fortified coconut oil or 3.5 g of natural coconut oil was weighed accurately into a 100-mL round-bottom flask. A fresh stock solution of cholesterol-d_7 (cholest-5-en-3-ol-25,26,26,26,27,27,27-d_7), consisting of about 22 mg in 22 mL of ethanol (both quantities weighed accurately to the nearest 0.001 mg), was prepared for each set of samples. Using a conditioned (Cohen et al., 1980) syringe, we transferred weighed aliquots of this solution into the four flasks containing the previously weighed coconut oil samples. The coconut oil was hydrolyzed by the AOAC method as modified. The hexane extract was allowed to evaporate, and the residue was redissolved in about 1 mL of methanol.

Each set of samples consisted of coconut oil samples from two vials of the fortified and two vials of the natural oil (NBS Special Publication 635). Three sets were independently prepared. Measurements were thus made on six vials each of the fortified and natural coconut oils.

Standards Preparation. From the same solution of labeled cholesterol that was used to spike the coconut oil, weighed aliquots were transferred with a conditioned syringe to 10 screw-capped tubes. A stock solution of cholesterol (SRM 911a), consisting of about 20 mg in 23 mL of ethanol (both quantities accurately weighed to the nearest 0.001 mg), was prepared for each set of samples. Weighed aliquots of this solution were transferred with a conditioned syringe into the screw-capped tubes, such that the weight ratio of unlabeled to labeled cholesterol was in the range of 0.77–1.34. These solutions were the standard mixtures.

Derivatization. Aliquots (100 μL) of the methanol solutions of standards and fortified coconut oils and the entire volume of the methanol solutions of the natural oil were evaporated at room temperature in a 0.3-mL Mininert vial. Aliquots of 100 μL of the derivatizing reagent *N,O*-bis(trimethylsilyl)acetamide were added to the residues of the standards and fortified oils, and aliquots of 50 μL of the derivatizing reagent were added to the residues of the natural oils. Vials were mixed and allowed to stand overnight.

Measurement Methods. Measurements were made following the definitive method for cholesterol (Cohen et al., 1980) except that the temperatures of the injection port, column, and interface to the mass spectrometer were 265, 230, and 245 °C, respectively. The instrumentation consisted of a gas chromatograph, a single-focusing magnetic sector mass spectrometer, and a control and data acquisition system specifically designed for isotope ratio measurements. The instrumentation and the measurement protocol have been thoroughly described (Cohen et al., 1980).

Fortified Oil Analysis. All fortified oil samples were analyzed by electron impact mass spectrometry for the measurement of the ratios of the molecular ions at m/z 458 and 465. Confirmatory measurements of the fortified oil samples were made by measuring the ratios of the ions at m/z 329 and 336 in electron impact mass spectrometry and by measuring the ratios of the ions at m/z 386 and 393 in ammonia chemical ionization mass spectrometry.

Natural Oil Analysis. All natural oil samples were analyzed by electron impact mass spectrometry for the measurement of the ratios of the molecular ions at m/z 458 and 465. Each sample was measured by the bracketing procedure (Cohen et al., 1980), but the protocol was modified by removing the requirement that sample duplicates had to agree within 1%. To remove the interference of previous injections of natural oils, it was necessary to bake out the GC column at 280 °C for 5 min after each injection of natural oil. Confirmatory measurements on the natural oil samples were not possible because of interferences at the masses used for these measurements.

RESULTS AND DISCUSSION

Certain requirements must be met for accurate and precise results to be obtained. Tests for complete hydrolysis of the cholesterol esters in coconut oil, for GC column memory effects, and for interferences must be performed. Each group of standards must also be checked for internal consistency.

Hydrolysis Study. A preliminary experiment established the length of time necessary for hydrolysis of the cholesterol esters. A sample of fortified coconut oil was mixed with labeled cholesterol and hydrolyzed as in the modified AOAC method. Aliquots of the solution were withdrawn during hydrolysis at 0.25, 0.5, 1, 2, and 4 h and extracted with hexane. An aliquot of the hexane was taken

Table I. Test of Consistency of Independently Prepared Sets of Standard Mixtures (SRM Cholesterol + Cholesterol-d_7)

std[a]	bracketed by	wt ratios, unlabeled/labeled: mean measd by ID/MS	wt ratios, unlabeled/labeled: weighed	diff, % sample	diff, % av
1-4	2-4, 2-8	0.9703	0.9693	−0.10	
1-9	2-8, 2-9	1.0267	1.0254	−0.13	0.13
2-8	1-4, 1-9	0.9939	0.9953	+0.14	
2-9	1-7, 1-9	1.1140	1.1155	+0.13	
3-8	2-4, 2-8	0.9446	0.9425	−0.22	
3-7	2-8, 2-9	1.0046	1.0021	−0.25	0.22
2-8	3-7, 3-8	0.9927	0.9953	+0.26	
2-9	3-4, 3-7	1.1140	1.1155	+0.13	
3-7	1-4, 1-9	1.0032	1.0021	−0.11	
3-4	1-7, 1-9	1.1450	1.1460	+0.09	0.11
1-4	3-7, 3-8	0.9680	0.9693	+0.13	
1-9	3-4, 3-7	1.0244	1.0254	+0.10	

[a] The first number is the set number; the second number is the sample number.

to dryness and the residue derivatized. The same experiment was run on natural oil, except that the entire hexane extract was taken to dryness and derivatized, and aliquots were withdrawn at 0.25, 0.5, 1.4, and 24 h. The unlabeled/labeled cholesterol intensity ratios measured on each set of samples were not statistically different by the Student's t-test at the 95% level with respect to hydrolysis time, which indicated that hydrolysis was complete in 15 min. A hydrolysis time of 30 min was therefore chosen, as is used with the AOAC method.

Memory Effects. We tested the cholesterol derivative for column memory effects. If a memory effect were present, injections of a sample or standard of one unlabeled/labeled ratio would affect the ratio measured for subsequent injections of other standards or samples. There was no evidence of a column memory effect with standards or the fortified coconut oil. Initially, there was a memory effect with the natural oils; it was eliminated by baking out the GC column after each injection of natural oil.

Standards Cross-Check. The accuracy of results for samples is limited by the accuracy of the standard mixtures used for calibration. Each set of standards was tested for internal consistency by bracketing each member of a set by the neighboring members of the *same* set. The weight ratio was then calculated and compared to the weighed value of the weight ratio. This cross-comparison among standards in each set indicated that the weight ratios of the calibration mixtures were accurate. No departure from linearity was observed for any of the three sets prepared.

Since samples were measured against standards made from the same labeled spiking solution, errors in preparing this solution drop out of the calculation. The cross-check of different sets of standards against each other assures that the unlabeled stock solutions were correctly weighed. This between-set consistency was tested by bracketing with the appropriate standards from *another* set each of two standards in each set that were used in measuring samples. The weight ratio was then calculated and compared to the weighed value of the weight ratio. The agreement between these values for each member of a set allows determination of a bias between sets. The results are shown in Table I. There is a bias of 0.10–0.18% between sets, which is within acceptable limits.

Sample Appearance. After derivatization, three of the natural oils (24/131, 35/96, and 39/71; box/vial number) had two liquid phases. The top phase was yellow and contained the cholesterol derivative; the bottom phase was pale yellow. The other three oils after derivatization contained one phase of dark brown liquid. This difference had no apparent effect on the results.

Table II. Measurements of the Concentration of Cholesterol in Fortified Coconut Oil in Milligrams/100 g (Measurement by EI at m/z 458/465)

set	box/vial	day 1	day 2	mean
1	16/32	64.37	64.43	64.40
1	13/97	64.72	64.38	64.55
2	11/129	64.64	64.68	64.66
2	18/21	64.63	64.46	64.54
3	8/43	64.85	64.41	64.63
3	12/28	64.54	64.17	64.36
			grand mean	64.52
			SD	0.12
			CV, %	0.19

Table III. Confirmatory Measurements of the Concentration of Cholesterol in Fortified Coconut Oil in Milligrams/100 g (Measurement by EI at m/z 329/336)

set	box/vial	day 1	day 2	mean
1	16/32	64.25	64.50	64.38
1	13/97	64.17	64.36	64.27
2	11/129	64.28	64.80	64.54
2	18/21	64.23	64.11	64.17
3	8/43	64.10	63.84	63.97
3	12/28	64.38	64.71	64.55
			grand mean	64.31
			SD	0.22
			CV, %	0.35
			diff from princ msmt, %	−0.32

Table IV. Confirmatory Measurements of the Concentration of Cholesterol in Fortified Coconut Oil in Milligrams/100 g (Measurement by NH_3 Chemical Ionization at m/z 386/393)

set	box/vial	day 1	day 2	mean
1	16/32	63.97	64.04	64.01
1	13/97	64.36	64.30	64.33
2	11/129	64.89	64.90	64.90
2	18/21	64.14	64.18	64.16
3	8/43	63.77	63.57	63.67
3	12/28	64.07	63.95	64.01
			grand mean	64.18
			SD	0.42
			CV, %	0.65
			diff from princ msmt, %	−0.53

Fortified Oil Results. All measurements on fortified oil met the criteria of the protocol; only one fortified oil (8-43) in the chemical ionization confirmation measurements required measurement on a third day. The results of the principal measurements on fortified oil are given in Table II, and the results of the confirmatory measurements are given in Tables III and IV. The principal and confirmatory measurements are not significantly different by the Student's t-test at the 95% confidence level.

Natural Oil Results. The results for natural oil are given in Table V. The results are less precise than for fortified oil but are satisfactory because the level of cholesterol in the natural oil is very low. Confirmatory measurements on the natural oil samples were not possible because of interferences at the masses of interest. Such interferences may have been present in the fortified oil samples; however, the cholesterol level in the fortified oil was sufficiently high that the interferences did not affect the measurement of the fortified oil.

Comparisons. The comparison of the gravimetric and measured concentrations of cholesterol added to the coconut oil is shown in Table VI. The gravimetric value is

Table V. Measured Concentration of Cholesterol in Natural Coconut Oil in Milligrams/100 g (Measurement by EI at *m/z* 458/465)

set	box/vial	day 1	day 2	mean
1	38/100	0.362	0.368	0.365
	24/131	0.325	0.334	0.330
2[a]	31/137	0.340	0.348	0.344
	35/96	0.334	0.335	0.334
3	39/71	0.330	0.339	0.335
	23/71	0.352	0.364	0.358
			grand mean	0.344
			SD	0.014
			CV, %	4.1

[a] For the natural oils in set 2, there were no standards covering the appropriate ratio range. Therefore, measurements for natural oils in set 2 were obtained using for day 1 the standards of set 1 and for day 2 the standards of set 3.

Table VI. Comparison of the Measured Concentration of Cholesterol Added to Coconut Oil with the Gravimetric Value in Milligrams/100 g

gravimetric method	
cholesterol added to oil	63.70
IDMS method	
total cholesterol in fortified oil	64.52
total cholesterol in natural oil	0.34
cholesterol added to oil (by difference)	64.18

63.70 mg of cholesterol/100 g of oil (Certificate of Analysis). The ID/MS value is 64.18 ± 0.11 mg of cholesterol/100 g of oil, which is about 0.8% higher than the gravimetric value. The difference is statistically significant; we have no explanation for it.

The cause of the discrepancy is probably not inhomogeneity of samples because that would mean a lower, not higher, ID/MS value compared to the gravimetric value. In addition, inhomogeneity is unlikely because the cholesterol was added to the oil as a solution in toluene, not as solid crystals. Further evidence that inhomogeneity is not the cause of the discrepancy is given by the very small set-to-set differences among the samples.

The ID/MS method is not likely to be inaccurate. The hydrolysis of the samples has been shown to be complete (and would in any case, if incomplete, mean a lower ID/MS value compared to the gravimetric value), the principal measurements show no statistical difference with the confirmatory measurements, and set-to-set differences for standards and samples were very small. Also, the method has been used to remeasure separately Standard Reference Material 909, a freeze-dried human serum, and the results were not significantly different from the previous results.

However, we know of no reason why the gravimetric value should be in error. Therefore, the source of this discrepancy remains unknown.

Conclusions. The concentration for total cholesterol in the fortified coconut oil is 64.2 ± 0.6 mg/100 g of oil (Certificate of Analysis). This value was calculated by placing equal weight on the principal IDMS measurements, the confirmatory IDMS measurements, and the gravimetric value. The associated uncertainty is expressed as 2 standard deviations of the reported value. At the workshop (NBS Special Publication 635), participants expressed a need for users to be accurate in measurement of a standard to within ±5% of the certified value at 95% confidence limits. The precision of the SRM is thus substantially better than required for the purpose for which it was developed: the measurement of cholesterol in foods.

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