Determination of Fat-Soluble Vitamins and Carotenoids in Standard Reference Material 3280 Multivitamin/Multielement Tablets by Liquid Chromatography with Absorbance Detection

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ABSTRACT

The concentrations of selected fat-soluble vitamins and carotenoids in Standard Reference Material (SRM) 3280 Multivitamin/Multielement Tablets have been determined by two independent liquid chromatographic methods with measurements performed by the National Institute of Standards and Technology (NIST). This SRM has been prepared as part of a collaborative effort between NIST and the National Institutes of Health's Office of Dietary Supplements (NIH/ODS). The SRM is also intended to support the Dietary Supplement Ingredient Database that is being established by the U.S. Department of Agriculture. The methods used at NIST to determine the concentration levels of vitamin A, E, and β-carotene in the SRM employed reversed-phase liquid chromatography with absorbance detection. The relative precision of these methods ranged from 2 % to 8 % for the analytes measured. SRM 3280 is primarily intended for use in validating analytical methods for the determination of selected vitamins, carotenoids, and elements in multivitamin/multielement tablets and similar matrices.

INTRODUCTION

As public health agencies try to link vitamin intakes from food and dietary supplements with resulting biological effects, discrepancies between the label value and actual vitamins and mineral content in a finished product have become important. The lack of specific requirements for content makes it difficult for public health agencies to know exactly how much of a given vitamin is being consumed through a particular dietary supplement. In 1994, the Dietary Supplement Health and Education Act (DSHEA) defined and regulated dietary supplements as foods (1) and required Good Manufacturing Practices for supplements. Good Manufacturing Practices, released by the Food and Drug Administration in 2007, require manufacturers to evaluate the identity, purity, quality, strength, and composition of their dietary supplements' ingredients and finished products (2, 3).

The development of well-characterized reference materials and reliable analytical methods are needed to verify ingredient concentrations and product quality. In support of the production of a Dietary Supplement Ingredient Database (4-6), that is being established by the U.S. Department of Agriculture (USDA) in conjunction with the National Institutes of Health/Office of Dietary Supplements (NIH/ODS), the National Institute of Standards and Technology (NIST) has developed Standard Reference Material (SRM) 3280 Multivitamin/Multielement Tablets for use in validating analytical methods for the determination of vitamins, carotenoids, and elements in dietary supplement tablets and similar matrices (7, 8). Manufacturers can use this reference material during method development and for quality assurance when assigning values to their in-house control materials. SRM 3280 was prepared as a non-commercial batch of multivitamin/multielement tablets using normal manufacturing procedures. The SRM is a direct-compression tablet formulation produced by blending a vitamin and mineral pre-mix with the remaining bulk of the formulation, compression, and tablet film coating. Details regarding the characterization of the SRM are described elsewhere (8).

Over the past 20 years, a number of analytical methods have been published for the determination of vitamin and carotenoid concentrations in multivitamin preparations (9-13). Many of these methods lacked specificity, could be used only for measurement of individual vitamins, required sample cleanup by open-column adsorption chromatography, and achieved quantitation by spectrophotometric or colorimetric analysis of column eluent. Recent advances in liquid chromatographic systems have enhanced capabilities for analysis of

multivitamin tablets/formulations. Current methods often allow simultaneous determination of multiple vitamins from a single preparation (14-18). Although these chromatographic systems have improved over the past two decades, the extraction of vitamins from encapsulated preparations still presents a challenge. The nature of the coating in which the vitamins are encapsulated impacts their extractability. Different manufacturers use different encapsulation techniques, which lead to differences in extraction requirements. Organic solvents such as dimethyl sulfoxide and pyridine (which could cause health concerns) are still commonly used in protocols for dissolution of the fat-soluble vitamins in multivitamin tablets.

The aim of this work was to develop reliable analytical methods based on different chromatographic approaches to ensure independence of the analytical results for the determination of fat-soluble vitamins and carotenoids in SRM 3280. The analytical development and performance characteristics of two independent analytical methods developed at NIST using liquid chromatography/ultraviolent absorbance detection (LC/UV) are described herein. The results from the LC/UV absorbance methods and the procedures that were used in this study to extract the vitamins and carotenoids from the sample matrix will also be discussed. The use of the results from these methods for the certification of SRM 3280 is described elsewhere (8).

MATERIAL AND METHODS[1]

Method 1: LC/UV-Visible Absorbance Detection of Vitamins A, E, and Select Carotenoids in SRM 3280

Standards and Calibration Solutions

Stock solutions of retinyl acetate (Sigma/Aldrich, St. Louis, MO, USA), *dl*-α-tocopheryl acetate (Sigma/Aldrich, St. Louis, MO, USA), lutein (Hoffmann-LaRoche, Nutley, NJ, USA), β-carotene (Sigma/Aldrich, St. Louis, MO, USA), δ-tocopherol (Sigma/Aldrich, St. Louis, MO, USA), and zeaxanthin (Hoffmann-LaRoche, Nutley, NJ, USA) were prepared by dissolving each compound in absolute ethanol that contained 30 mg/L butylated hydroxytoluene (BHT; added to prevent analyte oxidation). The concentrations of the vitamins and carotenoids in the stock solutions were determined spectrophotometrically using Beer's Law (19, 20).

[1]Certain commercial products are identified to specify adequately the experimental procedure. Such identification does not imply endorsement or recommendation by the National Institute of Standards and Technology, nor does it imply that the materials identified are necessarily the best available for the purpose.

The following extinction coefficients in absolute ethanol were used to determine the concentration of the stock solutions: 1560 dL/g·cm for retinyl acetate at 325 nm, 43.6 dL/g·cm for α-tocopheryl acetate at 284 nm, 2560 dL/g·cm for *trans*-β-carotene at 452 nm, 2765 dL/g·cm for lutein at 445 nm, and 2416 dL/g·cm for zeaxanthin at 452 nm (19). Working calibration solutions were prepared from the stock solutions using δ-tocopherol as the internal standard. Corrections for purity (mass fraction) were made based on the liquid chromatographic (LC) analysis of the stock solutions at the wavelengths at which the concentrations were determined. Four independent calibration solutions for each analyte were used to determine detector responses. Calibration curves were constructed using the ratio of analyte-to-internal-standard peak areas and were linear over the concentration ranges measured.

Sample Preparation

Six bottles of SRM 3280 were selected for analysis according to a stratified randomized sample scheme. The total content (30 tablets) of each bottle was ground for 10 min using an automated mortar grinder with agate mortar and pestle and Vulkollan scraper (Retsch RM-100; Newtown, PA, USA). The ground tablets were stored in each bottle prior to sample preparation, which was performed on the same day on which the contents were ground. Two test portions from each bottle of the SRM were weighed (about 2 g each) and mixed with about 15 g of 0.1 mol/L HCl solution in 50-mL polyethylene centrifuge tubes. The samples were simultaneously sonicated at 37 °C for about 25 min with vigorous intermittent shaking every 3 min to 5 min. Subsequently, about 2 g of the δ-tocopherol (internal standard) solution (8.22 mg/g in ethanol containing BHT) were added to the sample with additional mixing for about 5 min. About 35 mL of HPLC-grade hexane was added to the sample prior to being placed on a rotating shaker; the sample was allowed to shake overnight. After overnight shaking, each sample was centrifuged for 15 min at 315 radians per second (3000 revolutions per min). The initial hexane layer (which was golden yellow) was removed and placed in a flask that was covered in foil and stored in the freezer at -20 °C. To ensure complete extraction of the vitamins and carotenoids from the matrix, each sample was subsequently extracted with multiple (at least three) 35-mL portions of hexane until the organic layer was clear. Samples were sonicated at 37 °C for about 5 min prior to each subsequent extraction with hexane and placed on the rotating shaker for about 1 hour per extraction. All hexane layers were combined and agitated to ensure proper

mixing; 10 mL of the combined extracted sample was removed, evaporated to dryness using nitrogen, and reconstituted with 1 mL of ethanol containing BHT solution. The reconstituted extracts were vortex mixed for about 30 s and placed in polyethylene sample vials prior to LC analysis.

SRM 968c Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum, Level 1, (20) was prepared and analyzed for quality control. Prior to extraction, the serum sample was equilibrated to room temperature, reconstituted with 1.00 mL of HPLC-grade water, and sonicated for approximately 5 min. A 200-μL aliquot of serum was combined with an equal volume of ethanol containing the internal standard, δ-tocopherol (8.22 mg/g), and BHT (30 μg/mL) to precipitate the proteins from the serum matrix. Subsequently the analytes were extracted twice from the serum with hexane. The supernatant was removed from the extract and combined. The extract was then evaporated to dryness under a stream of nitrogen, and was reconstituted with 200 μL of ethanol containing 30 μg/mL BHT. The reconstituted extract was vortex mixed for about 30 s to ensure dissolution of the dry residue and placed in a sample vial prior to LC analysis.

Chromatographic Conditions

The LC system used for these measurements consisted of a ternary LC pump (Varian, Inc., Palto Alto, CA, USA), a programmable absorbance detector (Kratos Analytical, Ltd./ Shimadzu Corp., Kyoto, Japan), and an autosampler (Waters Corporation, Milford, MA, USA). Isocratic LC conditions using a 5 μm polymeric C_{18} column (4.6 x 250 mm; Vydac 201TP; Separations Group, Hesperia, CA, USA) at 25 °C was used for the separation. The mobile phase (listed as volume fractions) consisted of 4 % methanol containing 0.05 % triethylamine/96 % acetonitrile at a flow rate of 0.8 mL/min. UV/visible detection (deuterium lamp) at the following wavelengths was used: 325 nm at initial conditions for retinyl acetate; 284 nm at 7 min for δ-tocopherol and *dl*-α-tocopheryl acetate; and 450 nm at 13.5 min for β-carotene, lutein, and zeaxanthin.

Method 2: LC/UV-Visible Absorbance Detection of β-Carotene and Lutein in SRM 3280

Standards and Calibration Solutions

The concentration of individual ethanolic stock solutions of β-carotene (Sigma/Aldrich, St. Louis, MO, USA) and lutein (Hoffmann-LaRoche, Nutley, NJ, USA) were determined by spectrophotometry. Three aliquots of three independently weighed gravimetric dilutions were measured six times each in the spectrophotometer. An ethanolic solution of *trans*-β-apo-8'-carotenal (about 0.3 μg/mL; Hoffmann-LaRoche, Nutley, NJ) was used as an internal standard for lutein. A solution of *trans*-β-apo-10'-carotenal oxime (about 1.5 μg/mL) was used as the internal standard for β-carotene. The oxime was prepared from *trans*-β-apo-10'-carotenal by passing a concentrated solution through a preparative C_{18} column (Vydac 201TP, Separations Group, Hesperia, CA, USA) using 50:50 methanol:acetonitrile and collecting the fraction that contained the *trans*-β-apo-10'-carotenal oxime (21, 22). Four β-carotene calibrants and eight lutein calibrants were prepared by gravimetric dilution of the stock solution with the internal standard solution. Corrections for purity (mass fraction) were made based on the LC analysis of the stock solutions at the wavelengths at which the concentrations were determined. The response factor was calculated (using peak areas) for each calibrant; an average response factor was used for the determination β-carotene and lutein in SRM 3280.

Sample Preparation

Eight bottles of SRM 3280 were selected by a stratified random sampling scheme. Fifteen tablets from each bottle (containing 30 whole tablets) were ground for 5 min to 7 min in the automated mortar and pestle described for Method 1. Approximately 0.2 g to 0.6 g of the sample was then accurately weighed into a 50 mL polyethylene centrifuge tube and about 0.7 g of the ethanolic internal standard solution containing *trans*-β-apo-10'-carotenal oxime was added by mass via syringe to for the β-carotene measurements. Approximately 2.3 g of the ethanolic internal standard solution containing *trans*-β-apo-8'-carotenal was added by mass via syringe for the lutein measurements. A 10 mL aliquot of 1 % (volume fraction) ethylenediaminetetracetic acid (EDTA) solution was added to each sample and the centrifuge tube was immersed in a 41 °C water bath for 1 h to dissolve the encapsulation on some of the vitamins. The samples were put in an ultrasonic bath for 60 min. The aqueous solution was then extracted with about 20 mL to 30 mL of hexane for 60 min through rotational agitation on a rotating shaker at approximately

6 rad/s (60 rpm). The samples were centrifuged at 263 rad/s (2500 rpm) for 20 min, the hexane was removed from the aqueous solution, and the samples were immersed in an ultrasonic bath for 30 min. Another 20 mL aliquot was added to the vial and extracted on the rotating shaker for 60 min, then centrifuged and the hexane removed. This extraction was repeated five times.

Chromatographic Conditions

Analyses were performed on an LC system that consisted of a ternary pump and autosampler (Dionex Corp., Sunnyvale, CA, USA), and an ultraviolet/visible absorbance detector (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The carotenoids were detected at 450 nm. All mobile phase compositions are listed as volume fractions in percent.

Lutein. A 5 μm polymeric C_{30} carotenoid column (4.6 mm x 250 mm; YMC, Waters Corporation, Milford, MA, USA) was used for the separation of lutein in SRM 3280. The column temperature was held at 25 °C with a recirculating water bath. Solvent A was water with 2 mmol/L ammonium acetate and solvent B was acetone. A linear gradient was run from 60 % B to 100 % B in 70 min. The column was then re-equilibrated at the starting conditions for 15 min; the flow rate was 1.0 mL/min.

β-Carotene. A 5 μm C_{18} column (4.6 mm x 250 mm; Bakerbond, J.T. Baker, Phillipsburg, NJ, USA) was used for the separation of β-carotene in SRM 3280. The column temperature was held at 29 °C with a re-circulating water bath. Solvent A was acetonitrile, solvent B was methanol containing 0.05 mol/L ammonium acetate, and solvent C was ethyl acetate. Each of the three solvents contained 0.05% triethylamine (TEA) to enhance carotenoid recovery (23). The mobile phase program consisted of two linear gradients and an isocratic component. The initial mobile phase composition was 98% A/2% B and was ramped to 75% A/18% B/7% C in 10 min. A second linear gradient ran from this composition to 68% A/25% B/7% C in 5 min. This composition was held for 20 min; the system was then returned to initial conditions and re-equilibrated for 15 min. The flow rate was 1.5 mL/min.

RESULTS

Several preliminary studies were performed prior to the characterization of SRM 3280 for the measured fat-soluble vitamins and carotenoids reported in this paper. Analytical challenges such as examining the extractability of these analytes from their encapsulation, assessing the homogeneity and stability of the analytes in the material, identifying appropriate internal standards, and developing suitable chromatographic systems for separating the analytes of interest were evaluated.

Extraction of Fat-Soluble Vitamins and Carotenoids from the Matrix

The fat-soluble vitamins and carotenoids (retinyl acetate, β-carotene, ergocalciferol, phylloquinone, lutein, and dl-α-tocopheryl acetate) were added to the pre-mix for SRM 3280 by the manufacturer as formulated gelatin beadlets, therefore it was critical to use solvents that sufficiently dissolve the gelatin coating in the formulated tablets. The protocols described in this paper are reproducible and appear adequate for the extraction of the vitamins and carotenoids measured in SRM 3280. Prior to the adoption of the extraction procedures described herein, the ground vitamin tablets were mixed with water and dimethyl sulfoxide (DMSO), sonicated at 37 °C for 1 h to dissolve the gelatin coating, and extracted with hexane to determine the best extraction procedure for our work. Mixing the ground tablets in water prior to hexane extraction did not seem to dissolve the gelatin coating. The use of DMSO/water (3+1 volume fraction) appeared to dissolve the coating, but still required multiple hexane extractions (at least four) and very careful sample handling since DMSO passes readily through human skin (24). Mixing the ground tablets with EDTA (used in Method 2) followed by multiple hexane extractions (at least three) worked equally as well as extraction with 0.1 mol/L HCl (used in Method 1).

Studies were also performed at NIST to determine the completeness of the extraction of the analytes from the matrix. Samples were prepared according to the described extraction protocols using either EDTA or dilute HCl to dissolve the gelatin coating, followed by repeat organic solvent (hexane) extractions. Samples were extracted with hexane and analyzed by LC/absorbance detection until no traces of the analytes were detected in subsequent extracts. Our studies showed that at least three hexane extractions were needed to completely remove the vitamins and carotenoids from the matrix. A summary of these studies is presented in Table 1.

SRM 3280 was distributed to collaborating laboratories for analysis as part of the NIST Dietary Supplements Quality Assurance Program (25) which was established to help laboratories improve their measurement comparability for dietary supplements. For this intercomparison study, the collaborating laboratories chose various extraction approaches that were typically used in each laboratory to extract the analytes from the SRM. Each laboratory used NIST SRM 1849 Infant/Adult Nutritional Formula for quality control. This SRM, one of the NIST food-matrix SRMs, is characterized for proximates, fatty acids, vitamins, elements, amino acids, and nucleotides (26, 27). Data from the intercomparison study showed significant variability (some of which may be attributed to incomplete extraction) for some of the analytes measured. For example, results from the collaborating laboratories for retinol (which is the most polar of the vitamins being extracted and thus presents the greatest challenge to the extraction system) were 20 % to 30 % lower than those reported for retinol using the NIST protocol (Figure 1). Relative standard deviations for the retinol data from the collaborating laboratories were 10 % to 20 % (28).

Another parameter that was crucial to the extractability of the vitamins from the reference material was the mixing (contact) time that was allowed between the sample and the extractant. Two test portions from the same bottle were prepared using the same extraction protocol described in Method 1, except the overnight shaking step was eliminated. After each hexane extraction (four were performed) per sample, the samples were placed on the rotating shaker for 1 h prior to centrifugation, evaporation, reconstitution, and LC analysis. Based on results from these analyses, there was about a 5 % decrease in concentration levels for retinyl acetate and α-tocopheryl acetate when compared to the samples that had been allowed to shake overnight. There was no noticeable difference in the concentration levels for the carotenoids in either set of samples (i.e., overnight vs. no overnight shaking).

Analyte Homogeneity and Stability

The homogeneity of the fat-soluble vitamins and carotenoids in SRM 3280 was assessed by using the LC/absorbance methods described herein. SRM 3280 is provided as whole tablets. (Each tablet weighs approximately 1.5 g). At least 15 tablets must be ground to obtain a homogeneous sample (7, 8) because of tablet-to-tablet heterogeneity. Tablets were ground in a disk mill, which involved shaking in an orbital pattern for 6 min, or they were ground for 10 min

using an automated mortar and pestle (the technique described in this paper). An analysis of variance did not show inhomogeneity for the test portions analyzed. The relative within- and among-bottle precision of the LC/absorbance methods used for this work ranged from about 1 % to 2 % for the analytes measured.

Preliminary studies performed at NIST also showed that the measured fat-soluble vitamins and carotenoids in SRM 3280 were stable for at least four days following grinding. At a recent NIH/ODS Vitamin Methodology Workshop, these analytes were also reported to be stable in ground test samples for longer periods (6 months to 12 months) of time (29), although this has not been verified by NIST. The stability of these analytes in SRM 3280 will continue to be monitored at NIST. The Certificate of Analysis recommends the use of a freshly ground portion for vitamin analyses and that the reference material be stored at a controlled room temperature (20 °C to 25 °C) in an unopened bottle prior to use (7). Special precaution should also be taken to protect the analytes from light. For this work, all studies were performed under subdued lighting and when possible, the samples were fully shielded from light.

Internal Standards

The required properties of the internal standards for the characterization of SRM 3280 are that they be sufficiently close homologues of the analytes of interest so that the extractability from the matrix is mimicked and that they are readily separable from the analytes by the analytical approach used. The internal standards selected for the LC/absorbance approaches described in this paper were suitable and generally commercially available.

Analytical Approaches Used for the Determination of Fat-Soluble Vitamins and Carotenoids in SRM 3280

The methods used to determine the concentrations of fat-soluble vitamins and carotenoids in SRM 3280 employed two C_{18} stationary phases with different selectivities (see Table 2) as well as a polymeric C_{30} phase. Chromatograms from the analysis of SRM 3280 using the described NIST methods are illustrated in Figures 2 and 3**.** All analytes are well separated from matrix interferences. Polymeric C_{30} columns typically provide adequate separation of the geometric carotenoid isomers. The Bakerbond C_{18} column used for this work provided an alternative selectivity intermediate to the monomeric and polymeric stationary phase columns

(30-32). This column provided adequate separation for the *cis*-β-carotene isomers and was used to determine *trans*-, *cis*-, and total β-carotene in SRM 3280.

UV/visible absorbance detection at the selected wavelengths was adequate for the determination of all analytes of interest. The limit of quantitation for the analytes measured using UV/visible absorbance detection was 50 μg/g to 100 μg/g. The results for the analysis of this SRM using these different analytical approaches are summarized in Table 2. These data were combined with results from collaborating laboratories and the LC/mass spectrometry method developed at NIST for the analysis of SRM 3280 (33). The relative measurement precision for the methods described in this paper ranges from 2 % to 8 %.

CONCLUSIONS

The methods described in this paper have been successfully used to characterize SRM 3280 as part of a collaborative effort to produce the first NIST dietary supplement reference material for multivitamin/multielement tablets. This SRM was developed to support analytical method validation and quality control for the analysis of similar materials. The constituents in this SRM have been determined by reliable independent methods that support state-of-the-practice approaches for the measurement of the selected compounds.

ACKNOWLEDGMENT

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Table 1. Summary of Dissolution Studies for SRM 3280 Multivitamin/Multielement Tablets

Diluent	Coating dissolved during 1 h sonication at 37 °C	Hexane extractions needed[a]
Water	No	-
DMSO/water (3+1 volume fraction)	Yes	4
1 % EDTA[b]	Yes	3
0.1 mol/L HCl[b]	Yes	3

[a]Minimum number of hexane extractions needed to completely remove the vitamins and carotenoids from the ground tablets as indicated by no additional analyte extracted into the subsequent aliquot of solvent.

[b]Used in NIST sample preparation protocols.

Table 2. Summary of results (mass fractions in mg/g) from the analyses of SRM 3280 Multivitamin/Multielement Tablets for fat-soluble vitamins and carotenoids using the NIST analytical methods described in the text. Results have been corrected for purity relative to prepared calibration standards and for moisture content. One standard deviation of the mean is indicated in parentheses; the number of measurements is denoted by *n*.

Analyte	Method 1	Method 2		Assigned Value[a]
	LC/UV Polymeric C_{18} (n = 12)	LC/UV Intermediate C_{18} (n = 8)	LC/UV Polymeric C_{30} (n = 8)	
Retinol	0.921 (0.014)	-	-	0.78 ± 0.19[b]
α-Tocopherol	21.9 (0.4)	-	-	21.4 ± 3.5[b]
Lutein	0.242 (0.004)	-	0.162 (0.009)	0.205 ± 0.050
Zeaxanthin	0.005 (<0.001)	-	-	-
Trans-β-carotene	0.510 (0.011)	0.371 (0.029)	-	0.42 ± 0.10
Cis-β-carotene	0.078 (0.002)	0.066 (0.005)	-	0.072 ± 0.007[c]
Total β-carotene	0.587 (0.010)	0.438 (0.034)	-	0.514 ± 0.087

[a] The data in this table along with results from collaborating laboratories and the NIST LC/MS method described elsewhere (33) were used to obtain the assigned concentration values for the fat-soluble vitamins and carotenoids in SRM 3280. The NIST process of assigning values to its SRMs for chemical measurements is described in references 34 and 35. The uncertainty in the assigned values is expressed as an expanded uncertainty at the 95 % level of confidence (36-38).

[b]Assigned concentration values represent retinol and α-tocopherol equivalents for retinyl acetate and α-tocopheryl acetate, respectively.

[c]Results for Method 1 and Method 2 were used to assign value.

Figure 1. Results for the measurement of retinol in SRM 3280 from collaborating laboratories that participated in an interlaboratory comparison study as part of the NIST Dietary Supplements Quality Assurance Program (25). Error bars span ± 2 standard deviations around the laboratory mean values. The solid box encloses the expanded uncertainty at the 95 % level of confidence around NIST values. The consensus box (dashed lines) encloses ± 2 standard deviations around the consensus medians.

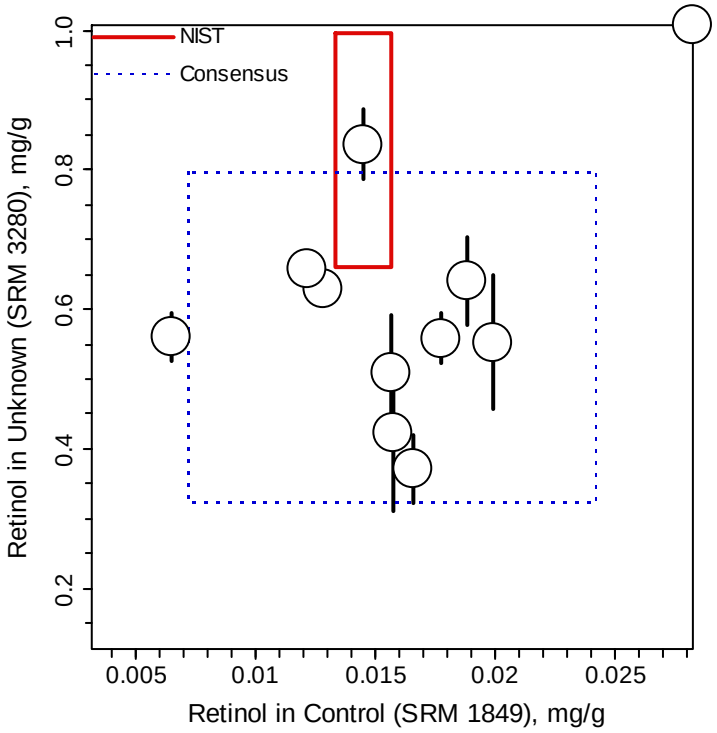


Figure 2. Chromatogram from the analysis of SRM 3280 using the LC/absorbance (Method 1) performed at NIST. Chromatographic conditions are described in the text.

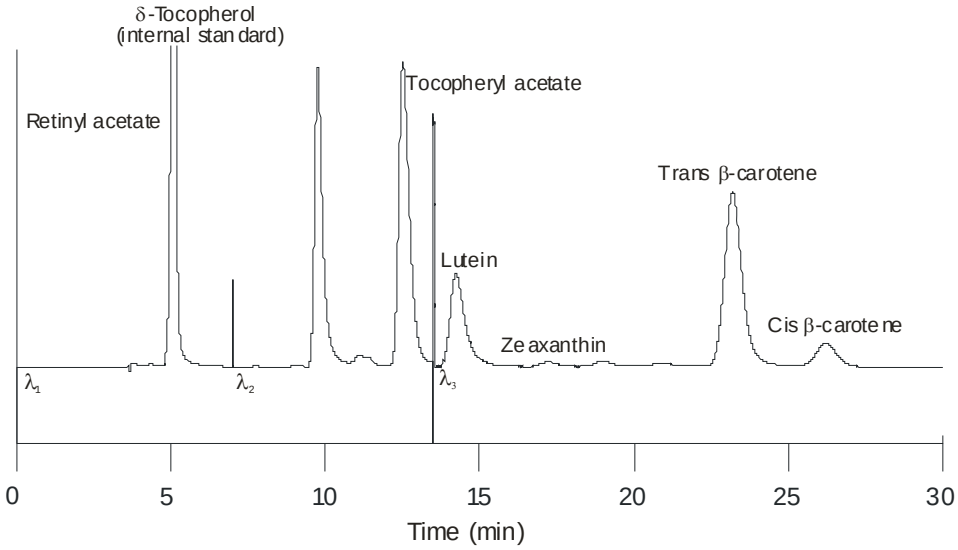
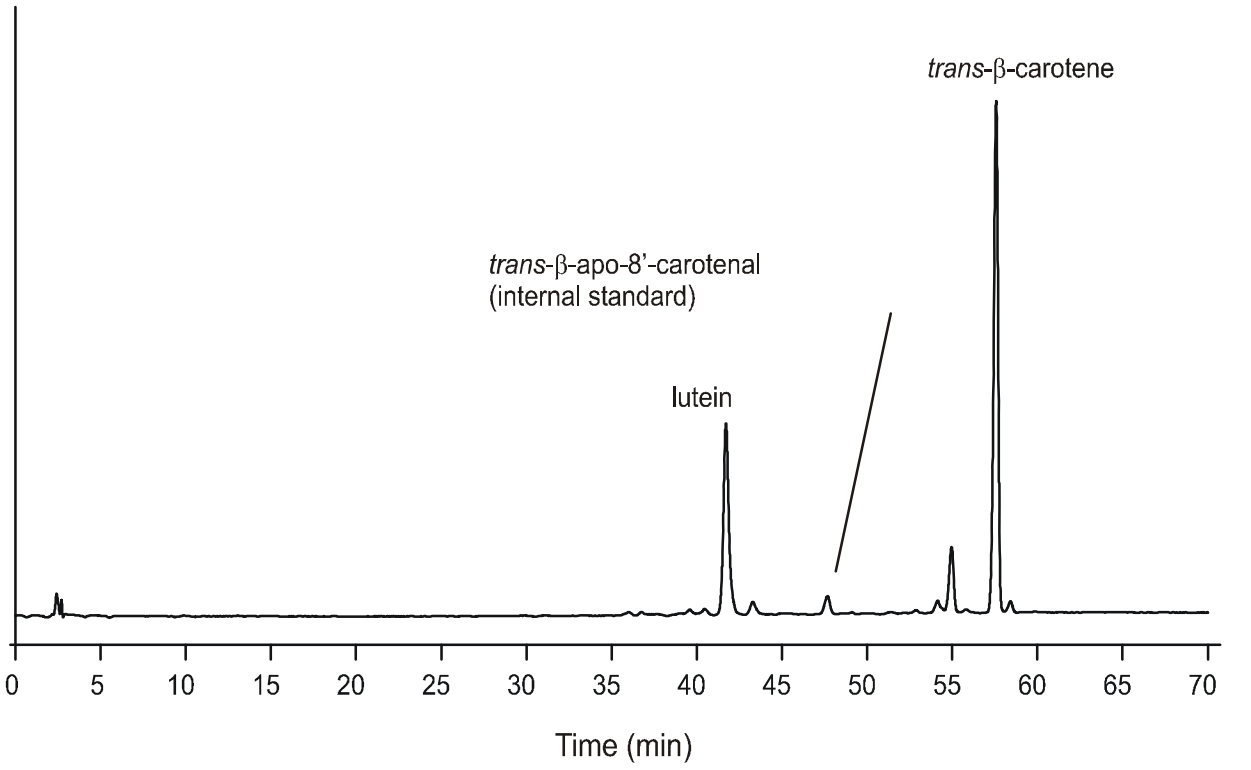
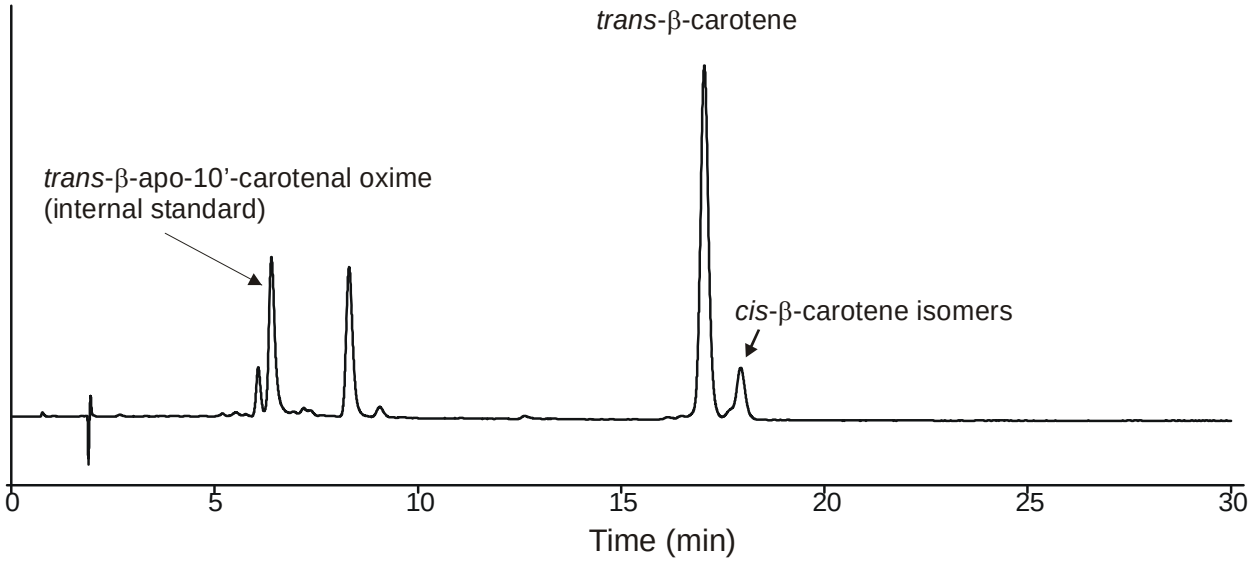


Figure 3. Chromatograms for the separation of carotenoids in SRM 3280 by LC/absorbance (Method 2) performed at NIST. Upper trace A: Analysis using a polymeric C_{30} carotenoid (YMC) column. Lower trace B: Analysis using an intermediate (Bakerbond) C_{18} column. Chromatographic conditions are described in the text.





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