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Measurements of the major isoforms of vitamins A and E and carotenoids in the blood of people with spinal-cord injuries

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Abstract

We used reversed-phase HPLC with diode array detection to simultaneously measure the major isoforms of vitamins A, E, and the carotenoids in serum from 55 healthy people with spinal cord injuries. Typically, the method measured retinol (vitamin A), α-tocopherol (vitamin E) and β-carotene, α-carotene, lutein, lycopene, and cryptoxanthin (carotenoids). γ-Tocopherol (vitamin E), 25-hydroxycalciferol (vitamin D), and the carotenoid zeaxanthin could also be measured when they were present in high concentrations. Healthy people with spinal cord injuries were more likely than similar people without injuries to have low concentrations of α-tocopherol, and to a lesser extent retinol and β-carotene.
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Keywords: Vitamins; Carotenes; Tocopherols; Carotenoids

1. Introduction

Over a million people worldwide have severe spinal cord injuries. We know very little about the dietary intakes and nutrient status of healthy people with spinal cord injuries. The limited data available suggests that people with spinal cord injuries are at greater risk for marginal nutrition than the general population [1–4]. Indeed, several studies have shown that people with severe physical disabilities have low serum concentrations of vitamins C [5] and D [6].

Vitamins A, E, D and the carotenoids are important fat-soluble nutrients found in human blood and tissues. Vitamin A is necessary for normal eyesight, growth, and embryonic development [7]. Vitamin A deficiency is arguably the most common preventable cause of blindness in the world, and a leading cause of infant mortality [8]. β-Carotene forms vitamin A. Furthermore, β-carotene and several other carotenoids such as lycopene and lutein have been strongly associated with degenerative disease prevention in epidemiological studies [9]. Vitamin E is the most abundant fat-soluble antioxidant, and is also associated with cancer and heart disease prevention [10]. Vitamin D is necessary for strong bones, and vitamin D deficiency causes a common bone deformity, rickets [11].

The terms ‘‘vitamins A’’, ‘‘vitamin E’’, ‘‘vitamin D’’, and ‘‘carotenoids’’ are actually not the names of single chemicals; instead, each of these terms represents a family of related compounds. ‘‘Vitamin A’’ consists of retinol, and also lesser amounts of retinal, retinyl palmitate, retinyl stearate, and other retinyl esters. ‘‘Vitamin D’’ consists of 25-hydroxy-

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calciferol, as well as minor metabolites such as 1,25-dihydroxycalciferol. Major forms of ''vitamin E'' include α-tocopherol, γ-tocopherol, as well as smaller quantities of δ-tocopherol. The ''carotenoids'' belong to a family of over 600 similar chemical compounds, the most familiar and abundant of these being β-carotene, α-carotene, lycopene, cryptoxanthin, lutein and zeaxanthin.

Several HPLC methods have been developed to assay β-carotene, retinol, and α-tocopherol [12–17]. These methods are inherently complex, since these nutrients differ in their concentrations, chemistries, spectral, and fluorescent properties. Early methods often relied on two chromatography column chemistries [12] or two modes of detection [13–15]. These methods were often difficult to maintain and to trouble-shoot. More recent methods have used single finely packed columns with diode array detectors and are relatively rapid and robust [16,17].

Recently scientists have focused attention on the potential of lycopene, lutein, and other carotenoids for the prevention of cancer and degenerative diseases [18]. This has necessitated the development of HPLC methods that differentiate and identify these other carotenoids [19–28]. This is a difficult problem in separation science, since over 20 carotenoids are detectable in normal human serum and many of the compounds have similar chemical, structural, and spectral properties [19,20]. At present, there are several methods that can reliably separate and measure retinol, α-tocopherol and four to six carotenoids of interest. However, all of these methods, including the one we report here, have drawbacks. The commonest separation problems are: (1) lutein and zeaxanthin cannot be separated at all [21–23]; (2) α-carotene and β-carotene migrate closely together and are misidentified or poorly quantitated by chromatography software [24,25]; (3) lycopene is degraded, and lycopene isomers are difficult to identify and measure [26]. When these separation problems are solved, as in the method we report, a fourth problem develops: run times are long [27,28].

We modified a reversed-phase HPLC method [28] to simultaneously measure the most abundant forms of vitamin A, D, E, and carotenoids found in human serum. Specifically, the method normally measures retinol, α-tocopherol, β-carotene, α-carotene, lycopene, lutein, and cryptoxanthin. It can also measure the carotenoids zeaxanthin and *cis*-β-carotene, γ-tocopherol and 25-hydroxycalciferol when these are present at high concentrations. The method gives baseline separation for all the nutrients of interest and is robust. The method has a long run time, but problems with the misidentification or poor quantitation of peaks are simple to target and resolve. We used this method to measure fat-soluble nutrients in healthy adult volunteers with spinal cord injuries.

2. Experimental

2.1. Subjects

This study was an observational survey of 55 healthy adult volunteers with spinal cord injuries of greater than 2 years duration. The Human Subjects–Institutional Review Committees of the University of California at Davis and the United States Department of Agriculture approved the study. Subjects were recruited from Contra Costa, Alameda, Yolo and Sacramento counties, CA, USA through advertisements, postings, and word-of-mouth. Subjects with spinal cord injuries had paraplegia or quadriplegia in approximately equal numbers (49% had paraplegia, 51% had quadriplegia). Most (60%) were male, which appears to be representative of people with spinal cord injuries nationwide. Their age averaged 41 ± 13 (SD) years; and the average number of years since their spinal cord injury was 19 ± 10. Although most subjects were Caucasian- (62%), the group also included Asian- (12%), Hispanic- (11%), Native-, and Arab-Americans. The most common causes of the spinal cord injuries were motor vehicle accidents (48%), gunshot wounds (14%) and sports-related injuries (14%).

2.2. Blood collection, processing and storage

Seventy ml of blood were collected from an antecubital vein following an overnight fast. Although spinal cord injury often results in skeletal and muscle deformities that can influence the location of veins and arteries, we obtained complete blood collections from everyone who volunteered for the study. Most of the blood collected was used for other

health and nutrient status tests that will be reported on elsewhere. Blood used for these analyses was collected into 10-ml red-top (serum) tubes via standard vacutainer. The blood collection tube was immediately placed into a covered ice bucket to protect the samples from heat and ambient light. Blood was centrifuged for 15 min at 3000 *g* on an RP5 Sorvall Centrifuge (Newtown, CT, USA) at 4 °C in a HP4 rotor within 3 h of collection, and then stored in tightly capped 2-ml plastic cryovials at −70 °C until use.

2.3. Chemicals

All solvents used were HPLC or reagent grade. Reagent-grade chemicals were purchased from Sigma (St. Louis, MO, USA), Fisher Scientific (Pittsburgh, PA, USA) and J.T. Baker (Phillipsburg, NJ, USA). Retinyl palmitate (>95% purity), retinol (>95% purity), retinol acetate (>90% purity), 25-hydroxycalciferol (>99% purity) and lutein (xanthophylls from alfalfa, 85% purity) were from Fluka (Milwaukee, WI, USA). 2,6-Di-*tert*.-butyl-*p*-cresol (BHT), α-tocopherol (98% purity), α-carotene (>90% purity), lycopene (>90% purity) and β-carotene (>97% purity) standards were from Sigma.

2.4. Preparation of solvents and HPLC standards

Our HPLC method is generally based on a previously published method used for buccal mucosal cells [28], but differs in almost all specific details. Mobile phases were made by mixing acetonitrile, tetrahydrofuran and methanol, then adding 10 g/l ammonium sulfate in HPLC grade water and 5 mg/l β-hydroxytoluene as an antioxidant. Solvent A contained acetonitrile–tetrahydrofuran–methanol–ammonium sulfate (85:5:5:5, v/v), and solvent B contained acetonitrile–tetrahydrofuran–methanol–ammonium sulfate (55:35:5:5, v/v). Each solvent was prepared fresh before use. The least stable component in this gradient is the tetrahydrofuran, which eventually turns yellow when exposed to air and light, increasing the baseline for the chromatographs. Tetrahydrofuran had to be purchased in amber-glass containers under argon for this reason. HPLC standards were prepared by dissolving ~1 mg standard into 10 ml toluene–ethanol (99:1 ethanol:toluene). Standards were prepared by mass, and stored at 4 °C for up to a week before use.

2.5. Sample preparation

Samples were thawed at room temperature under plastic sleeve-covered fluorescent lights to minimize sample degradation from exposure to UV light. Our standard method used 500 μl of human serum. After extraction, evaporation and resuspension, we end with a sample volume of 100 μl. This allows us to run duplicate determinations using a 20-μl injection volume. However, the extraction method can be used with essentially no modification in serum samples as small as 100 μl. Alternatively, the sample can be resuspended in a smaller volume to measure isoforms in lower concentrations. Samples were mixed with 1 ml 95% aqueous ethanol containing 5 mg/l BHT to denature the proteins, vortexed for 60 s, and then mixed with an equal volume of hexane. The hexane layer was removed and saved in 12×75 mm borosilicate test tubes, and the extraction repeated. Then the combined hexane layers were dried under a stream of nitrogen. Finally, the sample was resuspended in 100 μl solvent B, described above. Samples and standards were stable under these conditions for up to 1 week.

2.6. Equipment

Liquid chromatography was run on an Agilent 1100 gradient chromatograph with a binary pump, degasser, refrigerated autosampler, column heater, and diode array detection. Chromatographic analysis was run and interpreted with a Chemstation for LC 3D revision A.08.03 (847) for Agilent Technologies, running on a HP Kayak XM600 computer with Windows NT (Hewlett-Packard, Waldbronn, Germany).

2.7. Chromatography

HPLC was run as a gradient at 1.0 ml/min. Chromatography was run with a Prodigy 5 μm C_{18} ODS 3 100 Å pore 250×4.6 mm reversed-phase column (Phenomenex, Torrance, CA, USA). Total run time was 48 min. The gradient changed from 5

to 95% solvent B, as follows: 0 to 10.0 min, 5% solvent B; 10.0 to 29.0 min increasing linearly to 95% solvent B; 29.0 to 35.9 min, maintaining 95% solvent B; 35.9 to 36.0 min, abruptly decreasing to 60% solvent B; maintaining 60% solvent B from 36.0 to 44.9 min; then abruptly decreasing to 5% solvent B at 45 min. The column re-equilibrated with 5% solvent B from 45.0 to 48.0 min. Serum concentrations of nutrients were measured simultaneously by diode array detection at 325 nm (retinol and retinyl esters), 292 nm (tocopherols) and 452 nm (carotenoids). Standards for retinol, α-tocopherol, and β-carotene were analyzed after every fifth sample. Comparing retention times with those of the corresponding standards identified most peaks, and inspecting diode array three-dimensional plot scans (run from 250 to 500 nm wavelength) for wavelength maxima. Peaks that did not have standards (such as zeaxanthin and γ-tocopherol) were identified by their spectra and chromatographic characteristics (e.g. zeaxanthin is known to migrate closely with lutein under almost all chromatographic conditions).

2.8. Laboratory and data analyses

Health status indices (complete blood chemistries, human immunodeficiency virus antibody) were measured on a Roche/Hitachi/Boehringer Mannheim 902 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN, USA) with standard chemistries. 25-Hydroxycalciferol concentrations were measured by direct enzyme immunoassay (OCTEIA, Immunodiagnostic Systems, Boldon, UK) according to the manufacturer's instructions.

Data were evaluated by simple statistics (mean, standard deviation) and histograms were drawn and analyzed with Sigmaplot 2000 (SPSS, Chicago, IL, USA).

3. Results

Chromatographs showing the separation of chromatography standards are shown in Fig. 1. Most isoforms of fat-soluble nutrients were baseline separated, though some lycopene isomers were not. The chromatography conditions described easily separated and measured retinol, α-tocopherol, γ-tocopherol, lutein, lycopene, cryptoxanthin, α-carotene and β-carotene in all samples. Reproducibility was good, with RSDs for standard aliquots ($n=14$) of 1.8, 2.1, and 4.3% (for α-tocopherol, retinol and β-carotene). The detection limits, defined as three times baseline noise, were good and comparable to previously reported values for samples analyzed by diode array detection (with detection limits of 2, 0.6, and 1 μg/l for α-tocopherol, retinol, and β-carotene). Linearity of concentration curves was excellent up to at least 1000 times the detection limit, $r=0.99$, 0.98, and 0.99, respectively, for α-tocopherol, retinol and β-carotene. RSDs, detection limits, and linearity for α-carotene, lutein, zeaxanthin and cryptoxanthin were similar to those for β-carotene, while the RSD and detection limit for lycopene were worse (8% and 2 μg/l).

This is necessarily a complex chromatographic method. Although it is robust it contains some modifiers that improve the stability, separation and quantitation of carotenoids but also increase the chromatographic baseline. BHT, used as a carotenoid preservative, can form a peak migrating near the solvent front that is detectable at 254 and 292 nm. This peak is well separated from any of our peaks of interest and does not interfere with our assays. Further, concentrations of BHT greater than 200 mg/l also can increase background and interfere with detection limits. BHT does not interfere with our measurements. Tetrahydrofuran improves carotenoid separations, but it can cause even greater baseline deviations if it is oxidized. Lowest baselines are obtained when HPLC-grade tetrahydrofuran is purchased in small lots in brown bottles, stored under argon or helium, and mobile phase is prepared and discarded within a few days. Under these conditions, the baseline at 254 and 292 nm is low and stable. When tetrahydrofuran oxidizes, it increases the baseline at 254 and 292 nm. Mild oxidation, that occurs when mobile phases are prepared weekly, is easily observed (as shown in Figs. 1 and 2) but does not interfere with measurements. Greater oxidation, that would occur if mobile phases were prepared monthly or if oxidized tetrahydrofuran was used, could interfere with the determinations of γ-tocopherol concentrations.

25-Hydroxycalciferol does not have a good chromaphore, and must be analyzed at 254 nm.

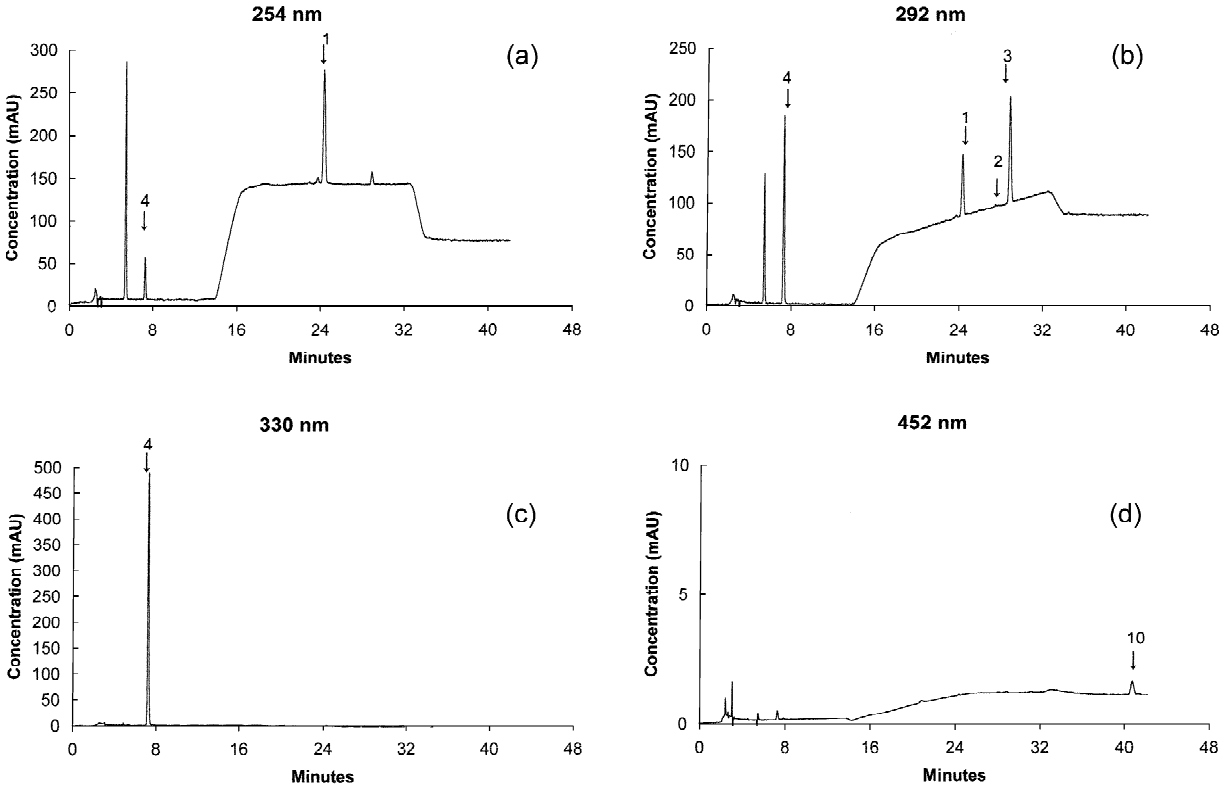

Fig. 1. Chromatograph of fat-soluble vitamin standards, run after every five samples. Chromatograph at (A) 254 nm; (B) at 292 nm; (C) at 325 nm; (D) at 452 nm. The peaks were identified as follows: (A) peak 1, 25-hydroxycalciferol; (B) peak 3, α-tocopherol; (C) peak 4, retinol; (D) peak 10, β-carotene.

Although its peak can be viewed in the diode array three-dimensional scan, several solvent components absorb in this area. Therefore 25-hydroxycalciferol can only be observed when it is present in higher than usual concentrations. The detection limit was ~10 μg/l. Direct enzyme immunoassay is currently a more sensitive technique for 25-hydroxycalciferol. Still, some subjects had measurable amounts of zeaxanthin, *cis*-β-carotene, and 25-hydroxycalciferol. Linearity was good, $r=0.95$. Reproducibility for the 25-hydroxycalciferol standard was poor, with an RSD of 13%; however, these peaks were also baseline-separated when present in sufficient concentrations to measure. A chromatograph from a typical sample from a well-nourished subject with spinal cord injuries is shown in Fig. 2.

No significant differences in nutrient serum concentrations were seen between men and women, presumably because of the large standard deviations seen for all groups. Serum α-tocopherol concentrations were low in our subjects with spinal cord injuries compared to national norms and to recommended concentrations thought to protect against heart disease (Table 1). Retinol and β-carotene concentrations were also somewhat low in subjects with spinal cord injuries. There are currently no recommended concentrations for lycopene or lutein, although these carotenoids are of emerging scientific interest.

4. Discussion

The method we developed for these analyses is generally based on a previously published method for measuring fat-soluble vitamins in buccal epithelial cells [28]; however, it differs in most details for three reasons. Firstly, we use a different HPLC

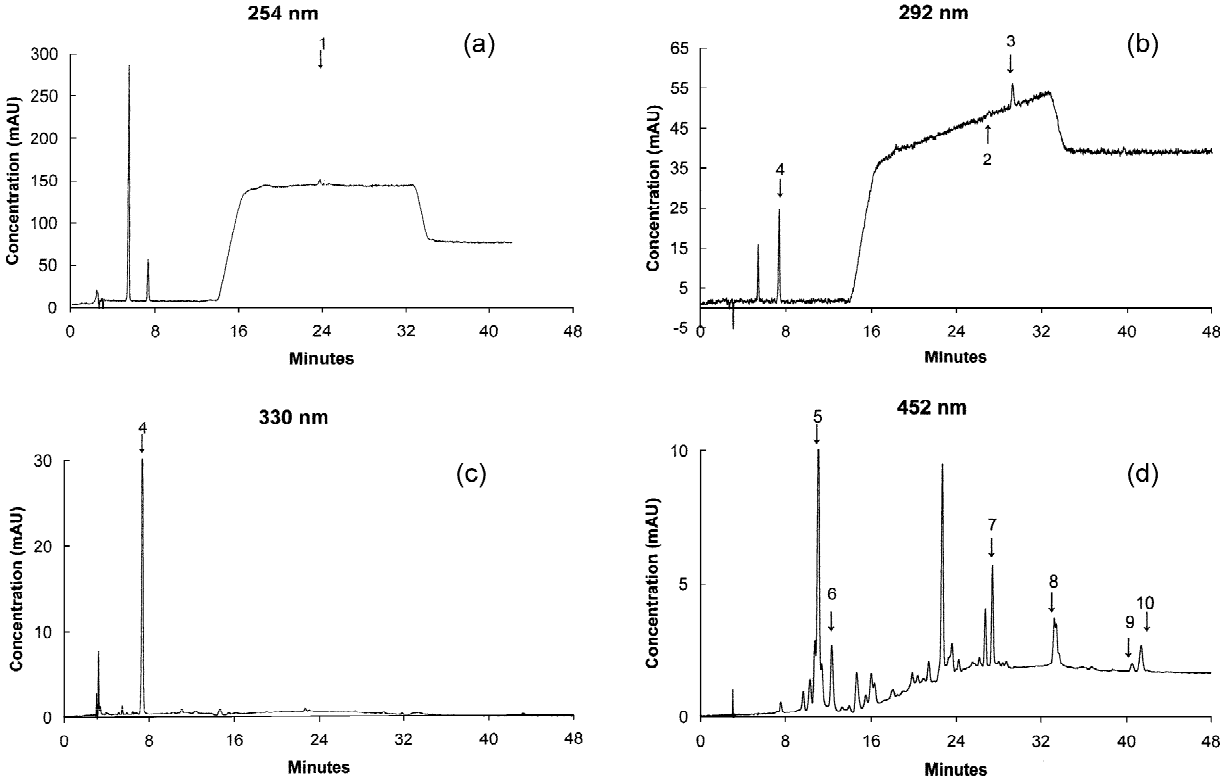

Fig. 2. Chromatograph of serum sample from a well-nourished subject with high concentrations of minor metabolites and carotenoids. Chromatograph at (A) 254 nm; (B) at 292 nm; (C) at 325 nm; (D) at 452 nm. The following peaks were identified: (B) peak 2, γ-tocopherol; peak 3, α-tocopherol; (C) peak 4, retinol; (D) peak 5, lutein; peak 6, zeaxanthin; peak 7, β-cryptoxanthin; peak 8, lycopene; peak 9, α-carotene; and peak 10, β-carotene.

system, diode array detector, column and software analysis program because these are what we had available to us. Secondly, like most HPLC methods published in the literature the buccal cell method appeared to have been developed for the inexperienced workforce and low labor costs associated with universities. Thus, it included several labor-intensive steps that impart only marginal value. For example, the original method calls for manual filtration of all solvents, while we use only HPLC grade solvents and an in-line filter. Similarly, the original method always calculates the concentrations of standards by their spectrophotometric maxima, and we buy highly purified standards and calculate their concentrations by mass. We have checked the purity of these standards; they have been in the range the manufacturer claims. Thirdly, our gradients and run times differ in small but significant ways from the original method. These minor changes increase the separation between β-carotene and α-carotene, and increase the probability that these peaks are identified correctly and measured accurately by our chromatography software.

We have seen no discussion of the role of chromatography software in the HPLC of vitamins and carotenoids, or on the potential problems caused with peak misidentification when the run-time is decreased. However, in our experience problems caused when our software misidentifies peaks or miscalculates peak areas are relatively common in carotenoid chromatography and can be time-consuming to correct. Our chromatography software, and indeed, most others, can identify peaks of similar spectral characteristics by their order of appearance (i.e. the 21st peak measured at 452 nm is always identified as ''β-carotene'') or by their time of

Table 1
Serum nutrient concentrations for subjects with spinal cord injuries compared to US national data

Nutrient	Subjects Mean (SD)	NHANES III[a] quartiles	Physiological cutoffs
Retinol	1.92	<1.64	<0.7 deficient
(μmol/l)	(0.66)	<1.99	<1.05 marginal
		2.12[b]	
		<2.37	
		≥2.37	
α-Tocopherol	17.3	<19.5	<20 marginal
(μmol/l)	(4.9)	<24.0	
		29.6[b]	
		<30.3	
		≥30.3	
β-Carotene	0.25	<0.17	<0.05 marginal
(μmol/l)	(0.14)	<0.28	
		0.43[b]	
		<0.45	
		≥0.45	

[a] National Health and Nutrition Survey of the United States of America, third survey, 1988–1994 [32].
[b] Mean.

appearance (i.e. the peak found between 41.6 and 42.5 min is ''β-carotene''). Since we typically analyze complex materials such as blood serum that may contain variable numbers of peaks, we must identify and quantitate peaks based on their time of appearance. This can be a problem during long chromatography runs, since carotenoid peaks gradually drift slightly as the column is conditioned, so the times during which they appear increase slightly. When peaks migrate close together at the end of the chromatography run, as α-carotene and β-carotene do, this drift can result in them being identified incorrectly, or missed entirely. Although β-carotene and α-carotene were baseline separated in the original method, this separation was small, and we had to review and reanalyze chromatographs regularly. Our modification results in a greater separation between the α- and β-carotene peaks, which meant we had to reanalyze and reinterpret chromatographs less frequently. For our laboratory, it is cost-effective to increase chromatography run-times by a few minutes when it results in decreased analysis time since we can run the chromatography system overnight inexpensively but reanalysis requires skilled labor.

Our results are consistent with the few other studies of nutrient concentrations of subjects with spinal cord injuries reported in the literature. Previous studies showed that people with spinal cord injuries had low concentrations of vitamin D [6]. Subjects with severe physical disabilities, including people with spinal cord injuries, polio, and cerebral palsy, had low serum concentrations of vitamin E [29] and vitamin C [5]. Furthermore, studies of induced spinal cord injuries in animals suggest that these injuries may increase metabolism and thus requirements for vitamin E and other antioxidants [30].

Our results show that subjects with spinal cord injuries are at risk for poor fat-soluble nutrient status, of which several of these nutrients are believed to protect against heart disease and cancer. Indeed, people with spinal cord injuries have shorter life spans than people without spinal cord injuries [31]. The reasons that people with spinal cord injuries have higher rates of heart disease and premature death have not been established. They are undoubtedly multi-factorial; however, it is reasonable to speculate from our data that poor diets may play a key role.

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