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New simplified procedures for the extraction and simultaneous high-performance liquid chromatographic analysis of retinol, tocopherols and carotenoids in human serum

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ABSTRACT

A short, simple extraction procedure and a sensitive reversed-phase high-performance liquid chromatographic assay, which utilizes isocratic elution and detection either by a photodiode-array detector or by two detectors set at 300 and 450 nm, have been developed to measure retinol, tocopherols and several carotenoids in human serum simultaneously. By relying on characteristic UV–visible spectra, seventeen carotenoids, retinol and α- and γ-tocopherols were identified in concentrated serum extracts by use of the three-dimensional data mode of a photodiode-array detector. The presence of some recently reported carotenoids in human serum has been confirmed.

INTRODUCTION

High-performance liquid chromatography (HPLC) is the most used method for analysis of retinoids, carotenoids and tocopherols in human serum or plasma and in other tissues [1,2]. Most published procedures employ one detector, set at the maximum absorption wavelength of the analyte under study. Because retinol absorbs maximally at 325 nm, tocopherols at 290 nm and the carotenoids around 450 nm, simultaneous analysis of these three classes of micronutrients by a single detector is not possible, although all these and other related compounds are usually extracted together from serum or other tissues. These nutrients can be simultaneously determined in a single extract by using more than one detector, of course, or by changing the detection wavelength during the run [3–13]. Although older photodiode-array (PDA) detectors were significantly less sensitive, and were more costly, than conventional one-wavelength detectors, currently available PDA detectors show sensitivity similar to single-wavelength detectors.

In this paper, we describe the use of a recently available PDA detector to identify and quantitate simultaneously retinol, tocopherols and carotenoids in human serum. We also describe a simple, rapid and highly efficient procedure for extracting these micronutrients from serum.

EXPERIMENTAL

Solvents and reagents

HPLC-grade acetonitrile and methanol and reagent-grade dichloromethane, dichloroethane, 2-propanol and 1-octanol were purchased from

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Fisher Scientific (Fairlawn, NJ, USA). Synthetic lutein, lycopene, β-carotene, α-tocopherol, γ-tocopherol and α-tocopheryl acetate were purchased from Sigma (St. Louis, MO, USA). β-Apo-8'-carotenal and canthaxanthin were purchased from Fluka (Ronkonkoma, NY, USA). β-Cryptoxanthin was isolated from red bell peppers [13]. All compounds were purified by HPLC, when necessary, before use [14,15].

HPLC system

The HPLC system consisted of the following equipment (Waters Associates, Milford, MA, USA): an automated sample injector (WISP 710B), a Model 510 pump and a Model 991 photodiode-array detector. The data were generated either in the three-dimensional (DT3) mode (250–550 nm region) or in the two-dimensional (DT2) mode (290, 325 and 450 nm). Data were stored and processed by a NEC Powermate SX Plus computer (NEC Technology, Boxborough, MA, USA). A Waters Model 5200 printer/plotter was used.

For routine quantitative analysis, the measurements were carried out in the DT2 mode, which required only 15–30 kbytes of disk space as compared with 3000–5000 kbytes in the DT3 mode.

In another procedure [14,15], instead of using the PDA detector, two ISCO (Lincoln, NE, USA) Model V4 detectors, one set at 450 nm (for carotenoids) and the other set at 300 nm (for retinol and tocopherols), were connected in series to the HPLC system. A dual-channel Shimadzu CR-4A integrator (Shimadzu, Columbia, MD, USA) was used for storing and recording the chromatograms.

The chromatographic analysis was performed on a Waters spherical 5-μm C_{18} "Resolve" column (30 cm × 3.9 mm I.D.). A guard column of C_{18} material (Upchurch Scientific, Omaha, NE, USA) preceded the main column. The mobile phase consisted of either acetonitrile–dichloromethane–methanol–1-octanol (90:15:10:0.1, v/v, system 1) [14,15] or acetonitrile–dichloromethane–methanol–water containing 0.1% ammonium acetate (90:10:5:2, v/v, system 2) or acetonitrile–dichloroethane–methanol containing 0.05% ammonium acetate (85:10:5, v/v, system 3). To prepare solvent systems 2 and 3, ammonium acetate [16] was first dissolved in water (system 2) or methanol (system 3), and then added to the rest of the solvent mixture. The flow-rate was 1 ml/min in HPLC systems 1 and 2, and 1.5 ml in system 3. Dichloroethane can replace the more volatile dichloromethane in solvent systems 1 and 2 without affecting the resolution or the retention times of the analytes.

Sample extraction

Human serum samples stored at −20°C or below were used. All extractions were performed at ice temperature in laboratories illuminated with yellow lights (Gold Fluorescent lamp) to minimize light-induced isomerization.

Method 1. Except for the internal standards, this method is similar to the one described previously from this laboratory [14,15]. In brief, 20–500 μl of serum were treated with twice the volume of ethanol and with 1 ml of ethyl acetate. One or all three of the internal standards, consisting of 4–7 μl each of tocopheryl acetate (16 μg/μl), canthaxanthin (2–3 ng/μl) and retinoic acid (10 ng/μl), were added. The mixture was vortex-mixed (30 s) and centrifuged (30 s) [16]. The supernatant solution was removed, and the pellet was broken up and extracted twice more with ethyl acetate (0.5–1 ml), then with hexane (0.5–1 ml) by vortex-mixing and centrifuging as before. The supernatant solutions were pooled, 500 μl of water were added and the mixture was vortex-mixed and centrifuged. The upper, organic layer was removed and evaporated to dryness under a stream of argon. The residue was dissolved in 100 μl of a mixture of dichloromethane–methanol (1:2, v/v). An aliquot (10–90 μl) of the extract was injected into the HPLC system.

Method 2. An aliquot (50–100 μl) of serum was pipetted out into a 1.5-ml Eppendorf Safe-Lock microcentrifuge tube (Brinkmann Instruments, Westbury, NY, USA). After twice the volume of isopropanol and the same volume of dichloromethane were added, the mixture was vortex-mixed briefly (5 s). Then 2–7 μl of the internal standards as described in method 1 were added.

The mixture was vortex-mixed for 1 min, then centrifuged in an Eppendorf microcentrifuge for 1 min, at 8100 *g*. (If a microcentrifuge is not available, the microcentrifuge tube can be lodged firmly in the mouth of a centrifuge tube and centrifuged in a regular centrifuge.) The clear supernatant solution was carefully drawn into a 250–500 μl syringe, and the volume was read and recorded. If desired, the final volume can be adjusted to a specific value by adding a mixture of isopropanol–dichloromethane (2:1, v/v). This is convenient because the volume of the extract will vary in each sample because of (i) differential loss of solvent during the vortex-mixing and centrifuging process and (ii) the presence of different amounts of precipitated proteins in different serum samples. Aliquots (25–100 μl) of the supernatant solution were injected into the HPLC system.

Analysis of PDA data

The data generated with the PDA detector on the DT3 mode were subjected to spectral analysis by monitoring the contour plots of chromatograms at 290 (for tocopherols), 325 (for retinol), 350 (for retinoic acid and phytofluene), 400 (for ζ-carotene), 450 (for most carotenoids) and 470 nm (for lycopene). The *y*-scale was adjusted to display full peaks. The entire region of each chromatogram thus obtained was scanned in the automatic mode for any well-defined UV–visible spectra in the 250–550 nm region. Any well-de-

TABLE I

LIST OF CAROTENOIDS, RETINOIDS AND TOCOPHEROLS DETECTED IN HUMAN SERUM (500 μl)

No.	Retention time (min)	λ_{max} (nm)			AU	Compound	μmol/l	(μg/dl)[a]
1	3.88		325		0.07	Retinol	2.78	(79.6)
2	4.46	415	443	470	0.006	ε,ε-Carotene-3,3'-dione[b]	0.08	(4.9)
3	4.72	415	442	470	0.008	3'-Hydroxy-ε,ε-caroten-3-one[b]	0.11	(6.6)
4	5.06	420	447	475	0.025	Lutein	0.35	(20.6)
5	5.28	420	450	478	0.025	Zeaxanthin	0.35	(20.7)
6	5.6		285		0.011	Unidentified		
7	6.56	430	455	485	0.012	Unidentified	0.17	(9.9)
8	7.22	420	448	475	0.02	2',3'-Anhydrolutein[b]	0.29	(16.5)
9	8.26	420	448	470	0.005	Unidentified	0.07	(4.5)
10	8.26		295		0.01	γ-Tocopherol	7.03	(292)
11	9.26		293		0.025	α-Tocopherol	17.0	(730)
12	9.26	415	447	475	0.01	α-Cryptoxanthin	0.15	(7.9)
13	9.96	425	452	480	0.015	β-Cryptoxanthin	0.24	(13.2)
14	12.5	442	474	505	0.045	Lycopene	0.51	(27.5)
15	14.56	420	445	470	0.002	Unidentified	0.03	(1.6)
16	15.86	440	465	495	0.002	γ-Carotene[b]	0.25	(1.3)
17	16.78	380	402	426	0.008	ζ-Carotene[b]	0.15	(8.2)
18	17.26	380	402	426	0.002	ζ-Carotene (*cis*)[b]	0.03	(2.0)
19	18.96	420	448	475	0.015	α-Carotene	0.21	(11.2)
20	20.1	335	350	370	0.007	Phytofluene[b]	0.07	(10.9)
21	20.32	425	455	485	0.03	β-Carotene	0.45	(24.35)

[a] Values were calculated based on $E(1\%,1\text{ cm})$ [17] = 2800 (α- carotene), 2592 (β-carotene), 3450 (lycopene), 2636 (α-cryptoxanthin), 2386 (β-cryptoxanthin), 2540 (zeaxanthin), 2550 (lutein), 3110 (γ-carotene), 2042 (ζ-carotene), 1350 (phytofluene), 72 (α-tocopherol) and 1850 (retinol). Other carotenoids were calculated based on $E(1\%,1\text{ cm})$ values of carotenoids shown in parentheses: 2, 3, 7, 8 (lutein) and 15 (α-carotene).

[b] Identification according to Khachick and co-workers, [18,19].

fined spectrum was recorded. The analytes were identified by comparing their UV–visible absorption characteristics with those of reference compounds [1,14–19]. The concentration of each identified analyte listed in Table I was calculated from its absorption unit (optical density) reading and known molar extinction coefficient values [1,14–19], and was corrected for the extraction efficiency.

The data generated with the PDA detector on the DT2 mode were reanalysed in the integrator mode to provide peak areas. The concentration of analytes under each peak, whether obtained with the PDA detector or with the two detectors, was determined from peak areas as described previously [13–15].

RESULTS

Fig. 1 shows the three-dimensional (3D, 90°, left) plot of the UV–visible spectra of the compounds present in 0.5 ml of human serum. The compounds were extracted according to method 1 and analysed with the PDA detector under DT3 mode by isocratic reversed-phase HPLC, using solvent system 1. Because retinol and the tocopherols absorbed very strongly as compared with the carotenoids, two different y-scales [0–0.12 AUFS for retinol and tocopherols (left) and 0–0.05 AUFS for carotenoids (right)] have been used to show the spectra. The chromatograms of the same assay plotted at 290 nm (for tocopherols), 325 nm (for retinol) and 450 nm (for carote-

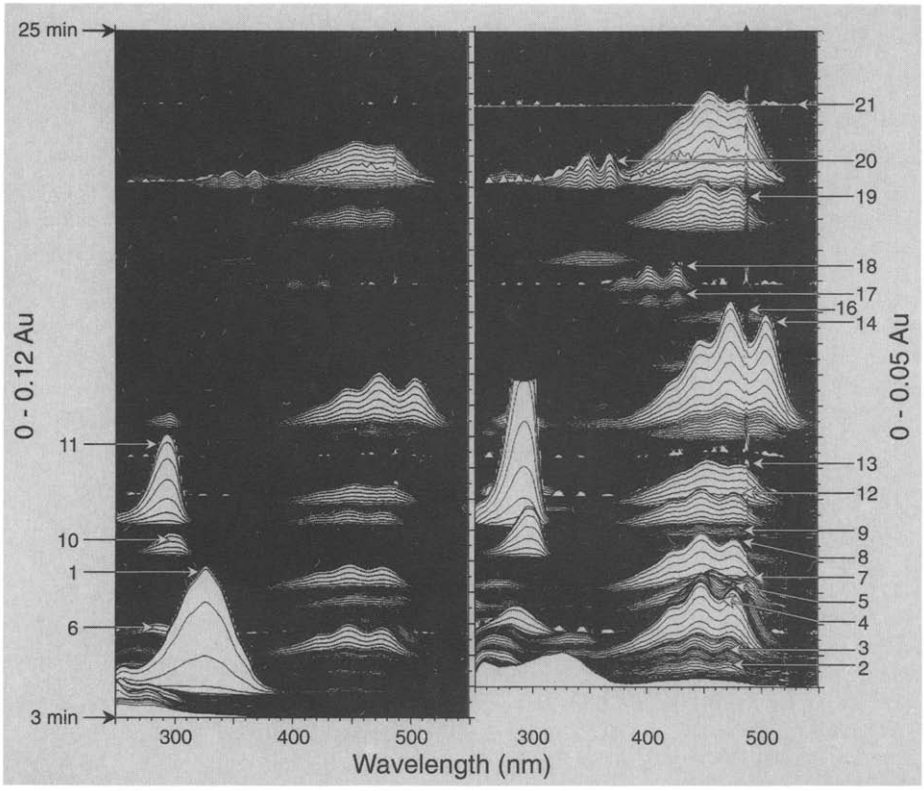

Fig. 1. Three-dimensional plot (90°, left) of UV–visible spectra of compounds present in human serum (500 μl), extracted by method 1. The compounds were separated by reversed-phase isocratic HPLC on a Waters 5-μm "Resolve" column (30 cm long) with a mobile phase of acetonitrile–dichloromethane–methanol–1-octanol (90:15:10:0.1, v/v, solvent system 1) at a flow-rate of 1 ml/min. Detection was carried out with a Waters Model 991 PDA detector. Peak 15 was hidden by peak 14 in this presentation. For peak identification, see Table I.

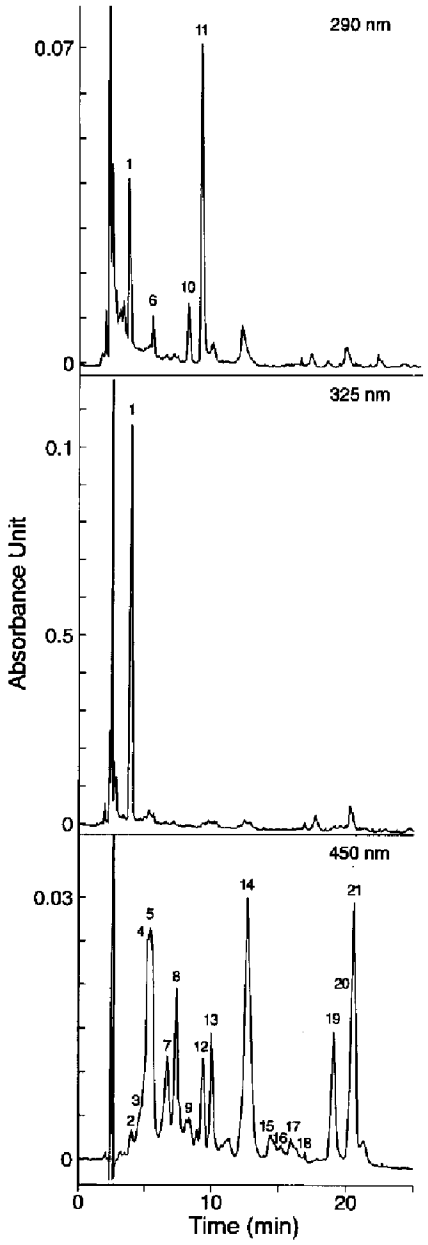

Fig. 2. Chromatograms obtained at 290 nm (for tocopherols), 325 nm (for retinol) and 450 nm (for carotenoids) of compounds in human serum (500 μl), extracted by extraction method 1. Detection and HPLC conditions are the same as described in the legend to Fig. 1. For peak identification, see Table I.

noids) are shown in Fig. 2. The contour plot and spectral analysis of the same serum extract resulted in the identification of seventeen carotenoids, retinol and α- and γ-tocopherols (Table I). As can be seen in Fig. 1 and Table I, several of the analytes coeluted (for example α-tocopherol and α-cryptoxanthin), but the absorption spectra were so different, and the maxima so far apart, that both compounds could readily be identified and quantitated. Based on thorough physicochemical characterization of minor carotenoids in serum by Khachik and co-workers [18,19], the presence in human serum of ε,ε-carotene-3,3'-dione, 3'-hydroxy-ε,ε-carotene-3-one, 2',3'-anhydrolutein, γ-carotene, ζ-carotene and phytofluene [18,19] was confirmed by use of characteristic absorption spectra and reported retention times in the present study. After correction for the extraction efficiency, the concentrations of these nutrients, calculated from the observed absorption units (AU) and known $E(1\%,1\ cm)$ values [17], are shown in Table I. Concentrations of serum carotenoids, also available as standards, were determined from peak areas of peaks in the chromatograms obtained under DT2 or DT3 mode. They were found to be almost identical (± 5%) to values determined from peak absorption units. By using the PDA detector, it was possible not only to record well-defined absorption spectra of the major analytes in serum, but also to determine the concentration of the analytes from the observed absorption unit (AU) values and known extinction coefficients. We found that, under our experimental conditions, the dilution factor (band broadening) with regard to the injected volume did not have any effect on quantitation.

The chromatograms shown in Fig. 3 were obtained from an injected aliquot of 50 μl equal to 12.5 μl of serum. Compounds were extracted by method 2 and analyzed by solvent system 1 by use of two detectors. Extraction of human serum by method 2, which avoids the evaporation and reconstitution steps in method 1, and analysis of the extract by HPLC, using solvent system 3 and the PDA monitored in the DT2 mode at 290, 325 and 450 nm, gave the chromatograms shown in Fig. 4.

The recoveries of internal standards in serum extracts in both methods 1 and 2 were satisfactory for tocopherols and carotenoids. In four different extractions by method 2, the recoveries of α-tocopheryl acetate and canthaxanthin were

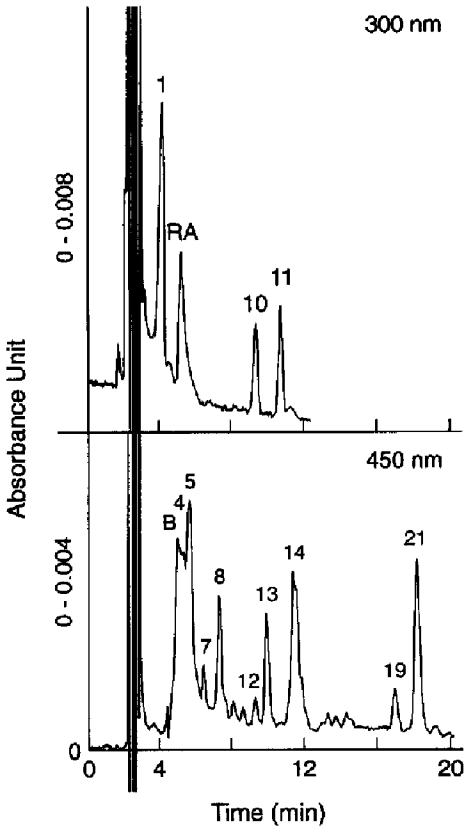

Fig. 3. Chromatograms obtained at 300 nm (for tocopherols and retinol) and 450 nm (for carotenoids) of compounds in human serum (50 μl injection, which is equal to 12.5 μl of serum) extracted by method 2. Detection was carried out with two ISCO V4 detectors. HPLC conditions are the same as described in the legend to Fig. 1. For peak identification, see Table I. RA = Retinoic acid; B = bilirubin.

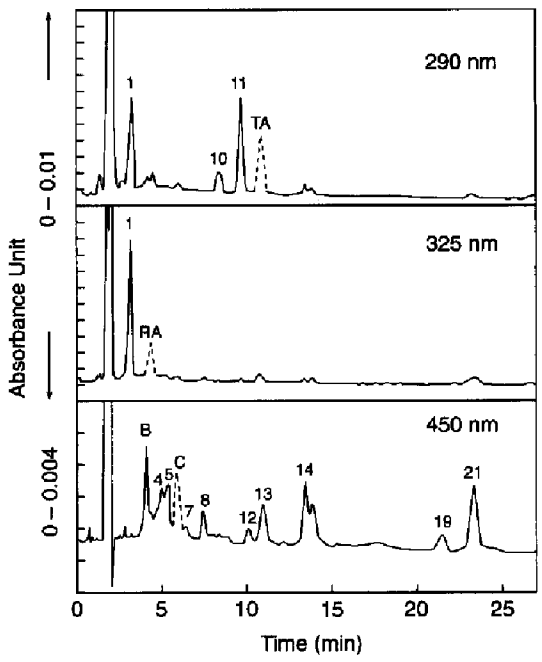

Fig. 4. Chromatograms obtained at 290 nm (for tocopherols), 325 nm (for retinol) and 450 nm (for carotenoids) of compounds in human serum (70 μl injection, which is equal to 25 μl of serum) extracted by method 2. HPLC analysis was carried out with solvent system 3 (acetonitrile–dichloroethane–methanol, 85:10:5, v/v, containing 0.05% ammonium acetate) at a flow-rate of 1.5 ml/min, and detection was carried out with a Waters Model 991 PDA detector. For peak identification, see Table I. TA = α-tocopheryl acetate; RA = retinoic acid; B = bilirubin; C = canthaxanthin.

94.6 ± 2.3% and 94.8 ± 9.0%, respectively. The efficiency of extraction of retinoic acid, however, was only 67.4 ± 1.5%.

The recoveries of added retinol, α-tocopherol and β-carotene, each representing a class of micronutrient in serum, were found in three replicate analysis to be 101 ± 4.7%, 91 ± 2.8% and 100 ± 6.1%, respectively. The within-run and between-run imprecision of method 2 was estimated by repeated analysis of three serum samples over a period of twelve days. The results are shown in Table II.

TABLE II

PRECISION OF THE EXTRACTION PROCEDURE (METHOD 2)

Analyte	Within-run comparison (mean ± S.D.) (μg/dl)	Between-run comparison (mean ± S.D.) (μg/dl)
Retinol	21.4 ± 0.57	23.6 ± 1.8
α-Tocopherol	1171 ± 205	1151 ± 75
β-Carotene	27.4 ± 0.82	26.1 ± 2.07

DISCUSSION

In this study, by using a PDA detector and a simplified method of extraction, retinoids, tocopherols and carotenoids were identified and quantitated simultaneously in an isocratic reversed-phase HPLC system.

HPLC solvent system 1 was satisfactory for analysis of serum extracted by method 1 [14,15], but was less satisfactory for use with serum extracted by method 2. In the latter case, polar 450-nm absorbing compounds eluted in a broad peak with the solvent front, or shortly thereafter. As a result, retinol, which also eluted on the final slope of the solvent front, was difficult to quantify.

In contrast, solvent systems 2 and 3 resolved retinol from the solvent front well and provided adequate separations of carotenoids and tocopherols. Method 2 also extracted bilirubin, which did not separate well from lutein in solvent system 1 (Fig. 3), but did in solvent systems 2 and 3 (Fig. 4). In addition to satisfactory resolution of micronutrients of interest, very good separation of retinol from the solvent front and partial resolution of polar carotenoids lutein and zeaxanthin were achieved in solvent system 3. Solvent system 3 is, therefore, the most suitable system for analysis of retinol, tocopherols and carotenoids in human serum, irrespective of the method of extraction.

In extraction method 2, it is not necessary to evaporate the extract and then reconstitute the residue in an appropriate solvent for injection into the HPLC system. Thus, the loss of micronutrients is minimized. Furthermore, because of the rapidity of the process, isomerization and oxidation of vulnerable carotenoids and retinoids is also minimized.

Retinol [20–22] and β-carotene [22–24] have been analysed by other procedures that require no evaporation of solvent before injection into the HPLC system. None of these methods, however, has determined retinol, tocopherols and carotenoids simultaneously. Creech Kraft *et al.* [25] and Eckhoff and Nau [26] used isopropanol for extracting retinoids from tissues. We found that isopropanol alone did not extract the hydrocarbon carotenoids at all well compared with a mixture of isopropanol and dichloromethane. We also found that ethyl acetate can replace dichloromethane.

A single extraction by method 2 resulted in recovery of 95–100% of the micronutrients as determined: (1) by the recovery of added β-carotene, retinol and α-tocopherol, each representing one class of micronutrients in serum; (2) by the recovery of internal standards added to serum; (3) by exhaustive extraction of the pellet left after the first extraction; and (4) by the recovery of tritium from the sera of rats dosed with radiolabelled [^{3}H]retinoids. It should, however, be noted that recoveries of added micronutrients in this type of analysis can be misleading, since the micronutrients added in solvents are readily extractable, in contrast to the protein-bound micronutrients in serum [27]. On the other hand, the inclusion of an internal standard during extraction and analysis of serum is useful, because several analytical errors may occur. One such common error is loss during evaporation, partitioning and redissolving of the extract for injection into the HPLC system. Although this error is unlikely to occur during extraction by method 2 in the present study, other errors can arise due to detector malfunction or column performance, which can be detected from the internal standard response.

When larger volumes of the extract (>100 μl) prepared by extraction method 2 were injected, unacceptably broad peaks resulted. On the other hand, when very small volumes (<20 μl) were analyzed with the PDA detector, the peaks due to minor carotenoids showed a low signal-to-noise ratio, thereby making quantitation difficult. However, retinol and α- and γ-tocopherols could be determined in as little as 10 μl of injected extract, equal to 3 μl of serum. Somewhat better sensitivity was obtained by the use of two detectors or, alternatively, by analyzing the concentrated serum extract obtained by method 1 with the PDA.

Although three internal standards, each representing one class of the micronutrients analyzed, were routinely used, a single internal standard, such as α-tocopheryl acetate, provides adequate

control for all of the analytes [12]. Recovery of retinoic acid was not satisfactory, and its use as internal standard is not recommended. None of the three internal standards interfered with any of the major analytes of interest. Furthermore, β-apo-8'-carotenal, which was not well resolved from lutein/zeaxanthin in solvent system 2, can nonetheless be used as an internal standard in other solvent systems.

The use of the PDA detector improves the analysis of carotenoids in serum. For example, although a sharp peak for lutein and zeaxanthin has been reported in several HPLC assays, the present study revealed that other carotenoids also coelute in this peak (Table I). Indeed, by using different solvent systems and columns, several keto and other carotenoids have been shown to coelute with lutein [18,19]. Not all the reported carotenoids were identified in the present study, largely because of their very low concentrations in serum. Carotenoids such as ζ-carotene and phytofluene, which show absorption maxima of 402 nm and 350 nm, respectively, will not be detected by single-wavelength detection at 450 nm, but can be fully analyzed with a PDA detector. A diffuse spectrum resembling that of phytoene (λ_{max} = 275, 286, 296 nm), which has been reported in human serum [18,19], was also detected immediately after the β-carotene peak. Sensitive PDA detectors should also be of use in the analysis of other light-absorbing micronutrients that can be extracted together with retinoids, carotenoids and tocopherols.

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