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Carotenoids, retinoids and alpha-tocopherol in human serum: identification and determination by reversed-phase HPLC

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Abstract: A rapid, simple and specific high performance liquid chromatographic procedure for assaying α- and β-carotene is described. The method also enables the simultaneous determination of retinol and DL-α-tocopherol in human serum. The same chromatographic procedure can be used to assay the major carotenoids in human serum, provided analyses are replicated and the effluent is monitored at 450 nm. The conditions described also enable determination of licopene, cryptoxanthine and lutein with zeaxanthine. An aliquot of 0.5 ml serum is deproteinized with ethanol (0.5 ml) and extracted with petroleum ether (0.75 ml). The petroleum ether extract is evaporated until dry and then redissolved immediately with 0.5 ml of an eluent mixture consisting of methanol–hexane (85:15, v/v). Aliquots of 50 μl are then injected onto a 250 × 4.6 mm column packed with Spherisorb ODS-2. Owing to its good reproducibility, the procedure can be used for assays with external standards. Clinical applications are described for cases of hypercarotinemia associated with endocrine dysfunctions such as hypothyroidism and diabetes.

Keywords: *Reversed-phase HPLC; carotenoids; retinoids; α-tocopherol; human serum.*

Introduction

Study of the procedures for analyzing blood retinol and other metabolically correlated vitamin constituents has increased considerably over the past five years, thanks to the development of methods based on high performance liquid chromatography (HPLC). HPLC techniques have already been described for the analysis of serum-retinol, retinoids and tocopherol [1–9] and, more recently, for the analysis of blood carotenoids [10–17]. However, not all authors have been able to suggest procedures by which retinoids, α-tocopherol and major serum carotenoids might be assayed in a single run. The need for a simultaneous assay has become apparent not only as a result of nutritional considerations [18–19], but also as a consequence of observations that some retinoids exhibit anticancer chemopreventive action [20–22]. Furthermore, epidemiological studies have shown a possible correlation between balanced diet and retinol and β-carotene blood levels on the one hand, and a lower risk of cancer incidence in humans on the other [23–26].

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The simultaneous assay of such vitamin constituents calls for particular technological features, the first of which have already been prioritized in earlier work from this laboratory [13]. These technological requirements are:

(a) The possibility of detecting effluents at different wavelengths, thanks to the fact that carotenoids, α-tocopherol and retinoids — though they have very close, or even coincident, retention times — do not interfere with each other either quantitatively or qualitatively. They can therefore each be assayed at the wavelength that provides the greatest specificity [13–14].

(b) The possibility of performing reversed-phase analysis with mobile phases made up entirely of organic solvents (the non-aqueous reversed-phase technique) [27] in order to avoid aqueous-organic eluents — as proposed earlier [15–16] — which would lead to the on-column precipitation of components, such as carotenoids, that are only very slightly soluble in such eluents.

In the opinion of the present authors, this second technological feature is especially relevant in view of the need for a mobile phase that can fully dissolve all components (and keep them dissolved) of the deproteinated serum petrol–ether extract residue. Carotenoids are in this class and present the greatest solubility problems.

The foregoing considerations are, it is believed, demonstrated by the procedure described in this paper. The authors hope that this may constitute a useful contribution to research aimed at the assay of vitamin constituents in blood, as already demonstrated in other papers [28–30].

Experimental

Materials and methods

Retinol was prepared by saponification of retinol palmitate (Merck for Biochemistry or Hoffman-La Roche) as per USP XXI [31]. The retinol was kept in alcoholic solution at −20°C for two months in vials sealed under nitrogen. Solution concentrations were checked spectrophotometrically by dilution in isopropanol prior to use. Absorbance was referred to the value of all-*trans* retinol, $A^{1\%}_{1cm}$ = 1835 at 326 nm.

The compounds d,l-α-tocopherol (Merck for Biochemistry — spectrophotometric check in ethanol solution referred to $A^{1\%}_{1cm}$ = 71 at 294 nm) and d,l-α-tocopherol acetate (Merck for Biochemistry — spectrophotometric check in ethanol solution referred to $A^{1\%}_{1cm}$ = 43 at 285 nm) were stored under nitrogen in closed containers in a refrigerator at −20°C.

Carotenoids: β-carotene (β,β-carotene) — Merck for Biochemistry — α-carotene (β,ε-carotene), lutein or xanthophyll (β,ε-carotene-3,3′-diol), zeaxanthin (β,β-carotene-3,3′-diol), *trans*-3R-cryptoxanthin (β,β-carotene-3-ol), lycopene (ψ,ψ-carotene), canthaxanthin (β,β-carotene-4,4′-dione) — all Hoffman-La Roche products were stored in a refrigerator at −80°C and used after prior preparation of mother solutions in benzene (concentrations between 0.1 and 1 mg ml^{-1}) which were stored separately under nitrogen in small test tubes in a refrigerator at −80°C. Concentrations were checked spectrophotometrically by diluting 100 μg aliquots with the assay solvent. The $A^{1\%}_{1cm}$ values used were as shown in Table 1.

Solutions were discarded after 60 days or whenever spectrophotometric checks revealed a drop in concentration. All operations were performed in reduced light conditions.

Table 1
Absorptivity values ($A_{1cm}^{1\%}$) used for compounds examined

Substance	Solvent	λ_{max} (nm)	$A_{1cm}^{1\%}$	Reference
β-carotene	hexane	451	2500	(34)
α-carotene	petroleum ether	445	2800	(32)
lutein	dioxane	453	2672	(33)
zeaxanthin	hexane	450	2340	(32)
trans-3R-cryptoxanthin	hexane	452	2460	(32)
lycopene	hexane	472	3450	(32)
canthaxanthin	cyclohexane	470	2250	(33)

Solvents

Benzene, pure or 96%-pure ethanol, petroleum ether and isopropanol were analytical grade and redistilled, if necessary. Ethyl ether and dioxane were purified to remove peroxides by passage through a Merck Activity 1 basic aluminium oxide column; *n*-hexane and methanol were HPLC grade.

Instrumentation

A Hewlett–Packard 1084 liquid chromatograph with HP VWD-79875A variable wavelength UV detector, complete with integrator–recorder and automatic injector were used. The injection volume was 50 μl.

The 4.6 × 250 mm columns used were packed with 5-μm Spherisorb ODS-2 (Phase Separations, UK or Bischoff, FRG) and fitted with a 2.1 × 30 mm guard column, packed with 30–40 μm Perisorb RP-18 (Merck). A methanol–*n*-hexane mixture (85:15, v/v) was used as eluent, prepared at the time of testing in a sufficient quantity for each specific test and run at a 1.0 ml min^{-1} flow rate and 25°C ambient temperature. It was considered that repeated degassing under vacuum could alter eluent composition.

Standards

To obtain calibration curves, aliquots of the mother solution pool (concentration 0.5–1.0 mg ml^{-1}) were drawn off and diluted with elution solvent to obtain the required concentration (ng per 50 μl), as indicated by each calibration curve range. Normally, four scalar dilutions were prepared, replicating each point four times.

Sample preparation and assay procedure

A quantity of serum (0.5 ml) was added to 0.5 ml of tocopherol acetate in ethanol solution (80 μg ml^{-1}). This constituted the internal standard. The solution was vortex-mixed for 10 s to facilitate deproteinisation. A volume 1.5 ml of 40°–70° b.p. petroleum ether (C. Erba) was added. Vortex-mixing or vigorous shaking was carried out for exactly 10 min, after which the solution was centrifuged at 6500 rpm for 15 min. After centrifuging, the clear petrol ether phase was transferred to a screw-capped 3-ml test tube using a thin Pasteur pipette. Exactly 1.2 ml of the extract, corresponding to 0.4 ml of serum, was drawn off, transferred to the injector vial and dried under nitrogen. The dry residue was immediately redissolved with the eluent in two phases as follows: (i) 0.1 ml of *n*-hexane was added, the solution was stirred for 10 s in an ultrasonic bath to solubilize the residue and then, (ii) 0.3 ml of methanol were added. Hence the sample solution was in the ratio of 1:1 with the serum. The quantity of sample solution enabled HPLC analysis to be replicated at least five times.

Each sample was subjected to five replications, according to the following two procedures. Procedure 1 (three replications) entailed first monitoring the effluent at 292 nm from start to 8.5 min (this wavelength enables free retinol, free tocopherol and tocopherol acetate internal standard to be assayed conveniently); then the detector was switched from 292 to 450 nm after 8.5 min in order to assay α- and β-carotene conveniently.

After that, in Procedure 2 (two replications), chromatography with detection at a wavelength of 450 nm was carried out, so that all carotenoids in the extract could be assayed selectively.

Column suitability test

The selectivity criterion chosen was based on the critical separation of α- and β-carotene peaks, which gave a resolution, $r = 0.97$ with the authors' column (plate number $N = 40970$/m calculated on the β-carotene peak). The test was run every 15 days of continued use.

Results and Discussion

Chromatographic procedure

Typical separations of vitamin constituents in a normal serum extract obtained with this method are shown in Figs 1 and 2. The chromatograms illustrate Procedure 1 (wavelength 292 and 450 nm in programmed succession) for the assay of retinol, α-tocopherol and α- and β-carotene, and Procedure 2 (450 nm) for the assay of total carotenoids.

Compounds were identified by comparing their retention times with those of the standards. Figures 3 and 4 obtained under the same conditions as above show the chromatograms of a complete mixture of standard compounds obtained under the specified operating conditions. Further identification was obtained by spiking a serum extract sample and checking the increase in peak height.

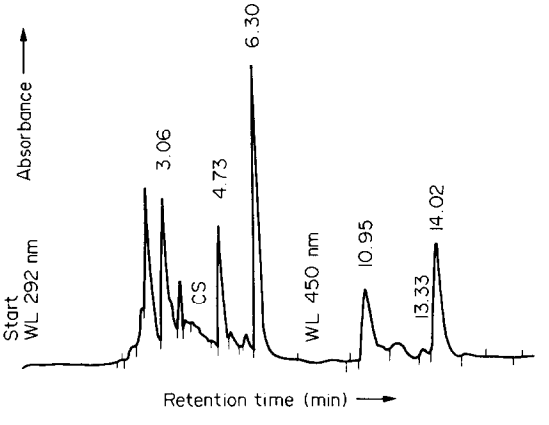

Figure 1
Chromatogram of a normal human serum extract, 50 μl (Procedure 1). For chromatographic conditions, see text. Chart speed: 1 cm min^{-1} for 3.8 min and then 0.5 cm min^{-1} to end of analysis; scale: 32×10^{-4} AU cm^{-1} (UV detector 292 nm for 8.5 min and then 450 nm). Key to peaks (retention times in min): all-*trans* retinol 3.06; d,l-α-tocopherol 4.73; d,l-α-tocopherol acetate (internal standard) 6.30; lycopene 10.95; α-carotene 13.33; β-carotene 14.02.

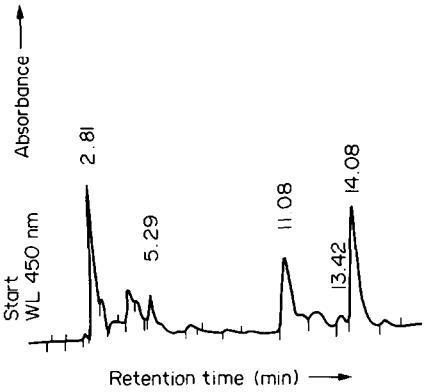

Figure 2
Chromatogram of a normal human serum extract, 50 μl (Procedure 2). For chromatographic conditions, see text. Chart speed: 0.5 cm min^{-1}, scale: 32 × 10^{-4} AU cm^{-1} (UV detector 450 nm). Key to peaks (retention times in min): zeaxanthin and/or lutein 2.81; *trans*-3R-cryptoxanthin 5.29; lycopene 11.08; α-carotene 13.42; β-carotene 14.08.

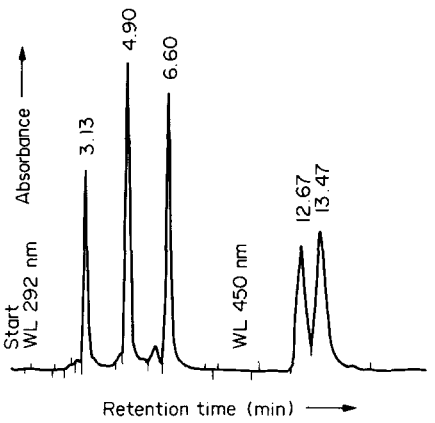

Figure 3
Chromatogram of a standard mixture, 50 μl (Procedure 1). For chromatographic conditions as in text. Chart speed: 0.5 cm min^{-1}; sensitivity: 32 × 10^{-4} AU cm^{-1} (UV detector 292 nm for 8.5 min and then 450 nm). Key to peaks (retention times in min): all-*trans* retinol 3.13; d,l-α-tocopherol 4.90; d,l-α-tocopherol acetate 6.60; α-carotene 12.67; β-carotene 13.47.

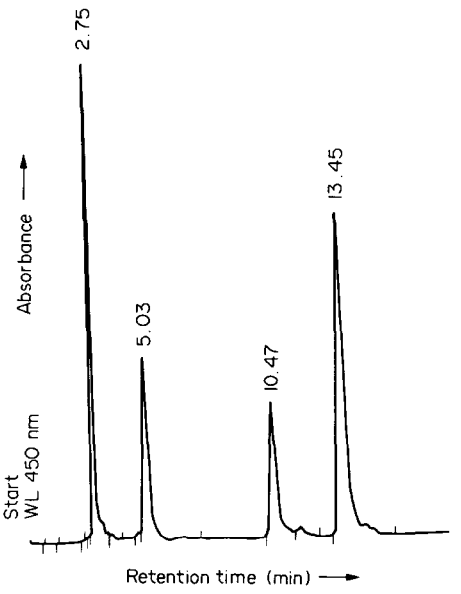

Figure 4
Chromatogram of a standard mixture, 50 μl (Procedure 2). For chromatographic conditions, see text. Chart speed: 0.5 cm min^{-1}; sensitivity: 32 × 10^{-4} AU cm^{-1} (UV detector, 450 nm). Key to peaks (retention times in min): zeaxanthin and/or lutein 2.75; *trans*-3R-cryptoxanthin 5.03; lycopene 10.47; β-carotene 13.45.

The chromatograms enable a clear identification of peaks corresponding to the compounds examined, even if some minor peaks due mostly to other oxygenated carotenoids appear as a complicating factor in the serum extracts.

Tocopherol acetate was used as the internal standard, because of the similarity of its liposolubility with that of the analytes; moreover, its retention time did not interfere with that of any of the components detected by Procedure 1.

Recovery

Recoveries were investigated according to two criteria: (a) addition of some analytes to a serum sample containing a relatively low analyte content; (b) verification on a second extract of serum samples already extracted.

As for criterion (a), serum was obtained from rats fed on a standard diet, relatively low in retinol and tocopherol (363.0 and 6733 ng ml^{-1}, respectively), or on a diet with no carotenoid content (laboratory diets). The following quantities of substances were added to each ml of serum: retinol, 694 ng; d,l-α-tocopherol, 10940 ng; β-carotene, 368 ng (approximately equal to the values per ml normally found in human serum samples using the present methods).

Recoveries (quantity found — basal quantity in serum/quantity added) were: 86.7% for retinol; 95.0% for α-tocopherol; and 88.9% for β-carotene. All were values obtained with excellent reproducibility for four test replicates.

These values were confirmed by criterion (b). After a first extraction according to the procedure described for the assay of analyte constituents, several samples of human serum were subjected to re-extraction and HPLC analysis. The chromatograms showed null, or nearly null, values for the analytes in the second set of extracts.

Calibration curves

The calibration curves for the constituents studied, determined by Procedure 1, and prepared as described in the experimental section, showed linearity to exist between the values of x (i.e. analyte quantities expressed in ng and y, the ratios, R, between integrator count for the analyte peak and that of tocopherol acetate). For assays performed according to Procedure 2 (carotenoid analysis), responses were given by

Table 2
Regressions equation data for compounds studied

Compound	Concentration	Linear regression analysis	
Retinol	(range 8–65 ng)	$y = [0.0144x + 0.0191]$	$r = 0.9955$
d,l-α-Tocopherol	(range 170–1350 ng)	$y = 0.0014x - 0.0360$	$r = 0.9969$
α-Carotene	(range 5–40 ng)	$y = 0.0309x + 0.0227$	$r = 0.9982$
β-Carotene	(range 6–50 ng)	$y = 0.0360x + 0.0238$	$r = 0.9983$
$y = R$ (ratio between sample response/internal standard response)			
x = analyte quantities expressed in ng			
Zeaxanthin	(range 6–50 ng)	$y = 1019.71x - 779.67$	$r = 0.9987$
Cryptoxanthin	(range 5–35 ng)	$y = 1003.34x - 379.42$	$r = 0.9985$
Lycopene	(range 5–40 ng)	$y = 852.99x - 927.95$	$r = 0.9986$
β-Carotene	(range 10–80 ng)	$y = 1121.90x - 861.65$	$r = 0.9996$

y = integrator counts.
x = analyte quantities expressed in ng.

analyte integrator counts. Table 2 gives the regression equation values calculated for the 7 compounds examined.

It is seen that an internal standard procedure was used for Procedure 1 that would safeguard against eventual accidental losses or variations in sample concentration. Procedure 2, on the other hand, requires that the procedure be performed with reference to an external standard. This was because it was neither easy nor convenient to insert a second internal standard that could be detected at 450 nm. However, because the problem under Procedure 2 was to assay replications on the same extract already examined under Procedure 1 for its β-carotene content, the area of the peak of this analyte obtained under Procedure 1 could be taken as a reference for possible corrections, whenever the peak area obtained by Procedure 2 differed by ±5% from that obtained under Procedure 1. In this way carotenoid assays were also safeguarded against errors due to loss or accidental variations in concentration.

Reproducibility

A reproducibility check was run on 3 samples of serum, replicating each one 5 times. Table 3 reports the analyte mean values, standard deviation (SD) and relative standard deviation (RSD).

SD and RSD values are not reported for α-carotene, because the values determined in the sample examined were very low (approx. 20 ng ml^{-1} of serum), i.e. just above the detection threshold for the volume injected. Hence, variability understandably appears to be very high.

As for the other six constituents, the RSD falls between 2.41 and 8.78% i.e. within acceptable limits for this type of determination [10, 11, 14, 16].

Table 3
SD and RSD values in 5 replicate analyses of 3 samples (A, B and C) of human serum

Compound		mean value	SD	RSD
Retinol	A	578.8	28.72	4.96%
(ng/ml)	B	644.5	44.90	6.97%
	C	559.7	30.53	5.46%
d,l-α-Tocopherol	A	9821.7	571.60	5.82%
(ng/ml)	B	12242.0	463.09	3.78%
	C	13718.6	1203.97	8.78%
Zeaxanthin and/or lutein	A	—	—	—
(ng/ml)	B	312.6	10.44	3.34%
	C	368.8	19.66	5.33%
Cryptoxanthin	A	—	—	—
(ng/ml)	B	100.1	7.26	7.25%
	C	103.8	8.65	8.33%
Lycopene	A	—	—	—
(ng/ml)	B	104.0	7.55	7.26%
	C	383.5	9.26	2.41%
β-Carotene	A	256.5	12.84	5.00%
	B	207.9	12.43	5.98%
	C	272.5	17.07	6.26%

Table 4
Vitamin constituents in 14 normal and 5 pathological serum samples assayed by HPLC*

Serum identification†	Retinol (ng ml^{-1})	d,l-α-Tocopherol (ng ml^{-1})	Zeaxanthine and/or lutein (ng ml^{-1})	Cryptoxanthine (ng ml^{-1})	Lycopene (ng ml^{-1})	α-Carotene (ng ml^{-1})	β-Carotene (ng ml^{-1})	Σ Unidentified carotenoids (ng ml^{-1})	Σ Total carotenoids (ng ml^{-1})	β-Carotene total carotenoids (%)
1 (F)	601.9	11553.5	125.4	331.3	207.7	29.6	155.6	118.2	967.8	16.1
2 (F)	483.4	9076.1	220.1	98.3	305.5	19.7	364.3	291.0	1273.8	28.6
3 (F)	537.2	12028.7	253.8	333.0	416.8	8.1	467.8	359.8	1839.3	25.4
4 (F)	352.1	15425.1	288.7	703.6	486.0	60.0	543.3	472.0	2425.1	22.4
5 (F)	458.1	12627.5	194.6	152.8	328.4	5.3	514.8	406.4	1602.3	32.1
6 (F)	545.8	12795.4	237.4	175.2	225.4	traces	75.8	205.2	919.0	8.2
7 (F)	873.1	14793.5	458.6	127.2	359.2	25.3	151.6	346.8	1468.7	10.3
8 (F)	644.5	12242.0	300.9	100.1	104.0	traces	207.9	151.2	864.1	24.1
9 (M)	1153.3	16425.6	365.9	145.6	486.9	12.8	104.6	364.2	1480.0	7.1
10 (M)	562.9	6956.6	236.2	112.6	291.4	36.7	229.8	219.2	1125.9	20.4
11 (M)	779.0	14650.6	360.4	195.4	809.8	27.7	472.7	466.7	2332.6	20.3
12 (F)	961.1	10734.1	205.6	51.0	652.8	8.2	238.3	305.4	1453.1	16.4
13 (F)	676.1	18647.9	326.5	179.7	475.6	31.3	578.7	787.5	2379.2	24.3
14 (F)	527.9	12736.9	396.8	98.5	386.2	42.0	278.9	250.2	1542.8	18.1
15 (M) (Diabetes)	467.1	15058.1	202.1	110.4	141.8	traces	318.7	252.3	1025.3	31.1
16 (M) (Diabetes)	1166.9	15723.3	229.1	94.6	341.6	2.6	155.6	296.4	1117.3	13.9
17 (F) (Hypothyroid)	586.4	23180.1	306.2	232.7	407.5	8.8	286.7	433.9	1667.1	17.2
18 (M) (Hypothyroid)	1122.9	24137.7	389.2	108.8	339.4	29.3	335.9	331.5	1534.1	21.9
19 (F) (Hypothyroid with myxoedema)	797.2	20981.8	689.4	1020.4	494.5	113.7	2495.3	443.1	5256.4	47.5
19 (F) (After 1 month's therapy)	785.3	17749.0	668.6	641.6	537.9	58.3	723.8	661.1	3291.5	22.0

*Pathology type indicated under serum identification number.
†M, male; F, female.

Clinical applications

Table 4 reports the content found for retinol, tocopherol and the carotenoids zeaxanthin + lutein, cryptoxanthin, lycopene.

α- and β-carotene in 14 normal and 5 pathologic and recuperating patients. It may be seen that the values for retinol and tocopherol agree with those found by other authors in this type of study [1, 9]. The present values were found to be at a higher level, in agreement with the high recoveries that this method affords. In the subjects examined, all on a normal, winter–spring diet for Italy, it was seen that β-carotene, which is the nutritionally most important carotenoid, thanks to its greater capability to convert biologically into retinol, does not always predominate. Comparably high levels were observed for lycopene and zeaxanthine plus lutein; which, owing to their close structural analogies (see table in experimental section), cannot be separated with this chromatographic system. The values for α-carotene were found to be very low in all subjects examined. Table 4 also includes the sum of unidentified carotenoid constituents, tentatively expressed as β-carotene in order to be referrable to that calibration curve. The values for total carotenoids and the fraction of β-carotene over total carotenoid content are also expressed.

Values found for the few pathological subjects, or those being treated (two cases of diabetes, three of hypothyroidism), show just how delicate the variations in the content of these vitamin constituents may be. Only deeper studies would enable such variations to be correlated with the pathological states found. As an example, Figs 5 and 6 give the chromatograms for subject No. 19, which show clearly an increase in the carotenoid components; the same subject after one month's thyroid extract therapy showed decreased values (cf. Table 4).

The total carotenoid values reported approximate to those found in normal subjects by the Neeld and Pearson spectrophotometric method [35] at 450 nm, as reported in the authors' previous paper [13].

Conclusions

The methods described enable better resolution of carotenoids and of α- and β-carotene, and also enable high and steady recoveries of β-carotene. This demonstrates the validity of the authors' hypothesis concerning the necessity to use an elution solvent

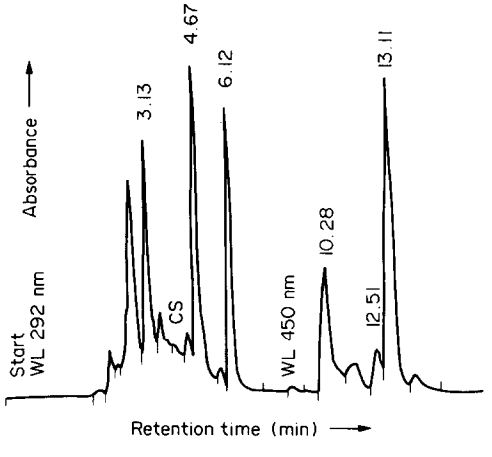

Figure 5
Chromatogram of a normal human serum extract (50 μl) from subject (no. 19) affected by myxoedema. For chromatographic conditions, see text. Chart speed: 1 cm min^{-1} for 3.8 min and then 0.5 cm min^{-1} to end of analysis; sensitivity: 32 × 10^{-4} AU cm^{-1} (UV detector 292 nm for 8.5 min and then 450 nm). Key to peaks (retention times in min): all-*trans* retinol 3.13; d,l-α-tocopherol 4.67; d,l-α-tocopherol acetate (internal standard) 6.12; lycopene 10.28; α-carotene 12.51; β-carotene 13.11.

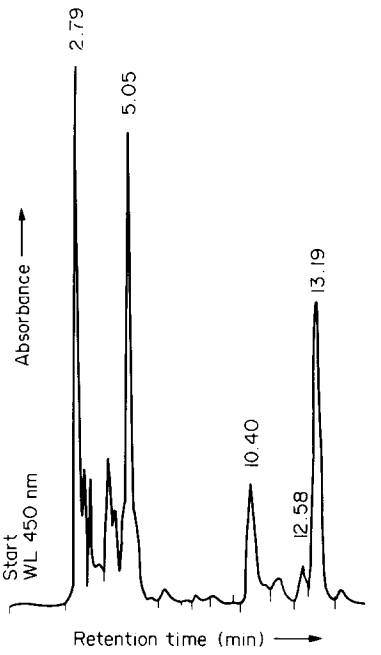

Figure 6
Chromatogram of human serum extract (50 μl) from subject (no. 19) affected by myxoedema. For chromatographic conditions, see text. Chart speed: 0.5 cm min^{-1}; sensitivity: 32 × 10^{-4} AU cm^{-1} (UV detector 450 nm). Key to peaks (retention times in min): zeaxanthine and/or lutein 2.79; *trans*-3R-cryptoxanthine + unidentified peak 5.05; lycopene 10.40; α-carotene 12.58; β-carotene 13.19.

that is capable of completely dissolving, and keeping dissolved, the petrol ether extract residue.

In the earlier method proposed by the authors [RP-8 column, acetonitrile eluent, 100%], the NARP technique provided good recoveries with β-carotene diluted standards, but these values diminished and varied whenever the serum sample analysis called for total redissolution of the petrol ether extraction residue.

Conversely, attempts to use solvents with high carotenoid dissolving powers (e.g. chlorinated solvents) or mixtures of the same to redissolve the injection residue, have given unsatisfactory results in that the chromatograms are influenced negatively. It is, therefore, absolutely necessary to inject the sample dissolved in its same elution solvent.

The authors have observed that serum samples may be stored frozen at −20°C for at least one month, provided thawing and refreezing are avoided by subdividing into sufficient aliquots for replicate analyses. Petrol ether extracts can be stored at 4°C for 8 h. A sample redissolved in the elution solvent must be analyzed within 4 h.

The applicability of the procedure is facilitated by the use of an isocratic elution solvent which thereby enables the automated analysis of samples to be achieved with ease. Absence of chlorinated solvents or acetonitrile in the eluent is a further guarantee of stability in the analysis of these easily oxidizable molecules.

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