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Simultaneous measurement of seven carotenoids, retinol and α-tocopherol in serum by high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatography (HPLC) procedure for the quantitative measurement in serum of seven carotenoids (lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, lycopenes, α-carotene and β-carotene), retinol, α-tocopherol and two internal standards (tocol and echinenone) has been developed. The geometric isomers, lutein and zeaxanthin, were completely separated as well as at least nine unidentified carotenoids. All compounds were resolved on an Adsorbosphere HS C_{18} (3 μm) column (250×4.6 mm I.D.) with a step gradient, 7.1 min after injection, from acetonitrile–methanol (60:40, v/v) containing 0.05% acetic acid to acetonitrile–methanol–dichloromethane (45.6:30.4:24, v/v) containing 0.04% acetic acid, in a total run time of 23 min. Chromatograms at four different wavelengths (292, 325, 450 and 473 nm) and spectra were monitored with a diode array detector. Because of its specificity and sensitivity for a large number of carotenoids, this procedure may be of interest for nutritional and epidemiological studies. Its speed and robustness make it suitable for analyses on large numbers of subjects.

Keywords: Carotenoids; Retinol; α-Tocopherol

1. Introduction

Since epidemiological studies have found negative relationships between the consumption of fruits and vegetables and several types of cancer [1–3], interest has been focused on the active compounds in these foods. Negative relationships were found between β-carotene in the diet or in plasma, and the risk of developing cancer [3–6]. It was therefore suggested that β-carotene may be a protective factor, although several studies indicated that its relation to cancer risk was weaker than that between cancer risk and vegetable and fruit consumption. Initially, research was focused on its activity as a precursor of vitamin A, but during the last ten years, interest has shifted towards its antioxidative properties [7,8] and its regulatory effects on cell communication, proliferation and differentiation [9–11]. The major antioxidative activity of β-carotene is the quenching of singlet oxygen [8,12,13]. Other carotenoids may protect in similar manner, but research was focused on β-carotene because of a lack of selective analytical methods for these compounds. With the introduction of new analytical methods, it was found that β-carotene is frequently not the most abundant carotenoid in serum [14–19] and that others, such as

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lutein and lycopene, have similar or even stronger antioxidant capacities [8,20]. α-Tocopherol, the principal and biologically most active tocopherol in the human body, reacts with peroxyl radicals to form a tocopheroxyl radical that interrupts the radical chain reaction [8,9,21]. Recently, it was found that α-tocopherol may act as an information transducer in the cell, interfering with cell proliferation [22]. Although a beneficial role in the prevention of atherosclerosis and cardiovascular disease has been established, dietary and serum vitamin E have been less consistently shown to be associated with cancer protection in epidemiological studies [23,24]. Even in large randomized trials with high doses of α-tocopherol and β-carotene supplements, based on the hypothesis that they were the main protective compounds from the diet, only a slight, or no, protective effect of α-tocopherol on cancer incidence was found. However, β-carotene was found to augment cancer incidence at certain sites, such as the lung [25,26].

Because of this inconsistency of the epidemiological results with the original hypotheses on protection by certain compounds, research now focuses on other compounds, the interactions between different compounds and the optimum, equilibrated concentrations of these compounds. This explains the need for sensitive, selective and rapid detection methods in serum and plasma.

High-performance liquid chromatography (HPLC) is now the most used analytical technique for the measurement of retinol, tocopherols and carotenoids. All of these compounds are lipophilic and can therefore be extracted together from plasma or serum. However, because of only slight differences in polarities, modern columns with high numbers of theoretical plates are necessary for their separation, and detection must be performed at separate wavelengths to optimize sensitivity. Several methods have been developed to measure different carotenoids in one run [14,17,27–31], or carotenoids together with α-tocopherol and retinol [18,19,32–44], in plasma, serum or tissue. Most carotenoids can be detected at 450 or 473 nm, but tocopherols and retinol can only be detected at 292 and 325 nm, respectively. Most of the above-mentioned methods therefore require the use of more than one detector [35,38,44], a multi-wavelength detector [18,33,37,41] or a change in the detection wavelength during the run [32,34,36,38,39,42]. Recently, methods for diode-array detectors (DADs) have been developed that monitor the chromatograms simultaneously at different wavelengths within a range from 280–500 nm [31,40,43]. DADs have an additional advantage in that the spectra of detected compounds can be stored and used in association with retention times for identification of compounds.

We combined the advantage of simultaneous detection by the DAD with the advantage of a step-gradient on selectivity and speed, in a new reversed-phase liquid chromatographic (HPLC) method. Retinol, α-tocopherol, two internal standards and sixteen carotenoids were separated. Among them, lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, lycopenes, α-carotene and β-carotene were identified and quantified under our analysis conditions. This method may be of use for qualitative and quantitative analysis and it opens up the possibility of including these biomarkers in epidemiological studies with large numbers of subjects, where low costs and speed of analyses are very important.

2. Experimental

Samples and standards were systematically protected from direct light. All manipulations and storage were performed in glass tubes, to avoid the influences of plasticizers.

2.1. Solvents and reagents

All of the solvents used were of HPLC grade quality (Merck, Darmstadt, Germany or Prolabo, Paris, France). Butylated hydroxy toluene (BHT) was obtained from Sigma (Sigma-Aldrich Chimie, l'Isle d'Abeau, France) and acetic acid was from Merck. Ultra-pure water (>18 MΩ) was produced in our laboratory (Elgastat UHQ II, Elga, Villeurbanne, France). Quantities lower than 1 ml were pipetted with an automatic dispenser.

2.2. Standards

Retinol, α-carotene and β-carotene were obtained from Sigma and lutein, zeaxanthin, canthaxanthin, α-tocopherol, β-cryptoxanthin and lycopene were generous gifts from Roche (Basle, Switzerland). Tocol (Lara Spiral, Couternon, France) was used as an internal standard for the products eluting with phase A (retinol, lutein, zeaxanthin, canthaxanthin) and α-tocopherol. Echinenone, a synthetic carotenoid (generously provided by Roche), was used as the internal standard for the carotenoids eluting with phase B (see Fig. 1).

Stock solutions of standards were made in batches every six months. Standards were weighed on an analytical balance (with 0.01 mg precision), dissolved in a mixture of hexane–dichloromethane (50:50, v/v) containing 0.025% BHT, then further diluted in a mixture of dichloromethane–methanol (50:50, v/v) and finally diluted in 100% methanol. Aliquots of 1 ml were stored at −80°C under nitrogen. Standards were considered pure when no other peak appeared on the chromatogram of the standard at twenty times the highest concentration used for the calibration standard. Only the β-carotene standard was found to contain 2–3% α-carotene, and was corrected for in weight, based on the relative surfaces of the two peaks. One stock solution was made for each internal standard; tocol and echinenone. Every day, 500 μl of each stock solution was diluted in methanol to a total volume of 10 ml (IS-2). For practical and stability reasons, calibration standards were prepared in two stock solutions: SEB1 containing retinol and β-carotene and SEB2 containing α-tocopherol and canthaxanthine. Combinations were chosen so as to avoid overlap of absorption maxima when verifying the concentrations in the calibration standards spectrophotometrically. The final calibration standard (SEB), prepared every day from thawed stock solutions, consisted of two volumes of IS-2 and one volume each of SEB1 and SEB2, resulting in final concentrations in 40 μl of pure SEB; 0.082 μg of retinol, 0.209 μg of α-tocopherol, 0.0153 μg of β-carotene, 0.00411 μg of canthaxanthine, 1 μg of echinenone and 1 μg of tocol. The qualitative standard (STD++), containing all of the compounds to be measured, was made in one single stock solution.

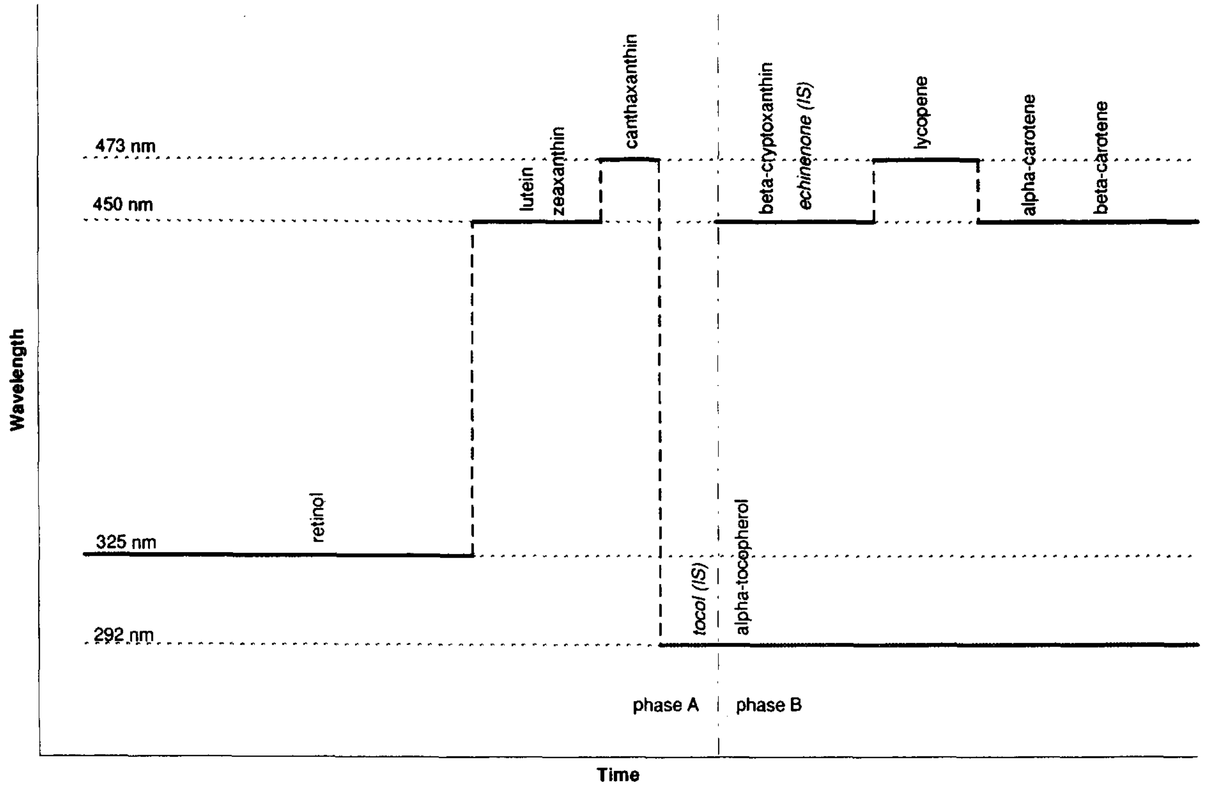

Fig. 1. Scheme of detection wavelengths, reconstituted in two channels with one internal standard.

2.3. Serum samples

Samples were extracted every day. Serum samples (200 μl) were first centrifuged, then 200 μl of internal standard (IS-2) and 200 μl of ethanol were mixed for 5 min in 5-ml glass tubes with plastic stoppers on an orbital shaker, avoiding contact between the samples and the plastic stoppers. Then, 200 μl of ultra pure water and 500 μl of hexane were added for a first extraction. After mixing for 10 min and centrifuging for 10 min at 2000 *g* at 17°C, 300 μl of the upper organic layer were transferred to 700 μl amber glass vials for HPLC (Interchim, Montlucon, France). Hexane (300 μl) was added to the serum for a second extraction and, after mixing for 10 min and centrifuging for 10 min at 4000 *g* at 17°C, 300 μl of the upper organic layer were added to the corresponding microtubes. The 600 μl of hexane were evaporated to moisture dryness in a rotary evaporator under vacuum in 15 min. The residue was dissolved in 300 μl of methanol–ethanol–hexane (88:10:2, v/v/v) and the was mixed for 10 min.

2.4. Chromatographic conditions

The HPLC system consisted of two isocratic pumps (model 420), an automatic sample injector (model 460) and a photodiode array detector (model 440), controlled by software for data acquisition and treatment (D450 version), all from Kontron (Kontron Instruments, Saint Quentin Yvelines, France). Data were collected in 3D mode and processed in 2D mode at four wavelengths (292, 325, 450 and 473 nm) for quantification. Two Adsorbosphere HS C_{18} 3 μm cartridges, one 100 mm×4.6 mm and one 150 mm×4.6 mm, connected with a "direct connect coupler" (all from Alltech France, Templeuve, France), were kept at 37°C in a water jacket placed over the column. Mobile phase mixture A consisted of 400 ml of methanol, 600 ml of acetonitrile and 500 μl of acetic acid. Mixture B consisted of 76% of phase A and 24% dichloromethane. Solvents were mixed and degassed by sonication for 5 min before use. During the run, they were kept in brown-glass bottles and filtered on-line through 0.45 μm filter discs (Whatman solvent IFD 0.2 μm, Maidstone, UK). The flow-rate was set at 0.9 ml/min. A step gradient to mobile phase B was introduced 7.1 min after injection. Under these conditions, tocol is the last peak to elute with mobile phase A. At 17.4 min, the columns are re-equilibrated with mobile phase A until the end of the run at 23 min. Injection volume on the column was 40 μl. Each run started with a blank of methanol to control the baseline. Calibration curves and internal standards were repeated at the end of each series for quality control. Detection wavelengths were as follows: 292 nm for tocol and α-tocopherol, 325 nm for retinol, 450 nm for lutein, zeaxanthin, β-cryptoxanthin, echinenone, α-carotene and β-carotene, and 473 nm for canthaxanthin and the lycopenes. Resolution was set at 4 nm. Peak identification was effected by comparing retention times and typical spectra (see Fig. 3) of standards. For quantification, the software was programmed with two channels reconstituted from the four acquisition wavelengths, using only one internal standard per reconstituted channel (Fig. 1).

2.5. Quality control

Within-day reproducibility of the method was determined regularly by analyzing a serum pool several times on the same day. For long term reproducibility control, the laboratory takes part in inter-laboratory controls from the External Quality Assurance Scheme for carotenes and vitamins A and E [45]. Routinely, every fifth sample was extracted and analyzed a second time; in case of doubt, the whole series was reanalyzed.

2.6. Quantification

Quantification was based on peak area, with a grouping of peak areas for the different isomers of lycopene and a vertical split of peaks when all-*trans*-β-carotene could not be separated completely from its *cis*-isomers. Primary calibration curves were measured for each wavelength, with α-tocopherol for 292 nm, retinol for 325 nm, β-carotene for 450 nm and canthaxanthin for 473 nm. Secondary calibration curves for other carotenoids were deduced from the extinction coefficients, as measured in the solvent mixture in which the compounds elute. Calibration curves are supposed to follow the equation for straight lines: $y=ax+b$ with $a=d\times\epsilon$, in which y

Table 1
Extinction coefficients ($E^{1\%}_{1CM}$), as measured at the detection wavelength, in the solvent mixture of elution and corresponding correction factors for secondary calibration curves

Compound	λ_{max}	λ_{det}	Solvent	$E^{1\%}_{1CM}$	Correction[a]	Curve[b]	I.S.[c]
Lutein	447	450	A	1463	1.74	B	T
Zeaxanthin	454	450	A	1677	1.52	B	T
Canthaxanthin	473	473	A	2260	1	C	T
β-Cryptoxanthin	453	450	B	2276	1.12	B	E
Lycopene	474	473	B	1621	1.39	C	E
α-Carotene	449	450	B	1524	1.67	B	E
β-Carotene	456	450	B	2544	1	B	E
Retinol	328	325	A	1780		R	T
α-Tocopherol	286	292	B	75.8		A	T
Tocol (I.S.)	285	292	A	87.99			
Echinenone (I.S.)	470	450	B	2316			

λ_{max}: wavelength (nm) of maximum absorption; λ_{det}: wavelength (nm) of detection.
[a] Calculated as the ratio of extinction coefficients.
[b] Primary calibration curve on which calculations are based. B=β-carotene; C=canthaxanthin; R=retinol; A=α-tocopherol.
[c] Internal standard. T=tocol; E=echinenone.

represents the quantity, in mmol/l, x is the measured absorption, a is the multiplying factor consisting of d, the dilution factor and ϵ, the extinction coefficient, and b represents the intercept (e.g., the absorption measured at zero concentration). Calibration curves for carotenoids, measured at the same wavelength, were deduced by replacing the extinction coefficients in the equation. Similar results are obtained by applying a correction factor to the measured calibration curve, calculated as the ratio of the extinction coefficients (primary curve carotenoid/secondary curve carotenoid). Extinction coefficients were measured at the wavelength of detection (λ_{det}, 450 or 473 nm), which might be different from the absorption maximum (λ_{max}) of the compound (Table 1).

3. Results and discussion

3.1. Method development

The method described in this paper separates and quantifies in one run, retinol, α-tocopherol and seven carotenoids plus two internal standards. In total, at least sixteen peaks are separated, absorbing at 450 and 473 nm and with the spectral characteristics of carotenoids. In Fig. 2, typical chromatograms are shown at the different wavelengths of detection and, in Fig. 3, typical spectra of different carotenoids are shown. Unfortunately, all peaks could not be identified definitively because of a lack of pure standards. However, even though we could not identify these peaks, separating them means that they do not interfere with identified peaks, which improves the specificity. All components are detected within 17 min and, with a re-equilibration period for the column in mobile phase A, one run takes about 23 min. The total run time is comparable to that of other methods in the literature, but, with this method, the number of components properly separated in this time is higher. Only 200 μl of serum is necessary, which is important for epidemiological and routine studies, where many analyses often have to be done on one small blood sample.

Although developed on the above-mentioned machines, the method can also be used on other, simpler configurations. The minimum requirements are: An isocratic pump with a remote switching valve, an automatic injector (preferably cooled) and a detector with at least two, but preferably four, simultaneous wavelengths in the 250–500 nm range. The need for simultaneous detection at different wavelengths is illustrated by α-tocopherol and β-cryptoxanthin that elute within a 5-s interval, but must be detected at 292 and 450 nm, respectively. Simultaneous light emission from deuterium and tungsten lamps, combining maximum energy at ultraviolet and visible wavelengths, can improve sensitivity in a manner

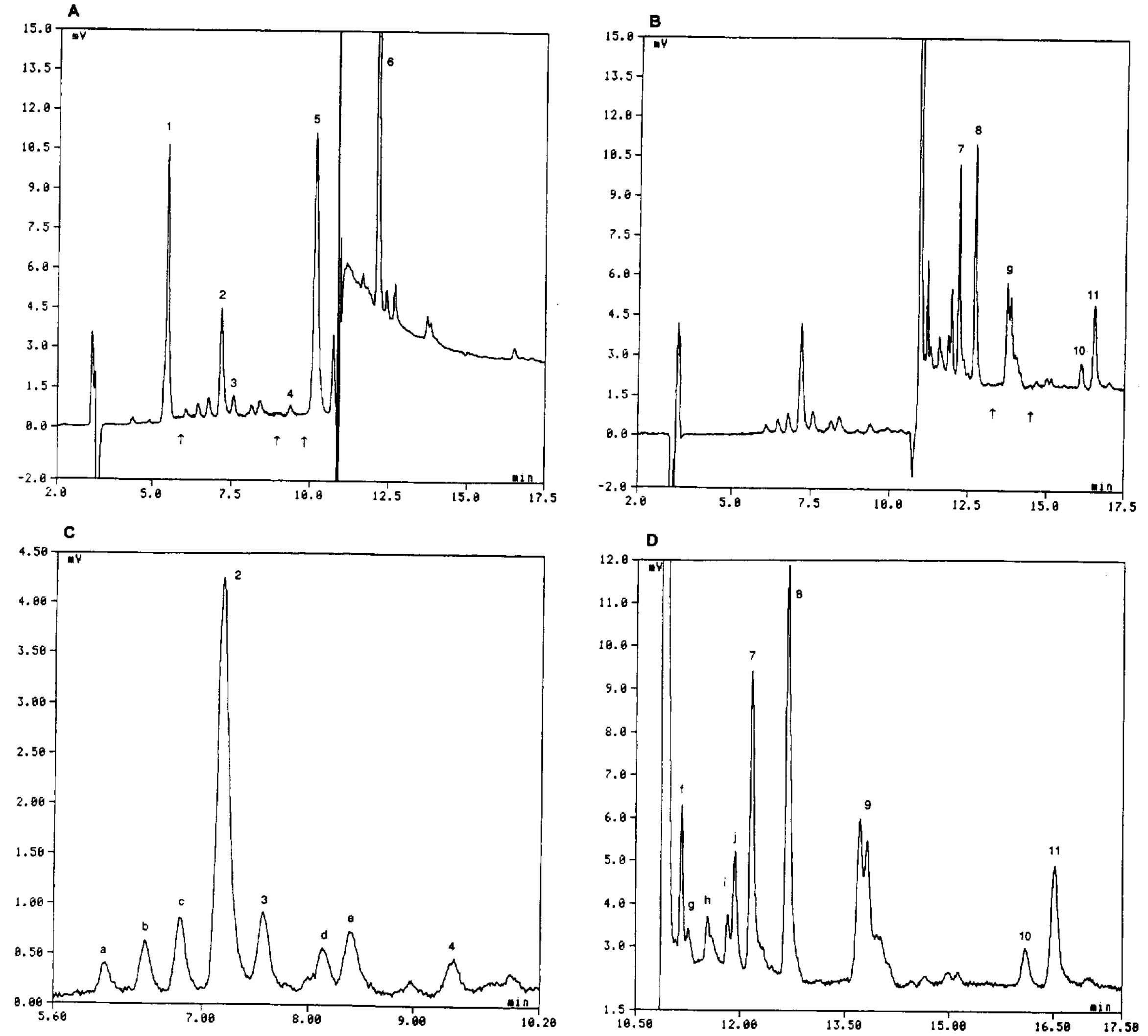

Fig. 2. Typical chromatograms in human serum. (A) Reconstitution of the four detection wavelengths on mobile phase A into one channel; (B) Reconstitution of the two detection wavelengths on mobile phase B into one channel; (C) Separation of lutein, zeaxanthin, canthaxanthin and unidentified carotenoids in mobile phase A, monitored at 450 nm; (D) Separation of compounds detected in mobile phase B at 473 nm. The identified compounds are the following: 1=retinol; 2=lutein; 3=zeaxanthin; 4=canthaxanthin; 5=tocol (I.S.); 6=α-tocopherol; 7=β-cryptoxanthin; 8=echinenone (IS); 9=different isomers of lycopenes; 10=α-carotene; 11=β-carotene. a–j are unidentified carotenoid peaks, absorbing at 450 and 473 nm with typical spectra. Arrows indicate changes in detection wavelength in reconstituted channels.

similar to that obtained using two detectors in line. We tested the method on two other machines with different configurations and every time, it was operable within one day, with comparable results.

At the first extraction, 200 μl of ultra-pure water was added to the deproteinized plasma. Without this extra water, the recoveries were low. Apparently, when the percentage of ethanol is too high, the separation of the serum–ethanol mixture from the hexane layer seems to be incomplete. Recently, our

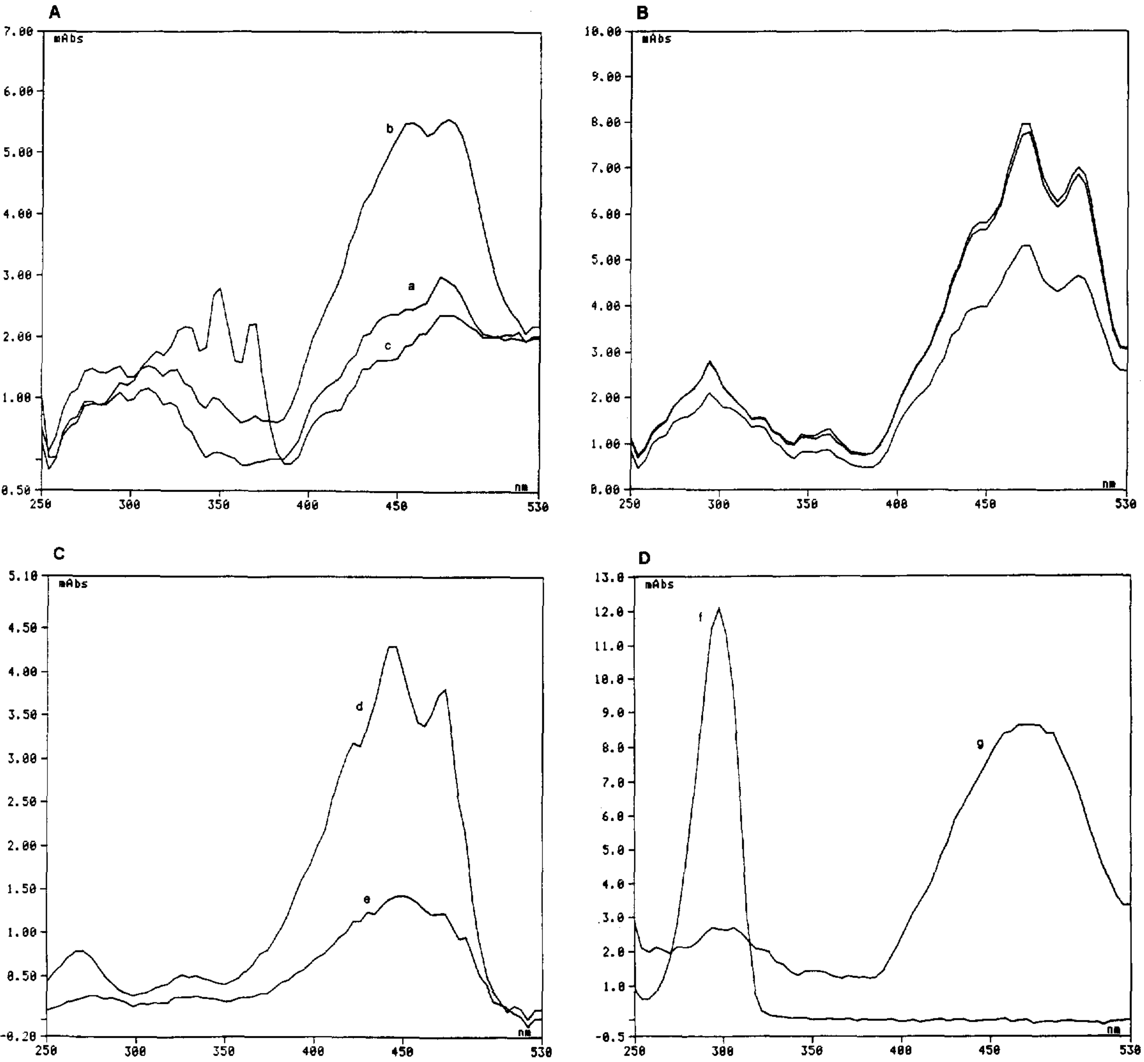

Fig. 3. Typical spectra of carotenoids detected in human serum. (A) α-carotene (a), β-carotene (b) and a *cis*-isomer of β-carotene (c) in mobile phase B; (B) Three isomers of lycopene in mobile phase B; (C) Lutein (d) and zeaxanthin (e) in mobile phase A; (D) Tocol (f) in mobile phase A and echinenone (g) in mobile phase B.

observation was confirmed by Jezequel-Cuer et al. [47]. Evaporated residues were found to be stable for 24 h at 4°C in the dark (results not published) and, once dissolved, samples could be preserved better at temperatures above 15°C, to avoid precipitation of the carotenoids. The stability of the stock solutions of calibration standards was verified spectrophotometrically and by HPLC, and quantities were found to decrease by less than 3% when stored at −80°C for six months. The relationship between quantity and absorption was linear in the range of the calibration standards (retinol up to 82 ng per peak; canthaxanthin up to 4.11 ng; α-tocopherol up to 209 ng and β-carotene up to 15.3 ng). In serum, linearity has been verified for concentrations that were twice as high. However, when stock solutions for calibration standards were made with such a high concentration (3 μg/ml), β-carotene precipitated under our storage conditions (−80°C) and the precipitate was not completely dissolved when thawed, leading to false calibration curves.

3.2. *Internal standards*

Double extraction resulted in recoveries of >92%, while a simple extraction recovered a maximum of 85% of lycopene and the carotenes, results corresponding to those of Cantilena and Nierenberg [17]. We selected the internal standards to have recoveries that were representative for the compounds. We found very low recoveries from serum spiked with more than 0.5% (v/v) hexane with carotenoids dissolved in it. Hexane in high concentrations seems to precipitate proteins with carotenoids bound to them, leading to low recoveries. Neither internal standard is naturally found in serum, unlike tocopherol acetate, which is largely used as an internal standard and is often used in medical care.

3.3. *Solvent mixtures*

The exact composition of the solvent mixture used to dissolve the residue for injection on the column and of mobile phases A and B was optimized in a previous series of experiments in order to combine satisfactory peak separation with low run time and optimum flow-rate. After evaporation, residues are dissolved in a methanol–ethanol–hexane mixture (88:10:2, v/v/v). The hexane (2%) makes the carotenoids more soluble. A higher percentage of ethanol decreased the separation of lutein and zeaxanthin, two geometric isomers. Mobile phase A is a mixture of acetonitrile and methanol, which has several advantages over methanol alone. A mixture of acetonitrile and methanol, which have different eluting forces but the same order of hydrophobicity, gives better resolution and has a lower viscosity, which makes it possible to work at 0.9 ml/min on a 25-cm column with a back pressure of only 105 bar. The presence of 0.05% acetic acid is to ensure that the medium remains acidic, to keep solutes in their non-ionized forms, leading to sharper peaks. The retention times of the carotenes are shortened by promptly increasing the percentage of the strong eluting dichloromethane up to 24% in mobile phase B. For the exact percentage of dichloromethane, a compromise had to be found between shortened retention times and resolution of all-*trans*-β-carotene from its *cis*-isomers. The extra time needed after each run for re-equilibration of the column with mobile phase A (5 min) is largely counterbalanced by the shorter retention times of the carotenes. The advantage of a step gradient over a linear gradient is that the composition of the solvent mixture in which the compound elutes is known exactly and its extinction coefficient can be measured spectrophotometrically in this solvent mixture.

3.4. *Extinction coefficients*

Table 1 shows the extinction coefficients for each compound, as measured spectrophotometrically at the detection wavelength in the solvent mixture for elution. Absorption maxima and calculated correction factors are given as well.

3.5. *Column*

To separate all solutes, both identified and unidentified, in serum samples from healthy subjects, which are very rich in lipophilic compounds, a 25-cm, 3 μm column was necessary. Shorter columns (15 and 20 cm) were tested to speed up the run, but did not give satisfactory resolution. Columns with a particle size of 3 μm have higher numbers of theoretical plates than columns with a particle size of 5 μm, but they are not very popular because of high back pressure. However, by using a solvent mixture without water, the viscosity is lower and the back-pressure is at a reasonable level. Most of the methods described in the literature do not separate the geometric isomers lutein and zeaxanthin, apart from those published by Epler et al. [38] and Krinsky et al [29]. The high selectivity of our method can be explained by the choice of column, a C_{18}, 3 μm with 20% carbon load. Krinsky et al. [29] used the same type of column, but it was only 15 cm long. Another explanation is the adaptation of the two mobile phases to the lipophilicity of two classes of compounds; a mixture of methanol and acetonitrile with 0.05% acetic acid for elution of the xanthophylls, followed by the introduction of a stronger eluent (25% dichloromethane) for elution of the more polar carotenes. In isocratic methods, where this adaptation is not possible and where strong elution conditions are used for β-carotene, the xanthophylls show little retention on C_{18} packing materials and, therefore, co-elute with each other and with other compounds [14,27,35,39,41,42,44].

3.6. Analysis of serum samples

Sensitivity, defined as the lowest measurable quantity of a compound in a volume of 40 μl, and calculated as the quantity that generates a peak with a height equivalent to a three-fold baseline noise range, was 0.8 ng for lutein, 0.7 ng for zeaxanthin, 0.1 ng for canthaxanthin, 0.5 ng for β-cryptoxanthin, 0.2 ng for lycopene, 0.7 ng for α-carotene, 0.4 ng for β-carotene, 1 ng for retinol and 2 ng for α-tocopherol. The intra-run C.V. in pooled serum ($n=8$) was 3.1% for retinol (mean 2.27 μmol/l), 2.9% for α-tocopherol (mean 47.9 μmol/l) and 3.6% for β-carotene (mean 0.82 μmol/l). Over a period of three months, no significant differences were found between the pairs ($n=25$) of double-analyzed samples for retinol, α-tocopherol and β-carotene (paired t-test, $P>0.01$). The regression ($n=84$) of values of retinol and α-tocopherol analyzed using our method and that of Catignani and Bieri [46] gave the equations $y=1.99\cdot10^{-2}+0.99x$ and $y=0.41+0.95x$ and correlations of 0.98 and 0.97, respectively.

A second isomer of vitamin E, γ-tocopherol, is not quantified here since it was generally very low in our population. However, after checking the molar extinction coefficient spectrophotometrically, this compound can be quantified since it is completely separated from other isomers (Fig. 4).

The method has already been used for more than 2000 samples from hospitalized and healthy subjects. Table 2 shows the results for the analyses of serum of 96 healthy French women aged 30–50 years. In comparison to other published values of studies with $n>25$ [19,37,48], the value ranges are comparable but the mean values are generally higher. This may be explained by differences between the analytical methods and by differences in the populations. Our population consisted only of women, who have higher carotenoid concentrations than men, especially in the age range 30–40 [48,49], and may have a diet richer in carotenoids, including supplements, than other populations. Serum concentrations correlated very well with dietary intake estimates (data will be published elsewhere). With lutein and lycopene found in comparable concentrations, our data confirm that β-carotene is not the only important carotenoid. Since it is now possible to measure other carotenoids as precisely, research should no longer focus only on β-carotene, as these carotenoids show pronounced differences in patterns between subjects, tissues [30] and foods [50], have different antioxidant capacities [20] and may differ in other functions at the cellular level.

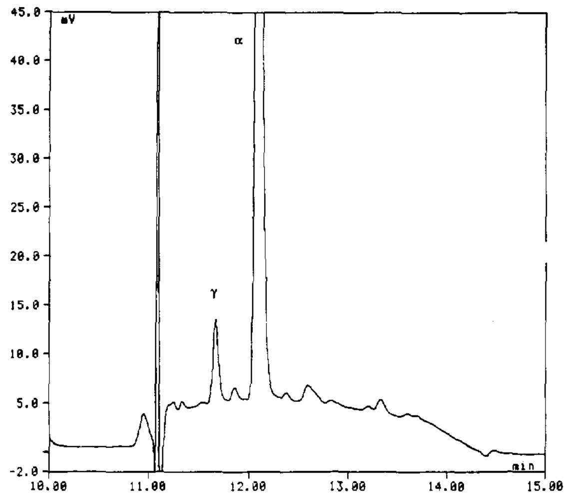

Fig. 4. Separation of γ-tocopherol and α-tocopherol, detected at 292 nm.

Acknowledgments

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Table 2

Concentrations of carotenoids, retinol and α-tocopherol (in μmol/l) in the serum of 96 healthy French women, age range 35–50 years, compared to other studies

Compound	This study		Olmedilla et al., 1994[a]	Krinsky et al., 1990[b]	Sowell et al., 1994[c]
	Mean±SD	5–95% range	Mean±SD	Mean±SD	Mean and 5–95% range
Lutein	0.71±0.30	0.28–1.16	0.24±0.21	0.33±0.12	0.36 (0.14–0.74)[d]
Zeaxanthin	0.09±0.05	0.01–0.19	0.07±0.04	0.08±0.03	[d]
Canthaxanthin	0.03±0.04	0.00–0.12			
β-Cryptoxanthin	0.35±0.27	0.06–0.92	0.60±0.47	0.21±0.10	0.22 (0.05–0.52)
Lycopene	0.56±0.43	0.14–1.64	0.42±0.24	0.38±0.14	0.40 (0.11–0.80)
α-Carotene	0.36±0.26	0.10–0.91	0.07±0.05	0.18±0.10	0.08 (0.02–0.22)
β-Carotene	0.81±0.45	0.30–1.79	0.37±0.23	0.63±0.38	0.34 (0.07–0.88)
Retinol	1.84±0.40	1.29–2.51	1.71±0.39		1.91 (1.05–2.97)
α-Tocopherol	33.0±6.67	21.73–46.44	32.65±7.4		25.7 (13.9–47.0)

[a] Concentrations in serum, $n=54$ women, age range 25–59 years [48].

[b] Concentrations in plasma, $n=5$, four men and one woman, age range 25–61 years, with an average of three individual samples taken within a 60-week period for each subject [29].

[c] Concentrations in serum, $n=3480$, men and women, age range 4–93 years [19].

[d] Sum of lutein and zeaxanthin, peaks not separated.

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