Serum carotenoids and retinoids in ferrets fed canthaxanthin

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*A high-performance liquid chromatographic method has been developed to analyze canthaxanthin (CX) with other carotenoids and retinoids in serum. Serum (100 μL) was extracted first with chloroform/methanol and then with hexane, using retinyl acetate and γ-carotene as the internal standards. The residue of the extract was resuspended into ethanol and injected onto an 8.3 × 0.46 cm 3-μm ODS column. The gradient system consisted of two solvents of acetonitrile/tetrahydrofuran/water with different ratios. The carotenoids and retinoids were measured at 450 nm and 340 nm, respectively. CX was detected in the serum of the ferrets fed CX (79% all-*trans *and 21%* cis *isomer, 50 mg/kg bodyweight by gavage for 1 month), but was not detected in the control group. The existence of CX in the serum was confirmed by its UV/visible absorption spectrum and by its negative chemical ionization/mass spectrum with an M^- ion at m/z 564. The serum level of all-*trans *CX in the experimental ferrets was 33.34 ± 3.33 nmol/L (n = 13) and that of* cis *CX was 72.48 ± 7.64 nmol/L (n = 13). There was no difference between the CX-fed group and the control group in the serum levels of retinol, retinyl esters, lutein, cryptoxanthin, or β-carotene. Retinyl esters in ferret serum represent 93% of total vitamin A level, which is more than 10 times higher than the vitamin A level in human blood. High retinyl esters level in the circulation of the ferret is a general phenomenon in carnivores.*

Keywords: carotenoids; canthaxanthin; retinoids; ferret; human; HPLC method

Introduction

Canthaxanthin (CX, 4,4′-diketo-β-carotene), a carotenoid without provitamin A activity, is used as a food colorant,[1] a treatment for photosensitivity disorders,[2,3] and an anticarcinogenic compound.[4–8] Increasing consumption of CX as an oral tanning agent and the observation of CX related retinal crystal deposition[9,10] have prompted careful examination of the biological effects of this compound.

High performance liquid chromatography (HPLC) has been widely used for the analysis of carotenoids in biological fluids since the 1970's.[11–13] The HPLC methods published recently separate carotenes (α-, β-carotene, lycopene) from retinol in human blood.[14–17] However, carotenoids with shorter retention times, mostly xanthophylls such as lutein, zeaxanthin, and cryptoxanthin, and retinoids with longer retention times, mostly retinyl esters such as retinyl oleate, retinyl palmitate, and retinyl stearate, are not separated adequately in those reverse phase isocratic HPLC systems.[14–16] Moreover, the gradient HPLC method developed for xanthophyll analysis[17] is not adaptable for CX analysis. Presently, there are no published methods that specifically address the analysis of CX in biological fluids.

The ferret has many anatomical and physiological features that are similar to humans.[18] Moreover, the ferret has been found to absorb intact β-carotene from the diet and has the capacity to store absorbed dietary

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β-carotene in tissues.[19] Therefore, the domesticated ferret appears to be a suitable laboratory animal model for the study of CX absorption and metabolism.

To analyze blood samples from ferrets that were fed CX with their diet, we developed a reverse-phase gradient HPLC method to separate CX from other carotenoids using human pooled blood. This method was then applied in the determination of serum carotenoid and retinoid levels in ferrets fed diets with or without CX.

Materials and Methods

Chemicals

All HPLC grade solvents were purchased from J.T. Baker Chemical Co. Philipsburg, NJ USA. HPLC solvents were routinely filtered through a 0.45 μm filter before use. Retinyl acetate, retinol, β-carotene (type IV), retinyl palmitate, fatty acyl chlorides (of arachidonate, stearate, oleate, myristate, linoleate, and laurate) were purchased from Sigma Chemical Co., St. Louis, MO USA. Retinyl esters were synthesized by reacting retinol with specific fatty acyl chlorides (vide supra).[20] γ-carotene, zeaxanthin, cryptoxanthin, and CX were gifts from Hoffmann-La Roche, Inc., Nutley, NJ USA. β-carotene was purified before use as a standard.[21] Other standards with impurity of less than 2% were used without further purification. Absolute alcohol was purchased from AAper Alcohol and Chemical Co., Shelbyville, KY USA.

Instrumentation

The HPLC system consisted of two Water 510 pumps, a model 715 Ultra-WISP autosampler, a 490E programmable multiwavelength detector, an 860 networking computer system (Waters Chromatography, Division of Millipore Corp., Milford, MA USA), and an LA75 Companion printer (Digital, Boston, MA USA). The detector was set at 340 nm for retinoids and 450 nm for carotenoids. The Waters 994 programmable photodiode array detector with a model 5200 terminal printer/plotter was used to measure the absorption spectra of compounds appearing in the HPLC chromatograms.

The gas chromatograph-mass spectrometer (GC-MS) consisted of a Model 5988A mass spectrometer and a Model 5890 series II GC that was equipped with a cool on-column injection port (Hewlett Packard, Andover, MA USA). The capillary column used for the analysis was a 1 m long, 0.22 mm i.d. aluminum clad fused silica column coated with HT-5, a special high temperature stationary phase (Scientific Glass Engineering, Inc., Austin, TX USA). The injection port temperature was initially set to 53° C and the column oven to 50° C. Both were programmed at 20° C/min to 350° C. Helium was used as the GC carrier gas. Methane was employed as the moderator gas for electron capture-negative ion chemical ionization mass spectrometry. The ion source temperature was 150° C and the ion source pressure was 0.5 torr of methane.

Other equipment included an ultrasonic bath (Branson, Shelton, CT USA), a bench top refrigerated centrifuge (Sorvall RT6000, E.I. DuPont, Wilmington, DE USA), and a nitrogen evaporator (N-Evaps; Organomation Associated Inc. Berlin, MA USA).

CX Feeding Study

Twenty-six ferrets (*Mustela putorius furo,* Marshall Farm, North Rose, NY USA) were used in this study. Thirteen ferrets were assigned to the treatment group, and in addition to a standard diet (vide infra), received 50 mg/Kg body weight per day of CX by gavage for 1 month. The CX (provided by Hoffmann-La Roche) was impregnated in water-dispersible beadlets (11.6% CX), and was determined to be 79% all-*trans* and 21% *cis*-isomer. The CX dosage was made by dispersing the CX beadlets into warm (40° C) water (1:10 wt/vol). The ferret diet consisted of Purina Ferret Chow #5280 (Ralston Purina, St. Louis, MO USA) that contained 1.9 μg of lutein, 0.5 μg of cryptoxanthin, trace amounts of β-carotene and 7.7 IU of vitamin A in 1 gram of diet. The diet was extracted following the procedure published in Chapter 43.020 in the Official Method of Analysis by the Association of Official Analytical Chemists, Inc., (1984), and analyzed by this HPLC method. The total fat content of the diet was 12% of weight. Control animals ($n = 13$) were fed the same diet and water dispersed beadlets but did not receive CX. All animals were fed the ferret chow diet ad libitum. Food consumption and body weights were measured weekly. The batch stability of the CX beatlets over time was checked by HPLC.

Chromatographic separation

The HPLC mobile phase was $CH_3CN/THF/H_2O$ (solvent A, 50:20:30, or solvent B, 50:44:6, vol/vol/vol, with 1% ammonium acetate in H_2O). The gradient procedure at a flow rate of 1 mL/min was as follows: 60% solvent A and 40% solvent B were used for 1 min followed by a 9-min linear gradient to 83% solvent B, a 4-min hold at 83% solvent B, then a 2-min linear gradient to 100% solvent B, a 2-min hold at 100% solvent B, and finally a 2-min gradient back to 60% solvent A and 40% solvent B. The column was a Pecosphere-3 C18 0.46 × 8.3 cm cartridge column with a 0.46 × 3.3 cm cartridge column as a guard column (Perkin-Elmer Inc., Norwalk, CT USA). An SSI 0.5-μm high pressure column filter (Rainin, Woburn, MA USA) was installed before the guard column.

Sample preparation

Pooled fasting blood samples from human volunteers (45 donors, age range 20–63 years) were used for method development. The blood sampling conformed to the guidelines of the Human Investigation Review Committee of Tufts University and the New England Medical Center. Serum was stored at −70° C in 0.5 mL aliquots until analyzed. Five samples were analyzed on each of 4 separate days from freshly thawed pooled serum aliquots. In addition, serum was obtained from the two groups of ferrets via jugular venipuncture before the daily gavage of CX and stored at −70° C until analyzed. All animal procedures were reviewed and approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

One hundred μL of serum were pipetted into a 13 × 100 mm test tube. Subsequently, 2 mL of $CHCl_3/CH_3OH$ (2:1 vol/vol) and 0.5 mL of saline (0.85%) were added and the tube's contents were vortexed for 10 seconds. The internal standard solution (60 ng of retinyl acetate and 50 ng of γ-carotene in 100 μL ethanol) was then added. The mixtures were vortexed intermittently for 20 sec and centrifuged (10 min, 800*g* at 4° C). The chloroform layer was transferred by a Pasteur pipet to a 10 × 75 mm test tube. The sample was extracted a second time using 3 mL of hexane. The hexane layer in the second extraction was removed and combined with the first extract. The extract was evaporated on a nitrogen evaporator with the water bath temperature at 35° C. The residue was redissolved in 100 μL of ethanol, vortexed

for 5 sec, and sonicated for 30 sec; and a 50 μL aliquot was injected onto the HPLC column. The extracts were kept at room temperature and were injected by autosampler within 12 hours. All sample processing was done under red light.

Pooled serum samples (6 mL) from the ferrets fed CX were extracted (vide supra) and the putative CX at peaks 5 and 6 from HPLC eluant was collected and evaporated to dryness. The residue was then resuspended in 50 μL of $CHCl_3$. A 1 μL aliquot was injected into GC-NCl/MS.

Standard curves

Retinyl acetate and γ-carotene were used as internal standards to monitor the extraction efficiency of retinoids and carotenoids, respectively. The standard curves were generated by injecting retinoids (retinol, retinyl palmitate) or carotenoids (lutein, canthaxanthin, lycopene, cryptoxanthin, α-carotene, β-carotene) in ethanol using a constant volume with increasing concentrations, and were plotted as peak area versus amount injected. Retinoid and carotenoid standards gave a linear calibration curve up to 70 μmol/L for retinyl palmitate and up to 27 μmol/L for CX, which is over the range of concentrations encountered in serum of ferrets and humans. The data were corrected individually by determining the recovery of the internal standard. The extinction coefficient at 470 nm of all-*trans* CX and *cis* CX used was 2200 and 1738 ($E^{1\%}_{1cm}$), respectively.[22]

Statistical methods

The Statview II program (Abacus Concepts, Inc., Berkeley, CA USA) was used for statistical analysis of the data. Differences were considered statistically significant at $P < 0.05$.

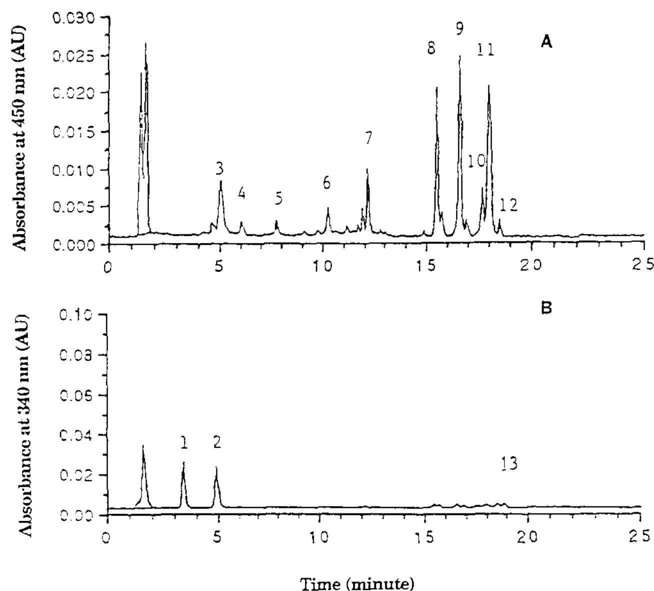

Figure 1 Representative HPLC chromatogram on a serum extract from human pooled blood. The detector was set at 450 nm (graph A) and 340 nm (graph B). Peaks are as follows: (1) retinol, (2) retinyl acetate (internal standard), (3) lutein (zeaxanthin), (4) unknown, (5) unknown, (6) unknown, (7) cryptoxanthin, (8) lycopene, (9) γ-carotene (internal standard), (10) α-carotene, (11) β-carotene, (12) *cis*-β-carotene, and (13) retinyl palmitate. Column: C18 0.46 × 8.3 cm cartridge column. Solvents: $CH_3CN/THF/H_2O$ (50:20:30, vol/vol/vol solvent A; 50:44:6, vol/vol/vol solvent B; 1% ammonium acetate in H_2O).

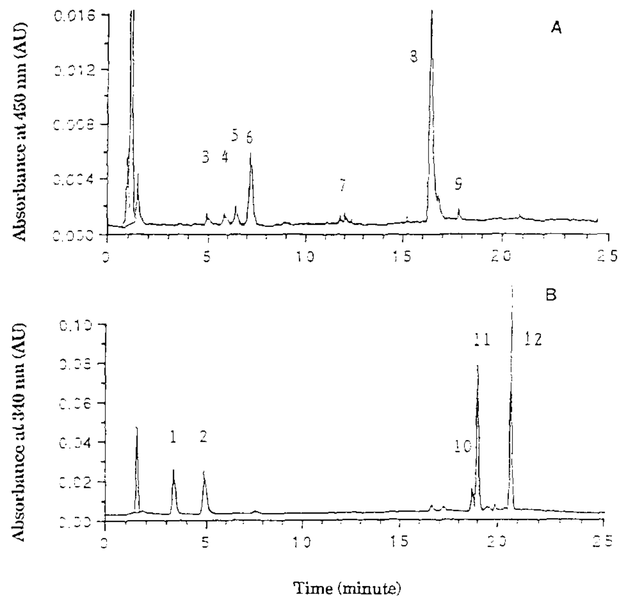

Figure 2 HPLC chromatogram from a serum extract from a ferret fed canthaxanthin for 1 month. The detector was set at 450 nm (graph A) and 340 nm (graph B). Peaks are as follows: (1) retinol, (2) retinyl acetate (internal standard), (3) lutein (zeaxanthin), (4) unknown, (5) all-*trans* canthaxanthin, (6) *cis* canthaxanthin, (7) cryptoxanthin, (8) γ-carotene (internal standard), (9) β-carotene, (10) retinyl oleate, (11) retinyl palmitate, and (12) retinyl stearate. Column and solvents are the same as in *Figure 1*.

Results

The chromatogram of a human serum pool extract showing the separation of carotenoids and retinoids is presented in *Figure 1*. In this chromatogram, peaks of xanthophylls (lutein and cryptoxanthin) were separated by an 8 min interval. Between these peaks, two unknown components (peaks 4 and 5 in *Figure 1A*) appeared at 6 and 8 min. This allowed enough interval for CX to appear between them (*Figure 2A*). Retinyl oleate, retinyl palmitate, and retinyl stearate were also separated using this method as shown in *Figure 2B*. Retinoic acid and retinal, which elute before and after retinol in this chromatogram, respectively, were not detected in 100 μL serum.

The method precision is presented in *Table 1*. The coefficient of variation (CV) for all compounds was below 8.4%. The recoveries of a known amount of retinyl palmitate and all-*trans* CX added to a human serum pool (initially without CX and a low level of retinyl palmitate) were 96.5% and 98.9%, respectively. The recovery of a known amount of β-carotene added to ferret serum (initially with a low level of β-carotene) was 103.3%. The extraction recovery was 91 ± 3% ($n = 9$) for retinoids using retinyl acetate as the internal standard and was 99 ± 6% ($n = 9$) for carotenoids using γ-carotene as the internal standard. The sensitivity of the assay was 3 ng/mL for retinoids and 2 ng/mL for carotenoids. Accuracy of this assay was assessed by applying this method to the analysis of reference samples ($n = 3$) from the National Institute of Standards and Technology. It showed a positive

deviation of 6.8 ± 2.0% for β-carotene and a positive deviation of 5.2 ± 1.7% for retinol.

The chromatogram of a typical ferret serum extract is presented in *Figure 2*. In this chromatogram, peaks 1, 3, 7, 9, 10, 11, and 12 have retention times identical to authentic retinol, lutein (zeaxanthin), cryptoxanthin, β-carotene, retinyl oleate, retinyl palmitate, and retinyl stearate, respectively. The UV spectra of peaks 11 and 12 showed maximum absorption at 328 nm, which represents a typical absorption spectrum of retinyl esters (data not shown). Peaks 5 and 6 were only detected in the ferrets fed CX. The retention times of peaks 5 and 6 matched those of standards of all-*trans* CX and *cis* CX, respectively. Under HPLC mobile phase conditions, the absorption spectrum of peak 5 showed maximum absorption at 472 nm and the spectrum of peak 6 showed the maximum absorption at 470 nm and a "*cis*-peak" at 372 nm (*Figure 3*). These observations provide the evidence that peak 5 is all-*trans* CX and that peak 6 is *cis* CX.[22] GC-NCl/MS of the HPLC fraction corresponding to HPLC peaks 5 and 6 confirmed the presence of CX in the serum. That is, the GC retention time of the putative CX samples were the same as the GC retention time of CX standards; the NCl mass spectra of the CX standards and the putative CX samples showed negative molecular ions at m/z 564. NCl was the only mass spectrometric ionization method that was sensitive enough to detect the small quantity of CX (2 pg/μL, quantified from the standard curve of CX in GC-NCl/MS) injected into the gas chromatograph (data presented separately).

The levels of retinoids and carotenoids in the sera of ferrets fed diets with and without CX is shown in *Table 2*. There was no difference between the CX-fed group and control group in the levels of retinol, retinyl esters, lutein, cryptoxanthin, and β-carotene. However, in the control group there were no detectable levels of CX in the serum, while in the group fed CX the serum levels of all-*trans* and *cis* CX were 33.34 ± 3.33 nmol/L and 72.48 ± 7.64 nmol/L, respectively.

Table 1 Analytical precision of retinol and β-carotene in a human serum pool by HPLC method

	n	Mean	SD μmol/L	CV %
Within day				
Retinol	5	1.938	0.123	6.3
Lutein	5	0.448	0.010	2.2
Cryptoxanthin	5	0.242	0.013	5.4
Lycopene	5	0.965	0.068	7.0
α-carotene	5	0.160	0.005	3.1
β-carotene	5	0.658	0.043	6.5
Between day				
Retinol	4	1.819	0.153	8.4
Lutein	4	0.447	0.027	6.0
Cryptoxanthin	4	0.255	0.006	2.4
Lycopene	4	0.956	0.047	4.9
α-carotene	4	0.165	0.004	2.4
β-carotene	4	0.685	0.030	4.4

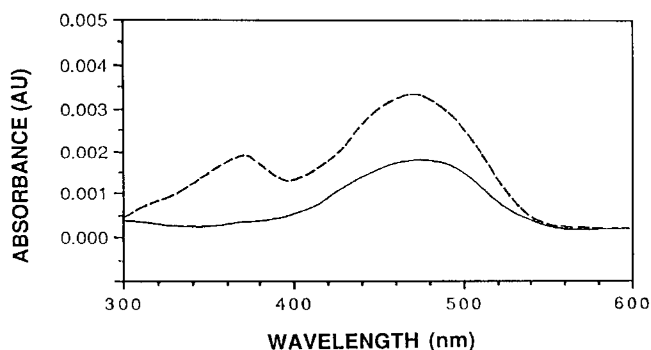

Figure 3 Spectra of peak 5 (solid line) and peak 6 (dashed line) in the chromatogram of *Figure 2*, taken from the diode-array detector during HPLC analysis.

Table 2 Levels of retinoids and carotenoids in the sera of ferrets fed with/without canthaxanthin

	CX-fed group (n = 13)	Control group (n = 13)	P Value
Retinoids	Mean (SEM) in μmol/L		
Retinol	1.83 (0.12)	2.20 (0.17)	0.09
Retinyl oleate	1.60 (0.21)	1.77 (0.29)	0.63
Retinyl palmitate	10.50 (1.15)	11.58 (1.90)	0.63
Retinyl stearate	15.74 (2.03)	16.16 (2.86)	0.91
Total esters	27.84 (3.30)	29.51 (5.02)	0.78
Carotenoids	Mean (SEM) in nmol/L		
Lutein	22.18 (2.57)	27.98 (3.64)	0.21
Trans canthaxanthin	33.34 (3.33)	0.0	<0.0001
Cis canthaxanthin	72.48 (7.64)	0.0	<0.0001
Cryptoxanthin	27.76 (2.65)	19.91 (4.13)	0.12
β-carotene	13.73 (1.12)	11.97 (2.82)	0.57

Discussion

The HPLC method separates all-*trans* CX, *cis* CX (peaks 4 and 5) and the other carotenoid components in both human serum and ferret serum (*Figures 1 & 2*). In a 26-min run, seven carotenoids (lutein, CX, *cis* CX, cryptoxanthin, lycopene, α-carotene, and β-carotene) and four retinoids (retinol, retinyl oleate, retinyl palmitate, and retinyl stearate) can be quantified. The high recovery of added CX, retinyl palmitate, and β-carotene demonstrates that this gradient HPLC procedure is well suited for the analysis of all retinoids and carotenoids in the extract. Although retinol, lutein (zeaxanthin), cryptoxanthin, and β-carotene were detected in both ferret and human serum, lycopene and α-carotene were only detected in human serum.

By using retinyl acetate and γ-carotene to monitor the extraction recovery, we found the extraction efficiency in the carotenoid channel was slightly higher than that in the retinoid channel (99% versus 91%). The molecular structure of γ-carotene consists of two dissimilar terminal groups: one terminal group is a β-ionone ring, which is half of the β-carotene molecule; the other terminal group is an open chain, which is

half of the lycopene molecule. γ-carotene eluted between lycopene and β-carotene. The amount of γ-carotene added to the mixture was compatible with the level of β-carotene and did not interfere with the analysis of β-carotene. Therefore, using γ-carotene as an internal standard for the carotenoid channel instead of using retinyl acetate for all components in the serum ensures the precision of the method. It is noticed that the retinyl acetate co-elutes with lutein in this chromatography. For samples with high lutein and zeaxanthin content, other compounds that elute at 10 min in the chromatogram (for example, retinal *o*-ethyloxime) may be used.

In the extraction procedure, we used $CHCl_3/CH_3OH$ to denature the protein and extract the serum components into $CHCl_3$ before re-extracting the sample with hexane. This double extraction gave an extraction efficiency greater than 90%. After the extracts were evaporated and redissolved in ethanol, we did not allow the extracts to sit at room temperature longer than 12 hours because evaporation of the solvent from the extract gives an abnormally high recovery (over 100%) and results in high errors. Our data showed variance of the mean value ($n = 4$) for a sample analyzed at 0 hr and at 14 hours was −0.16% for retinol, +1.1% for lutein, +4.8% for cryptoxanthin, −0.12% for lycopene, −3.2% for α-carotene, and +2.8% for β-carotene. Therefore, under these conditions, we found that it was not necessary to add any antioxidant during the extraction as reported by Craft et al.[23]

Retinyl oleate, retinyl palmitate, and retinyl stearate level were extremely high in ferret serum; the ratio of retinyl oleate, retinyl palmitate, and retinyl stearate was 5.9:38.5:55.6. These three components represent 93% of total vitamin A levels in ferret serum. In contrast, in fasted human blood retinyl palmitate is the dominant ester form and represents less than 3% of total circulating vitamin A.[24] Retinol levels in ferret serum and in human serum are nearly identical (*Tables 1 & 2*). This observation supports the previous reports by Clausen et al.,[25] Wilson et al.,[26], and Schweigert et al.[27,28] that high vitamin A ester levels in blood are a general phenomenon in carnivores and are not a sign of vitamin A intoxication.

The occurrence of CX in every ferret fed CX shows that the ferret was able to absorb CX intact. Moreover, we did not find a significant difference in serum level of lutein (zeaxanthin) between two groups of ferrets (*Table 2*). Serum retinol and retinyl esters did not show significant changes in concentration in either group of ferrets (CX fed group or control group). Therefore, we did not detect any significant amount of CX metabolite other than *cis* CX under the wavelength of 340 nm and 450 nm.

In the serum of CX-fed ferrets, *cis* CX was the predominant isomer form. The recovery of all-*trans* CX when added to a human serum pool was 98.9%, which eliminated the possibility of isomerization during handling, extraction, and the HPLC process. In the diet, the percentages of the *cis* CX versus all-*trans* CX was 21% and 79%, respectively. However, the percentage of *cis* CX versus all-*trans* CX in the serum of the ferrets was 68% versus 32%, respectively. The observation of *cis* CX formed from all-*trans CX* in vivo has been reported in rats and in monkeys by Mathews-Roth et al.[29] and in chicks by Mayne et al.[30] We do not know whether the profile of *cis* CX and all-*trans* CX in ferret serum is due to physico-chemical properties (solubility and crystalizability) of *cis* carotenoids, to bio-isomerization of CX, or both.

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