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Analytical Methods Section

Separation and determination of carotenoids, retinol, retinal, and their dehydro forms by isocratic reversed-phase HPLC

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A high-performance liquid chromatographic (HPLC) procedure has been developed to separate two retinol forms (retinol$_1$ and retinol$_2$ or dehydroretinol), their corresponding retinal forms and carotenoid pigments commonly found in fish tissues. A reversed-phase C_{18} column was used with an isocratic solvent system (acetonitrile/dichloromethane/methanol/water/propionic acid, 71 : 22 : 4 : 2 : 1, v/v/v/v/v). Prior to HPLC analysis fatty tissues were defatted with acetone/methanol (1 : 1, v/v), at –80°C or on silica gel column. This method gave satisfactory resolution of polar compounds (retinol and retinal forms, astaxanthin and phoenicoxanthin or zeaxanthin) and an acceptable elution time for less polar molecules (retinyl palmitate, α- and β-carotene). Using this method, levels of retinoid forms and some carotenoids were determined in eyes, blood and eggs of mature rainbow trout (*Oncorhynchus mykiss* Walbaum).

INTRODUCTION

Fish and other cold-blooded vertebrates are known to possess vitamin A$_1$ forms (retinol and retinal) plus their dehydro homologues (vitamin A$_2$) and many carotenoids in their organs and tissues (Morton & Creed, 1939; Goodwin, 1951; Grangaud *et al.*, 1963). Carotenoids, especially xanthophylls, could be provitamin A$_1$ and A$_2$ (Grangaud *et al.*, 1962; Gross & Budowski, 1966). However, some of the xanthophylls (astaxanthin, canthaxanthin and zeaxanthin) can be specific precursors of retinol$_1$ or retinol$_2$ depending on the fish species (Barua & Goswami, 1977; Schiedt *et al.*, 1985; Katsuyama & Matsuno, 1988).

High-performance liquid chromatography (HPLC) is known to be a powerful tool in carotenoid research. In recent years, many isocratic or gradient elution reversed-phase HPLC methods have been published for the separation of xanthophylls and provitamin A carotenoids in algae, fruit and vegetable sources (Zakaria *et al.*, 1979; Wright & Shearer, 1984; Khachick *et al.*, 1986; Gregory *et al.*, 1987; Zonta *et al.*, 1987; Khachick & Beecher, 1988; Wills *et al.*, 1988).

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Many researchers have used a similar method to determine retinol, retinyl esters and various carotenoids in human plasma or serum (Driskell *et al.*, 1982; Broich *et al.*, 1983; Tangney, 1984; Bieri *et al.*, 1985; Miller & Yang, 1985; Stacewicz-Sapuntzakis *et al.*, 1987) since reversed-phase HPLC offers several advantages over normal-phase methods for carotenoid determinations (Nelis & De Leenheer, 1983). However, normal phase was used with success to separate vitamin A$_1$ forms and their dehydro homologues (Zonta & Stancher, 1984) and was subsequently adapted to analyze fish tissues (Schiedt *et al.*, 1986; Katsuyama & Matsuno, 1988). Isocratic elution was preferred to gradient elution since it gave precise and reproducible results (Rosset *et al.*, 1982).

Only two reports appear to refer to reversed-phase HPLC methods for determining retinol$_1$ and retinol$_2$ (Al-Kalifah, 1986) or retinoid and carotenoid forms (Guillou *et al.*, 1989) in fish tissues. In the latter report the authors could not separate astaxanthin from lutein or zeaxanthin and the identification of these xanthophylls seems to be important for the determination of carotenoid metabolic pathways.

The aim of the present study was to develop an isocratic reversed-phase HPLC technique in order to simplify the analysis of the major vitamin A forms (retinol$_1$, retinol$_2$ and their retinal forms) and various types of carotenoids found in fish. To improve this

method, three types of fish tissues were used: eyes which generally contain only vitamin A forms (retinals and retinols), serum which possesses low levels of lipids, carotenoids and retinoids, and eggs which contain high concentrations of lipids, carotenoids and retinoids.

MATERIALS AND METHODS

Equipment

The HPLC system included a liquid chromatograph (Varian model 5000), and integrator (Varian model 4270), an injector (Rheodyne model 7125) with a 20 μl loop and a variable wavelength detector (Spectra-Physics model SP 8440XR). The column was a 300 mm × 3·9 mm Nova-Pak C_{18} (4 μm) with a Guard-Pak precolumn module (Waters).

Standards, solvents and HPLC procedures

Standards of carotenoids (astaxanthin, α- and β-carotene and β-apo-8'-carotenoic acid ethyl ester (CAEE), canthaxanthin, citranaxanthin, crustaxanthin, cryptoxanthin, echinenone, isocryptoxanthin, isozeaxanthin, lutein, phoenicoxanthin, zeaxanthin) and retinoids ($retinal_1$, $retinal_2$, $retinol_1$, $retinol_2$, retinyl acetate, retinyl palmitate, retinyl propionate) were obtained from Hoffmann–La Roche Co., Basle, Switzerland. Pigments and retinoids (5·0 mg) were dissolved in 100 ml of toluene/methanol mixture (1 : 1), containing 500 mg litre^{-1} of BHT (butylated hydroxytoluene, Sigma, St Louis, USA) to produce 50 μg ml^{-1} standards. These stock solutions were stable for at least four months in the dark at –20°C. These were further diluted with the mobile phase to give working standards. HPLC-grade solvents (Carlo Erba, Milan, Italy) were degassed by vacuum filtration prior to use and water was double distilled. Both retinoids and carotenoids were separated using acetonitrile/dichloromethane/methanol/water/propionic acid (71 : 22 : 4 : 2 : 1, v/v/v/v/v) as mobile phase. The flow rate was 1·0 ml min^{-1}.

Absorption maxima of carotenoids and retinoids dissolved in the authors' mobile phase were determined by their respective absorption spectrum made with a spectrophotometer (Beckman, model 25) between 280 and 550 nm.

During the first 10 min of analysis, detection of carotenoid pigments was performed at 450 nm and retinoid forms at 352 nm ($retinol_2$ maximum absorbance in the mobile phase). When detection of retinyl palmitate was required it was switched to 326 nm. Two runs were needed to quantify retinoids and carotenoids as the UV-visible detector used had only one channel. Retinoids and carotenoids were quantified by the integration of their peak area. The column and the mobile phase were thermoregulated at 0°C by a recirculating system to avoid variations in peak heights and retention times caused by ambient temperature changes. Under this condition separations were optimized. In order to evaluate the performance of the HPLC system during the analysis, a mixture of retinoids and carotenoids (external standards) was injected at various intervals during the day.

Extraction procedures

Tissues (eyes, eggs and serum), taken from mature female rainbow trout (*Oncorhynchus mykiss* Walbaum) fed a diet containing astaxanthin, were stored at –80°C in the dark. Two eyes weighing 1·7 g, eggs (3 g) and serum (200 μl) were used for duplicate extraction procedures. As retinoids and carotenoids are lipophilic compounds, it was interesting to determine their concentrations in relation to the total lipid content of fatty tissues. Hence, lipid extractions were performed according to the methods of Folch *et al.* (1957) for eggs (200 mg litre^{-1} of BHT was added to Folch solution), Stacewicz-Sapuntzakis *et al.* (1987) for serum and Tsin *et al.* (1985) for eyes. Internal standard was used for lipid or pigment extraction procedures without difficulty. Three internal standards have been tested for the determination of carotenoid and retinoid forms: CAEE, retinyl acetate and retinyl propionate.

Egg samples were defatted by the precipitation of lipids in acetone/methanol (1 : 1) solution maintained at –80°C for at least six hours, followed by rapid vacuum filtration (Müller-Mulot *et al.*, 1976) and evaporation to dryness. All samples were kept in the dark at –30°C under nitrogen until analyzed. They were then dissolved in acetonitrile/dichloromethane (2 : 1), solution and filtered through Millex-HV (0·45 μm) filter (Millipore) before chromatographic injection.

Statistical analysis was made according to Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

Isocratic reversed-phase offers many advantages in the determination of free carotenoids and retinoids over normal-phase HPLC methods (Rosset *et al.*, 1982; Nelis & De Leenheer, 1983). The method described here allowed the separation and quantification of $retinol_1$, $retinol_2$, their retinal forms, retinyl esters and various carotenoids in animal tissues differing in their biochemical compositions.

Elution of retinoids and principally carotenoid compounds is influenced by minor variations in room temperature (Stacewicz-Sapuntzakis *et al.*, 1987). Theoretically, better peak resolution or augmentation of the selectivity factor (α) could be obtained by lowering temperatures (Rosset *et al.*, 1982). By decreasing the temperature from 20°C to 0°C, peak separation of astaxanthin and phoenicoxanthin or zeaxanthin was greatly improved but retention times of all compounds were increased by 10 to 50%.

The retention time, capacity factor (k') and maximum wavelength absorption of carotenoid and retinoid standards chromatographed in our conditions are given in Table 1.

Table 1. Retention times, capacity factor (k') and maximal absorption wavelengths of carotenoid and retinoid standards.[a] Operating system = 300 mm × 3·9 mm Nova-Pak C_{18} column (4 μm): acetonitrile/dichloromethane/methanol/water/propionic acid (71/22/4/2/1, v/v/v/v/v) as mobile phase and spectrophotographic solution; flow rate = 1 ml min^{-1}; temperature = 0°C

Compound	Capacity factor k'	Retention time (min)	Absorption maxima (nm)		
Crustaxanthin (3,4,3',4'-tetrahydroxy-β-carotene)	0·66	3·65	460		488
Astaxanthin (3,3'-dihydroxy-4,4'-diketo-β-carotene)	0·99	4·37		480	
Isozeaxanthin (4,4'-dihydroxy-β-carotene)	1·30	5·05	452		478
Lutein (3,3'-dihydroxy-α-carotene)	1·53	5·57	448		476
Phoenicoxanthin (3-hydroxy-4,4'-diketo-β-carotene)	1·70	5·95		475	
Zeaxanthin (3,3'-dihydroxy-β-carotene)	1·94	6·47	454		482
Canthaxanthin (4',4'-diketo-β-carotene)	2·81	8·39		472	
CAEE (β-apo-8'-carotenoic acid ethyl ester)	4·30	11·65		445	
Citranaxanthin (β-apo-8'-carotenal)	4·46	12·02		468	
Isocryptoxanthin (4-hydroxy-β-carotene)	5·60	14·53	427		450
Cryptoxanthin (3-hydroxy-β-carotene)	6·26	15·98	454		480
Echinenone (4-keto-β-carotene)	7·97	19·73		460	
α-Carotene	19·60	45·33	448		476
β-Carotene	21·64	49·80	454		482
$Retinol_2$ (3,4-dehydroretinol)	0·51	3·33		352	
$Retinal_2$ (3,4-dehydroretinal)	0·66	3·65		390	
$Retinol_1$	0·75	3·85		326	
$Retinal_1$	0·95	4·30		377	
Retinyl acetate	1·15	4·72		326	
Retinyl propionate	1·42	5·32		326	
Retinyl palmitate	26·06	59·54		326	

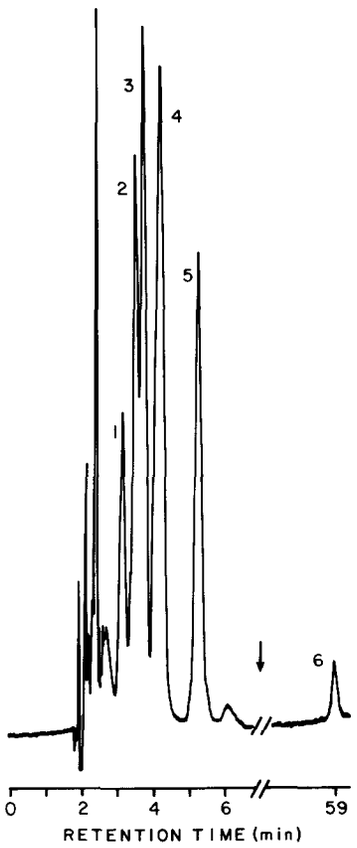

Fig. 1. Chromatogram of retinoid standards at a concentration of 2·0 μg ml^{-1}. The arrow indicates change of detection wavelength from 352 to 326 nm (15 Milli absorbance unit full scale) and diminution of chart speed (0·5 to 0·1 cm min^{-1}). Chromatographic conditions are given in Table 1. Peak identifications: 1, $retinol_2$; 2, $retinal_2$; 3, $retinol_1$; 4, $retinal_1$; 5, retinyl propionate; 6, retinyl palmitate.

Retinoid standards separation

The separation of retinoid standards, retinol, retinal and retinyl ester is shown in Fig. 1. $Retinol_1$ and retinyl esters possess the same absorption maximum (326 nm) whereas $retinol_2$ and retinal forms have higher absorption maxima (Table 1). Retention times of retinals, retinols and retinyl acetate or propionate (internal standards) were too close to allow a wavelength switch during their elution (Table 1). To determine the best wavelength that should be used for the detection and quantification of these compounds, four wavelengths (400, 352, 340 and 326 nm) were tested. At 326 nm, $retinol_2$ and retinal forms showed a very weak absorption response compared to $retinol_1$ and retinyl esters. The peak area of $retinol_2$ was almost half of those of $retinol_1$ and retinyl acetate or retinyl propionate at 340 nm, but at 352 nm all five compounds were well detected (Fig. 1). Moreover, it was observed that between 345 and 360 nm interfering absorption of astaxanthin and other pigments was generally very low. For these reasons, 352 nm was the wavelength used during the first part of the run and switched to 326 nm after retinyl propionate elution (approximately 10 min.) in order to obtain maximum detection sensitivity for retinyl palmitate. No $retinol_1$ or retinyl esters could be detected at 400 nm whereas the absorption of retinal forms was very good. This wavelength can therefore be used for the specific detection and quantification of retinal forms.

In order to determine the linearity of the detection response as a function of different internal standards and retinoid ratios, some retinoid standard curves were determined by mixing 1·5 μg ml^{-1} of retinyl propionate

Table 2. Linear regression statistics of some retinoid and carotenoid standard curves

Variable \ Compounds:	$Retinol_1$	$Retinol_2$	Retinyl palmitate	Asta-xanthin	Phoenico-xanthin	Zeaxanthin	Canthaxanthin	Echinenone	β-Carotene
Slope	91·57[a]	71·42	65·69	67·04	66·98	91·83	83·41	66·18	88·93
Standard error	1·22	2·61	2·90	2·49	1·06	1·80	1·11	0·64	1·91
Intercept	−4·62	0·58	−4·39	2·51	1·01	3·41	−5·47	−2·72	−10·53
SE	4·45	9·50	10·52	5·97	2·55	4·32	2·68	1·55	4·60
Correlation coefficient	99·93	99·47	99·23	99·46	99·90	99·85	99·93	99·96	99·82
SE of the estimate	3·890	8·308	9·202	5·129	2·193	3·715	2·301	1·332	3·951

[a] Note that all data were multiplied by 100 to save table room.

(internal standard) to various concentrations of retinoids (0·25–3·0 μg ml^{-1}). The ratio of peak areas (retinoid/internal standard) was plotted against respective retinoid concentrations. Linear regression data for three retinoid curves are shown in Table 2. A *t*-test with eight degrees of freedom indicates that intercept = 0 for all curves.

Carotenoid standard separations

Astaxanthin has been known to present some erratic chromatographic behaviour like asymmetric peaks and low plate counts. Addition of 1·5% formic acid in the mobile phase has been observed to restore peak shape and column efficiency (Nelis & De Leenheer, 1983). In the authors' system, 1% propionic acid in the mobile phase gave good resolution of astaxanthin (Fig. 2a).

The chromatographic pattern of the 10 carotenoid standards is shown in Fig. 2b. Although the separation of zeaxanthin and lutein was possible under the authors' conditions, it was not possible to separate a mixture of these two pigments in the presence of phoenicoxanthin. Since the retention times of these compounds are slightly different (Table 1), it is nevertheless possible to make a preliminary identification of one of these carotenoids in a chromatographic separation from an unknown sample. A thin-layer chromatographic separation (Stahl, 1969) and a UV-visible light absorption spectra will permit to identify the unknown molecules more precisely.

It is well known that a better quantification of compounds is achieved when wavelength detection is set near the peak of the absorption spectrum (Johnson & Stevenson, 1978). In these conditions a small change in the detector response produces only a negligible error on absorbance but becomes quite significant when it is set on the steep rise or fall of the wavelength spectrum. As it was impossible to shift wavelength detection to the respective wavelength detection of the respective maximum of all compounds, the unique detection wavelength, 450 nm, used in carotenoid analysis was chosen, as an acceptable compromise, according to wavelength absorption maxima of most carotenoid compounds in our mobile phase. Astaxanthin, phoenicoxanthin and canthaxanthin have shown good response at 450 nm in spite of their respective maximum absorption wavelengths of 480, 475 and 472 nm (Fig. 2b).

Standard curves for carotenoids were established as for retinoids. The concentration of internal standard (CAEE) was fixed (1·0 μg ml^{-1}), whereas concentrations of other carotenoid pigments were varied from 0·2 to 2·0 μg ml^{-1}. Table 2 presents the linear regression data obtained for six carotenoid curves. All curves intercept at zero like curves for retinoids.

Application

In order to demonstrate the possible application of this isocratic reversed-phase HPLC method for carotenoid and retinoid analyses of biological materials, three different tissue types were chosen.

Since retention times of carotenoids and retinoids were very stable (few hundredths of a minute difference) during the analysis peaks, these were identified on the basis of relative retention times of the components in the sample and those of the standard compounds.

Table 3. Levels of retinoid and carotenoid compounds determined in three tissues of female rainbow trout and percentage of internal standard recovery

Tissues \ Compounds:	$Retinol_1$	$Retinol_2$	$Retinal_1$	$Retinal_2$	Astaxanthin	Phoenicoxanthin	Canthaxanthin	Retinyl propionate	CAEE
								% of recovery	
Serum[a]	—	5·82	—	—	8·31	2·24	—	99·0	92·2
Eyes[b]	—	7·41	5·44	12·08	—	—	—	98·3	91·0
Eggs[b]	1·61	2·03	—	—	28·63	1·73	1·38	83·4	82·2

[a] μg ml^{-1}.
[b] μg g^{-1} of fresh tissue.

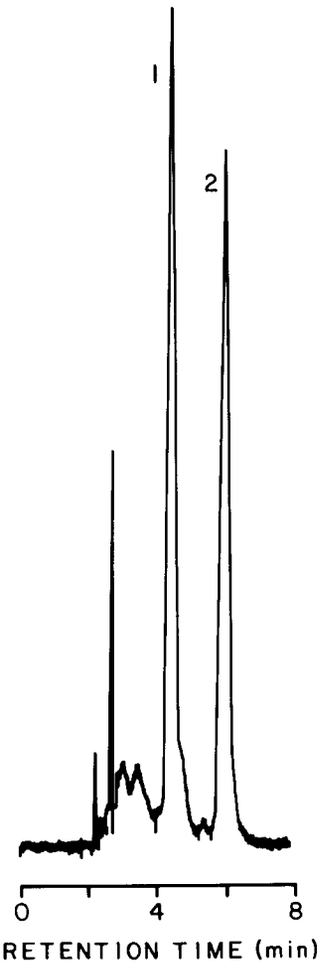

Fig. 2a. Chromatogram of astaxanthin (1) and phoenicoxanthin (2) standards at a concentration of 2·0 μg ml^{-1}.

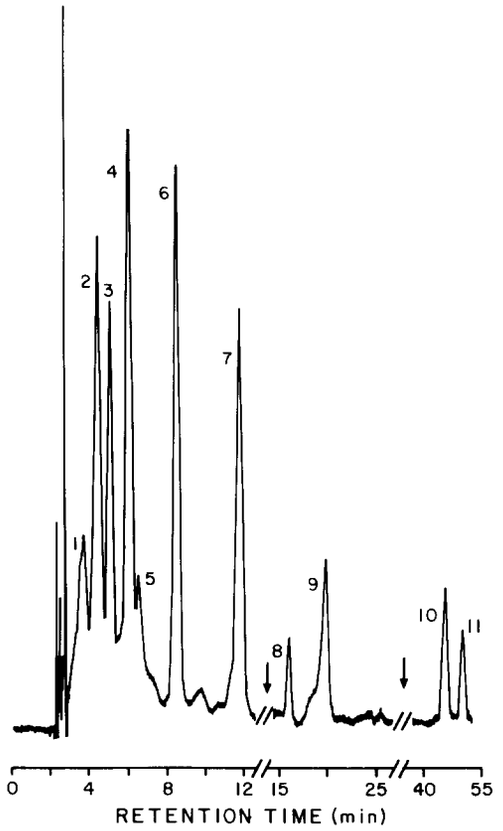

Fig. 2b. Chromatogram of 10 carotenoid standards (2·0 μg ml^{-1}). Detection wavelength: 450 nm (15 Milli absorbance unit full scale). Arrows indicate change of chart speed (0·5 to 0·1 cm min^{-1}). Chromatographic conditions are given in Table 1. Peak identifications: 1, crustaxanthin; 2, astaxanthin; 3, isozeaxanthin; 4, phoenicoxanthin; 5, zeaxanthin from isozeaxanthin fraction; 6, canthaxanthin; 7, CAEE (internal standard); 8, cryptoxanthin; 9, echinenone: 10, α-carotene; 11, β-carotene.

To achieve this, thermoregulation of the column and mobile phase is essential.

The levels of carotenoids and retinoids determined and the percentage of internal standard for each tissue are presented in Table 3. Unfortunately, no data are available concerning the precision of repeated analysis on the same tissue sample. The lowest percentage of internal standard recovery was found in eggs samples. The delipidation procedure used may probably explain this result.

Samples of rainbow trout eggs which contained high lipid content were delipidated before chromatographic analysis to avoid column fouling due to lipid compounds. A typical chromatogram of retinoids extracted from rainbow eggs is shown in Fig. 3a. Note that astaxanthin and an unknown carotenoid were detected at 352 nm. The profile of carotenoid compounds found in the same egg extract but at a higher dilution is given in Fig. 3b.

It is very important to note that most of the carotenoids have some absorption in the near UV region, where retinoids have maximum absorption. This is the major problem for simultaneous quantification of carotenoid and retinoid forms with HPLC methods using UV-visible spectrophotometry. Stacewicz-Sapuntzakis *et al.* (1987) emphasized that, under their conditions, high β-carotene concentration in a sample would perturb retinyl palmitate detection. The presence of *cis*-β-carotene, which absorbs at 313 nm, and similar retention times for these two compounds, was invoked. This problem could not exist in the present authors' method because retention times of these two compounds were very different (Table 1). Guillou *et al.* (1989) recently reported the probable conversion of astaxanthin into retinol$_2$ in rainbow trout gonads. They misinterpreted their results because under their chromatographic conditions astaxanthin and retinol$_2$ had exactly the same retention time. This astaxanthin interfering absorption during retinoid analysis produced an artifact and, therefore, led to overestimation of retinol$_2$ concentrations from astaxanthin-fed fish. This problem has been corrected with the new chromatographic conditions described. Astaxanthin and retinol$_1$ or retinol$_2$ are not eluted at the same time (Table 1) and these peaks are separated as shown in Fig. 3a. The present conditions allow quantification of retinol forms more accurately even if the sample contains high concentration of polar carotenoids.

The best way to determine what is the best internal standard for a given tissue is to know in advance what

Fig. 3a. Chromatographic separations of retinoid extracted from 3·0 g of eggs taken from astaxanthin-fed rainbow trout. The sample was delipidated and diluted with 5 ml of acetonitrile/dichloromethane (2 : 1, v/v) solution before injection. Chromatographic conditions are given in Table 1 and Fig. 1. Peak identifications: 1, retinol$_2$; 2, retinol$_1$; 3, astaxanthin (interfering peak); 4, unknown; 5, retinyl propionate (internal standard).

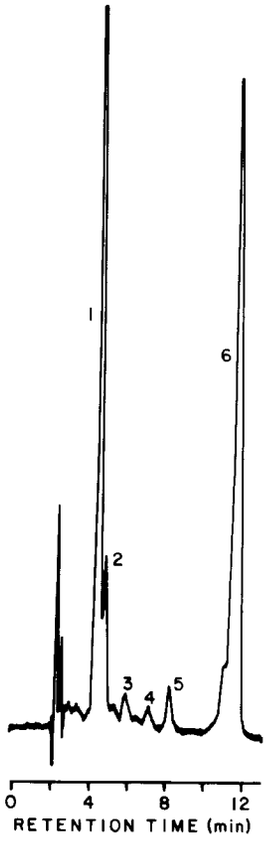

Fig. 3b. Carotenoid chromatogram profile from the same sample but at higher dilution (1/15). Chromatographic conditions are given in Table 1 and detection was made at 450 nm (15 Milli absorption unit full scale). Peak identifications: 1, astaxanthin; 2, unknown; 3, phoenicoxanthin; 4, unknown; 5, canthaxanthin; 6, CAEE (internal standard).

are the major carotenoid and retinoid forms that may be found in the sample. Retinyl acetate or retinyl propionate could be used as vitamin A internal standards because their respective retention times differ from those of the major carotenoids (astaxanthin, phoenicoxanthin, canthaxanthin) generally found in fish tissues. In eye samples, retinyl propionate is preferred because its elution is slightly longer, giving a better peak resolution between retinal$_1$ and the internal standard.

Recovery of the internal standard principally depends on loss during extraction procedures and on the precision of the dilution before chromatographic analysis. A recovery efficiency of 75–105% allows to quantify carotenoids and retinoids with the internal standard method.

In conclusion, the isocratic reversed-phase HPLC method presented here provides a good compromise between a satisfactory resolution of most important polar vitamin and provitamin A molecules in fish such as retinol and retinal forms, astaxanthin, phoenicoxanthin and reasonable retention times for less polar ones like β-carotene and retinyl palmitate (45·3 and 59·5 min). For these reasons, this method seems to be suitable for simultaneous determination of retinoids and carotenoids in a large range of tissues and organs.

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