ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 901–909

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Ultrasensitive determination of β-carotene in fish oil-based supplementary drugs by HPLC-TLS

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Received 29 December 1998; received in revised form 27 April 1999; accepted 6 May 1999

Abstract

This research study demonstrates that thermal lens spectrometry (TLS) combined with efficient isocratic HPLC separation provides an excellent analytical means to profile and quantify carotenes in fish body oils and possibly also in vegetable oils. In particular, a highly sensitive, selective, simple and rapid method for the determination of ultratraces of β-carotene in fish oil-based supplementary drugs has been proposed. The analyte could be determined reliably and precisely in the presence of a complex matrix including other fat-soluble vitamins (A, D and E), polyunsaturated fatty acids, sterols and other pigments by matrix-matched calibration in cod liver oil. The suitability of the method is also evidenced by favourable analytical figures of merit for β-carotene such as a linearity range of 1–120 ng ml^{-1}, LOD of 0.58 ng ml^{-1}, recovery of 101.4 ± 3.3% and measurement repeatability of 4.1%. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: β-Carotene determination; Fish body oil preparations; HPLC-TLS

1. Introduction

During last two decades, increasing attention has been paid to the role of fat-soluble vitamins with possible cancer-preventing activity (provitamins and vitamins A, vitamin E). Carotenoids, members of the terpenoid family of compounds and characterized by their polyunsaturated nature, comprise one of the main groups of natural pigments occurring in fruits, vegetables as well as in some animal products and seafoods. Hydrocarbon carotenoids (or carotenes), e.g. lycopene and β- and α-carotenes are mainly present in foods. Xanthophylls (or oxygenated carotenoids) include cryptoxanthin, the major pigment of citrus fruits, astaxanthin and canthaxanthin found in algae and in salmonid flesh, and lutein and zeaxanthin as common carotenoids in plants and algae.

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PII: S0731-7085(99)00185-5

Quite recently, the research, originally focused on the activity of β-carotene as a precursor of vitamin A, has shifted towards studies of its antioxidative capacity. Although β-carotene has been postulated as a singlet oxygen quenching agent [1,2] recent investigations have put the emphasis on other carotenoids, such as lutein and lycopene that are capable of even stronger antioxidant activity [2,3]. Such developments make carotenoid research still an actual and challenging task.

Marine organisms are very rich carotenoid sources. For example, β-carotene is a naturally occurring yellow-orange pigment which can be derived from saline microalgae, marine phytoplankton and some plant-derived oils. Levels of β-carotene ranging from 200 to 1700 ppm were reported for blue-green and green algae [4,5]. A herring may contain as much as 700 ppb of carotenoids [4].

HPLC, notably reversed-phase, has proven particularly useful for carotenoid profiling in algae, higher plants, food, animal tissues, and human systemic fluids [6–9]. HPLC identification of carotenoids in rainbow trout tissues, brine shrimp and in marine and lake phytoplankton has been reported by several authors [9–16].

Thermal lens spectrometry (TLS), one of the laser photothermal detection schemes, has already proven an ultrasensitive detection method that has been successfully applied in environmental [17,18] and biomedical research [19]. Recently, its application for analysis of β-carotene in human plasma has been reported [20]. Unlike photoacoustic spectroscopy [21], TLS has to the best of our knowledge not yet been applied for drug analysis in general, and for determination of carotenoids in pharmaceutical preparations in particular. Today there is a growing interest directed toward supplementary drugs and dietetic products based on fish body oils as the latter are very rich in polyunsaturated fatty acids. In the work described here, the efficient HPLC separation and ultrasensitive TLS detection were combined to obtain carotene patterns in such samples.

2. Materials and methods

2.1. Samples

All samples analyzed in this study were commercial products purchased from local pharmacies:

sample 1: cod liver oil [22];
sample 2: cod liver oil [23];
sample 3: vitamins $A + D_2$ in cod liver oil [23] (one capsule stated to contain 3750 IU retinyl palmitate, 370 IU ergocalciferol and 37.5 mg fish oil);
sample 4: concentrate of standardized salmon oil (one capsule of 1000 mg stated to contain 18% EPA, 12% DHA and 7 mg vitamin E);
sample 5: refined concentrate of fish oil (one capsule of 500 mg stated to contain 18% EPA, 12% DHA and 20 mg vitamin E, fish source not disclosed);
sample 6: concentrate of natural marine fish oil (one capsule of 1000 mg stated to contain 18% EPA, 12% DHA and 5 IU D-α-tocopherol, fish source not disclosed).

Pools of samples 4–6 were prepared by combining and homogenizing the contents of 30–60 capsules. A pool of sample 3 was prepared from 200 pearls. The pools 3–6 were stored in tightly closed Falcon tubes; all samples were protected from light and kept at +4°C. Samples were dissolved in the diluent prior to chromatographic runs.

2.2. Standards

Standard mixture of carotenoids and β-carotene standard were kindly provided by the Department of Human Nutrition, Wageningen University and Research Centre (The Netherlands), while retinyl propionate, retinyl palmitate, cholecalciferol and DL-α-tocopheryl acetate were obtained from Pliva (Croatia). Cholesterol and the mixture of chlorophylls *a* and *b* (oil-soluble, E140) were the products of Farmacija (Croatia) and International Flavors and Fragrances (USA), respectively.

Standards were protected from light and stored at −20°C prior to use and at +4°C during the

work. Working standards were prepared by diluting the stock solution with mobile phase and with 20 × diluted sample 2.

2.3. Solvents, mobile phase and diluent

All solvents (HPLC grade) used were the products of Labscan (Ireland).

Mobile phase was a mixture methanol–tetrahydrofuran (90:10, v/v). The diluent was prepared as an equivolume mixture of the mobile phase and chloroform.

2.4. Equipment

The 100-μl (Kloehn, USA) and 2500-μl syringes (Hamilton, USA) were used for volume measurements.

2.4.1. HPLC-TLS

Experimental apparatus combining the HPLC and a dual beam thermal lens detector was constructed in the laboratory. Details of an experimental set-up for conventional thermal lens spectrometry were described previously [20,24–26].

The HPLC system included the isocratic LC pump 250 (Perkin Elmer, USA), manual injection switching valve (Rheodyne, USA) with a 10-μl loop, the C_{18} bonded, polymeric stationary phase (VYDAC 218TP54 column, length 250 mm, internal diameter 4.6 mm, particle size 5 μm, pore diameter 30 nm, Separations Group, USA), solvent delivery system and the analytical flow cell Milton Roy SM 400 (length 1 cm, diameter 1 mm, UK).

2.5. Analytical procedures

2.5.1. HPLC-TLS measurements

Five to 40-fold diluted samples were chromatographed isocratically at a mobile phase speed of 1 ml min^{-1} (pressure 4.1–5.2 MPa) at ambient temperature (22–24°C). The detection wavelength was 476 nm. The peak on the chromatogram with t_r corresponding to β-carotene was processed in an area mode.

2.6. Evaluation of analytical results

Calibration lines were obtained (number of points 6–10) by the least squares method using the computer package LSQANAL [27]. An analysis of the parallelism of calibration lines was carried out using the computer programs TWOLINE and TWOLINE1 [27].

Limit of detection (LOD) expressed as the minimum detectable concentration is derived from the relation: $LOD = 3\sigma_B/a$, where 'a' is the slope of the calibration line and σ_B is the S.D. of 10 measurements of the 'field' blank [28].

Results for recovery and for β-carotene concentration are given as mean $\pm \sigma$ values.

3. Results and discussion

3.1. Carotenoid pattern

Peaks in samples 1–6 (Fig. 1b) were identified against external standards (Fig. 1a). An unidentified peak observed in all samples at 14.3 min might be possibly attributed to fatty acid esters. A very strong signal at ca 200 s observed in sample 3 might at least partly be ascribed to a high concentration of vitamin A. However, the same signal in sample 4 may be assigned to lutein and/or zeaxanthin as well. A peak at 300 s in samples 4 and 6 remains unidentified. A peak at 6.0 min appeared in all samples but with varying intensity (it is very strong in samples 4 and 6) and might be attributed to some xanthophyll, possibly canthaxanthin, astaxanthin or echinenone in the case of sample 4. In samples 4–6, a contribution from α-tocopherol/α-tocopheryl esters should not be excluded.

However, peaks with $t_r = 10.2$ and 11.6 min were found characteristic for salmon oil (sample 4) only. The first peak was assigned to β-carotene, probably *trans* isomer, whereas the second one might be attributed to some *cis* isomer of β-carotene or to lycopene. A peak corresponding to β-carotene co-eluted with synthetic β-carotene. The presence of β-carotene in salmon oil (sample 4) is in accordance with the fact that algae from the genus *Dunaliella*, known to contain carotenes,

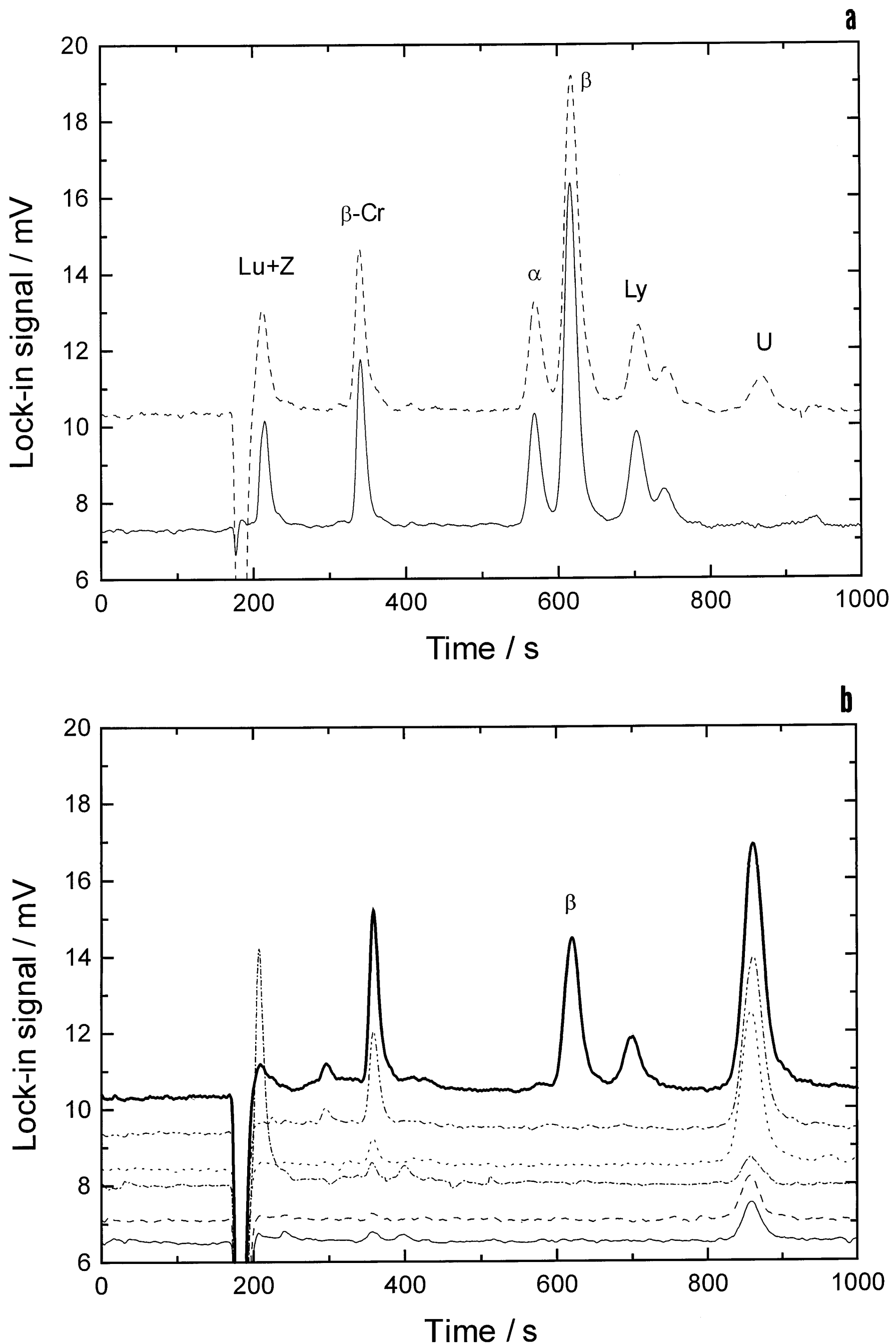

Fig. 1. Typical chromatograms of standard mixture and samples. (a) Standard mixture (concentrations of components in ng ml^{-1}: retinol 71.9, lutein 2.1, zeaxanthin 2.15, β-cryptoxanthin 8.04, α-carotene 5.9, β-carotene 21.4, and lycopene 92.9) in mobile phase (solid line) and in 20 × diluted sample 2 (dashed line). (b) Samples 1–6 diluted 20 × with diluent: sample 1 (solid line), sample 2 (dashed line), sample 3 (dot–dash line), sample 4 (thick solid line), sample 5 (dotted line), sample 6 (dot–dot–dash line). Legend: α, α-carotene; β, β-carotene; β-Cr, β-cryptoxanthin; Lu, lutein; Ly, lycopene; U, unidentified; Z, zeaxanthin. For the sake of clarity, chromatograms are vertically shifted.

xanthophylls, phytoenes and phytofluenes [15,29], are often part of the diet fed to farmed salmons.

Our findings are in accordance with the facts that in reversed-phase HPLC, xanthophylls elute prior to carotenes, ζ- and α-carotene elute before β-carotene while phytoene and phytofluene which might partially co-elute with β-carotene should not contribute significantly to the overall absorption at 476 nm due to their low ε at this wavelength [30]. Results concerning fast elution of retinol and retinyl acetate as well as retention times of α-tocopherol, lycopene, lutein, zeaxanthin, β-cryptoxanthin, echinenone, α- and β-carotenes comparable to our own were also obtained by several authors under similar experimental conditions [31–33].

Moreover, our results are supported by those obtained by numerous authors concerning the suitability of a polymeric C_{18} phase and methanol-based solvent for separation of carotenoids, e.g. Refs. [31,33]. Despite the fact that analogous systems have also been reported to provide separation of *trans* and *cis* isomers of some carotenes [29,34–38], the C_{30} stationary phase designed recently should be used for efficient resolving of geometric isomers [36,38,39].

Some algae possess the ability to accumulate carotenoids particularly astaxanthin, canthaxanthin, lutein, zeaxanthin and β-carotene [5,12,15,29]. Since salmonids are unable to synthesize carotenoids de novo or to synthesize them from other compounds, they depend on a dietary intake. Astaxanthin and canthaxanthin are therefore often found as major pigments of wild salmonid flesh, where they are deposited unchanged, whereas considerable amounts of their metabolites may be found in other tissues, such as skin and liver [40–42]. Ingested astaxanthin is reduced to zeaxanthin and possibly lutein, and canthaxanthin via echinenone to β-carotene in the Atlantic salmon and the rainbow trout [41]. Unlike rainbow trout, in salmon, *Salmo salar*, dietary astaxanthin and canthaxanthin are deposited more efficiently in flesh than in skin. Analysis of salmon skin indicates the abundance of xanthophyll esters such as that of lutein and zeaxanthin, followed by astaxanthin and adonixanthin esters, idoxanthin, canthaxanthin, echinenone and β-carotene. The latter, detected in a salmon fed canthaxanthin-supplemented diet, was found to contribute to total carotenoids by 25%; the actual concentration of β-carotene in salmon skin is therefore about 1 ppm. It follows that, depending on the diet composition, β-carotene in salmonids might originate from β-carotene ingested as such and/or formed as a metabolic product of ingested canthaxanthin.

The liver is a major storage organ for vitamin A; however, it is not yet known whether reduction of carotenoids and their transformation into vitamin A occur in the liver of rainbow trout in analogy with the cleavage of β-carotene into retinal in the liver of warm-blooded animals [40,43]. This might explain different chromatographic patterns recorded for cod liver oils (samples 1–3) and fish body oils (samples 4–6) (Fig. 1b).

Naturally occurring hydroxy carotenoids (e.g. lutein, zeaxanthin, β-cryptoxanthin) and epoxy carotenoids are usually esterified with fatty acids [44]. Therefore, carotenoid extracts from natural sources suspected to contain carotenol acyl esters are usually saponified to remove the fatty acids and liberate the parent carotenoids. Saponification can result in the destruction and/or structural transformation of some carotenoids. Ng and Tan [30] reported a 4% increase of β-carotene level in the extract of palm oil after saponification which lasted for 15 h. The same treatment, however, was responsible for the loss attributed to the degradation of all-*trans*-β-carotene to its *cis* isomers. Therefore, HPLC methods separating various classes of carotenoids without saponification step can be highly advantageous and provide valuable information on the identity and the levels of these compounds in their natural state. There are reports on attempts to avoid saponification. For example, Elton-Bott and Stacey [45] simply dissolved cod liver oil in a mobile phase prior to HPLC separation of fat soluble vitamins while Pfund et al. [46] extracted β-carotene from soya oil and brine samples into dichloromethane before voltammetric determination. In this work, an overnight saponification with 2.5 mol l^{-1} KOH resulted in somewhat lower resolution of peaks, but neither have the peaks eluting between 300 and 800 s in chromatogram of sample 4 changed,

nor have new peaks appeared. Therefore, the saponification step was abandoned and the samples were prepared by simple dissolution in the diluent.

3.2. Analytical performance

3.2.1. Accuracy

Calibration functions for β-carotene in sample 2, mobile phase and diluent as well as standard addition of β-carotene to sample 4 are depicted in Fig. 2. Intercepts of calibration lines were not found statistically different from zero at the 95% probability level. Analysis of parallelism of lines is presented in Table 1.

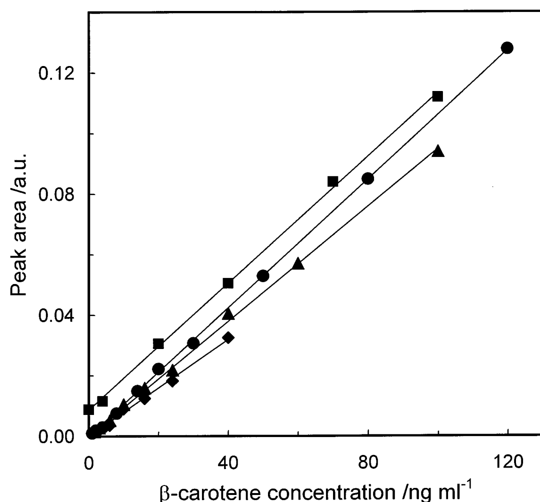

Fig. 2. Standard addition of β-carotene to 20 × diluted sample 4 (■) and calibration lines for β-carotene in 20 × diluted sample 2 (●), in mobile phase (▲), and in diluent (◆).

Table 1
t-Values for compared slopes of standard addition line and ideal calibration lines[a]

	Calibration line in 20 × diluted sample 2	
	Slope	P-value
Line of standard addition in 20 × diluted sample 4	1.104	N.S.
Calibration line in mobile phase	4.909	<0.001
Calibration line in diluent	12.886	<0.0001

[a] See also Fig. 2; NS, not significant.

A recovery of 101.4 ± 3.3% was evaluated from the slopes of the calibration line in sample 2 and the line of standard addition in sample 4. For reliable determination of β-carotene in salmon oil preparation, calibration in a cod liver oil as a matrix might be used. This makes the method of standard addition redundant. The rotational effect of major constituents, a phenomenon well known in determination of trace analytes in complex matrices, resulted in significantly reduced slope of calibration lines in diluent and in mobile phase compared to that in sample 2, namely in cod liver oil (Fig. 2).

3.2.2. Linearity range and sensitivity

Regression analysis of the plots showing a peak area versus concentration of β-carotene produced excellent linear relationships with coefficients of correlation of ≥ 0.999 (Fig. 2). A linear response was obtained for the concentration of β-carotene in cod liver oil, i.e. in sample 2 ranging from 1 to 120 ng ml^{-1}. Sensitivity expressed as a slope of calibration line ± S.D. is $a = 1.0619 \times 10^{-3} \pm 4.7775 \times 10^{-6}$ ml ng^{-1}, $r = 0.99991$, and the residual sum of squares about the line, R.S.S. = 5.1×10^{-6}.

3.2.3. Limit of detection

Twenty-fold diluted cod liver oil (sample 2) served as a 'field' blank; the LOD value for β-carotene was estimated to be 0.58 ng ml^{-1}. This was also confirmed by obtaining a S/N ratio of 7.2 when adding 1.0 ng ml^{-1} β-carotene to the blank. Taking into account the complexity of the sample, this LOD agrees well with the value obtained in human plasma extract under the same experimental conditions [20].

Due to such a low LOD the new method is capable of analyzing highly diluted fish oil preparations (e.g. diluted up to hundred times). This reduces the risk of contamination of the octadecylsilane type of bonded stationary phase by lipids. After about hundred consecutive analyses of 10–20 times diluted samples, an increase of pressure of 28% was observed indicating no need for reconditioning of the column.

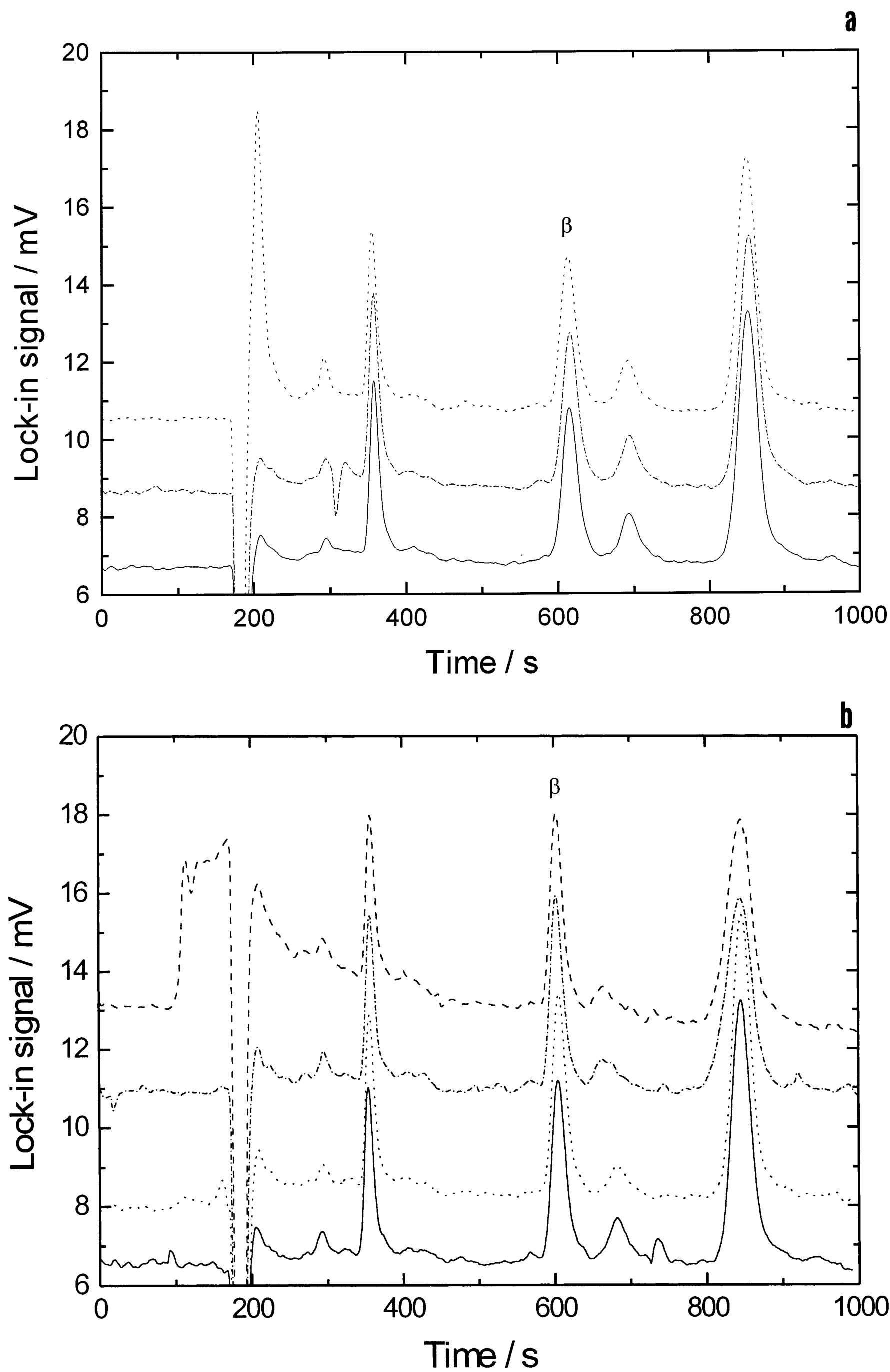

Fig. 3. Selectivity in the presence of fat-soluble vitamins, chlorophylls and cholesterol. Chromatograms of 20 × diluted sample 4: alone (solid line), and in the presence of (a) 38.6 mmol l^{-1} DL-α-tocopheryl acetate (dash–dot line) and 5.0 mmol l^{-1} retinyl palmitate + 8.2 mmol l^{-1} retinyl propionate (dotted line); (b) 69.4 mmol l^{-1} cholecalciferol (dotted line), 55.6 mmol l^{-1} cholesterol (dot–dash line) and 0.3 mmol l^{-1} chlorophylls $a+b$ (dashed line). Legend: β, β-carotene. For the sake of clarity, chromatograms are vertically shifted.

3.2.4. Precision

Repeatability and intermediate precision of β-carotene measurements in 20 × diluted sample 4 expressed as R.S.D. value were 4.1% ($n = 7$) and 5.7% ($n = 3$), respectively. Precision data are reflective of the fact that a laboratory-made device is prone to instability of the laser, misalignment, the effect of mechanical vibrations and temperature fluctuations; all of which are expected to be improved in a commercial instrument. Results are in agreement with previous reports on TLS measurements [20,24].

3.2.5. Selectivity

Selectivity was evaluated in the presence of a many-fold excess of fat-soluble vitamins, chlorophylls and cholesterol (Fig. 3a,b). Vitamin E did not affect the β-carotene peak of sample 4 even at a molar excess of 2.4×10^6 times but co-eluted with a peak at 6.0 min. Moreover, the presence of retinyl esters, cholecalciferol, chlorophylls and cholesterol, in the respective molar excess of $(3.1–5.1) \times 10^5$, 4.3×10^6, 1.9×10^4 and 3.5×10^6 times above that of β-carotene, caused no significant change of the β-carotene peak in sample 4.

Selectivity was also evidenced through the chromatograms from cod liver oils (samples 2 and 3) declared to contain vitamins A, D_3 and D_2, respectively (Fig. 1b).

4. Conclusions

The ultrasensitive and selective method proposed here for determination of β-carotene in complex matrices of simply diluted fish body oil preparations has evidenced the feasibility of avoiding the saponification step. Due to efficient HPLC separation, carotenes could be detected in the presence of other fat-soluble vitamins, such as retinyl palmitate and propionate, cholecalciferol, ergocalciferol and DL-α-tocopheryl acetate. It is known that vitamins A and D are characteristic for fish oils, while DL-α-tocopherol is often used as an antioxidant. Moreover, it was possible to determine β-carotene in the presence of other carotenes such as lycopene. Cod liver oil was used as an appropriate blank matrix due to its complex nature resembling that of fish body oils. Highly sensitive TLS detection enabled the reliable quantification of ultratraces of β-carotene in fish body oils: a concentration of 172.0 ± 5.9 ng ml^{-1} ($n = 7$) was determined in salmon oil (sample 4). The actual measurements of 20 × diluted sample 4 were performed at a S/N ratio of 64.4.

Fish body oils, each having their own and unique carotene distribution depending on the dietary intake, might easily and accurately be characterized by the proposed method. This enables one to deduce the fish source used for body oil preparation. In principle, the control of processing and storage of carotenoid-containing oils is also possible.

As the analytical method developed here also seems applicable to vegetable oils, quality control of such products in regard to the content of carotenes might therefore be simple and cost effective. This possibly extends the applicability of the method for the control of supplementary drugs with β-carotene dissolved in vegetable oil matrices and for carotene profiling of plant-derived oils themselves. Investigations in this direction are already underway.

Acknowledgements

The Visiting Scientist Fellowships granted to the first two authors by the Wageningen University and Research Centre, The Netherlands, are greatly appreciated. Research described in this paper is also being carried out within the frame of Inco Copernicus project (ER BIC 15CT96/003) funded by the European Community. We are indebted to J. Marincel and I. Aničić for helpful discussions as well as to the staff of Fidifarm (Gornja Pušća, Croatia), Alkaloid (Skopje, Macedonia) and Milsing (Zagreb, Croatia) for their cooperation.

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