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Journal of Chromatography B, 718 (1998) 47–54

JOURNAL OF CHROMATOGRAPHY B

Determination of *trans*-β-carotene and other carotenoids in blood plasma using high-performance liquid chromatography and thermal lens detection

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Received 1 April 1998; received in revised form 13 July 1998; accepted 13 July 1998

Abstract

Thermal lens spectrometry (TLS) was applied for the detection of β-cryptoxanthin, α-carotene, *trans*-β-carotene, and lycopen in blood plasma. This combined high-performance liquid chromatography–TLS (HPLC–TLS) method was validated by comparison with HPLC–UV–Vis analysis of blood plasma under identical chromatographic conditions and by comparing the results obtained from an independent, standard HPLC procedure for determination of carotenoids in blood plasma samples. The results demonstrated good agreement with the target values for carotenoids in an in-laboratory control sample and confirmed the accuracy of the HPLC–TLS technique. Limits of detection for blood plasma samples were 70 pg/ml for β-cryptoxanthin, 85 pg/ml for α-carotene, 100 pg/ml for *trans*-β-carotene, and 120 pg/ml for lycopen. This represents a 100-fold improvement compared to the HPLC analysis with UV–Vis detection.

Keywords: Thermal lens spectrometry; Carotenoids

1. Introduction

As a precursor of vitamin A *trans*-β-carotene is essential to human health. In addition, it has been observed that compounds from the group of carotenoids, abundant primarily in fresh fruit and vegetables, act as natural lipid-soluble anti-oxidants. The concentrations of each carotenoid compound in body fluids such as blood plasma differ and range typically from 10 to 750 ng/ml (2×10^{-8}–1.5×10^{-6} mol/l) [1–5]. As such they provide important information on the nutritional and health status of the human body.

The determination of carotenoids is usually carried out by HPLC using UV–Vis spectrophotometric detection [1–8]. The limit of detection (LOD) of this technique is about 10 ng/ml. The levels of *trans*-β-carotene and other carotenoids in blood plasma of different individuals, depending on the nutritional habits and other activities, are, however, often below

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0378-4347/98/$19.00
PII: S0378-4347(98)00347-8

the LOD attainable by the HPLC technique. For example, smoking was found to reduce the levels of *trans*-β-carotene in blood plasma down to 10 ng/ml and lower [9–12]. Measurements of such low concentrations are therefore essential when investigating the effects of external factors and nutrition on the carotenoid levels in the human body and its health status.

Therefore, at these low concentrations the application of a detection technique with sensitivity higher than conventional spectrophotometry is essential. For compounds with low fluorescence quantum yields, such as most carotenoids, thermal lens spectrometry (TLS) [13] is clearly the method of choice. Furthermore, the favourable optothermal properties (low thermal conductivity and high temperature coefficient of the refractive index) of organic solvents used as eluents in HPLC separation of carotenoids [14] are complimentary for reaching the extreme sensitivity of the technique, as confirmed in several applications [15].

In view of research on the photo-physical processes associated with light absorption, attempts have already been made to improve the detection limits for carotenoids involved in such processes [16]. The TLS technique provided an LOD of 3.7 ng/ml (6.9×10^{-9} mol/l). The potential advantages of the technique were hindered, however, by the relatively low pump laser power and its instability. Furthermore, the reported results were obtained from synthetic standard solutions and the performance of the TLS detection has so far not been verified by the analysis of carotenoids in real biological samples.

As already mentioned, there is interest in a more sensitive and reliable analytical technique and improved limits of detection in carotenoid analysis. Therefore, the objective of this work was to validate the TLS as a detection technique in HPLC analysis of *trans*-β-carotene and other carotenoids in blood plasma, and to improve and evaluate the LOD of the method.

2. Experimental

2.1. Reagents and standards

The solvents methanol (LabScan), tetrahydrofuran (THF, LabScan), and hexane (Rethburn) were all HPLC grade. Stock solutions of pure carotenoids and the mixed standard stock solution were prepared from commercially available solutions of individual carotenoids. The concentrations of carotenoids in the mixed standard stock solution were: 181.2 ng/ml lutein (Roth), 68.4 ng/ml zeaxanthin (Roth), 99.6 ng/ml β-cryptoxanthin (Roth), 92.8 ng/ml α-carotene (Sigma), 416.4 ng/ml *trans*-β-carotene (Sigma), 157.6 ng/ml lycopen (Sigma).

The stock solution was stored in the refrigerator at +5°C. Working standards were prepared daily from the stock solution by appropriate dilution with the eluent (mobile phase).

2.2. Sample preparation

Carotenoids were extracted from 0.5 ml blood plasma samples with hexane, following the addition of 0.5 ml methanol to precipitate the proteins. After evaporation of the solvent at room temperature in a stream of nitrogen, the solid residue was dissolved in 0.25 ml 50% THF in MeOH. Butylated hydroxy toluene (BHT, Rethburn), an antioxidant, was added (100 mg/l) to extracts and standards. When immediate analysis was not possible the extracts were stored in a deep freezer at −80°C. Prior to analysis the extracts were appropriately diluted by the mobile phase.

2.3. Chromatographic conditions

The HPLC separation of carotenoids was performed on a 4.6×250 mm Vydac 218TP54 column (5 μm particle size, pore diameter 30 nm). A 10 μl loop was used for injection unless stated otherwise. A 10% solution of THF in MeOH at a 1 ml/min flow-rate was used as mobile phase. A TSP Spectra System HPLC chromatograph (equipped with a P2000 pump, UV3000 detector, and AS300 autosampler) was used for measurements based on UV–Vis detection at 450 nm. In the case of TLS detection a Perkin-Elmer isocratic pump LC 250 was used and the samples were injected manually.

2.4. TLS detection

All thermal lens spectrometric measurements were performed on a dual beam, mode mismatched TLS instrument using an experimental setup similar to

that described in detail previously [17,18]. A Lexel M85 Ar-ion laser tuned to 476 nm (60 mW) was used as a pump beam source, while a Melles Griot 05-LHP-153 He–Ne laser provided the probe beam of 7 mW power at 632.8 nm. The pump beam was modulated by a Stanford SR540 mechanical chopper at 41 Hz. The intensity of the probe beam was monitored by a Thorlabs 201/5797227 photo-diode connected to an Ithaco 3961B Lock-in amplifier with a pre-set 3 s time constant. A 1 cm long SM400 Milton Roy HPLC cell connected to the outlet of the chromatographic column was used instead of a conventional spectrophotometric sample cell.

3. Results and discussion

Preliminary TLS measurements of *trans*-β-carotene standard solutions prepared in the mobile phase, but without chromatographic separation, confirmed that extremely low limits of detection can be expected for carotenoids when using a specific laser wavelength (476 nm), power (60 mW), and mobile phase (10% THF in MeOH). The LOD achieved for *trans*-β-carotene in a 1-cm spectrophotometric cell was 40 pg/ml (7.4×10^{-11} mol/l). The LOD was calculated as the concentration of *trans*-β-carotene that produced a thermal lens signal three times higher than the standard deviation of the background signal (blank). In an actual chromatographic analysis, when injecting a 10 μl sample onto the column, this would corresponds to 40 fg of *trans*-β-carotene, which exceeds by more than two orders of magnitude the lowest amounts of *trans*-β-carotene normally detected when using HPLC with UV–Vis detection for the analysis of carotenoids. At such low concentrations any adsorption of carotenoid compounds on the column would lead to erroneous analytical results. Therefore, the study of possible adsorption of *trans*-β-carotene and other carotenoids on the column was initially our major concern.

To elucidate the eventual effects of adsorption on the column a series of chromatograms of mixed standard solutions at different dilution was recorded. Good resolution was achieved for β-cryptoxanthin, α-carotene, *trans*-β-carotene, and lycopen, while lutein and zeaxanthin co-eluted and were observed as the first peak in the chromatogram (Fig. 1). The absolute response factors for various carotenoids

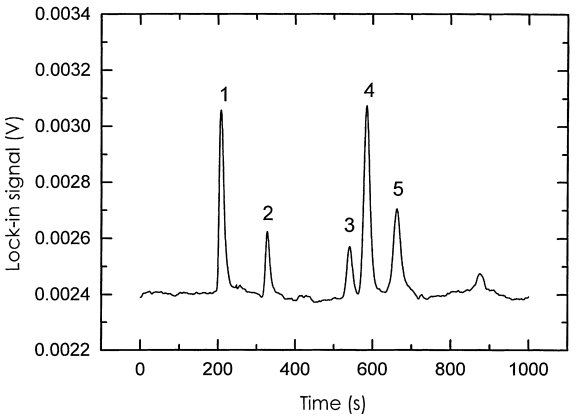

Fig. 1. Chromatogram of a carotenoid mixture obtained by injecting 10 μl of a 25-times diluted mixed standard stock solution. Compounds eluted in the following order: **1** – lutein+ zeaxanthin, **2** – β-cryptoxanthin, **3** – α-carotene, **4** – *trans*-β-carotene, and **5** – lycopen.

were calculated from the areas of the corresponding chromatographic peaks. The results, summarised in Table 1, demonstrate no significant adsorption on the column within the investigated range of injected carotenoids. At five-fold dilution these amounts are already about 10 times lower compared to those injected with the lowest concentration of the standard used in the routine procedure for analysis of carotenoids in blood plasma by HPLC with UV–Vis detection [1,7,8]. The deviations between the absolute response factors at different dilutions for a particular carotenoid are within the margins of reproducibility of the validation procedure, which reflects the reproducibility of HPLC–TLS measurements and the uncertainty introduced by diluting the standards.

The reproducibility of the HPLC–TLS technique was verified by 10 consecutive injections of a 20-times diluted mixed standard stock solution. The results of the reproducibility test are presented graphically in Fig. 2, which shows the variations in peak areas for each carotenoid during the reproducibility test performed over a 3.5 h time period. The relative standard deviations are generally between 4 and 5%, which is expected for TLS measurements. The relative standard deviation for lycopen is, however, more than 12%. This can be attributed to the longer retention time of lycopen and to the associated larger dispersion in the chromatographic system which contributes to uncertainty of

Table 1
Absolute response factors (ARF), expressed as (peak area/pg)×1000, for β-cryptoxanthine (β-Cryx), α-carotene (α-Car), *trans*-β-carotene (t-β-Car), and lycopen (Lyc) at different concentrations

Compound	Injected at 5× dilution	ARF				
		Dilution				
		5×	10×	20×	30×	40×
β-Cryx	199 pg	69.8	77.7	71.9	88.9	85.9
α-Car	185 pg	73.8	72.5	77.8	90.5	83.7
t-β-Car	833 pg	73.5	72.5	77.3	87.9	79.3
Lyc	630 pg	55.0	51.4	51.4	58.4	64.5

the peak area. Since lycopen is the least stable among the investigated carotenoids its decomposition during the reproducibility test might additionally contribute to larger standard deviations in lycopen concentration compared to those observed with other carotenoids.

To evaluate the linearity between the peak area and the concentration, the dynamic range and the LOD of the HPLC–TLS method, calibration curves for investigated carotenoid compounds were prepared by injections of 0–70-times diluted mixed standard stock solution. The results, collected in Table 2, indicate good linearity and a dynamic range exceeding two orders of magnitude for all carotenoids investigated. The differences in dynamic ranges are due to different concentrations of individual carotenoids in the mixed standard solutions used for the preparation of calibration curves. However, because of the very similar absorption coefficients of different carotenoids, dynamic ranges from about 1 to 420 ng/ml can be expected for each carotenoid under the specified conditions. The LOD values reported in Table 2 differ quite significantly for each individual carotenoid and reflect the differences in their absorption coefficients as well as the background signal noise levels at the positions of corresponding chromatographic peaks. The reported LODs represent a two orders of magnitude improvement when compared to LODs for the HPLC procedure with UV–Vis detection and over a 10-fold improvement compared to previously reported values for HPLC with TLS detection [16].

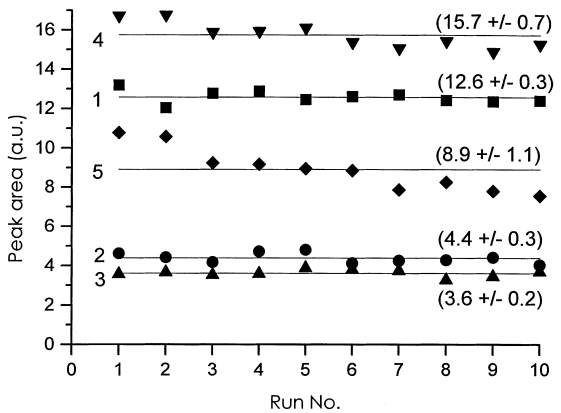

Fig. 2. Peak areas from 10 consecutive injections of a 20-times diluted mixed carotenoid standard stock solution. Solid lines indicate the mean peak area value and the numbers in brackets represent mean values and related standard deviations for each carotenoid (the corresponding peak numbers are displayed on the left-hand side of each line).

As a final step in the validation of the HPLC–TLS technique, a pool blood plasma sample, used as an in-laboratory control sample, was analyzed by employing TLS and UV–Vis detection under identical HPLC conditions. A typical HPLC–TLS chromatogram obtained at 50-times dilution of the pooled blood plasma sample extract is shown in Fig. 3. Chromatographic peaks of α-carotene and *trans*-β-carotene are well resolved, while the peaks of β-cryptoxanthin and lycopen overlap slightly with

Table 2
Dynamic ranges, regression coefficients (*R*) of calibration curves and LOD values for HPLC–TLS determination of β-cryptoxanthine (β-Cryx), α-carotene (α-Car), *trans*-β-carotene (t-β-Car), and lycopen (Lyc) in a mixed standard solution

Compound	Dynamic range (ng/ml)	*R*	LOD (pg/ml)
β-Cryx	1.4–100	0.9973	57
α-Car	1.3–93	0.9993	65
t-β-Car	6–416	0.9995	80
Lyc	2.3–158	0.9983	70

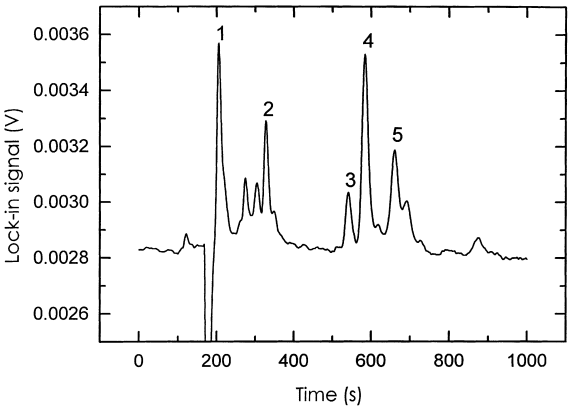

Fig. 3. Chromatogram of a 50-times diluted extract from a pooled blood plasma sample obtained by HPLC with TLS detection. Peak identifications: **1** – lutein+zeaxanthine, **2** – β-cryptoxanthine, **3** – α-carotene, **4** – *trans*-β-carotene, and **5** – lycopen.

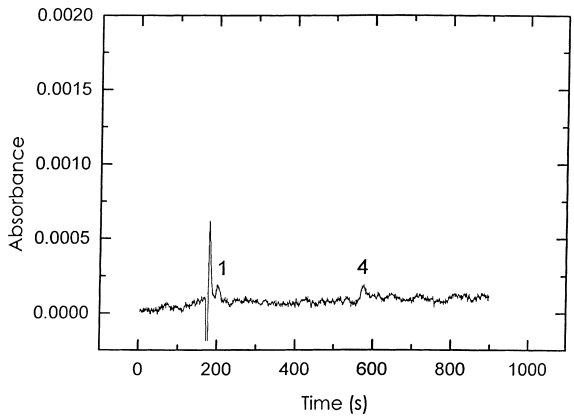

Fig. 4. Chromatogram of a 50-times diluted extract from a pooled blood plasma sample obtained by HPLC with UV–Vis detection. Identified peaks: **1** – lutein+zeaxanthine, **4** – *trans*-β-carotene.

unidentified interfering compounds. Quantification of lutein and zeaxanthin, which coelute, is, however, impossible due to the decrease of the signal. This originates from the difference in refractive indexes of the mobile phase and the solvent used to dissolve the solid residue after the extraction of carotenoids and evaporation of hexane (50% THF in MeOH). The change in refractive index distorts the probe beam and causes a loss of photothermal signal when the solvent from the injected sample reaches the HPLC cell.

Very important, however, is the fact that the chromatographic peaks are well above the baseline noise, which is not the case for the HPLC–UV–Vis technique. As shown in Fig. 4, at the same dilution of the pooled blood plasma sample extract, the concentrations of β-cryptoxanthine, α-carotene and lycopen are already below the LOD values of UV–Vis detection. The only peak observed in the chromatogram is the peak of *trans*-β-carotene. Its concentration is, however, close to the LOD provided by the HPLC–UV–Vis technique.

Based on the previously discussed calibration curves, the concentrations of carotenoids were calculated from chromatograms obtained at different dilutions of pooled blood plasma sample extracts (Tables 3 and 4). The target values needed for comparison purposes were taken from records on measurements with a chromatographic procedure which was previously verified and applied on a routine basis as a standard method for the determination of carotenoids in blood plasma by HPLC with UV–Vis detection. The procedure is actually a combination of various methods for the determination of carotenoids in blood plasma [1,7,8].

The agreement between the TLS values for a particular carotenoid at different dilutions is very good except for the 50-times dilution where, compared to the results at other dilutions, consistently lower values (11–25%) were obtained for all carotenoids. When all data for diluted samples analyzed by HPLC–TLS are inspected (Tables 1 and 3), no trend of decreasing values for increasing dilution factor can be observed. Therefore, the most probable explanation for the lower results at 50-times dilution is a systematic error introduced during this particular dilution step. Taking into account that the results for diluted samples in Table 3 are single measurement values, the range of concentrations spanning about two standard deviations of the average value is still reasonably good and acceptable at these extremely low concentrations.

The results of both techniques demonstrate good agreement between measured concentrations and the target values for α-carotene and *trans*-β-carotene obtained by the independent standard HPLC–UV–Vis procedure. The HPLC–TLS technique also gives good agreement for target values of β-cryptoxanthine, while the corresponding values from UV–Vis detection are about 25% too low. An even larger

Table 3
Concentrations of carotenoids in the in-laboratory control sample obtained by the HPLC–TLS technique. All values for diluted samples were recalculated for the original blood plasma sample. The number of measurements taken to obtain the average concentration value of a particular carotenoid is indicated within subscript parentheses

Compound	Target value and (range) (ng/ml)	Found (ng/ml)					
		Average value	Dilution				
			2×	10×	20×	30×	50×
β-Cryx	252 (210–301)	$251\pm26_{(5)}$	278	250	258	266	207
α-Car	109 (76–128)	$111\pm9_{(5)}$	115	120	109	114	97
t-β-Car	457 (390–510)	$437\pm46_{(5)}$	490	433	433	463	367
Lyc	Not available	$239\pm20_{(5)}$	242	257	240	249	205

Table 4
Concentrations of carotenoids in the in-laboratory control sample obtained by the HPLC–UV–Vis technique. All values for diluted samples were recalculated for the original blood plasma sample. The number of measurements taken to obtain the average concentration value of a particular carotenoid is indicated within subscript parentheses. The average of two replicate analyses is given for each dilution

Compound	Target value and (range) (ng/ml)	Found (ng/ml)					
		Average value	Dilution				
			2×	10×	20×	30×	50×
β-Cryx	252 (210–301)	$185\pm59_{(8)}$	180	182	203	216	≤LOD
α-Car	109 (76–128)	$108\pm33_{(4)}$	94	121	≤LOD	≤LOD	≤LOD
t-β-Car	457 (390–510)	$429\pm115_{(10)}$	372	405	418	473	477
Lyc	Not available	$120\pm36_{(4)}$	145	96	≤LOD	≤LOD	≤LOD

discrepancy (a factor of two) between the TLS and UV–Vis results was observed for lycopen, but cannot be compared to target values for lycopen, which were not available from the independent HPLC–UV–Vis procedure. Furthermore, despite the manual injection of samples and additional dilution of plasma samples (which, in principle, both introduce additional uncertainty into the measurements when compared to the standard procedure) all relative standard deviations of HPLC–TLS values are actually within 10%. The HPLC–TLS results are also well within the tolerance range which represents a standard deviation for the concentrations of carotenoids obtained by daily HPLC–UV–Vis analysis of a pooled plasma control sample over a period of several years. This, however, was not the case with the HPLC–UV–Vis results obtained under chromatographic conditions identical to HPLC–TLS (Table 4). As already noted (Fig. 4), some carotenoids could not be detected at higher dilution, due to the higher LODs of UV–Vis detection. For the same reason the HPLC–UV–Vis results exhibit a standard deviation of up to 30% which is about three times larger compared to the HPLC–TLS results. This, as well as the inaccuracy of HPLC–UV–Vis already discussed for the case of β-cryptoxanthine and lycopen, stems from the fact that at the dilution of blood plasma samples used in this experiment the concentrations of some carotenoids were close or even below the LOD values of UV–Vis detection in blood plasma samples (given in Table 5 together with the LOD values for HPLC–TLS detection). Because of the matrix effect, the LOD values for

Table 5
LOD values for HPLC–UV–Vis and HPLC–TLS determination of β-cryptoxanthine (β-Cryx), α-carotene (α-Car), *trans*-β-carotene (t-β-Car), and lycopen (Lyc) in blood plasma

Compound	LOD (ng/ml)	
	HPLC–UV–Vis	HPLC–TLS
β-Cryx	6.1	0.070
α-Car	7.8	0.085
t-β-Car	11	0.100
Lyc	13	0.120

determination of carotenoids in blood plasma by HPLC–TLS are higher compared to those obtained from mixed standards prepared in the eluent (Table 2), as expected. However, these results still represent an improvement of about 100 times compared to the HPLC–UV–Vis technique used in this work. Furthermore, it should be mentioned that despite the dilution factor of 50 and the five times smaller injection loop volume, which reduced the amounts of carotenoids injected onto the column by 250 times compared to the independent HPLC–UV–Vis procedure [12], the TLS technique still enabled accurate detection of carotenoids after HPLC separation.

4. Conclusions

It has been demonstrated that TLS is a suitable detection technique for HPLC analysis of *trans*-β-carotene and other carotenoids in complex samples such as blood plasma. Validation of the technique confirmed the good reproducibility and accuracy of HPLC–TLS, as demonstrated by the good agreement between the results from HPLC–UV–Vis and HPLC–TLS measurements, and the target values for the in-laboratory control sample.

The advantages of TLS compared to UV–Vis detection include higher sensitivity and about a 100-times lower LOD for carotenoids in blood plasma. This enables the accurate determination of carotenoids at sub-nanogram per millilitre concentrations in samples which cannot be analyzed by the HPLC–UV–Vis technique due to insufficient sensitivity.

Furthermore, HPLC–TLS allows injection of smaller volumes and smaller sample quantity. This minimizes the problem of incompatibility between the solvent used to dissolve the dry residue, remaining after the extraction and evaporation of hexane, and the mobile phase. A larger injection loop (50 μl), such as used with the independent standard procedure that provided the target values, affects the resolution of the separation considerably and requires a mobile phase with a smaller concentration of THF (2%) in MeOH and, therefore, considerably longer times for completion of the analysis (45 min). Additional improvements in the reduction of both the time needed for analysis and consumption of solvents are possible when applying TLS detection to the microbore column HPLC, where the possibility of tight focusing of laser beams is a significant advantage in comparison to conventional light sources used in UV–Vis detection.

The emphasis in this work was placed on the sensitivity rather than the selectivity of the analytical procedure. It has to be pointed out, however, that the selectivity of the technique can be easily improved by applying a different eluent and possibly also gradient elution. With this included, the interfering effects of some compounds [6], which affect the accuracy of the technique in the case of samples such as fruit and vegetable juices, will be avoided. Therefore, by selecting appropriate separation conditions the HPLC–TLS technique could be used for samples other than blood plasma. Some possible applications which will be investigated in the near future include determination of carotenoid patterns for verification of fruit juice authenticity, measurements of carotenoids associated with the biological production in non-polluted sea-water and the identification of pollution sources based on carotenoid patterns in water.

Acknowledgements

This research was supported in part by the Ministry of Science and Technology of the Republic of Slovenia and partly by the Inco-Copernicus grant of EC (ERBIC 15 CT 961003).

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