Journal of Chromatography A, 1231 (2012) 59–65

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of lutein by high-performance thin-layer chromatography using densitometry and screening of major dietary carotenoids in food supplements

Z. Rodić [a], B. Simonovska [a], A. Albreht [a], I. Vovk [a,b,*]

[a] National Institute of Chemistry, Laboratory for Food Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia
[b] EN-FIST Centre of Excellence, Dunajska 156, SI-1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 2 January 2012
Received in revised form 24 January 2012
Accepted 25 January 2012
Available online 4 February 2012

Keywords:
HPTLC
Food supplements
Carotenoids
Lutein
HPTLC–MS

ABSTRACT

The main problem in the densitometric determination of carotenoids is their rapid degradation during and immediately after chromatography, respectively. In this study, we show that 15 ng of lutein, lycopene and β-carotene standards applied on C_{18} RP high-performance thin-layer chromatography (HPTLC) plates pre-developed with dichloromethane–methanol 1:1 (v/v) remained stable for 1 h after the development of chromatogram using methanol–acetone 1:1 (v/v) with 0.1% of 2-*tert*-butylhydroquinone (TBHQ), which is a substantial improvement of their stability. An HPTLC quantification procedure for free lutein, with densitometry at 450 nm based on the developed method described above, was established and validated. Repeatabilities of the chromatography expressed by the relative standard deviation (RSD) from 6 applications of lutein standard at 5, 15 and 25 ng were 3.41, 1.33 and 1.65%, respectively. The best fit calibration curve from 5 ng to 30 ng of lutein was polynomial. Limit of detection (1.5 ng) and limit of quantification (5 ng) were the best achieved so far. With these chromatographic conditions dietary carotenoids lutein esters, lycopene, free lutein and β-carotene from food supplements were also well separated and were identified by visible absorption spectra scanned in situ and by mass spectra. Some additional developing solvents with the same type of chromatographic layer are proposed for the fast separation of lutein esters from free lutein in food supplements.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Carotenoids are considered one of the most important groups of natural pigments. They contribute yellow to red color to many flowers, fruits and vegetables. Beside in plants, carotenoids are biosynthesized in some fungi, bacteria and algae, while their occurrence in animals and humans is dependent on the dietary intake. Carotenoids are beneficial to human health owing to their antioxidant properties and provitamin A activity of some of them. Carotenoids structurally belong to the tetraterpenes and are classified into two subgroups, carotenes which are pure hydrocarbons (i.e. lycopene, α- and β-carotene), and oxocarotenoids or xanthophylls (i.e. lutein, zeaxanthin and β-cryptoxanthin), which possess at least one oxygen atom in their molecule. Xanthophylls with hydroxyl groups can be found in nature in the form of higher fatty acid esters [1].

In our research a particular attention was given to lutein, a non-provitamin A xanthophyll, which plays an essential role in eye health. Together with its structural isomer zeaxanthin it is concentrated in the yellow spot of the eye retina (*macula lutea*) and acts as a blue light filter and thus can contribute to the prevention of age-related macular degeneration and cataracts [2]. The major dietary sources of lutein are green leafy vegetables [3–5], such as spinach [6] or kale [7], squashes and several other vegetables, some fruits [4] and egg yolks [5]. When dietary intake of lutein is not sufficient, it can be supplemented by dietary supplements [2]. In food supplements lutein occurs in two forms, as free lutein or a mixture of lutein diesters with saturated higher fatty acids. Inappropriate labeled products regarding lutein are often found on the market [8].

Nowadays, HPLC is the technique of choice for the analysis of carotenoids [9,10], although due to their chromophores carotenoids can easily be detected by TLC. Furthermore, there are some advantages of HPTLC over HPLC in qualitative and quantitative analysis, such as lower solvent consumption, minimal sample preparation, and concurrent analysis of high throughput with minimal costs, just to name a few of them [11]. Recently a review article regarding TLC analysis of carotenoids in plant and animal samples was published where the need for studying the application of scanning densitometry in quantification of carotenoids was emphasized [12]. TLC analysis of carotenoids is demanding due to their instability. They are namely prone to isomerization, oxidation, and degradation when exposed to light, heat, oxygen, or acids.

* Corresponding author at: National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia. Tel.: +386 14760341; fax: +386 14760300.
E-mail address: irena.vovk@ki.si (I. Vovk).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2012.01.085

Sorbent itself can play a crucial role as its active surface can accelerate the degradation of carotenoids [9]. On C_{18} reversed-phase silica gel plates, where carotenoids are more stable than on ordinary silica gel, methanol, acetonitrile, acetone and petrol ether or *n*-hexane are used in different combinations and ratios as developing solvents for their separation [8,13–16]. There are some articles describing quantitative HPTLC analysis of lutein [8,9,13–17]. However, these methods lack sensitivity and selectivity, so there are possibilities for improvements, especially because fast, selective and sensitive methods are needed for the separation and quantification of carotenoids present in food supplements.

The aim of this work was to develop an HPTLC method for densitometric determination of lutein. Special attention was devoted to the enhancement of lutein stability during and after the chromatography. By using the same method, screening of major dietary carotenoids in food supplements was made and their structure confirmed by mass spectra and in situ visible spectra. Distinguishing lutein esters from free lutein on C_{18} RP HPTLC plates by using some additional developing solvents is also presented.

2. Experimental

2.1. Chemicals and standards

All solvents were of analytical grade. Acetone and 2-*tert*-butylhydroquinone (TBHQ) (≥97%) were from Sigma–Aldrich (St. Louis, MO, USA), methanol was from J.T.Baker (Deventer, The Netherlands), dichloromethane, ethyl acetate, *n*-hexane, tetrahydrofuran (THF), triethylamine (TEA) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) (≥99%) were purchased from Merck (Darmstadt, Germany). Lutein (95%) was from Extrasynthèse (Genay, France), β-carotene (≥97%) was from Fluka (Buchs, Switzerland) and lycopene was from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of standard solutions

Lutein standard stock solution was prepared by dissolving approximately 1 mg of accurately weighed lutein standard in 10 mL of ethyl acetate containing 0.1% BHT. Exact lutein concentration in stock solution was rechecked spectrophotometrically (Lambda 45 UV/VIS Spectrometer, Perkin Elmer, Waltham, USA), considering molar absorption coefficient of lutein in ethyl acetate at 446 nm 143,900 L mol^{-1} cm^{-1} [18]. Working solutions were prepared by appropriate dilution of standard stock solution with ethyl acetate containing 0.1% BHT to concentrations of lutein 0.5, 1, 2 and 5 ng/μL. Standard solutions of β-carotene and lycopene were prepared by dissolving lycopene in acetone containing 0.1% BHT and β-carotene in ethyl acetate containing 0.1% BHT. Concentration of working standard solutions of lycopene and β-carotene was 5 ng/μL. Standard solutions were kept in tightly sealed storage vials (National Scientific Company, USA) at −80 °C.

2.3. Sample preparation for carotenoid screening in food supplements

Six different brands of lutein food supplements were purchased from local drug stores. Brands 1, 2, 4 and 5 were in the form of hard gelatin capsules filled with powder, while brands 3 and 6 were tablets. In all cases lutein was declared to be present as free lutein. Samples were taken out from the capsules, frozen in liquid nitrogen and pulverized by Mikro-Dismembrator S (Sartorius, Göttingen, Germany) using a frequency of 1700 min^{-1} for 1 min. Approximately 8–32 mg of each sample was accurately weighed and transferred into 10 mL volumetric flasks. Lutein and other carotenoids were extracted by ethyl acetate containing 0.1% BHT in ultrasonic bath for 30 min. After extraction samples were centrifuged and the supernatant was filtered through 0.45 μm Millex-MV hydrophobic polyvinylidene difluoride (PVDF) membrane filter (Millipore, Billerica, MA, USA). The obtained sample test solutions were then applied on HPTLC plate.

2.4. Evaluation of lutein, β-carotene and lycopene stability on the C_{18} RP HPTLC plates

Stability of lutein was evaluated on 4 cm × 10 cm reversed phase C_{18} RP HPTLC glass-backed plates with layer thickness 0.20 mm (Merck, Art. No.: 1.05914.0001). Plates were used without predevelopment or they were predeveloped with methanol–dichloromethane 1:1 (*v*/*v*) or methanol–dichloromethane 1:1 (*v*/*v*) containing 0.5% TEA and dried for 20 min at 100 °C. On each plate 15 ng of lutein was applied in two 6 mm bands 12 mm from side edges and 10 mm from the bottom edge. Plates were developed ascendently at ambient temperature to a distance of 7 cm in 10 cm × 10 cm twin trough chamber (Camag, Muttenz, Switzerland) lined with filter paper, and saturated for 30 min with 5 mL of developing solvent in each trough. Developing solvents were methanol–acetone 1:1 (*v*/*v*) with or without addition of 0.5% TEA, 0.1% BHT or 0.1% TBHQ. After development, plates were dried under a stream of cool air from a hair dryer. Stability of lutein was evaluated by densitometry immediately after development and after 15, 30 and 60 min, meanwhile the plates were stored in the darkness at room temperature. The mean value of peak area from two applications was considered for the final result. Stability of β-carotene (15 ng) and lycopene (15 ng) was estimated under the best conditions obtained for lutein on plate predeveloped with methanol–dichloromethane 1:1 (*v*/*v*) and using methanol–acetone 1:1 (*v*/*v*) with 0.1% TBHQ as developing solvent.

2.5. Thin-layer chromatographic analysis

Thin-layer chromatographic analyses were performed on 20 cm × 10 cm C_{18} RP HPTLC glass-backed plates with layer thickness 0.20 mm (Merck). Before use the plates were pre-developed to the top using dichloromethane–methanol 1:1 (*v*/*v*) and dried for 20 min at 100 °C in the oven. Solutions of lutein, β-carotene and lycopene standards and sample test solutions were applied on the plates as 6 mm bands 20 mm from the side edges, 10 mm from the bottom and 8.8 mm apart by Automatic TLC Sampler 4 (Camag, Muttenz, Switzerland) equipped with a 25-μL microsyringe. Plates were developed ascendently at ambient temperature to a distance of 7 cm in twin trough chamber 20 cm × 10 cm (Camag) lined with filter paper, and saturated for 30 min with 10 mL of developing solvent methanol–acetone 1:1 (*v*/*v*) with 0.1% TBHQ in each trough. Developing time was 7 min. After development, plates were dried under a stream of cool air from a hair dryer.

The plates were documented by Camag Digistore 2 documentation system (Camag) in white light transmission mode. Afterwards tracks were scanned by Camag TLC scanner 3 in absorption/reflectance mode at λ = 450 nm and spectra were recorded from 400 nm to 600 nm with tungsten source. Slit dimensions were: length 4 mm, width 0.3 mm, and scanning speed was 20 mm/s. Quantitative evaluation was established through peak area. Both instruments were controlled by the winCATS program (Version 1.4.1.8154).

2.6. Mass spectrometry

TLC–MS interface (Camag) was used for the elution of free lutein, lutein esters, lycopene and β-carotene from the C_{18} RP HPTLC plate using methanol–ethyl acetate 3:1 (*v*/*v*) at 0.5 mL/min; 0.2% acetic

Table 1
Influence of the sorbent impregnation and addition of TEA, BHT or TBHQ to the developing solvent on stability of lutein standard (15 ng) applied on the C_{18} RP HPTLC plates after development.

Time [min]	Peak area at time t/peak area at t_0 = 0 min × 100%											
	A_x	A_y	A_z	B_x	B_y	B_z	C_x	C_y	C_z	D_x	D_y	D_z
0	100	100	100	100	100	100	100	100	100	100	100	100
15	33	66	69	61	83	84	60	77	76	96	97	99
30	20	35	47	33	58	66	49	58	62	86	94	97
60	17	20	31	21	29	38	31	41	44	76	91	91

Pre-developing solvents:
x) None.
y) Methanol–dichloromethane 1:1 (v/v).
z) Methanol–dichloromethane 1:1 (v/v) + 0.5% TEA.
Developing solvent:
A) Methanol–acetone 1:1 (v/v).
B) Methanol–acetone 1:1 (v/v) + 0.5% TEA.
C) Methanol–acetone 1:1 (v/v) + 0.1% BHT.
D) Methanol–acetone 1:1 (v/v) + 0.1% TBHQ.

acid in methanol at 0.1 mL/min was added to the effluent prior to injecting the solution into the ion trap LCQ MS system (Thermo Finnigan, San Jose, CA, USA) controlled by Thermo Finnigan Excalibur software. Atmospheric-pressure chemical ionization (APCI) source in positive mode was employed for the scanning of mass spectra of the compounds. The capillary and APCI probe were maintained at 200 °C and 400 °C, respectively. The source voltage was set to 5.5 kV and the sheath and auxiliary gas flow rates were set to 20 and 0 a.u. (arbitrary units), respectively.

3. Results and discussion

3.1. Stability of the carotenoids on the plate after the development

It was found that chloroplast pigments degraded less on C_{18} bonded silica gel and cyano-functionalized silica gel than on the ordinary silica gel [13,17]. To increase stability of carotenoids on silica gel, neutralization of acidity by addition of 0.1–0.5% TEA to the developing solvent was recommended [19]. In order to achieve more reliable TLC analysis of carotenoids, we studied the effect of plate impregnation in the pre-development stage and addition of TEA or antioxidants (BHT or TBHQ) to the developing solvent on stability of lutein applied on the plate. BHT, the well-known synthetic antioxidant used in preservation of foods and cosmetics, is often used in analytical procedures for keeping sensitive compounds, such as carotenoids, stable in every step of the analysis, from the preparation of sample test solutions [3,20] to the quantification by HPLC [21,22]. TBHQ is a related antioxidant with similar usage in foods and cosmetics, but it is much less employed in analytics. It was proposed to be used as an additive during extraction of carotenoids from spinach [23], however, it was never intended for the stabilization of carotenoids in TLC. The results of our stability study are summarized in Table 1. On C_{18} RP HPTLC plates without pre-development lutein decomposed rapidly and 15 min after the development with methanol–acetone 1:1 (v/v) only 33% of lutein remained unaltered. If the plates were pre-developed with dichloromethane–methanol 1:1 (v/v) the deterioration of lutein occurred to a lesser extent and after 15 min only 34% of lutein degraded. The stability of lutein was not significantly increased by addition of TEA to the pre-developing solvent. Nevertheless, the stability of lutein on the sorbent was substantially prolonged by addition of 0.5% TEA, 0.1% BHT or 0.1% TBHQ to the developing solvent methanol–acetone 1:1 (v/v). TBHQ proved to be the best choice as it preserved lutein almost intact for 15 min after plate development and if plates were pre-developed with dichloromethane–methanol 1:1 (v/v) only 9% of lutein degraded in 1 h after the development, which should enable quantitative determination of lutein by densitometry. Moreover, the peaks in the densitograms were sharp and symmetrical, without tailing or fronting, which is supposed to be a common problem in TLC analysis of carotenoids [19]. Incorporation of BHT or TBHQ into the sorbent at the pre-development stage was not suitable due to the appearance of secondary front and fronting of lutein peaks in the densitograms. Chromatographic conditions at which lutein was the most stable were applied also to study stability of lycopene and β-carotene. As it is evident from Table 2, all carotenoids show similar degradation trend in the first hour.

Instability of carotenoids on TLC plates prevents a wider use of scanning densitometry. From the major dietary carotenoids, β-carotene proved to be very unstable on silica gel and C_{18} reversed-phase but incorporation of 2% of BHT in the layer markedly improved its stability. However, this measure influenced the selectivity of the chromatographic system [24]. Stability of photosynthetic pigments for several days at 4 °C in dark was also studied on HPTLC CN plates [17], but the results obtained are not reliable for routine densitometry since the needed short-time stability (e.g. 30 min) was calculated from the decay equation in their conditions. The loss of lutein was estimated to be much lower than measured in our study at similar TLC conditions (no predevelopment, no additive): 2.3% and 80% loss after 30 min, respectively (Table 1). The main reason for the big difference in losses was the amount of lutein applied which was in our case only 15 ng and in their case about 2 μg.

3.2. Quantification of lutein

Quantification of free lutein (Fig. 1) was performed according to the procedure described in Section 2.5. The calibration plot, the peak area vs. the amount of lutein, derived from 6 applications of lutein standard solution in the range of 5–30 ng per band was polynomial (Fig. 2). Other authors worked in the range of 80–320 ng of lutein at the determination of lutein in dietary supplements [8] and 40–160 ng of lutein to determine the content of lutein in *Pomacea*

Table 2
Stability of β-carotene (15 ng), lycopene (15 ng) and lutein (15 ng) standards on the C18 RP HPTLC plates pre-developed with methanol–dichloromethane 1:1 (v/v); developing solvent methanol–acetone 1:1 (v/v) with 0.1% TBHQ.

t [min]	Peak area at time t/peak area at t_0 = 0 min × 100%		
	β-Carotene	Lycopene	Lutein
0	100	100	100
15	99	99	99
30	95	95	97
60	87	88	91

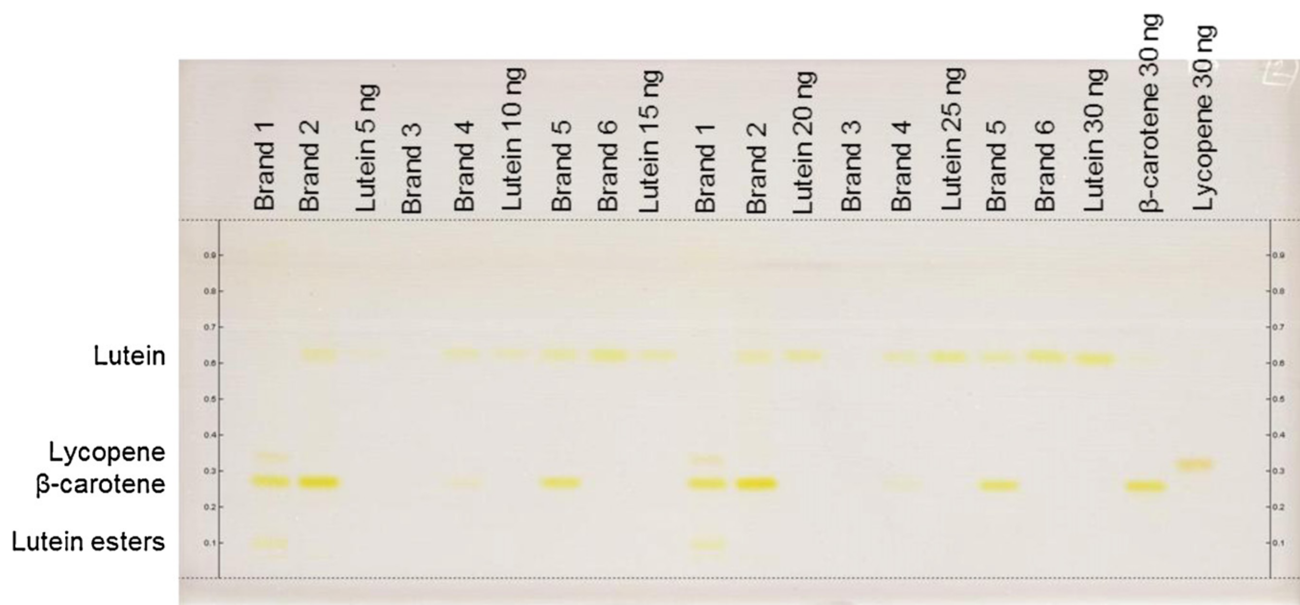

Fig. 1. C_{18} RP HPTLC plate with sample test solutions and lutein standards of lutein, β-carotene and lycopene developed by methanol–acetone 1:1 (v/v) + 0.1% TBHQ (lutein esters R_F = 0.04, β-carotene R_F = 0.24, lycopene R_F = 0.32 and lutein R_F = 0.68).

bridgesii [14] and *Biomphalaria glabrata* and *Helisoma trivolvis* (Colorado and Pennsylvania strains) snails [16]. Quantification of the main chloroplast pigments from spinach by HPTLC scanning densitometry immediately after the development was performed on a C_{18} layer [13]. The calibration range was from 20 ng to 160 ng for lutein and β-carotene. Since the standards were co-chromatographed in lanes next to the samples it was probably considered that the degradation could be kept at a minimum by scanning immediately after the development and, in this case, the accuracy and the precision of the results were not affected significantly. However, working in the conditions without degradation of carotenoids as in our case is obviously more favorable: the intermediate precision performed by 18 applications of lutein standard solution, with 6 applications near LOQ (5 ng), 6 application at the middle range (15 ng) and 6 applications at the upper range of the calibration plot (25 ng) resulted in the following RSD at each level: 3.40%, 1.33% and 1.65%, respectively. The limit of detection (LOD) and limit of quantification (LOQ) of lutein estimated on the basis of signal to noise ratio (S/N) according to equations LOD = 3 × S/N and LOQ = 10 × S/N were found to be 1.5 ng and 5 ng, respectively, which is far lower LOQ than 40 ng [8] or 7 ng [17]. Visually, 5 ng of each carotenoid was detectable.

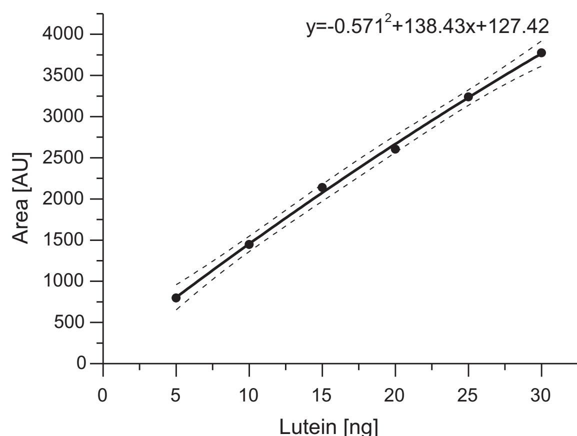

Fig. 2. Polynomial calibration curve with 95% confidence limits for determination of lutein.

3.3. Screening of carotenoids in food supplements

The chromatographic method was expected to be selective for the determination of free lutein in food supplements. The only compound which was expected to interfere in the determination of lutein was its structural isomer zeaxanthin, which may be present in some food supplements as a minor component. Interferences caused by zeaxanthin could be identified by visible spectroscopy as its absorption maximum is expected to be at 455 nm.

The developed HPTLC method, described in Section 2.5 was used to establish carotenoid profiles of some food supplements and to find eventual interference with determination of free lutein. It was found that major carotenoids present in analyzed food supplements were lutein esters, lycopene, β-carotene and free lutein, and they were separated as sharp symmetrical bands as shown in Fig. 1. Lycopene had a dark orange, almost red, appearance, while β-carotene, lutein and lutein esters appeared yellow. In situ visible spectra presented in Fig. 3 were scanned from 400 nm to 600 nm. As expected, spectra of lutein and lutein esters were very similar. Absorption maximum of lutein on the C_{18} RP HPTLC plate occurred at 450 nm which was a few nm higher in comparison to its absorption maximum in ethyl acetate (λ_{max} = 446 nm) or ethanol (λ_{max} = 445 nm) [18]. The spectrum of β-carotene was somewhat deformed compared to the spectrum in *n*-hexane obtained by a spectrophotometer probably because of the partial oxidation of the compound on the plate but the maximum occurred at about 455 nm, as expected. Lycopene is the most unstable amongst the major dietary carotenoids but a satisfactory spectrum with the maximum at about 475 nm was obtained as the consequence of the stabilization of lycopene on the plate. The chromatographic conditions in which lutein was not stable were not suitable for scanning in situ UV–VIS spectra. The absorption maxima were moved to shorter wavelengths (from 449 nm to 428.5 nm), at 2.3 μg and 71 ng applied, respectively [17], presumably due to the formation of degradation products on the plate and the appearance of lutein spectrum was changed compared to the spectrum in solution. At the optimum conditions presented in this paper lutein gave the characteristic three peak spectra.

The carotenoid profiles of the chosen lutein food supplements were scanned also by densitometer at 450 nm and the

Table 3
Screening of lutein in selected six food supplements brands.

Brand	Lutein			Other carotenoids	
	Declared		Found	Declared	Found
	Form	[mg/unit]	Form		
1	Lutein	1.5	Lutein esters	β-Carotene, lycopene	β-Carotene, lycopene
2	Lutein	6	Lutein	β-Carotene	β-Carotene
3	Lutein	0.25	Not found	β-Carotene, lycopene	None
4	Lutein esters	0.5	Lutein	β-Carotene	β-Carotene
5	Lutein	3	Lutein	β-Carotene	β-Carotene
6	Lutein	Not declared	Lutein	None	None

densitograms, where peaks of lutein esters, β-carotene, lycopene, and free lutein were sharply resolved on the base line, are presented in Fig. 4. Mixture of lutein esters appeared in one sharp band. Optimized HPTLC system is suitable for the fast screening of the studied carotenoids in different food supplements as shown in Table 3. In brand 1 lutein was present esterified, though the claimed content was 1.5 mg of free lutein together with 6 mg of β-carotene and 1.5 mg of lycopene per capsule. Despite of label claim in brand 3: 250 μg free lutein, 2 mg β-carotene, 300 μg lycopene per tablet, neither free lutein nor other carotenoids were found. Brand 4 contained lutein instead of claimed lutein esters and also β-carotene as declared. Brands 2 and 5 contained lutein and β-carotene as claimed on the label, while brand 6 contained only lutein as declared. Some producers fail to declare the actual form of lutein in food supplements which means that either free lutein, lutein esters, or both could be present. TLC is well suited for a fast differentiation between both forms. Some simple developing solvents for the separation of lutein esters from free lutein on C_{18} RP HPTLC plates are presented in Table 4.

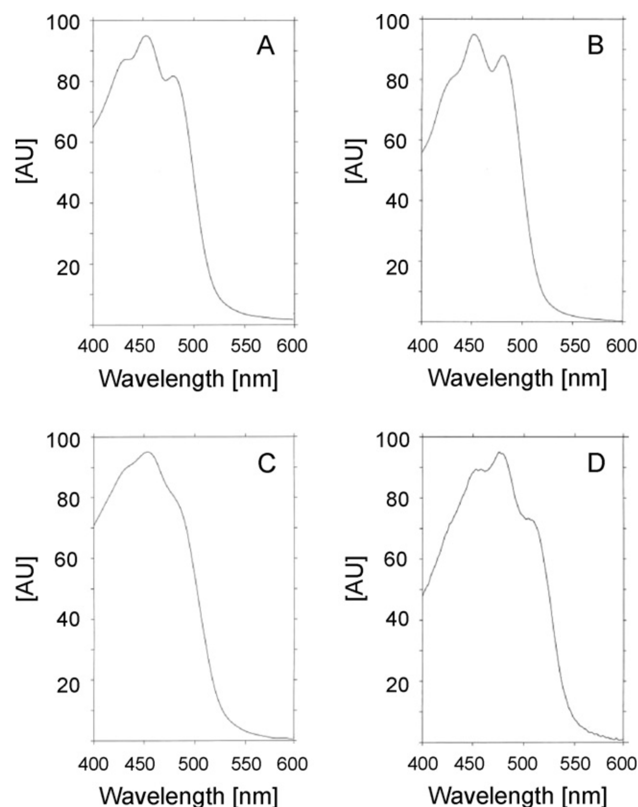

Fig. 3. Spectra of lutein esters (A), free lutein (B), β-carotene (C) and lycopene (D), scanned in visible spectral range on C_{18} RP HPTLC plate developed in methanol–acetone 1:1 (v/v) + 0.1% TBHQ.

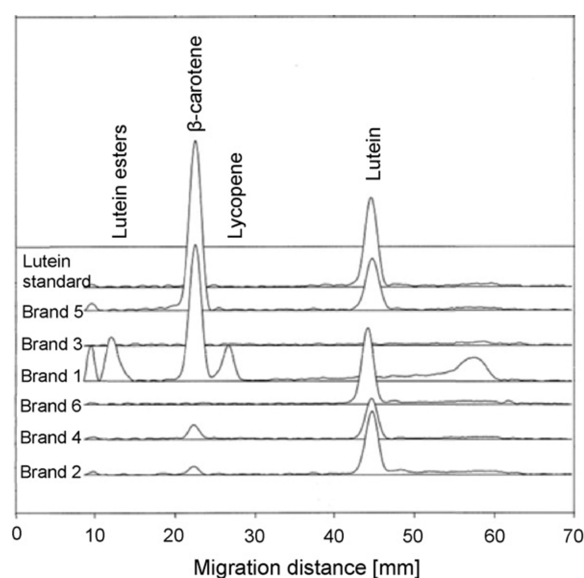

Fig. 4. Densitograms recorded at 450 nm representing 30 ng of lutein standard and carotenoid profiles of tested brands of lutein food supplements on C_{18} RP HPTLC plate developed with methanol–acetone 1:1 (v/v) + 0.1% TBHQ.

Table 4
Separation of lutein esters from free lutein on the C_{18} RP HPTLC plates.

Developing solvent	R_F of free lutein	R_F of lutein esters
Methanol–n-hexane 7:3 (v/v)	0.67	0.13
Methanol–acetone 7:3 (v/v)	0.65	0.04
Methanol–acetone 4:6 (v/v)	0.75	0.16
Methanol–acetone–THF 6:3:1 (v/v)	0.76	0.20
Methanol–acetone–THF 7:2.5:0.5 (v/v)	0.70	0.08
Methanol–acetone–THF 6:4:2 (v/v)	0.79	0.20

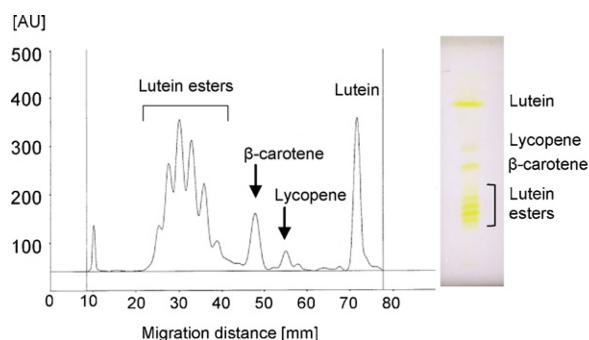

Fig. 5. Partial separation of lutein esters and separation of free lutein on the C_{18} RP HPTLC plate developed in methanol–acetone–n-hexane 1:1:1 (v/v).

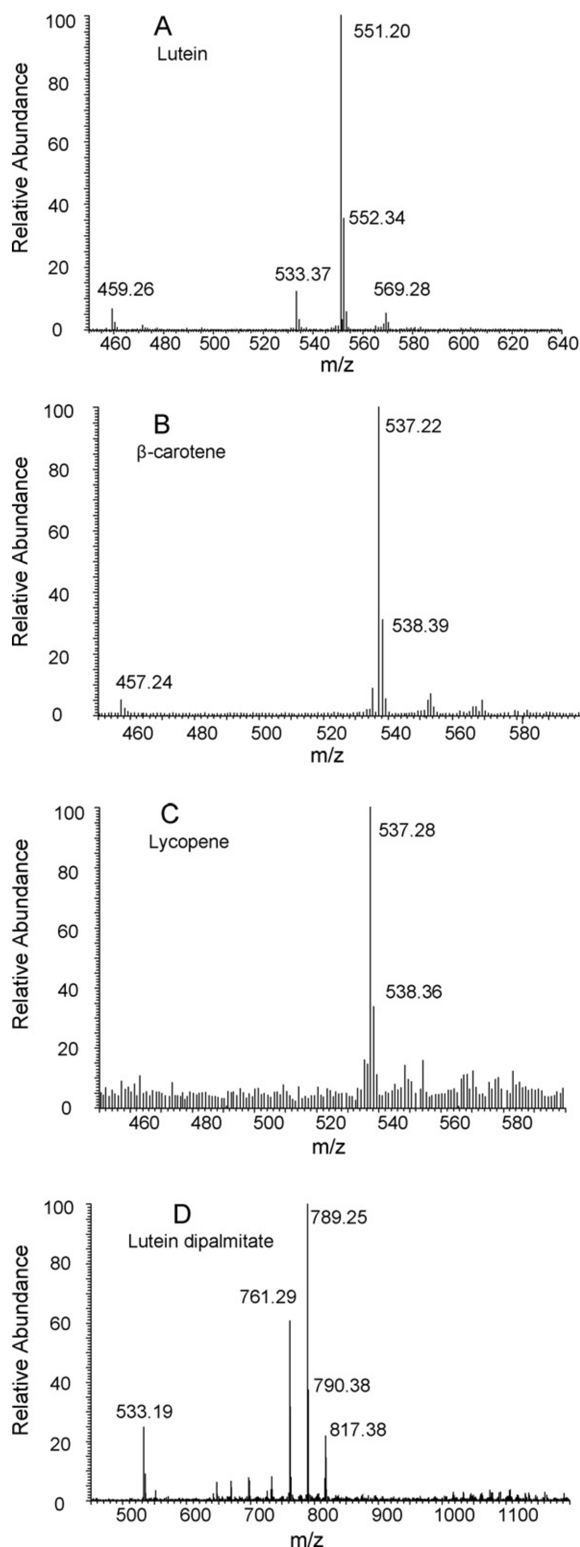

Fig. 6. Mass spectra of lutein (A), β-carotene (B), lycopene (C) and lutein dipalmitate (D) obtained after elution from C_{18} RP HPTLC plate developed in methanol–acetone 1:1 (*v*/*v*) + 0.1% TBHQ.

As it is presented on the densitogram and image (Fig. 5), with a three component developing solvent methanol–acetone–*n*-hexane 1:1:1 (*v*/*v*), the mixture of lutein esters originating from marigold (*Tagetes erecta*), world's main source of lutein for food supplements, appeared at least partially resolved. The group of lutein esters was also well separated from the free lutein, lycopene and β-carotene.

For the determination of lutein in a food supplement the preparation of test solution is the first and very important part of the method. It is usually impossible to apply one preparation method to different types of samples, since they can differ considerably. The preparation method must be developed in separate experiments for each type of sample. Quantitative extraction of relatively high amounts of lutein requires, in most cases, high volumes of organic solvents. Further investigation of extraction conditions is needed to develop a complete method for the quantitative determination of lutein in different food supplements.

3.4. TLC–MS

Identity of the bands in the chromatogram of food supplements was additionally confirmed by mass spectrometry, i.e. by use of Camag TLC–MS interface, which enables direct coupling of TLC to MS without scratching the spots from the sorbent. Fig. 6 shows the mass spectra of free lutein, lutein esters (lutein dipalmitate), β-carotene and lycopene. For lutein, pseudo-molecular ion $[M+H]^+$ and the most abundant fragment ion $[M+H-H_2O]^+$ were clearly observed at *m*/*z* 569.3 and 551.2, respectively (Fig. 6A). Pseudo-molecular ions $[M+H]^+$ for β-carotene (Fig. 6B) and lycopene (Fig. 6C) were observed at *m*/*z* 537.2 and 537.3, respectively. Lutein and zeaxanthin are structurally very similar, they differ only in the position of one double bond on the rings. Only lutein contains an allylic hydroxyl group, which is easier to eliminate than the other hydroxyl group at the secondary carbon atom with saturated neighboring C—C bonds. The formed ion at *m*/*z* 551.2 is stabilized that is why it appears as the most abundant peak in the MS of lutein. In the APCI MS of zeaxanthin the pseudo-molecular ion at *m*/*z* 569.3 occurs as the main peak. Pseudo-molecular ion for lutein dipalmitate (Fig. 6D) was not obtained, but the most abundant peak at *m*/*z* 789.2 corresponds to the fragment $[M+H-C_{15}H_{33}CO-H_2O]^+$.

4. Conclusions

Our results show that HPTLC can be a very powerful technique in qualitative and quantitative analysis of carotenoids. The introduced HPTLC method for the determination of free lutein is accurate, precise and selective and it can be readily employed in routine quality control of lutein in food supplements, foods and plant materials. Furthermore it is also useful in preliminary experiments for carotenoids screening in food supplements. Selected and optimized HPTLC conditions enabled analysis at far lower amounts of lutein than reported so far. All the stability experiments were performed with lutein as a representative of carotenoids. At the optimum TLC conditions for lutein stability, all other carotenoids from the dietary supplements also exhibited increased stability on the plate. Additionally, a new Camag TLC–MS interface was proven as a useful tool for direct identity confirmation or structure elucidation of carotenoids resolved on a TLC plate.

Acknowledgements

This study was supported by a grant from Slovenian Research Agency and EN-FIST Centre of Excellence (Dunajska 156, SI-1000 Ljubljana, Slovenia).

References

[1] G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Vol. 1A, Birkhäuser, Basel, 1995, p. 13.
[2] R.L. Roberts, J. Green, B. Lewis, Clin. Dermatol. 27 (2009) 195.
[3] O. Sommerburg, J.E.E. Keunen, A.C. Bird, F.J.G.M. van Kuijk, Br. J. Ophthalmol. 82 (1998) 907.
[4] E. Murillo, A.J. Meléndez-Martínez, F. Portugal, Food Chem. 122 (2010) 167.
[5] A. Perry, H. Rasmussen, E.J. Johnson, J. Food Compos. Anal. 22 (2009) 9.

[6] R. Aman, R. Carle, J. Conrad, U. Beifuss, A. Schieber, J. Chromatogr. A 1074 (2005) 99.
[7] C.H. Azevedo, D.B. Rodriguez-Amaya, J. Sci. Food Agric. 85 (2005) 591.
[8] J. Sechrist, J. Pachuski, J. Sherma, Acta Chromatogr. 12 (2002) 151.
[9] A.J. Meléndez-Martínez, I.M. Vicario, F.J. Heredia, J. Food Compos. Anal. 20 (2007) 638.
[10] S.M. Rivera, R. Canela-Garayoa, J. Chromatogr. A (2010), doi:10.1016/j.chroma.2011.12.025.
[11] J. Sherma, J. Chromatogr. A 880 (2000) 129.
[12] A. Zeb, M. Murkovic, J. Planar Chromatogr. 23 (2010) 94.
[13] J. Sherma, C.M. O'Hea, B. Fried, J. Planar Chromatogr. 5 (1992) 343.
[14] J.A. Jarusiewicz, B. Fried, J. Sherma, Comp. Biochem. Physiol. B 143 (2006) 244.
[15] T. Hayashi, H. Oka, Y. Ito, T. Goto, N. Ozeki, Y. Itakura, H. Matsumoto, Y. Otsuji, H. Akatsuka, T. Miyazawa, H. Nagase, J. Liq. Chromatogr. Relat. Technol. 26 (2003) 819.
[16] R.T. Evans, B. Fried, J. Sherma, Comp. Biochem. Physiol. B 137 (2004) 179.
[17] M.H. Daurade-Le Vagueresse, M. Bounias, Chromatographia 31 (1991) 5.
[18] N.E. Craft, J.H. Soares, J. Agric. Food Chem. 40 (1992) 431.
[19] G. Britton, in: M. Waksmundzka-Hajnos, J. Sherma, T. Kowalska (Eds.), Thin Layer Chromatography in Phytochemistry, CRC Press, New York, 2008, p. 543.
[20] G.E. Lester, G.J. Hallman, J.A. Pérez, J. Agric. Food Chem. 58 (2010) 4901.
[21] D. Kopsell, M.G. Lefsrud, D.E. Kopsell, J. Agric. Food Chem. 54 (2006) 7998.
[22] D.J. Hart, K.J. Scott, Food Chem. 54 (1995) 101.
[23] K. Černelič, B. Simonovska, I. Vovk, in: J. Blattaná, A. Horna, T. Zima, A. Zobel (Eds.), Abstract Book and Final Programme of 10th International Nutrition & Diagnostics Conference, Prague, 2010, p. 101.
[24] M.J. Cikalo, S.K. Poole, C.F. Poole, J. Planar Chromatogr. 5 (1992) 200.