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New simple spectrophotometric assay of total carotenes in margarines ☆

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Abstract

Direct and reliable spectrophotometric method for assaying total carotenes (TC) in margarines with the minimum of sample manipulation is proposed. For the first time saponification step used in determination of carotenes in margarines was omitted leading to a substantial cost saving and reduction of time needed to complete the analysis. The resulting analytical procedure is characterized in details in terms of the figures of merit. The method is sensitive, precise and accurate; for both, standard additions and calibration in soybean oil, recovery ranges between 98 and 102%. For the most accurate analyses the approach of standard additions is preferred but for quick routine analyses this latter can be replaced by the calibration in soybean oil. Limit of detection value ($LOD = 3S.D._B/a$, where $S.D._B$ is the standard deviation of the blank, "a" is the slope of calibration line) as low as 12 μg TC/100 g was achieved in soybean oil enabling the sensitive detection. Concentration of TC in margarines declared as being coloured with β-carotene (carotene) ranges between 0.3 and 0.9 mg/100 g while in carrot extract-coloured margarine TC is 0.2 mg/100 g.

Keywords: Margarines; Total carotenes; Spectrophotometry; Quality control

1. Introduction

The carotenoids are widespread naturally occuring antioxidants the importance of which is related to their functions. They act as provitamin A and food colorants [1] and are being extensively studied for their potential role in reducing the risk for cancer and other chronic diseases [2,3]. The quenching of singlet oxygen is the major antioxidative activity of β-carotene [4].

Due to their system of conjugated double bonds carotenoids are extremely reactive and consequently unstable. Precaution steps taken during the isolation and analysis include the protection from light, avoiding the exposure to oxygen, use of antioxidants (e.g. BHT, pyrogallol, vitamin E), operation at reduced temperatures and the need for completing the analysis in the shortest possible time. For foods with a high fat content the saponification is being employed prior to (or after) extraction in order to hydrolyze the carotenoid esters and remove fatty material. Although this optional step facilitates subsequent separation, identification and quantification of carotenes, it prolongs the time of analysis and might also lead to a degradation of carotenoids. Hence the saponification in the analytical procedure should be omitted whenever possible.

Carotenoids are used as colorants [e.g. β-carotene (E160a), annatto dye-stuffs norbixin and bixin (E160b), lycopene, lutein, capsanthin, etc.], in foodstuffs such as beverages, cookies, cereals, margarines, butter, cheese, etc. Natural carotenoid pigments (carrot extract, palm oil, saffron, annato or paprika) or synthetic carotenoids are used to impart yellow colour to margarines. Antioxidant fortified margarine increases the antioxidant status in humans [5].

Quantification of carotenoid colorants in foodstuffs is therefore very important from nutritional, epidemiological and food quality points of view. High performance liquid chromatography (HPLC) is a demonstrated powerful tool in the field of carotenoid research especially in the analysis of complex mixture of carotenoids [6,7]. For example, HPLC was applied following the saponification and solvent extraction for the analysis of butter and margarine [8] or cheese [9], for the study of margarine following gel permeation isolation step [10] or cheese after SPE [11]. Accelerated solvent extraction was developed to precede HPLC analysis of carotenoids in various foodstuffs [12]. Likewise, photometric determination of β-carotene in butter after saponification and solvent extraction was reported [13].

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In this paper we propose a new, simple and direct spectrophotometric method for determination of total carotenes (TC) in margarines. It should be emphasized that in this study the saponification step was for the first time omitted in the analysis of carotenes, leading to cost saving, shortening of analysis time and preservation of carotenes present in the sample. The method is in extenso characterized in terms of analytical performances.

2. Experimental

2.1. Chemicals, samples and instruments

Betatene® 20% Soy (Cognis Australia Pty Ltd., Cheltenham, Vic., Australia) and β-carotene 30% FS (Roche, Basel, Switzerland) were used as carotene standards. Betatene® 20% Soy is a suspension of a mixture of β- and α-carotene in soybean oil obtained from extracts of the alga *Dunaliella salina* (λ_{max} 446–448 nm); β-carotene 30% FS is a suspension in vegetable oil (λ_{max} 450–452 nm). Both standards were gifts: the former from Dr. Christina Gaertner from Cognis GmbH, Düsseldorf, Germany, and the latter from Zvijezda (Zagreb, Croatia).

Reference margarines (RM1 and RM2) and their respective blanks (margarines without β-carotene, real blanks) were obtained from Zvijezda. The same is true for few margarines (samples 3, 4, 8, 9) and respective blanks with no water added. All other margarines were purchased in the supermarkets in different European countries (samples 1, 2, 5–7, 10–17).

Soybean oil, the product of Zvijezda, was purchased in the supermarket.

Reference margarines (RM1, RM2) and their real blanks were prepared in a pilot device. For production of RM1 and RM2 β-carotene FS 30% colorant was accurately weighted (0.26 and 0.60 g, respectively) into the exactly weighted margarine blank (20.0 kg) and blended. No other sources of carotenes were used during the production of reference margarines. Blanks contained tocopherol acetate (0.01%) and the mixture of vitamins A palmitate and D_3 (0.004%) (all from Roche).

Samples 1–4, 7–15 and 17 were coloured with β-carotene, specimens 5 and 16 with carrot extract and carotene, respectively, while sample 6 contained no specified colorant. Samples contained 34–80% total fats and oils; ratio of saturated to unsaturated fatty acids 1:2 or 1:3. All samples (1–17) were enriched with vitamins A and D; with the exception of specimens 6, 12, 13 and 15, all other samples contained vitamin E as well. Finally, samples 2, 3, 6, 16 and 17 were enriched with olive oil.

The *n*-hexane p.a. (Kemika, Zagreb, Croatia) was used as a solvent for extraction.

The spectra were recorded on Agilent 8453 (Hewlett-Packard, Waldbronn, Germany) spectrophotometer equipped with PC-HP 845x UV–vis system that was recalibrated prior to use.

2.2. Sample preparation

Margarines were thoroughly homogenized using a mixer and then kept at +4 °C. Prior to use they were equilibrated at room temperature overnight and homogenized again manually. An accurately weighted quantity of margarine or its blank or soybean oil (pure or after addition of carotene standard) was dissolved in 10–20-fold volume excess of *n*-hexane. The level of dilution was chosen such to achieve the optimum absorbance of the hexane extract. The mixture, shaken mechanically for 15–30 min with generated yellowish suspension was centrifuged at 3000 rpm for 10 min. The upper, clear, yellow, hexane layer and the lower, turbid colourless aqueous layer separated with a white precipitate remaining at the interface. Most of margarines without aqueous phase added readily dissolved in hexane; in some cases little, white precipitate remained. Likewise, soybean oil readily dissolved in hexane.

In all cases, the hexane layer/solution was collected to record spectrum; the absorbance value at λ_{max} within 443–451 nm region served to calculate TC.

Real blanks and soybean oil served as blanks; their absorbance at the respective wavelength served for purposes of the background correction.

2.3. Analytical procedures

Initially, β-carotene FS or Betatene® was added to the real blank or soybean oil to produce an interdilution. Such interdilution was then used to prepare respective working standards and the spiked samples; the latter were prepared fresh daily. To assure accurate results when using calibration in soybean oil, the use of fresh, daily prepared interdilutions is a necessity.

Three calibration methods were used and compared, i.e. the calibration in real blank, soybean oil and the method of standard additions. In the first two approaches working standards were prepared by accurate weighing of the respective interdilution of β-carotene FS 30% or Betatene® into soybean oil or real blank. As to the standard additions the same interdilutions were weighed-into margarines (reference margarines and samples). After thorough homogenisation the mixture obtained was subjected to the procedure already described under Section 2.2.

Carotene standards and interdilutions as well as reference margarines were stored at −18 °C; margarine samples were kept at +4 °C. All sample manipulations were performed under the from-light-protected conditions.

The final concentration of added TC in calibration studies (expressed per ml of hexane solution) was about 0.17, 0.34, 0.50, 0.67, 1.00, 1.34, 1.67 μg ml^{-1} at 1 + 10 dilution level, and 0.17, 0.25, 0.34, 0.50, 0.67 and 0.84 μg ml^{-1} at 1 + 20 dilution level (see Tables 1 and 2 and Fig. 2). In standard additions the final concentration of added TC in hexane solution was 0, 0.17, 0.25, 0.34, 0.50 and 0.67 μg ml^{-1} for 1 + 20, and 0, 0.17, 0.34, 0.50, 0.67, 1.00 and 1.34 μg ml^{-1} at 1 + 10. Each point in the standard additions or calibration lines refers to an independent calibration sample obtained by the complete analytical procedure; the absorbance value is the average of at least three repetitive measurements. "Collective" calibration lines presented in Fig. 2 were obtained using the data for each-day calibrations for over more than a month.

Recovery as a measure of accuracy was estimated in two ways: from TC assayed by the suggested method versus declared concentration in reference margarines RM1 and RM2, and from

Table 1
Accuracy data for standard additions and calibration methods (λ_{max} = 451 nm)

Reference sample		Standard addition[a]				
Code/dilution level	TC declared (mg/100 g)	Regression line[c], $a \pm$ S.D.$_a$ (ml μg^{-1})/TC added (μg ml^{-1})/*N*/*R*/RSS	Total carotenes found (n = 6)[d]			
			Mean ± S.D. (mg/100 g)	Recovery mean ± S.D. (%)	e_{max} (±%)	R.S.D. (%)[g]
RM1/1 + 10	0.39	0.269 ± 0.002/0–1.34/32/0.9990/1.0×10^{-3}	0.39 ± 0.02	99.8 ± 3.9	4	3.9
RM2/1 + 20	0.90	0.281 ± 0.004/0–0.67/32/0.9971/7.9×10^{-4}	0.88 ± 0.03[j]	97.5 ± 3.5	7	3.6
Reference sample		Calibration in real blank[a]				
Code/dilution level	TC declared (mg/100 g)	Regression line[e], $a \pm$ S.D.$_a$ (ml μg^{-1})/TC conc. (μg ml^{-1})/*N*/*R*/RSS	Total carotenes found (n = 6)[e,f]			
			Mean ± S.D. (mg/100 g)	Recovery mean ± S.D. (%)	e_{max} (±%)	R.S.D. (%)[g]
RM1/1 + 10	0.39	0.264 ± 0.004[h]/0.17–1.67/13/0.9987/5.9×10^{-4}	0.40 ± 0.01	102.0 ± 3.7	7	3.6
RM2/1 + 20	0.90	0.292 ± 0.004[k]/0.17–0.84/12/0.9989/1.3×10^{-4}	0.87 ± 0.01[l]	96.2 ± 0.9	5	0.9
Reference sample		Calibration in soybean oil[a,b]				
Code/dilution level	TC declared (mg/100 g)	Regression line[d], $a \pm$ S.D.$_a$ (ml μg^{-1})/TC conc. (μg ml^{-1})/*N*/*R*/RSS	Total carotenes found (n = 6)[d,f]			
			Mean ± S.D. (mg/100 g)	Recovery mean ± S.D. (%)	e_{max} (±%)	R.S.D. (%)[g]
RM1/1 + 10	0.39	0.263 ± 0.004[i]/0.17–1.67/44/0.9954/5.3×10^{-3}	0.39 ± 0.02	99.8 ± 4.8	6	4.8
RM2/1 + 20	0.90	0.277 ± 0.003[m]/0.17–0.84/68/0.9960/2.2×10^{-3}	0.92 ± 0.04[l,j]	102.4 ± 4.0	6	3.9

a, slope of the regression line; *N*, number of points; *n*, number of independent analyses; R.S.D., relative standard deviation; *R*, coefficient of correlation; RSS, residual sum of squares; TC, total carotenes.
[a] Addition of β-carotene FS.
[b] Addition of Betatene®.
[c] Background correction with both, soybean oil and real blank.
[d] Background correction with soybean oil.
[e] Background correction with real blank.
[f] Calculated from ideal calibration lines.
[g] Intermediate precision.
[h] Not significant difference between slopes compared to standard addition method: $t(a_1a_2) = 1.115$, t(d.f. 40, p 0.05) = 2.021.
[i] Not significant difference between slopes compared to standard addition method: $t(a_1a_2) = 1.124$, t(d.f. 70, p 0.05) = 1.994.
[j] p = 0.05 (limit).
[k] Not significant difference between slopes compared to standard addition method: $t(a_1a_2) = 1.476$, t(d.f. 40, p 0.05) = 2.021.
[l] p = 0.03 (significant difference).
[m] Not significant difference between slopes compared to standard addition method: t $(a_1a_2) = 0.739$, t(d.f. 100, p 0.05) = 1.984.

Table 2
Sensitivity, precision and limiting data

	λ_{max} (nm)								
	Standard additions[a,b]				Calibration in real blanks[c,d,e], 451	Calibration in soybean oil[a,b,e]			
	451	443	446	449		451	443	446	449
1 + 10 dilution level (added TC: 0.17–1.68 μg ml^{-1})									
Sensitivity ($a \pm$ S.D.$_a$) (ml μg^{-1})/N/R									
β-Carotene FS	0.264 ± 0.014 (n = 12)	–	0.259 ± 0.0003 (n = 3)	–	0.270 ± 0.002[f]/ 32/0.9995	0.257 ± 0.002[f]/ 39/0.9986		0.239 ± 0.003/ 5/0.9998[g]	–
Betatene®	–		–		–	0.259 ± 0.005/ 5/0.9992		0.253 ± 0.006/ 5/0.9991[g]	–
Repeatability R.S.D.$_{max}$ (%) (n = 3)	4.4	–	0.5	–	4.6	4.8	–	4.3	–
Intermediate precision R.S.D.$_{max}$ (%) (n = 6)	3.9	–	–	–	3.7	5.4	–	3.9	–
LOD (μg/100 g)[h]	–	–	–	–	9.7 (n = 18)[i]	21.9 (n = 28)[j]	–	35.8 (n = 9)[j]	–
LOQ (μg/100 g)[h]	–	–	–	–	32.5 (n = 18)[i]	72.9 (n = 28)[j]	–	119.4 (n = 9)[j]	–
1 + 20 dilution level (added TC: 0.17–0.84 μg ml^{-1})									
Sensitivity ($a \pm$ S.D.$_a$) (ml μg^{-1})/N/R									
β-Carotene FS	0.285 ± 0.009 (n = 27)	–	–	–	0.297 ± 0.002 [k]/35/0.9991	0.276 ± 0.002 [l,m,k]/57/0.9992	0.252 ± 0.010 [l]/10/0.9939[g]	0.263 ± 0.004 [m]/10/0.9989	0.273 ± 0.003/ 11/0.9992
Betatene®	–	0.274 ± 0.002 (n = 3)	0.286 ± 0.003 (n = 2)	0.276 ± 0.001 (n = 3)	–	0.274 ± 0.004/ 10/0.9992	0.257 ± 0.008/ 10/0.9959[g]	0.274 ± 0.004/ 10/0.9992	0.276 ± 0.004/ 10/0.9992
Repeatability R.S.D.$_{max}$ (%) (n = 3)	8.0	1.6	3.0	0.9	6.2	6.3	2.0	3.1	0.9
Intermediate precision R.S.D.$_{max}$ (%) (n = 5–7)	3.6	–	–	–	3.7	3.9	–	5.9	–
LOD (μg/100 g)[h]	–	–	–	–	30.6 (n = 20)[i]	38.3 (n = 30)[j]	31.1 (n = 8)[j]	31.6 (n = 8)[j]	36.4 (n = 8)[j]
LOQ (μg/100 g)[h]	–	–	–	–	102.0 (n = 20)[i]	127.7 (n = 30)[j]	103.6 (n = 8)[j]	105.4 (n = 8)[j]	121.2 (n = 8)[j]

a, slope of the regression line; LOD, limit of detection (3S.D.$_B$ criterion); LOQ, limit of quantitation (10S.D.$_B$ criterion); *N*, number of points; *n*, number of independent lines or number of independent analyses; *R*, coefficient of correlation; R.S.D., relative standard deviation.

[a] Addition of β-carotene FS or Betatene®.
[b] Background correction with soybean oil.
[c] Addition of β-carotene FS.
[d] Background correction with real blank.
[e] Calculated from ideal calibration lines.
[f] Significant difference between slopes: $t(a_1 a_2) = 4.961$, t(d.f. 70, p 0.05) = 1.994.
[g] Calculated from non-ideal calibration lines.
[h] Calculated on the basis of intermediate S.D.$_B$ values (measured on different days).
[i] Calculated from real blank.
[j] Calculated from soybean oil.
[k] Significant difference between slopes: $t(a_1 a_2) = 8.201$, t(d.f. 90, p 0.05) = 1.986).
[l] Significant difference between slopes: $t(a_1 a_2) = 2.413$, t(d.f. 60, p 0.05) = 2.000.
[m] Significant difference between slopes: $t(a_1 a_2) = 2.868$, t(d.f. 60, p 0.05) = 2.000).

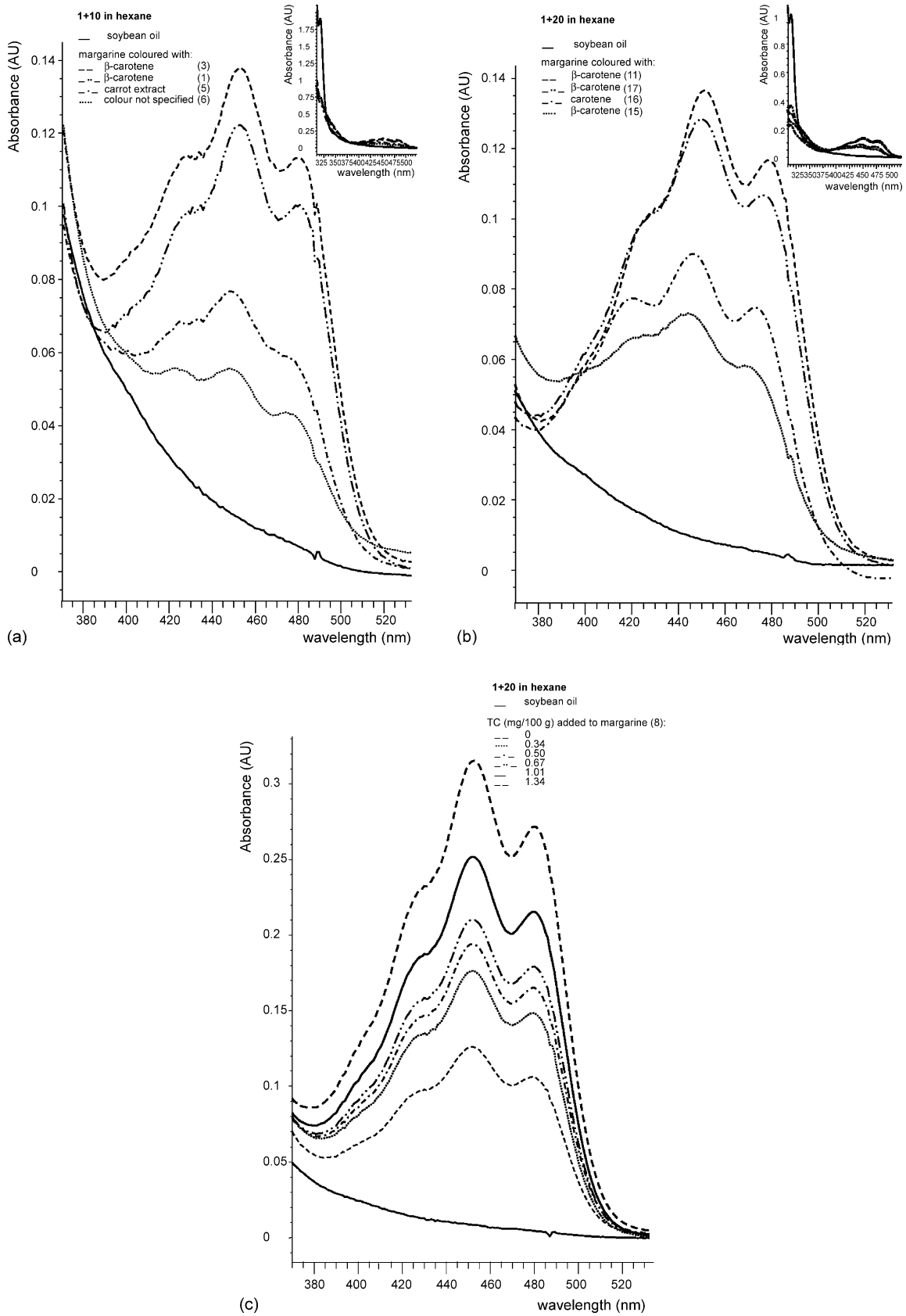

Fig. 1. Spectra of representative margarines and soybean oil in hexane (sample codes in parentheses) at: (a) 1 + 10 dilution level and (b) 1 + 20 dilution level; (c) standard additions to margarine coloured with β-carotene (diluted 1 + 20). Insets: spectra down to 310 nm.

the calibration line-to-line of standard additions slopes ratio (see Tables 1 and 2).

Precision is expressed as repeatability (within-a-batch precision) and as intermediate precision (between-batches precision, precision obtained by the same analyst and instrument during several days) (see Tables 1–3), for all samples (margarines 1–17, RM1 and RM2).

The LOD (limit of detection) and LOQ (limit of quantitation) values were calculated as $3S.D._B/a$ and $10S.D._B/a$, with $S.D._B$ and "a" being the standard deviation of the signal of the blank (soybean oil or respective real blank) and the slope of the calibration line, respectively.

Student's t-test and testing of parallelism of regression lines were used for statistical analysis of the results obtained.

3. Results and discussion

Our preliminary results revealed that acceptable, time-saving analyses are achievable even after 15 min extraction (with hexane); however, for highly accurate results the extraction process should be prolonged to 30 min (Tables 1–3, Figs. 1 and 2). Extraction with hexane has already been utilized for determination of β-carotene, vitamin A and vitamin E in butter, margarine or milk prior to photometric [13] or HPLC measurement [8,14]; however, in all these cases the saponification step was included in analysis of carotenes. In this report saponification step was obviated and the resulting, direct extraction-spectrophotomeric procedure for total carotenes characterized in details.

Three approaches were applied and compared: method of standard additions, calibration in real blank and the calibration in soybean oil. From the standpoint of practicality calibration in real blank is not of much use. Therefore, the most of the forthcoming discussion concentrates on comparing the calibration in soybean oil to that of the standard additions method.

Diversity of isomers of carotenes is expected in margarine samples. The reason for this is two-fold: (i) the presence of isomers in colorants and (ii) the conditions used during the production process. Our method aims for determination of the concentration of total carotenes (usually α- and/or β-carotene) including all their geometric isomers. We find this approach good enough for routine control analyses of margarines. Spectra recorded from hexane extracts of margarines featured the characteristic three-peak carotenoid pattern; analytical measurements were performed at a wavelength of middle peak. It is well known that most of *cis*-isomers are characterized by a blue shift relative to all-*trans*-β-carotene [15,16]. Among the samples declared as coloured with β-carotene, most of them exhibited λ_{max} at 451 nm (samples 1–4, 7–14) indicating the dominance of *trans*-isomer; in some specimens the shift of λ_{max} to lower wavelengths (sample 15 at 443 nm and sample 17 at 449 nm) was observed suggesting the *cis*-isomerization of β-carotene. Samples 5, 6 and 16 all absorbed at 446 nm; for sample 5 this confirmed the presence of α-carotene from carrot extract declared as colorant. In case of sample 16 (declared as being coloured with carotene) this might be either α-carotene and/or *cis*-isomers of β-carotene. The same applies to sample 6 with no specified colorant.

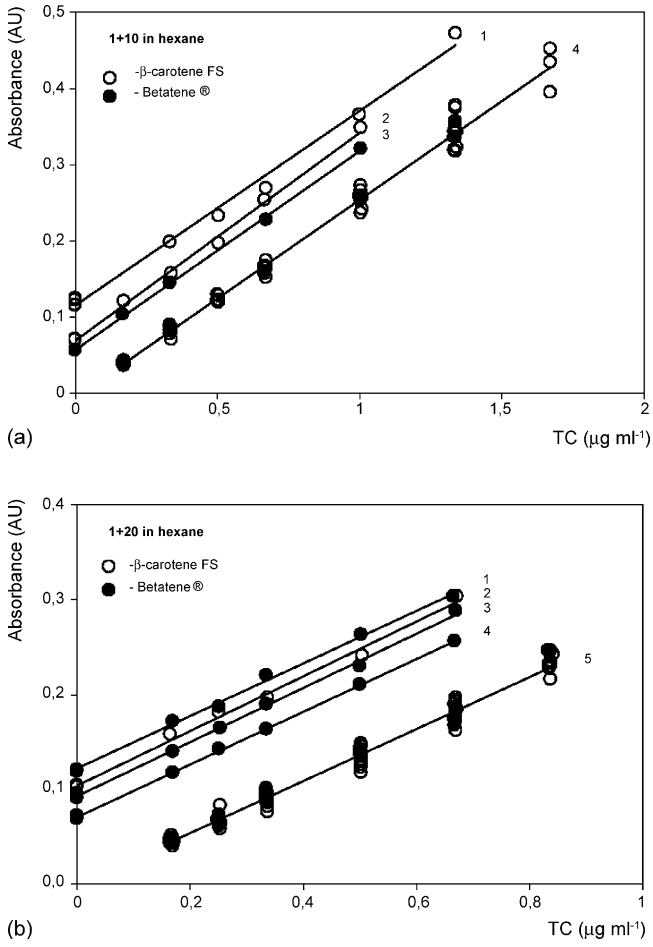

Fig. 2. Standard additions and "collective" calibration lines in soybean oil (TC concentration expressed in μg per ml of hexane solution). (a) 1 + 10 dilution level; standard additions to: 1, margarine 3 (451 nm), $N = 8$, $A = 0.255\gamma + 0.115$, $R = 0.9966$; 2, margarine 4 (451 nm), $N = 8$, $A = 0.273\gamma + 0.070$, $R = 0.9987$; 3, margarine 5 (446 nm), $N = 7$, $A = 0.259\gamma + 0.058$, $R = 0.9997$; 4, calibration in soybean oil (451 + 446 nm), $N = 60$, $A = 0.259\gamma - 0.006$, $R = 0.9950$. (b) 1 + 20 dilution level; standard additions to: 1, margarine 17 (449 nm), $N = 8$, $A = 0.278\gamma + 0.121$, $R = 0.9986$; 2, margarine 13 (451 nm), $N = 8$, $A = 0.289\gamma + 0.104$, $R = 0.9974$; 3, margarine 16 (446 nm), $N = 7$, $A = 0.285\gamma + 0.093$, $R = 0.9990$; 4, margarine 15 (443 nm), $N = 8$, $A = 0.277\gamma + 0.071$, $R = 0.9999$; 5, calibration in soybean oil (451 + 443 + 446 + 449 nm), $N = 129$, $A = 0.275\gamma - 0.001$, $R = 0.9937$.

The spectra of analyzed margarines alone and after standard additions are presented in Fig. 1a–c. Fig. 2a and b show standard additions lines as well as the "collective" calibration lines in soybean oil.

3.1. Analytical figures of merit

3.1.1. Linearity

High R's values (>0.995) and low RSS values (1×10^{-4}–5×10^{-3}) confirmed strong linear correlation in the concentration range from 0.17 to 1.67 μg TC ml^{-1} hexane solution at 1 + 10 dilution level, and from 0.17 to 0.84 μg TC ml^{-1} hexane solution at 1 + 20 dilution level, for standard additions and calibrations (Tables 1 and 2).

Table 3
Total carotenes in margarines

Sample code/λ_{max}	Standard addition[a]		Calibration in real blank[b,c]		Calibration in soybean oil[a,c]	
	Mean ± S.D. (mg/100 g) (*n*)	R.S.D. (%)[d]	Mean ± S.D. (mg/100 g) (*n*)	R.S.D. (%)[d]	Mean ± S.D. (mg/100 g) (*n*)	R.S.D. (%)[d]
1 + 10 dilution level						
1/451	–	–	0.40 ± 0.003 (3)[e]	0.8	0.39 ± 0.003 (3)[e]	0.7
2/451	–	–	0.40 ± 0.004 (3)[e]	1.1	0.42 ± 0.004 (3)[e]	1.1
3/451	0.48 ± 0.02 (3)[e]	4.4	0.41 ± 0.01 (3)[e]	2.2	0.45 ± 0.02 (6)[e]	5.4[f]
4/451	0.25 ± 0.001 (3)[e]	0.2	0.27 ± 0.01 (6)[e]	3.7[f]	0.27 ± 0.01 (6)[e]	3.7[f]
5/446	0.23 ± 0.001 (3)[g]	0.5	–	–	0.23 ± 0.002 (3)[g]	0.9
6/446	–	–	–	–	0.19 ± 0.01 (9)[e,g]	3.6[f]
1 + 20 dilution level						
7/451	–	–	0.82 ± 0.01 (3)[e]	0.9	0.90 ± 0.01 (3)[e]	0.9
8/451	0.86 ± 0.04 (3)[e]	5.0	0.86 ± 0.03 (6)[e]	3.7[f]	0.87 ± 0.03 (6)[e]	3.7[f]
9/451	0.74 ± 0.02 (5)[e]	2.3[f]	0.75 ± 0.02 (3)[e]	2.7	0.77 ± 0.02 (7)[e]	2.2[f]
10/451	0.78 ± 0.02 (2)[e]	2.8	0.74 ± 0.01 (3)[e]	1.1	0.82 ± 0.03 (5)[e]	3.3[f]
11/451	0.86 ± 0.03 (2)[e]	3.6	0.81 ± 0.01 (3)[e]	6.2	0.90 ± 0.03 (5)[e]	3.3[f]
12/451	0.60 ± 0.002 (3)[e]	0.3	–	–	0.65 ± 0.02 (6)[e]	2.3[f]
13/451	0.73 ± 0.004 (3)[e]	0.5	–	–	0.75 ± 0.004 (3)[e]	0.6
14/451	0.68 ± 0.05 (3)[e]	8.0	–	–	0.68 ± 0.04 (3)[e]	6.3
15/443	0.51 ± 0.01 (3)[g]	1.6	–	–	0.51 ± 0.01 (3)[g]	2.0
16/446	0.65 ± 0.02 (2)[g]	3.0	–	–	0.69 ± 0.04 (5)[g]	6.0[f]
17/449	0.89 ± 0.01 (3)[g]	0.9	–	–	0.90 ± 0.01 (3)[e,g]	0.8

n, number of independent analyses; R.S.D., relative standard deviation; TC, total carotenes.
[a] Background correction with soybean oil.
[b] Background correction with real blank.
[c] Calculated from ideal calibration lines.
[d] Repeatability.
[e] Addition of β-carotene FS.
[f] Intermediate precision.
[g] Addition of Betatene®.

3.1.2. Sensitivity

Sensitivity was expressed as the slope of the calibration line (Tables 1 and 2). It is evident that at 1 + 20 dilution level the slopes of calibration lines are significantly higher than that at 1 + 10 dilution level ($p = 0.001$).

Considering both dilution levels and all analytical wavelengths the sensitivity (slope) ranged between 0.26 and 0.29 ml μg^{-1} for standard additions and from 0.24 to 0.28 ml μg^{-1} for the calibration in soybean oil. Testing for parallelism between standard addition lines and "collective" calibration lines at constant dilution level at 451 nm revealed no significant slope differences (Table 1).

3.1.3. Accuracy

Accuracy was estimated using the reference samples. The mean recovery of 99.6 ± 2.4% indicates that accurate analyses can be achieved by either standard additions or calibrations. Data from Table 1 also shows that in terms of favourable recovery (97.5–102.4%) and maximal relative error (4–7%), calibration in soybean oil is comparable to standard additions method at 451 nm; this makes standard additions redundant at this wavelength. This was confirmed by recovery value estimated from the calibration-to-standard additions slope ratio at 451 nm ($n = 5$) of 101.9 ± 2.4%, for calibration in soybean oil.

3.1.4. Calibration

From Table 2 it is evident that slopes obtained with β-carotene FS and Betatene® as calibrants in soybean oil, do not differ significantly regardless the analytical wavelength; hence they are interchangeable. However, the slope of the calibration line in soybean oil might drop significantly at 443–446 nm compared to 451 nm with either calibrant.

Soybean oil and the real blank used as media for correction of background in both, standard additions or the calibration method might be used interchangeably. For the sake of practicality soybean oil was chosen for correction of background.

For most accurate analyses the use of standard additions is recommended; at 451 nm it may be replaced with calibration in soybean oil. However, Fig. 2a and b show that upon calibration samples in soybean oil measured during more than a month in the spectral range of 443–451 nm, using both carotene standards, good regression lines could be obtained. This shows that the method is rugged enough and that a single, universally applicable calibration line in soybean oil could be used for quick routine analyses over extended period of time.

3.1.5. Limiting values

Based upon between-runs $S.D._B$ values, the estimated LOD of TC in soybean oil ranged between 22 and 36 μg TC/100 g at 1 + 10 dilution level and from 31 to 38 μg TC/100 g at 1 + 20

dilution level (Table 2). However, the LOD can be lowered to 6 μg TC/100 g in a real blank and to 12 μg TC/100 g in soybean oil if calculation is based upon within-a-run $S.D._B$ values.

3.1.6. Precision

At both dilution levels precision data (Table 2, and other tables) found for method of standard additions and for calibration in soybean oil is comparable. The repeatability imprecision ranged between 0.2 and 8% while intermediate imprecision was 4–6%. Keeping in mind the complexity of samples the obtained imprecision is acceptable.

3.2. Concentration of TC in margarines

Overall, 17 margarines were analyzed (Table 3). The concentration of TC ranged between 0.3 and 0.9 mg/100 g margarine in samples declared as being coloured with β-carotene (samples 1–4, 7–15, 17) or carotene (sample 16). In margarines coloured with carrot extract (sample 5) as well as in the sample with no colorant declared (sample 6) concentration of TC was 0.2 mg/100 g product. These values show a good agreement with data provided by other authors who reported the concentrations of carotene of 0.1–1.3 mg/100 g [17] and 0.39 mg/100 g [18] in margarines, and of 0.3 mg/100 g [8,18,19] and 0.8–1.4 mg/100 g [19] in butter and carotene-enriched butter, respectively.

Reader is reminded that palm oil as well as olive oil, both often used in the manufacturing process of margarines could markedly contribute to the TC content.

4. Conclusions

Simple, rapid and reliable spectrophotometric method for determination of total carotenes (TC) in margarines is proposed. Unlike other methods, the novel approach eliminates the need for the time-consuming saponification step. The new method is applicable in the analysis of margarines declared as being coloured with carotenes. Both approaches, the standard additions and calibration in soybean oil are sensitive, accurate and precise enough to allow their application for the quality control of margarines, process control during production of margarines, as well as in the nutritional and epidemiological studies.

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