Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 104 (2007) 439–444
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

On the monitoring of carotenogenesis by *Blakeslea trispora* using HPLC

Fani Mantzouridou, Maria Z. Tsimidou *

Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

Received 25 July 2006; received in revised form 19 September 2006; accepted 28 September 2006

Abstract

A rapid, isocratic RP-HPLC method for the determination of the carotenoids produced by *Blakeslea trispora* is described. The mixture of acetone:acetonitrile, 60:40, v/v, found appropriate for the cellular triacylglycerol analysis, was also successfully used for the separation of lycopene, γ-carotene and β-carotene. The method was validated for β-carotene using an olive oil triacylglycerol fraction devoid of carotenoids. Recovery study (300 mg/kg oil) was 99%. RSDr and RSD_L were satisfactory (<4%). The limit of quantification was found to be 4.56 ng/5 μL and the system was linear in the range 2.0–30.0 ng/μL.

Keywords: *Blakeslea trispora*; Carotenogenesis; β-Carotene; Intracellular lipids; RP-HPLC; Quality control

1. Introduction

Blakeslea trispora has gained the interest of investigators in the production of carotenoids. All-*trans* β-carotene, lycopene and γ-carotene have been repeatedly reported as the only coloured carotenoids synthesized by this mold. The research so far indicated that carotenogenesis using *B. trispora* may be manipulated in favor of a particular carotenoid by genetic and chemical activation/inhibition of the different biosynthetic steps. As a result, mixtures varying in the relative amounts of the above mentioned carotenoids are accumulated (Bhosale, 2004; López-Nieto et al., 2004; Mehta, Obraztsova, & Cerdá-Olmedo, 2003).

A careful literature survey showed that in most works carotenogenesis is monitored using spectrometric procedures for the estimation of total carotenoids (e.g. Govind, Amin, & Modi, 1982; Lampila, Wallen, Bullerman, & Lowry, 1985; Thomas & Goodwin, 1967). HPLC procedures have been used mainly for the characterization of carotenoids at the stage of maximum carotenoid accumulation in cells (Kim, Seon, & Park, 1997; López-Nieto et al., 2004; Mehta et al., 2003) and not to collect information about the carotenogenesis pattern in the course of fermentation.

Carotenoid HPLC analysis is a rather interesting case since elution systems seem to be numerous though not so dramatically different to each other. This is due to the fact that natural extracts usually contain a wide array of carotenoids differing in polarity. For this reason, the systems found in literature most of the times tackle specific needs, i.e. complete characterization of the carotenoids found in a test mixture (Cserhati, Forgacs, Morais, & Mota, 2000; Echavarri-Erasun & Johnson, 2002; Eitenmiller & Landen, 1999; Oliver & Palou, 2000). Both normal and reversed-phase HPLC systems have been developed and applied with success so far, the latter being the mode of choice for obvious reasons. Aqueous RP-HPLC elution systems usually are based on acetone whereas non-aqueous systems are usually comprised of acetonitrile, methanol or mixtures of these solvents. Less polar organic solvents are then augment selectivity of the system.

In our effort to introduce RP-HPLC in kinetic studies of carotenogenesis using *B. trispora*, we observed that the so far applied protocols are time and solvent consuming. Indeed, as evidenced, reported β-carotene retention times on ordinary C18 columns are longer than 15 min (e.g. López-Nieto et al., 2004; Nam, Cho, & Rhee, 1988;

* Corresponding author. Tel.: +30 2310 997796; fax: +30 2310 997779.
E-mail address: tsimidou@chem.auth.gr (M.Z. Tsimidou).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.09.051

Sandmann, Woods, & Tuveson, 1990; Shlomai, Ben-Amotz, & Margalith, 1991). Taking into account that the carotenoid pattern consists only of lycopene, γ- and β-carotene, and that acetone and acetonitrile are key-solvents in carotenoid analysis, though in different modes, we examined whether mixtures of the two solvents could be used for the purpose of the study. Thus, the mixture acetone:acetonitrile, 60:40, v/v, found appropriate for the separation of triacylglycerol species – the main components of intracellular lipid bodies of the mold – using our laboratory facilities, was also tested for the separation of its carotenoids. Preliminary data were very promising, so that analytical requirement elements for method validation set by the EURACHEM Guide (1998) were then examined and values for the necessary limits were produced. The method can be used in the parallel study of carotenoid and triacylglycerol composition (on or off line) in the course of carotenogenesis by *B. trispora.*

2. Experimental

2.1. Samples, standards and solvents

Lipid extracts of *Blakeslea trispora* cells grown in culture media enriched with refined olive pomace oil or soybean oil (Mantzouridou, Tsimidou, & Roukas, 2006) were stored under nitrogen in a freezer at −18 °C until analysis. Fresh carrots obtained from the local market were immediately cut into small pieces and ground into juice with a food processor. All-*trans* β-carotene standard (for biochemistry, purity 97%) was purchased from Merck (Darmstadt, Germany). All-*trans* lycopene (redivivo™ 10% FS, DSM, Nutritional Products Ltd., Basel, Switzerland) was a gift of BASF Aktiengesellschaft (Ludwigshafen, Germany) to our laboratory. Chemicals and solvents were of appropriate purity and various suppliers.

2.2. Instrumentation

RP-HPLC for the analysis of carotenoids was performed using a solvent delivery system consisted of two Marathon IV Series HPLC pumps (Rigas Labs, Thessaloniki, Greece), a Rheodyne injection valve (model 7125) with a 20 μL fixed loop (Rheodyne, Cotati, CA) and a Linear UVIS-206 diode array multiple-wavelength detector (Linear Instruments, Fermont, CA). Column temperature was controlled using a model 505 LC column oven (Rigas Labs). The data from the detector were processed with the chromatographic software EZChrom (Sci Software, Inc, San Ramon, CA, USA). RP-HPLC of triacylglycerols was achieved on a SSI liquid chromatograph (model 300; Scientific Systems Inc., State College, PA) equipped with a SSI pulse damper (model LP-21 LO pulse) and a RID-6A refractive index detector (Shimadzu Co., Kyoto, Japan). A Hewlett-Packard, Model HP 3396 Series II electronic integrator (Avondale, PA) was used for recording and quantifying the chromatographic peaks. Injection was by means of Rheodyne 7125 injector (Cotati, CA) with a 20 μL loop. Column temperature was controlled using an SSI model 207 column oven. Absorbance measurements were recorded on a HITACHI U-2000 spectrophotometer (Hitachi Ltd, Tokyo, Japan).

2.3. Extraction of cellular lipids

Lipids were removed from the mold cells after cell rupture by freezing and thawing, using liquid nitrogen, and then by manual grinding in the presence of quartz sand until complete cell breakage occurred. The preparation was examined under a phase contrast microscope (Nikon E 200, USA) to ensure complete destruction. Magnification ×100 was used for image analysis (Matrox Inspector version 3.0, Matrox Electronic Systems Ltd., Canada). Cellular lipid extraction with chloroform-methanol (2:1, v/v) mixture was performed three times (each session lasted 1 h) using the Folch method (Folch, Lees, & Slane-Stanley, 1957). The lower chloroform phase was then centrifuged at 10.000*g* for 10 min to remove cells, passed through a water-free Na_2SO_4 layer and rotary-evaporated to dryness. Carotenoid extraction from carrot juice was carried out in a manner similar to a published procedure (Taungbodhitham, Jones, Wahlqvist, & Briggs, 1998). The whole extraction procedure was protected from light exposure. Adequate repeatability for both extraction techniques was obtained (RSD values less than 5.0%, $n = 3$).

2.4. HPLC separation, identification and quantification of carotenoids

2.4.1. Preparation of standard solutions and samples

Stock solution of all-*trans* β-carotene standard (50 mg/L) was prepared by dissolving the standard first in a small quantity of tetrahydrofuran and then in acetone; working standards solutions of all-*trans* β-carotene were daily prepared from the stock solution by appropriate dilution with acetone. A stock standard solution of lycopene (300 mg/L) was prepared in hexane according to manufacturer's instructions. An aliquot of the above stock standard solution was evaporated to dryness and further diluted with acetone to obtain a working standard solution of 15 ng/μL. Storage of stock solutions never exceeded four days. Lipid extracts from *B. trispora* (5% w/v) were first dissolved in a small quantity of tetrahydrofuran and then in acetone and filtered through 0.45 μm membrane filter (Schleicher & Schuell, Dassel, Germany) just before HPLC analysis. Extract of carrot juice was appropriately dissolved in acetone and used for qualitative analysis of carotenoids. Care was taken to exclude direct light exposure of samples and standard solutions throughout the analytical procedure.

2.4.2. Chromatographic analysis and peak identification

Acetone:acetonitrile (60:40, v/v) was used as the eluent. Separation was achieved isocratically on a Nucleosil C18,

5 μm (250 × 4 mm i.d.) column (Macherey-Nagel, Düren, Germany) at a 1.2 mL/min flow rate. Column temperature was set at 30 °C. The injection volume was 5 μL. Column was carefully conditioned between two injections with the elution mixture for 15 min. Backpressure level at *ca.* 1000 psi was maintained with careful washing throughout the analysis period.

Spectral data in the region 380–700 were recorded using the diode array detector. Peak identification and purity were based on spectral characteristics (absorption maxima and peak ratios), retention time, and peak spiking with authentic standards. The height of the largest wavelength absorption band was expressed as a percentage of the middle one. The baseline was drawn at the valley minimum between these two peaks (Davies, 1975).

2.4.3. Preparation of standard curve

The standard curve for β-carotene (as ng/5 μL versus peak area) was calculated by linear regression analysis in the range 2.0–30.0 ng/μL. The response was detected at 453 nm. Injections for both standards and samples were made in duplicate. Control of standard solutions was carried out spectrophotometrically (2590 at 450 nm in hexane).

2.5. Method validation

To validate the method, an olive oil triacylglycerol fraction devoid of carotenoids (subsequently called "oil"), was prepared by a method repeatedly applied in our laboratory (Psomiadou & Tsimidou, 1999). This fraction was used as the lipid substrate where needed. Recovery study was carried out for β-carotene (300 mg/kg oil level of addition). The precision of the method was evaluated by replicate analysis of the same sample used for the estimation of recovery under intralaboratory reproducibility and repeatability conditions. For the evaluation of the method repeatability and reproducibility, the standard deviation and the respective relative standard deviation – SDr (RSDr), SD_L (RSD_L) – were calculated. Calculation of the instrumental limit of detection (LOD) was based on integration of peak areas recorded at the elution time of the analyte by 5-fold injection of the sample solvent (acetone). The detection limit was expressed as the concentration of the analyte the response of which was equivalent to the base line noise increased by three times the standard deviation. The instrumental limit of quantification (LOQ) was calculated as the limit of detection increased by ten times the standard deviation. Linearity was tested for the range of concentrations reported in 2.4.3.

2.6. Chromatographic analysis of cellular TAGs

The elution system, column and flow rate used were the same as the ones described for carotenoid analysis. The injection volume was 10 μL. Column temperature was set at 30 °C. The refractive index detector was set at a full-scale sensitivity 16×10^{-6} RIUFS (refractive index units full scale). Column was carefully conditioned between two injections with the elution mixture for 15 min. Identification of the main triacylglycerol species and peak assignment to equivalent carbon numbers (ECNs) were based on separation of authentic olive oil and soybean oil triacylglycerols.

3. Results and discussion

3.1. Method potential

In the course of RP-HPLC separation of intracellular TAG species at elution conditions optimized for olive oil triacylglycerols (acetone:acetonitrile, 60:40, v/v), the presence of several unknown peaks in the beginning of the chromatogram was observed (data not shown). In an attempt to locate carotenoids, refractive index detector was replaced by a UV–Vis diode array multiple-wavelength detector and peaks were scanned in the visible region. At 453 nm, a satisfactory separation of three peaks was evidenced. All peaks, corresponding to carotenoids on the basis of spectral characteristics, were recorded in less than 10 min (Fig. 1). Peak identification was achieved by comparison of retention time with that of standards, and confirmed by spiking and comparison of spectral data (Table 1). Owing to the lack of availability of standard γ-carotene, its identification was solely based on elution order and spectral data in the visible region with regards to published information (Britton, 1992). Carotenoid pigments eluted in the order all-*trans* lycopene < all-*trans* γ-carotene < all-*trans* β-carotene in agreement with previous reports for retention on reversed phase columns (Khachik, Beecher, & Lusby, 1989; Sandmann et al., 1990). The retention factor k' values for all carotenoids discussed so far are presented in Table 1. All k' values were considered satisfactory for the purpose of the study on the basis of standard operational procedure criteria (Snyder & Kirkland, 1979) and also after

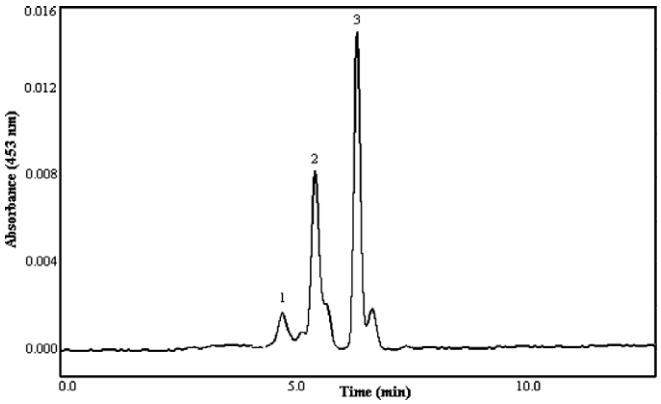

Fig. 1. RP-HPLC lipid profile in *Blakeslea trispora* cells grown in soybean oil enriched substrate at 453 nm (1, lycopene; 2, γ-carotene; 3, β-carotene) (chromatographic conditions as in Section 2).

Table 1
Chromatographic and spectral characteristics of *Blakeslea trispora* carotenoids

Peak no.[a]	Analyte	k[b]	Spectral data for the HPLC eluent			
			Max. wavelength (found/reported[c]) (nm)			Peak height ratio found[d]
			I	II	III	
1	lycopene	1.11	448/448	474/474	505/505	38
2	γ-carotene	1.42	439/439	462/461	492/491	27
3	β-carotene	1.82	(429)/(429)[e]	453/452	479/478	20

[a] As in Fig. 1.
[b] Retention factor $(k) = (t_r - t_m)/t_m$, where t_r = retention time of the pigment peak and t_m = retention time of an unretained component.
[c] Determined in acetone (Britton, 1992).
[d] Peak height ratio 100 III/II for carotenoids (Davies, 1975).
[e] Parenthesis indicates a shoulder.

comparison with absolute retention times (Fig. 1) and chromatogram quality of published works (Barba, Hurtado, Mata, Ruiz, & Tejada, 2006; Chen, Tai, & Chen, 2004; Granelli & Helmersson, 1996; Hart & Scott, 1995; Khachik et al., 1989; Zakaria & Simpson, 1979). The presence of a shoulder eluting immediately after all-*trans*-β-carotene could be attributed to one or more β-carotene *cis*-isomers (O' Neil & Schwartz, 1992). Similarly, *cis*-isomers of lycopene and γ-carotene may be responsible for the shoulders that followed the respective all-*trans*-forms. Considering that the presence of stereoisomers, other than all-*trans* ones, is not expected in *B. trispora* cells (López-Nieto et al., 2004) *cis*-ones can be found in small amounts in the lipid extracts of most of the molds solely because of isomerization during sample treatment (Echavarri-Erasun & Johnson, 2002). Each shoulder peak was co-quantified with the respective main peak in the subsequent analysis.

To satisfy curiosity we examined whether α- and β-carotene could be separated as follows. We spiked the *B. trispora* extract with an extract of carrot juice known to contain both α- and β-carotene. In the chromatogram of the spiked sample, peak distortion for β-carotene indicated that separation of α- and β-carotene was not possible without modification of the chromatographic system. However, since α-carotene is not synthesized by *B. trispora*, this was not considered as a burden for the aim of the study. Using the above acetone:acetonitrile mixture, the chromatographic separation of lycopene, γ-carotene and β-carotene becomes a rapid, simple and low-cost procedure. Ten years ago Tereshina and coworkers (Tereshina, Memorskaya, & Feofilova, 1994) argued about the need of such a method when they published an express spectrophotometric method for the determination of lycopene and β-carotene contents in standard model mixtures or in extracts from *B. trispora* cells. There is no doubt about the merits of our proposal in comparison to that one or even with comparison to other time and solvent consuming protocols used so far for the analysis of carotenoids of *B. trispora* or even of other microorganisms. The fact that the expected carotenoid pattern is simple should not be overlooked. The carotenoids produced by industrial *B. trispora* strains are of great practical significance as food and feed additives. For these reasons our proposal could be easily adopted by the industry to monitor the steps of the biosynthesis of carotenoids as well as to meet analytical needs for labeling requirements of the end product.

3.2. Method validation

Taking into account current analytical requirements (EURACHEM, 1998), our efforts were then focused on method validation for β-carotene, one of the two major hydrocarbons produced by *B. trispora*.

The method was tested for linearity, LOD and LOQ, recovery and precision. Linearity between the concentration and peak area was determined by analyzing five standard solutions of the analyte (2.0–30.0 ng/μL). A high correlation coefficient ($r^2 = 0.999$) was found for the standard curve and the regression equation for all-*trans*-β-carotene was $y = 6246.5 \times -24{,}974$. The RSD of the slope and the intercept were below 4.0%. Correlation coefficient of the calibration curve and the other calculated regression parameters confirmed good linearity. Our reservation about the suitability of acetone:acetonitrile mixture due to low relative solubility of β-carotene (Craft, 1992) was not proved as critical as expected. Thus, LOD value for β-carotene (4.17 ng/5 μL) was satisfactory when compared with those reported for optimized but time consuming elution schemes (Table 2). The determined LOQ value (4.56 ng/5 μL) was 5-fold lower than that achieved in the spectrophotometric approach of Tereshina and coworkers (1994) indicating applicability of the procedure for the aim of the study, i.e. monitoring of carotenogenesis in *B. trispora*. The fact that retention time was several folds lower than those shown in Table 2 is stressed. Using similar types of columns retention time of β-carotene usually ranges between 14 and 40 min. Its rapid elution using methanol:acetonitrile, 90:10, v/v +9 μM triethylamine (Barba et al., 2006) was not considered appropriate in our case since γ-carotene could not be separated in the presence of lycopene.

Our opinion was reinforced by the satisfactory precision and recovery control data. Repeatability and reproducibility relative standard deviation for β-carotene (300.0 mg/kg oil) was less than 4.0% ($n = 5$). Mean recovery (%) of β-car-

Table 2
LOD values for β-carotene with regards to characteristics of earlier methods and to those described in this paper

Column	Eluent	LOD (ng/5 μL)	Retention time (min)	Reference
Partisil-PXS-5/ODS, 5 μm	MeCN:$CHCl_3$ (92:8)	1.50	14.0	Zakaria and Simpson (1979)
Spherisorb ODS2 metal-free, 5 μm	MeCN:MeOH:CH_2Cl_2 (75:20:5, v/v/v) containing 0.05% triethylamine	0.23	22.0	Hart and Scott (1995)
μBondapack C18, 10 μm	MeOH/MeCN (90:10, v/v) + triethylamine, 9 μM	0.80	6.0	Barba et al. (2006)
Hypersil ODS (C18), 5-μm	Linear gradient of solution P[a]:H_20:MeOH (10:10:80, v/v) and acetone: MeOH (20:80, v/v)	0.50–1.50	17.0	Mantoura and LLevellyn (1983)
Hypersil ODS (C18), 5 μm	Linear gradient of MeOH:MeCN (90:10, v/v) and acetone (100%)	0.32	28.0	Palmisano et al. (1988)
Zorbax SB (C18), 5 μm	Linear gradient of MeOH:MeCN (80:20, v/v) to MeOH:MeCN (60:40, v/v)	0.125	40.0	Lee et al. (2004)
Nucleosil (C18), 5 μm	Acetone:MeCN (60:40, v/v)	4.17	7.0	New method

[a] The ion-pairing reagent (solution P) consisted of 1.5 g of tetrabutyl-ammonium acetate and 7.7 g of ammonium acetate in 100 ml water (Mantoura & LLevellyn, 1983).

otene at 300.0 mg/kg was satisfactory (99.3%, $n = 5$). The above data are satisfactory not only for monitoring the bioprocess but also for determining the absolute content in the end product.

3.3. Application of HPLC methodology to monitor carotenogenesis in *B. trispora* grown in refined olive pomace or soybean oil enriched substrates

The proposed HPLC method was used for monitoring the individual carotenoid changes in extracts of *B. trispora* cells in distinct phases of the fermentation process (e.g. at intervals of 48 h to 8 days) described in a previous study (Mantzouridou et al., 2006). As shown in Fig. 2, it was possible to examine the effect of various substrates, such as refined olive pomace oil and soybean oil, on carotenogenesis and control accumulation of a particular carotenoid during fermentation. Using this rapid procedure, having no lipid sample pre-treatment other than dissolution in the injection solvent, information is obtained on the mode of action of chemical and genetic activators/inhibitors of carotenogenesis by the mold. In terms of quantitative information, quantification of total β-carotene or even % expression of *cis-trans* isomers was satisfactory. At the stage of maximum carotenoid accumulation β-carotene level, expressed as mg/g biomass dry weight, ranged from 22.5 ± 0.6 (olive pomace oil) to 89.7 ± 4.9 (soybean oil). The amount of β-carotene *cis*-isomers varied from 9% to 12% of the total content. Our findings are in agreement with the size of reported values for β-carotene level under different fermentation conditions (Estrella Castro et al., 2003; Kim et al., 1997; Mehta et al., 2003).

As a conclusion, our proposal stands as a rapid RP-HPLC system suitable for (a) monitoring carotenogenesis in *B. trispora* strains, (b) purity control of β-carotene and quantification in the respective end product and (c) simultaneous characterization of the triacylglycerol profile of the lipid cell extract of the mold. The method is promising for routine industrial work, for academic research and official control requirements.

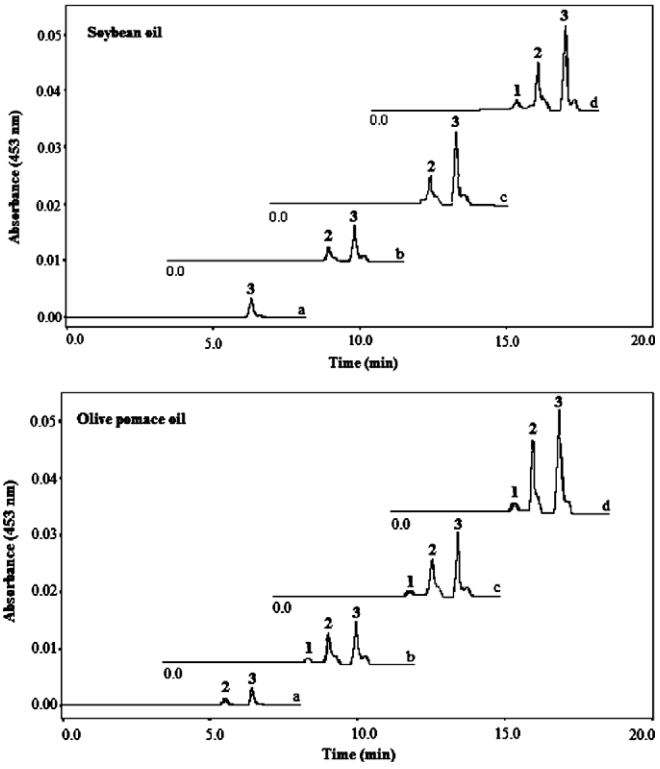

Fig. 2. Changes in the chromatographic profile of carotenoids in extracts of *Blakeslea trispora* cells in the course of fermentation in substrate enriched with soybean oil and olive pomace oil; 1, lycopene; 2, γ-carotene; 3, β-carotene; fermentation period: a, 2; b, 4; c, 6; and d, 8 days (chromatographic conditions as reported in Experimental section).

Acknowledgements

Dr. P. Tarantili is acknowledged for participation as a second analyst in the reproducibility experiment. F.M. thanks IKY for a postdoctoral scholarship.

References

Barba, A. I. O., Hurtado, M. C., Mata, M. C. S., Ruiz, V. F., & Tejada, M. L. S. (2006). Application of a UV–vis detection–HPLC method for a rapid determination of lycopene and β-carotene in vegetables. *Food Chemistry, 95*, 328–336.

Bhosale, P. (2004). Environmental and cultural stimulants in the production of carotenoids from microorganisms. *Applied Microbiology and Biotechnology, 63*, 351–361.

Britton, G. (1992). Carotenoids. In G. A. F. Hendry & J. D. Houghton (Eds.), *Natural food colorants* (1st ed., pp. 141–181). London: Blackie.

Chen, J. P., Tai, C. Y., & Chen, B. H. (2004). Improved liquid chromatographic method for determination of carotenoids in Taiwanese mango (*Mangifera indica* L.). *Journal of Chromatography A, 1054*, 261–268.

Craft, N. E. (1992). Relative solubility, stability, and absorptivity of lutein and β-carotene in organic solvents. *Journal of Agriculture and Food Chemistry, 40*, 431–434.

Cserhati, T., Forgacs, E., Morais, M. H., & Mota, T. (2000). Liquid chromatography of natural pigments. *Biomedical Chromatography, 14*, 281–286.

Davies, B. H. (1975). *Chemistry and biochemistry of plant pigments*. London: Academic Press.

Echavarri-Erasun, C., & Johnson, E. A. (2002). Fungal carotenoids. *Applied Mycology and Biotechnology, 2*, 45–85.

Eitenmiller, R. R., & Landen, W. O. (1999). *Vitamin analysis for the health and food science* (p. 3). Boca Raton: CRC Press.

Estrella Castro, A., Marcos Rodriguez, A. T., Peiro Cezon, E. E., Collados De La Vieja, A., Costa Perez, J., Esteban Morales, M., & Gonzalez De Prado, J. E. (2003). Method for the production of β-carotene. Patent EP 1306444.

Folch, J., Lees, M., & Slane-Stanley, J. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry, 226*, 497–509.

Govind, N. S., Amin, A. R., & Modi, V. V. (1982). Stimulation of carotenogenesis in *Blakeslea trispora* by cupric ions. *Phytochemistry, 21*, 1043–1044.

Granelli, K., & Helmersson, S. (1996). Rapid high-performance liquid chromatographic method for determination of β-carotene in milk. *Journal of Chromatography A, 721*, 355–358.

Hart, D. J., & Scott, K. J. (1995). Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chemistry, 54*, 101–111.

Khachik, F., Beecher, G. R., & Lusby, W. R. (1989). Separation, identification, and quantification of the major carotenoids in extracts of apricots, peaches, cantaloupe, and pink grapefruit by liquid chromatography. *Journal of Agricultural and Food Chemistry, 37*, 1465–1473.

Kim, S. W., Seon, W. T., & Park, Y. H. (1997). Enhanced production of β-carotene from *Blakeslea trispora* with Span 20. *Biotechnology Letters, 19*, 561–562.

Lampila, L. E., Wallen, S. E., Bullerman, L. B., & Lowry, S. R. (1985). The effect of strain and type of whey on the production of β-carotene and other parameters. *Lebensmittel Wissenshaft und Technologie, 18*, 366–369.

Lee, B. L., Su, J., & Ong, C. N. J. (2004). Monomeric C18 chromatographic method for the liquid chromatographic determination of lipophilic antioxidants in plants. *Journal of Chromatography A, 1048*, 263–267.

López-Nieto, M. J., Costa, J., Peiro, E., Méndez, E., Rodriguez-Sáiz, M., De La Fuente, J. L., et al. (2004). *Applied Microbiology and Biotechnology, 66*, 153–159.

Mantoura, R. F. C., & LLevellyn, C. A. (1983). The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high performance liquid chromatography. *Analytica Chimica Acta, 151*, 297–314.

Mantzouridou, F., Tsimidou, M. Z., & Roukas, T. (2006). Performance of crude olive pomace oil and soybean oil during carotenoid production by *Blakeslea trispora* in submerged fermentation. *Journal of Agricultural and Food Chemistry, 54*, 2575–2580.

Mehta, J., Obraztsova, I. N., & Cerdá-Olmedo, E. (2003). Mutants and intersexual heterokaryons of *Blakelsea trispora* for production of β-carotene and lycopene. *Applied Environmental Microbiology, 69*, 4043–4048.

Nam, H. S., Cho, S. Y., & Rhee, J. S. (1988). High-performance liquid chromatographic analysis of major carotenoids from *Rhodotorula glutinis*. *Journal of Chromatography, 448*, 445–447.

O'Neil, C. A., & Schwartz, S. J. (1992). Chromatographic analysis of cis/trans carotenoids isomers. *Journal of Chromatography, 624*, 235–252.

Oliver, J., & Palou, A. (2000). Chromatographic determination of carotenoids in foods. *Journal of Chromatography A, 881*, 543–555.

Palmisano, A. C., Cronin, S. E., & Des Marais, D. J. J. (1988). Analysis of lipophilic pigments from a phototrophic microbial mat community by high performance liquid chromatography. *Journal of Microbiological Methods, 8*, 209–217.

Psomiadou, E., & Tsimidou, M. (1999). On the role of squalene in olive oil stability. *Journal of Agricultural and Food Chemistry, 47*, 4025–4032.

Sandmann, G., Woods, W. S. R., & Tuveson, W. (1990). Identification of carotenoids in *Erwinia herbicola* and in a transformed *Escherichia coli* strain. *Federation of European Microbiological Societies Microbiology Letters, 71*, 77–82.

Shlomai, P., Ben-Amotz, A., & Margalith, P. (1991). Production of carotene stereoisomers by *Phycomyces blakesleeanus*. *Applied Microbiology and Biotechnology, 34*, 458–462.

Snyder, L., & Kirkland, J. J. (1979). *Introduction to modern liquid chromatography*. New York: Wiley.

Taungbodhitham, A. K., Jones, G. P., Wahlqvist, M. L., & Briggs, D. R. (1998). Evaluation of extraction method for the analysis of carotenoids in fruits and vegetables. *Food Chemistry, 63*, 577–584.

Tereshina, V. M., Memorskaya, A. S., & Feofilova, E. P. (1994). Express method for determining the lycopene and β-carotene content. *Microbiology, 63*, 630–633.

The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics. (1998). EURACHEM Guide, ISBN 0-948926-12-0.

Thomas, D. M., & Goodwin, T. W. (1967). Studies on carotenogenesis in *Blakeslea trispora* – I. *General observations on synthesis in mated an unmated strains. Phytochemistry, 6*, 355–360.

Zakaria, M., & Simpson, K. (1979). Use of reversed-phase high-performance liquid chromatographic analysis for the determination of provitamin A carotenes in tomatoes. *Journal of Chromatography, 176*, 109–117.