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Simultaneous determination of carotenes and tocopherols in ATBC drinks by high-performance liquid chromatography

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Abstract

A simple method for the simultaneous extraction and HPLC determination of provitamin A and vitamin E derivatives in vitamin supplemented (ATBC) drinks has been developed. The method allowed the complete separation of α-, β-, γ-, and δ-tocopherol, α-tocopheryl acetate, all-*trans*-α-carotene, all-*trans*-β-carotene, and the 9-*cis* and 13-*cis* isomers of β-carotene in less than 50 min. Recovery of analytes and reproducibility of HPLC determination proved to be excellent. Eight commercial ATBC drinks were investigated for their tocopherol and carotene contents. UV detection was sufficiently sensitive for the detection of all compounds. Due to the ease of handling, the analytical system may find application both in the food and pharmaceutical industry and in the Food Inspection Board.

Keywords: Carotene isomers; Tocopherols; ATBC drinks; Vitaminized drinks; Reversed-phase HPLC

1. Introduction

Functional foods are a rapidly growing segment of the food market. Currently estimated at $80 billion, this market is assumed to have grown to $500 billion by 2010 (Kuzminski, 1999). Among the vitaminized drinks, so-called ATBC drinks have experienced growing popularity. After their launch in 1993, almost 10 million liters were sold on the German market in 1996 (Latz-Weber, 1997). ATBC drinks are supplemented with ascorbic acid (vitamin C), tocopherol (vitamin E), and β-carotene (provitamin A) which are considered as protective micronutrients (Charleux, 1996). With respect to their juice content, ATBC drinks are classified as vitaminized juices and nectars or as vitaminized refreshment drinks (Carle, 1999).

For the production of ATBC drinks, gelatine formulations of synthetic β-carotene are usually employed to enhance water solubility. The provitamin A moiety of ATBC drinks may also originate from carrot juice as a natural source of β-carotene. 'Vitamin E' is a collective term encompassing α-, β-, γ-, and δ-tocopherol and the corresponding tocotrienols (Drotleff & Ternes, 1999). Their antioxidant effect is determined by the degree and position of methyl substitution. Besides the free forms, tocopherols are available as their acetate esters, or, less common, as their hemisuccinate esters. The tocopheryl esters are usually applied for the purpose of fortification since they are less prone to oxidative degradation, whereas the free forms are used as chemical antioxidants (Johnson, 1995). The potential of improving tocopherol levels in the diet has recently been reviewed by Bramley et al. (2000).

Although a number of methods for the determination of carotenes and tocopherols have been reported (e.g. Darnoko, Cheryan, Moros, Jerrel, & Perkins, 2000; De Greyt, Petrauskaite, Kellens, & Huyghebaert, 1998; Garcia-Plazaola & Becerril, 1999; Gimeno, Calero, Castellote, Lamuela-Raventos, de la Torre, & Lopez-Sabater, 2000; Hess, Keller, Oberlin, Bonfanti, & Schuep, 1991; Kurilich et al., 1999; Manzi, Panfili, & Pizzoferrato, 1996; Salo-Väänänen, Ollilainen, Mattila, Lehikoinen, Salmela-Mölsä, & Piironen, 2000; Strohschein, Pursch, Lubda, & Albert, 1998; Ye, Landen, & Eitenmiller, 2000), the application to the analysis of functional food such as ATBC drinks has not yet been described. Furthermore, separation of carotene stereoisomers which display different biological activities has not been considered in most cases, or *cis*-isomers could not be unambiguously assigned (Lietz & Henry, 1997).

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To ensure a high throughput, a rapid method would be desirable both for the producing industry and the Food Inspection Board.

Recently, a method for the quantitative determination of carotene stereoisomers in carrot juices and ATBC drinks has been developed (Marx, Schieber, & Carle, 2000). This method has now been extended to the simultaneous extraction and determination of carotenes and tocopherols. Since tocopherols differ in their biological activity (Traber, Serbinova, & Packer, 1999), with α-tocopherol being the most active, we aimed at complete resolution of α-, β-, γ-, δ-tocopherol, and α-tocopheryl acetate.

2. Materials and methods

2.1. Materials and reagents

ATBC drinks were purchased from local markets. All chemicals used (Merck, Darmstadt, Germany) were of reagent grade. HPLC solvents were of gradient grade. All-*trans*-β-carotene (type II), (±)-α-tocopherol, (+)-γ-tocopherol and (+)-α-tocopheryl acetate were obtained from Sigma (St. Louis, USA), (±)-β-tocopherol and (±)-δ-tocopherol were from Merck (Darmstadt, Germany). All-*trans*-α-carotene was a gift from Hoffmann La Roche Ltd. (Basel, Switzerland). 9-*cis*-β-Carotene and 13-*cis*-β-carotene were obtained by iodine-catalyzed photoisomerization of all-*trans*-β-carotene according to a protocol described by Zechmeister (1962). The *cis*-isomers formed were purified by preparative HPLC using a C_{30} stationary phase Carotenoids (S5 250 mm×10 mm i.d.) from YMC (Wilmington, USA). The isomers were identified by their chromatographic behaviour, their UV/VIS and ^{1}H NMR spectra. Purity was checked by spectrophotometry (UV/VIS and second order derivative spectra), and by re-chromatography. HPLC analyses revealed that standard solutions of 9-*cis* and 13-*cis*-β-carotene contained 4 and 11%, respectively, of the all-*trans*-isomer, owing to back-isomerization of mono-*cis*-β-carotene isomers (Schierle, Härdi, Faccin, Bühler, & Schüep, 1995). To exclude overestimation especially of 13-*cis*-β-carotene, the back-isomerization was compensated for by calculation of the relative contributions of the *cis*- and *trans*-isomers to the total absorbance of the stock solutions.

2.2. HPLC

The HPLC system (Shimadzu, Kyoto, Japan) was equipped with a system controller SCL-10Avp, an auto injector SIL-10 ADvp, a pump FCV-10ALvp, a solvent delivery module LC-10ATvp, a column oven CTO-10Avp, and diode array detector SPD-M10Avp. Chromatographic analysis was performed using an analytical scale (250 mm×4.6 mm i.d.) C_{30} reversed phase column with a particle size of 5 μm (YMC, Wilmington, USA).

Eluent A consisted of methanol: *tert*-butyl methyl ether (MTBE):water (81:15:4,v:v), eluent B was prepared by mixing MTBE, methanol and water (90:6:4,v:v). Separation was performed at a column temperature of 15 °C by a linear gradient from 100% A to 56% B within 50 min at a flow rate of 1 ml/min. Identification of carotenes and tocopherols was based on retention time, co-injection with standards, and UV-VIS spectra. Quantification was carried out by external standards for all-*trans*-β-carotene, all-*trans*-α-carotene, 9-*cis*-β-carotene, 13-*cis*-β-carotene, (±)-α-tocopherol, and (+)-α-tocopheryl acetate monitored at their spectral maximum: all-*trans*-β-carotene (453 nm), all-*trans*-α-carotene (445 nm), 9-*cis*-β-carotene (445 nm), 13-*cis*-β-carotene (445 nm), α-tocopherol (292 nm), and α-tocopheryl acetate (285 nm). Calculation of concentrations was based on linear calibration graphs.

2.3. Sample preparation

ATBC drinks (5 ml) were extracted in an amber glass separatory funnel with a mixture of acetone and hexane (1:1, v:v). The emulsion formed was removed by adding 50 ml sodium chloride solution (10%, wt:v). After separation, the hexane layer was washed with sodium chloride solution (50 ml) to remove acetone. The extract was finally dried with sodium sulfate (2%). Hexane was evaporated in vacuo (T < 30 °C, 150 mbar), the residue was dissolved in 2-propanol and adjusted to a volume of 5 ml. Aliquots of 20 μl were used for HPLC analysis.

2.4. Recovery studies

Recovery studies were performed by adding 1 and 2 ml of an ethanolic solution of α-tocopherol (100 mg/l) to 5 ml of sample 8. For the determination of α-tocopheryl acetate recovery, 1 ml of an ethanolic solution of α-tocopheryl acetate (200 mg/l) was added to 5 ml of sample 1. Recovery studies for carotenes were previously carried out with carrot juice by spiking 1 ml of juice with 1 and 2 ml of a solution of all-*trans*-β-carotene (20 mg/l in 2-propanol), and with 1 and 2 ml of a solution of 13-*cis*-β-carotene (4 mg/l in 2-propanol), respectively (Marx, Schieber, & Carle, 2000). Contents of carotenes and tocopherols were determined prior to recovery studies. All determinations were performed in duplicate.

3. Results and discussion

The method presented in this study allowed the common extraction of carotenes and tocopherols from ATBC drinks with a mixture of acetone and hexane. No

further purification steps such as saponification were required. As described previously, recovery of all-*trans*-β-carotene was 97–105%, for the *cis*-isomer a recovery of 90–93% was found (Marx et al., 2000). Recovery of α-tocopherol and of α-tocopheryl acetate was 97–98 and 100%, respectively.

The separation of tocopherols, especially of the isomers β- and γ-tocopherol, by conventional reversed-phase HPLC systems has proved difficult in the past. Therefore, normal-phase HPLC has been applied in most cases for the separation of all tocopherols (De Greyt et al., 1998; Psomiadou & Tsimidou, 1998; Salo-Väänänen et al., 2000). However, normal-phase systems tend to be disadvantageous with respect to column stability, reproducibility of retention times, and time required for equilibration (Dionisi, Prodolliet & Tagliaferri, 1995; Gimeno et al., 2000; Shin & Samuel-Godber, 1993). Recently, Garcia-Plazaola and Becerril (1999) established a new reversed-phase HPLC method for the simultaneous determination of all major photosynthetic pigments of higher plants, including carotenoids, tocopherols, and chlorophylls; however, without considering *cis*-isomers of carotenoids and without separation of β- and γ-tocopherol.

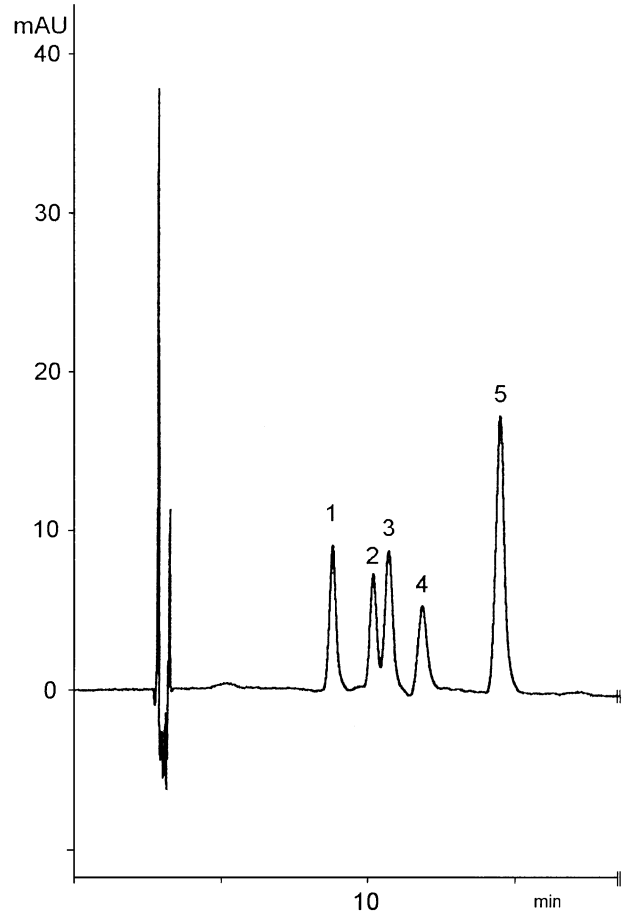

Fig. 1. Separation of a reference mixture of synthetic tocopherols by reversed-phase HPLC (280 nm). (1) δ-tocopherol, (2) γ-tocopherol, (3) β-tocopherol, (4) α-tocopherol, (5) α-tocopheryl acetate.

Strohschein et al. (1998) were the first to achieve complete separation of α-, β-, γ-, and δ-tocopherol, and α-tocopheryl acetate using a C_{30} stationary phase. Since NMR detection was performed in that study, isocratic elution was necessary, resulting in peak tailing especially of late eluting compounds, i.e. α-tocopherol and α-tocopheryl acetate. Therefore, this method is not suitable for routine analysis. Darnoko et al. (2000) reported the simultaneous HPLC analysis of palm carotenoids and tocopherols also using a C_{30} stationary phase. Unfortunately, β- and γ-tocopherol could not be separated, and 13-*cis*-β-carotene was not considered in this study. The applicability of C_{30} stationary phases to food analysis has recently been reviewed by Sander, Sharpless, and Pursch (2000).

Using gradient elution at a column temperature of 15 °C, the separation of all tocopherols and α-tocopheryl acetate with sharp peaks was achieved in the present study (Fig. 1). The elution order (δ-, γ-, β-, α-tocopherol, α-tocopheryl acetate) is consistent with the results obtained by Strohschein et al. (1998). The separation of tocopherols and carotenes extracted from a vitaminized drink (sample 8) is shown in Fig. 2. As can be seen, the separation of carotene stereoisomers was not adversely affected by the slightly changed chromatographic conditions in comparison to the previously described method (Marx et al., 2000). Although fluorescence detection is considerably more sensitive, UV detection proved to be sufficient for the quantitative determination of tocopherols.

The analytical system was applied to the quantification of lipid-soluble vitamins and provitamins, respectively, of eight commercially available ATBC drinks. As can be seen from Table 1, all-*trans*-β-carotene was found to be the predominant carotene in all samples investigated. Amounts of all-*trans*-α-carotene were considerably lower except for sample 8 which displayed about half the amount of all-*trans*-β-carotene. These findings indicate that, with the exception of sample 8, only small amounts of carrot juice were used for the production of these ATBC drinks. With respect to the *cis*-isomers, only samples 3 and 4 displayed elevated contents of 9-*cis*- and 13-*cis*-β-carotene which are supposed to originate from thermally and light-induced isomerization of all-*trans*-β-carotene during processing and storage (Chen, Peng & Chen, 1995, 1996). In most samples, the β-carotene contents determined exceeded the values specified on the label. This results from stability overages which need to be added to compensate for losses during storage, thus maintaining the vitamin content over the specified shelf life (Carle, 1999; Marx et al., 2000).

From Table 2 it becomes evident that seven out of eight samples exclusively contained α-tocopheryl acetate

and small amounts of α-tocopherol. The latter is supposed to originate from ester hydrolysis during production and storage which is favoured by the acidic milieu of the ATBC drinks (for pH values, see Table 1). As a consequence of drastic thermal conditions an elevated α-tocopherol level was found in sample 3. This assumption is also supported by the relatively high content of 13-*cis*-β-carotene determined in sample 3. Considering

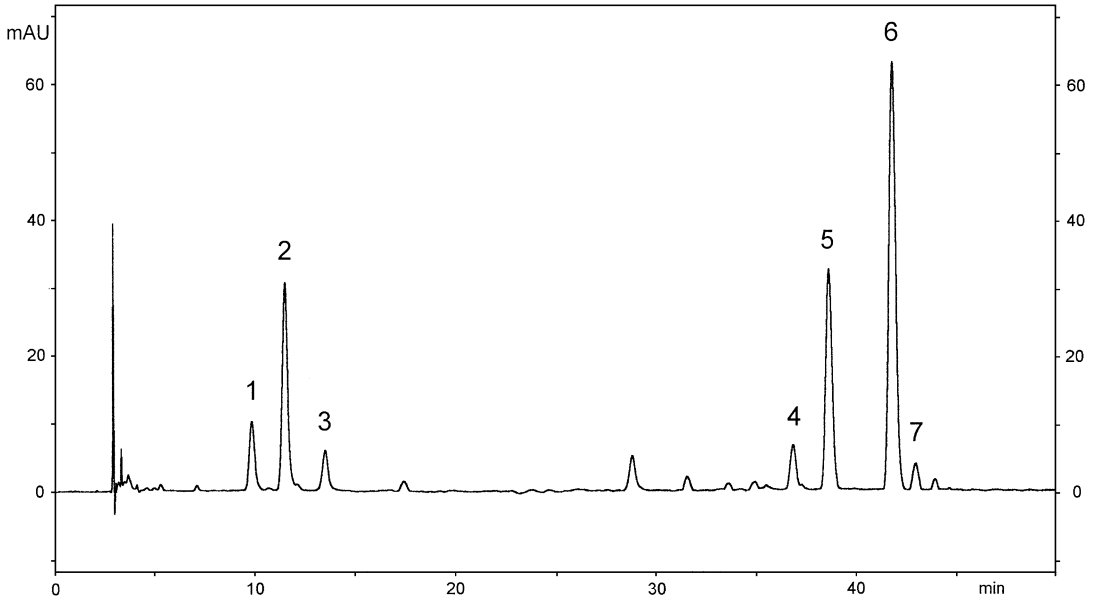

Fig. 2. Separation of tocopherols and carotenes extracted from an ATBC drink (sample 8, spectrum maximum plot). (1) δ-tocopherol, (2) γ-tocopherol, (3) β-tocopherol, (4) 13-*cis*-β-carotene, (5) all-*trans*-α-carotene, (6) all-*trans*-β-carotene, (7) 9-*cis*-β-carotene.

Table 1
Product specification and carotene content of commercial ATBC drinks[a]

Sample	pH	Declaration of carotene content mg/l	All-*trans*-α-carotene mg/l	All-*trans*-β-carotene mg/l	9-*cis*-β-carotene mg/l	13-*cis*-β-carotene mg/l
1	3.4	20.6	1.8±0.1	28.8±0.6	0.9±0.0	1.8±0.1
2	3.4	14.4	2.1±0.1	27.2±0.7	0.8±0.0	1.8±0.1
3	3.8	36.0	1.7±0.1	49.8±1.3	2.0±0.1	7.3±0.6
4	3.4	28.8	1.8±0.1	25.0±1.1	1.6±0.0	4.8±0.1
5	3.4	29.0	2.1±0.1	42.2±1.4	1.0±0.0	1.9±0.0
6	3.5	29.0	1.4±0.0	32.7±1.1	0.9±0.0	1.7±0.1
7	3.7	24.0	4.5±0.3	24.3±1.8	0.6±0.0	2.3±0.1
8	4.0	24.0	11.3±0.6	22.1±1.2	1.2±0.1	2.5±0.1

[a] Contents given in the table are mean values±standard deviation of three replications

Table 2
Product specification and tocopherol content of commercial ATBC drinks[a]

Sample	Declaration of vitamin E content mg/l	α-tocopherol mg/l	γ-tocopherol mg/l	δ-tocopherol mg/l	α-tocopheryl acetate mg/l	Calculated vitamin E content[b] mg/l
1	43.0	5.4±0.1	n.d.[c]	n.d.	69.3±1.0	51.8
2	30.0	5.3±0.3	n.d.	n.d.	42.9±1.1	34.0
3	75.0	10.2±0.7	n.d.	n.d.	132.6±2.3	98.6
4	60.0	6.0±0.4	n.d.	n.d.	81.5±1.4	60.6
5	60.0	6.7±0.5	n.d.	n.d.	108.6±4.8	79.5
6	61.0	5.7±0.2	n.d.	n.d.	81.9±3.1	60.6
7	50.0	4.7±0.5	n.d.	n.d.	73.3±1.8	53.8
8	50.0	49.8±2.5	174.0±3.0	59.5±1.3	n.d.	69.0

[a] Contents given in the table are mean values ± standard deviation of three replications
[b] Calculations based on the following conversion factors: γ-tocopherol, 0.1; δ-tocopherol, 0.03; α-tocopheryl acetate, 0.67 (Traber et al., 1999)
[c] n.d.: not detectable (detection limits: α-tocopherol, 4 mg/l; γ-tocopherol, 4 mg/l; δ-tocopherol, 4 mg/l, α-tocopheryl acetate, 8 mg/l)

the biological activity of DL-α-tocopheryl acetate being 67% of D-α-tocopherol (Traber et al., 1999), the vitamin E content was calculated for samples 1–7 and compared with the declaration. As for the carotenes, tocopherol contents exceeded the values specified on the label due to stability overages.

A completely different tocopherol pattern was found in sample 8 which contained α-, γ-, and δ-tocopherol. Synthetic tocopheryl acetate was absent. The vitamin E moiety is supposed to originate from extracts of natural vegetable oils, e.g. soybean oil. The specified vitamin E content is covered by α-tocopherol, whereas the biological activity of γ- and δ-tocopherol as vitamers is comparatively low (Traber et al., 1999). The latter compounds serve as natural antioxidants rather than as vitamins.

4. Conclusion

A method for the simultaneous extraction and quantitative determination of carotene isomers and tocopherols in ATBC drinks by HPLC is provided. With minor modifications, the analytical system may also be applied to other functional foods based on lipid-soluble vitamins. In this study, UV detection proved to be sufficiently sensitive for the detection of all compounds investigated. Only if quantification of trace amounts of tocopherols is necessary, fluorescence detection may be required. This method is valid for stability control and monitoring of content uniformity of products, respectively. It may also be useful for authenticity control of vegetable oils. Due to the ease of handling, the analytical system is expected to find application both in the food and pharmaceutical industry and in the Food Inspection Board.

References

Bramley, P. M., Elmadfa, I., Kafatos, A., Kelly, F. J., Manios, Y., Roxborough, H. E., Schuch, W., Sheehy, P. J. A., & Wagner, K.-H. (2000). Vitamin E. *Journal of the Science of Food and Agriculture*, *80*, 913–938.

Carle, R. (1999). Physical and chemical stability of ATBC-drinks. *Fruit Processing*, *9*, 342–349.

Charleux, J.-L. (1996). Beta-carotene, vitamin C, and vitamin E: the protective micronutrients. *Nutrition Reviews*, *54*, S109–S114.

Chen, B. H., Peng, H. Y., & Chen, H. E. (1995). Changes of carotenoids, color, and vitamin A contents during processing of carrot juice. *Journal of Agricultural and Food Chemistry*, *43*, 1912–1918.

Chen, H. E., Peng, H. Y., & Chen, B. H. (1996). Stability of carotenoids and vitamin A contents during storage of carrot juice. *Food Chemistry*, *57*, 497–503.

Darnoko, D., Cheryan, M., Moros, E., Jerrel, J., & Perkins, E. G. (2000). Simultaneous HPLC analysis of palm carotenoids and tocopherols using a C-30 column and photodiode array detector. *Journal of Liquid Chromatography & Related Technologies*, *23*, 1873–1885.

De Greyt, W. F., Petrauskaite, V., Kellens, M. J., & Huyghebaert, A. D. (1998). Analysis of tocopherols by gas-liquid and high-performance liquid chromatography: a comparative study. *Fett/Lipid*, *100*, 503–507.

Dionisi, F., Prodolliet, J., & Tagliaferri, E. (1995). Assessment of olive oil adulteration by reversed-phase high-performance liquid chromatography/amperometric detection of tocopherols and tocotrienols. *Journal of the American Oil Chemists' Society*, *72*, 1505–1511.

Drotleff, A. M., & Ternes, W. (1999). *Cis/trans* isomers of tocotrienols—occurrence and bioavailability. *European Food Research and Technology*, *210*, 1–8.

Garcia-Plazaola, J. I., & Becerril, J. M. (1999). A rapid high-performance liquid chromatography method to measure lipophilic antioxidants in stressed plants: simultaneous determination of carotenoids and tocopherols. *Phytochemical Analysis*, *10*, 307–313.

Gimeno, E., Calero, E., Castellote, A. I., Lamuela-Raventós, R. M., de la Torre, M. C., & López-Sabater, M. C. (2000). Simultaneous determination of α-tocopherol and β-carotene in olive oil by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A*, *881*, 255–259.

Hess, D., Keller, H. E., Oberlin, B., Bonfanti, R., & Schuep, W. (1991). Simultaneous determination of retinol, tocopherols, carotenes and lycopene in plasma by means of high-performance liquid chromatography on reversed phase. *International Journal of Vitamin and Nutrition Research*, *61*, 232–238.

Johnson, L. E. (1995). Food technology of the antioxidant nutrients. *Critical Reviews in Food Science and Nutrition*, *35*, 149–159.

Kurilich, A. C., Tsau, G. J., Brown, A., Howard, L., Klein, B. P., Jeffery, E. H., Kushad, M., Wallig, M. A., & Juvik, J. A. (1999). Carotene, tocopherol, and ascorbate contents in subspecies of *Brassica oleracea*. *Journal of Agricultural and Food Chemistry*, *47*, 1576–1581.

Kuzminski, L. N. (1999). Issues and pressures for food and beverage research and development in the 21st century. *Critical Reviews in Food Science and Nutrition*, *39*, 1–11.

Latz-Weber, H. (1997). Innovative Getränkebranche—Neue Verbrauchertrends. *Flüssiges Obst*, *64*, 185–187.

Lietz, G., & Henry, C. J. K. (1997). A modified method to minimise losses of carotenoids and tocopherols during HPLC analysis of red palm oil. *Food Chemistry*, *60*, 109–117.

Manzi, P., Panfili, G., & Pizzoferrato, L. (1996). Normal and reversed-phase HPLC for more complete evaluation of tocopherols, retinols, carotenes and sterols in dairy products. *Chromatographia*, *43*, 89–93.

Marx, M., Schieber, A., & Carle, R. (2000). Quantitative determination of carotene stereoisomers in carrot juices and vitamin supplemented (ATBC) drinks. *Food Chemistry*, *70*, 403–408.

Psomiadou, E., & Tsimidou, M. (1998). Simultaneous HPLC determination of tocopherols, carotenoids, and chlorophylls for monitoring their effect on virgin olive oil oxidation. *Journal of Agricultural and Food Chemistry*, *46*, 5132–5138.

Salo-Väänänen, P., Ollilainen, V., Mattila, P., Lehikoinen, K., Salmela-Mölsä, E., & Piironen, V. (2000). Simultaneous HPLC analysis of fat-soluble vitamins in selected animal products after small-scale extraction. *Food Chemistry*, *71*, 535–543.

Sander, L. C., Sharpless, K. E., & Pursch, M. (2000). C_{30} Stationary phases for the analysis of food by liquid chromatography. *Journal of Chromatography A*, *880*, 189–202.

Schierle, J., Härdi, W., Faccin, N., Bühler, I., & Schüep, W. (1995). Geometrical isomers of β,β-carotene. In G. Britton, S. Liaaen-Jensen, & H. Pfander (Eds.), *Carotenoids. Vol. 1A: isolation and analysis* (pp. 265–272). Basel: Birkhäuser.

Shin, T.-S., & Samuel-Godber, J. (1993). Improved high-performance liquid chromatography of vitamin E vitamers on normal-phase columns. *Journal of the American Oil Chemists' Society*, *70*, 1289–1291.

Strohschein, S., Pursch, M., Lubda, D., & Albert, K. (1998). Shape selectivity of C_{30} phases for RP-HPLC separation of tocopherol isomers and correlation with MAS NMR data from suspended stationary phases. *Analytical Chemistry*, *70*, 13–18.

Traber, M. G., Serbinova, E. A., & Packer, L. (1999). Biological activities of tocotrienols and tocopherols. In L. Packer, M. Hiramatsu, & T. Yoshikawa (Eds.), *Antioxidant food supplements in human health* (pp. 55–71). San Diego: Academic Press.

Ye, L., Landen, W. O., & Eitenmiller, R. R. (2000). Liquid chromatographic analysis of all-*trans*-retinyl palmitate, β-carotene, and vitamin E in fortified foods and the extraction of encapsulated and nonencapsulated retinyl palmitate. *Journal of Agricultural and Food Chemistry*, *48*, 4003–4008.

Zechmeister, L. (1962). *Cis-trans isomeric carotenoids, vitamins A and arylpolyenes* (pp. 51–54). Vienna: Springer.