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Quantitative determination of carotene stereoisomers in carrot juices and vitamin supplemented (ATBC) drinks

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Abstract

For the quantification of α- and β-carotene including the *cis*-isomers of β-carotene in carrot juices and vitamin supplemented (ATBC) drinks, a rapid and artefact-free method was developed. The analytical procedure involves the extraction of carotenes with a mixture of acetone-hexane and their determination by HPLC using a C_{30} stationary phase. No saponification prior to HPLC is required. The method was applied to the determination of carotenes in commercially available carrot juices, and, for the first time, in ATBC drinks. In carrot juices, α-carotene contents ranged from 19.9 to 49.4 mg/l and for all-*trans*-β-carotene from 32.8 to 84.8 mg/l. Relative amounts of *cis*-isomers, calculated as percentages of all-*trans*-β-carotene, were up to 16.2%. High relative amounts (31.8–44.5%) were found in ATBC drinks exclusively composed of synthetic β-carotene. In contrast, ATBC drinks containing carrot juice as a natural source of β-carotene showed significantly lower isomerization (6.7–13.6%), which is in the range of plain carrot juices. Technological implications as well as nutritional consequences of these findings are discussed.

1. Introduction

Epidemiological studies provide growing evidence that carotenoids and other antioxidants may protect humans against certain types of cancer (Steinmetz & Potter, 1996), and cardiovascular diseases (Gaziano, Manson, Buring & Hennekens, 1992). The role carotenoids play in protecting plants and animals from excess sunlight has also been recently observed in humans (Biesalski, Hemmes, Hopfenmuller, Schmid & Gollnick, 1996). Therefore, a minimum of five servings a day of vegetables and fruits, especially green and yellow vegetables and citrus fruits, is recommended (National Research Council [NRC], 1989). According to Biesalski (1995), healthy adults who are not exposed to any particular oxidative stress should consume 2–4 mg β-carotene daily.

Although consumers are increasingly aware of diet related health problems (Gilbert, 1997), a large group of the population lacks a generous intake of fruits and vegetables (Krebs-Smith, Cook, Subar, Cleveland, Friday & Kahle, 1996). As an alternative, recommended daily allowance of carotenoids may be met by the intake of functional foods (beverages) such as ATBC drinks which are supplemented with ascorbic acid (vitamin C), tocopherol (vitamin E), and β-carotene (provitamin A). The provitamin A moiety of ATBC drinks may also originate from carrot juice as a natural source of β-carotene. With respect to the juice content, ATBC drinks are classified as vitaminized juices and nectars, or as vitaminized refreshment drinks.

During technological treatment and processing of food such as canning, drying, cooking, and storage, all-*trans*-carotenoids are partly converted to their *cis*-isomers. Exposure of all-*trans*-β-carotene to light predominantly leads to the formation of the 9-*cis*-isomer (Chen, Peng & Chen, 1996), whereas 13-*cis*-β-carotene is mainly formed by thermal treatment (Chen, Peng & Chen, 1995). From a technological point of view, the occurrence of *cis*-isomers is correlated with a decrease of colour intensity (Bauernfeind, 1981). The nutritional consequences of these conversions may be the reduction of vitamin A activity (Rodriguez-Amaya & Tavares, 1992), and alterations regarding their bioavailability and their antioxidative properties (Ben-Amotz & Levy, 1996; Jimenez and Pick, 1993). Investigation of the effects of various technological steps on the degree of

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isomerization is, therefore, of great interest. For this purpose, the availability of adequate analytical techniques, including sample preparation and unambiguous determination by liquid chromatography, must be considered a prerequisite.

In spite of the great number of publications dealing with carotenoid analysis in fresh and processed fruits and vegetables, there is no generally accepted method for the isolation and determination of carotenoids, especially of their *cis*-isomers. As a consequence, results obtained by various investigations and studies are, if any, difficult to compare. Establishing databases of individual carotenoids and their *cis*-isomers, as suggested by Ben-Amotz and Fishler (1998), also requires reliable and accurate analytical techniques for their determination. The reported methods of carotene isomer separation and quantification are tedious, time-consuming, and often nonreproducible. The official method for the determination of total carotenoids in fruit juices does not allow the differentiation of individual stereoisomers (International Union of Fruit Juice Producers [IFU], 1991).

The purpose of the present study was to establish a rapid and reliable method for the quantitative determination of α- and β-carotene including the stereoisomers of β-carotene in carrot juices and ATBC drinks.

2. Materials and methods

2.1. Materials and reagents

Carrot juices and ATBC drinks were purchased from local markets or obtained from juice producing companies. All chemicals used (Merck, Darmstadt, Germany) were of reagent grade. HPLC solvents were of gradient grade.

All-*trans*-α-carotene (type V) and all-*trans*-β-carotene (type II) were obtained from Sigma (St. Louis, USA), 9-*cis*-β-carotene, 13-*cis*-β-carotene, and 15-*cis*-β-carotene were provided by Hoffmann La Roche Ltd. (Basel, Switzerland). The internal standard β-apo-8′-carotenal was from Fluka (Basel, Switzerland).

2.2. HPLC

The HPLC system (Shimadzu, Kyoto, Japan) was equipped with an auto injector SIL-10 ADvp, a system controller SCL-10Avp, a solvent delivery module LC-10 ATvp, a pump FCV-10ALvp, a column oven CTO-10Avp, and a 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). HPLC conditions were as follows: 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 of carotenoids was achieved by a linear gradient from 100% A to 56% B within 50 min at a flow rate of 1 ml/min. Carotenoid isomers were identified by their retention and their spectral data. Except for 13-*cis*-β-carotene, individual carotenoid peaks were monitored at their spectral maximum: all-*trans*-β-carotene (452 nm); all-*trans*-α-carotene (445 nm); 9-*cis*-β-carotene (445 nm); 13-*cis*-β-carotene (471 nm); β-apo-8′-carotenal (462 nm).

Quantification was carried out both by external standards and by standard addition of β-apo-8′-carotenal. Purity of the standards was checked before use. All standards displayed their characteristic absorption spectra and were eluted as individual peaks. The concentrations of the standard solutions were determined spectrophotometrically using the following extinction coefficients (Britton, 1995; Schierle, Härdi, Faccin, Bühler & Schüep, 1995): all-*trans*-β-carotene (2592 at 450 nm); all-*trans*-α-carotene (2725 at 446 nm); 9-*cis*-β-carotene (2550 at 445 nm); 13-*cis*-β-carotene (2050 at 443 nm); β-apo-8′-carotenal (2640 at 457 nm). Carotene isomers were dissolved in hexane containing 2% (v/v) dichloromethane, the internal standard was dissolved in light petroleum. For HPLC calibration, standard solutions were dried with nitrogen and their original volumes restored with 2-propanol. Aliquots of 20 μl were used for HPLC. The standard curves were linear and covered the concentration ranges of all samples.

2.3. Sample preparation

Carrot juices and ATBC drinks (1–5 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 water (50 ml) to remove acetone. Butylated hydroxytoluene (BHT) was added as an antioxidant to reach a final concentration of 0.1%, and the extract was dried with sodium sulfate (2 g). Hexane was evaporated in vacuo ($T < 30°C$, 150 mbar), the residue was dissolved in isopropanol and made up to a volume of 25 ml. Aliquots of 20 μl were used for HPLC analysis.

3. Results and discussion

The chromatographic separation of carotenoid stereoisomers isolated from a carrot juice sample and of β-apo-8′-carotenal used as an internal standard is shown in Fig. 1. As can be seen, baseline separation was achieved for all compounds of interest. A purity check conducted by liquid chromatography–diode array detection revealed that ζ-carotene coeluted with 13-*cis*-β-carotene. Therefore, the determination of 13-*cis*-β-

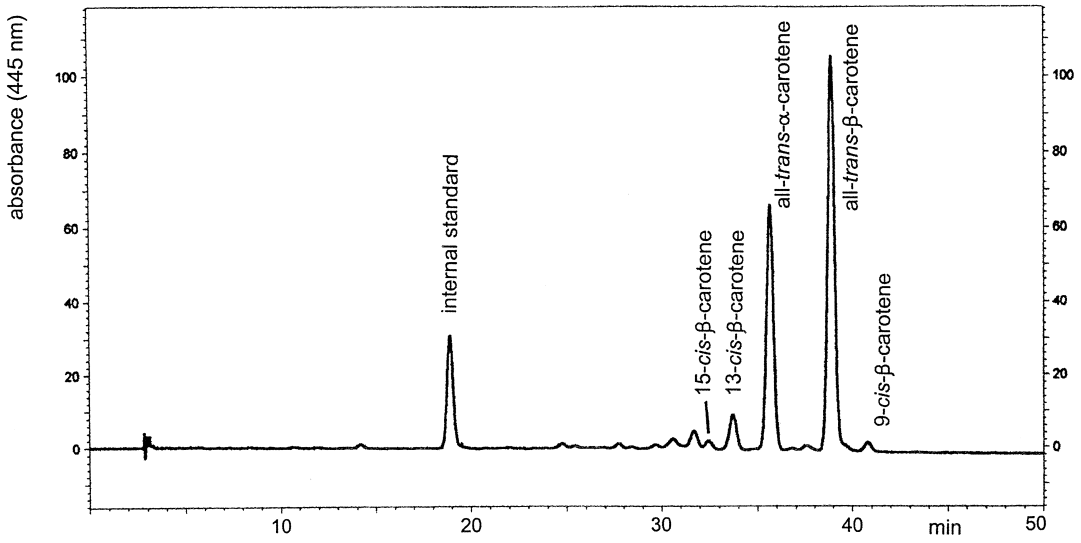

Fig. 1. Separation of α-carotene and β-carotenene stereoisomers isolated from a carrot juice sample, and of β-apo-8′-carotenal used as an internal standard.

carotene was performed at 471 nm. The amounts of 15-*cis*-β-carotene could not be quantified since not enough reference material was available.

It has been reported that hot saponification may lead to the formation of *cis*-isomers (Kimura, Rodriguez-Amaya & Godoy, 1990). Since lipids coextracted with carotenoids did not interfere with the compounds of interest in the chromatographic runs, no saponification step was required during sample preparation. The use of chlorinated solvents, e.g. chloroform and methylene chloride, may also promote the formation of artefacts (Pesek, Warthesen & Taoukis, 1990). If diethyl ether or tetrahydrofuran are employed, the formation of peroxides must be avoided.

Therefore, no chlorinated solvents were used for extraction and chromatographic separation of carotenoids. A mixture of acetone and hexane proved to be highly suitable for the extraction of analytes, as observed by complete decolorization of the aqueous layer. Since all steps of sample preparation were carried out with amber glassware within 20 min, no light-induced isomerization was monitored, although photosensitizing properties of acetone were described (Kagan, 1993).

Recovery of analytes was exemplified for all-*trans*-β-carotene and 13-*cis*-β-carotene. For all-*trans*-β-carotene a recovery of 97–105% was determined, for the *cis*-isomer a recovery of 90–93% was found. To prove artefact-free sample preparation, fresh carrots were homogenized and treated as described above. Chromatographic analysis showed that the amount of *cis*-isomers was $<0.1\%$.

In our study, a polymeric C_{30} stationary phase was used for the analysis of β-carotene stereoisomers. This column was specifically developed for the separation of carotenoids (Sander, Epler Sharpless, Craft & Wise, 1994) and has been successfully applied to the isolation of geometrical isomers of β-carotene (Emenhiser, Englert, Sander, Ludwig & Schwartz, 1996) and the separation of carotenoid isomers in biological extracts (Emenhiser, Simunovic, Sander & Schwartz, 1996) and in fresh and processed fruits and vegetables (Lessin, Catigani & Schwartz, 1997).

Amounts of carotenoids and pH values determined in commercially available carrot juices and ATBC drinks are presented in Table 1. In carrot juices, amounts of all-*trans*-α-carotene ranged from 19.9 mg/L (sample 1) to 49.4 mg/L (sample 3). Concentrations of all-*trans*-β-carotene ranged from 32.8 mg/l to 84.8 mg/l. With respect to the *cis*-isomers, amounts of 0.9 mg/l to 3.5 mg/L were found for 9-*cis*-β-carotene, and 2.8 mg/l to 9.6 mg/L for 13-*cis*-β-carotene.

α-Carotene was only detected in those ATBC drinks that contained carrot juice, with concentrations ranging from 0.6 to 1.5 mg/l. Amounts of all-*trans*-β-carotene were between 17.3 and 29.7 mg/l. The concentrations of the *cis*-isomers ranged from 0.6 to 2.5 mg/l for 9-*cis*-β-carotene, and from 1.1 mg/L to 8.0 mg/L for 13-*cis*-β-carotene. Relative amounts of *cis*-isomers were calculated as percentage of all-*trans*-β-carotene. In juices, up to 16.2% *cis*-isomers were found. Minimum amount of *cis*-isomers was 4.0%. Significantly higher percentages of *cis*-isomers (31.8–44.5%) were determined in ATBC drinks. The pH values in carrot juices were between 4.5 and 5.7, whilst ATBC drinks had lower pH values ranging from 3.2 to 3.9.

The production of carrot juices involves various technological steps that may affect their carotenoid contents and isomeric composition (Handschuh, 1995). The raw material is usually blanched to inactivate pectin esterase. Since carrots are low-acid products (pH 5.5–6.5), acidification is usual to avoid sterilization conditions.

Table 1
Product specification and carotenoid content of carrot juices and ATBC drinks

Samples	pH	Declaration of carotenoid 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	Relative amount of *cis*-isomers (%)[a]
Carrot juices							
1	4.6	45.0[b]	19.9	32.8	1.0	2.8	11.6
2	5.3	50.0[c]	24.6	54.0	1.0	2.8	7.0
3	5.3	0[e]	49.4	83.9	3.5	9.6	15.6
4	5.2	0[e]	38.2	71.4	3.4	8.2	16.2
5	5.2	146.0[b]	45.3	84.8	2.0	8.8	12.7
6	4.5	140.0[b]	43.1	78.3	0.9	8.7	12.3
7	5.7	0[e]	40.4	69.2	n.d.	2.8	4.0
ATBC drinks							
1	3.9	20.0[d]	n.d.[f]	23.6	2.5	8.0	44.5
2	3.2	20.0[d]	n.d.	17.3	0.8	4.7	31.8
3	3.4	20.6[d]	0.7	20.1	0.6	1.1	8.5
4	3.5	29.0[d]	0.6	29.7	0.6	1.4	6.7
5	3.4	28.8[d]	1.5	26.4	1.1	2.5	13.6

[a] Calculated as percentage of all-*trans*-β-carotene. Amounts given in the table are mean values of two measurements.
[b] Total carotenoids.
[c] β-carotene.
[d] Provitamin A.
[e] 0 Missing.
[f] n.d.: not detectable.

For stabilization, carrot juices are pasteurized and sterilized by tyndallization, respectively.

The results of our investigations show that in carrot juices the amounts of 13-*cis*-β-carotene consistently exceed those found for the 9-*cis*-isomer (Table 1). Regarding the relatively high pH values of most of the the juices investigated, elevated heat load had to be applied for preservation. Since 13-*cis*-β-carotene is mainly formed by thermal treatment (Chen et al., 1995), the presence of 13-*cis*-β-carotene is assumed to result from heat treatment during juice production.

The formation of 9-*cis*-β-carotene may not be attributed to acidification measures because no correlation between the amounts of 9-*cis*-β-carotene and the pH value could be found. More likely, a conversion of *cis*-isomers via all-*trans*-β-carotene might have taken place during storage (Pesek et al., 1990). Storage conditions largely affect the isomeric composition of carotenoids. Chen et al. (1996) found that storage of carrot juice in the dark leads to the formation of the 13-*cis*-isomers of lutein, and α- and β-carotene, whereas exposure to light facilitates the formation of the 9-*cis*-isomers. Since *cis*-isomers of carotenoids have not been found in fresh carrots (Godoy & Rodriguez-Amaya, 1998), the contribution of the raw material to the *cis*-isomer content seems to be negligible.

Due to its pronounced lipophilic properties β-carotene is virtually insoluble in water and ethanol. With respect to their application in foods, only few solvents, e.g. citrus oils, triglycerides or vitamin E may be considered. Commercial β-carotene preparations contain about 30% micro-crystalline all-*trans*-β-carotene with a crystal size of 3–10 μm. The crystals are suspended in vegetable oil (Klaeui, 1981). For the production of ATBC-basic material β-carotene and vitamin E are dissolved in a hot mixture of weighting agent (e.g. sucrose–acetate–isobutyrate) and lipophilic solvent and finely dispersed by homogenization in the aqueous phase containing a hydrocolloid solution, and syrup or fruit juice concentrate and antioxidants (Lueddecke & Horn, 1986). Heating the aqueous phase prevents rapid recrystallization.

Two of the five ATBC drinks examined in the present study exclusively contained synthetic β-carotene which was probably emulsified as described above. Consequently, both samples showed significantly higher relative amounts of *cis*-isomers (31.8 and 44.5%, respectively) than the other ATBC drinks (6.7%–13.6%). These findings suggest that hot dissolution of β-carotene plays an important role in the isomerization of the raw material. According to our own investigations, high-pressure homogenization (250–300 bar) also facilitates the formation of 9-*cis*-β-carotene during manufacture of ATBC drinks (Carle, 1999).

In comparison to carrot juices, pH values of the ATBC drinks were substantially lower. Therefore, pasteurization conditions are sufficient for the preservation of these products. This fact might explain the findings that most of the ATBC drinks display smaller amounts of 13-*cis*-β-carotene than carrot juices. It is concluded that the presence of 13-*cis*-β-carotene in ATBC drinks is a consequence of hot dissolution of β-carotene rather than of pasteurization of the finished product. According to investigations of Chen et al. (1995), acidification

of carrot juice to pH 4.0 had virtually no effects on carotene isomerization.

ATBC drinks containing carrot juice as a natural source of β-carotene display a significantly lower isomerization rate. Since also β-carotene stability of plain carrot juices during storage was significantly better than in ATBC drinks, a protecting matrix effect of carrot juice with respect to *trans-cis*-isomerization of carotenoids is assumed (Carle, 1999). From a nutritional point of view, the *cis*-isomers have been associated with a reduced vitamin A activity (Rodriguez-Amaya & Tavares, 1992) and alterations with respect to their bioavailability and their antioxidative properties (Ben-Amotz & Levy, 1996; Jimenez & Pick, 1993). Although relative amounts of *cis*-isomers are much higher in ATBC drinks exclusively produced from synthetic β-carotene, there is virtually no difference in absolute amounts of *cis*-isomers that are ingested via carrot juices and ATBC drinks. Nevertheless, the conversion of β-carotene, especially in ATBC drinks, should be generally minimized by using adequate technological treatment.

For ATBC drinks, the declaration of the vitamin content has to be guaranteed during the specified shelf life. From a technological point of view, stability overages have to be added to counteract losses of carotenoids during storage. For the consumer the problem arises that an exact calculation of the provitamin A intake cannot be realized. Therefore, admissible deviations from the amounts specified should be fixed. The Association of German Chemists recommends that a deviation of $\pm 30\%$ for provitamin A and vitamin E should be tolerated. Overages of 50% of the specified vitamin content, however, should not be exceeded (N.N., 1998).

As can be seen in Table 1, amounts of carotenoids in carrot juices were specified either as total carotenoids, or as β-carotene. In three samples a declaration of carotenoids was missing. In ATBC drinks, carotenoids were expressed as provitamin A. Both in ATBC drinks and in carrot juices, the amounts of carotenoids determined were in part lower than specified on the label. The deviations observed may be due to differences in analytical methodology employed for the extraction and quantification of carotenoids, or they may be a consequence of carotenoid degradation during storage. This confirms the necessity of establishing standardized methods for carotenoid analysis.

4. Conclusion

The method described allows the rapid isolation and quantitative determination of all-*trans*-α- and all-*trans*-β-carotene including the *cis*-stereoisomers of β-carotene. Due to the ease of sample preparation, the method may be used both in industry, i.e. quality control, and by food inspection authorities. Thus, it provides the basis to more accurately assess the carotenoid content of processed foods.

Our study clearly shows that during the production of carrot juice and ATBC drinks a conversion of all-*trans*-β-carotene to its *cis*-isomers takes place to a different extent. It will be of interest to investigate which of the technological steps applied contribute most to these isomerization reactions in order to draw conclusions for optimized production. The role of chromoplast degradation and its possible effects on the isomerization of carotenoids needs further study. These investigations, including semi-industrial processing of carrot juice and ATBC drinks, are currently under way.

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