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REVIEW ARTICLE

Analytical tools for the analysis of β-carotene and its degradation products

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Abstract

β-Carotene, the precursor of vitamin A, possesses pronounced radical scavenging properties. This has centered the attention on β-carotene dietary supplementation in healthcare as well as in the therapy of degenerative disorders and several cancer types. However, two intervention trials with β-carotene have revealed adverse effects on two proband groups, that is, cigarette smokers and asbestos-exposed workers. Beside other causative reasons, the detrimental effects observed have been related to the oxidation products of β-carotene. Their generation originates in the polyene structure of β-carotene that is beneficial for radical scavenging, but is also prone to oxidation. Depending on the dominant degradation mechanism, bond cleavage might occur either randomly or at defined positions of the conjugated electron system, resulting in a diversity of cleavage products (CPs).

Due to their instability and hydrophobicity, the handling of standards and real samples containing β-carotene and related CPs requires preventive measures during specimen preparation, analyte extraction, and final analysis, to avoid artificial degradation and to preserve the initial analyte portfolio. This review critically discusses different preparation strategies of standards and treatment solutions, and also addresses their protection from oxidation. Additionally, *in vitro* oxidation strategies for the generation of oxidative model compounds are surveyed. Extraction methods are discussed for volatile and non-volatile CPs individually. Gas chromatography (GC), (ultra)high performance liquid chromatography (U)HPLC, and capillary electrochromatography (CEC) are reviewed as analytical tools for final analyte analysis. For identity confirmation of analytes, mass spectrometry (MS) is indispensable, and the appropriate ionization principles are comprehensively discussed. The final sections cover analysis of real samples and aspects of quality assurance, namely matrix effects and method validation.

Keywords: β-Carotene, cleavage products, extraction and analysis methods, *in vitro* oxidation methods, quantification and validation

Abbreviations: ACN, acetonitrile; AIBN, 2,2´-azobisisobutyronitrile; AMVN, 2,2´-azobis(2,4´-dimethylvaleronitrile); APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; β-CC, β-cyclocitral; β-IO, β-ionone; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CCE cell, controlled-current electrolyte cell; CD, cyclodextrin; CE, capillary electrophoresis; CEC, capillary electrochromatography; CPs, cleavage products of β-carotene; DCM, dichloromethane; DHA, dihydroactinidiolide; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; DMSO, dimethylsulfoxide; DVB, divinyl benzene; EI, electron impact; ES, electrospray; ESI, electrospray ionization; GC, gas chromatography; HCD, headspace co-distillation; HS-SPME, headspace solid-phase microextraction; IS, internal standard; LLE, liquid-liquid extraction; LLME, liquid-liquid micro extraction; LOX, lipoxygenase; MCPBA, m-chloroperbenzoic acid; MS, mass spectrometry; NP(-HPLC), normal phase (HPLC); O/W, oil-in-water; PML, polymorphonuclear leukocytes; P_{OX}, oxidation potential; ROS, reactive oxygen species; RP(-HPLC), reversed phase (HPLC); SBSE, stir bar sorption extraction; SD, steam distillation; SDE, simultaneous distillation extraction; SFE, supercritical fluid extraction; SIM, single ion monitoring; SPE, solid-phase extraction; TFA, trifluoroacetic acid; THF, tetrahydrofuran; UHPLC, ultrahigh performance liquid chromatography

Introduction

β-Carotene is a fat-soluble natural pigment, mainly found in plants [1]. After its uptake, β-carotene is enzymatically cleaved in the intestinal mucosa by β-carotene 15,15´-monooxygenase (BCO1) at the central double bond [2–4], yielding two molecules of vitamin A (retinol), which are further converted enzymatically into the vision pigment retinal. β-Carotene is therefore also called pro-vitamin A. Apart from its action as a pro-vitamin, β-carotene has been demonstrated to induce a large number of favorable biological effects. It acts as an antioxidant by scavenging peroxyl radicals, thus inhibiting lipid peroxidation [5–7], and exerts antimutagenic effects which can be attributed either to its antioxidant action, that is, in combination with γ-irradiation [8], or to the induction of xenobiotic metabolizing enzymes [9–12]. Moreover, it has been demonstrated to induce cell cycle arrest and apoptosis [13], and to inhibit chemically induced neoplastic transformation by the stimulation of cell communication via gap junctions [14]. Furthermore, β-carotene has also been found to modulate immune function by stimulating the killing activity of neutrophils in blood [15].

These effects, together with the observation that the increased uptake of carotenoids via fruits and vegetables reduces the risk for degenerative diseases such as

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cardiovascular diseases [16–18], macular degeneration [19], cataract formation [20,21], and certain types of cancer [22], led to the assumption that β-carotene supplementation may prevent these diseases.

β-carotene paradox

A major chemoprevention trial – the Beta-Carotene and Retinol Efficacy Trial (CARET) Study – involving a total of 18,314 smokers, former smokers, and workers exposed to asbestos, however, resulted in 388 new cases of lung cancer during the average follow-up period of 4 years. Compared to the placebo group, the active-treatment group had a relative risk of lung cancer of 1.28, whereas no significant differences were found for other types of cancer. Furthermore, the relative risk of death from lung cancer was 1.46, and that of death from cardiovascular diseases, 1.26. For this reason, the trial was stopped 21 months ahead of schedule [23,24]. These results are consistent with those of the Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) Study involving 29,133 male smokers [25].

To explain these findings, Wang and Russell [26] suggested that β-carotene metabolites are responsible for the carcinogenic response in the lungs of smokers. There has also been some speculation whether β-carotene supplementation might impair response to radiation therapy, for instance in treatment of prostate gland cancer, with the risk of a second primary cancer. The impact of β-carotene might be related to the selected radiation therapy, for example, external radiation or brachytherapy. In the study by Margalit et al., no detrimental effect was revealed. The authors assumed that high reactive oxygen species (ROS) concentrations that were produced in radiation therapy overwhelmed the scavenging capacity of β-carotene [27].

A survey of compounds that have been identified as CPs of β-carotene and are addressed in the following sections is given together with Chemical Abstract Service (CAS) registry numbers in Table I, with assignment to major analytical methods. The numbering of C-atoms in β-carotene and related CPs is in accordance with the IUPAC rules [28]. Sommerburg et al. [29] further postulated that oxidants released by activated polymorphonuclear leukocytes (PML) cause nonenzymatic cleavage of carotenoids, and demonstrated that β-carotene is degraded in the culture medium of activated PML. During the oxidative burst of activated PML, superoxide radicals and hydrogen peroxide are released via the action of NADPH oxidase. Hydrogen peroxide is involved in the formation of the highly reactive oxidant, hypochlorous acid, with the reaction catalyzed by myeloperoxidase of neutrophils [30,31]. Hypochlorous acid treatment of β-carotene therefore served as a model for neutrophil-derived degradation which causes the formation of volatile short-chain metabolites such as β-cyclocitral (β-CC), β-ionone (β-IO), 5,6-epoxy-β-ionone, dihydroactinidiolide (DHA), and 4-oxo-β-ionone, as well as long-chain metabolites, such as β-apo-8´-carotenal and β-apo-12´-carotenal [29]. A mixture of oxidative CPs and β-apo-8´-carotenal have further been shown to be genotoxic in primary rat hepatocytes [4,32–34], indicating that these CPs may be responsible for the increased risk of lung cancer.

Degradation of β-carotene to volatile CPs

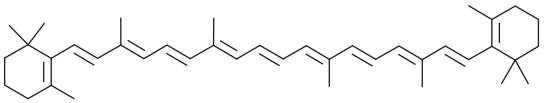
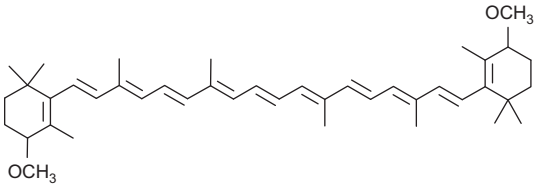
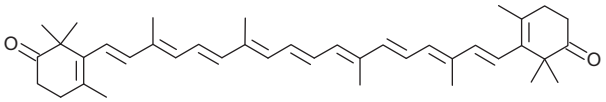
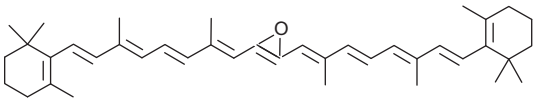
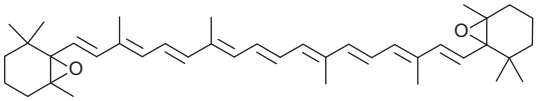
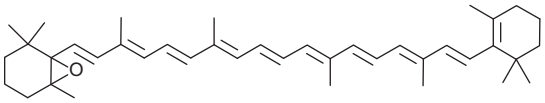
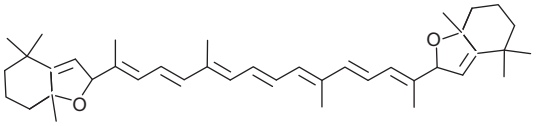
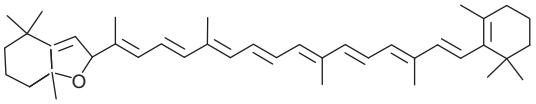
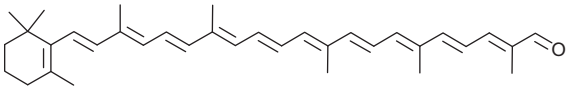
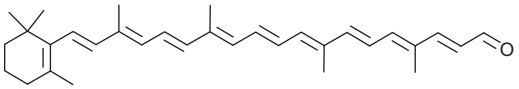
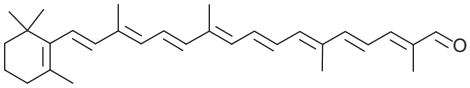
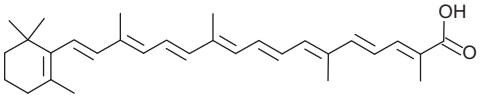
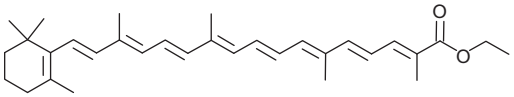
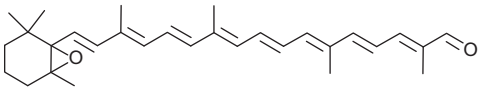
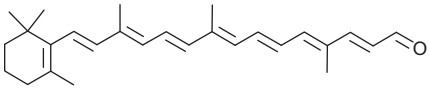
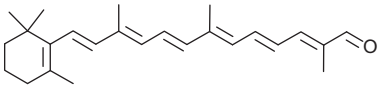
β-IO, 5,6-epoxy-β-ionone, and DHA have been identified as the major volatile CPs of β-carotene that are generated by radical attack and subsequent cleavage at the C9–C10 bond. However, the isomeric structure of β-carotene seems to determine the relative abundances of these CPs. All-*trans*-β-carotene favors the generation of β-CC and β-apo-8´-carotenal. A high content of 9-*cis*-β-carotene in the initial product increased the percentage of DHA in the final portfolio of CPs. This indicates a distinct degradation pathway for *cis*-variants of β-carotene that differs from that of all-*trans*-β-carotene [35]. Generally, short-chain CPs are major aroma compounds in flowers, fruits, and vegetables [36]. Therefore, sample treatment and analysis of these compounds covered in subsequent sections will also refer to mixtures of flavor compounds that comprise, for instance, β-IO and β-CC as prominent representatives.

Orange-fleshed sweet potatoes with their high content of β-carotene (325 μg/g dry weight) represent ideal candidates to study degradation of β-carotene in food and generated CPs. Decomposition of β-carotene has been investigated on the basis of dried chips made from sweet potatoes, also addressing degradation kinetics under different storage conditions, that is, temperature (10°C–40°C), water activity, and oxygen level. High temperature was observed to promote degradation, with 90% of initial β-carotene lost at 40°C within 54 days. 5,6-epoxy-β-carotene showed an even higher susceptibility to temperature. Both compounds followed first order reaction kinetics. Moreover, low water activities promoted degradation, although to a less pronounced extent than temperature. This points to autoxidation rather than to an enzymatic reaction catalyzed by peroxidase, the main enzyme contained in potatoes. The highest degradation rates were encountered when increasing the oxygen level (0–21% oxygen). The time course of the formation of volatile CPs, that is, β-CC, β-IO, 5,6-epoxy-β-ionone, and DHA, has been given for different water activities and oxygen contents showing analyte-dependent characteristics in their formation. β-IO and 5,6-epoxy-β-ionone possessed a similar time profile, whereas formation of DHA was retarded under different water activities. Similarly, under different oxygen concentrations, DHA reached the highest concentrations when the other CPs were already in decline [37].

In vitro oxidation and preparation of oxidation products of β-carotene

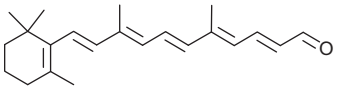
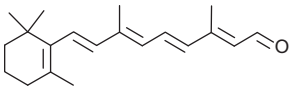
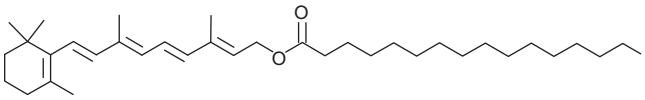
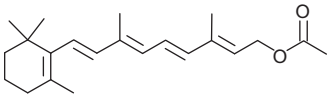
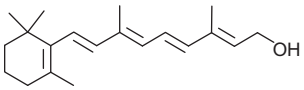
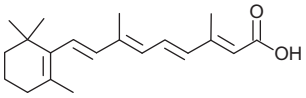
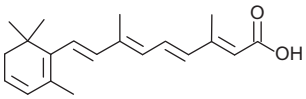
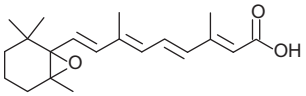
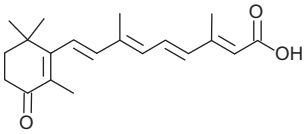
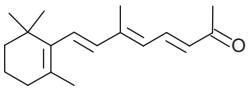
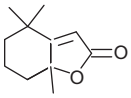
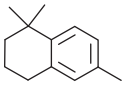
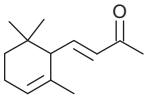
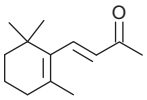
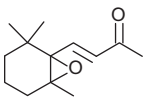
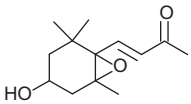
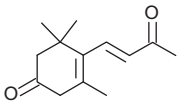
Different strategies have been applied in the model oxidation of β-carotene for the generation of CPs. These approaches are discussed individually in the subsequent sections. For a better orientation, a general survey is provided in Figure 1. Moreover, a recent review discusses the stability of β-carotene and describes different oxidation

Table I. Chemical structure, name, and Chemical Abstract Service (CAS) registry number of β-carotene and the most import cleavage products that are also addressed in the text. The numbering of C-atoms is in accordance with IUPAC rules [28] as exemplified on β-carotene attached to this table. For reasons of simplicity, only all-*trans* isomers are depicted.

Chemical structure	Name	CAS Nr.
	β-carotene	7235-40-7
	4,4´-dimethoxy-β-carotene	6895-71-2
	β-carotene-2,2´-dione	63596-39-4
	15,15´-epoxy-β-carotene	132541-62-9
	5,6,5´,6´-diepoxy-β-carotene	864-94-8
	5,6-epoxy-β-carotene	1923-89-3
	5,8,5´,8´-diepoxy-β-carotene	6821-09-6
	5,8-epoxy-β-carotene	515-06-0
	β-apo-4´-carotenal	5056-12-2
	β-apo-6´-carotenal	5056-13-3
	β-apo-8´-carotenal	1107-26-2
	β-apo-8´-carotenoic acid	1962-15-8
	β-apo-8´-carotenoic acid ethyl ester	1109-11-1
	5,6-epoxy-β-apo-8´-carotenal	41548-56-5
	β-apo-10´-carotenal	640-49-3
	β-apo-12´-carotenal	1638-05-7

(*Continued*)

Table I. (*Continued*)

Chemical structure	Name	CAS Nr.
	β-apo-14´-carotenal	6985-27-9
	retinal = β-apo-15-carotenone	116-31-4
	retinyl palmitate	79–81-2
	retinyl acetate	127-47-9
	retinol	68-26-8
	retinoic acid	302-79-4
	3,4-didehydro-retinoic acid	4159-20-0
	5,6-epoxy-retinoic acid	13100-69-1
	4-oxo-retinoic acid	38030-57-8
	β-apo-13-carotenone	17974-57-1
	dihydroactinidiolide (DHA)	15356-74-8
	1,1,6-trimethyltetraline (TMT) = α-ionene	475-03-6
	α-ionone	127-41-3
	β-ionone (β-IO)	79-77-6
	5,6-epoxy-β-ionone	23267-57-4
	3-hydroxy-5,6-epoxy-β-ionone	38274-01-0
	3-oxo-β-ionone	117048-10-9

(*Continued*)

Table I. (*Continued*)

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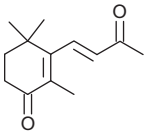
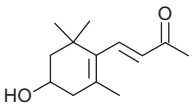
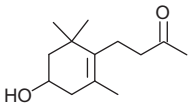
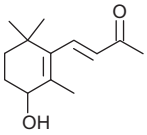
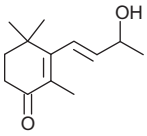
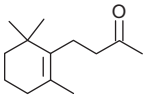
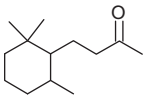
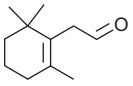
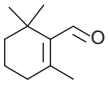
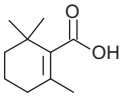
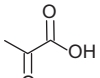
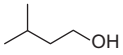
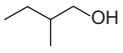
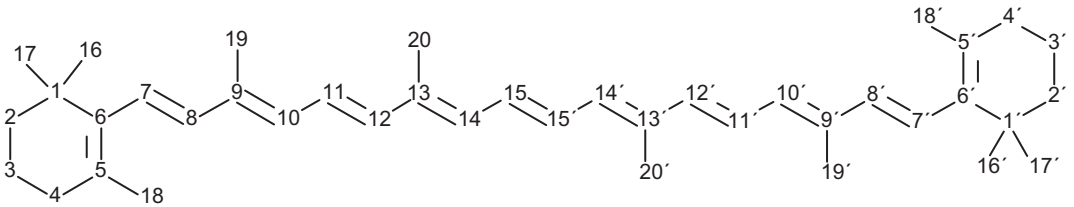
	4-oxo-β-ionone	29790-29-2
	3-hydroxy-β-ionone	50281-38-4
	3-hydroxy-7,8-dihydro-β-ionone	131544-22-4
	4-hydroxy-β-ionone	14398-34-6
	4-oxo-β-ionol	29790-30-5
	dihydro-β-ionone	17283-81-7
	tetrahydro-β-ionone	6138-85-8
	β-homocyclocitral	472-66-2
	β-cyclocitral (β-CC)	432-25-7
	2,6,6-trimethyl-cyclohexene-1-carboxylic acid	564-24-9
	pyruvic acid	127-17-3
	3-methylbutan-1-ol	123-51-3
	2-methylbutan-1-ol	137-32-6

Compounds from β-carotene to β-apo-13-carotenone as listed consecutively in the table are preferably analyzed by RP-(U)HPLC (either with C18 or C30 columns) with detection performed either with DAD or MS (for example, ion-trap, triple-quadrupole, Orbitrap). Hyphenation between HPLC and MS is preferably with APCI and ESI. Volatile CPs (covering analytes from dihydroactinidiolide to 2-methylbutan-1-ol in Table I) are analyzed by GC-EI-MS.

Numbering of C-atoms according to IUPAC rules [28]



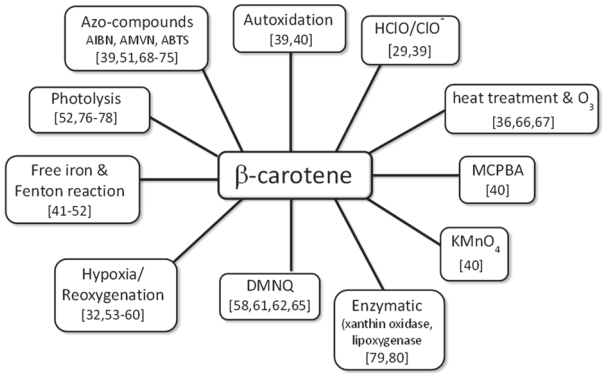


Figure 1. Survey of *in vitro* methods applied in the generation of CPs of β-carotene, mostly serving as models to mimic *in vivo* oxidation of β-carotene. Due to practical considerations, the presented scheme is based on the applied initiators of oxidation and the applied techniques, and not on the underlying reaction mechanism. Further details are given in the text.

mechanisms employed in the degradation of β-carotene as well as related CPs [38].

Autoxidation and oxidation with hypochlorite

Handelman et al. [39] addressed the degradation profiles of β-carotene by application of different oxidation strategies. They investigated (i) autoxidation in toluene under either air or oxygen at 37°C or 60°C, (ii) peroxyl radical-induced oxidation by 10 mmol/L azobisisobutyronitrile (AIBN), (iii) a combination of autoxidation with addition of 10 mmol/L AIBN, and (iv) degradation by 1.25 mmol/L sodium hypochlorite (NaOCl) [39]. Actually, the oxidation with AIBN refers to degradation with peroxyl radicals, a mechanism that is separately discussed in a subsequent section. Although not exactly meeting the degradation reactions addressed in this section, AIBN results are prepended to allow for a more comprehensive comparison of data as given by the authors. The addition of 0.1 µmol/L and 10 µmol/L α-tocopherol under air-saturated conditions and heating to 60°C for 120 min suppressed the oxidation of β-carotene by 4% and 92%, respectively. Profiles of oxidation products were similar for autoxidation and oxidation induced by AIBN, whereas the hypochlorite-driven degradation process provided a different profile. The reaction mechanism with oxygen and AIBN shares features with lipid peroxidation and comprises products of the β-apocarotenal series, that is, β-apo-10´-, β-apo-12´-, and β-apo-14´-carotenal, retinal, but also β-apo-13-carotenone and β-carotene-5,6-epoxide. However, β-apo-8´-carotenal was absent. Under the selected conditions, Handelman et al. observed that the 7´,8´-bond was most stable. Products induced by hypochlorite comprised CPs of the polyene carbonyl series. Thus, Handelman et al. concluded that "*Different forms of active oxygen may each yield a unique set of oxidation products* …" [39]. For autoxidation of β-carotene in model systems, low moisture as well as aqueous conditions have been tested by comparison of controls stored in the dark, with model samples exposed to standardized fluorescent light and screening for degradation products between 1 and 36 days. Both for low-moisture and aqueous systems, no differences between irradiated and non-irradiated conditions were revealed except a promotion of *cis*-isomerization of the initial all-*trans*-β-carotene [40].

Based on the decomposition strategy of Handelman et al. [39], Sommerburg et al. [29] degraded β-carotene in 1/10 and 1/100 (mol/mol) mixtures of HOCl/ClO^-, either in methanolic solutions or an emulsion containing 70% glycerol in 30% water. The final emulsion was prepared by spiking the glycerol-water mixture with an appropriate amount of β-carotene dissolved in soybean oil. The reaction solution, prepared in a 1/100 ratio (β-carotene/NaOCl), provided β-IO, 5,6-epoxy-β-ionone, β-CC, DHA, and 4-oxo-β-ionone (Figure 2), whereas long-chain CPs were absent. In aqueous emulsions that also contained soybean oil, degradation was different and β-carotene was not completely degraded, even at the applied 1/100 ratio (Figure 2). Higher soybean contents decelerated the degradation progress. Increasing the NaOCl content increased the concentration of β-apo-12´-carotenal and retinal, whereas β-apo-8´-carotenal ceased. β-apo-4´-carotenal was not formed [29].

β-carotene oxidation with free iron and copper, and the Fenton reaction

Sy et al. [41] added 1.5 µmol/L–1.5 mmol/L Fe^{2+} and Fe^{3+} to a micelle solution of pH 4.37 that contained β-carotene. The reaction was for 2 h. Fe^{2+} reacted with O_2, forming a perferryl species ($Fe^{3+}-O_2^{\bullet}$). This perferryl compound can decompose to Fe^{3+} and superoxide or conjugate with β-carotene, thus forming a radical that leads to isomerization, epoxide CPs, or cleavage of the polyene chain. Fe^{3+} showed a delayed onset of β-carotene degradation. This has been explained by a previous reduction to Fe^{2+} since the latter is considerably more oxidizing than Fe^{3+} under the selected conditions. CPs have been identified with UPLC-DAD-MS [41]. Similarly, oxidation of β-carotene was done by means of iron or copper. Identified CPs comprised β-CC, β-IO, DHA, 3-oxo-β-ionone, 6-hydroxy-α-ionone, and trans-β-ionone-5,6-epoxide. The portfolio of CPs resembled the oxidation by radicals (using a mixture of Tris, xanthine, and hypoxanthine) that was performed in parallel, except that 3-oxo-β-ionol was formed [42].

In the presence of H_2O_2, Fe^{2+} is oxidized to Fe^{3+}. This reaction is named after Henry John Horstman Fenton [43], who published an article on the oxidation of tartaric acid in the presence of ferrous iron and hydrogen peroxide in 1893, involving the generation of the highly reactive hydroxyl radical, according to the following equation:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

Several studies underpin the importance of the generated hydroxyl radicals for oxygen toxicity at the cellular and plasma levels [44,45], and their formation has been confirmed by electron spin resonance (ESR) studies [46,47].

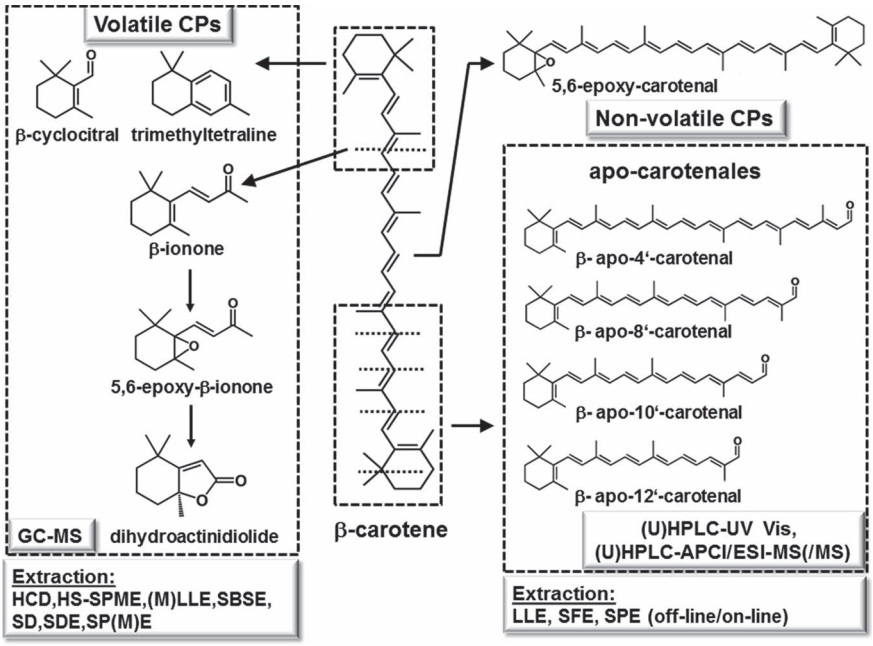


Figure 2. Degradation pathways of all-*trans*-β-carotene (by HOCl according to [29]) resulting in non-volatile (β-apo-carotenales) and volatile cleavage products (CPs). Analysis methods and preceding extraction methods applied for the identification and quantification of the respective CPs are stated. Details are given in the text.

Furthermore, the hydroxyl radical has been shown to be involved in lipid peroxidation [48], in particular as the initiating radical [49]. In this context, it has to be mentioned that Polyakov et al. [50] demonstrated that carotenoids can enhance the radical yield of the Fenton reaction via the reduction of Fe^{3+}. Moreover, the Fenton reaction entailed a fast β-carotene bleaching with a 50% loss within 5 min [51]. Hydroxyl radicals that have been produced by laser photolysis of a specific reagent were also shown to produce a β-carotene radical [52].

Hypoxia/reoxygenation

Hypoxia and reoxygenation are important factors in clinical conditions, such as myocardial ischemia, stroke, and organ transplantation predisposing to tissue injury [53]. This injury is mediated by reactive oxygen and nitrogen species, which have been shown to be elevated not only in the reoxygenation phase [54], but also during hypoxia. For example, a 5-fold increase of ROS detected by the fluorescent probe dichlorofluorescin (DCF) was reported for smooth muscle cells of the rat pulmonary artery [55]. The major sources of ROS production are two enzymes: xanthine dehydrogenase-oxidase and NADPH oxidase. The activities of xanthine dehydrogenase-oxidase are highest in the liver and the intestine [56]. During reoxygenation, xanthine dehydrogenase is oxidized and converted to xanthine oxidase, producing the superoxide radical and hydrogen peroxide [53]. Endothelial cells on the other hand express a membrane NADPH oxidase, which is an important source of ROS during ischemia [57]. Hypoxia/reoxygenation has been employed to induce oxidative stress in a variety of cellular systems, that is, cerebral endothelial cells [58], microglial cells [59], isolated cardiac myocytes [60], or primary rat hepatocytes [32].

2,3-Dimethoxy-1,4-naphthoquinone

2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) is a redox-cycling quinone. Its toxicity depends on ROS production via one-electron reduction, leading to the formation of the respective semiquinone radical. The semiquinone radical then interacts with molecular oxygen to form the superoxide radical and its dismutation product hydrogen peroxide [61,62]. Several cytosolic flavoenzymes, such as NADPH-cytochrome P450 reductase or NADH-cytochrome b reductase, have been reported to catalyze these one-electron reductions [61,63,64]. DMNQ has been employed to induce oxidative stress in a variety of cellular systems, that is, cerebral endothelial cells [58] or lung epithelial cells [65].

Oxidation by heat treatment and ozone

Others have oxidized β-carotene and β-IO by thermal degradation and a combined thermal-ozone treatment [36,66]. This procedure is of particular relevance in the food industry, where ozone is applied as an antimicrobial agent and food processing frequently includes heating steps. If food contains β-carotene either naturally, added as an antioxidant, or as a colorant additive, β-carotene breakdown products might be generated by these treatments and also consumed. Treatment was conducted with 0.80–2.54 ppm ozone at flow rates of 1 L/min for 7 h. CPs identified by HPLC-ion-trap mass spectrometry (MS) comprised epoxides and carbonyl compounds as major oxidation classes, including β-apo-14´-carotenal, β-apo-12´-carotenal, and

their respective 5,6-epoxides. Moreover, β-apo-15-carotenal and 3,7,11,11-tetramethyl-10,15-dioxo-hexadec-2,4,6,8-tetraenal were identified as intermediates that were further oxidized. Criegee´s biradical also takes a prominent role in ozone-driven oxidative degradation, for example, by degradation of β-CC to pyruvic acid. Detailed degradation pathways are given elsewhere [66]. Zeb and Murkovic [67] have oxidized β-carotene dissolved in corn oil at 110°C in a Rancimat system, at an air flow of 20 L/h for 1–4 h. The CPs identified after this treatment comprised β-apo-8´-carotenal, β-apo-6´-carotenal, 5,6-epoxy-β-apo-8´-carotenal, β-carotene-2,2´-dione, 5,6-epoxy-β-carotene, 5,8-epoxy-β-carotene and 5,6,5´,6´-diepoxy-β-carotene (Figures 3A and B). Furthermore, triacylglycerols of the corn oil were also oxidized under these conditions promoted by β-carotene, producing various hydroperoxide degradation products (Figure 3C). Analysis was done by HPLC-APCI-MS for β-carotene and its CPs, whereas HPLC-ESI-MS was applied for the characterization of the products of triacylglycerol degradation [67].

Oxidation with m-chloroperbenzoic acid and potassium perchlorate

Rodriguez and Rodriguez-Amaya [40] tested different chemical approaches for oxidation of β-carotene. These reactions comprised treatment with m-chloroperbenzoic acid (MCPBA) for epoxidation, oxidative degradation with potassium perchlorate ($KMnO_4$), and autoxidation [40]. Addition of MCPBA to a two-phase solution of β-carotene in dichloromethane (DCM) and an aqueous NH_4HCO_3 portion entailed an epoxidation of carbon double bonds and decreased β-carotene concentration to 22% of its original content. Various mono- and di-epoxides of β-carotene were generated, with identification of seven degradation products by UV-absorbance spectra and MS. Without NH_4HCO_3 in the reaction solution, a rearrangement of 5,6- to 5,8-epoxides was induced. β-Carotene treatment with $KMnO_4$ generated a portfolio of apocarotenals by cleavage of carbon double bonds, but also formed epoxides and semi-β-carotenones. The reaction mechanism, which also involved a preceding isomerization, is described in the paper [40].

Oxidation with free radicals generated by azo-compounds

Azo-compounds have been used as initiators of radical formation, namely, peroxyl radicals. The stimulation of these azo-compounds is initiated via UV irradiation or heating. AIBN and AMVN are preferably used [51,68]. The thermal degradation pathway of AIBN is discussed elsewhere [69]. Reaction mechanisms with carotenes, proposed in this context, comprise peroxyl radical addition, electron capture, and hydrogen abstraction. The latter explain modifications at the C4 and C4´ atoms, leading to CPs that are methoxylated or ethoxylated in these positions when using methanol or ethanol as solvents, respectively [51]. Handelman et al. [39] used AIBN for oxidation of β-carotene. Details have been given in the previous section *Autoxidation and oxidation with hypochlorite* for the sake of completeness, since in their paper, AIBN oxidation was compared with autoxidation and HOCl oxidation. Salgo et al. [70] also used AIBN to oxidize β-carotene in benzene at 60°C for 4 h. HPLC separation and analysis of collected fractions allowed for the identification of

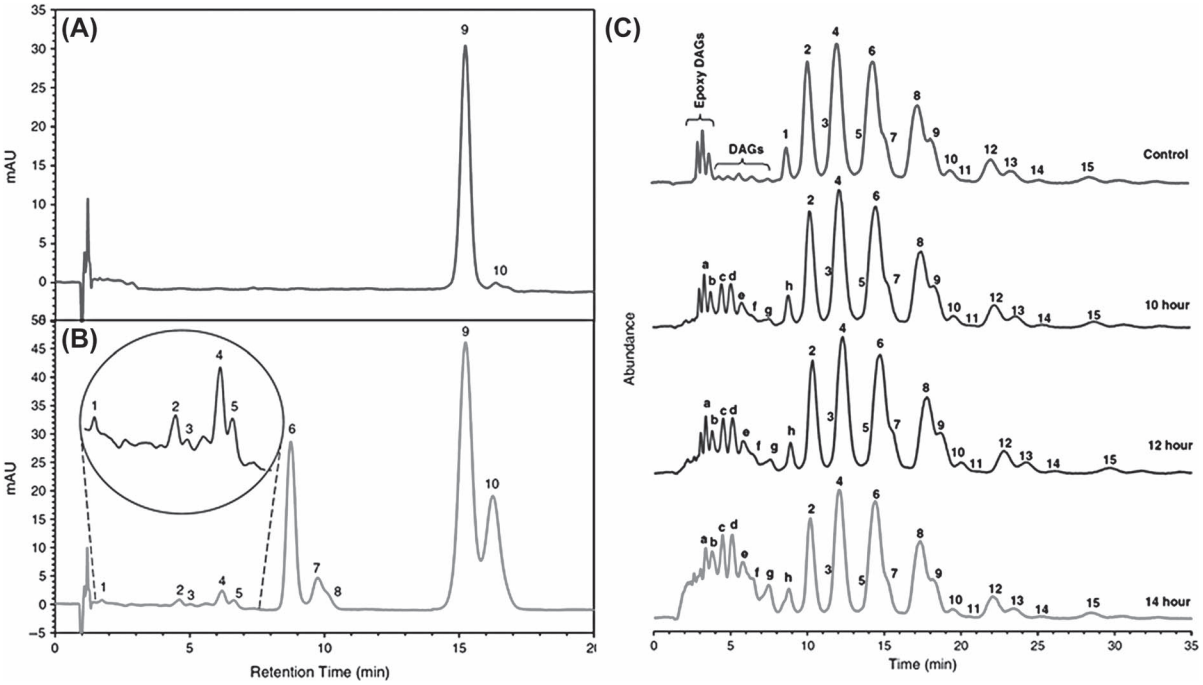


Figure 3. HPLC separation profiles of β-carotene and its oxidation products. (A) All-*trans*-β-carotene standard, (B) β-carotene oxidation products and (C) total ion current chromatograms of corn oils showing control, and oxidized samples for 10, 12 and 14 h in the Rancimat at 110°C. Reprinted from [67], © 2011, with permission from Elsevier.

β-apo-13-carotene, β-apo-15-carotenal, β-apo-14´-, β-apo-12´-, and β-apo-10´-carotenal, as well as 5,6-epoxy-β-carotene,11,15´-cyclo12,15-epoxy-11,12,15,15´-tetrahydro-β-carotene. Rodrigues et al. [71] employed thermal decomposition of AIBN at 41°C to compare different carotenoids for the peroxyl radical scavenger properties, for example, β-carotene, β-apo-12´-, β-apo-10´-, and β-apo-8´-carotenal as well as 15-*cis*- and 9-*cis*-β-carotene [71]. Similarly, AIBN (37°C for 90 min) was applied to test the antioxidant capacity of β-carotene for inhibiting lipid peroxidation of fatty acids and malondialdehyde formation [72]. Moreover, 2,2´-azobis(2-amidinopropane) dihydrochloride has also been used to deliver peroxyl radicals, whereas 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) provided a synthetic ABTS radical. Substitution of the β-ionone ring in position 4 and 4´, that is, substitution by a carbonyl or keto group, increased the peroxyl radical scavenging capability [73].

Kennedy and Liebler [74] induced oxidation of β-carotene by formation of peroxyl radicals via thermolysis of azobis(2,4-dimethylvaleronitrile) (AMVN), generating 5,6-epoxy- and 15,15´-epoxy-β-carotene as major degradation products. Formation of epoxides was considered to occur via a peroxyl radical adduct. Adhikari et al. [75] generated halogenated alkylperoxyl radicals in a microemulsion system by pulse radiolysis with 7-MeV electrons. Depending on the solvent system selected, different halogenated alkylperoxyl radicals were generated, for example, $CCl_3OO^{\bullet}$, $CBr_3OO^{\bullet}$, $CHCl_2OO^{\bullet}$, and $CHBr_2OO^{\bullet}$.

Free radical formation by photolysis

Photo-induced formation of β-carotene radical cations (β-carotene$^{\bullet+}$) occurs from excited states of β-carotene [52]. Both singlet ($1B_u^+$, $1B_u^-$) and triplet ($1^3B_u^+$) states of β-carotene that were induced by photo-excitation were shown to directly transfer electrons to chloroform, thus forming β-carotene$^{\bullet+}$ [76]. Konovalova et al. [77] have shown the favorable effects of solvents with high electron-accepting properties, such as CCl_4, on the formation of β-carotene$^{\bullet+}$ after photo-excitation by laser photolysis. The formation of β-carotene$^{\bullet+}$ was accompanied by a concomitantly generated solvent radical anion (for example, $CCl_4^{\bullet-}$). Photolysis of β-carotene in chloralkane solvents, such CCl_4, $CHCl_3$, CH_2Cl_2, or in CS_2 produced paramagnetic compounds due to β-carotene cation radicals and solvent anion radicals. The lifetime of these species was extended under low temperature conditions (in other words, 77 K). Interaction of both radical species are explained in their paper, including the generation of donor-acceptor complexes and "solvent-separated radical ion pairs" [77]. Everett et al. [78] used pulse radiolysis to generate nitrogen dioxide radicals ($NO_2^{\bullet}$), thiyl radicals ($RS^{\bullet}$), and thiylsulfonyl radicals ($RSO_2^{\bullet}$) that reacted with β-carotene and formed β-carotene$^{\bullet+}$, β-carotene adduct radicals, and polyene radical cations. Detailed reaction pathways are given in their paper [78].

Enzymatic oxidation: xanthine oxidase and lipoxygenase

Complementary to autoxidation, a co-oxidation of β-carotene with xanthine oxidase was performed in biphasic media. Since free radicals do not enter the organic phase, an aqueous phase had to be implemented, with micelles acting as a solubility mediator. Under these conditions, β-carotene concentration diminished to less than 50% of the initial content within 2 h. At 45 mg/L β-carotene, the β-IO yield was highest, probably due to an optimal ratio between β-carotene and free radicals. Whereas β-IO yield was higher in the presence of 10% n-hexane, the opposite was true for the secondary degradation product 5,6-epoxy-β-ionone. This is most likely due to the protective environment of n-hexane against radicals that are derived from oxygen [79].

Furthermore, oxidation of β-carotene by oxidized polyunsaturated fatty acids has been described. Their oxidation was shown to occur by lipoxygenases (LOX). This proposed LOX-related co-oxidation of β-carotene has to be considered when handling biological samples or formulations prepared in biological matrices, for example, in soybean oil. The assumed co-oxidation mechanism has been mimicked by spiking model solutions that contained β-carotene and linoleic acid with different LOX isoforms. In parallel, hydroxyperoxidation activity of LOX was determined in the presence of linoleic acid, without the addition of β-carotene. The reaction was stopped by acidification in both cases. The hydroxyperoxidation activity is different between LOX isoforms and other species. The proposed reaction mechanism proceeds on the assumption of LOX-triggered formation of linoleylperoxyl radicals. These peroxyl radicals attack β-carotene randomly along its polyene chain, with the formation of β-carotene radicals and peroxycarotenoid radicals. The identification of CPs revealed volatile degradation products, for example, β-CC and β-IO, and more than 50 non-volatile products with an UV absorbance maximum (λ_{max}) >350 nm, which excludes constituents of linoleic acid or enzyme origin. Non-volatile CPs identified by HPLC-MS comprised six β-apocarotenals, four 5,6-epoxy-β-carotenals, and three 5,8-epoxy-β-carotenals. The authors provided a degradation scheme of β-carotene under the selected conditions that implemented 31 CPs [80]. Actually, the derived *in vitro* reaction mechanism might serve as an appropriate simulation of the *in vivo* situation in lungs under pro-oxidative conditions.

Limitations in the solubility of β-carotene

The hydrophobicity of β-carotene and most of its oxidation derived CPs constitutes a pronounced challenge for their effective application in cell cultures and thus requires potent solubilizers. β-Carotene is practically insoluble in water and hardly soluble even in methanol, acetonitrile, (ACN), or dimethylsulfoxide (DMSO) [81–83]. Storage in ethanol at − 80°C might lead to precipitation of carotenoids [84]. Solubility is improved in hexane, chloroform,

and DCM, with the highest solubility of all tested solvents seen in tetrahydrofuran (THF) [81–83]. This provides an essential input for the selection of appropriate solvents applicable in the extraction, purification, and chromatographic separation of these analytes [82,83]. Stability testing by UV-photometry over 10 days revealed pronounced degradation of β-carotene in cyclohexanone and DCM, where only 32–34% of the initial absorbance prevailed. Remarkably, in a mixture of THF and BHT, 97% of the original absorbance was still recovered, thus giving the highest β-carotene stability within all tested solvents [83]. Tresczanowicz et al. [85–87] provided a comprehensive overview of β-carotene solubility in various binary solvent systems. Hagiwara et al. [88] used a mixture of DCM and hexane for dissolving β-carotene. Van Meulebroek et al. used a mixture of ACN/methanol/chloroform (18/7.5/74.5, v/v/v) for dissolving β-carotene and β-apo-8´-carotenal [89]. However, the aforementioned solvents are mostly not compatible with biological systems, for example, cell cultures. Moreover, solvent contamination with hydroperoxides and hydrochloride has been described that may decompose target analytes already in stock solutions [82]. Thus, solubilizers that act as mediators to ensure miscibility with aqueous culture media and foster an efficient cellular uptake, without inducing detrimental effects at the cellular level or interfering with biological effects expected for β-carotene and its CPs, are required. Both a pre-cleaning procedure as well as a solvent change might become mandatory during sample preparation, to assure compatibility with final analysis techniques allowing for subsequent analytical separation and detection.

Application- and treatment solutions

Organic solvents

For *in vitro* experiments, β-carotene has been delivered in DMSO (< 1% in cell culture medium) or as β-carotene-containing beadlets [90], in THF (0.05–0.5%) [91], and an aqueous suspension composed of 0.02% THF and 2.5 μmol/L α-linoleic acid [91] to target cells, for example, bronchial epithelial cell lines [90], and human intestinal and lung cell lines [91]. Similar strategies have been utilized for CPs, such as β-apocarotenals [91]. A concentration of 0.3% ethanol in cell growth medium was used for the preparation of treatment solutions that contained various β-apocarotenoic acids [92]. In terms of THF, carcinogenic activity has been revealed, leading to adenoma or carcinoma in kidney and hepatocellular neoplasms in some rat and mouse strains [93]. THF has also been shown to induce a release of lactate dehydrogenase [91]. When THF was inhaled, higher cytochrome P450 levels were induced in the liver of female mice and a higher mitotic index was reported [94]. Stock solutions of β-carotene prepared in THF and stored at − 80°C were stable for 1 month [95].

Solvents applied in treatment and extraction were passed over an aluminum oxide column for removal of peroxide traces in some instances, for example, for THF [91,96]. However, this was shown to promote the conversion of 5,6-epoxy-β-carotene to 5,8-epoxy-β-carotene, possibly by traces of HCl, as suggested by the authors. Similarly, 5,6,5´,6´-diepoxy-β-carotene was isomerized to 5,8,5´,8´-diepoxy-β-carotene [97]. Moreover, BHT has been added frequently as an anti-oxidant (0.025%) [91]. However, in solutions intended for treatment of cell cultures, BHT will counteract pro-oxidative effects of CPs and thus has to be avoided.

Cyclodextrin solutions

Other approaches have used more sophisticated strategies, such as solubilization in cyclodextrin (CD) solutions. CDs form cyclic structures composed of a variable number of glucopyranose moieties. Therein, CDs possess a rigid hydrophobic cavity providing a favorable and water-protective microenvironment for β-carotene, whereas hydroxyl groups of the glucose moieties protrude into the circumfluent bulk solution and ensure the water wettability of CD [98,99]. Although frequently applied as chiral selectors, CDs have also been used to generally improve the solubility of hydrophobic compounds in aqueous solutions [100]. Different carotenoids have been tested for their capability to form host-guest complexes with CD variants. Remarkably, binding stoichiometries greater than 1:1 have been observed in aqueous solutions. This has been related to aggregation of CDs by hydrogen bonds via their external hydroxyl groups [101]. Randomly methylated CD variants showed reduced propensity to aggregate and enhanced solubility properties. Nevertheless, carotenoids will not entirely be incorporated in the hydrophobic cavity but rather their cyclohexene moiety, preferably when carrying a substituent. Thus, β-CD is most appropriate due to the close steric fitting. Unfortunately, contrary to lutein and zeaxanthin, β-carotene showed no improved solubility with methylated CD [101]. On the contrary, acidic variants, such as β-carotene-8´-oic acid, showed improved water solubility in the presence of β-CD applied under appropriate pH conditions. This has been confirmed by NMR and UV-Vis absorption spectroscopy. In parallel, antioxidant protection against peroxyl radicals was lost, whereas oxidation by Fenton reaction was still observed. This has been related to different localization of sites attacked by peroxyl radicals and Fe^{3+} [102].

(Nano-)Emulsions

Emulsions and systems providing solid-liquid particles have been applied in the delivery of β-carotene to food. These delivery systems have been reviewed elsewhere and provide useful insights that might also be used in the preparation of standard and treatment solutions [38]. Alternatively, addition of bovine serum albumin (BSA) and subsequent formation of a protein-β-carotene complex has been used to prepare aqueous emulsions [103]. Moreover, β-carotene is also available in commercial formulations, such as solutions prepared in soy-bean extracts [104]

or in sunflower oil [105]. Similarly, standard reference materials of other fat-soluble vitamins have been prepared in coconut oil [106]. However, the application of these formulations requires confidence in the batch-to-batch consistency and might suffer from ill-defined matrix composition and degradation processes of emulsions, such as Ostwald ripening and droplet coalescence [107].

Recently, nano-emulsions of β-carotene have been produced using different techniques of preparation [105,108,109]. Preparation was mostly as oil-in-water (O/W) emulsions. Yuan et al. prepared O/W emulsions of β-carotene using triglycerides of medium chain length, that is, chain lengths of C8–C10 [108], or sunflower oil [105]. Thereby, β-carotene was first dissolved in the oil fraction at 140°C with subsequent addition of ultrapure water and Tween 20, the latter acting as emulsifier. The solution was finally homogenized by a high speed homogenizer [105,108]. A statistical evaluation of the effect of different preparation variables was done by the so-called response surface methodology, revealing that the concentration of β-carotene and the homogenization temperature governs the particle size. Remarkably, parameters that downsized particles also reduced the emulsion stability. Due to their higher surface energy and surface area, smaller particles are more prone to coalescence [108]. When comparing different emulsifiers, that is, Tween 20, 40, 60, and 80, Tween 20 was found to provide the smallest particles with the narrowest size distribution. This is related to the higher hydrophilicity of Tween 20 compared to the other selected Tween species that contrariwise enhanced the stability of the particles. In case of small diameters, the particle number and the Brownian motion as well as collision and coalescence propensity are increased. Storage for over 4 weeks at + 4°C decreased the β-carotene concentration in the nano-emulsion by 14–25% [105]. Tan and Nakajima [110] applied an emulsification-evaporation strategy with β-carotene first dissolved in hexane. This solution was then transferred into aqueous Tween 20, and hexane was removed by rotary evaporation. In this initial step, 1–8% of β-carotene was lost. Due to the susceptibility of β-carotene against oxygen, light, and heat, the decrease in concentration becomes intelligible. Besides an increased surface area of particles, the formation of free radicals during high pressure homogenization has been discussed as a cause for β-carotene decrease. After a 12-week storage period, only 25–56% of the initial β-carotene concentration was encountered [110].

Nanodispersions

Other studies have employed solution displacement for preparation of nanodispersions of β-carotene, by applying biocompatible polymers. Thereby, β-carotene was dissolved in acetone, with subsequent polymer addition. This solution was dropped into an aqueous phase that contained an emulsifier. By subsequent evaporative concentration, acetone diffused in the aqueous phase and finally evaporated. The loss of acetone is considered to entail a collapse of the colloid that surrounds particle intermediates, with subsequent formation of nanoparticles. Their core is formed by polymer with dissolved β-carotene therein, whereas the emulsifier constitutes the shell. The preparation protocol ensured stable nanodispersions with a narrow particle size distribution that stayed stable over a storage period of 5 months (at + 4°C) [109].

Relation between in vitro bioavailability and applied solvent

The formulation of β-carotene and CP solutions for their application in cell cultures determines not only the cellular uptake, but also the time-dependent availability of analytes for treatments that run over several hours. This has been demonstrated when comparing treatment solutions of volatile CPs prepared in Tween 20 and THF, respectively. Both were added to cell culture media with incubation over 3 h at 37°C, respectively. Losses of volatile CPs were higher in THF-based solutions, due to the higher volatility of THF and subsequent precipitation and/or evaporation of hydrophobic volatile CPs (Figure 4) [111].

Handling and storage of β-carotene and CP (stock) solutions

To prevent isomerization and degradation during handling of samples and standards, (stock) solutions of β-carotene and long-chain CPs, for example, β-apocarotenals, have to be prepared and stored under light protection and a protective atmosphere by using inert gases, such as argon or nitrogen, and stored at − 20°C to − 80°C [81,91,112]. Due to their thermal instability evaporation below 40°C is recommended, if necessary, during extraction [81]. Additionally, sample handling under orange, yellow, or red light has been recommended [81,90,113], and exposure to extreme pH values should be avoided [112]. The effect of light exposure and storage duration on β-carotene stability has been assessed elsewhere by multilinear regressions [114]. However, isomerization is more likely in standard and extraction solutions than in biological matrices that provide a more protective milieu [81]. Moreover, addition of antioxidants is recommended [82,114], but has to be restricted to extracted solutions and solutions ready for injection in the respective analytical system. Addition to treatment solutions applied for *in vitro* investigation of oxidative effects has to be omitted in order to prevent interfering protective artifacts.

Butylated hydroxyanisole (BHA, also known as E320) [42,90] and butylated hydroxytoluene (BHT, also known as E321) – mostly at 0.01–0.1% [42,81,89,112] – are frequently applied antioxidants in this context. Analytical standard solutions have been prepared, for instance, in THF, with 1% BHT [115]. Handelman et al. outlined that a BHT film on glass walls might be required during sample preparation, especially during evaporation processes, to prevent a degradation catalyzed by silanol groups [39]. Ascorbic acid has also been assigned a

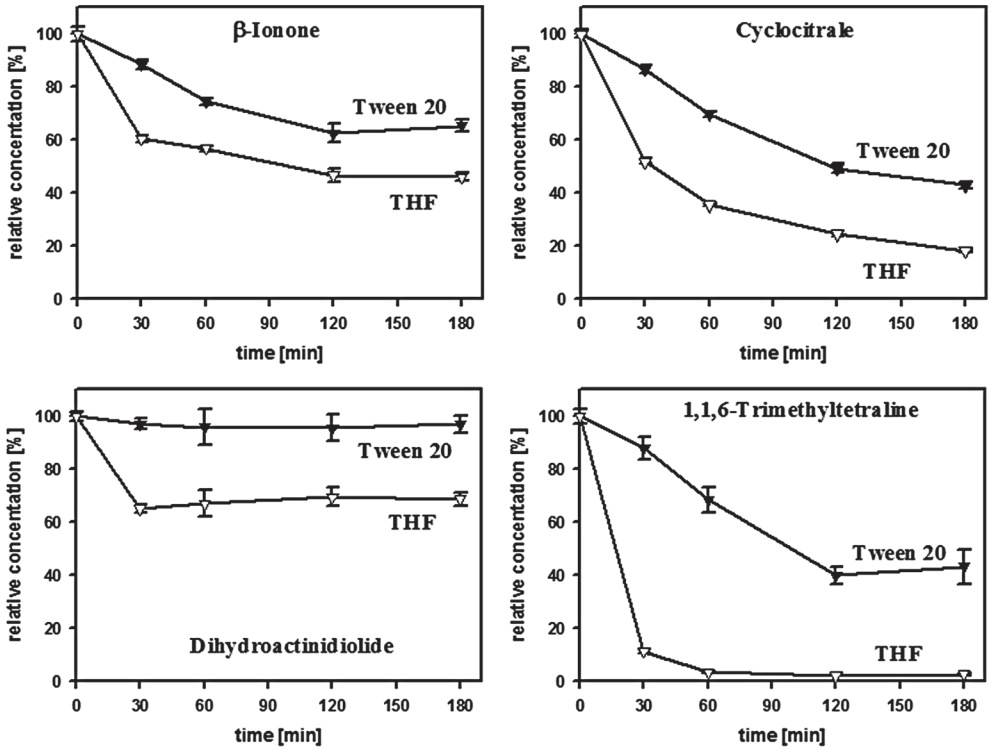


Figure 4. Time-dependent losses of individual volatile CPs in spiked cell culture medium (MEM) over an incubation period of 180 min. Spiked MEM contained either 1.0% (v/v) THF or 0.25 mmol/L Tween 20. Spiked MEM was extracted prior to incubation (referred to as 100%) and after 30, 60, 120, and 180 min of incubation, and analyzed by GC-MS. Reprinted from [111], under the terms of Creative Commons Attribution License.

protective role due to its contribution to the reduction of lipid hydroperoxides [114].

Analytical methods

Extraction methods

The divergent physicochemical properties of β-carotene and its CPs [111,116] imposes ambitious demands on extraction methods, particularly when considering the simultaneous extraction of β-carotene and related volatile and non-volatile CPs. A plethora of methods has been employed for β-carotene and CP extraction from different biological matrices. For better orientation, a methodic overview is provided in Figure 5. To our knowledge, extraction and preconcentration have been performed separately for volatile and non-volatile CPs up to now. This is mostly related to the delicate challenge in reconciling the diverse physicochemical properties with a combined extraction and preconcentration strategy, preferably tackling all relevant CPs.

Extraction of β-carotene and non-volatile CPs

Rodriguez et al. used a mixture of ethanol/*tert*-butylmethylether/THF (9/5/1, v/v/v) for micro-scale liquid-liquid extraction (LLE) of β-carotene, various β-apocarotenals, 5,6-epoxy-β-carotenal, retinol, and retinal from cell culture media [90]. The same solvent mixture has been used for analyte extraction from cell cultures of human lung and intestine cell lines, and also from cell lysates, by 10-fold dilution with 25 µL of culture medium. ^{14}C-labeled β-carotene indicated recoveries between 94.2% and 98% for culture medium and cells, respectively [91]. Others used chloroform/methanol (2/1, v/v) either for single-step extraction or combined with subsequent extraction of β-carotenoic acids from cell pellets and culture medium with hexane [92]. LLE with diethylether from reaction mixtures containing β-carotene and various long-chain β-apocarotenals, 5,6-epoxy-, and 5,8-epoxy-β-apocarotenals has been described as well [80]. Rodriguez et al. applied acetone for carotenoid extraction from fruits, followed by partitioning in petroleum ether, evaporation to dryness, and reconstitution in acetone [40], whereas Rentel et al. extracted β-carotene with DCM, followed by a 10-fold preconcentration after evaporation with N_2 and reconstitution in the same solvent [117]. Van Meulebroek et al. optimized an extraction of β-carotene from tomatoes, applying a fractional factorial design by means of a software program using a so-called G-efficiency as an optimization criterion. In total, eight extraction parameters were statistically evaluated. Therein, the extraction solvent, a secondary extraction phase, the clean-up step, and the avoidance of evaporation exerted positive effects on recovery. Unfortunately, the applicability of the extraction procedure was not tested for CPs [89]. LLE of human serum with hexane, subsequent evaporation, and re-solubilization in DCM, ACN, and methanol or ethanol has been applied successfully [88,118]. Alternatively, serum was mixed

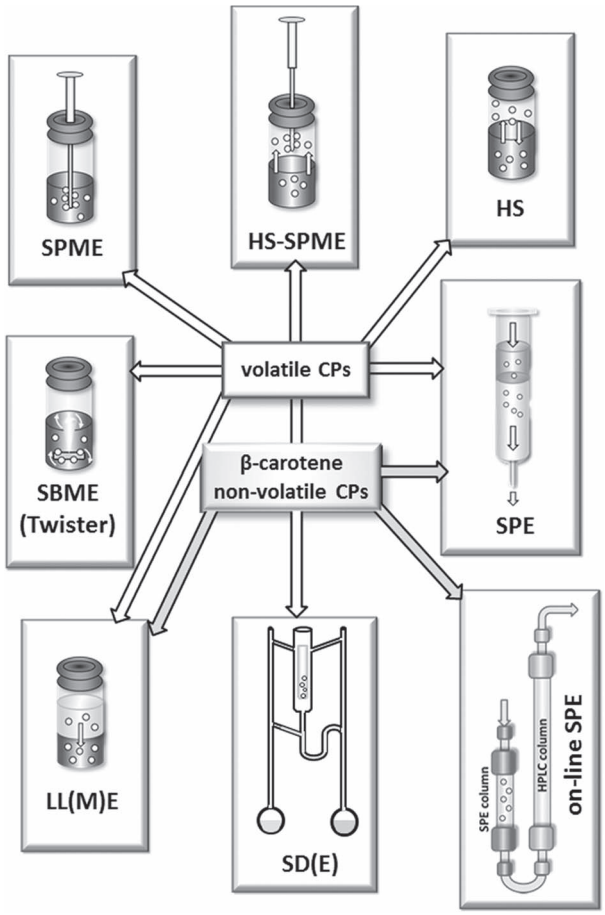


Figure 5. Survey of frequently used methods applied in the extraction and pre-concentration of β-carotene and CPs, differentiating between volatile and non-volatile CPs. Further description including experimental details and abbreviations are given in the text.

with ethanol and extracted with a 1/5 ratio of methylene chloride/hexane. After evaporation to dryness, residues were reconstituted in a 1/1 mixture of ethanol/THF for subsequent injection in HPLC [119]. Repetitive LLE of α- and β-carotene from serum with hexane followed by evaporation and subsequent reconstitution in methanol has been applied by Hagiwara et al. [88]. Although the applicability of this method for CPs has not been demonstrated by the authors, it seems likely – at least for long-chain β-apocarotenals.

Accelerated solvent extraction (ASE) at 40°C and 70 bar has been applied successfully for extraction of β-carotene and β-apo-8´-carotenal, applying different solvent systems [120]. Recently, Nakornriab et al. [121] described supercritical fluid extraction (SFE) with CO_2 at 6,000 psi and 45°C to 60°C, off-line hyphenated to HPLC-ESI-MS as an innovative extraction method for β-carotene. The SFE method allowed for recoveries between 92% and 101%, while enabling rapid extraction time and avoiding solvent contamination.

Nakagawa et al. [95] extracted α- and β-carotene from human erythrocytes. After centrifugation and removal of plasma and buffy coats, cells were diluted with water. Addition of pyrogallol (80 mmol/L) and KOH (1.8 mol/L) improved the extraction efficiency. After sonication, extraction was done with 0.1% SDS and hexane/DCM (5/1, v/v) also including BHT. Subsequent to sonication, vortexing, and centrifugation, the supernatant was subjected to a solvent change and finally passed over a Sep-Pak silica cartridge for removal of matrix compounds [95].

Saponification

Saponification, mostly with KOH, was used to remove interfering di- and triglycerides prior to the final analysis step. Moreover, the quality of HPLC chromatograms was improved by this clean-up step. Unfortunately, saponification simultaneously hydrolyzes retinyl palmitate and other retinyl esters, and thus prevents their individual analysis [122]. After saponification, Heudi et al. [122] extracted all-*trans*-retinol and 13-*cis*-retinol from certified milk powder (SRM 1846) and from a manufacturer-specific reference material by solid-phase extraction (SPE) with Chromabond XTR® phase. Elution was done by n-hexane containing BHT [123]. Hosotani and Kitagawa [124] carried out saponification with 10 mol/L KOH and heating to 70°C to increase the amount of β-carotene and retinol recovered in human serum and rat liver. The increase in recovery was 1.5–2.7-fold for β-carotene, and 1.2-fold for retinol. Analytes were extracted with n-hexane; the extraction protocol has been given in detail. The authors also compared the extraction efficiency of n-hexane, chloroform, and ethyl acetate and tested different sample solvents, showing their effect on the determined concentrations [124].

Extraction of volatile CPs of β-carotene

Short-chain CPs of β-carotene, such as β-IO and β-CC, impose an additional challenge in quantitative extraction, due to their volatility. In general, different extraction methods have been developed and applied for volatile compounds, as described in a recent review [125]. LLE and liquid-liquid microextraction (LLME) have been compared using a stock solution of reference volatile compounds. Whereas recovery for β-CC was comparable for both methods, LLE provided considerably higher recovery for β-IO than LLME, that is, 70% versus 26%. Authors have stated limited LLME efficiencies for highly polar and apolar compounds. Volatile compounds identified in fruit samples encompassed, among others, DHA, and two isomers of 3-OH-7,8-dihydro-β-ionone, 3-OH-β-ionone and 3-OH-5,6-epoxy-β-ionone [126]. Fujise et al. [127] applied headspace solid-phase microextraction (HS-SPME) using an SPME fiber of divinylbenzene (DVB)/carboxen/polydimethyl siloxane (PDMS). After a 20 min extraction (at 60°C) in the headspace of the sample, the fiber was transferred to the injector block of a gas chromatograph. Follow-on oxidation products, such as 3-methyl-1-butanol and 2-methyl-1-butanol, were identified as well [127]. The same type of SPME fiber was used by de Pinho et al. for the extraction of β-IO and β-CC from wine, with subsequent thermal desorption to GC-MS [128]. HS-SPME with a DVB/PDMS fiber was applied for semi-quantitative

determination of volatile CPs, including β-CC, β-homocyclocitral, α- and β-IO, dihydro-β-ionone, and DHA [129]. SPME with a DVB/carboxen/PDMS fiber was also applied to extract β-CC, β-IO, 5,6-epoxy-β-ionone, and DHA [37].

Others have applied HS-SPE with ice-cooled Tenax columns and elution with diethylether [80]. Stir bar sorptive extraction (SBSE) has also been applied in the extraction of volatile CPs. Thereby, a so-called twister, that is, a glass stir bar with a sorptive phase (primarily polydimethylsiloxane) attached to its surface, is applied. The principle of SBSE is outlined elsewhere [130,131]. Briefly, the twister is placed in the sample solution and stirred for a defined period of time, with extraction efficiency governed by analyte partition between the sorptive twister phase and the bulk solution. Under the SBSE conditions selected by Caven-Quantrill and Buglass [132], extraction was almost complete after 2 h. The stirring speed influenced the extraction. Further analysis was performed by thermal desorption and cryofocusing with liquid nitrogen prior to the transfer of analytes into gas chromatography (GC). Recovery data were compared with simultaneous distillation-extraction (SDE) using a Likens-Nickerson apparatus [125,132]. Thereby, a water phase (including the sample) and a water-insoluble extraction solvent are heated in separated flasks with resulting vapors mixing in a joined chamber. Solvents condense again in their respective flasks, dissolving and thus partitioning analytes according to their respective hydrophobicity. The continuous performance of distillation/extraction results in a high extraction efficiency [125]. Recovery of β-IO was 95% and 51% for SDE and SBSE, respectively. However, the applied β-IO concentration was 40-fold lower in SBSE [132]. Peng et al. [133] compared steam distillation (SD), SDE, and headspace-co-distillation (HCD) in terms of their extraction efficiency for CPs, including, for example, β-retinol, β-CC, β-IO, 5,6-epoxy-β-ionone, dihydro-β-methylionone, tetrahydro-β-ionone, DHA, and ionene. SD has been classified as time- and solvent-consuming, bearing the risk for contaminations and sample loss. In case of SDE, a heating under "open-air condition" has given rise to speculation about possible analyte oxidation and generation of artifact compounds. This assumption is related to some abundant compounds in SDE that soar over time. HCD is most appropriate for small and highly volatile compounds [133]. Furthermore, purge-and-trap has been used. Thereby, the sample was purged with dry nitrogen at 35°C, and volatile compounds were retained on an SPE Tenax trap. Analytes were eluted with hexane-ether (50/50, v/v). This approach has been compared with headspace (HS)-SPME, addressing, for example, β-IO [134].

Combined extraction of β-carotene, volatile, and non-volatile CPs

To our knowledge, an SPE method with simultaneous extraction of volatile and non-volatile CPs together with β-carotene has not been published until recently. Our group has developed and validated an SPE method applicable for major volatile and non-volatile CPs and β-carotene. Thereby, a commercial reversed-phase phenyl sorbent was applied in SPE. The extraction principle is primarily based on π-π-interactions of the analytes with the phenyl rings of the sorbent. Samples, that is, model solutions and cell culture media, were passed through the SPE cartridges at a flow rate ≥ 2.0 mL/min. Elution was done with 2.0 mL of 10% (v/v) THF in n-hexane. Recoveries were between 67% and 105% for volatile CPs (β-CC, β-IO, DHA, and 1,1,6-trimethyltetraline) and between 66% and 102% for β-carotene and non-volatile CPs (major β-apocarotenals, and 5,6-epoxy-β-carotene). The final eluate was split and treated differently, depending on the subsequent analysis system, that is, GC-EI-MS or UHPLC-DAD-ESI-Orbitrap MS [111,116].

On-line SPE

Wyss and Bucheli [135] applied an on-line sample extraction method combined with RP-HPLC. After deproteination with ethanol, plasma samples were directly injected into an HPLC system and passed over LiChrospher 100 RP-18 pre-columns (with 5 μm particles) for on-line SPE of retinoids. Ethanol supported the removal of co-extracted lipids from the stationary phase, and reduced isomerization effects. Pre-columns were automatically replaced, either when exceeding a backpressure threshold or after 200 injections. This approach allows for a high degree of automation, prevents evaporative losses of extraction solvents, and the need for manual solvent change after extraction. To reduce analyte solubility despite the high sample ethanol content after deproteination, on-line addition of ammonium acetate was done to promote on-line SPE. This allowed for a sample injection in the mL range. Backflush purging of pre-columns prevented their clogging and contamination of analytical columns with proteins and particles [135].

Separation of β-carotene and its cleavage products

Gas Chromatography

Gas chromatography of volatile CPs without derivatization

GC with mass spectrometric detection (MS) and preceding electron impact (EI) ionization is the preferred method for analysis of volatile CPs. This method has been employed for the analysis of short-chain CPs (from 5,8-epoxy-β-ionone to 2-methylbutan-1-ol; see Table I). Thereby, a variety of GC columns and different temperature gradients have been used to separate various mixtures of volatile CPs, without preceding derivatization. GC-MS was performed on BPX5 capillary columns, with a temperature gradient from 50°C to 280°C. Identification of analytes was done via fragmentation by EI, with an electron energy of 70 eV [80]. Others used a Zebron ZB-1

column for GC-MS analysis with an ion trap mass spectrometer, running a temperature gradient from 40°–300°C for the analysis of volatile CPs derived from β-carotene by degradation with HClO, including β-CC, β-IO, 5,6-epoxy-β-ionone, DHA, and 4-oxo-β-ionone [29]. Nonier et al. used a DB-5MS^{TM} column, applying a temperature gradient from 60°C–310°C with an EI of 70 eV to identify degradation products of β-carotene generated by oxidation with copper or a radical reaction. A comprehensive list of generated β-carotene degradation products as well as their EI fragments is given elsewhere [42]. Waché et al. applied a CP Wax 58CB column with a 4°C/min temperature gradient from 90°C to 220°C coupled to an ion-trap mass spectrometer, to identify volatile CPs, such as β-IO, 5,6-epoxy-β-ionone, and DHA [35]. Others used a STABIL WAX-DA column and a thermal gradient from 40°C–220°C at 2°C/min to separate β-IO and β-CC as degradation products in port wine [128].

In terms of GC-MS analysis of the main aromatic compounds of plants, the major focus was on β-CC and β-IO subsequent to various extraction methods (see section "*Extraction of volatile CPs of β-carotene*"). GC separations were performed on a DB-5MS Varian capillary and a temperature profile between 45°C and 240°C [134], or a VF-5ms Varian column applying a gradient from 40°C to 220°C. In the latter case, GC-EI-ion trap MS was used for analyte fragmentation and analysis [136]. Separation of CPs extracted from sweet potato chips was done by a DB-WAX column [37].

VF-5ms (from Varian) and STABILWAX-DA (from Restek) have been employed to separate and identify β-CC, β-homocyclocitral, α-ionone, 2,3-epoxy-α-ionone, dihydro-β-ionone, β-IO, and DHA in *Catharanthus roseus* [129]. β-IO and β-CC have also been determined as odorous compounds in spiked water samples and river water by GC-ion trap MS using a DB-5 capillary column [137]. Volatile CPs have been analyzed by GC-EI-Q ion trap MS using a HP-5MS column with 5% diphenylsiloxane monomer as stationary phase. Different temperature gradients were applied for the analysis of (i) neutral and (ii) acidic and alkaline fractions [133]. GC with flame ionization detection (FID) and MS detection have been applied for the analysis of volatile flavor compounds, including β-IO and derivatives, and DHA in fruits. A SolGel-Wax capillary column was employed in both cases [126]. CPs stemming from cyanobacteria were analyzed by means of a DB-624 column [127]. Our group has used a DB-20 WAX column from Agilent to separate β-CC, β-IO, DHA, and 1,1,6-trimethyltetraline (TMT), including two internal standards. A temperature gradient that raised the column temperature from 65°C to 220°C was used to separate analytes within 24 min (Figure 6) [111].

Gas chromatography of CPs after derivatization

Reinersdorff et al. analyzed a non-volatile CP, that is, retinol, by GC-MS after its derivatization with N,O-bis(trimethylsilyl)-trifluoroacetamide to trimethylsilylretinol

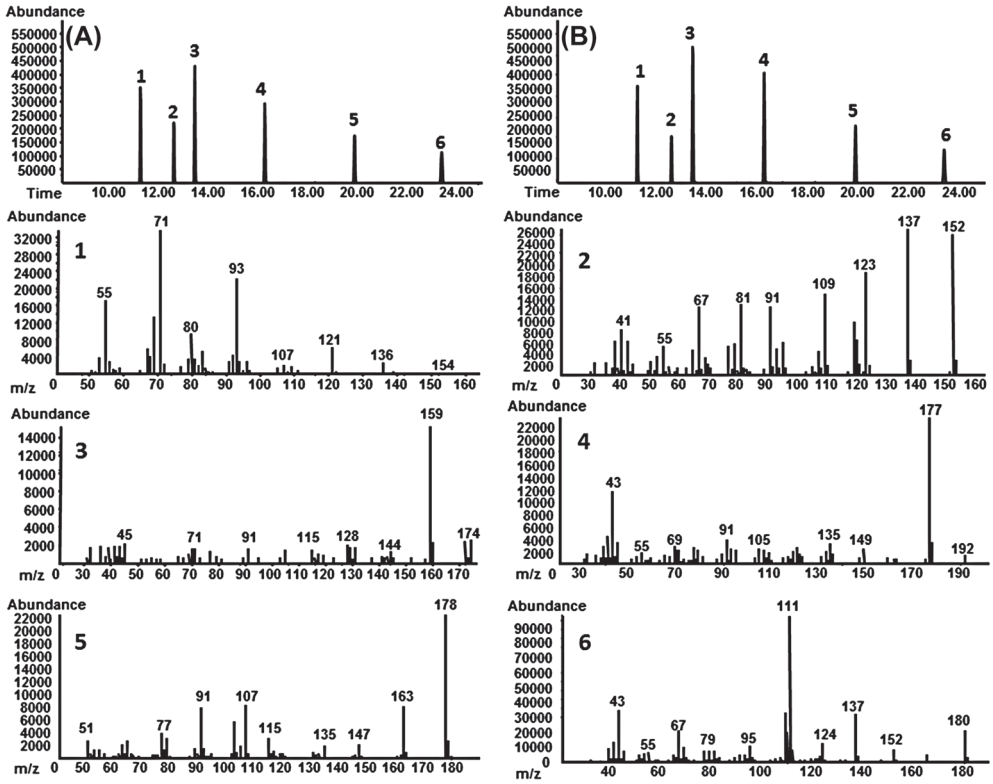


Figure 6. Chromatograms and MS spectra of CPs and internal standards (IS). (A) Chromatogram for a CP standard solution (50 µg/mL of each CP dissolved in 10% (v/v) THF in n-hexane); (B) Chromatogram for CP standard solution after SPE (expected concentrations for individual CPs correspond to (A)). 1–6 MS spectra of CPs derived from (A): 1 linalool (IS), 2 β-cyclocitral (β-CC), 3 1,1,6-trimethyltetraline (TMT), 4 β-ionone (β-IO), 5 methylisoeugenol (IS), and 6 dihydroactinidiolide (DHA). Reprinted from [111], under the terms of Creative Commons Attribution License.

using a DB-1 capillary GC column. Fragment ions were produced by a methane-reagent gas via chemical reaction, producing anhydroretinol [138]. Similarly, Handelman et al. derivatized retinol extracted from human plasma with N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide after HPLC-purification. Fragmentation of derivatized retinol was done by EI with 70 eV [139].

High performance liquid chromatography – HPLC

Normal-phase HPLC

McCaffery et al. found normal-phase HPLC with a linear gradient (using n-hexane, dioxane, and isopropanol) advantageous for the separation of retinal, retinol, retinoic acid, didehydro-retinoic acid, and 4-oxo-retinoic acid within 20 min, since it allowed for the inclusion of high concentrations of BHT [140]. Heudi et al. separated all-*trans*- and 13-*cis*-retinol with NP-HPLC on a silica-based column in isocratic mode, applying hexane/dioxane/2-propanol (69.7/3.0/0.3, v/v/v) as mobile phase [123].

Reversed-phase HPLC – General separation aspects

High performance liquid chromatography in reversed-phase mode (RP-HPLC) is the preferred separation technique for β-carotene and its hydrophobic non-volatile CPs [81]. Thereby, C18 columns are employed frequently. The application of more hydrophobic C30 columns also allows for a differentiation between isomeric variants of β- and α-carotene, that is, all-*trans*- and various *cis*-isomers [84,117,141,142], but is also applied for the separation of retinol, retinyl acetate, retinyl palmitate, and β-carotene, or β-apo-8´-carotenal, β-apo-8´-carotenoic acid ethyl ester, and β-carotene [120,122,143]. Isomeric variants can additionally be distinguished by a so-called hypsochromic shift in the UV absorbance maximum of *cis*-variants in comparison to all-*trans* forms. Moreover, a novel *cis*-band occurs in the UV spectrum at ~340 nm [141]. In terms of 5,6-epoxy and 5,8-epoxy-β-carotene that cannot be differentiated by MS, UV absorbance allows for a distinction due to their different absorbance maxima, that is, 449 nm and 430 nm, respectively [40].

For separation in RP-HPLC, mobile phases with non-aqueous properties are applied frequently using ternary gradients [81,89,116]. The previously addressed limited solubility in solvents (see "*Application- and treatment solutions – Organic solvents*") routinely used in RP-HPLC, that is, methanol and ACN, requires the addition of supportive solvents, such as THF [81,116], n-hexane, and isopropanol [123,140], to improve solubility and chromatographic peak shapes. Other solvents applied in UHPLC comprised DCM [124,144], dioxane [123,140], acetone [117], *tert*-butylmethyl ether [90,91], 1-octanol [124], methylene chloride [145], and the implementation of further additives such as BHT [140] and triethylamine [90].

The UV-Vis absorbance maxima (λ_{max}) of β-carotene and its related CPs highly depend on the applied solvents, for example, 448 nm in hexane, with up to 462 nm observed in benzene, chloroform, and toluene when measuring β-carotene [83]. When β-carotene was dissolved in hexane or methanol, a comparison of λ_{max} showed differences in the intensity but not in the apex of the UV-Vis absorbance spectra. Moreover, when testing the UV-Vis detector response for up to ~13 µmol/L β-carotene, the slope of the calibration curve leveled off at 5 µmol/L in methanol, but appeared still linear in hexane. This has been related to the formation of β-carotene microcrystals at 3 mg/L in methanol [146]. The following paragraphs provide more details of (U)HPLC separation systems applied for the distinction of β-carotene and non-volatile CPs.

RP-HPLC – Separation Conditions

Sommerburg et al. applied an Ultrasphere C18 column with 5 µm particles using a mixture of ACN/THF/methanol/ammonium acetate for separation of β-apo-4´-, β-apo-8´-, β-apo-12´-carotenal, and retinal. Unfortunately, neither chromatograms nor retention times were provided [29]. Franssen-van Hal et al. applied a Vydac RP 18 column in combination with gradient elution with two eluents composed of ACN/*tert*-butylmethylether/ammonium acetate prepared in different ratios to separate β-carotene, retinoic acid, retinal, retinyl palmitate, β-apo-4´-, β-apo-8´-, β-apo-10´-, and β-apo-12´-carotenal within 30 min [91]. Zhu et al. separated ^{13}C-labeled β-carotene and retinyl palmitate on a YMC carotenoid C30 column with 3 µm particles [122]. A C30 column with 3 µm particles and a linear quaternary gradient with a flow rate of 1.0 mL/min was also applied for the separation of β-carotene, β-apo-8´-carotenal, and other carotenoids [89]. Barua and Olson employed RP-HPLC with a 3 µm Microsorb-MV column and gradient elution with water, methanol, ammonium acetate, and DCM to separate retinoids, for example, all-*trans*-5,6-epoxy-retinoic acid, retinoic acid, retinol, and retinal [144]. Rodriguez et al. used a C18 column from Vydac to separate β-apocarotenals, all-*trans*-retinal, all-*trans*-retinol, and 5,6-epoxy-β-carotene in isocratic mode, with ACN, *tert*-butylmethylether, ammonium acetate, and triethylamine [90]. Arora et al. used 5 µm octadecyl silica particles and a linear gradient for separation of β-apocarotenals, epoxy-β-carotenes, and 4-nitro-β-carotene, with UV and triple quadrupole mass spectrometric detection in negative atmospheric pressure chemical ionization (APCI) mode [96]. Wyss and Bucheli [135] utilized two C18 columns of LiChroCART (packed with endcapped Superspher 100 RP-18) coupled in series. This design was required to assure both separation of retinoic acids and analytes from the plasma matrix. Target analytes comprised 13-*cis*-retinoic acid, all-*trans*-retinoic acid, and their corresponding 4-oxo metabolites in plasma samples from different species. In total, 14 endogenous retinoids could be separated within a total sequence time of 38 min (including on-line SPE) [135]. Thibeault et al. [145] used a Thermo Spherisorb ODS2 (= C18) column with 3 µm particles, with a preceding guard column and biocompatible titan frits to separate all-*trans*-α-, all-*trans*-β-, and *cis*-β-carotene (isomer not specified), retinol, and tocopherols

within 15 min. The guard column was replaced every 500 runs. Isocratic elution was done with ACN/methylene chloride/methanol (7/2/1) including 20 mmol/L ammonium acetate, with either UV diode array (DAD) or fluorescence detection. Inclusion of ammonium acetate improved peak shape and repeatability of chromatographic separations for α- and β-carotene. This has been related to the neutralization of HCl traces stemming from methylene chloride that might cause degradation. However, at an ammonium acetate concentration of 50 mmol/L, salt precipitation effects were observed over time [145]. Hosotani and Kitagawa [124] employed a Resolve 5-µm spherical C18 column. Isocratic elution was done by ACN/DCM/methanol/1-octanol. β-carotene was detected at 451 nm, whereas retinol was recorded at 325 nm [124].

Ultrahigh performance liquid chromatography–UHPLC

Rivera et al. [147] used an ACQUITY UPLC BEH C18 column with 1.7 µm particles and a binary gradient with ACN, methanol, and water. Retention times were between 4.81 and 10.07 min for β-apo-8´-carotenal and β-carotene, respectively [147]. Paliakov et al. separated retinol and β-carotene, two tocopherols, and CoQ10 on an ACQUITY Shield RP18 BEH column (1.7 µm particle size) within 2 min by using a gradient composed of water, ACN, methanol, and 2-propanol [148]. Arnold et al. [149] employed an Express RP Amide column (2.7 µm particles) to separate all-*trans*-retinoic acid and four isomers within 15 min. In comparison to C8 and C18 columns, the amide column was superior and provided baseline resolution when using gradient elution applying a mobile phase composed of water, ACN, methanol, and formic acid [149]. Sy et al. used a C18 column with 1.8 µm particles and a binary gradient to separate 5,6-epoxy-, 5,8-epoxy-, 5,6,5´,6´-diepoxy-, 5,8,5´,8´-diepoxy- and 5,6,5´,8´-diepoxy-β-carotene as well as β-apo-8´-, β-apo-12´-, β-apo-14´-carotenal, β-apo-15-carotenal and β-apo-13-carotenone within 18 min. Detection was with DAD and APCI-ion trap MS [41]. We have used a Kinetex® C18 column with 1.7 µm core-shell particles to separate β-carotene, 5,6-epoxy-β-carotene, β-apo-4´-, β-apo-8´-, β-apo-10´-, and β-apo-12´-carotenal within 5.5 min. A quaternary gradient composed of ultrapure water, ACN, trifluoroacetic acid (TFA), and THF was used. UV detection was executed at a data rate of 40 Hz, due to the small peak widths (Figure 7). ESI-Orbitrap MS was affiliated in series [116].

Capillary electrophoresis

Due to the pronounced hydrophobicity of β-carotene, capillary electrophoresis (CE) separations in aqueous media are inappropriate. Instead, capillary electrochromatography (CEC), a chimeric separation mode including features of CE and HPLC, has been applied. Thereby, selectivity of HPLC is combined with the exceptional efficiency of CE [150,151], which is based on the flat flow-profile of the electro osmotic flow [152,153]. Sander et al. applied CEC with a stationary C30 phase. Both isocratic separations and gradient elution CEC were carried out employing methanol/*tert*-methyl butylether/background electrolyte (35%/60%/5%, v/v/v) and acetone/background electrolyte (95%/5%, v/v) for isocratic mode, and acetone/background electrolyte (gradient from 80%/20% to 99%/1%, v/v) for gradient elution. Background electrolyte was 1 mmol/L sodium borate in both cases. Separation conditions allowed for baseline separation of 13-*cis*-α-, 13´-*cis*-α-, 13-*cis*-β-, all-*trans*-α-, 9-*cis*-α-, 9´-*cis*-α-, all-*trans*-β-, and 9-*cis*-β-carotene within 20 min. Unfortunately, no CPs have been included in the standard solutions and sample mixtures tested [154]. Adalid et al. [155] applied methacrylate ester-based monolithic columns in CEC instead of conventionally packed CEC columns, which provide several advantages, as outlined by the authors. A mixture of 35%THF/30% ACN/30% methanol/5% 5 mmol/L Tris (pH 8.0) (v/v/v/v) was applied as

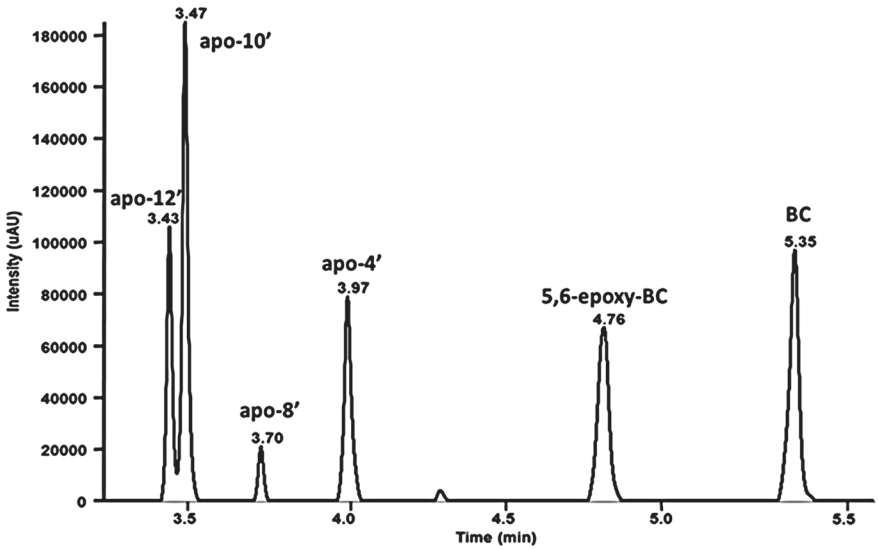


Figure 7. UHPLC chromatogram acquired at 460 nm for a standard solution containing all-*trans*-β-carotene (BC) and CPs at 1.0 µg/mL. Reprinted from [116], under the terms of Creative Commons Attribution License.

mobile phase. β-carotene and β-apo-8´-carotenal (applied as internal standard) were separated within 6 min [155].

Detection of β-carotene and its cleavage products

Mass spectrometry

General aspects

Characterization of β-carotene and related CPs by their retention times and UV-Vis spectra might be inadequate for their unequivocal identification [156]. Highly accurate mass information is mostly indispensable. In this context, hyphenation of chromatographic separation systems to MS is progressively applied. Fragmentation of analytes by MS/MS or higher order MS (MS^n), and the comparison with theoretical isotope patterns in case of high-resolution MS systems, offer further decisive tools for an improved reliability of data in analyte identification (Figure 8) [89,116]. A discussion of the principles of electrospray ionization (ESI) in MS is beyond the scope of this review. The interested reader is thus directed to comprehensive reviews that tackle this aspect [157–160]. Unfortunately, β-carotene, like other nonpolar analytes, lacks protonation and deprotonation sites and thus ionization in ESI-MS is cumbersome [117,123]. Frequently, this prevents adequate sensitivity or even detection at all [161].

Ionization modes in mass spectrometry for analysis of β-carotene and CPs

Different ionization modes have been applied in MS including APCI, atmospheric pressure photoionization (APPI), and ESI [147]. A comparison of APCI and APPI is given elsewhere [162,163]. Briefly, whereas ESI is appropriate for polar and ionic compounds, APPI provides favorable performance for nonpolar analytes. The efficiency of different ionization modes was compared in the direct infusion mode with standard solutions prepared in 70% ACN/30% methanol (v/v). β-apo-8´-carotenal was detected as $[M + H]^+$ with m/z 417.5 in all three ionization modes, whereas β-carotene was detected as $[M + H]^+$ with m/z 537.7 in the APCI mode, and as cationic radical ion $[M^\bullet]^+$ with m/z 536.7 in ESI and APPI. The signal intensity in APPI can be increased by so-called dopants, that is, additives that are mostly added post-column. Dopants can easily be ionized and support the ionization of analytes. When comparing the effect of acetone, toluene, anisole, and chlorobenzene as dopants, the latter enhanced signal intensities of β-carotene and β-apo-8´-carotenal by a factor of 1.6 and 3.5, respectively, in comparison to dopant-deficient systems [147]. Moreover, APCI-MS provided a wide dynamic range and linear detector response over four orders of magnitude, which was not achieved in ESI-MS that shows a continuous loss in sensitivity with increased analyte concentrations [116,143]. This has to be considered when analyzing β-carotene together with CPs that are accessible to ESI-MS detection, as long as they possess ionization sites.

Atmospheric pressure chemical ionization

APCI in the positive ionization mode has been applied to identify retinoic acid and four isomers by means of UHPLC-MS/MS. The negative ionization mode provided a ~10-fold impaired sensitivity. Although higher sensitivity was yielded for the analytes by ESI-MS based on the availability of ionizable groups, this approach was not applied by the authors due to matrix effects present when analyzing human serum. The identity of retinoic acid and

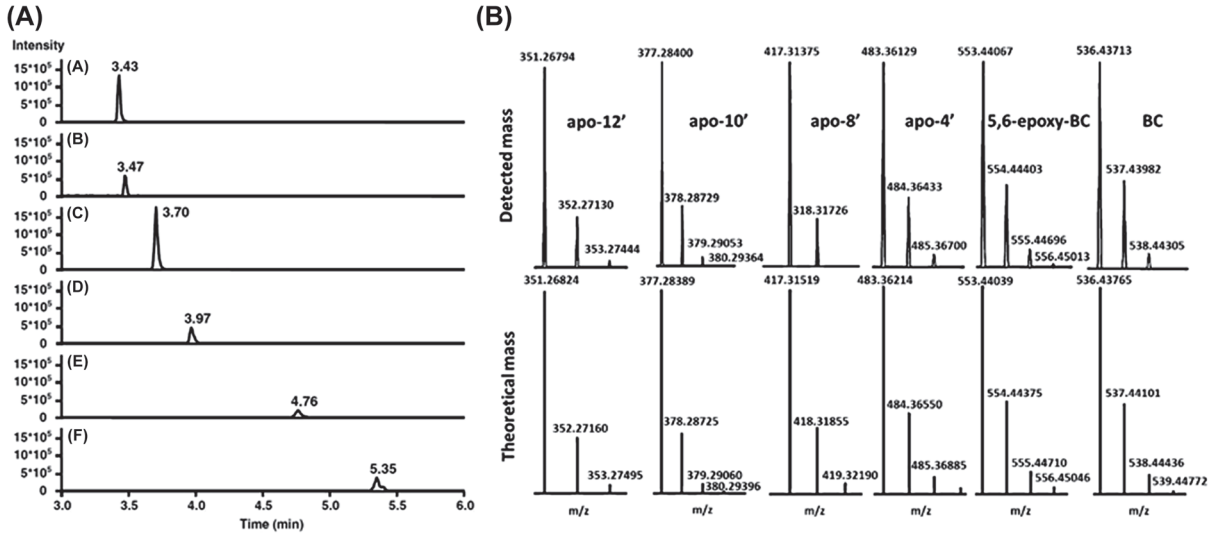


Figure 8. (A) Extracted ion current chromatograms of a standard solution containing all-*trans*-β-carotene and target CPs at 1.0 µg/L. Identity of peaks: A, β-apo-12'-carotenal, B, β-apo-10'-carotenal, C, β-apo-8'-carotenal, D, β-apo-4'-carotenal, E, 5,6-epoxy-β-carotene (BC), and F, β-carotene (BC). (B) Detected masses and isotope distribution for β-carotene and target β-apocarotenals including a comparison with theoretical masses and theoretical isotope distribution by means of the software option provided. Reprinted from [116], under the terms of Creative Commons Attribution License.

isomers extracted from human serum was confirmed by MS/MS [149]. Similarly, APCI in positive mode has been used for detection of all-*trans*- and 13-*cis*-retinol. Both provided a protonated ion of m/z 269.1, that is, $[M\text{-}H_2O + H]^+$, but differed in their retention times under NP-HPLC conditions [123]. Others applied HPLC-APCI-quadrupole ion-trap MS, using up to MS^4 to identify retinoids [140]. Wang et al. used RP-HPLC-APCI-MS for the analysis of β-carotene and retinyl palmitate in human serum extracts [164]. HPLC with APCI-MS in positive ionization mode revealed compounds interfering with $^{13}C_{10}$-labeled β-carotene in blank samples, such as feces and serum. Thus, Zhu et al. also applied APCI in negative ionization mode, where the interfering peak was absent. However, signal intensity in single ion monitoring (SIM) mode was reduced approximately by a factor of six [122]. Others used APCI-MS for analysis of α- and β-carotenes [84,88]. Van Meulebroek et al. applied APCI- Exactive Orbitrap MS equipped with a high energy collision dissociation (HCD) cell and APCI-TSQ triplequadrupole MS for analysis of β-carotene in tomatoes [89]. HPLC-APCI-MS was also used to characterize CPs after degradation of β-carotene at 110°C in a Rancimat [67].

Electrospray ionization β–carotene – formation of radical cations

Even neutral analytes with highly conjugated systems can be ionized with high efficiency in ESI-MS under well-selected frame conditions, employing in-solution ionization for example, by Lewis acids, halogens, and metal salts [156,165]. Therefore, initial attempts in ESI-MS analysis referred to post-column reactions with oxidizing agents, including halogenated solvents [156], or silver salts [117,141]. For instance, post-column addition of a $AgClO_4$ solution via a liquid junction with a final Ag^+ concentration of 5 µmol/mL in the solution allowed for the formation of silver-adducts $[M + Ag]^+$ and radical cations $[M^{\bullet}]^+$ of β-carotene. Both ion types showed different fragmentation patterns, whereby $[M + Ag]^+$ provided a higher number of fragment ions that were still complexed by Ag^+. Moreover, stability of Ag^+ adducts was increased in the presence of *cis*-configurations of β-carotene [117].

Electrospray ionization – principles of radical cation formation

The formation of radical ions does not involve reactions comprising the loss or gain of protons, typically encountered in ESI. Consistently, they are rarely observed in ESI-MS if appropriate measures are not taken [166]. Generally, radical cations can be generated in ESI-MS either (i) by in-solution oxidation under the influence of oxidizing reagents in the bulk solution, or (ii) by oxidation events at the solvent/electrospray (ES) needle interface [166]. Besides, the detection of radical ions is also a question of their stability in solution [165]. First investigations on the radical formation process in ESI-MS have been performed on model compounds, such as metallocenes, metalloporphyrins, and polycyclic aromatic hydrocarbons (PHAs), where removal of single electrons became the predominant process [161,166].

Ad (i): In-solution oxidation was enhanced by post-column addition of halogenated solvents, also including TFA in the mobile phase. Signal intensity and repeatability were best with application of 0.02–0.25% (v/v) 2,2,3,3,4,4,4,-heptafluoro-1-butanol. When different TFA concentrations were tested within a range of 0.01% and 0.5% (v/v), 0.1% (v/v) TFA was found to be the most efficient in the formation of radical cations of β-carotene in 70% ACN/30% *tert*-butylether (v/v). Higher TFA concentrations apparently impaired ES formation, quenched gas phase ions of β-carotene, or prevented their release from sprayed aerosol droplets. However, TFA did not contribute via its acidity [156].

Ad (ii): The formation of radical ions can also be achieved by a conventional ESI source that acts as a controlled-current electrolyte (CCE) cell. Thereby, the ES current and the related potential at the sprayer interface have to be selected to be sufficiently high for an oxidation of neutral target analytes [167,168]. Due to the applied electric field at the ES device (in other words, spray needle), ions of the same charge polarity (e.g., cations) are selectively lost via the Taylor cone and the formed jet stream that nebulizes into aerosol droplets. These droplets undergo further reduction in size due to solvent evaporation until they reach the so-called Raleigh stability limit, and then show (asymmetric) fission [160]. Countercharged ions (in other words, anions in the described case) will remain and accumulate in the solution of the separation line. Theoretically, this should disrupt the spray process over time. Since this is not observed, apparently a "charge balancing" event occurs, that is, an electrochemical process that produces either cations or neutralizes the piled anions. Besides an oxidation of the material of the ES needle, compounds in the separation medium can be oxidized as well, forming radical ions [168,169]. The presented case describes the so-called positive ionization mode in MS, whereas in the negative ionization mode, the situation is inverted, with loss of anions and charge balance occurring via reduction reactions [168].

Charge balancing in ESI-MS – contribution to the formation of radical cations

As an integral part of "charge balancing", the outlined oxidation reaction in positive ionization mode (or reduction reaction in negative ionization mode) creates a faradaic current that equals the ES current that is carried by the charge transfer via ES. Relevant aspects are discussed as examples for positive ionization, but are equally valid for the negative ionization mode. Charge balancing requires a sufficient amount of electroactive species to be oxidized at the interface between the metallic sprayer surface and the bulk solution. Oxidizable species comprise analytes, electrolytes, and/or contaminants present in the bulk solution as well as the sprayer material itself

[168,169]. Blades et al. described the oxidation of OH^- and H_2O as part of the "charge balancing process". The concomitant production of H^+ is most prominent in aqueous systems [169]. The material of the ES electrode thus also determines the efficiency of analyte oxidation [168]. This was demonstrated when stainless steel and platinum capillaries were compared [167].

The tendency of neutral molecules to release an electron is related to the energy of the highest occupied molecular orbital (HOMO). Thus, HOMO determines the half-wave potential ($E_{1/2ox}$) that characterizes the oxidation propensity of individual compounds [161]. The relation between radical cation formation and the molecular orbital level is discussed elsewhere [170]. The electric potential required for radical cation detection always has to exceed the onset potential of ES and depends on the analyte of interest [166]. A slow flow rate of the bulk solution, for example, of the mobile phase, was shown to improve the ionization efficiency by an enhanced transport of analytes toward the metal/solution interface where formation of radical ions takes place. Thus, the accessibility of the analyte depends on its diffusion time, and therefore the diffusion coefficient and the diffusion distance that is determined by the capillary diameter. Concomitantly, the propensity of radical ion formation of non-polar analytes depends on (i) the ES current, (ii) the concentration and redox potential of concurrent species in the bulk solution, as well as (iii) the selected flow rate of the mobile phase [168]. Moreover, formation of gas-phase ions must not be hampered in order to detect radical ions in MS [167]. Electrolytes with low ion suppression, such as lithium triflate, provide higher signal intensities of analyte radical ions due to the lower propensity of lithium triflate in forming gas-phase ions. This allows for higher ES currents, either by increased ion strength or the application of higher ES voltages. Both strategies enhance oxidation of analytes according to the charge balancing, as outlined above [167]. Similar effects can be assumed for β-carotene, although this has been exemplified on other neutral compounds with conjugated systems.

Formation of radical cations of β-carotene in ESI-MS

As outlined in the previous sections, the fundamental results for neutral compounds provide an improved understanding of the radical cation formation process for carotenoids, namely β-carotene. According to voltammetric measurements, analytes with an oxidation potential $P_{OX} < 0.3$ V are highly prone to radical ion formation, whereas a higher P_{OX} reduces the efficiency in the generation of radical ions and higher analyte concentrations are required to produce radical ions in sufficient amounts [165]. Voltammetric studies of β-carotene, which possesses a peak P_{OX} of ~0.75 V, showed radical cation formation followed by a further oxidation event with generation of a dication at a slightly higher potential. Flow injection experiments with β-carotene injected into a stream of 0.1% TFA in methylene chloride at a low flow rate of 10 µL/min resulted in a TFA-caused oxidation of β-carotene to a dication $[M]^{2+}$, with subsequent proton loss giving $[M^{2+}\text{-}H^+]^+$ according to

$$[M] \rightarrow [M]^{2+} \rightarrow [M^{2+}\text{-}H^+]^+ + H^+.$$

The dication had a half-life of 6.3 s in the applied solvent system. Other solvent combinations failed in detecting ions related to β-carotene, most likely due to an extenuated lifetime [165].

Indeed, an electrochemical oxidation process at the metal-liquid surface was shown to occur for β-carotene [156]. Moreover, the importance of selecting an appropriate solvent composition for achieving high signal intensities for molecular cations has been addressed before [161,166]. The addition of TFA to the mobile phase to assure solution-phase oxidation of neutral analytes with highly conjugated electron systems has been described elsewhere [156,165]. Apparently the inclusion of TFA in the mobile phase can represent a crucial aspect to achieve adequate sensitivity for β-carotene [166]. The composition of the mobile phase determines the sort of ions generated. A combination of methanol and TFA, for instance, was shown to generate $[M^{\bullet}]^+$. Indeed, the addition of TFA was shown to entail a pronounced increase in the signal intensity of β-carotene radical ions [156]. In some instances, the favorable effect of TFA has been related to a stabilization effect on radical ions with a concomitant extension of their lifespan [161,166]. In-solution consumption of radical cations has been reported for protic as well as nucleophilic solvents (e.g., water and methanol) and nucleophilic additives, such as acetate. This is one explanation of why a binary solvent mixture composed of methylene chloride and TFA – that is frequently applied in direct infusion and flow injection experiments – but also dried ACN, resulted in highest signals for radical ions. The stabilization effect of TFA has been related to "…*an interaction between the non-nucleophilic CF3 group of TFA and the cation*…" [161,171]. This solvation by TFA prevents nucleophilic attacks of the radical cation [161]. Xu et al. observed no increase in radical cation signals with application of TFA, but the effect seems to be analyte-specific [166].

The answer to the general question of whether radical cations are produced in sufficient amounts (i) in solution or (ii) at the solvent-ES needle interface, and are then sufficiently stabilized to allow for a substantial transfer to the gas phase and then MS, depends on the appropriate selection of instrumental settings, for example spray voltage and temperature, the setup of the ES source, and on the properties of applied solvents (in other words, promoting the formation and stabilization of radical cations) [156,161,166]. Further reviews on the formation of radical ions from neutral organic compounds, also discussing β-carotene and related degradation products, are given elsewhere [172,173].

ESI-MS application in β-carotene measurements

β-Carotene was analyzed with ESI-quadrupole time-of-flight (Q-TOF) MS in positive and negative ionization mode hyphenated to RP-HPLC and by applying collision-induced dissociation (CID). Beside the radical cation $[M^{\bullet}]^+$, MS/MS provided daughter ions at m/z 444, that is, $[M^{\bullet}\text{-}92]^+$, due to the loss of toluene, and also at m/z 495, 457, and 413 [121]. The application of TurboIonspray ionization operating at up to 500°C in positive ionization mode allowed for the formation of radical ions $[M^{\bullet}]^+$ by electron loss, circumventing the need for in-solution oxidation. Operation at increased temperatures enhanced the intensity of $[M^{\bullet}]^+$ [115]. We have applied UHPLC hyphenated to ESI-LTQ-Orbitrap MS. β-Carotene was detected as a radical cation, whereas β-apocarotenals occurred as $[M + H]^+$. Identification was based on the high mass accuracy (< 2 ppm) that was realized and by comparison of theoretical and experimental isotope distribution of the analytes (Figure 8) [116].

Spectroscopic methods

The structural configuration and vibration properties of β-carotene have been addressed by Raman and Fourier transform infrared (FTIR) spectroscopy. Data provided information on the angle taken by the β-ionone rings relative to the molecular plane as well as angles and lengths of individual C–C bonds. This allowed for a distinction between the all-*trans*-β-carotene and a *cis*-β-carotene variant [174]. Probably because FT-Raman spectroscopy has been applied to distinguish *cis*/*trans*-isomers of carotenes it would be applicable for quality control [175], for example, of standards and treatment solutions.

Identification of β-carotene was also done in real samples, such as pumpkin and carrot root, where, in comparison to the standard, a shift in wavenumber has been observed and related to protein interaction. Although not demonstrated, authors have addressed the option for an *in situ* measurement of epoxy-carotenoids [175].

Measurement of biological samples

Plants and insects

β-CC, β-IO, and β-methylionone have been determined in kale before and subsequent to herbivore attack, and after mechanical damage. Measurements also included content of CPs in butterfly larvae of *Pieris brassicae* when fed with kale leaves [136]. Volatile CPs were also identified in flue-cured tobacco leaves including β-CC, 2,3-dehydro-α-ionone, β-IO, 5,6-epoxy-β-ionone, dihydro-β-methylionone, tetrahydroionone, α-ionone, DHA, and tetrahydroactinidiolide [133]. Furthermore, β-CC and β-IO were determined in cyanobacteria as volatile CPs of β-carotene. 2,6,6,-trimethylcyclohexene-1-carboxylic acid and two aliphatic CPs occurred as secondary oxidation products of β-CC [127].

Food and Beverage

Fruits are rich in antioxidant compounds, such as carotenes, and their content depends on the stage of fruit maturity. Some biological antioxidants are even localized in parts that are discarded during fruit processing when producing nectar, syrup, and puree. Carotenoids for instance are mostly located in fruit peels [176,177]. In food and beverages, natural antioxidants are progressively replacing their synthetic counterparts, that is, BHT and BHA [177]. β-Carotene, retinol, retinyl acetate, and retinyl palmitate have been determined in juices enriched with vitamins A and C, so-called ACE juices [178]. Rodriguez and Rodriguez-Amaya detected 10 CPs of β-carotene in processed acerola and mango juices and in dried apricots [40]. Rodriguez-Comesaña et al. analyzed 13 beverages to which β-carotene had been added. In fact, determined concentrations were up to a factor of 30 higher than the labeled content. For labeling, manufacturers apparently only considered added vitamin, but not the contribution of natural vitamin content [179]. Fleshman et al. revealed β-apo-8´-, β-apo-10´-, β-apo-12´-, and β-apo-14´-carotenal together with β-apo-13-carotenone in orange-fleshed melons (that is, "Orange Dew" and cantaloupe "Cruiser") (Figure 9). The average content was between 176.3 and 242.8 μg β-carotene/g dry weight. For individual CPs, the average content was approximately between 20 and 45 pmol/g wet weight [180]. β-Carotene, 5,6,5´,6´-diepoxy-β-carotene, and an unidentified epoxy-β-carotenoid were identified in mango. Further carotenoids were determined but not specified [144]. β-CC and β-IO have been identified as CPs of β-carotene at up to 0.5 and 0.2 μg/L in port wine, respectively. β-IO contributes to the wine aroma [128]. Additionally, β-IO was identified in Huxelrebe grape juice derived from Muscat grapes [132]. β-IO, isomers of 3-hydroxy-7,8-dihydro-β-ionone, 3-hydroxy-β-ionone, 3-hydroxy-5,6-epoxy-β-ionone, and DHA were identified in white peach and grape tomato [126]. β-Carotene, 5,6-epoxy-β-carotene, β-CC, β-IO, 5,6-epoxy-β-ionone, and DHA have also been identified in chips made from sweet potatoes [37]. β-Carotene was also determined in Thai black rice, although CPs have not been addressed [121]. 5,6-Epoxy-β-carotene, β-carotene (E160), β-apo-8´-carotenal (E 160 e), and β-apo-8´-carotenoic acid ethyl ester (E 160 f) are all listed as "carotenoid food additives" (CFAs) according to the EU legislation [120,181]. Together with other CFAs, they have been extracted from a spiked composite real sample. Analysis was done by RP-HPLC-APCI-MS in positive mode [120].

Cell culture models

Primary rat hepatocytes

β-Carotene, selected β-apocarotenals, and volatile CPs have been addressed in cell culture medium of primary

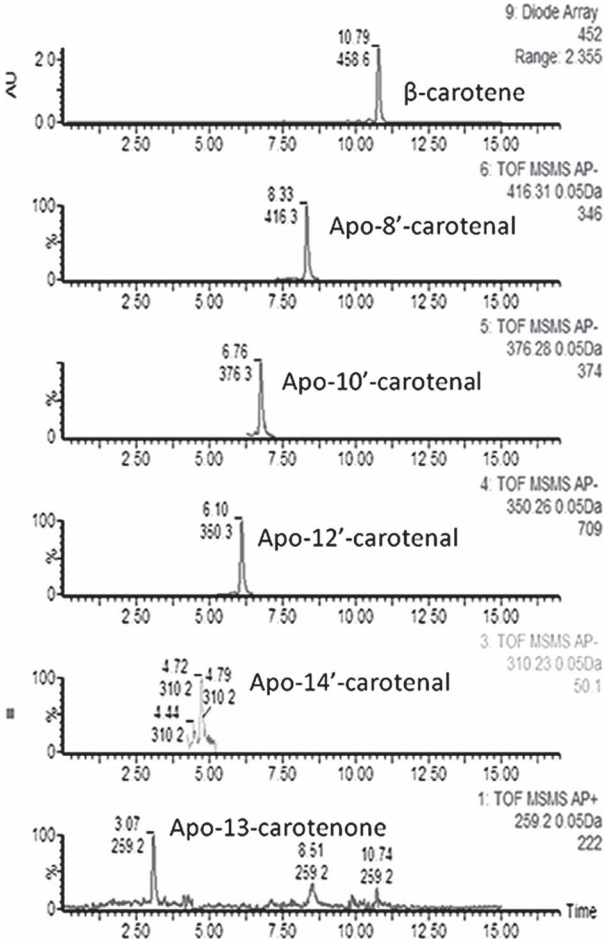


Figure 9. β-apocarotenoids and β-carotene in the 'Orange Dew' melons. The top chromatogram shows the absorbance at 452 nm, and the other chromatograms show the β-apocarotenoids at their respective masses. Reprinted with permission from [180]. © 2011, American Chemical Society.

rat hepatocytes after a joint SPE of these compounds [111,116]. After the addition of 10 µmol/L β-carotene to cell cultures, oxidative stress was induced by different means, including DMNQ, H_2O_2, and Fe(II)lactate. Incubation time was 3 h after addition of the treatment solution. Control treatments without cells under otherwise identical conditions revealed that 5,6-epoxy-β-carotene was not formed by cells but rather by autoxidation. β-Apo-4´-carotenal seemed to be generated by mediation of primary target cells since it was absent in control samples, but the extent of production seemed to follow an individual cell response [116].

Human bronchial and intestinal cell lines

Rodriguez et al. [90] incubated human bronchial epithelial cells with 5 µmol/L all-*trans*-β-carotene in DMSO. After addition of the treatment solution to the cell culture medium, DMSO content was below 1%. Analytes were extracted by LLE from the medium and isocratically separated on a C18 column, with identification based on retention times and UV-VIS spectra. Beside all-*trans*-, 9-*cis*- and 13-*cis*-β-carotene were detected. The metabolites identified comprised β-apo-4´-, β-apo-8´-, and β-apo-12´-carotenal, 5,6-epoxy-β-carotene (Figure 10), as well as retinol and retinal (the latter two in all-*trans* form). After 24 h of incubation, the highest intracellular concentrations of β-carotene were detected with no essential increase over time. After 72 h, 10–15% of the initially applied amount of β-carotene was present. Both identified *cis*-isomers of β-carotene were assumed to have been formed intracellularly, which has also been presumed for the β-apocarotenals and the identified epoxy product. Authors stated a low-activity pathway for eccentric cleavage since the abundances of CPs were low. The time-course of the intracellular development of CP concentrations is also given [90]. The group of Keijer measured intracellular levels of β-carotene and related CPs in colorectal, intestinal, and lung carcinoma cell lines. Cell lines were exposed to 1 µmol/L β-carotene for a period between 2 h and 3 weeks. The intracellular β-carotene content increased over time. The final β-carotene concentrations were cell-line-specific, and varied between 1.5 and 54.7 µmol/L. Keijer and co-workers postulated an active uptake mechanism in cell lines which showed highest β-carotene concentrations. In addition to β-carotene, seven metabolites were detected including *cis*-carotene, retinol, three epoxy-β-carotenes, and two retinyl esters. The observed metabolite profile was cell-line-specific [91]. Immortalized human bronchial cells have been incubated with β-carotene under simultaneous gas phase treatment with cigarette smoke. Identified oxidation products of β-carotene comprised 5,6-epoxy-, 5,8-epoxy-β-carotene, β-apo-14´-, β-apo-12´-, β-apo-10´-carotenal, and 4-nitro-β-carotene. The latter was addressed as a unique marker for oxidation of β-carotene with cigarette smoke [96].

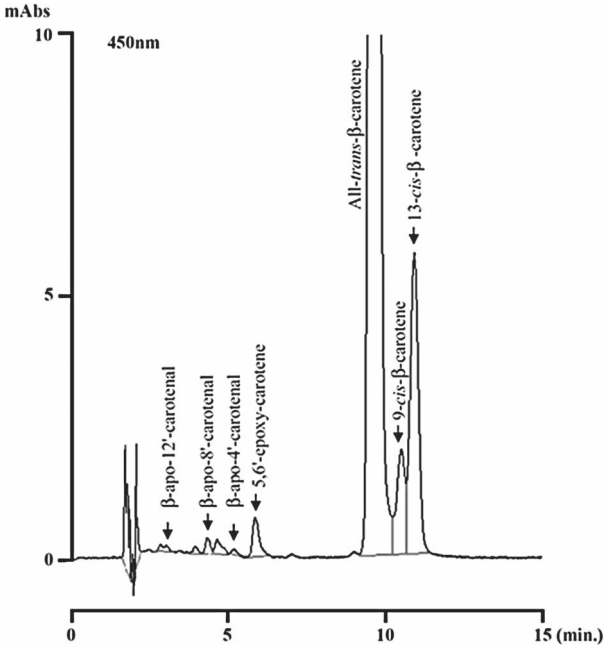


Figure 10. Representative chromatogram of the β-carotene isomers and metabolites analyzed in the cells by HPLC. Reprinted from [90], © 2005, with permission from Elsevier.

Stimulated leukocytes from human blood

Freshly prepared human PML (10×10^6 cells/mL) stimulated with phorbol myristate acid were incubated with 1 μmol/L β-carotene (for degradation) and 100 μmol/L–10 mmol/L β-carotene for identification, all in soybean oil. With 100 μmol/L β-carotene, mostly short-chain volatile CPs were identified but no β-apocarotenals. When 10 mmol/L β-carotene was applied, retinal, β-apo-8´-, and β-apo-12´-carotenal were present as well [29].

Serum, plasma, and blood

Van Breemen et al. analyzed retinoids, that is, retinol and retinyl palmitate, in human serum, after protein precipitation with ethanol and LLE with n-hexane. Retinol concentrations were between 1.04 and 1.56 mmol/L [143]. Barua and Olson determined α- and β-carotene, retinoic acid, retinol, and retinyl acetate in human serum [144]. Both crystalline 5,6-epoxy- and 5,8-epoxy-β-carotene dissolved in corn oil and soaked into bread were administered orally at 9.1 μmol to a human volunteer. Another volunteer consumed a ripe mango fruit known to contain 5,6-epoxy-β-carotene. The analysis of capillary plasma before and 3 h after oral application indicated 5,6-epoxy-β-carotene as the major carotenoid, with a concentration of up to 2.29 μmol/L. This corresponds to 61% of the orally supplemented dose. Additionally, retinyl palmitate was detected [97]. Olmedilla-Alonso et al. compared 166 pairs of serum and lithium-heparin plasma samples from healthy volunteers for comparability of determined concentrations of α- and β-carotene, and retinol [119]. Nakagawa et al. determined α- and β-carotene in human plasma and erythrocytes by RP-HPLC-DAD-APCI-MS after off-line SPE. This revealed an approximately 16-fold lower concentration in erythrocytes in comparison to plasma. Moreover, differences in carotenoid content of plasma and erythrocytes were determined for men and women [95]. Hosotani and Kitagawa measured β-carotene, retinol, and retinyl palmitate in human serum and rat liver [124]. Zeng et al. investigated the human carotenoid metabolism on male volunteers by orally providing a single dose of 100 μg 4,4´-dimethoxy-β-carotene, ethyl-β-apo-8´-carotenoate and β-apo-8´-carotenal in peanut oil, respectively. Blood was collected at defined time points. Concentration development and peak concentrations were compared. β-Apo-8´-carotenal was not determined in pronounced amounts, but rather β-apo-8´-carotenol and β-apo-8´-carotenyl palmitate. The authors also proposed a degradation pathway for β-apo-8´-carotenal [182].

Feces and urine

Zhu et al. performed a diet intervention study with pharmacological doses of $^{13}C_{10}$-β-carotene and $^{13}C_{10}$-β-retinyl palmitate targeting for a steady-state level in serum. Whereas $^{13}C_{10}$-β-carotene was detected in serum and feces, $^{13}C_{10}$-β-retinyl palmitate was only observed in serum [122]. ^{14}C-β-carotene was administered orally to a male volunteer by a single dose of 306 μg. Extraction of ^{14}C-β-carotene and radioactively labeled neutral and acidic CPs was done from plasma, feces, and urine, respectively. Neutral and acidic extractable compounds were distinguished by different sample extraction procedures. Both classes were fractionated by RP-HPLC using the isocratic mode with subsequent analyte determination by accelerator MS that can measure the ratios of $^{14}C/^{12}C$. Due to saponification, retinyl esters were transformed to retinol. Within the first 2 days after oral intake, 57.4% was excreted via feces, whereas 42.6% of ^{14}C-β-carotene was absorbed. Further cumulative losses from day 4 via urine and feces were 0.98 and 8.98% of the original dose, respectively [183].

Matrix effects

Matrix effects, that is, effects that entail different signal intensities and thus an altered slope of the calibration curve when comparing calibration data derived from model samples (calibration standards) with real samples, will impair accuracy and precision of quantitative data and detection sensitivity. An adverse, or more rarely, enhancing impact on the ionization efficiency in MS, that is, ion suppression or ion enhancement, represent causative reasons and will entail a reduction or increase in the signal intensity [113,184,185]. The degree of ion suppression (or enhancement) can thus differ between different biological materials, animal species, and biological lots, and might also be influenced by conditions and duration of storage. Thereby, absolute and relative matrix effects have been distinguished [184]. The exact mechanism of matrix effects in MS is still disputed. Most likely, matrix compounds coexisting with analytes affect the ionization and spray processes. In the latter case, the fission process forming continuously smaller droplets might be hampered by matrix compounds that influence pH, electrolyte concentration, and solvent composition of the sprayed solution and thus of ES droplets [185,186]. Processes that are likely to prevent analytes from forming either an individual ion or a charged cluster in the gas phase are discussed elsewhere [186].

The immanent importance of matrix effects has also been raised in a "Guidance for Industry", addressing method validation issued by the FDA and related organizations. Therein, a method validation based on the relevant biological matrix (or matrices) is suggested [187]. Moreover, matrix effects are not only related to the original sample but depend also on the sample preparation and the selected ionization technique and thus the ES-interface applied in MS [184,185]. Matuszewski et al. [184] described a validation strategy where (i) neat solvents were spiked, or the sample matrix was fortified with analytes (ii) after or (iii) before the sample extraction procedure. An assessment of the respective signals allowed for a differentiation between matrix effect, recovery, and "process efficiency". A comparison of signals derived from (ii) and (i) provides the absolute matrix effect. In case of a

direct comparison of signals that are derived from different matrix batches spiked with analytes subsequent to the extraction, relative matrix effects can be addressed. The inclusion of an internal standard (IS) can compensate for this effect as long as the ratio between IS and analyte signal is maintained. The same authors have also addressed a comparison of slopes of the calibration curves but have not provided the statistical evaluation procedure [184]. In our recent paper, a statistical evaluation of calibration slopes acquired for volatile CPs of β-carotene from model solutions and spiked cell culture medium was done on a significance level of 5% [111]. Some authors have proposed replacing plasma with phosphate buffered saline (PBS) either alone or in combination with 5% serum albumin, to mimic plasma matrix during calibration [135].

Internal standards

IS compounds are added to standard solutions and samples for reasons of correction in the quantification step. As given elsewhere, an IS has to meet several requirements in order to execute its task appropriately [82]. Different compounds have been used as IS when analyzing β-carotene and its related CPs, including methylisoeugenol [35,111] and linalool [111], both preferably for volatile CPs, whereas echenelon [95,120,145], squalene [88], and also β-apo-8´-carotenal have been applied for non-volatile CPs [42]. In the latter case, it has to be assured that this compound is not among the generated CPs. In addition, isotope-labeled $^{13}C_6$-β-carotene was employed as IS [122]. Moreover, deuterated compounds, for example, 13-*cis*-retinoic acid-d_5, 4-oxo-13-*cis*-retinoic acid-d_3, and all-*trans*-retinol-d_6 have been applied as IS when analyzing retinoic acid and related isomers [113,149]. Retinyl acetate has been used as IS as well, but its absence in the sample has to be confirmed [97,119,143,144,188]. Moreover, retinal-O-ethyloxime and β-apo-12´-carotenal-O-t-butyloxime have been employed as IS for retinol and β-carotene [189]. Acitretin was applied as IS for retinoic acids and related 4-oxo metabolites [135].

Method validation and analyte quantification

The ICH guideline Q2(R1) [190], an IUPAC guideline [191], as well as an industrial guideline [187] outline the parameters that should be addressed during method validation (mostly for single laboratory validation). Relevant validation issues comprise trueness, precision (including repeatability and intermediate precision, and in some cases even reproducibility), specificity or selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, sensitivity, range, and robustness (sometimes also stated as ruggedness). Furthermore, recovery and stability testing have been listed [187]. All cited guidelines also include suggestions on how testing should be executed practically, and provide equations for calculation of validation parameters [187,190,191]. Moreover, method testing can comprise full validation and partial validation. The latter has to be executed if, for instance, changes in instruments, sample processing, matrix, or method transfer has been done [187]. In addition, calculation of measurement uncertainty is briefly addressed as well [191], with a comprehensive introduction given elsewhere [192].

In the context of estimation of method trueness, usually stated as bias, and thus also for quantification, strictly speaking, an accepted reference value is required. Ideally, trueness should be derived from certified reference materials (CRMs) that are traceable to international standards and provide an (expanded) combined standard uncertainty [191,192]. However, disposability of CRMs is limited and mostly restricted to selected analytes and/or analyte combinations available in defined matrices. Recently, the National Institute of Standard and Technology (NIST) has – together with other institutions – prepared and characterized standard reference material (SRM) 3276 delivering carrot extract in oil that provides, *inter alia*, certified values for *trans*-α-, *trans*-β-, and total β-carotene [84], including expanded uncertainties. The SRM also contained BHT for stability improvement of the analytes [84]. Variations in the matrix composition and their effects on method validation should be given particular attention and be addressed separately [191]. Details have been outlined in a previous section. However, with a few exceptions [111,116,184], this has mostly been disregarded.

Publications mostly cover selected validation aspects, such as precision of retention times, peak areas and recoveries, LOD, LOQ, or working range. Some selected cases are discussed exemplarily in this paragraph. For method validation, Breithaupt included LOD, LOQ, calibration based on peak areas (covering 0.5–25 mg/L), and recovery based on LLE, that is, $>97.9\%$, for β-carotene, β-apo-8´-carotenal, and β-apo-8´-carotenoic acid ethyl ester [120]. On-line SPE coupled with RP-HPLC allowed for recoveries between 92.1% and 93.5% for 13-*cis*-retinal, all-*trans*-retinal, and related 4-oxo metabolites, with a precision of 0.9–8.7% ($n = 6$). Inter-assay accuracy was between 92.5% and 96.7%. Authors tested a working range of 0.3–100 ng/mL and also the stability of analytes in plasma under different storage conditions. Whereas analyte content decreased over 9 months at $-20°C$, this was not the case at $-80°C$. Human plasma samples could even be stored for 2 years under these conditions [135]. Hosotani and Kitagawa [124] validated a RP-HPLC-UV-Vis method for determination of β-carotene, retinol, and retinyl palmitate in human serum and rat liver tissue. Recovery of β-carotene was from $99.7 \pm 3.0\%$ to $107.1 \pm 6.2\%$, whereas retinol and retinyl palmitate were recovered at $92.8 \pm 7.8\%$ and $98.8 \pm 7.3\%$ (both as retinol, due to sample saponification), with $n = 6$ in both cases. Precision of determined analyte concentrations was from 4.7% to 7.1% for intra-day, and from 2.7% to 6.4% for inter-day assay [124].

However, full validation of analytical methods applied for the identification and quantification of β-carotene and CPs are rare, not to mention comprehensive and correct statistical evaluations therein. Paliakov et al. [148] validated their UHPLC-UV method by determining accuracy, preci-

sion, linearity, LOD, LOQ, sample stability, and recovery. Moreover, they also evaluated matrix effects by comparing slopes of calibration curves. However, validation addressed β-carotene but not related CPs. Van Meulebroek et al. [89] presented a fully validated UHPLC-APCI-Orbitrap (Exactive) MS method for analysis of carotenoids in tomatoes. Data were compared to UHPLC-DAD-MS/MS results acquired with a TSQ triple-quadrupole MS. The Orbitrap Exactive MS instrument was operated at a resolution of 100 000 (full width at half-maximum, FWHM) and used a high energy collision dissociation (HCD) cell at 40 eV for fragmentation. However, HCD was not applied during actual measurements. The system provided high mass resolution and improved mass accuracy. The selected mass extraction window was < 5 ppm, since mass deviation was < 1.5 ppm for β-carotene. Precision of retention times in UHPLC hyphenated to APCI-Orbitrap MS or UV-Vis-MS/MS was $< 0.58\%$. The selectivity/specificity of the Orbitrap MS approach surpassed that of UV-Vis and triple-quadrupole MS. All three methodic approaches were validated and compared in terms of LOD and LOQ (based on six-point calibration curves) and linearity was determined between 0.5 and 5.0 ng/μL in case of β-carotene. The coefficient of determination r^2 served apparently as an indicator of linearity. Due to the previously addressed absence of appropriate CRMs, trueness and recovery were derived from spiking experiments, applying 1.00, 2.00, and 3.00 ng/μL for either method [89].

Contrary to all other validation approaches published so far in the context of β-carotene and CPs, our groups have jointly published two comprehensive papers providing full validation of a GC-EI-MS method for volatile CPs and an UHPLC-DAD-ESI-Orbitrap MS method for non-volatile CPs and β-carotene, with preceding off-line SPE for all analytes [111,116]. The GC-EI-MS method covered β-CC, β-IO, DHA, and 1,1,6-trimethyltetraline. Validation covered instrumental aspects including repeatability and inter-day precision of retention times and IS-corrected peak areas. Instrumental linearity testing covered 0.5–4.5 μg/mL and 1.0–50 μg/mL for all analytes. Homoscedasticity was statistically confirmed at 1.0 μg/mL and 50 μg/mL. LOD and LOQ calculations were based on calibration curves [111]. Contrary to most publications, linearity was not evaluated by the erroneously used coefficient of determination that only provides information about the correlation between concentration and the measured signal [193]. Instead, the Mandel fitting test was used for statistical evaluation [111,194]. Additionally, intra-day and inter-day precision as well as linearity, LOD, and LOQ were also determined with spiked culture medium for the entire analysis method including preceding off-line SPE. Moreover, specificity was determined by extracting a blank matrix after incubation with primary target cells, all with concomitant statistical evaluation. Slopes for (i) instrumental and (ii) SPE-implementing calibration curves, were compared for significance of differences [111].

An equally comprehensive validation concept has been pursued for the UHPLC-DAD-ESI-Orbitrap MS approach, addressing β-carotene and non-volatile CPs. Again, (i) instrumental validation of UHPLC-DAD and UHPLC-ESI-Orbitrap MS alone as well as (ii) in combination with off-line SPE, were performed. Linearity testing comprised a range of 0.025–5.00 μg/mL, tested either entirely or subdivided into three concentration domains. Besides the Mandel fitting test, significance testing of the intercept as well as homoscedasticity testing were performed. Moreover, relevant parameters, including specificity, were additionally determined for off-line SPE-UHPLC-DAD-ESI-Orbitrap MS. Inter-day recoveries were compared by one-way ANOVA. SPE recoveries were additionally crosschecked after addition of 10% (v/v) fetal calf serum, thus modifying the matrix [116].

Conclusions

The pro-oxidative and carcinogenic effects that have been encountered for some proband groups within two comprehensive intervention trials with β-carotene have fostered the interest in revealing the causative rationale. Thereby, cleavage products (CPs) of β-carotene have moved into scientific focus. The *in vivo* situation in humans has been mimicked by *in vitro* and animal models. In either case, analytical tools are indispensable in revealing generated CPs, for their unambiguous identification as well as for their quantification in biological matrices. Moreover, this also requires the characterization of standard and treatment solutions to determine the analyte content, and the stability and batch consistency of these solutions. Analytical methods that mostly miss provision of mass information, preferably with high accuracy, fail to confirm compound identity unequivocally. This refers to chromatographic methods combined with UV detection. UV spectra represent at best a co-decisive parameter for analyte identification. Ideally, mass information derived from MS measurements should be complemented by the fragmentation pattern achieved by MS^2 or MS^n, and the isotope pattern. Together with retention times and UV spectra orthogonal property arrays become disposable, allowing an enhanced validity of identification.

Besides identification, quantification is of equal relevance. Strictly speaking, this requires the availability of CRMs covering individual target matrices and including β-carotene and relevant CPs. CRMs provide a certified reference value with a related combined standard uncertainty for either analyte. Polemically stated, the restricted availability of CRM matrices for β-carotene (not even addressing CPs) can currently be considered as one of the major limitations. Since biological hypotheses have to be tested statistically, analytical results form their scientific backbone. Inappropriate and non-validated methods will result in misleading conclusions and wrong explanation models. Moreover, the analytical decision tree is foliated with a plethora of pitfalls. Thus, an appropriate analytical approach has to consider (i) preparation, stability, and

quality control of treatment and standard solutions, (ii) sample preparation and extraction, (iii) selection of adequate separation methods and detection principles, (iv) full-method validation with comprehensive statistical evaluation, as well as (v) related quantification aspects (e.g., matrix effects, related bias, and uncertainties). Generally, the selected analytical pathway might already predetermine the analyte portfolio that can be addressed. In this context, a selected sample preparation and extraction strategy might fail, for instance, in picking all relevant analytes, particularly low-abundance species. The situation might be aggravated by limitations of method sensitivity. The physicochemical properties of the target compounds determine the pre-concentration strategy and applicable separation strategy. β-Carotene and long-chain CPs are mostly separated by RP-HPLC, preferably employing C18 columns. In case a differentiation between *cis*- and *trans*-isomers is requested, C30 columns are applied. Due to its favorable separation performance, UHPLC can be considered a prospective ascending technique. Detection is mostly done with DAD and APCI-MS or ESI-MS. Due to their volatility, short-chain CPs are analyzed with GC-MS and EI ionization preferentially.

Moreover, the limited number of commercial standards imposes analytical constraints. Reinforced efforts in broadening the availability of commercial standard compounds in defined quality, together with instrumental and methodological improvements, prepare the ground for deeper insights in the β-carotene paradox. Consequently, this calls for an interdisciplinary alliance, for example, between analytical chemistry, cell biology, physiology, and pharmacology. This will entail progressive amendments in either field, fostered by the interactive cooperation.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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