

Review

An Updated Overview on the Use of the β -Carotene Bleaching Method in Assessing the Antioxidant Activity of Compounds

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Abstract

Compounds with antioxidant properties have recently gained popularity. Globally, in parallel with the search for new sources of antioxidants, research is being conducted on methods for assessing antioxidant properties. The aim of this review article is to systematize and update knowledge about one of the most popular methods for testing antioxidant properties—the β -carotene bleaching method. This article presents the most important information regarding this method. It discusses, among other things, the basic reaction mechanism used to assess antioxidant properties, the properties of the model antioxidant— β -carotene, the measurement procedure and reagent preparation, and, importantly, factors that the analyst should consider when interpreting the final result. Furthermore, the article reviews applications of the β -carotene method. The information presented should be helpful in obtaining consistent and reliable results using this method and contribute to the standardization of antioxidant testing methods.

Keywords: β -carotene bleaching assay; antioxidant properties; hydrogen atom transfer methods; parameters differentiating antioxidant activity



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1. Introduction

Compounds exhibiting antioxidant properties are very popular in both the food and cosmetics industries, as well as in pharmaceutical sectors. This popularity stems from their ability to delay or inhibit the production of free radicals, capture free radicals, convert free radicals into less toxic compounds, delay the formation of secondary toxic active species, interrupt the chain propagation reaction (chain-breaking antioxidants), protect other molecules against oxidation, and strengthen the endogenous antioxidant defense system through synergy with other antioxidants and chelate metal ions [1]. However, it is important to remember that the effectiveness of antioxidant research depends on the use of reliable methods for assessing their properties.

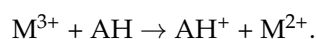
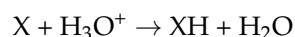
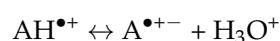
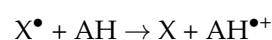
Many methods have been developed for assessing the antioxidant potential of individual compounds and their mixtures, but there is no universal method that provides unambiguous results. Each method has its own unique mode of action, each with its advantages and disadvantages. Therefore, when determining antioxidant properties, several methods should be used. Generally, these methods can be divided into primary (additive) and secondary (post-additive). In additive methods, the oxidation process occurring in the tested sample is retarded by the antioxidants present. In post-addition methods, the amount of antioxidant present is determined after a certain time has passed since the reaction with the test compound. This amount is inversely proportional to its activity.

In another perspective on the division of methods for assessing antioxidant properties, it should be noted that spectrophotometric methods are particularly popular. In these methods, antioxidants neutralize the radicals responsible for harmful oxidation processes through two main reaction mechanisms [2–5]:

1. Single Electron Transfer, Abbreviated as SET;
2. Hydrogen Atom Transfer, HAT.

Both mechanisms produce the same final product, but the kinetics and potential for side reactions are differed. Furthermore, both mechanisms can occur simultaneously, but one always dominates. This predominance is determined, among other factors, by the antioxidant's properties, solubility in the solvent used for testing, the partition coefficient, and the solvent system used. It should be added that the effectiveness of an antioxidant and its main reaction mechanism are primarily determined by two parameters: bond dissociation energy (BDE) and ionization potential (IP).

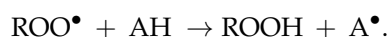
SET-based methods measure the ability of a potential antioxidant to transfer a single electron, leading to the reduction in various compounds, including metals, carbonyl groups, and radicals. The idea of the SET mechanism is described by the following general reactions, where $X^\bullet$ —radical, AH—antioxidant being a single electron donor, M—metal ions [6,7]:



SET reactions are observed by a color change in the reaction mixture containing the oxidant and antioxidant. The most commonly used oxidants include 2,2-diphenyl-1-picrylhydrazyl radical (DPPH $^\bullet$) in the DPPH method and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical (ABTS $^{\bullet+}$) in the ABTS method. The former is one of the most commonly used methods. According to the Scopus database, as of November 14, nearly 36,000 articles were indexed, in which the terms "DPPH method" and "antioxidant activity" appear simultaneously in the publication title, the publication body or are indicated by the author(s) as keywords. It is worth noting that the DPPH radical is ready-to-use (it does not require prior preparation as in the ABTS method) and is characterized by relatively high stability. Alcoholic solutions of this reagent are typically used, but the radical should be used in excess of the antioxidant to ensure the full amount of antioxidant reacts. During the reaction, the color of the solution changes from purple to light yellow, that is monitored spectrophotometrically in the wavelength range (λ) of 515–540 nm. Exposure to light and oxygen should be limited during the measurement. Furthermore, the pH and solvent should be carefully selected, as they can reduce the absorption of the radical solution [6–8]. In the second method (according to the Scopus database, nearly 11,000 publications were indexed in the same period, where the terms "ABTS method" and "antioxidant activity" appear simultaneously in the title, in the body of the publication, or are indicated by the author(s) as keywords), the ABTS cation radical (ABTS $^{\bullet+}$) can be generated in chemical reactions (e.g., with $K_2S_2O_8$ or MnO_2) or enzymatic reactions (e.g., with horseradish peroxidase or myoglobin). It should be emphasized that chemical methods are more demanding due to the long reaction time. During the reaction, the blue-green color of the solution disappears, and its intensity decreases proportionally to the antioxidant content in the solution. Radical scavenging is usually monitored at 734 nm [6–8]. Without going into detail about other methods for testing antioxidant properties based on the SET mechanism, it is worth mentioning their names [7]: the ferric

ion reducing antioxidant parameter (FRAP) method (using similar search criteria, the full text of the method was used instead of the abbreviation, approximately 160 publications were indexed in Scopus database), the cupric ion reducing antioxidant capacity (CUPRAC) method (301 publications were indexed), and the dimethyl-p-phenylenediamine dihydrochloride (DMPD) cation radical method (Scopus indexed 16 publications from 2006 to 2024).

HAT-based methods assess the neutralization capacity of an antioxidant through hydrogen donation. The relative reactivity of an antioxidant in these methods is determined by the bond dissociation energy of the hydrogen-containing group. It should be noted that the presence of reducing agents, including metals, can complicate HAT assays and lead to falsely high apparent reactivity [8]. The principle of HAT-based methods involves monitoring the kinetics of competitive reactions between the tested antioxidant(s) and a model antioxidant, which compete with each other in their reaction with the peroxy radical. In the literature, the model antioxidant (e.g., fluorescent protein, β -carotene) is called a “molecular probe” or “protected molecule”, and its amount in the tested system is precisely defined [6,9,10]. These reactions are monitored in two measurement systems. One, called the “control system”, contains only the model antioxidant (molecular probe) and the neutralized peroxy radical. The second system contains, in addition to the above-mentioned components, also the tested antioxidant(s). The HAT mechanisms in these methods proceed according to the general equation, where $\text{ROO}^\bullet$ —peroxy radical, AH —antioxidant being a hydrogen atom donor [6]:



Methods involving the HAT mechanism include [6,11,12]

- Oxygen radical absorbance capacity (ORAC);
- Total radical trapping antioxidant parameter (TRAP);
- Inhibition of induced low density lipoproteins (LDL) oxidation;
- Total oxyradical scavenging capacity assay (TOSCA);
- Crocin bleaching assay;
- Chemiluminescent assay;
- Hydroxyl radical antioxidant capacity (HORAC) and the title method;
- β -carotene bleaching assay.

Figure 1 summarizes the number of publications indexed in the Scopus database regarding the use of HAT-based methods. The data presented refer to publications in which the full names of the techniques (not abbreviations) appear in the publication title, in the body of the publication, or are indicated by the author(s) as a keyword (as of 14 November 2025).

The main aim of this review is to take a closer look at one of the oldest (the first mention of this method comes from Marco in 1968 [13]) and, at the same time, the most popular methods (compare the heights of the bars in Figure 1) for testing antioxidant properties based on the HAT mechanism—the β -carotene bleaching method (abbreviated as the β -carotene method). As mentioned earlier, the most popular and most commonly used methods include the DPPH and ABTS methods, respectively. Like the β -carotene method, these methods are spectrophotometric, with the difference that they are based on the SET mechanism. Numerous review articles have been devoted to both methods. However, a comprehensive study compiling information on the β -carotene method is lacking, hence the idea for this study.

This literature review was conducted by systematically collecting, reviewing, and collating information (with a significant share of recent papers) from available online databases, such as Google Scholar, Scopus, Web of Science, PubMed, and Science Direct.

The search was limited to English. Article abstracts were pre-screened prior to full-text analysis. The collected information was analyzed in detail to ensure that this article serves as a compendium of general knowledge about the β -carotene bleaching method, its implementation procedure, factors to consider during its implementation, and examples of its application.

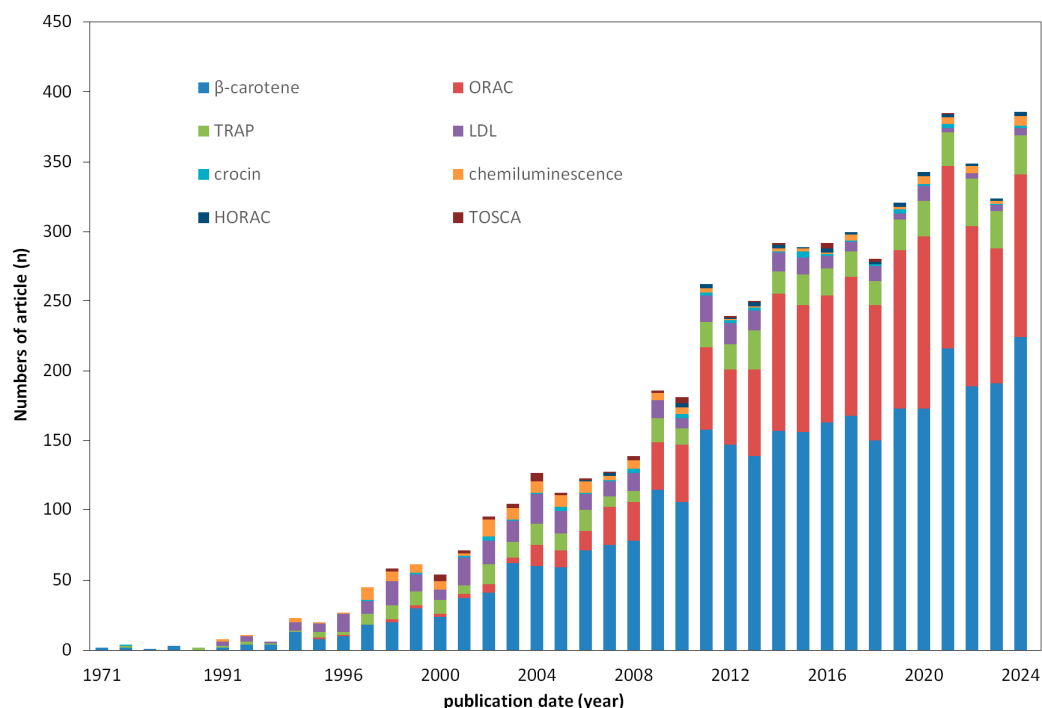


Figure 1. Scopus database search results showing the number of articles published over the years (up to 2024) regarding the use of HAT-based methods for testing antioxidant properties.

2. β -Carotene as Molecular Probe in β -Carotene Bleaching Assay

β -carotene, the 3D structure of which is shown in Figure 2, is a compound classified as a carotenoid, a naturally occurring pigment produced by photosynthetic organisms to protect against photodynamic damage [14]. In addition to plants, β -carotene is produced by certain microorganisms: fungi, microalgae, and bacteria [15]. Popular fungal strains include *Blakeslea trispora* and *Rhodotorula glutinis*, while microalgae such as *Dunaliella salina* are characterized by high beta-carotene production. Bacteria such as *Erwinia uredovora*, *Pantoea agglomerans*, and some strains of *Serratia marcescens* can also produce it.

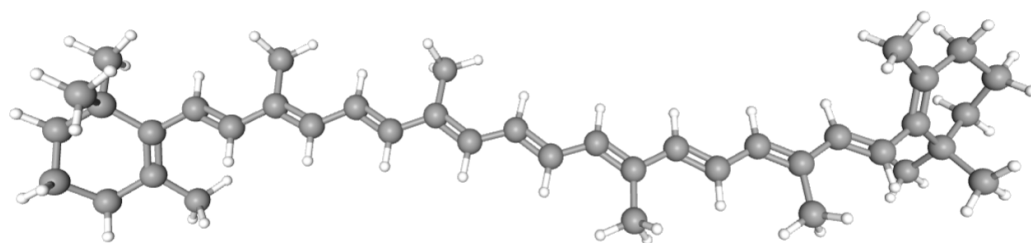


Figure 2. Three-dimensional model of the chemical structure of the β -carotene conformer (IUPAC name: 1,3,3-trimethyl-2-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)-3,7,12,16-tetramethyl-18-(2,6,6-trimethylcyclohexen-1-yl)octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]cyclohexene) [16].

β -Carotene: naturally occurs as the *trans* isomer. Yet, under the influence of light (especially UV radiation), heat, and the presence of enzymes, one of the double bonds can isomerize to the *cis* position, which causes a change in the molecular structure. Both

the *cis* and *trans* forms of β -carotene absorb light, but their absorption spectra differ due to differences in molecular structure [17,18]. The *trans* form is characterized by a strong yellow-orange color (absorption maximum 450–470 nm). The *cis* form shows a distinct additional peak at a shorter wavelength (around 330–340 nm). Additionally, the *cis* form is characterized by lower color intensity than *trans*- β -carotene due to the lower extinction coefficient.

This carotenoid is capable of quenching singlet oxygen. It is also characterized by its ability to be easily oxidized by free radicals. This is due to the long chain of conjugated double bonds present in the molecule, which give it an intense orange color (see Figure 2) [19,20]. These double bonds are susceptible to attack by free radicals. In a methodological context, its antioxidant activity can be easily monitored spectrophotometrically [21–23]. When β -carotene reacts with free radicals, the conjugated double bond system is broken, causing the molecule to lose color. This “bleaching” is a visual indicator of the consumption of β -carotene in the reaction with free radicals. It also allows researchers to effectively assess the antioxidant activity of the tested antioxidant(s) [21–23]. The extent to which a substance can inhibit β -carotene fading is directly related to its antioxidant activity. The weaker the bleaching, the stronger the antioxidant properties. Finally, the use of β -carotene as a molecular probe in antioxidant studies is supported by both economic (it is relatively cheap and readily available) and ecological considerations, as it is considered a substance generally recognized as safe (GRAS) [21–23]. Moreover, it can be used with equal effectiveness both in studies of processes occurring in living organisms, such as humans, animals, or plants (*in vivo*), and those conducted under controlled laboratory conditions (*in vitro*) [24,25]. However, it should be noted, as emphasized in many publications, that β -carotene is neither a preventive antioxidant nor a conventional chain reaction-terminating antioxidant. Its antioxidant activity is associated with low oxygen partial pressure, whereas at high oxygen partial pressure it acts as a pro-oxidant [26].

3. Principle of the Method

The main principle of the method is to monitor two competitive reactions in which β -carotene, as a model antioxidant, and a test antioxidant compete for the peroxide radical [27]. As a result, the antioxidant properties of the tested sample (containing β -carotene, the tested antioxidant(s), and peroxy radicals) are always assessed against a control sample (containing β -carotene and peroxy radicals but not the tested antioxidant). In both cases, changes in β -carotene absorbance value over time are monitored, and, as already noted, the color changes result from the rupture of the π -coupling by the addition reaction of radicals to the C=C bond of β -carotene [28]. The smaller the color change in the tested sample compared to the control system, the stronger its antioxidant activity. In other words, the tested antioxidant strongly protects the reference molecule, β -carotene, against oxidation (degradation).

β -carotene is a weak antioxidant; therefore, when other antioxidants are present in solution, they react first protecting the β -carotene structure by intercepting the attack of reactive oxygen species before they can attack and destroy the β -carotene. Only when these are depleted, and peroxy radicals are still present in the measurement system, does β -carotene take over the antioxidant function. In this case, depending on the antioxidant's potency, its consumption over time is lower (higher) than in the control sample, corresponding to the stronger (weaker) antioxidant—compare the positions of the curves in Figure 3 [29].

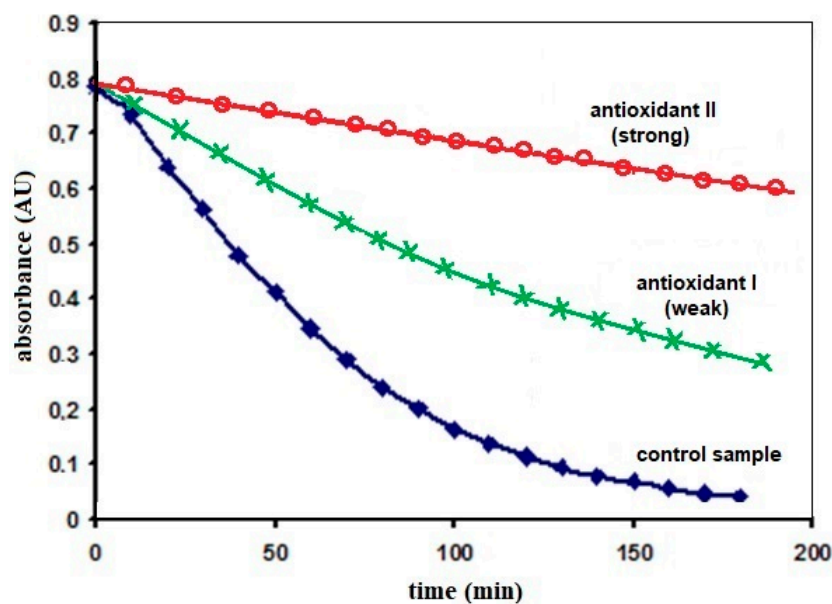


Figure 3. Examples of possible changes in β -carotene absorbance values over time, monitored in a sample containing the model antioxidant and a neutralized peroxide radical (control sample, blue line) and test samples (in addition to the above-mentioned components, also containing the tested antioxidant) with weaker (green line) and stronger (green line) (red curve) antioxidant properties (based on the results of our own research, detailed description—see text above).

Considering the possible transformation of *trans*- β -carotene to the *cis*- form during the reaction, it should be added that the observed in this method degradation of β -carotene in both the control sample and the test sample may be additionally related not only to the process of β -carotene oxidation by linoleic acid but may also result from the above-mentioned isomerization, which results in a color change in the emulsion. It should be noted here that in both the control and test samples, the changes in the β -carotene structure caused by isomerization should occur to the same extent. This is related to the fact that both samples are heated in the same way (45 °C) for the same period of time.

Figure 4 illustrates the processes involved in the color change in β -carotene. The discoloration of the orange–yellow or dark yellow β -carotene solution is caused by radicals (lipids or lipid peroxide radicals) generated during the immediate oxidation of fatty acids present in an aqueous emulsion of linoleic acid and β -carotene (a lipophilic oxidizable substrate) (part A) [30,31]. Once formed, lipids or lipid peroxide radicals attack β -carotene (part B), causing discoloration. This discoloration is caused by the “loss” of a conjugated double bond system through an addition reaction of the radicals to the C=C bond or by the substitution of a radical to the β -ionone ring located at each end of the long chain of 11 conjugated double bonds in the β -carotene molecule [32,33]. The reaction results in the formation of radical adducts, examples of which are provided in the figure. Each of these adducts is a colorless form stabilized by resonance with electrons located on the carbon atom. However, it should be noted that the exact position of the electron (its delocalization in the resulting radical structure) is unknown (hence, the drawing shows intermediate bonds instead of double bonds, indicating charge dispersion). The radical can be located on any secondary C in the conjugated system. Undoubtedly, as a result of adduct formation, the concentration of β -carotene in the test solution (referred to as the control sample) decreases. In the presence of the antioxidant, β -carotene competes for the formation of the mentioned adducts [24,28].

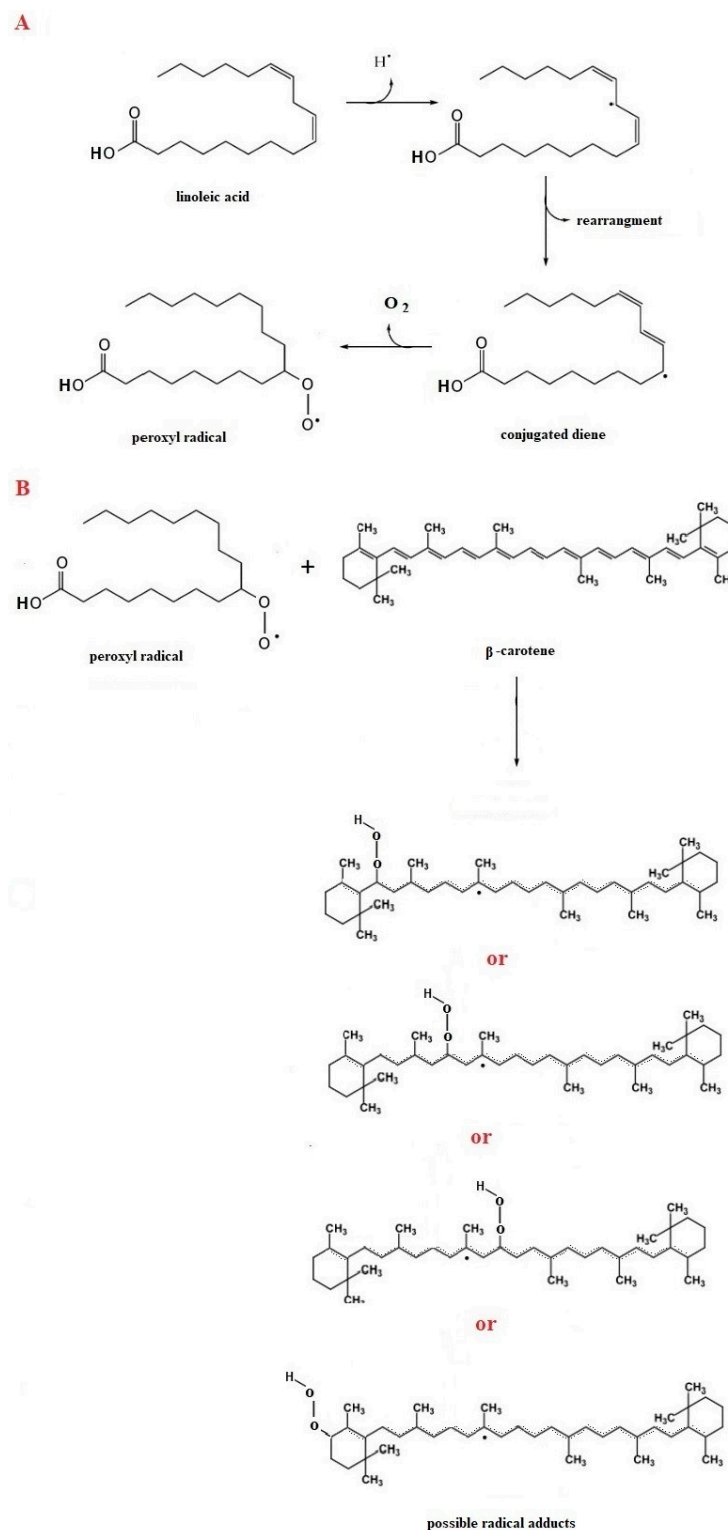
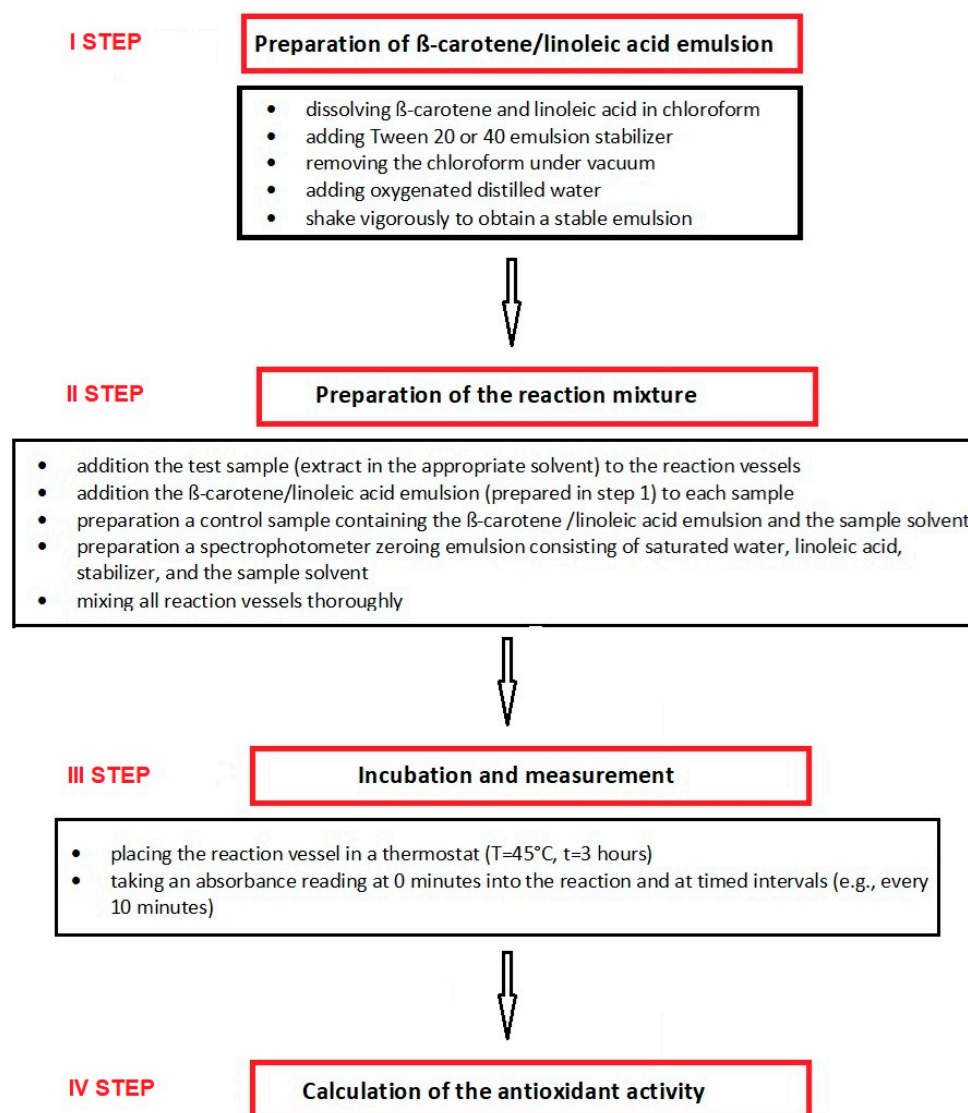


Figure 4. Reactions showing the oxidation of linoleic acid (**A**) and the possible formation of various radical adducts between β -carotene and a peroxy radical (**B**) [30–33].

4. Procedure

The β -carotene bleaching method was first proposed by Marco (1968) [13] to prevent autooxidation of emulsified linoleic acid in extracts. Over time, the technique has undergone several modifications aimed at facilitating its application (simplified the operation) [34] or converting it to microplates form [35]. Regardless of the modification, the β -carotene bleaching method is carried out in several stages, which are presented in Scheme 1 [36].

The most important step is the preparation of the test reagent (See Step I in the scheme). For this purpose, a chloroform solution of β -carotene is prepared; the chloroform is evaporated under reduced pressure in the presence of linoleic acid and Tween (an emulsion stabilizer). During this time, the water is saturated with oxygen (usually 100 mL of water is saturated with oxygen for 30 min at a controlled flow of 100 mL/min), because the presence of oxygen and the elevated measurement temperature favor the oxidation of linoleic acid and the production of peroxide radicals. The resulting radical can either bleach the β -carotene or abstract a proton from the radical scavenger (antioxidant) [37]. Finally, the residue left after evaporation of the chloroform is gently combined with oxygenated water.



Scheme 1. Stages for testing the antioxidant properties of solutions using the β -carotene bleaching method.

It should be noted that there is no standardization of antioxidant testing methods, including the β -carotene method. This means that studies using the same method are conducted under various, sometimes significantly different, conditions (e.g., different reaction monitoring times and temperatures). These factors, along with the different ways of presenting the obtained results, make direct comparisons difficult. Table 1 summarizes examples from the literature illustrating how measurements are performed and results interpreted using the β -carotene bleaching method. Table 2, in turn, proposes the optimal conditions for the procedure, according to the authors.

Table 1. Literature examples of measurement conditions and results presentation for the β -carotene bleaching method.

Reagents	Sample Type	Sample Preparation	Volume of Emulsion [mL]/Volume of Sample [mL] (Emulsion to Sample Ratio)	Measurement Conditions	Manner of Expressing Results	Ref.
1 mL of β-carotene solution (c = 0.2 mg/mL in CHCl_3) 0.02 mL of linoleic acid 0.2 mL of Tween 20 100 mL of distilled H_2O 0.2 mL of sample (c = 1 mg/mL in 70% EtOH *)	Vegetables	- Vegetables were cleaned, washed and boiled in H_2O. - Vegetables were air-dried under the fan and cut into small pieces and homogenized - Extraction with 70% EtOH.	5:0.2 (25:1)	T = 45 °C λ = 470 nm t = 2 h	Equation (3)	[37]
1 mL of β-carotene solution (c = 0.5 mg/mL in CHCl_3) 0.025 mL of linoleic acid 0.2 g of Tween 40 100 mL of distilled H_2O (saturated with O_2—30 min, 100 mL/min) 0.35 mL of sample (c = 2 mg/mL in MeOH *)	Herbs	- Extraction of the dried material with hexane, then chloroform, and then methanol in the Soxhlet apparatus (6 h for each solvent). - Suspension of the methanol extract in CHCl_3 to obtaining water-soluble and water-insoluble subfractions. - Lyophilization of the obtained extracts.	2.5:0.35 (7:1)	T = 25 °C λ = 490 nm t = 48 h	Absorbance vs. time	[38]
1 mL of β-carotene solution (c = 0.5 mg/mL in CHCl_3) 0.025 mL of linoleic acid 200 mg of Tween 40 100 mL of distilled H_2O (saturated with O_2—30 min, 100 mL/min) 0.3 mL of sample (dissolved of residual after evaporation in water or EtOH * or DMSO *)	Herbs	- Herbs were dried and ground. - Hydrodistillation to obtain essential oil. - Extraction of plant material with ethanol. - Extraction of plant material with water (boiling for 20 min). - Filtration of the extracts. - Evaporation to dryness.	2.5:0.3 (8.3:1)	T = 50 °C λ = 470 nm t = 2 h	Equation (4)	[39]
1 mL of β-carotene solution (c = 0.2 mg/mL in CHCl_3) 20 mg of linoleic acid 200 mg of Tween 40 50 mL of aerated distilled H_2O 0.2 mL of sample about different concentrations (dissolved in MeOH *)	Herbs	- Steam distillation of herb to obtain a essential oils. - An aliquot of the sample was mixed with ethanol.	5:0.2 (25:1)	T = 50 °C λ = 470 nm t = 2 h	Equations (1) and (2)	[40]

Table 1. Cont.

Reagents	Sample Type	Sample Preparation	Volume of Emulsion [mL]/Volume of Sample [mL] (Emulsion to Sample Ratio)	Measurement Conditions	Manner of Expressing Results	Ref.
1 mL of β-carotene solution (c = 0.5 mg/mL in CHCl_3) 0.025 mL of linoleic acid 200 mg of Tween 40 100 mL of distilled H_2O (saturated with O_2—30 min, 100 mL/min) 0.35 mL of BHT (c = 0.005 mg/mL in MeOH *)	Standard antioxidant (BHT)	Dissolving the appropriate amount of antioxidant in methanol.	2.5:0.35 (7.1:1)	T = 45 °C λ = 470 nm t = 3 h	Equations (1) and (2)	[41]
1 mL of β-carotene solution (c = 0.2 mg/mL in CHCl_3) 0.045 mL of linoleic acid 400 mg of Tween 40 100 mL of aerated distilled H_2O 0.5 mL of sample (c = 0.00625–0.8 mg/mL in 90% EtOH *)	Herbs	- Herbs were dried and ground. - Extraction of plant material with 90% EtOH *. - Filtration and concentration of extract under reduced pressure at 35 °C.	4.5:0.5 (9:1)	T = 40 °C λ = 470 nm t = 2 h	Equation (4)	[42]
1 mL of β-carotene solution (c = 0.5 mg/mL in CHCl_3) 0.025 mL of linoleic acid 0.2 mL of Tween 40 50 mL of hydrogen peroxide (H_2O_2) 0.04 mL of sample (about different concentrations in H_2O)	Herbs (medicinal plants)	- The ground, dried herb was mixed with boiling distilled water and boiled for 20 min. - Filtration of the mixture. - Drying the mixture at 45 °C.	0.16:0.04 (4:1)	T = 50 °C λ = 470 nm t = 2 h	Equations (1) and (2)	[43]
2 mL of β-carotene solution (c = 1 mg/mL in CHCl_3) 40 mg of linoleic acid 400 mg of Tween 40 100 mL of aerated distilled H_2O 0.1 mL of sample (0.003–0.05 mg/mL in EtOH *)	Pigments	- Astaxanthin extraction from shrimp shells using EtOH * using a laboratory blender. - The shrimp shell residue was vacuum filtered and evaporated under vacuum at 40 °C using a rotary evaporator. - The resulting concentrate was stored at −20 °C until further analysis.	0.1:0.1 (1:1)	T = 50 °C λ = 470 nm t = 2 h	Equation (3)	[44]

Table 1. Cont.

Reagents	Sample Type	Sample Preparation	Volume of Emulsion [mL]/Volume of Sample [mL] (Emulsion to Sample Ratio)	Measurement Conditions	Manner of Expressing Results	Ref.
1 mL of β-carotene solution ($c = 0.5$ mg/mL in CHCl_3) 0.025 mL of linoleic acid 200 mg of Tween 40 100 mL of aerated distilled H_2O 0.35 mL of sample ($c = 2$ mg/mL in DMSO *)	Herbs	- Hydrodistillation of the dry, aerial parts of the herb to obtain the essential oils which were dried and stored at $+4$ °C. - Extraction of the aerial parts using MeOH * in a Soxhlet apparatus. - Filtration of the obtained extracts, evaporation of the solvent to obtain a waxy substance. - The extracts were suspended in water and separated with chloroform to obtain water-soluble (polar) and water-insoluble (nonpolar, chloroform) fractions. The extracts were concentrated, dried, and stored in the dark at $+4$ °C until analysis.	2.5:0.35 (7.1:1)	$T = 50$ °C $\lambda = 470$ nm $t = 2$ h	Equation (4)	[45]
1 mL of β-carotene solution ($c = 0.5$ mg/mL in CHCl_3) 0.025 mL of linoleic acid 200 mg of Tween 40 100 mL of aerated distilled H_2O 0.35 mL of sample ($c = 0.2$–1 mg/mL in EtOH *)	Plants	- The plant material was dried and ground. - Maceration by using distilled H_2O. - Filtration of the obtained extract. - Lyophilization to dry power.	(2.5:0.35) 7.1:1	$T = 40$ °C $\lambda = 470$ nm $t = 2$ h	Equations (1) and (2)	[46]
1 mL of β-carotene solution ($c = 1.0$ mg/mL in CHCl_3) 0.025 mL of linoleic acid 200 mg of Tween 20 50 mL of aerated distilled H_2O 0.06 mL of sample ($c = 5$ mg/mL in MeOH *) with 10% DMSO *)	Plants	- Extraction of the aerial part of herbs using a maceration technique with n-hexane, MeOH *, and H_2O. - Filtration of the obtained supernatants and evaporation of the organic solvent to get dry extracts. - Dehydration of aqueous extracts using lyophilization process.	0.25:0.06 (4.2:1)	$T = 50$ °C $\lambda = 492$ nm $t = 1.75$ h	Equation (3)	[47]
1 mL of β-carotene solution ($c = 2.0$ mg/mL in CHCl_3) 20 mg of linoleic acid 200 mg of Tween 40 50 mL of aerated distilled H_2O 0.2 mL of sample ($c = 200$ mg/mL in 50% MeOH * with HCOOH)	Food products	- Extraction of the tested cakes using a 50% MeOH * solution with 0.1% HCOOH in an ultrasonic bath. - Centrifugation and filtration of the obtained extracts.	5:0.2 (25:1)	50 470 2	Equation (6)	[48]

Table 1. Cont.

Reagents	Sample Type	Sample Preparation	Volume of Emulsion [mL]/Volume of Sample [mL] (Emulsion to Sample Ratio)	Measurement Conditions	Manner of Expressing Results	Ref.
1 mL of β-carotene solution ($c = 0.1$ mg/mL in CHCl_3) 20 mg of linoleic acid 200 mg of Tween 40 50 mL of aerated distilled H_2O 0.2 mL of sample (in MeOH *)	Plants	The washed and dried material was hydro distilled to obtain the essential oil.	5:0.2 (25:1)	$T = 50^\circ\text{C}$ $\lambda = 470$ nm $t = 2$ h	Equations (1) and (2)	[49]
1 mL of β-carotene solution ($c = 0.2$ mg/mL in CHCl_3) 40 mg of linoleic acid 200 mg of Tween 80 100 mL of aerated distilled H_2O 0.1 mL of sample ($c = 0.02$–1 mg/mL in EtOH *)	Algae	- Depigmentation of dried and ground brown algae using formaldehyde. - Extraction with H_2SO_4. - Alkalization of the resulting precipitate with Na_2CO_3. - Removal of insoluble particles by centrifugation, filtration, and dialysis. - Lyophilization of the dialysate.	3:0.1 (30:1)	$T = 40^\circ\text{C}$ $\lambda = 470$ nm $t = 2$ h	Equation (3)	[50]
2 mL of β-carotene solution ($c = 0.4$ mg/mL in CHCl_3) 50 mg of linoleic acid 400 mg of Tween 40 50 mL of aerated distilled H_2O 0.01 mL of sample (2 mg/mL in MeOH *)	Plants	- Ozonation process of the studied shoots at various times. - Drying the obtained samples. - Extraction of lignin by maceration in H_2O or alkali. - Filtration of the extracts, and their lyophilization.	(0.25:0.01) 25:1	$T = 45^\circ\text{C}$ $\lambda = 470$ nm $t = 2$ h	Equations (1) and (2)	[51]
1 mL of β-carotene solution ($c = 1$ mg/mL in CHCl_3) 0.02 mL of linoleic acid 0.184 mL of Tween 40 10 mL of 50 mM H_2O_2 50 mL of distilled H_2O 0.04 mL of sample (different extracts) ($c = 0.0025$ – 0.08 mg/mL in MeOH *)	Propolis	- Propolis samples were cut into small pieces and extracted with 20 mL of 80% EtOH *. - Centrifugation and collection of the supernatant. - Evaporation to dryness and dissolution in MeOH */H_2O. - The resulting extract was subjected to liquid–liquid extraction using a hexane/CHCl_3 mixture. - The obtained extracts were evaporated to dryness and dissolved in MeOH *. - The hexane/chloroform dry extract was fractionated and in each fractions the solvent was evaporated.	4:0.04 (100:1)	$T = 40^\circ\text{C}$ $\lambda = 470$ nm $t = 2$ h	Equations (1) and (2)	[52]

Table 1. Cont.

Reagents	Sample Type	Sample Preparation	Volume of Emulsion [mL]/Volume of Sample [mL] (Emulsion to Sample Ratio)	Measurement Conditions	Manner of Expressing Results	Ref.
1 mL of β-carotene solution ($c = 0.2$ mg/mL in CHCl_3) 0.025 mL of linoleic acid 0.200 mL of Tween 20 50 mL of aerated distilled H_2O 0.05 mL of sample ($c = 0.003$–0.12 mg/mL in MeOH *)	Plants	- The powdered rhizomes were dried extracted with EtOH * to obtain a brown color crude extract. - Then, a portion of the extract was fractionated with hexane. - The residue insoluble in hexane was partitioned with CHCl_3:H_2O. - The chloroform layer was filtered, and the solvent was evaporated. - Analysis of the ethanol, hexane, and chloroform extracts after evaporation of solvent and dissolution in MeOH *.	0.2:0.05 (4:1)	T = 40 °C $\lambda = 470$ nm t = 3 h	Equation (3)	[53]
3 mL of β-carotene solution ($c = 0.1$ mg/mL in CH_3Cl) 40 mg of linoleic acid 400 mg of Tween 40 100 mL of aerated distilled H_2O 0.3 mL of sample ($c = 0.25$ mg/mL in MeOH *)	Plants	- Extraction of the homogenized sample with EtOH *. - Centrifugation and removing the solvent under nitrogen to dryness.	4.5:0.3 (15:1)	T = 50 °C $\lambda = 470$ nm t = 1.7 h	Equation (3)	[54]

* dimethyl sulfoxide (DMSO); ethanol (EtOH); methanol (MeOH).

Table 2. Proposed measurement conditions for the β -carotene bleaching assay.

Parameters/Factors	Proposed Conditions	Reason
Wavelength	450–470 nm	The given length range corresponds to the maximum in the absorption spectrum of β -carotene
Measuring time	2–3 h	The measurement of the absorbance is continued until the color of β -carotene disappears [30]
Sample solvent	Mainly alcohols, acetone	The mentioned solvents mix well with the emulsion and also have a low ability to block hydrogen in the process of its transport from the antioxidant to the radical [41]
pH	5.5–7.0	At 45 °C, the time required to achieve a given degree of bleaching in the control sample is very long at low pH (3.0–4.0), changes rapidly from pH 4.0–5.5, remains essentially constant from pH 5.5 to 7.5, and then slowly increases from pH 7.5 to 11.0. Therefore, the range of 5.5–7.5 appears to be the best option for ensuring stable change over a reasonable time [20,55]
Temperature	45 °C	This temperature has little effect on the spontaneous oxidation of β -carotene, allows for the observation of different levels of antioxidants (weaker, stronger), and produces the fewest side effects at this temperature [20]
Reagent ratio v of emulsion/v of sample	From 50:1 to 25:1	Our studies have shown that changing the volume ratio of the reactants towards reducing the amount of emulsion and increasing the volume of the sample leads to worse antioxidant properties, even by more than 10% when the ratio changes from 50:1 to 5:1 (with the same amount of antioxidant dissolved in a different amount of solvent) [41].
Way of expressing results	Equations (1) and (2)	These are the two equations most commonly found in the literature. They represent the changes in β -carotene absorbance in the control and test samples and relate these changes to the initial absorbance in a logarithmic manner.

As the examples presented (see Table 1) demonstrate, the β -carotene bleaching method is used under various measurement conditions, which undoubtedly makes it difficult, if not impossible, to compare the results of different research teams. However, the lack of a uniform presentation of results is not the only problem with this method. Its infamous reputation also stems from the low reproducibility of the obtained results. Furthermore, emulsion preparation is time-consuming and labor-intensive, and susceptible to interference from various factors (including the solvent used, the influence of metal ions, pH, and temperature) [20,41]. As a result, as Figure 1 shows, the applicability of the β -carotene method, measured by the number of publications indexed in the Scopus database, has not increased over the last five years and remains at a comparable level of approximately 330 publications per year.

5. Presentation of the Final Results

The question arises how to interpret the obtained results and correctly express antioxidant activity using this method. The literature provides various methods for presenting final results [56], including for the β -carotene bleaching method. This leads to results that are difficult to interpret and compare, especially when the same antioxidant is tested using the same method but a different method for presenting final results. This is a significant problem not only for the β -carotene method. Below, we summarize the most well-known equations, collected from the literature and used to present final results. They are presented

in order of popularity. However, the authors' experience indicates that Equations (1) and (2) are more reliable.

The basic manner of presenting results is to determine the rate of β -carotene bleaching (R), both in the control and in the tested samples, according to the following equation [57,58]:

$$R = [\ln(a/b)/t], \quad (1)$$

where $\ln$ = natural log, a = absorbance at time "0", and b = absorbance at time "t".

In turn, the antioxidant activity (expressed as inhibition percent, $I\%$) can be determined from the below equation, where R is the bleaching rate [57,58]:

$$I (\%) = (R_{\text{control}} - R_{\text{sample}}) / R_{\text{control}} \times 100\%. \quad (2)$$

The higher the value of this parameter, the better the antioxidant properties of the tested sample. When interpreting the results, it can also be assumed that the higher the % inhibition, the greater the degree of protection of β -carotene from degradation in the test sample compared to its degradation in the control sample.

In addition to the method mentioned above (Equations (1) and (2)), antioxidant activity determined by this method is calculated directly from absorbance alone [44,59–61]:

$$\%AOA = [1 - (As(0) - As(t)) / (Ac(0) - Ac(t))] \times 100\%, \quad (3)$$

where %AOA—the percentage antioxidant activity, $Ac(0)$ is the absorbance of the control sample at $t = 0$ min, $Ac(t)$ is the absorbance of the control sample at t , $As(0)$ is the absorbance of the test sample at $t = 0$ min, and $As(t)$ is the absorbance of the test sample at t . The total measurement time (t) ranges from 60 min to 120, 150, 180 or 210 min, and absorbance measurements are performed at different time intervals (every 10 min, every 15 min, every 25 min) [44,59–61].

You can also find papers [62–64] in which the antioxidant activity (percent inhibition, $I\%$) of the samples was calculated using the following equation:

$$I\% = (A_{\beta\text{-carotene}}(t) / A_{\beta\text{-carotene}}(0)) \times 100\%, \quad (4)$$

where $A_{\beta\text{-carotene}}(t)$ is the absorbance of β -carotene remaining in the samples after definite time of testing (t), and $A_{\beta\text{-carotene}}(0)$ is the absorbance of β -carotene at the beginning of the experiments.

Using each of the methods presented, based on the relationship between the determined % inhibition values and time, the so-called IC_{50} value can be determined. It should be noted that the IC_{50} is the concentration of an antioxidant/sample that, in this case, provides 50% protection against β -carotene oxidation. The lower the IC_{50} value when comparing different samples based on this parameter, the better the antioxidant properties.

In the literature, there are also other approaches to expressing test results using the β -carotene bleaching method, for example, using standards of compounds with known antioxidant properties [56,65]. This approach was used by Dapkevicius et al. in [66], who reported relative antioxidant activities (RAA) calculated based on the dependencies:

$$RAA = \text{Absorbance of sample} / \text{Absorbance of BHT}. \quad (5)$$

These researchers used butylhydroxytoluene (BHT) as the antioxidant standard, and the changes in β -carotene absorption in the tested sample and in the sample containing BHT were measured both after 28 and 56 h, noting that the values they obtained were smaller when the period after which the measurement was made was longer. It should be noted that the higher the RAA value, the greater the antioxidant properties.

Yet another, relatively new way of determining antioxidant properties is the so-called antioxidant activity coefficient (AAC) proposed in the work of Andrade et al. [67]. It is calculated based on the following equation:

$$\text{AAC} = [\text{As}(t) - \text{Ac}(t) / \text{Ac}(0) - \text{Ac}(t)] \times 1000, \quad (6)$$

where $\text{As}(t)$ is the absorbance of the sample at definite time, $\text{Ac}(t)$ is the absorbance of the control at definite time, and $\text{Ac}(0)$ is the absorbance of the control at 0 min. The higher the value of this parameter, the better the antioxidant properties.

6. Factors Influencing the Assessment of Antioxidant Properties Estimated by the β -Carotene Method

As mentioned above, a number of factors influence the assessment of antioxidant capacity using the β -carotene bleaching method [20,41]. Among the most frequently encountered in the literature are temperature, hydrogen ion concentration (pH), solvent, and metal ions. These are discussed in the individual paragraphs below.

Temperature is an important factor that should be controlled both during reagent preparation and the measurement itself. Temperature has been observed to increase the rate of β -carotene bleaching (regardless of whether linoleic acid is present in the measurement system) and also influence solvent evaporation. In the original description of the method presented by Marco, a measurement temperature range of 50–55 °C was used. Today, most applications of this method use a temperature of 45 °C. According to Prieto et al. [20], using this temperature significantly reduces spontaneous β -carotene oxidation, leads to better differentiation between different antioxidant levels, is significantly more sensitive to prooxidant effects, and significantly reduces temperature gradients, which is extremely important in microplate applications. It should be noted that using a lower temperature than originally proposed (50–55 °C) extends the analysis time but reduces side effects. At the expense of extending the measurement time, statistically better results are obtained. On the other hand, using higher temperatures shortens the analysis time but is subject to the possibility of errors resulting from excessive β -carotene oxidation, and furthermore, the reaction conditions move away from those in which antioxidants have practical significance.

The β -carotene method is often used to assess the antioxidant properties of plant extracts, which may differ in the content and type of natural acids. Studies conducted on BHT have shown that, in environments with varying pH, antioxidant activity of BHT is higher in more acidic environments [55]. It should be noted that these results were obtained relative to a control sample that did not contain hydrogen ions. As mentioned in the introduction, antioxidant activity in the β -carotene method is determined by comparing two competing chemical reactions involving the tested antioxidant and/or β -carotene (a model antioxidant) [68]. The main component of the reaction medium is an emulsion of β -carotene/linoleic acid in water with the generated peroxide radicals ($\text{LOO}\bullet$). During the test, changes in β -carotene concentration are monitored in the control sample and in the sample containing the tested antioxidant. β -carotene reacts with peroxide radicals in the sample immediately after the antioxidant is depleted. Given the above, to determine whether the presence of hydrogen ions affects BHT or β -carotene (this was not fully known from monitoring the test sample), subsequent studies were conducted to monitor changes in beta-carotene in a control sample (without BHT). The data indicated that increasing hydrogen ion concentration reduced the loss of β -carotene in the control sample. This observation contradicts Valgimigli's explanation [69], who reported that the presence of small amounts of acid accelerates the reaction between antioxidants and peroxide radicals. The results indicate that increasing hydrogen ion concentration leads to the neutralization of peroxide radicals, which cannot be true. The most likely explanation for

the observed correlations is that the presence of acid in the measurement system slows down the formation of peroxide radicals. This process occurs both in the presence and absence of an antioxidant. Furthermore, in [55] it was demonstrated that the type of cations and anions (buffer components) also influences the observed antioxidant properties. This is also confirmed by the results of studies conducted by Prieto et al. [20], in which the effect of pH on β -carotene was studied in buffered solutions (pH 3.5–(0.5)–11.0) at various concentrations of BHA, 0–(0.5)–5 μ M, in the presence and absence of linoleic acid. They show that no hypso- or bathochromic shifts in the β -carotene absorption spectrum were observed within the pH range studied. In the control sample, however, the time required to achieve a given degree of whitening was very long at low pH, confirming that hydrogen ions influence the oxidation of linoleic acid. The authors of the aforementioned article further state that the best range for testing this method, which guarantees stable results within a reasonable time, is a pH range of 5.5 to 7.5.

The *solvent* plays an important role in assessing the antioxidant properties of compounds. In the β -carotene method, the reaction medium is an aqueous emulsion, and when the sample is poorly soluble in water, it is necessary to use other solvents, such as alcohols (methanol, ethanol), acetone, or 1,4-dioxane. The solvent not only affects solubility but also participates in the peroxy radical neutralization reaction, which, as mentioned earlier, is historically considered the HAT mechanism. According to the literature [2,70], the addition of hydrogen from the antioxidant to the peroxy radical precedes the formation of a complex between the antioxidant and the peroxide radical, resulting in the phenolic hydrogen being hydrogen bonded to the peroxide radical. This hydrogen is then transferred as a proton from the phenol to the ion pair on the peroxide radical, along with the conjugate movement of an electron from the phenolic oxygen 2p ion pair to the orbital containing the unpaired electron on the peroxide radical, so that the electron moves between two formally non-bonding orbitals [71]. A solvent that is a stronger hydrogen acceptor (e.g., dioxane) can hinder this process and thus slow down the reaction rate, which may translate into the observed lower antioxidant properties. Furthermore, the type of antioxidant solvent, due to its different hydrophobicity, can affect the dispersion (or solubility) of the emulsion or its components, which can consequently change the reaction rate. An important aspect concerns the solvent is the so-called “polar paradox” [72] related to the fact that due to hydrophobic repulsion, non-polar antioxidants readily accumulate closer to the lipid environment in which oxidation takes place, hence their observed higher antioxidant activity in oil-water emulsion. Regarding the effect of solvent on antioxidant properties assessed using the β -carotene method, another aspect must be considered. The lack of standardized methods for measuring antioxidant properties means that tests are performed using varying volume ratios of the reagents. Such a variable volume ratio of the tested antioxidant solution to the β -carotene/linoleic acid emulsion also significantly impacts the assessment of antioxidant activity [41].

The presence of *metal ions* in plant extracts is a consequence of their natural occurrence in plants. Studies also show that the presence of metals in the measurement system affects antioxidant properties estimated using the β -carotene method. In most cases, increasing metal ion concentration accelerates the oxidation process [20,41]. However, none of the tested metals promoted β -carotene bleaching (in the absence of linoleic acid). This clearly suggests that in all cases the observed activity was not related to β -carotene bleaching, but to the production of radicals by the fatty acid. One possible explanation for this phenomenon is the decomposition of hydroperoxides formed after the neutralization reaction, observed in the presence of metal ions [73,74].

Typical factors that significantly modify the assessment of antioxidant properties using the β -carotene method are presented above. However, it is important to realize that there

are more factors and controversies surrounding them. For example, some authors use initiators to initiate the radical formation reaction. However, according to Prieto et al. [20], their use is unnecessary, as the reaction proceeds smoothly without them. Others cite the size of the formed micelles as a factor differentiating the results. However, according to [20], this parameter is irrelevant if centrifugation or ultraviolet irradiation is used after reagent preparation (before measurement). Similarly, according to [20], initial oxygen saturation does not cause significant differences in the bleaching process, and the saturation step can be omitted.

7. Application

As mentioned, the β -carotene method was first used to test the autoxidation of emulsified linoleic acid. However, as the procedure was refined and simplified, its application range expanded significantly. Currently, it is used to study various matrices, and while liquid samples are directly analyzed, in the case of solid matrices, in accordance with the methodology of their processing, after preliminary grinding, most often using a mill, they are subjected to extraction. Each case requires an individual and differentiated approach, as demonstrated, among others, in Table 1. A literature review shows that this method is used to determine the antioxidant properties of, among others,

- Plant (including herbs, spices, vegetables, fruits and seaweed) extracts obtained from roots [32,75], leaves [76–79], stems [78], flowers [80], buds [81], seed [82,83], fruits [84,85] and seaweed species [86] are used for research.
- Essential oils [87–90];
- Food products (for example honey, propolis, beans) [91–95] and enriched food product [96,97];
- Beverages (functional drinks, juice) [98–100];
- Food waste and industrial waste [44,101–107];
- Synthetic compound [108–112];
- Components so called “active packing” [113–116];
- Mushrooms [117,118];
- Microorganisms (cyanobacteria) [119];
- Cosmetic products [120];
- Natural pigments [121];
- Edible oils [122];
- Animal proteins [123,124].

8. Conclusions

The aim of this review article is to systematize and update knowledge on the use of the β -carotene bleaching method in antioxidant studies based on the HAT mechanism. This article explains the basics of β -carotene’s reaction with free radicals and the causes of discoloration of this molecular probe. It is emphasized that the use of this molecule in antioxidant studies is supported by both economic and ecological considerations. β -carotene is considered a substance generally recognized as safe (GRAS). Furthermore, it can be used with equal effectiveness both in studies of processes occurring in living organisms (in vivo) and in studies conducted under controlled laboratory conditions (in vitro). Given the lack of standardized conditions for conducting antioxidant studies using this method, the authors propose an optimal methodology, in their opinion, that will help obtain consistent, reliable, and easily comparable results between research groups. The authors present the influence of relevant parameters that significantly differentiate the final analytical results. Therefore, this methodology should be considered a specific guide for studying the antioxidant properties of simple and complex systems using the β -carotene

method, ensuring the effective identification of sources of naturally occurring antioxidants and the design of new antioxidant compounds for future use as health-promoting agents and/or safe food additives.

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Abbreviations

The following abbreviations are used in this manuscript:

AAC	Antioxidant Activity Coefficient
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AH	Antioxidant
AOA	Antioxidant Activity
BDE	bond dissociation energy
BHA	Butylhydroxyanisole
BHT	Butylhydroxytoluene
CUPRAC	cupric ion reducing antioxidant capacity method
DMSO	Dimethyl sulfoxide
DMPD	dimethyl-p-phenylenediamine dihydrochloride
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOH	Ethanol
FRAP	ferric ion reducing antioxidant parameter method
GRAS	generally recognized as safe
HAT	Hydrogen Atom Transfer
HORAC	Hydroxyl Radical Antioxidant Capacity
IC	Inhibition Concentration
IP	ionization potential
LDL	Low Density Lipoprotein
MeOH	Methanol
ORAC	Oxygen Radical Absorbance Capacity
RAA	Relative Antioxidant Activities
ROS	Reactive Oxygen Species
SET	Single Electron Transfer
TOSCA	Total Radical Scavenging Capacity Assay
TRAP	Total Radical Trapping Antioxidant Parameter

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