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Review

Evaluation of Spectrophotometric Methods for Assessing Antioxidant Potential in Plant Food Samples—A Critical Approach

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Abstract: Spectrophotometric antioxidant assays can generally be divided into two fundamental categories: single electron transfer (SET)-based assays and hydrogen atom transfer (HAT)-based methods. In SET-based assays, the progression of the electron exchange reaction is determined by the redox potential of the substrates. In contrast, HAT-based methods assess the antioxidant's ability to transfer a hydrogen atom to a radical, thereby stabilizing it. The objective of this article is to provide a critical evaluation of antioxidant spectrophotometric assays. Assessing the antioxidant potential of food should involve multiple assays to ensure accuracy and reliability. A positive correlation among different methods enhances the validity of the results. Moreover, antioxidants may interact with other food components, such as amino acids, potentially leading to inaccurate outcomes—as observed in the Folin–Ciocalteu assay. Among the various techniques, CUPRAC and ORAC exhibit greater repeatability and reagent stability compared to other assays. Furthermore, these methods are considered superior due to their closer resemblance to in vivo conditions. In contrast, approaches such as $ABTS^+$, DPPH, FRAP, and Folin–Ciocalteu are often criticized for their non-physiological environments. There is a pressing need to establish a standardized method that, to the greatest extent possible, reflects in vivo conditions and can serve as a reference standard for other assays.

Keywords: antioxidant potential; plant food; spectrophotometric assay

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1. Introduction

Reactive oxygen species (ROS), which are generated during normal metabolic processes—primarily in the respiratory chain—pose a significant health risk. In mammals, the energy essential for life is produced in the form of adenosine triphosphate (ATP) through the utilization of oxygen (O_2). This process involves the electron transport chain (ETC), located in the inner mitochondrial membrane, and results in the generation of both ATP and ROS [1]. Moreover, environmental factors, chronic diseases, and dietary habits may contribute to elevated ROS levels in the body [2]. The most common ROS include the superoxide anion ($^{\bullet}O_{2-}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) [3–6]. These species are implicated in the development of various diseases, including cancer [7].

Over the course of evolution, humans have developed both endogenous and exogenous defense mechanisms against ROS. Endogenous defense mechanisms primarily involve enzymes that regulate ROS levels in living organisms, including superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GPx) [8]. Exogenous antioxidants, such as phenolic compounds (e.g., hydroxybenzoic acid and hydroxycinnamic acid), are obtained through the diet and play a crucial role

in counteracting ROS [9–11]. These compounds are predominantly found in plant-based foods, particularly in fruits, vegetables, and herbs [12–15].

The activity or functional capacity of food compounds is assessed using various analytical approaches [16]. Numerous methods for analyzing antioxidant potential have been extensively described in the scientific literature. These methods are generally classified into two main categories: chromatographic and spectrophotometric techniques. Commonly used chromatographic methods for evaluating antioxidant activity include high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS), liquid chromatography–electron capture dissociation (LC–ECD), and gas chromatography–mass spectrometry (GC–MS) [17–22]. This article focuses on spectrophotometric methods, discussing their respective advantages and limitations. Given their widespread use and the substantial discrepancies among spectrophotometric assays, a critical evaluation is warranted.

Antioxidant assays are generally classified into two fundamental types: single electron transfer (SET)-based assays and hydrogen atom transfer (HAT)-based methods. In SET-based assays, the progression of the electron exchange reaction is governed by the redox potential of the substrates. Consequently, antioxidants react with an oxidizing agent rather than directly with free radicals [23]. This reaction is typically characterized by a color change in the solution, which reflects the concentration of antioxidants. The magnitude of absorbance change at a specific wavelength correlates with the concentration of antioxidants in the sample.

Electron transfer-based methods include the $ABTS^+$ assay, cupric reducing antioxidant power (CUPRAC) assay, ferric reducing antioxidant power (FRAP) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and the Folin–Ciocalteu (FC) assay [24,25]. Hydrogen transfer-based assays exploit the antioxidant's ability to donate a hydrogen atom to a free radical, thereby stabilizing it. The activity and capacity of an antioxidant can be assessed using reaction kinetics by measuring changes in the absorbance curve in the presence or absence of a reducing agent. This category includes the oxygen radical absorbance capacity (ORAC) assay [26]. These methods are characterized by substantial variability among individual assays. To date, no standardized spectrophotometric approach exists for assessing antioxidant potential.

This article focuses on a specific niche: the assessment of antioxidant potential in plant-derived foods. Although previous reviews have discussed spectrophotometry as a method for evaluating antioxidant potential, they either concentrate on methodological discrepancies without comparing actual results or address antioxidant activity across all food types without differentiation [11,27,28]. The aim of this article is to critically evaluate the most commonly used spectrophotometric assays for determining antioxidant potential in plant-based food samples. The selected methods—FRAP; CUPRAC; DPPH; $ABTS^+$; Folin–Ciocalteu; and ORAC—were chosen for their widespread use in food analysis and methodological diversity. A thorough evaluation of these techniques is essential for identifying reliable, physiologically relevant, and reproducible approaches that can serve as reference standards in antioxidant capacity assessment, particularly in the context of diverse food matrices and their implications for human health.

2. Materials and Methods

During the preparation of this narrative review, scientific articles published after 2019 were retrieved from the PubMed, ScienceDirect, and Scopus databases and analyzed. Older articles were included occasionally, only when they made a significant contribution to the field. The search terms included "antioxidant potential assay" in combination with "fruit" and "vegetable" in the title, keywords, or abstract. The search using "an-

tioxidant potential assay" and "fruit" yielded 25,984 results, while the combination with "vegetable" produced 16,576 articles. The results were further refined by including the names of specific spectrophotometric methods: "FRAP" (1295 results), "CUPRAC" (681 results), "DPPH" (1908 results), "$ABTS^{+}$" (1408 results), "Folin–Ciocalteu" (5698 results), and "ORAC" (1503 results). From this selection, the most relevant articles were analyzed. The inclusion criteria were as follows:

- The study evaluated antioxidant potential using spectrophotometric assays;
- The samples analyzed were derived from plant-based foods.

A total of 149 articles met these criteria and were included in the final analysis.

3. Plant-Derived Antioxidants in In Vitro Assays

According to common knowledge, plant-derived foods are a natural source of antioxidants. These foods can adequately meet nutritional requirements for antioxidant vitamins, such as A, C, and E [29–31]. Plant tissues are significant sources of exogenous antioxidants, including vitamins (ascorbic acid, tocopherols) and carotenoids (xanthophylls and carotenes) [9,32,33]. Other bioactive compounds, such as flavonoids, lignans, stilbenes, and condensed tannins, belong to the large phenolic family [11]. Phenolic compounds exhibit strong antioxidant activity in in vitro spectrophotometric assays, primarily due to their chemical structure and the presence of aromatic rings capable of stabilizing or delocalizing unpaired electrons. Additionally, while these compounds share the same basic aromatic structure, the position and number of phenolic groups influence their antioxidant activity [34]. Plant tissues also contain by-products with antioxidant potential, such as bioactive peptides [35,36]. The antioxidants used as standards in the methods discussed may be either natural or synthetic chemical compounds (Figure 1). These antioxidants typically have organic (e.g., Trolox, ascorbic acid) or inorganic (e.g., $FeSO_4$) chemical structures. Their activity in in vitro methods relies primarily on two mechanisms: donating a hydrogen atom or accepting a free radical [16]. The following section will describe specific spectrophotometric assays used to assess the antioxidant potential of plant-derived food samples.

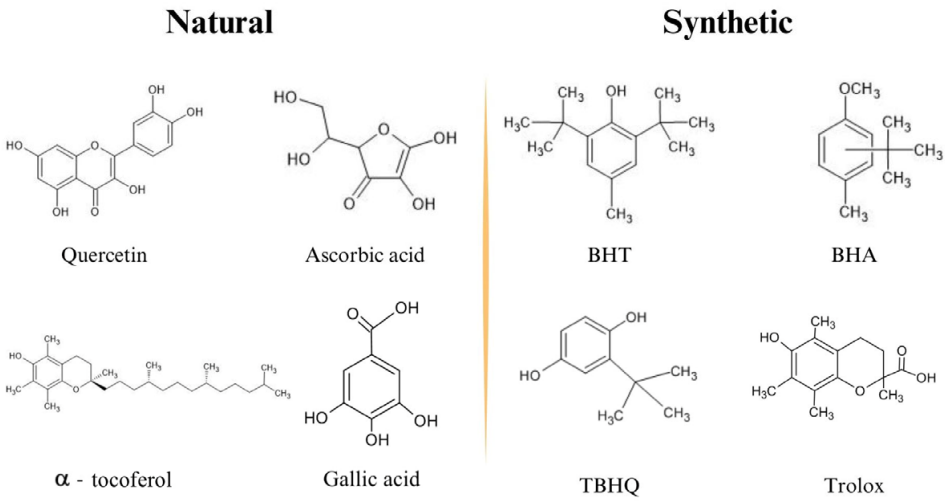


Figure 1. Division of chemical compounds with organic structure commonly used as reference substances in methods for assessing antioxidant potential. BHT—butylated hydroxytoluene; BHA—butylated hydroxyanisole; TBHQ—tert-butylhydroquinone.

4. Electron Transfer-Based Assays

All ET-based assays involve the donation of a single electron from an antioxidant to a free radical, characterized by the presence of an unpaired electron [22]. This mechanism underlies the methods discussed in this chapter, namely FRAP, CUPRAC, DPPH, $ABTS^{+}$, and FC.

4.1. Ferric-Reducing Antioxidant Power

4.1.1. Working Principle of FRAP Assay

The FRAP assay was developed in the 1990s by Benzie and Strain [37]. In their 1996 publication, they introduced a novel method for measuring antioxidant capacity based on the reduction of Fe^{3+} (ferric) to Fe^{2+} (ferrous). This method was designed to be simple, rapid, and reproducible. The FRAP assay is based on electron transfer to an iron (Fe) complex with 2,4,6-tripyridyl-s-triazine (TPTZ):

$$Fe^{3+} + TPTZ \rightarrow [Fe^{2+} - TPTZ]^{+}$$

The reduced form of the complex exhibits a dark blue color. Its intensity is proportional to the antioxidant concentration in the sample. The reaction is carried out at a low pH (<4), maintained by an acetate buffer at pH 3.6 [37]. The reduced complex reaches its maximum absorption at 593 nm. An increase in absorbance values corresponds directly to the content of antioxidants in the sample. The results are expressed as moles of Fe^{2+} equivalents per unit mass of the sample [38,39].

The FRAP reagent consists of an acetate buffer, TPTZ, and $FeCl_3$ and is prepared ex tempore, immediately prior to analysis [40]. This solution was originally used to assess the antioxidant power of blood plasma based on its ability to reduce Fe^{3+} ions to Fe^{2+} ions [37]. Currently, the FRAP assay is used to evaluate ferric-reducing antioxidant power by measuring total antioxidant capacity in both biological fluids and food samples [41,42].

The FRAP assay is widely recognized as a simple and cost-effective method, making it suitable for use in various laboratory settings [43]. The reaction is highly reproducible, and the results directly correlate with the molar concentration of antioxidants present. Notably, no concentration-dependent stoichiometric variations have been observed, indicating that the $Fe(TPTZ)_3{}^{+}$ concentration in the FRAP reagent does not influence the final results [37]. Moreover, no decrease in absorbance was observed for at least 30 min, indicating the absence of agents that inhibit Fe^{3+} reduction or cause Fe^{2+} reoxidation. However, these conclusions were based on studies analyzing plasma antioxidant activity [37]. Although Fe^{3+} naturally occurs in plant-based foods [44], no research has specifically investigated its potential role as an interfering factor in the FRAP assay. However, the presence of such ions in extracts may affect the measured antioxidant potential [45]. Therefore, it remains unclear whether the iron content in plants affects the reaction mechanism. Additionally, it has been shown that the results of the FRAP assay may vary depending on the solvents used, potentially contributing to discrepancies in findings [46].

4.1.2. A Comparative Analysis of Methodological Approaches in Studies on Plant Materials

Although the approach to conducting the FRAP method is similar across studies, it is not identical (Table 1), with most using a measurement wavelength of 593 nm [38,47,48] and some reporting 595 nm [39]. Results are typically expressed as the concentration of antioxidants with ferric-reducing ability equivalent to a standard compound, usually $FeSO_4$, per 100 g of sample [38,48]. This consistency in reporting units is considered an advantage of the FRAP assay, as it facilitates comparison of results across studies. The type and duration of incubation vary, which may be attributed to differences in the

food matrices used in the studies [39,49,50]. Furthermore, the extraction and incubation conditions of antioxidant compounds are influenced by the composition of the matrix, which may include proteins, fatty acids, fiber, and polysaccharides [47,48]. The efficiency of antioxidant extraction depends on the chemical properties of the matrix; therefore, factors such as temperature, incubation time, and specific reagents used during extraction may vary [51,52]. These discrepancies may affect the final results obtained by the FRAP assay, with the type of extraction being one of the main factors contributing to significant variations in findings [53–55].

The FRAP reagent plays a crucial role in the reaction between $Fe(TPTZ)^{3+}$ and antioxidants. It is prepared ex tempore, and significant variations in its composition have been reported (Table 1). However, no conclusive data are available to determine whether these differences significantly affect the final assay outcomes.

Table 1. Comparison of methodological approaches in the FRAP assay.

Tested Material	Standard	Substrates	Type and Time of Incubation	Wavelength	Source
Various kinds of fruits	$FeSO_4$	1.8 mL FRAP reagent (2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$, 25 mL of 300 mM acetate buffer (pH = 3.6), 40 µL of tested sample, 0.2 mL MQ	10 min, 37 °C	593	[48]
Apple	$FeSO_4$	2 mL of FRAP reagent (10 mL of 10 mM TPTZ in HCl, 10 mL of 20 mM $FeCl_3$, 100 mL of acetate buffer pH = 3.6), 50 µL of tested sample	10 min, room temperature	593	[47]
Oat β-glucans	millimolar Fe^{2+}	3 mL of FRAP reagent (1 mL of 20 mM $FeCl_3 \cdot 6H_2O$, 1 mL of 10 mM TPTZ in 40 mM HCl, 10 mL of 300 mM acetate buffer, pH = 3.6), 0.1 mL of sample, 0.3 mL of MQ	4 min, 37 °C	593	[38]
Wine	$FeSO_4 \cdot 7H_2O$	1 mL of FRAP reagent (1 mL of 20 mM $FeCl_3 \cdot 6H_2O$, 1 mL of 10 mM TPTZ in 40 mM HCl, 10 mL of 300 mM acetate buffer, pH = 3.6), 50 µL of tested sample, 0.45 mL of MQ	5 min, darkness, 37 °C	593	[49]
Radish	Ascorbic acid	0.19 mL of FRAP reagent (2.5 mL of 20 mM $FeCl_3$, 2.5 mL of 10 mM TPTZ, 2.5 mL of 10 mM acetate buffer pH = 3.6), 10 µL of tested sample	10 min, 37 °C	593	[50]
Tomato juice and carrot juice	BHT	0.6 mL of FRAP reagent (50 µL of 20 mM $FeCl_3$, 50 µL of 10 mM TPTZ, 0.5 mL of 300 mM acetate buffer pH = 3.6), 100 µL of extract	6 min, 25 °C	595	[39]
Various meals in kindergarten	Ascorbic acid	3 mL of FRAP reagent (20 mM $FeCl_3$, 10 mM TPTZ in 40 mM HCl, 300 mM acetate buffer pH = 3.6), 100 µL of extract	10 min, room temperature	593	[56]
Maple syrup	$FeSO_4$	210 µL of FRAP reagent (20 mM $FeCl_3$, 10 mM TPTZ in 40 mM HCl, 300 mM acetate buffer pH = 3.6), 30 µL of extract	8 min, 37 °C	593	[57]

TPTZ—2,4,6-tripyridyl-s-triazine; BHT—2,6-di-tert-butyl-4-hydroxytoluene; MQ—Milli-Q water; HCl—hydrochloric acid.

4.2. Cupric Reducing Antioxidant Capacity (CUPRAC)

4.2.1. Working Principle of CUPRAC Assay

The CUPRAC assay was originally developed at the beginning of the XXI century to determine the antioxidant potential of plant compounds (e.g., ascorbic acid or quercetin) [58]. The tested extract is combined with an aqueous solution of copper(II) chloride ($CuCl_2$), an ethanolic solution of neocuproine, and an ammonium acetate buffer at pH 7. This leads to the formation of the $[Cu(Nc)_2]^+$ complex, which is responsible for the observed color change [59]. After an incubation period of 30–60 min, absorbance is measured at a wavelength of 450 nm. Upon addition of the extract or standard compound to the reagents, the color changes from green-blue to yellow-orange. The underlying principle of the method is the reduction of copper ions from Cu^{2+} to Cu^+ by antioxidants. The intensity of the resulting yellow-orange color is proportional to the antioxidant content in the test sample. The reaction involved in the CUPRAC method is as follows [59]:

$$n\ Cu(Nc)_2^{2+} + n\text{-electron reductant} \leftrightarrow n\ Cu(Nc)_2^{+} + n\text{-electron oxidized product} + n\ H^+$$

The CUPRAC assay is considered superior to other methods for measuring antioxidant potential because it operates at pH 7, which approximates physiological pH [60]. Furthermore, it has been demonstrated that CUPRAC is relatively unaffected by solvent effects in alcohol-water mixtures of varying compositions [46]. However, some studies have reported contrasting results, which were attributed to differences in the solvents used [61]. Methanolic and ethanolic extracts showed significantly higher antioxidant potential, as measured by the CUPRAC assay, compared to acetone-based extracts [61]. The short incubation time (30 min) at room temperature in the dark appears to be sufficient, yielding satisfactory results for most biologically relevant antioxidants, including ascorbic acid, gallic acid, and quercetin. Longer incubation times may be required for compounds such as naringin or naringenin, likely due to their glycosidic forms [58,60,62,63]. The CUPRAC assay is characterized by good linearity over a wide range of concentrations of standard substances, reagent stability, simplicity, and low cost. Additionally, it can be applied to both lipophilic and hydrophilic antioxidants [60,64].

4.2.2. Comparison of Methodological Approaches in Previous Studies of Plant Materials

The procedure and concentration of reagents in the CUPRAC assay are consistent across the literature, with most researchers adhering to the original protocol presented by Apak et al. [58], as applied in apricot [65], green tea [64], and cherries [66] (Table 2). The standard assay involves $CuCl_2$ (10 mM aqueous solution), neocuproine (7.5 mM ethanolic solution), and an ammonium acetate buffer at pH 7, combined with the tested antioxidant. However, some studies have reported alternative reagent concentrations [67]. For example, Puangbanlang et al. used 5 µL of $CuCl_2$ (150 mM), 5 µL of neocuproine (600 mM), 5 µL of ammonium acetate at pH 7, and 25 µL of the tested sample [67]. In some cases, $CuSO_4 \cdot 5H_2O$ is used as a substitute for $CuCl_2$ [63]. Although the stoichiometric ratio between copper ions and the chelator compound tends to be preserved, incubation time varies between 20 and 50 min, depending on the food matrix and experimental conditions (e.g., temperature, light exposure) [63,68]. Since reaction time plays a critical role in the accuracy of the results, optimizing the incubation period is essential when analyzing new food matrices [63,69].

Table 2. Comparison of previous approaches in the CUPRAC assay.

Tested Material	Standard	Substrates	Type and Time of Incubation	Wavelength	Source
Synthetic mixture of plant antioxidants	Trolox	1 mL of aqueous solution of $CuCl_2$ (10 mM), 1 mL of ethanolic solution of neocuproine (7.5 mM), 1 mL of ammonium acetate buffer at pH = 7, 1.1 mL of tested sample	20 min, 50 °C in water bath	450 nm	[58]
Synthetic mixture of plant antioxidants/green tea extracts	Trolox	1 mL of aqueous solution of $CuCl_2$ (10 mM), 1 mL of ethanolic solution of neocuproine (7.5 mM), 1 mL of ammonium acetate buffer at pH = 7, 1.1 mL of tested sample	30 min, room temperature	450 nm	[46]
Blackcurrant	Ascorbic acid	1 mL of aqueous solution of $CuCl_2$ (10 mM), 1 mL of ethanolic solution of neocuproine (7.5 mM), 1 mL of ammonium acetate buffer at pH = 7, 1.1 mL of tested sample	20 min, 50 °C in water bath	450 nm	[62]
Beetroot	Trolox	1 mL of aqueous solution of $CuCl_2$ (10 mM), 1 mL of ethanolic solution of neocuproine (7.5 mM), 1 mL of ammonium acetate buffer at pH = 7, 1.1 mL of tested sample	30 min, room temperature	450 nm	[68]
Tea extracts	Ascorbic acid	2 mL of 1 g/L $CuSO_4 \cdot 5H_2O$, 2 mL of 0.25% solution of neocuproine in ethanol, 6 mL of H_2O	Mixed in rotator for 20 min	450 nm	[63]
Tea, wine, and fruit juices	Gallic acid	5 µL of $CuCl_2$ (150 mM), 5 µL of neocuproine (600 mML), 5 µL of ammonium acetate at pH = 7, 25 µL of examined sample	30 min, room temperature	450 nm	[67]
Apricot	Trolox	1 mL of aqueous solution of $CuCl_2$ (10 mM), 1 mL of ethanolic solution of neocuproine (7.5 mM), 1 mL of ammonium acetate buffer at pH = 7, 1.1 mL of tested sample	30 min, room temperature	450 nm	[65]

$CuCl_2$—copper chloride.

4.3. DPPH

4.3.1. Operating Principle

The development of the DPPH assay dates back to the XX century [70]. Since then, this method has undergone several modifications aimed at improving its stability and reproducibility. Solvents used in the procedure, such as methanol, can influence the reaction mechanism by affecting hydrogen atom release, often favoring SET over HAT [26].

The DPPH assay measures the reduction of the α-diphenyl-β-picrylhydrazyl radical by antioxidants. This radical solution has a deep violet color, which serves as the reference in spectrophotometric measurements [71]. Upon reduction, its color intensity decreases. As a result, an inverse relationship is observed between absorbance and antioxidant concentration. The extent of decolorization is stoichiometrically related to the number of electrons transferred during the reaction. However, the absorbance of the working DPPH solution should not exceed 0.8 ± 0.02, in accordance with the Lambert–Beer law [70]. The reaction involved in this method was presented in Figure 2 [42].

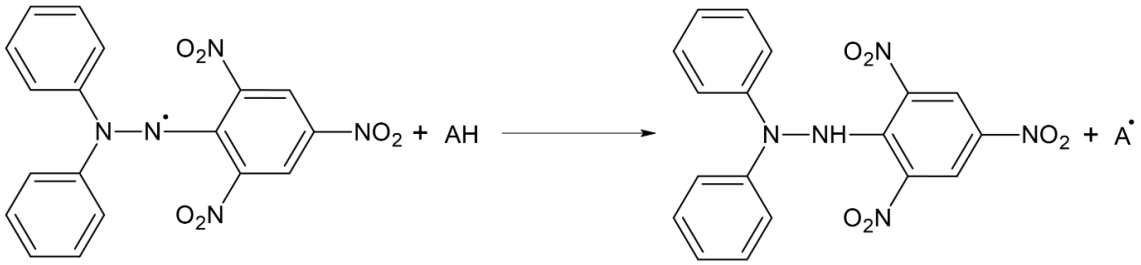


Figure 2. The reaction involved in the DPPH assay.

Despite involving relatively stable free radicals, the absorbance of both the stock and working DPPH solutions is not constant, even when stored at −70 °C. Therefore, a fresh working solution must be prepared prior to each analysis to ensure reliable results. This is essential for constructing an accurate calibration curve, which must be based on the same working solution used for sample analysis [71]. All solutions should be protected from light by storing them in the dark and using amber flasks or tubes during analysis. This precaution is necessary because the DPPH radical is highly sensitive to light and unstable under such conditions [72]. To date, no optimal storage conditions have been identified that would ensure long-term stability of the DPPH solution [73].

A major limitation of the DPPH assay is the absence of a standardized unit of measurement. Results are commonly reported as a percentage of antioxidant activity, as Trolox equivalents, or as IC_{50} (half maximal inhibitory concentration) [71]. These variations make it difficult to compare results across different studies. Moreover, expressing results as a percentage of antioxidant activity fails to account for the initial concentration of the DPPH solution, which significantly affects the outcome. The DPPH radical is also pH-sensitive, with the optimal range for reproducible results being between 5.0 and 6.5 [74]. Another limitation is that DPPH is an artificial and non-physiological radical, so the reactions it undergoes do not reflect those occurring in the human body, where antioxidants exert their biological effects [42]. Furthermore, DPPH has limited biological relevance because of its low operating pH (~5) and structural dissimilarity to naturally occurring reactive free radicals [73].

4.3.2. Comparison of Methodological Approaches Used in Previous Studies of Plant Materials

In the DPPH assay, several inconsistencies exist, ranging from the preparation of the stock DPPH solution to the calculation of measurement units. The concentrations of both the stock and working DPPH solutions vary across studies (Table 3). Such variations can compromise linearity, especially when the concentration of the dissolved substance is too high. Ideally, the concentration of the working solution should range between 50 and 100 μM to achieve an absorbance of 0.8 ± 0.02 [71]. However, some studies either do not report the concentration of the stock DPPH solution or use concentrations significantly higher than 100 μM [75]. These discrepancies in DPPH concentrations make it difficult to compare results across different studies. The absorbance measured in this assay depends on the initial DPPH concentration, which directly affects the final results.

The most significant inconsistency in the DPPH method concerns the way results are reported. Results are most often expressed as the percentage of neutralized radicals, i.e., the percentage activity (I %) of the tested antioxidant [68,76,77]. It is expressed by the formula

$$I\,\% = (A_0 - A_s)/A_0 \times 100$$

A_0—absorbance of control
A_s—absorbance of sample

A key disadvantage of this unit is that it does not account for the initial concentration of the DPPH solution, which directly affects the final absorbance measurement [71]. Moreover, the percentage activity provides no information about the actual amount or concentration of antioxidants in the test sample. Additionally, variations in the volumes of extracts used for analysis are often not accounted for when calculating results in this unit. Therefore, if the concentration of the test DPPH solution is unknown and the volume of extract varies, comparing results across studies may lead to erroneous conclusions [26,71]. An alternative unit is milligrams of Trolox equivalents per 100 g of sample (mg TE/100 g) [69]. However, this approach also fails to consider the initial concentration of the DPPH solution during result conversion. Some studies have reported results using both units—percentage activity and mg TE/100 g—which allows for comparison of discrepancies between them [68,69]. In addition, the results presented in these two units were not always directly proportional.

Due to the instability of the DPPH radical, the test solution should be prepared freshly (ex tempore) and used as soon as possible. When not in use, it should be stored in the dark at refrigeration temperature. Using the same test solution is crucial to ensure consistent absorbance values in both the calibration curve and sample measurements. Only under such conditions can the results be reliably referenced to the standard curve [71].

These discrepancies and limitations affect both the comparability and interpretation of DPPH assay results. Moreover, many researchers optimize various methodological factors, which further complicates comparison of the obtained results. Nonetheless, such discrepancies and inconsistencies are common to all spectrophotometric methods used to assess antioxidant potential.

Table 3. Previous approaches in DPPH assay.

Test Material	Standard	Substrates	Concentration of DPPH Working Solution	Type and Time of Incubation	Units	Wavelength	Source
Beetroot	Nd	3.9 mL DPPH, 0.1 mL of extract	60 µM	30 min, darkness, 37 °C	%	515	[76]
Carrot-orange juice	Nd	2 mL of juice sample, 2 mL DPPH	60 µM	30 min, darkness, room temperature	%	517	[78]
Edible roots	BHT	2.5 mL DPPH,	100 µM	30 min	%, IC_{50}	517	[77]
Carrot	TBHQ	2 mL DPPH, 0.5 mL of extract	1.5 mM	30 min, darkness, room temperature	%, IC_{50}	517	[79]
Cocoa beans	Trolox	3.9 mL DPPH, 0.1 mL extract	60 µM	30 min	TEC	515	[80]
Grape	Ascorbic acid	2.97 mL DPPH, 0.03 mL of extract	15 µM	4 h, room temperature	AAE ascorbic acid eq	515	[81]
Garlic	Ascorbic acid	0.5 mL extract, 0.5 mL DPPH	250 µM	30 min, darkness, room temperature	AAE	517	[82]
Grape	Trolox	0.01 mL extract, 3 mL DPPH	150 µM	60 min, darkness, room temperature	TE	517	[83]
Tea fruit	BHT	1 mL extract, 3 mL DPPH	100 µM	30 min, 30 °C, darkness	%, IC_{50}	517	[84]
Wine	Trolox	0.075 mL extract, 1.425 mL DPPH	100 µM	60 min, darkness, room temp	TE	515	[49]
Tea	Ascorbic acid	1 mL extract, 1 mL DPPH	200 µM	30 min	%	517	[85]
Tea	Quercetin, EGCG	0.1 mL extract, 2.4 mL DPPH	60 µM	30 min, darkness	%	516, 525	[86]

Table 3. *Cont.*

Test Material	Standard	Substrates	Concentration of DPPH Working Solution	Type and Time of Incubation	Units	Wavelength	Source
Tomato juice and carrot juice	α-tocopherol	1.0 mL extract, 0.5 mL DPPH	300 µM	15 min	mol α-TE/mol	540	[86]
Chlorella vulgaris	Nd	25 µL of extract, 975 µL DPPH	60 µM	30 min, darkness	%, IC_{50}	517	[87]

IC_{50}—the concentration of a substance required to inhibit 50% of the DPPH radical activity, ND—no data; BHT—butylated hydroxytoluene; EGCG—epigallocatechin gallate; DPPH—2,2-diphenyl-1-picrylhydrazyl; TBHQ—tert-butylhydroquinone; TE—Trolox equivalent.

4.4. ABTS$^+$ Assay

4.4.1. Mechanism of Action

The 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assay is based on the generation of the green-blue ABTS$^{\bullet+}$ radical, which can be reduced by antioxidants. The reduction of the radical results in a decrease in the color intensity of the solution, and this change is proportional to the reductant content in the test sample (Figure 3). In spectrophotometric measurements, the wavelength used is 734 nm [72]. The ABTS$^{\bullet+}$ radical reacts with potassium persulfate in a 2:1 molar ratio; thus, the ABTS concentration should be at least twice that of potassium persulfate in the reaction mixture [88,89].

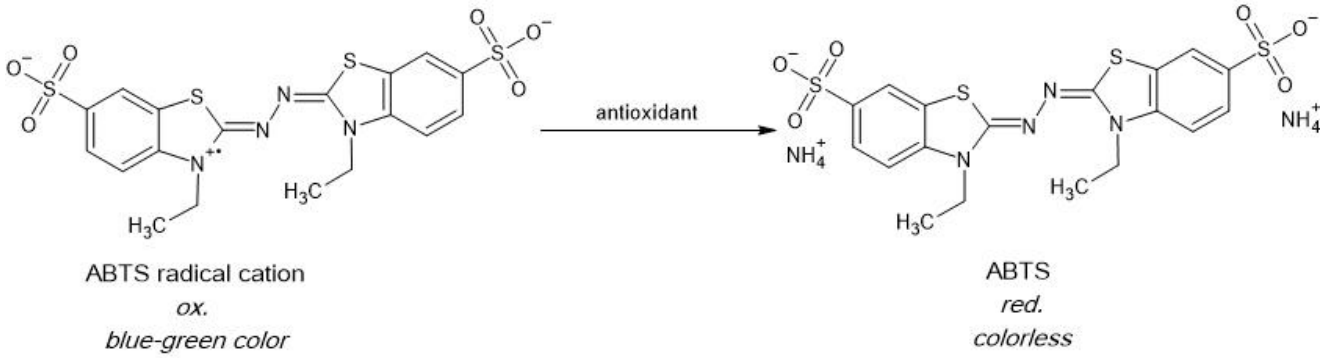


Figure 3. The scheme of reaction occurring between the ABTS radical and the antioxidant.

The ABTS$^{\bullet+}$ radical is soluble in both aqueous and organic solvents, which is a key advantage of the assay, as it enables the determination of both lipophilic and hydrophilic antioxidants. However, because the choice of solvent can affect the reaction kinetics [90], results obtained using different media should not be directly compared [73]. The influence of various solvents on reaction kinetics was investigated using butylated hydroxytoluene (BHT) as the antioxidant agent [91]. The reaction kinetics and the concentration of unreacted ABTS$^{\bullet+}$ after 60 min depended on the type of solvent used. The fastest ABTS$^{\bullet+}$/BHT reaction was observed in 1-propanol, while the slowest occurred in methanol. Additionally, the use of propranolol-1 resulted in a lower percentage of unreacted ABTS$^{\bullet+}$ compared to other solvents [91].

Different solvents may also require adjustments to the measurement wavelength and incubation time to achieve comparable results. It has been shown that an optimal incubation time for ABTS$^{\bullet+}$ is 6 h [88]. Nevertheless, ABTS$^{\bullet+}$ has been shown to remain stable in stock solution (with potassium persulfate) for up to two days when stored in the dark at room temperature [88]. Among the two assays that employ free radicals (DPPH and ABTS), the superior stability of ABTS$^{\bullet+}$ offers a distinct advantage.

4.4.2. Comparison of Methodological Approaches in Previous Research on Plant Materials

The first protocol for the ABTS assay was developed by Re et al. [88]. In their method, ABTS was dissolved in water to a concentration of 7 mM, and 2.45 mM potassium persulfate was added, resulting in the formation of the $ABTS^{\bullet+}$ cation radical and a green-blue color change. The reaction mixture was incubated in the dark at room temperature for 12 to 16 h. After incubation, the $ABTS^{\bullet+}$ solution was diluted with ethanol to achieve an absorbance of 0.7 ± 0.02 at 734 nm. Subsequently, 1 mL of $ABTS^{\bullet+}$ solution was mixed with 10 µL of the ethanolic plant extract, and absorbance was measured at 734 nm after 1 to 6 min, with the entire procedure conducted at 30 °C [89]. This protocol has been widely adopted in subsequent studies [56,92–97]. However, in the following years, researchers attempted to optimize this assay, which led to numerous methodological discrepancies (Table 4). Variations have been reported in the concentrations and absorbance values of the stock solution [98–100], as well as in the measurement wavelength [98–102]. Additionally, discrepancies were observed in the incubation time of the $ABTS^{\bullet+}$ solution, which in some cases was shorter than the recommended minimum of 6 h [100,103].

Table 4. Comparison of previous approaches in the ABTS assay.

Tested Material	Standard	Substrates	Type and Time of Incubation	Wavelength	Source
Apricot	Trolox	1 mL of 10% ABTS radical diluted in ethanol, 4 mL of ethanol, 0.1–0.5 mL of sample extract,	6 min, room temperature	734 nm	[65]
Various plant beverages	Gallic acid	1.425 mL of ABTS working solution [equal volumes of ABTS solution (mM/L) and potassium persulfate solution (2.45 mM) were mixed, and after 16–20 h in the dark, it was diluted with water at the ratio 1:30], 0.075 mL of sample	10 min, room temperature	734 nm	[67]
Meals in kindergarten	Ascorbic acid	3 mL of ABTS working solution [equal volumes of ABTS solution (7 mM) and potassium persulfate solution (2.45 mM) were mixed and after 16 h in the dark at 5 °C, it was diluted with water at the ratio 1:50], 0.1 mL of sample	30 min in darkness, room temperature	734 nm	[56]
Various fruits and vegetables	Ascorbic acid	0.98 mL of ABTS working solution (equal volumes of: 2.5 mM of ABTS solution, 1 mM of 2,2-azobis(2-amidinopropane)dihydrochloride), and 10 mM PBS were mixed and incubated in a water bath at 68 °C for 40 min, then cooled to room temperature and diluted with PBS until an absorbance of 0.65 ± 0.02 was achieved	10 min, 37 °C, water bath	734 nm	[72]
Synthetic mixture of plant antioxidants	Trolox	227 µL of ABTS working solution [equal volumes of ABTS solution (7 mM) and potassium persulfate solution (145 mM) were mixed, and after 16 h in the dark at room temperature, it was diluted with PBS until absorbance of 0.70 ± 0.02], 22.7 µL of sample	6 min, room temperature	734 nm	[104]

ABTS—2,2′-azino-bis-3-ethylbenzthiazoline-6-picrylhydrazyl; PBS—phosphate buffered saline.

4.5. Folin–Ciocalteu Method

4.5.1. Principle of the Method

The Folin–Ciocalteu method is considered one of the most widely used assays in laboratories worldwide for quantifying the total polyphenol content [105]. It is based on the oxidation of hydroxyl groups in phenolic compounds by the Folin–Ciocalteu reagent (FCR) [106]. The blue color of the test solution corresponds to the reduced form of the FCR. The intensity of this hue is directly proportional to the concentration of phenolic compounds in the analyzed sample. An alkaline environment (pH ≈ 10), usually achieved by using an aqueous solution of sodium carbonate (Na_2CO_3) or sodium hydroxide (NaOH), accelerates the reaction. Under these conditions, phenols are more readily deprotonated to phenolates, which have a lower redox potential and thus react more efficiently with FCR. During the redox reaction, molybdenum (Mo) ions in the reagent are reduced from the +6 to the +5 oxidation state, leading to the characteristic blue color of the mixture [107]. Accordingly, the FC assay operates via the SET mechanism and assesses the overall reducing capacity of the sample, using the phenolic content as a primary indicator of antioxidant potential [108]. The general reaction mechanism between phenolic compounds and FCR can be represented as follows:

MoO_4^{2-} (in the reagent, yellow color) −−phenolic compounds−−> MoO_3^{2-} (reduced form, blue color)

The popularity of the FC assay is attributed to its simplicity, speed, and low cost. However, a major limitation of the FC assay is its lack of specificity. Originally developed for protein quantification [106,109], the method is not selective for phenolic compounds. The Folin–Ciocalteu reagent (FCR) can react with hydroxyl groups found in various non-phenolic compounds, such as tryptophan and hydroxylamine [28]. Additionally, reducing agents other than phenolic compounds—such as ascorbic acid and sulfites—can also interfere with FCR reactivity [102]. Due to this lack of specificity, the FC assay should not be directly referred to as a method for determining total phenolic content (TPC) [105]. Another important limitation of the FC assay is the absence of a standardized protocol. Significant methodological variations across studies hinder the direct comparison of results obtained from different experiments.

Due to a lack of specificity and inconsistencies in methodology, it is difficult to properly estimate the TPC in analyzed foods. Consequently, the content of the TPC may be overestimated or underestimated. This may be the reason for the erroneous determination of the antioxidant potential of food products.

4.5.2. Comparison of Analytical Approaches Plant-Based Studies

Organic solvents are commonly employed for extraction purposes; however, their selection varies among studies (Table 5). The most commonly used solvents are n-hexane, acetone, methanol, and ethanol, often combined with Milli-Q water [35,110]. The influence of solvent type and concentration on the efficiency of polyphenol extraction has been extensively investigated. For instance, higher polyphenol content was observed in extracts obtained using 70% ethanol in water compared to those obtained with 70% methanol, absolute ethanol, or absolute methanol [111,112]. Similarly, another study found 50% methanol to be more effective than acetone or ethanol at various concentrations [113]. Ammar et al. [114] concluded that greater solvent polarity enhances extraction efficiency. In their study, the effectiveness of the diluents followed the order: methanolic extract > ethanolic extract > acetone extract > diethyl ether extract > ethyl acetate extract > hexane extract [114].

Table 5. Comparison of previous approaches in FC assay.

Tested Material	Standard	Substrates	Type and Time of Incubation	Wavelength	Source
Soybean, green tea extracts	Gallic acid	0.25 mL of extract, 0.25 mL FCR and 4.5 mL 20% Na_2CO_3	25 min, room temperature	730 nm	[115]
Strawberries	Gallic acid	0.05 mL of extract, 0.43 mL MQ, 0.02 mL FCR, 0.05 mL 20% Na_2CO_3, 0.45 mL MQ	60 min, room temperature	725 nm	[116]
Black tea	Gallic acid	1 mL of diluted extract, 1 mL of 3-fold-diluted FCR, 2 mL of 35% Na_2CO_3	30 min, room temperature	700 nm	[117]
Vegetable juices	Gallic acid	0.2 mL of extract, 1.5 mL of 10-fold-diluted FCR, 1.5 mL of 6% Na_2CO_3	90 min, room temperature	725 nm	[118]
Berries	Gallic acid	0.5 mL of extract, 2.5 mL of FCR, 2 mL 7.5% Na_2CO_3	60 min, room temperature	765 nm	[119]
Tomato and apple juices	Quercetin	0.5 mL of extract, 2.4 mL MQ, 0.1 mL FCR, 2 mL 2% Na_2CO_3	60 min, room temperature	750 nm	[120]
Fruit juices	Gallic acid	0.1 mL of extract, 0.5 mL MQ, 0.1 mL FCR, 1 mL of 7% Na_2CO_3, 0.5 MQ	90 min	760 nm	[121]
Pickled Chinese cabbage	Gallic acid	0.5 mL of extract, 2.5 mL FCR, 2 mL of 7.5% Na_2CO_3,	60 min, darkness	760 nm	[122]
Cabbage: white, Chinese, and red	Gallic acid	0.05 mL of extract, 0.1 FCR, 2 mL 1% Na_2CO_3,	90 min, room temperature	750 nm	[123]
Dovyalis caffra	Gallic acid	0.3 mL of extract, 0.5 mL of FCR, 5.8 mL of MQ, 1.5 mL of 20% Na_2CO_3	2 h, room temperature	760 nm	[124]
Tea extracts	Ascorbic acid	0.1–0.3 mL of tea sample, 0.5 mL of FCR, 0.5 mL of 30% NaOH	30 min, room temperature	630 nm	[63]
Buckwheat	Gallic acid	0.3 mL of sample, 1.5 mL of FCR, 1.2 mL of 7.5% Na_2CO_3	30 min, room temperature	765 nm	[125]

FCR—Folin–Ciocalteu Reagent; FC—Folin–Ciocalteu assay; MQ—Milli-Q water.

Therefore, solvents with higher polarity, particularly when mixed with water, tend to yield more efficient polyphenol extraction.

5. Hydrogen Atom Transfer-Based Assays

5.1. Oxygen Radical Absorbance Capacity (ORAC)

5.1.1. Method Principle

The oxygen radical absorbance capacity (ORAC) method, developed by Cao, Alessio, and Cutler [126], is based on measuring the decrease in the fluorescence of fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one). Moreover, the ORAC assay enables the assessment of peroxyl radical scavenging, with radicals generated by 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH). This compound generates peroxyl radicals that oxidize fluorescein; however, in the presence of antioxidants, degradation is delayed, and fluorescence is maintained.

In this system, AAPH acts as a peroxyl radical initiator, fluorescein serves as the molecular probe, and the antioxidant functions as the radical scavenger. Phosphate buffer is used as a blank sample [127]. The ORAC reaction mechanism is illustrated in Figure 4.

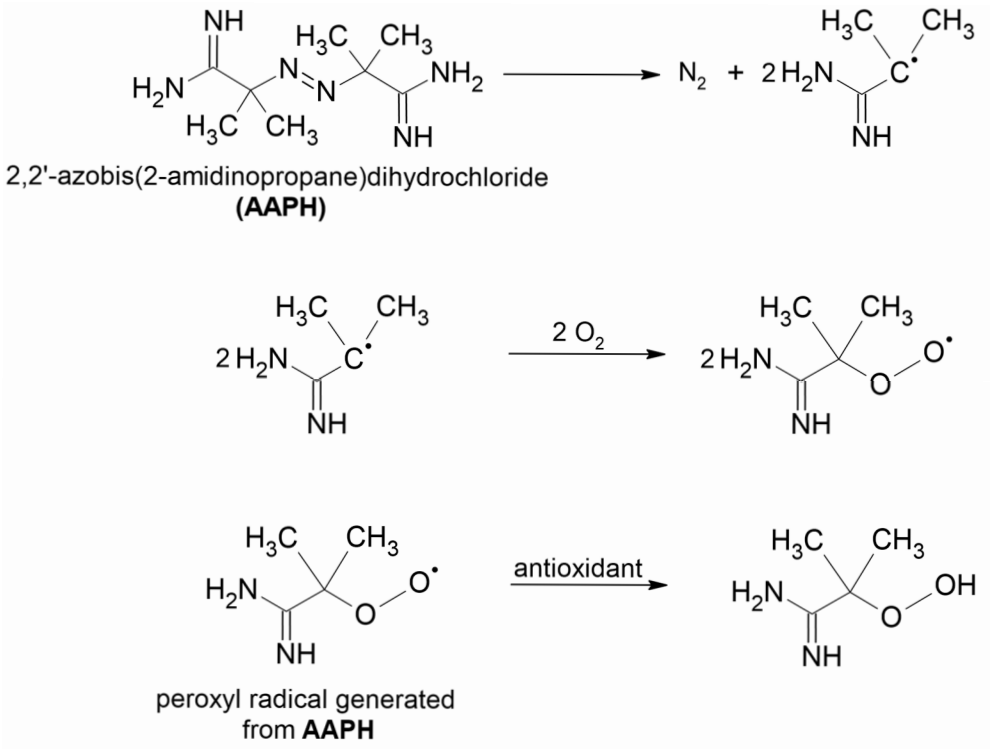


Figure 4. The reactions occurring during the ORAC assay. AAPH—2,2′-azobis(2-methylpropionamidine) dihydrochloride.

The ORAC assay measures both hydrophilic and lipophilic antioxidant capacities against biologically relevant peroxyl radicals. This feature makes ORAC superior to other spectrophotometric assays for assessing antioxidant potential, as the radicals used in the test are more representative of those found in biological systems [128,129]. The ORAC scale is used by the United States Department of Agriculture (USDA) to compare the antioxidant activity of various foods [130]. Notably, other oxidants present in the body, such as singlet oxygen, are not accounted for in the ORAC assay [25]. However, the assay may be affected by secondary reactions related to the radical chain-breaking process or the presence of metal ions [131]. Elements like Cu, Fe, zinc (Zn), and aluminum (Al) can reduce the antioxidant capacity measured by the ORAC assay. This effect is attributed to the formation of phenol-metal complexes. The release of phenolic compounds can increase the antioxidant potential of the test sample [132]. Moreover, the use of solubility enhancers, such as cyclodextrins, in the ORAC assay may interfere with the measurement of antioxidant activity, as demonstrated in previous studies [133,134]. Nevertheless, cyclodextrins are still used in the ORAC assay [135].

5.1.2. Comparative Analysis of Methodological Approaches in Previous Studies on Plant Materials

In the original ORAC protocol, β-phycoerythrin (β-PE) was used as the fluorescent probe; however, it exhibited several disadvantages, including photosensitivity and batch-to-batch variability. Consequently, fluorescein was adopted as a replacement for β-PE, effectively eliminating these issues [136]. Pyrogallol red has also been evaluated as a fluorescent probe; however, the results obtained showed no correlation with ORAC-fluorescein values. Moreover, the antioxidant capacity measured with fluorescein was significantly higher than that obtained with pyrogallol red [137]. Although the ORAC assay follows a relatively standardized methodology, variations in wavelengths have been reported across studies (Table 6). There is a need to establish the most suitable wavelength to enable better

comparison of results across studies. In most studies, the wavelength is optimized within each laboratory and depends on the food matrix (Table 6). These discrepancies can directly influence the results, which are expressed as the net area under the curve (AUC). The rate of fluorescence loss in the blank sample is compared with that of the test sample [24].

Table 6. Comparison of previous approaches in the ORAC assay.

Tested Material	Standard	Substrates	Type and Time of Incubation	Wavelength	Source
Various plants extracts	Trolox	0.3 mL of extract, 75 mM phosphate buffer (pH 7.4), 1.8 mL of fluorescein (70 nM), 0.9 mL of AAPH (12 mM)	60 min, 27 °C	530 nm	[138]
Polyphenols	Trolox	20 µL of sample (75 mM phosphate buffer (pH 7.0) mixed with 10 mg of test sample), 0.2 mL of fluorescein (94.4 nM), 75 µL of AAPH (31.7 mM)	10 min, 37 °C	528 nm	[139]
Oranges	Trolox	50 µL of extract, 75 mM phosphate buffer (pH 7.0), 50 µL of fluorescein (78 nM), 25 µL of AAPH (221 mM)	15 min, 37 °C	535 nm	[136]
Berries	Trolox	100 µL of extract, 75 mM phosphate buffer (pH 7.4), 60 µL of fluorescein (200 nM), 40 µL of AAPH (60 mM)	10 min, 37 °C	527 nm	[140]
Maple syrup	Trolox	120 µL of extract, 75 mM phosphate buffer (pH 7.4), 30 µL of fluorescein (400 nM), 50 µL of AAPH (200 mM)	15 min, 37 °C	527 nm	[57]
Chlorella vulgaris	Trolox	100 µL of extract, 75 mM phosphate buffer (pH 7.4), 800 µL of fluorescein (40 nM), 100 µL of AAPH (400 mM)	nd	520 nm	[87]
Acai pulp	Trolox	100 µL of extract, 75 mM phosphate buffer (pH 7.0), 2.6 mL of fluorescein (40 nM), 300 µL of AAPH (200 mM)	30 min, 37 °C	520 nm	[141]

AAPH—2,2′-Azobis (2-methylpropionamidine) dihydrochloride; nd—no data.

6. Summary and Future Challenges

This article critically reviewed widely applied spectrophotometric methods for evaluating antioxidant potential. A key challenge in this area is the lack of standardized protocols, leading to considerable variability in standards, solvents, extraction procedures, incubation times, and assay conditions [142,143]. These methodological differences can influence reaction kinetics, potentially leading to inconsistent and inaccurate results.

Furthermore, the use of varying measurement units within the same assay hinders the direct comparison of results. This is especially noticeable in the DPPH method, where a wide range of units are in use. To date, no consensus has been reached on a universal unit for reporting antioxidant capacity.

In addition, extrapolating in vitro findings to in vivo conditions remains challenging due to significant differences in radical species and pH levels between experimental assays and the human body [26]. Nevertheless, some methods show greater biological relevance. The CUPRAC assay, for instance, operates at a near-physiological pH, while the ORAC method targets peroxyl radicals commonly present in the human body. These characteristics may improve the applicability of in vitro results to in vivo conditions.

The popularity of spectrophotometric techniques stems largely from their simplicity, low cost, and ease of use. According to the Association of Official Analytical Collaboration (AOAC), existing guidelines mainly focus on validation procedures [144]. This may explain

why most researchers tend to optimize nearly all parameters of a given method, including incubation time, temperature, solvent type, and wavelength.

Undoubtedly, the evaluation of antioxidant activity should not be performed by a single assay. The use of multiple techniques, each representing different reaction mechanisms of bioactive compounds, increases the likelihood of accurately assessing their antioxidant capacity. A positive correlation between results from different methods strengthens the validity of the overall outcomes.

Nevertheless, significant differences in methodology still pose a major problem to comparing data across studies. Variations in reagent selection, experimental protocols, and reaction conditions remain key factors contributing to this lack of consistency (Table 7). Among the methods discussed, CUPRAC has shown the highest consistency. This was confirmed in a study quantifying measurement uncertainty, which reported a relative uncertainty of ±3.05% at a 95% confidence interval based on apple juice analysis [145].

In contrast, the DPPH method exhibits the greatest variability among all the approaches, encompassing both methodological differences and units that do not accurately reflect the true antioxidant activity. Research indicates that any alteration in these elements can significantly raise uncertainty levels, with relative standard deviations reaching up to 11%, depending on the tested substance [146–148]. Nonetheless, the results of antioxidant potential assays are generally positively correlated [69,149], supporting the rationale for using multiple assays to obtain a more reliable assessment.

Among radical-based techniques, $ABTS^{\bullet+}$ demonstrates greater repeatability and chemical stability compared to DPPH, which is highly susceptible to environmental factors. Moreover, none of the current spectrophotometric assays fully replicate physiological conditions, as the reactive species and chemical reagents employed are not naturally occurring in vivo. Still, certain protocols—such as ORAC and FRAP—incorporate biologically meaningful elements like peroxyl radicals and iron ions. These are, however, constrained by non-physiological pH levels: 3.6 in FRAP and 10 in the Folin–Ciocalteu assay. In contrast, the CUPRAC method operates at pH 7.0, aligning more closely with human biological conditions and offering an advantage among SET-based techniques.

Table 7. The advantages and disadvantages of antioxidant spectrophotometric assays.

Method	Feature	Advantage	Limitation	Explanation
FRAP	pH		X	pH = 3.6 below physiological pH
	Wavelength	X		593 nm Repeatability
	Biological molecules	X		The method includes Fe ions, which naturally occur in the body
	Substrates used in the method		X	Various molar ratios between FRAP reagent and the sample
CUPRAC	pH	X		pH = 7 similar to physiological pH
	Wavelength	X		450 nm Repeatability
	Biological molecules		X	There is a lack of biologically relevant molecules
	Substrates used in the method	X		Repeatability of substrates and the molar ratio among reagents

Table 7. *Cont.*

Method	Feature	Advantage	Limitation	Explanation
DPPH	pH		X	pH = 5.0–6.5 below physiological pH
	Wavelength		X	Various e.g., 515, 516, 517, 525, 540
	Biological molecules		X	There is a lack of biologically relevant molecules
	Substrates used in the method		X	Lack of a standardized concentration of DPPH solution
$ABTS^{+}$	pH		X	pH ~ 4.5–7.0 (near neutral): however, large pH deviations may affect the reaction
	Wavelength	X		734 nm Repeatability
	Biological molecules		X	There is a lack of biologically relevant molecules
	Substrates used in the method		X	Variability in the concentrations of ABTS radical in the working solution
FC	pH		X	pH = 10 above physiological pH
	Wavelength		X	700–760 nm—results can differ significantly (high discrepancy)
	Biological molecules		X	There is a lack of biologically relevant molecules
	Substrates used in the method		X	Lack of unification FCR can interact with hydroxyl groups present in various compounds, not just phenols
ORAC	pH	X		pH = 7.0–7.4 similar to physiological pH
	Wavelength		X	e.g., 528, 530, 535 nm High discrepancy
	Biological molecules	X		It allows for the assessment of the scavenging activity against peroxyl radicals
	Substrates used in the method		X	Divergent concentrations of AAPH

A further obstacle is the inconsistent reporting of antioxidant capacity, often using multiple units within a single assay framework. This inconsistency hampers data interpretation and cross-study comparisons.

Currently, no standard method exists for evaluating the antioxidant potential of plant-based foods. There is an urgent need to establish a reference method to guide and unify future analytical approaches.

7. Conclusions

Numerous spectrophotometric methods are available for assessing the antioxidant potential of plant-based foods, primarily due to their simplicity and low cost. However, substantial methodological inconsistencies have been reported across different assays. Given the widespread application of in vitro spectrophotometric techniques, there is an urgent need to standardize current protocols or to establish a universal reference method.

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References

1. Bhatti, J.S.; Bhatti, G.K.; Reddy, P.H. Mitochondrial Dysfunction and Oxidative Stress in Metabolic Disorders—A Step towards Mitochondria Based Therapeutic Strategies. *Biochim. Biophys. Acta* **2017**, *1863*, 1066. [CrossRef] [PubMed]
2. Christ, A.; Lauterbach, M.; Latz, E. Western Diet and the Immune System: An Inflammatory Connection. *Immunity* **2019**, *51*, 794–811. [CrossRef]
3. Huang, M.Z.; Li, J.Y. Physiological Regulation of Reactive Oxygen Species in Organisms Based on Their Physicochemical Properties. *Acta Physiol.* **2020**, *228*, e13351. [CrossRef]
4. Tan, Y.; Duan, Y.; Chi, Q.; Wang, R.; Yin, Y.; Cui, D.; Li, S.; Wang, A.; Ma, R.; Li, B.; et al. The Role of Reactive Oxygen Species in Plant Response to Radiation. *Int. J. Mol. Sci.* **2023**, *24*, 3346. [CrossRef] [PubMed]
5. Brieger, K.; Schiavone, S.; Miller, F.J.; Krause, K.H. Reactive Oxygen Species: From Health to Disease. *Swiss Med. Wkly.* **2012**, *142*, w13659. [CrossRef]
6. Cardoso, M.A.; Gonçalves, H.M.R.; Davis, F. Reactive Oxygen Species in Biological Media Are They Friend or Foe? Major In Vivo and In Vitro Sensing Challenges. *Talanta* **2023**, *260*, 124648. [CrossRef]
7. Srinivas, U.S.; Tan, B.W.Q.; Vellayappan, B.A.; Jeyasekharan, A.D. ROS and the DNA Damage Response in Cancer. *Redox Biol.* **2019**, *25*, 101084. [CrossRef]
8. Forman, H.J.; Zhang, H. Targeting Oxidative Stress in Disease: Promise and Limitations of Antioxidant Therapy. *Nat. Rev. Drug Discov.* **2021**, *20*, 689–709. [CrossRef] [PubMed]
9. Pérez-Torres, I.; Castrejón-Téllez, V.; Soto, M.E.; Rubio-Ruiz, M.E.; Manzano-Pech, L.; Guarner-Lans, V. Oxidative Stress, Plant Natural Antioxidants, and Obesity. *Int. J. Mol. Sci.* **2021**, *22*, 1786. [CrossRef]
10. Albarracin, S.L.; Stab, B.; Casas, Z.; Sutachan, J.J.; Samudio, I.; Gonzalez, J.; Gonzalo, L.; Capani, F.; Morales, L.; Barreto, G.E. Effects of Natural Antioxidants in Neurodegenerative Disease. *Nutr. Neurosci.* **2012**, *15*, 1–9. [CrossRef]
11. Christodoulou, M.C.; Orellana Palacios, J.C.; Hesami, G.; Jafarzadeh, S.; Lorenzo, J.M.; Domínguez, R.; Moreno, A.; Hadidi, M. Spectrophotometric Methods for Measurement of Antioxidant Activity in Food and Pharmaceuticals. *Antioxidants* **2022**, *11*, 2213. [CrossRef] [PubMed]
12. Podsedek, A. Natural Antioxidants and Antioxidant Capacity of Brassica Vegetables: A Review. *LWT–Food Sci. Technol.* **2007**, *40*, 1–11. [CrossRef]
13. Khan, N.; Mukhtar, H. Tea Polyphenols in Promotion of Human Health. *Nutrients* **2018**, *11*, 39. [CrossRef] [PubMed]
14. Rasines-Perea, Z.; Teissedre, P.L. Grape Polyphenols' Effects in Human Cardiovascular Diseases and Diabetes. *Molecules* **2017**, *22*, 68. [CrossRef]
15. Gorzynik-Debicka, M.; Przychodzen, P.; Cappello, F.; Kuban-Jankowska, A.; Gammazza, A.M.; Knap, N.; Wozniak, M.; Gorska-Ponikowska, M. Potential Health Benefits of Olive Oil and Plant Polyphenols. *Int. J. Mol. Sci.* **2018**, *19*, 686. [CrossRef] [PubMed]
16. Xiao, F.; Xu, T.; Lu, B.; Liu, R. Guidelines for Antioxidant Assays for Food Components. *Food Front.* **2020**, *1*, 60–69. [CrossRef]
17. Olasunkanmi, A.A.; Fadahunsi, O.S.; Adegbola, P.I. Gas Chromatography-Mass Spectroscopic, High Performance Liquid Chromatographic and In-Silico Characterization of Antimicrobial and Antioxidant Constituents of Rhus Longipes (Engl). *Arab. J. Chem.* **2022**, *15*, 103601. [CrossRef]
18. Meng, M.; Wan, H.; Bao, Y.; He, Y.; Li, C.; Wan, H. Rapid Identification, Quantitation, and Antioxidant Activity Evaluation of the Components in Guanxin Shutong Capsule with Liquid Chromatography and Mass Spectrometry. *J. Pharm. Biomed. Anal.* **2023**, *224*, 115194. [CrossRef]
19. Kumar, Y.; Singhal, S.; Tarafdar, A.; Pharande, A.; Ganesan, M.; Badgujar, P.C. Ultrasound Assisted Extraction of Selected Edible Macroalgae: Effect on Antioxidant Activity and Quantitative Assessment of Polyphenols by Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS). *Algal Res.* **2020**, *52*, 102114. [CrossRef]

20. Luo, J.; Liang, L.; Xie, Q.; Qiu, Y.; Jiang, S.; Yang, Y.; Zhu, L.; Fu, Y.; Chen, S.; Wang, W.; et al. Differential Analysis of Phytochemistry and Antioxidant Activity in Five Citrus By-Products Based on Chromatography, Mass Spectrometry, and Spectrum-Effect Relationships. *Food Chem. X* **2023**, *20*, 101010. [CrossRef]
21. Jimoh, M.O.; Jimoh, M.A.; Kerebba, N.; Bakare, O.O.; Senjobi, C.T.; Saheed, S.A.; Kadye, R.; Prinsloo, E.; Laubscher, C.P. Liquid Chromatography–Mass Spectrometry and XCELLigence Real Time Cell Analyzer Revealed Anticancer and Antioxidant Metabolites in *Trianthema portulacastrum* L. (Aizoaceae). *Phytomed. Plus* **2024**, *4*, 100550. [CrossRef]
22. Yang, X.; Li, Y.; Shao, Q.; Li, Z.; Chun, Z.; Wang, Y.; Zhou, Y.; Chen, R. Screening, Fingerprinting, and Identification of Phenolic Antioxidants in *Persicaria chinensis* (L.) H. Gross by Liquid Chromatography–Electrochemical Detection and Liquid Chromatography–Tandem Mass Spectrometry. *J. Chromatogr. B* **2025**, *1250*, 124387. [CrossRef] [PubMed]
23. Gulcin, İ. Antioxidants and Antioxidant Methods: An Updated Overview. *Arch. Toxicol.* **2020**, *94*, 651–715. [CrossRef]
24. Prior, R.L.; Wu, X.; Schaich, K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302. [CrossRef]
25. Apak, R.; Özyürek, M.; Güçlü, K.; Çapanoğlu, E. Antioxidant Activity/Capacity Measurement. 1. Classification, Physicochemical Principles, Mechanisms, and Electron Transfer (ET)-Based Assays. *J. Agric. Food Chem.* **2016**, *64*, 997–1027. [CrossRef]
26. Schaich, K.M.; Tian, X.; Xie, J. Reprint of "Hurdles and Pitfalls in Measuring Antioxidant Efficacy: A Critical Evaluation of ABTS, DPPH, and ORAC Assays". *J. Funct. Foods* **2015**, *18*, 782–796. [CrossRef]
27. Munteanu, I.G.; Apetrei, C. Analytical Methods Used in Determining Antioxidant Activity: A Review. *Int. J. Mol. Sci.* **2021**, *22*, 3380. [CrossRef] [PubMed]
28. Tan, J.B.L.; Lim, Y.Y. Critical Analysis of Current Methods for Assessing the in Vitro Antioxidant and Antibacterial Activity of Plant Extracts. *Food Chem.* **2015**, *172*, 814–822. [CrossRef]
29. Shahidi, F.; Pinaffi-Langley, A.C.C.; Fuentes, J.; Speisky, H.; de Camargo, A.C. Vitamin E as an Essential Micronutrient for Human Health: Common, Novel, and Unexplored Dietary Sources. *Free Radic. Biol. Med.* **2021**, *176*, 312–321. [CrossRef]
30. See, X.Z.; Yeo, W.S.; Saptoro, A. A Comprehensive Review and Recent Advances of Vitamin C: Overview, Functions, Sources, Applications, Market Survey and Processes. *Chem. Eng. Res. Des.* **2024**, *206*, 108–129. [CrossRef]
31. Ribaya-Mercado, J.D.; Maramag, C.C.; Tengco, L.W.; Dolnikowski, G.G.; Blumberg, J.B.; Solon, F.S. Carotene-Rich Plant Foods Ingested with Minimal Dietary Fat Enhance the Total-Body Vitamin A Pool Size in Filipino Schoolchildren as Assessed by Stable-Isotope-Dilution Methodology. *Am. J. Clin. Nutr.* **2007**, *85*, 1041–1049. [CrossRef] [PubMed]
32. Charlton, N.C.; Mastyugin, M.; Török, B.; Török, M. Structural Features of Small Molecule Antioxidants and Strategic Modifications to Improve Potential Bioactivity. *Molecules* **2023**, *28*, 1057. [CrossRef] [PubMed]
33. Igielska-Kalwat, J.; Gościańska, J.; Nowak, I. Carotenoids as Natural Antioxidants. *Postep. Hig. Med. Dosw.* **2015**, *69*, 418–428. [CrossRef] [PubMed]
34. Cömert, E.D.; Gökmen, V. Evolution of Food Antioxidants as a Core Topic of Food Science for a Century. *Food Res. Int.* **2018**, *105*, 76–93. [CrossRef]
35. Xu, D.P.; Li, Y.; Meng, X.; Zhou, T.; Zhou, Y.; Zheng, J.; Zhang, J.J.; Li, H. Bin Natural Antioxidants in Foods and Medicinal Plants: Extraction, Assessment and Resources. *Int. J. Mol. Sci.* **2017**, *18*, 96. [CrossRef]
36. Lorenzo, J.M.; Munekata, P.E.S.; Gómez, B.; Barba, F.J.; Mora, L.; Pérez-Santaescolástica, C.; Toldrá, F. Bioactive Peptides as Natural Antioxidants in Food Products—A Review. *Trends Food Sci. Technol.* **2018**, *79*, 136–147. [CrossRef]
37. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Anal. Biochem.* **1996**, *239*, 70–76. [CrossRef]
38. Du, B.; Xu, B. Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) of β-Glucans from Different Sources with Various Molecular Weight. *Bioact. Carbohydr. Diet. Fibre* **2014**, *3*, 11–16. [CrossRef]
39. Müller, L.; Fröhlich, K.; Böhm, V. Comparative Antioxidant Activities of Carotenoids Measured by Ferric Reducing Antioxidant Power (FRAP), ABTS Bleaching Assay (ATEAC), DPPH Assay and Peroxyl Radical Scavenging Assay. *Food Chem.* **2011**, *129*, 139–148. [CrossRef]
40. Hayes, W.A.; Mills, D.S.; Neville, R.F.; Kiddie, J.; Collins, L.M. Determination of the Molar Extinction Coefficient for the Ferric Reducing/Antioxidant Power Assay. *Anal. Biochem.* **2011**, *416*, 202–205. [CrossRef]
41. Gliszczynska-Swiglo, A. Antioxidant Activity of Water Soluble Vitamins in the TEAC (Trolox Equivalent Antioxidant Capacity) and the FRAP (Ferric Reducing Antioxidant Power) Assays. *Food Chem.* **2006**, *96*, 131–136. [CrossRef]
42. Rumpf, J.; Burger, R.; Schulze, M. Statistical Evaluation of DPPH, ABTS, FRAP, and Folin-Ciocalteu Assays to Assess the Antioxidant Capacity of Lignins. *Int. J. Biol. Macromol.* **2023**, *233*, 123470. [CrossRef] [PubMed]
43. Avelino, F. Ferric Reducing Antioxidant Power (FRAP). In *Antioxidant Methods*; Academic Press: New York, NY, USA, 2024; pp. 123–133. [CrossRef]
44. van Wonderen, D.; Melse-Boonstra, A.; Gerdessen, J.C. Iron Bioavailability Should Be Considered When Modeling Omnivorous, Vegetarian, and Vegan Diets. *J. Nutr.* **2023**, *153*, 2125–2132. [CrossRef]

45. Tomova, T.; Petkov, V.; Slavova, I.; Stoyanov, P.; Argirova, M. Naturally Present Metal Ions in Plants Could Interfere with Common Antioxidant Assays. *MethodsX* **2020**, *7*, 100995. [CrossRef] [PubMed]
46. Çelik, S.E.; Özyürek, M.; Güçlü, K.; Apak, R. Solvent Effects on the Antioxidant Capacity of Lipophilic and Hydrophilic Antioxidants Measured by CUPRAC, ABTS/Persulphate and FRAP Methods. *Talanta* **2010**, *81*, 1300–1309. [CrossRef]
47. Samid, I.; Khouya, T.; Elbouny, H.; Sellam, K.; Alem, C. Quantification of Phytochemical and Antioxidant Properties of Aqueous Apple Extracts from the High Atlas Mountains in Morocco and Their Anti-Inflammatory Effect on Wistar Rats. *Sci. Afr.* **2023**, *22*, 01897. [CrossRef]
48. Guo, C.; Yang, J.; Wei, J.; Li, Y.; Xu, J.; Jiang, Y. Antioxidant Activities of Peel, Pulp and Seed Fractions of Common Fruits as Determined by FRAP Assay. *Nutr. Res.* **2003**, *23*, 1719–1726. [CrossRef]
49. Liang, Z.; Zhang, P.; Ma, W.; Zeng, X.A.; Fang, Z. Physicochemical Properties, Antioxidant Activities and Comprehensive Phenolic Profiles of Tea-Macerated Chardonnay Wine and Model Wine. *Food Chem.* **2024**, *436*, 137748. [CrossRef]
50. Li, W.; Zhang, Y.; Deng, H.; Yuan, H.; Fan, X.; Yang, H.; Tan, S. In Vitro and in Vivo Bioaccessibility, Antioxidant Activity, and Color of Red Radish Anthocyanins as Influenced by Different Drying Methods. *Food Chem. X* **2023**, *18*, 100633. [CrossRef]
51. Adrar, N.; Gulsunoglu-Konuskan, Z.; Ceylan, F.D.; Capanoglu, E. Overview and Trends in Electrochemical Sensors, Biosensors and Cellular Antioxidant Assays for Oxidant and Antioxidant Determination in Food. *Talanta* **2025**, *283*, 127058. [CrossRef]
52. Michiels, J.A.; Kevers, C.; Pincemail, J.; Defraigne, J.O.; Dommes, J. Extraction Conditions Can Greatly Influence Antioxidant Capacity Assays in Plant Food Matrices. *Food Chem.* **2012**, *130*, 986–993. [CrossRef]
53. Thaipong, K.; Boonprakob, U.; Crosby, K.; Cisneros-Zevallos, L.; Hawkins Byrne, D. Comparison of ABTS, DPPH, FRAP, and ORAC Assays for Estimating Antioxidant Activity from Guava Fruit Extracts. *J. Food Compos. Anal.* **2006**, *19*, 669–675. [CrossRef]
54. Thavamoney, N.; Sivanadian, L.; Tee, L.H.; Khoo, H.E.; Prasad, K.N.; Kong, K.W. Extraction and Recovery of Phytochemical Components and Antioxidative Properties in Fruit Parts of Dacryodes Rostrata Influenced by Different Solvents. *J. Food Sci. Technol.* **2018**, *55*, 2523. [CrossRef]
55. Orosz-Tóth, M.; Nemes-Kun, A.; Lowy, D.A.; Csihon, Á.; Sándor, Z.; Kincses, I.; Holb, I.J. Comparison and Intercorrelation of Extraction Methods for Polyphenol Content and Antioxidant Capacity of Scab-Resistant Apple Cultivars. *Agronomy* **2022**, *12*, 289. [CrossRef]
56. Berlic, M.; Jug, U.; Battelino, T.; Levart, A.; Dimitrovska, I.; Albreht, A.; Korošec, M. Antioxidant-Rich Foods and Nutritional Value in Daily Kindergarten Menu: A Randomized Controlled Evaluation Executed in Slovenia. *Food Chem.* **2023**, *404*, 134566. [CrossRef]
57. Xiao, L.; Liu, J.; Hua, M.Z.; Lu, X. Rapid Determination of Total Phenolic Content and Antioxidant Capacity of Maple Syrup Using Raman Spectroscopy and Deep Learning. *Food Chem.* **2025**, *463*, 141289. [CrossRef]
58. Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S.E. Novel Total Antioxidant Capacity Index for Dietary Polyphenols and Vitamins C and E, Using Their Cupric Ion Reducing Capability in the Presence of Neocuproine: CUPRAC Method. *J. Agric. Food Chem.* **2004**, *52*, 7970–7981. [CrossRef] [PubMed]
59. Cárdenas, A.; Gomez, M.; Frontana, C. Electrochemical Method to Quantify Antioxidants Employing Cupric Reducing Antioxidant Capacity, CUPRAC. *Procedia Chem.* **2014**, *12*, 62–65. [CrossRef]
60. Özyürek, M.; Güçlü, K.; Apak, R. The Main and Modified CUPRAC Methods of Antioxidant Measurement. *Trends Anal. Chem.* **2011**, *30*, 652–664. [CrossRef]
61. Emu, S.A.; Dulal, M.A.; Kali, T.D.; Chadni, M.S.; Rasul, M.G.; Mondal, M.N.; Ahsan, M.E.; Khan, M.; Shah, A.K.M.A. Effects of Extracting Solvents on Phytochemical, Antioxidant, and Antibacterial Activity of Some Seaweeds from the Bay of Bengal Offshore Island. *Food Humanit.* **2023**, *1*, 1157–1166. [CrossRef]
62. Granato, D.; Fidelis, M.; Haapakoski, M.; dos Santos Lima, A.; Viil, J.; Hellström, J.; Rätsep, R.; Kaldmäe, H.; Bleive, U.; Azevedo, L.; et al. Enzyme-Assisted Extraction of Anthocyanins and Other Phenolic Compounds from Blackcurrant (*Ribes nigrum* L.) Press Cake: From Processing to Bioactivities. *Food Chem.* **2022**, *391*, 133240. [CrossRef] [PubMed]
63. Krylova, E.; Gavrilenko, N.; Saranchina, N.; Gavrilenko, M. Novel Colorimetric Sensor for Cupric Reducing Antioxidant Capacity (CUPRAC) Measurement. *Procedia Eng.* **2016**, *168*, 355–358. [CrossRef]
64. Özyürek, M.; Bektaşoğlu, B.; Güçlü, K.; Güngör, N.; Apak, R. Simultaneous Total Antioxidant Capacity Assay of Lipophilic and Hydrophilic Antioxidants in the Same Acetone–Water Solution Containing 2% Methyl-β-Cyclodextrin Using the Cupric Reducing Antioxidant Capacity (CUPRAC) Method. *Anal. Chim. Acta* **2008**, *630*, 28–39. [CrossRef] [PubMed]
65. Güçlü, K.; Altun, M.; Özyürek, M.; Karademir, S.E.; Apak, R. Antioxidant Capacity of Fresh, Sun- and Sulphited-Dried Malatya Apricot (Prunus Armeniaca) Assayed by CUPRAC, ABTS/TEAC and Folin Methods. *Int. J. Food Sci. Technol.* **2006**, *41*, 76–85. [CrossRef]
66. Bayrakçeken Güven, Z.; Alshehri, O.; Yüce, N.; Bakan, E.; Demirci, B.; Yilmaz, M.A.; Ertas, A.; Basaran, A.A. Chemical Composition, Nutritional Values, Elemental Analysis and Biological Properties of Prunus Mahaleb L.: From Waste to New Potential Sources for Food, Cosmetic and Drug Industry. *Food Biosci.* **2023**, *53*, 102632. [CrossRef]

67. Puangbanlang, C.; Sirivibulkovit, K.; Nacapricha, D.; Sameenoi, Y. A Paper-Based Device for Simultaneous Determination of Antioxidant Activity and Total Phenolic Content in Food Samples. *Talanta* **2019**, *198*, 542–549. [CrossRef]
68. Brzezińska-Rojek, J.; Sagatovych, S.; Malinowska, P.; Gadaj, K.; Prokopowicz, M.; Grembecka, M. Antioxidant Capacity, Nitrite and Nitrate Content in Beetroot-Based Dietary Supplements. *Foods* **2023**, *12*, 1017. [CrossRef]
69. Knez, E.; Hałasa, R.; Turecka, K.; Ośko, J.; Kadac-Czapska, K.; Waleron, K.; Grembecka, M. Microbiological Safety and Health Properties of Marketed Fermented Root Vegetables. *Appl. Sci.* **2024**, *15*, 121. [CrossRef]
70. Blois, M.S. Antioxidant Determinations by the Use of a Stable Free Radical. *Nature* **1958**, *181*, 1199–1200. [CrossRef]
71. de Menezes, B.B.; Frescura, L.M.; Duarte, R.; Villetti, M.A.; da Rosa, M.B. A Critical Examination of the DPPH Method: Mistakes and Inconsistencies in Stoichiometry and IC50 Determination by UV–Vis Spectroscopy. *Anal. Chim. Acta* **2021**, *1157*, 338398. [CrossRef]
72. Floegel, A.; Kim, D.O.; Chung, S.J.; Koo, S.I.; Chun, O.K. Comparison of ABTS/DPPH Assays to Measure Antioxidant Capacity in Popular Antioxidant-Rich US Foods. *J. Food Compos. Anal.* **2011**, *24*, 1043–1048. [CrossRef]
73. Mota, J.C.; Almeida, P.P.; Freitas, M.Q.; Stockler-Pinto, M.B.; Guimarães, J.T. Far from Being a Simple Question: The Complexity between in Vitro and in Vivo Responses from Nutrients and Bioactive Compounds with Antioxidant Potential. *Food Chem.* **2023**, *402*, 134351. [CrossRef] [PubMed]
74. Zheng, L.; Lin, L.; Su, G.; Zhao, Q.; Zhao, M. Pitfalls of Using 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Assay to Assess the Radical Scavenging Activity of Peptides: Its Susceptibility to Interference and Low Reactivity towards Peptides. *Food Res. Int.* **2015**, *76*, 359–365. [CrossRef] [PubMed]
75. Karimi, N.; Ghanbarzadeh, B.; Hajibonabi, F.; Hojabri, Z.; Ganbarov, K.; Kafil, H.S.; Hamishehkar, H.; Yousefi, M.; Mokarram, R.R.; Kamounah, F.S.; et al. Turmeric Extract Loaded Nanoliposome as a Potential Antioxidant and Antimicrobial Nanocarrier for Food Applications. *Food Biosci.* **2019**, *29*, 110–117. [CrossRef]
76. Ravichandran, K.; Ahmed, A.R.; Knorr, D.; Smetanska, I. The Effect of Different Processing Methods on Phenolic Acid Content and Antioxidant Activity of Red Beet. *Food Res. Int.* **2012**, *48*, 16–20. [CrossRef]
77. Moulick, S.P.; Jahan, F.; Islam, M.B.; Bashera, M.A.; Hasan, M.S.; Islam, M.J.; Ahmed, S.; Karmakar, D.; Ahmed, F.; Saha, T.; et al. Nutritional Characteristics and Antiradical Activity of Turmeric (*Curcuma longa* L.), Beetroot (*Beta vulgaris* L.), and Carrot (*Daucus carota* L.) Grown in Bangladesh. *Heliyon* **2023**, *9*, e21495. [CrossRef]
78. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT–Food Sci. Technol.* **1995**, *28*, 25–30. [CrossRef]
79. Ma, T.; Tian, C.; Luo, J.; Zhou, R.; Sun, X.; Ma, J. Influence of Technical Processing Units on Polyphenols and Antioxidant Capacity of Carrot (*Daucus carrot* L.) Juice. *Food Chem.* **2013**, *141*, 1637–1644. [CrossRef]
80. Azusena Castillejos-Mijangos, L.; Gabriela Meza-Márquez, O.; Osorio-Revilla, G.; Jiménez-Martínez, C.; Gallardo-Velázquez, T.; Massieu, W.; Cda Miguel Stampa, E.; Unidad Profesional Adolfo López Mateos, C.; Gustavo Madero, A.A.; de México, C.C.; et al. Identification of Variety and Prediction of Chemical Composition in Cocoa Beans (*Theobroma cacao* L.) by FT-MIR Spectroscopy and Chemometrics. *Foods* **2023**, *12*, 4144. [CrossRef]
81. Guaita, M.; Motta, S.; Messina, S.; Casini, F.; Bosso, A. Polyphenolic Profile and Antioxidant Activity of Green Extracts from Grape Pomace Skins and Seeds of Italian Cultivars. *Foods* **2023**, *12*, 3880. [CrossRef]
82. Farhat, Z.; Scheving, T.; Aga, D.S.; Hershberger, P.A.; Freudenheim, J.L.; Hageman Blair, R.; Mammen, M.J.; Mu, L. Antioxidant and Antiproliferative Activities of Several Garlic Forms. *Nutrients* **2023**, *15*, 4099. [CrossRef] [PubMed]
83. Stabrauskiene, J.; Marksa, M.; Ivanauskas, L.; Viskelis, P.; Viskelis, J.; Bernatoniene, J. Citrus × Paradisi L. Fruit Waste: The Impact of Eco-Friendly Extraction Techniques on the Phytochemical and Antioxidant Potential. *Nutrients* **2023**, *15*, 1276. [CrossRef]
84. Wang, Y.; Huang, S.; Shao, S.; Qian, L.; Xu, P. Studies on Bioactivities of Tea (*Camellia sinensis* L.) Fruit Peel Extracts: Antioxidant Activity and Inhibitory Potential against α-Glucosidase and α-Amylase in Vitro. *Ind. Crops Prod.* **2012**, *37*, 520–526. [CrossRef]
85. Wang, Y.; Yang, Z.; Wei, X. Antioxidant Activities Potential of Tea Polysaccharide Fractions Obtained by Ultra Filtration. *Int. J. Biol. Macromol.* **2012**, *50*, 558–564. [CrossRef]
86. Pekal, A.; Pyrzynska, K. Effect of PH and Metal Ions on DPPH Radical Scavenging Activity of Tea. *Int. J. Food Sci. Nutr.* **2015**, *66*, 58–62. [CrossRef] [PubMed]
87. Tomassi, E.; Arouna, N.; Caruso, M.G.; Girgenti, A.; Picone, P.; Nuzzo, D.; Pucci, L. Fermentation of Chlorella Vulgaris and Aphanizomenon Flos-Aquae Biomass Improves the Antioxidant Profile. *LWT* **2025**, *215*, 117183. [CrossRef]
88. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
89. Platzer, M.; Kiese, S.; Herfellner, T.; Schweiggert-Weisz, U.; Miesbauer, O.; Eisner, P. Common Trends and Differences in Antioxidant Activity Analysis of Phenolic Substances Using Single Electron Transfer Based Assays. *Molecules* **2021**, *26*, 1244. [CrossRef] [PubMed]
90. Tian, X.; Schaich, K.M. Effects of Molecular Structure on Kinetics and Dynamics of the Trolox Equivalent Antioxidant Capacity Assay with ABTS+●. *J. Agric. Food Chem.* **2013**, *61*, 5511–5519. [CrossRef]

91. Dawidowicz, A.L.; Olszowy, M. The Importance of Solvent Type in Estimating Antioxidant Properties of Phenolic Compounds by ABTS Assay. *Eur. Food Res. Technol.* **2013**, *236*, 1099–1105. [CrossRef]
92. Ramírez-Anaya, J.D.P.; Samaniego-Sánchez, C.; Castañeda-Saucedo, M.C.; Villalón-Mir, M.; De La Serrana, H.L.G. Phenols and the Antioxidant Capacity of Mediterranean Vegetables Prepared with Extra Virgin Olive Oil Using Different Domestic Cooking Techniques. *Food Chem.* **2015**, *188*, 430–438. [CrossRef] [PubMed]
93. Gorinstein, S.; Jastrzebski, Z.; Leontowicz, H.; Leontowicz, M.; Namiesnik, J.; Najman, K.; Park, Y.S.; Heo, B.G.; Cho, J.Y.; Bae, J.H. Comparative Control of the Bioactivity of Some Frequently Consumed Vegetables Subjected to Different Processing Conditions. *Food Control* **2009**, *20*, 407–413. [CrossRef]
94. Wojdyło, A.; Oszmiański, J.; Czemerys, R. Antioxidant Activity and Phenolic Compounds in 32 Selected Herbs. *Food Chem.* **2007**, *105*, 940–949. [CrossRef]
95. Siddhuraju, P. The Antioxidant Activity and Free Radical-Scavenging Capacity of Phenolics of Raw and Dry Heated Moth Bean (*Vigna aconitifolia*) (Jacq.) Marechal Seed Extracts. *Food Chem.* **2006**, *99*, 149–157. [CrossRef]
96. Pellegrini, N.; Serafini, M.; Colombi, B.; Del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. Total Antioxidant Capacity of Plant Foods, Beverages and Oils Consumed in Italy Assessed by Three Different In Vitro Assays. *J. Nutr.* **2003**, *133*, 2812–2819. [CrossRef] [PubMed]
97. Hu, M.; Skibsted, L.H. Antioxidative Capacity of Rhizome Extract and Rhizome Knot Extract of Edible Lotus (*Nelumbo nuficera*). *Food Chem.* **2002**, *76*, 327–333. [CrossRef]
98. Awika, J.M.; Rooney, L.W.; Waniska, R.D. Anthocyanins from Black Sorghum and Their Antioxidant Properties. *Food Chem.* **2005**, *90*, 293–301. [CrossRef]
99. Larrosa, M.; Llorach, R.; Espín, J.C.; Tomás-Barberán, F.A. Increase of Antioxidant Activity of Tomato Juice Upon Functionalisation with Vegetable Byproduct Extracts. *LWT–Food Sci. Technol.* **2002**, *35*, 532–542. [CrossRef]
100. Leong, L.P.; Shui, G. An Investigation of Antioxidant Capacity of Fruits in Singapore Markets. *Food Chem.* **2002**, *76*, 69–75. [CrossRef]
101. Moreno-Montoro, M.; Olalla-Herrera, M.; Gimenez-Martinez, R.; Navarro-Alarcon, M.; Rufián-Henares, J.A. Phenolic Compounds and Antioxidant Activity of Spanish Commercial Grape Juices. *J. Food Compos. Anal.* **2015**, *38*, 19–26. [CrossRef]
102. Soong, Y.Y.; Barlow, P.J. Antioxidant Activity and Phenolic Content of Selected Fruit Seeds. *Food Chem.* **2004**, *88*, 411–417. [CrossRef]
103. Khanam, U.K.S.; Oba, S.; Yanase, E.; Murakami, Y. Phenolic Acids, Flavonoids and Total Antioxidant Capacity of Selected Leafy Vegetables. *J. Funct. Foods* **2012**, *4*, 979–987. [CrossRef]
104. Di Matteo, A.; Lavorgna, M.; Russo, C.; Orlo, E.; Isidori, M. Natural Plant-Derived Terpenes: Antioxidant Activity and Antibacterial Properties against Foodborne Pathogens, Food Spoilage and Lactic Acid Bacteria. *Appl. Food Res.* **2024**, *4*, 100528. [CrossRef]
105. Raposo, F.; Borja, R.; Gutiérrez-González, J.A. A Comprehensive and Critical Review of the Unstandardized Folin-Ciocalteu Assay to Determine the Total Content of Polyphenols: The Conundrum of the Experimental Factors and Method Validation. *Talanta* **2024**, *272*, 125771. [CrossRef]
106. Kaluza, W.Z.; McGrath, R.M.; Roberts, T.C.; Schroder, H.H. Separation of Phenolics of *Sorghum bicolor* (L.) Moench Grain. *J. Agric. Food Chem.* **1980**, *28*, 1191–1196. [CrossRef]
107. Pękal, A.J. *Wpływ Doboru Procedury Analitycznej Na Wyznaczanie Właściwości Antyutleniających Próbek Żywności*; Uniwersytet Warszawski Wydział Chemii: Warszawa, Poland, 2014.
108. Ignat, I.; Volf, I.; Popa, V.I. A Critical Review of Methods for Characterisation of Polyphenolic Compounds in Fruits and Vegetables. *Food Chem.* **2011**, *126*, 1821–1835. [CrossRef]
109. Prakash Jain, B.; Pandey, S.; Goswami, S.K. Protein Estimation by Folin-Ciocalteau (Lowry) Method. In *Protocols in Biochemistry and Clinical Biochemistry*; Academic Press: New York, NY, USA, 2025; pp. 93–95. [CrossRef]
110. Insanu, M.; Nayaka, N.M.D.M.W.; Solihin, L.; Wirasutisna, K.R.; Pramastya, H.; Fidrianny, I. Antioxidant Activities and Phytochemicals of Polar, Semi-Polar, and Nonpolar Extracts of Used and Unused Parts of Carica Papaya Fruit. *Biocatal. Agric. Biotechnol.* **2022**, *39*, 102270. [CrossRef]
111. Mikucka, W.; Zielińska, M. Effect of the Solvent on the Extraction of Polyphenols from Distillery Stillage and on Their Antioxidant Activity. *Acta Univ. Lodziensis. Folia Biol. Oecol.* **2021**, *17*, 54–62. [CrossRef]
112. Xiang, Z.; Liu, L.; Xu, Z.; Kong, Q.; Feng, S.; Chen, T.; Zhou, L.; Yang, H.; Xiao, Y.; Ding, C. Solvent Effects on the Phenolic Compounds and Antioxidant Activity Associated with Camellia Polyodonta Flower Extracts. *ACS Omega* **2024**, *9*, 27192–27203. [CrossRef] [PubMed]
113. Elnour, A.A.; Mirghani, M.E.S.; Kabbashi, N.A.; Musa, K.H. Effect of Solvent Types on Phenolics Content and Antioxidant Activities of Acacia polyacantha gum. *Int. Food Res. J.* **2017**, *24*, 369–377.

114. Ammar, A.F.; Baeshen, M.; Aqlan, F.M.; Siddeeg, A.; Chamba, M.V.M.; Afifi, M.; Almulaiky, Y.Q.; Alayafi, A. Solvent Effects on Antioxidant Activities and Phenolic Contents of the Alhydwan (Boerhavia Elegana Choisy) Seed Flour. *J. Food Meas. Charact.* **2018**, *12*, 2121–2127. [CrossRef]
115. Chen, L.Y.; Cheng, C.W.; Liang, J.Y. Effect of Esterification Condensation on the Folin–Ciocalteu Method for the Quantitative Measurement of Total Phenols. *Food Chem.* **2015**, *170*, 10–15. [CrossRef] [PubMed]
116. Lester, G.E.; Lewers, K.S.; Medina, M.B.; Saftner, R.A. Comparative Analysis of Strawberry Total Phenolics via Fast Blue BB vs. Folin–Ciocalteu: Assay Interference by Ascorbic Acid. *J. Food Compos. Anal.* **2012**, *27*, 102–107. [CrossRef]
117. Turkmen, N.; Sari, F.; Velioglu, Y.S. Effects of Extraction Solvents on Concentration and Antioxidant Activity of Black and Black Mate Tea Polyphenols Determined by Ferrous Tartrate and Folin–Ciocalteu Methods. *Food Chem.* **2006**, *99*, 835–841. [CrossRef]
118. Wootton-Beard, P.C.; Moran, A.; Ryan, L. Stability of the Total Antioxidant Capacity and Total Polyphenol Content of 23 Commercially Available Vegetable Juices before and after in Vitro Digestion Measured by FRAP, DPPH, ABTS and Folin–Ciocalteu Methods. *Food Res. Int.* **2011**, *44*, 217–224. [CrossRef]
119. Zhang, M.Q.; Zhang, J.; Zhang, Y.T.; Sun, J.Y.; Prieto, M.A.; Simal-Gandara, J.; Putnik, P.; Li, N.Y.; Liu, C. The Link between the Phenolic Composition and the Antioxidant Activity in Different Small Berries: A Metabolomic Approach. *LWT* **2023**, *182*, 114853. [CrossRef]
120. Shaghaghi, M.; Manzoori, J.L.; Jouyban, A. Determination of Total Phenols in Tea Infusions, Tomato and Apple Juice by Terbium Sensitized Fluorescence Method as an Alternative Approach to the Folin–Ciocalteu Spectrophotometric Method. *Food Chem.* **2008**, *108*, 695–701. [CrossRef]
121. Alim, M.A.; Karim, A.; Shohan, M.A.R.; Sarker, S.C.; Khan, T.; Mondal, S.; Esrafil, M.; Linkon, K.M.M.R.; Rahman, M.N.; Akther, F.; et al. Study on Stability of Antioxidant Activity of Fresh, Pasteurized, and Commercial Fruit Juice during Refrigerated Storage. *Food Humanit.* **2023**, *1*, 1117–1124. [CrossRef]
122. Guo, J.; Wang, C.; Liu, L.; Xu, K.; Li, B.; Zhang, L.; Xu, H.; Lei, H. Antioxidant Activities and Volatile Compounds of Chinese Cabbage Sauce Prepared by the Combination of Lactobacillus Plantarum and Functional Oligosaccharides. *Food Biosci.* **2023**, *54*, 102854. [CrossRef]
123. Parada, R.B.; Marguet, E.; Campos, C.; Vallejo, M. Improved Antioxidant Capacity of Three Brassica Vegetables by Two-Step Controlled Fermentation Using Isolated Autochthone Strains of the *Genus leuconostoc* spp. and *Lactiplantibacillus* spp. *Food Chem Mol. Sci.* **2023**, *6*, 100163. [CrossRef]
124. Waweru, D.M.; Arimi, J.M.; Marete, E.; Jacquier, J.C.; Harbourne, N. Chemical and Antioxidant Characterization of Dovyalis Caffra and Dovyalis Abyssinica Fruits in Kenya. *Heliyon* **2022**, *8*, 11064. [CrossRef] [PubMed]
125. Bani, C.; Peñas, E.; Baron, G.; Martínez-Villaluenga, C.; Mercogliano, F.; Aldini, G.; Piazza, S.; Di Lorenzo, C.; Restani, P. Characterization of the Phenolic Profile and in Vitro Antioxidant Potential of Different Varieties of Common Buckwheat (*Fagopyrum esculentum* Moench) and Tartary Buckwheat (*Fagopyrum tataricum* (L.) Gaertn.). *LWT* **2025**, *215*, 117261. [CrossRef]
126. Cao, G.; Alessio, H.M.; Cutler, R.G. Oxygen-Radical Absorbance Capacity Assay for Antioxidants. *Free Radic. Biol. Med.* **1993**, *14*, 303–311. [CrossRef]
127. Huang, D.; Boxin, O.U.; Prior, R.L. The Chemistry behind Antioxidant Capacity Assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856. [CrossRef]
128. Prior, R.L. Oxygen Radical Absorbance Capacity (ORAC): New Horizons in Relating Dietary Antioxidants/Bioactives and Health Benefits. *J. Funct. Foods* **2015**, *18*, 797–810. [CrossRef]
129. Esfandi, R.; Willmore, W.G.; Tsopmo, A. Structural Characterization of Peroxyl Radical Oxidative Products of Antioxidant Peptides from Hydrolyzed Proteins. *Heliyon* **2024**, *10*, 30588. [CrossRef]
130. Bhagwat, S.; Haytowitz, D.B.; Holden, J.M. USDA Database for the Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods. In *American Institute for Cancer Research Launch Conference*; Nutrient Data Laboratory, Beltsville Human Nutrition Research Center: Beltsville, MD, USA, 2007.
131. Sadowska-Bartosz, I.; Bartosz, G. Evaluation of The Antioxidant Capacity of Food Products: Methods, Applications and Limitations. *Processes* **2022**, *10*, 2031. [CrossRef]
132. Knez, E.; Kadac-Czapska, K.; Grembecka, M. Effect of Fermentation on the Nutritional Quality of the Selected Vegetables and Legumes and Their Health Effects. *Life* **2023**, *13*, 655. [CrossRef]
133. Navarro-Orcajada, S.; Conesa, I.; Matencio, A.; Rodríguez-Bonilla, P.; García-Carmona, F.; López-Nicolás, J.M. The Use of Cyclodextrins as Solubility Enhancers in the ORAC Method May Cause Interference in the Measurement of Antioxidant Activity. *Talanta* **2022**, *243*, 123336. [CrossRef] [PubMed]
134. Nakano, T.; Hayashi, S.; Ochiai, Y.; Shirakawa, H.; Wu, H.; Endo, H.; Yu, H. Modification of the Oxygen Radical Absorbance Capacity Assay and Its Application in Evaluating the Total Antioxidative State in Fish. *Adv. Redox Res.* **2022**, *6*, 100049. [CrossRef]
135. Folch-Cano, C.; Jullian, C.; Speisky, H.; Olea-Azar, C. Antioxidant Activity of Inclusion Complexes of Tea Catechins with β-Cyclodextrins by ORAC Assays. *Food Res. Int.* **2010**, *43*, 2039–2044. [CrossRef]

136. Zulueta, A.; Esteve, M.J.; Frígola, A. ORAC and TEAC Assays Comparison to Measure the Antioxidant Capacity of Food Products. *Food Chem.* **2009**, *114*, 310–316. [CrossRef]
137. Alarcón, E.; Campos, A.M.; Edwards, A.M.; Lissi, E.; López-Alarcón, C. Antioxidant Capacity of Herbal Infusions and Tea Extracts: A Comparison of ORAC-Fluorescein and ORAC-Pyrogallol Red Methodologies. *Food Chem.* **2008**, *107*, 1114–1119. [CrossRef]
138. Dudonné, S.; Vitrac, X.; Coutiére, P.; Woillez, M.; Mérillon, J.M. Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. *J. Agric. Food Chem.* **2009**, *57*, 1768–1774. [CrossRef]
139. Ishimoto, H.; Tai, A.; Yoshimura, M.; Amakura, Y.; Yoshida, T.; Hatano, T.; Ito, H. Antioxidative Properties of Functional Polyphenols and Their Metabolites Assessed by an ORAC Assay. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 395–399. [CrossRef]
140. He, Y.; Liu, J.; Hua, M.Z.; Singh, K.; Lu, X. Determination of Antioxidant Capacity and Phenolic Content of Haskap Berries (*Lonicera caerulea* L.) by Attenuated Total Reflectance-Fourier Transformed-Infrared Spectroscopy. *Food Chem.* **2025**, *463*, 141283. [CrossRef]
141. Vargas, V.; Saldarriaga, S.; Sánchez, F.S.; Cuellar, L.N.; Paladines, G.M. Effects of the Spray-Drying Process Using Maltodextrin on Bioactive Compounds and Antioxidant Activity of the Pulp of the Tropical Fruit Açai (*Euterpe oleracea* Mart.). *Heliyon* **2024**, *10*, 33544. [CrossRef]
142. Mirzadeh, M.; Arianejad, M.R.; Khedmat, L. Antioxidant, Antiradical, and Antimicrobial Activities of Polysaccharides Obtained by Microwave-Assisted Extraction Method: A Review. *Carbohydr. Polym.* **2020**, *229*, 115421. [CrossRef]
143. Kumari, N.; Kumar, M.; Radha; Lorenzo, J.M.; Sharma, D.; Puri, S.; Pundir, A.; Dhumal, S.; Bhuyan, D.J.; Jayanthy, G.; et al. Onion and Garlic Polysaccharides: A Review on Extraction, Characterization, Bioactivity, and Modifications. *Int. J. Biol. Macromol.* **2022**, *219*, 1047–1061. [CrossRef]
144. SMPR, NEW AOAC. AOAC SMPR 2011.011 Standard Method Performance Requirements for in Vitro Determination of Total Antioxidant Activity in Foods, Beverages, Food Ingredients, and Dietary Supplements. *J. AOAC Int.* **2012**, *95*, 1557. [CrossRef] [PubMed]
145. Kılınçer, M.; Çiçek, G.; Özyürek, M.; Apak, R. Uncertainty Estimation for Total Antioxidant Capacity Measurement of Apple Juice Using Main CUPRAC Method. *J. Chem. Metrol.* **2022**, *16*, 28–37. [CrossRef]
146. Yeo, J.D.; Shahidi, F. Critical Re-Evaluation of DPPH Assay: Presence of Pigments Affects the Results. *J. Agric. Food Chem.* **2019**, *67*, 7526–7529. [CrossRef] [PubMed]
147. Sharma, O.P.; Bhat, T.K. DPPH Antioxidant Assay Revisited. *Food Chem.* **2009**, *113*, 1202–1205. [CrossRef]
148. Shimamura, T.; Sumikura, Y.; Yamazaki, T.; Tada, A.; Kashiwagi, T.; Ishikawa, H.; Matsui, T.; Sugimoto, N.; Akiyama, H.; Ukeda, H. Applicability of the DPPH Assay for Evaluating the Antioxidant Capacity of Food Additives—Inter-Laboratory Evaluation Study. *Anal. Sci.* **2014**, *30*, 717–721. [CrossRef]
149. Chaves, N.; Santiago, A.; Alías, J.C. Quantification of the Antioxidant Activity of Plant Extracts: Analysis of Sensitivity and Hierarchization Based on the Method Used. *Antioxidants* **2020**, *9*, 76. [CrossRef]

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