

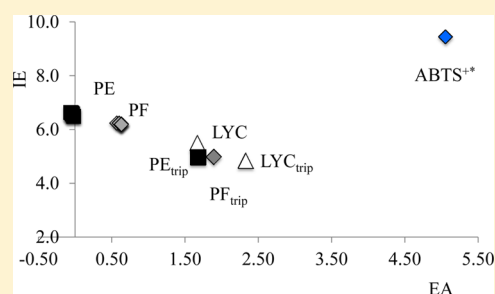
Free Radical Scavenging Properties of Phytofluene and Phytoene Isomers as Compared to Lycopene: A Combined Experimental and Theoretical Study

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ABSTRACT: Free radical scavenging properties of Lycopene (LYC), Phytofluene (PF), and Phytoene (PE) are studied experimentally, considering the electron transfer mechanism and using the ABTS^{•+} decoloration assay. Density Functional Theory is used to obtain the theoretical UV–visible spectra of ABTS (neutral and cation) and to determine the free radical scavenger capacity in terms of the electron donor mechanism and the deactivation of singlet oxygen. The experimental data agreed very well with the theoretical results. Considering both mechanisms, LYC, PF, and PE can be considered good free radical scavengers, with LYC turning out to be the best and PE the worst antiradical. However, the triplets that formed following the deactivation of singlet oxygen presented similar capacity for donating electrons. In the case of triplets, the antiradical capacity of LYC, PF, and PE is similar in terms of the electron donor mechanism. Although the results indicated that PF and PE are not as effective antiradicals as LYC, which is to be expected due to the number of conjugated double bonds, they do present a higher antioxidant capacity than expected considering the small number of conjugated double bonds.



INTRODUCTION

Fruit and vegetables contain secondary metabolites, known as carotenoids.^{1–4} Carotenoids can act as free radical scavengers that may help prevent oxidative stress. One of the antiradical substances found in fruit such as watermelon, tomato, guayaba, grapefruit, and papaya is lycopene (ψ,ψ -carotene, LYC), a red carotenoid that is also thought to provide health benefits.^{5,6} LYC is used as an additive to enhance food color and is also interesting as a functional ingredient, in terms of potential biological effects.⁷ In addition to lycopene, over 700 colored carotenoids considered as good antioxidants have been described in the literature. To analyze the properties of colored carotenoids, there have been reports concerning the UV–visible spectra.^{8–11}

Some foods also contain two colorless carotenoids in significant quantities: phytofluene (7,8,11,12,7',8'-hexahydro- ψ,ψ -carotene, PF) and phytoene (7,8,11,12,7',8',11',12'-octahydro- ψ,ψ -carotene, PE), both considered oddities within the carotenoid group. These molecules are the first compounds in the biosynthetic pathway of colored carotenoids.⁵

Several studies have been conducted to investigate the oxidation/antioxidant properties of colored carotenoids in relation to their chemical structure,^{12,13} although both colorless carotenoids (PF and PE) have been largely ignored, as their chemical structures suggest that they are not as effective antioxidants as the other carotenoids. The main structural difference between LYC, PF, and PE relates to the number of conjugated double bonds (cdb). LYC has 11 cdb, while PF and

PE have 5 and 3, respectively (see Figure 1). These differences are expected to affect not only the UV–vis absorption, but also

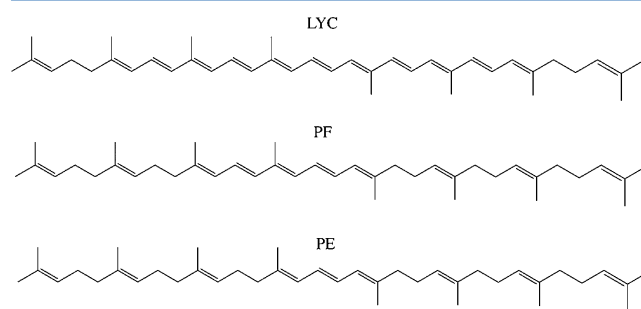


Figure 1. Molecular structure of lycopene (LYC), phytofluene (PF), and phytoene (PE).

the chemical properties and interactions with other molecules that produce oxidative stress. The scarcity of studies analyzing the physicochemical properties of PF and PE compared to other dietary carotenoids is surprising, as they are found in the plasma and tissues of humans and other animals.¹⁴ Some previous studies have suggested that they may be involved in several biological functions, such as protection against

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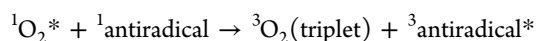
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oxidation, UV-light induced erythema, and skin inflammation, either in isolation or in tomato products also containing LYC and other compounds.^{15–18} Nevertheless, studies concerning the oxidative stability of PF and PE are very limited and should thus be encouraged, in order to improve the understanding of their chemical properties and to determine the capacity of these molecules for scavenging free radicals.

LYC *cis–trans* isomers were theoretically investigated previously.¹⁹ Apparently, *trans* isomers are slightly more stable than *cis* isomers, with 15-*cis* isomers rating as the least stable in all cases. Antiradical properties, in terms of electron transfer mechanism are very similar for both kinds of isomers. The *cis* isomers represent as effective antiradicals as the *trans* isomers. In spite of these results, no similar studies concerning PF and PE are available, as the electron transfer capacity of these substances has not been reported previously.

Studies concerning the antiradical properties of LYC establish that this substance is able to act as a free radical scavenger, transferring one electron or deactivating the singlet oxygen molecules, by the following mechanism:



The asterisk indicates that singlet oxygen and triplet antiradicals both represent excited states. In this context, an energy transfer process mediates the exchange of electrons. The interaction of antiradicals with singlet oxygen is known to occur mainly by physical quenching. This takes place by the direct energy transfer from the highly energetic and harmful ${}^1\text{O}_2$ to the antiradical. As a result, ground state oxygen and a triplet antiradical (excited states) are produced. The triplet antiradical is not very reactive and any excess energy is dissipated to the environment, in the form of heat. The excited antiradical dissipates its energy and returns to the ground state energy level. The antiradical can act several times in the quenching of singlet oxygen since it remains intact. In contrast to physical quenching, the chemical interaction between singlet oxygen and the carotenoids are thought to be of much lower importance.²⁰ In this sense, lycopene has been proposed as a very efficient quencher among carotenoids.²¹

Experimental investigations suggest that PF and PE are involved in bioactivity, but little is known about the mechanism and also about any real benefits that these substances may contribute to humans.¹⁴ For this reason, the main goal of this investigation is to study the antiradical properties of PF and PE. The experimental values for the Trolox Equivalent Antioxidant Capacity (TEAC) are reported and likewise a theoretical study about isomers, focusing on the electron transfer mechanism and the reaction with ${}^1\text{O}_2$. In this study, LYC is included for comparison.

METHODOLOGY

Experimental Methodology. The antioxidant capacity of carotenoid standards was assessed by applying the 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation decolorization assay.²² The $\text{ABTS}^{\bullet+}$ radical cation was produced by reacting an aqueous solution of ABTS (7 mM) with potassium persulfate (2.45 mM, final concentration) and allowing the mixture to stand in the dark at room temperature, for 12–16 h. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma) was used as a standard. Measurements were taken individually, using different concentrations (in triplicate) of LYC, PF and PE. The experimental results

concerning the Trolox Equivalent Antioxidant Capacity (TEAC) of PF and PE were obtained using the synthetic radical ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt). ABTS is a colorless substance that loses an electron and forms a cationic radical molecule in the form of a blue-green colored radical ($\text{ABTS}^{\bullet+}$) substance. This radical is used to follow the reaction with antioxidants, using a spectrophotometer. Typically, there is a drop in the absorbance at the near-infrared region at 734 nm in the case of solutions of the radical, following the addition of chemical species to the reaction medium.²² The results are finally expressed as TEAC by comparing with known quantities of Trolox (a synthetic water-soluble vitamin E analogue). The TEAC can be interpreted as the millimolar concentration of a Trolox solution, with an antioxidant capacity equivalent to a 1.0 mM solution of the antioxidant being tested. This is considered as a parameter that makes possible the comparison of the relative ability of electron- or hydrogen-donating antioxidants for scavenging $\text{ABTS}^{\bullet+}$ compared with that of Trolox.²³

Computational Details. Geometry optimizations and frequency calculations have been undertaken, using the B3LYP²⁴ functional in conjunction with the 6-31G(d) basis set. Energies were improved by single point calculations using the m062x²⁵ functional with 6-311+G(d,p) basis set. Local minima were identified by the absence of imaginary frequencies. The UV–visible spectra were computed with time-dependent density functional theory (TDDFT). All electronic calculations were performed with Gaussian 09 program series.²⁶ There are studies that analyze the performance of B3LYP Density Functional methods for the geometry optimization of organic molecules. It was reported that the best ground-state geometries are obtained with B3LYP.²⁷ In another study,²⁸ authors analyzed 622 organic compounds and conclude that the mean absolute errors in the heats of formation obtained with B3LYP/6-31G(d) is similar to the errors obtained with other functionals and basis. According to this, and due to the good agreement between experimental and theoretical results reported here, we can be confident about the results that we obtained. In order to compute vertical Ionization Energy (IE) and vertical Electron Affinity (EA), further single-point calculations are necessary. IE is calculated as the difference between the energy of the cation and the neutral molecule, assuming that both of these have the ground-state nuclear configuration of the neutral molecule. EA is also calculated as vertical, and represents the energy difference between the neutral and the anion, calculated with the ground-state nuclear configuration of the neutral molecule. IE and EA are used to compare the electron donor–acceptor capacity of the antioxidants with the capacity of $\text{ABTS}^{\bullet+}$. For this purpose, a map that was presented previously^{29–31} is constructed from IE and EA (Figure 2). This map makes it possible to compare

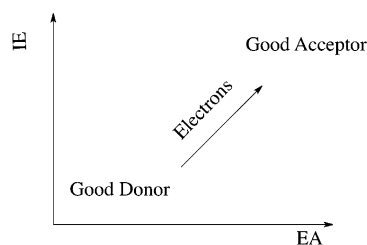


Figure 2. Full Electron Donor–Acceptor Map (FEDAM).

different molecules as either electron donors or acceptors. Small values of IE mean that molecules are good electron donors. Large values of EA represent molecules that are good electron acceptors. Accordingly, molecules situated in the lower left corner represent good electron donors, and those situated in the upper right corner are good electron acceptors. Electrons will be transferred from species located down to the left of the map, to species located up to the right.

RESULTS AND DISCUSSION

UV–visible Spectra of ABTS and ABTS^{•+}. The assessment of the total antioxidant capacity of substances is determined with reference to the decolourization of the ABTS radical cation, given that this is proportional to the antioxidant activity of the studied substances. In order to analyze the decolourization of the ABTS^{•+}, experimental and theoretical spectra of ABTS and ABTS^{•+} are obtained. For the theoretical spectrum, the optimized structures of ABTS and ABTS^{•+} are used. Figure 3 reports the UV–visible spectra

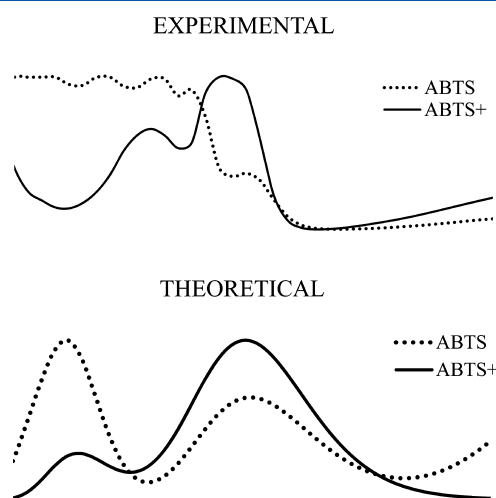


Figure 3. Experimental and theoretical UV–visible spectra of ABTS (neutral and cation).

(experimental and theoretical) of ABTS and ABTS^{•+}. In both spectra (experimental and theoretical), decolourization is evident. For ABTS, the UV–visible spectra present a maximum between approximately 200 and 360 nm and then a smaller peak at 430 nm. The analyses of the ABTS^{•+} spectra indicate that there is a small peak between 300 and 350 nm and a maximum at 400–420 nm. Theoretical and experimental results agreed very well. Some discrepancies are evident in terms of secondary signals, but in the case of λ_{max} experimental and theoretical data are in accordance.

TEAC. The absorbance changes of the ABTS^{•+} solution as a function of the amount of carotenoids added (LYC, PF, and PE) are presented in Figures 4–6, and the TEAC values in Table 1.

As evident, the decrease in the absorbance of the radical cation solution, following the addition of the carotenoids is linear within the ranges tested; that is ranging from 0 to 5 μg in the case of LYC and from 0 to 3 μg in the case of PE and PF (Figures 4–6). Predictably, considering their chemical structures (specifically, from their number of conjugated double bonds), TEAC capacity is as follows: LYC > PF > PE (Table 1). Interestingly, the TEAC values for PF and PE (2.57 and

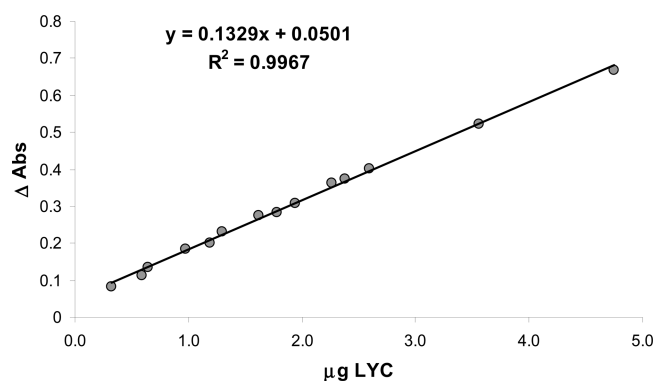


Figure 4. Representation of the absorbance changes of the ABTS^{•+} solution as a function of the amount of LYC added.

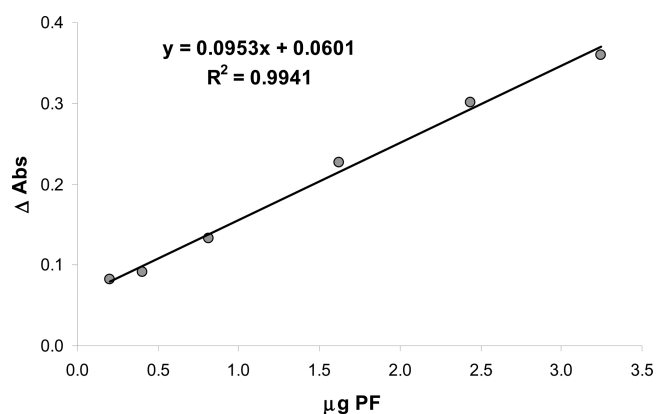


Figure 5. Representation of the absorbance changes of the ABTS^{•+} solution as a function of the amount of phytofluene (PF) added.

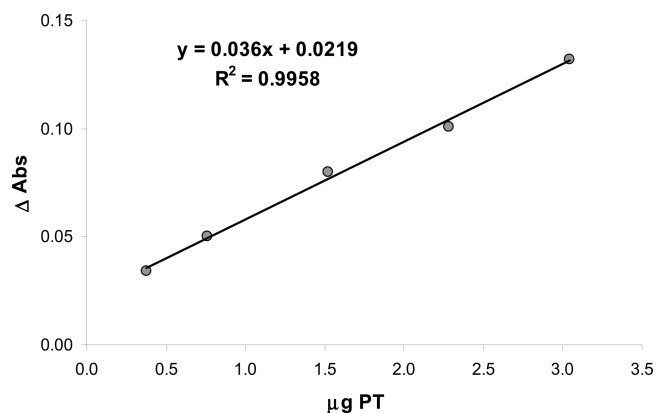


Figure 6. Representation of the absorbance changes of the ABTS^{•+} solution as a function of the amount of phytoene (PE) added.

Table 1. Experimental Values for the Trolox Equivalent Antioxidant Capacity (TEAC). Theoretical Singlet-Triplet Energy Differences and ΔE (HOMO–LUMO) in eV.

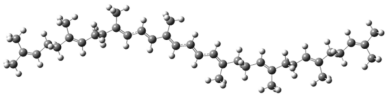
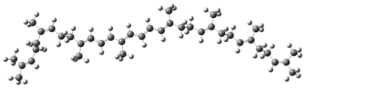
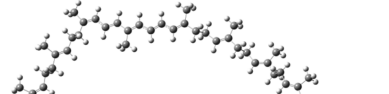
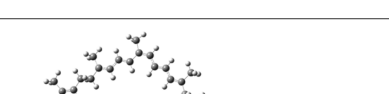
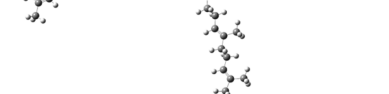
molecule (cdb)	TEAC (mM Trolox)	ΔE (singlet–triplet)	ΔE (HOMO–LUMO)
LYC (11)	3.52	0.63	0.08
PF (5)	2.57	1.22	0.11
PE (3)	1.02	1.74	0.15

1.02, respectively) are much higher and very similar to those reported for α -tocopherol (a classical dietary lipid-soluble

antioxidant). In fact, the experimental PF value for TEAC is virtually identical to that reported for β -carotene, traditionally considered as a very good carotenoid free radical scavenger.²² In summary, our results indicate that PF and PE's capacity for scavenging ABTS^{•+} is less than that of LYC, predictable when differences in their double bond chromophores are considered; however, their TEAC are surprisingly higher than might have been previously expected, considering the number of conjugated double bonds. Although the *in vitro* total antioxidant capacity of chemical substances is dependent upon many factors, it appears that the study of the oxidation/antioxidant capacity of PE and PF under other conditions deserves further attention.

Geometry Optimization and Electron Transfer Reaction. The optimized structures of all possible cis–trans isomers of PE and PF are reported in Tables 2 and 3. Relative

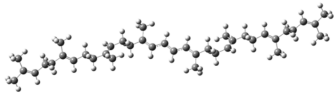
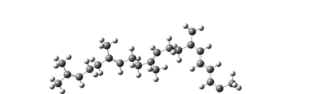

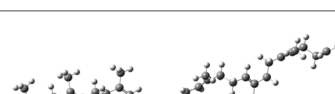
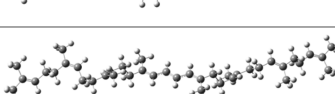
Table 2. Phytofluene (PF) Optimized Structures (B3LYP/6-31G(d)) and Relative Energies (m062x/6-311+G (d,p)) with Respect to the Most Stable Structure (all-trans)

Name	Optimized Structures	ΔE (kcal/mol)
(All-trans)-phytofluene		0.0
(5-cis)-phytofluene		0.1
(9-cis)-phytofluene		-0.1
(13-cis)-phytofluene		0.8
(15-cis)-phytofluene		2.0

energies with respect to the most stable structure are included. The all-trans isomers are slightly more stable than the cis isomers, with the 15-cis isomers rating as the least stable in all cases. For PF, the 9-cis isomer is more stable than the all-trans for 0.1 kcal/mol. Among all the systems reported here, the energy difference is less than the limit of accuracy for the calculation and all isomers appear to have similar stability.

One of the mechanisms for radical scavenging discussed in the literature^{29–32} is that of the electron transfer reaction. The electron donor–acceptor capacity of LYC, PF, and PE using

Table 3. Phytoene (PE) Optimized Structures (B3LYP/6-31G(d)) and Relative Energies (m062x/6-311+G (d,p)) with Respect to the Most Stable Structure (all-trans)

Name	Optimized Structure	ΔE (kcal/mol)
(All-trans)-phytoene		0.0
(13-cis)-phytoene		0.1
(9-cis)-phytoene		0.2
(5-cis)-phytoene		1.4
(15-cis)-phytoene		2.0

the FEDAM (Full Electron Donor–Acceptor Map) is analyzed. Results are reported in Figure 7. IE and EA are obtained in gas

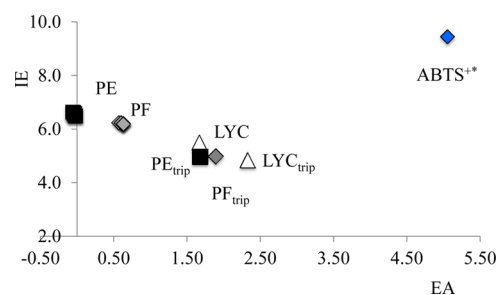


Figure 7. Full electron donor–acceptor map for LYC, PF, and PE in the gas phase. ABTS^{•+} is included for comparison.

phase for all the isomers of PF and PE. It is apparent that all the cis–trans isomers of PE are located in the same position at the FEDAM. A similar situation is revealed in the case of the cis–trans isomers of PF. This means that the cis–trans isomers have the same stability and also the same electron donor–acceptor capacity. Comparing LYC, PF, and PE, it can be concluded that PE isomers are less effective for transferring electrons than PF isomers; and LYC is a better electron donor–acceptor

molecule than PF and PE. In terms of the electron transfer reaction, LYC represents the best and PE the worst antiradical.

The experimental TEAC was determined using $\text{ABTS}^{\bullet+}$. In order to compare theoretical predictions with experimental results, $\text{ABTS}^{\bullet+}$ is also included in the FEDAM of Figure 7. This cationic radical is located upwards and to the right of LYC, PF, and PE. This means that it will accept electrons from these molecules, and therefore the best electron donor will be the best antiradical. Comparing these three molecules, the best electron donor is LYC, followed by PF and finally PE. In summary, LYC will react more with $\text{ABTS}^{\bullet+}$ than with PF and PE. PE is the less reactive substance against $\text{ABTS}^{\bullet+}$. These results totally coincide with the experimental results reported in Table 1.

Deactivating the Singlet Oxygen Molecule. The capability of these three molecules (LYC, PF, and PE) to deactivate the singlet oxygen molecule is related to the singlet–triplet energy difference. In this process, there is an energy transfer from $^1\text{O}_2$ to the antiradical and subsequently the triplet antiradical is formed. The lower the singlet–triplet energy difference becomes, the better the substance as an antiradical, in terms of this mechanism. In Table 1, the singlet–triplet energy differences are reported. Evidently, LYC presents the lower energy difference followed by PF. PE presents the greatest energy difference. According to this mechanism, LYC is also the best antiradical and PE the worst one, agreeing with the experiment.

It could be that these molecules act as antiradicals by applying two mechanisms: deactivation of singlet oxygen (triplets are formed as a consequence), and electron transfer from the triplets formed to the free radicals. In order to define whether the triplets are reactive in terms of the electron transfer mechanism, we calculated IE and EA of the triplets and we localize these molecules in the FEDAM of Figure 7. Triplets are better electron donors than singlets and, consequently, triplets are better antiradical molecules in terms of the mechanism of electron transfer. Differences in terms of electron-donor capacity between triplets are less than the differences between the singlets, indicating that the antiradical capacity of LYC, PF, and PE (triplets) is similar. Apparently, LYC, PF, and PE can deactivate the singlet oxygen forming triplets. In this process, LYC is more reactive than PF and PE. Once the triplets are formed, they can react, donating an electron to other free radicals rather than to singlet oxygen. As electron donors, the triplets of LYC, PF, and PE are similar. These results may explain the astounding free radical scavenger capacity of PF and PE.

Molecular Orbitals. Figures 8–10 report the Molecular Orbital Pictures of the frontier orbitals of the three molecules being studied. As expected, LYC presents more π bonding orbitals than those of PF or PE. PE has less π orbitals because the number of double bonds is lower. In all cases, HOMO and LUMO are similar. This indicates that the shape of the molecular orbitals has little influence in the HOMO–LUMO gap. Other differences are that LYC presents two degenerated orbitals that correspond to π bonding orbitals of nonconjugated C–C double bonds. PF and PE show two pairs of two degenerated orbitals that are also π bonding orbitals, localized at the nonconjugated double bonds. These molecular orbital images may be very useful for analyzing other reaction mechanisms for scavenging free radicals, for example radical adduct formation.

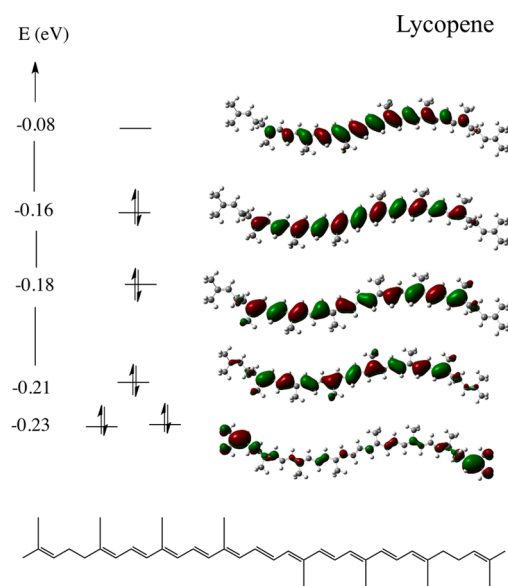


Figure 8. Molecular orbital picture of LYC.

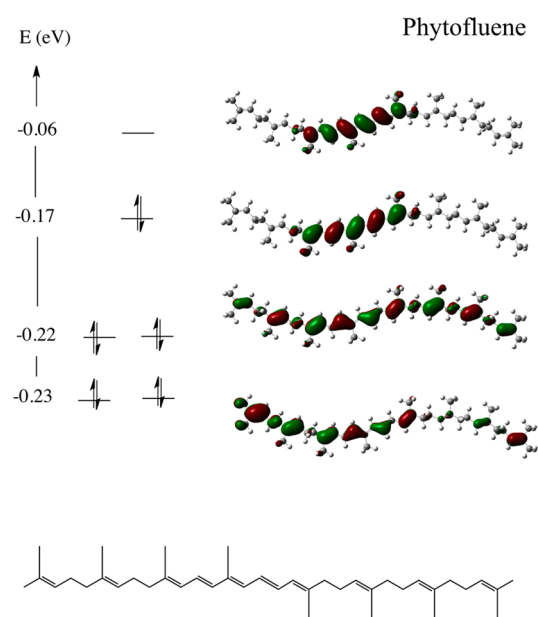


Figure 9. Molecular orbital picture of PF.

CONCLUSIONS

Experimental and theoretical results concur very well. UV–visible spectrum of ABTS (neutral and cation) effectively indicates the decolouration of $\text{ABTS}^{\bullet+}$.

In order to scavenge free radicals, two reaction mechanisms are analyzed: electron transfer and deactivation of singlet oxygen. Considering electron transfer, LYC, PF, and PE represent effective free radical scavengers against $\text{ABTS}^{\bullet+}$, with LYC representing the best and PE the worst antiradical. In order to deactivate the singlet oxygen, it is necessary to analyze the singlet–triplet energy difference that must be small, in order to act as an effective free radical scavenger. LYC presents the lowest value and PE the highest. Consistent with the two reaction mechanisms that were analyzed, LYC is the best free radical scavenger and PE the worst. However, the triplets that are formed following the deactivation of singlet oxygen present similar capacity for donating electrons. In the case of the

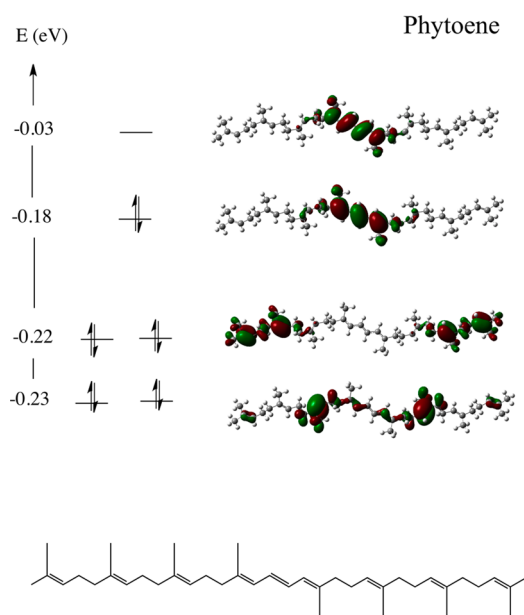


Figure 10. Molecular orbital picture of PE.

triplets, the antiradical capacity of LYC, PF, and PE is similar, considering electron donor mechanism.

Although PF and PE are not as effective antiradicals as LYC, to be expected due to the number of conjugated double bonds, they present considerably higher antioxidant capacity, considering the small number of conjugated double bonds. More studies concerning the behavior of these colorless carotenoids under different oxidation conditions will be interesting for evaluating their potential use as food ingredients, for instance as possible health-promoting carotenoids that do not greatly modify the color of the product. The antioxidant capacity of PF (whose TEAC when measured for this study turned out to be identical to that reported for β -carotene, and 2.5-fold higher than that of α -tocopherol in the reference paper by Re et al.²²) is particularly noteworthy and warrants further study.

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Notes

The authors declare no competing financial interest.

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