From food to mobility: investigating a screening assay for new automotive antioxidants using the stable radical DPPH

Analytical Chemistry

From Food to Mobility: Investigating a Screening Assay for New Automotive Antioxidants Using the Stable Radical DPPH

Clare L. Higgins,[a] Sorin V. Filip,[b] Ashfaq Afsar,[a] Howard M. Colquhoun,[a] and Wayne Hayes*[a]

By taking inspiration from the food industry, an assay was investigated as a potential screening tool to test the efficiency of new phenolic antioxidants. The method was based on the spectrophotometric measurement of the stable free radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) which, in its radical form, has an absorbance maxima at 515 nm. The disappearance of this absorbance band, upon reaction with an antioxidant, was monitored to reveal the kinetic pathway of the reaction, which was defined simply as either fast, medium or slow. Adaptation of the assay was attempted for application to biofuels and oil-based automotive fluids whereby the effect of polar and non-polar solvents on the kinetics of the reaction was investigated. In addition, the stoichiometry of the radical scavenging reaction was also analysed to give an insight into the structure-activity relationships of phenolic antioxidants.

Introduction

Oxidation is an important issue not only for the oil and automotive industries but also for the food industry, where the oxidation of lipids is responsible for changes in the color, flavor, texture, nutritional quality and safety of foods.[1–2] In addition, the presence of radical species in foodstuffs has been found to contribute to the ageing process of human tissues and to the development of various pathological disorders.[2–4] It is therefore necessary for this industry to protect food lipids and human tissues against free radicals by introducing antioxidants of natural or synthetic origin. Naturally-occurring antioxidants, particularly those derived from fruit or vegetable extracts, have gained increasing interest among the scientific community because epidemiological studies have indicated that frequent intake of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer.[5–6] Furthermore, there is widespread agreement that some commonly used synthetic antioxidants such as 2,6-di-*tert*-butyl-4-methylphenol (BHT, **1**) and 2-*tert*-butyl-4 hydroxyanisole (BHA, **2**) (Figure 1) need to be replaced with natural antioxidants because of their toxicity and potential health risks.[7–8] Hence, recent years have seen a significant increase in the use of methods for estimating the radical scavenging efficiencies of a wide range of natural products.[9–11]

1 2

Figure 1. Structures of the synthetic antioxidants BHT (**1**) and BHA (**2**).

A wide range of spectrophotometric assays have thus been developed with convenient methodologies which allow rapid quantification of antioxidant capacities and which lend themselves to high-throughput analysis.[12–16] Free radical scavenging is one of the known mechanisms by which antioxidants inhibit oxidation and consequently the most widely-used assays utilize generated or stable radical species such as 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS, **3**) and 1,1-diphenyl-2 picryl hydrazyl (DPPH, **4**) (Figure 2).[11,17–18] Both assays are based on an electron transfer process and involve the reduction of a colored oxidant, which can be monitored readily by spectrophotometry.

More recent developments of the ABTS assay are based on the generation of the blue/green $ABTS^+$ chromophore through an initial reaction with potassium persulfate ($K_2S_2O_8$).[19] This is a relatively long-lived radical cation and can subsequently be reduced by an antioxidant or hydrogen donor.[9] This process can be monitored by use of UV-vis spectroscopy, with multiple absorbance intensities reported at 415, 645, 734 and 815 nm.[19–21] An advantage of this assay is that it is suitable for

[a] *Dr. C. L. Higgins, Dr. A. Afsar, Prof. H. M. Colquhoun, Prof. W. Hayes*
Department of Chemistry
University of Reading
Whiteknights, Reading, RG6 6AD, UK
E-mail: w.c.hayes@reading.ac.uk
[b] *Dr. S. V. Filip*
BP Formulated Products Technology
Research & Innovation
Pangbourne, UK

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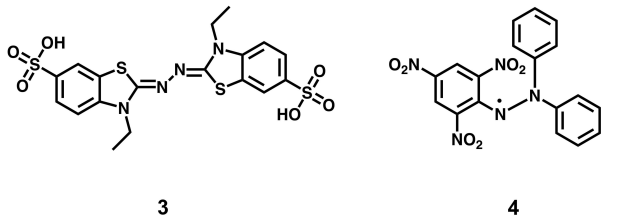
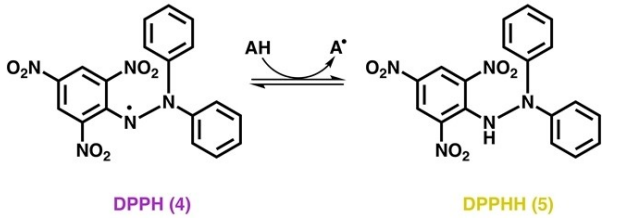


Figure 2. Structures of the spectrophotometric assay species ABTS (**3**) and DPPH (**4**).

strongly colored samples as the absorbance can be measured outside the visible spectral range using the absorbances centred on either 734 or 815 nm.[9] Moreover, this species is water-soluble which is of particular importance when analyzing biological systems. In contrast, the DPPH assay is based on the reduction of the commercially available, stable radical DPPH (**4**) to 1,1-diphenyl-2-picrylhydrazine (DPPHH, **5**) (Scheme 1).[22–23] The DPPH assay shows a color change from deep purple to pale yellow upon reaction. This color change can be monitored by UV-vis spectroscopy by noting the decrease in intensity of the characteristic absorbance maximum at 515 nm (see Supporting Information (SI), Figure S1).[22–23]

Even though these assays may have limitations regarding biological application, there is an opportunity for the methods to be applied to other industries. Currently, in the development of new fuel and lubricant technologies, the performance of a new antioxidant is measured by blending the candidate compound into a hydrocarbon base oil or fuel and using accelerated oxidative conditions to assess the hydrocarbons' resistance to oxidation.[24–28] Exposure to typical conditions actually found in an engine is important, but such methods can require relatively large amounts of the antioxidant and significant blend volumes, which is not always practical. Oxidative stability tests also require the use of specialized and expensive instrumentation, with analytical techniques including pressurized differential scanning calorimetry, Rancimat analysis and bespoke oxidation tests which often have lengthy testing procedures.[29–31] Through the exploitation of the DPPH assay, it is proposed that a suitable screening method to evaluate the performance of previously synthesized antioxidants[29–31] as radical scavengers could be developed and applied in the development stages of biofuels and automotive fluid technologies. The DPPH assay has a significant advantage over the ABTS



Scheme 1. Reduction of DPPH free radical (**4**) by an antioxidant AH to DPPHH (**5**).

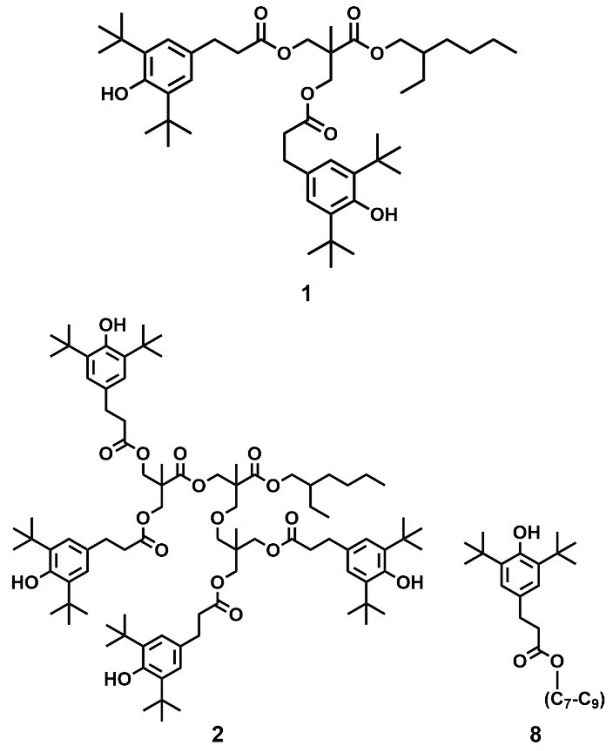


Figure 3. Structures of first generation (**6**) and second generation (**7**) dendritic polyphenols plus the industrial antioxidant Irganox L135 (**8**).

assay in that the radical species is generated directly, thus eliminating the need to introduce additional chemical species into the reaction medium.[23] The DPPH assay could provide a convenient and high-throughput analysis of potential new antioxidants by giving an estimation of which candidates possess radical scavenging capabilities while also probing key antioxidant structure-activity relationships.

Results and Discussion

Radical scavenging analysis using different solvents. The previously-synthesized[29] dendritic polyphenols **6** and **7** alongside the industrial antioxidant Irganox L135 (**8**) (see Figure 3) were analysed for antioxidant activity and compared to the phenolic antioxidant BHT (**1**). Solutions of each antioxidant, at the same molar concentration, were prepared in methanol using the general procedure for radical scavenging experiments (see Experimental section in the SI).

The percentage of DPPH radical remaining was calculated by converting the absorbance to concentration ($mol\,dm^{-3}$) using Equation 1, derived from the calibration plot Figure S2 (see SI). The percentage was subsequently calculated using Equation 2 and plotted against time to produce a 'time-scavenging' graph.

$$Abs_{515nm} = 1.0059[DPPH] + 1 \times 10^{-4} \quad (1)$$

$$\%\ of\ DPPH\ remaining = \frac{[DPPH]_{t=x}}{[DPPH]_{t=0}} \times 100 \quad (2)$$

The time-scavenging graph (Figure 4) showed successful reduction of the DPPH radical (**4**) by all three antioxidants. From the kinetic profile for BHT (**1**), a 'slow' reaction was observed with DPPH (**4**) and the time taken to reach a steady state was greater than one hour, corresponding well to that reported by Brand-Williams and co-workers.[23] Structural inspection of these antioxidants suggested that under equimolar conditions compound **7**, possessing four phenolic groups, would show the best radical scavenging capabilities followed by **6** (two phenolic groups) and finally BHT (**1**) with just one phenolic group.

The results shown in Figure 4 confirmed this trend, showing that **7** had the most efficient radical scavenging profile by scavenging a higher percentage of DPPH radicals in a shorter time than **6** and BHT (**1**). However, even after reaching a steady state, *ca.* 10% of the DPPH radical still remained. It should be noted that the %[DPPH] remaining values in a certain antiradical concentration do depend on the concentration of DPPH.

It was expected that compounds **6** and **7** would scavenge all of the DPPH radicals as there was an excess of radical scavenging sites compared to the number of moles of DPPH radicals. It is therefore postulated that the system under these experimental conditions could reach equilibrium (Scheme 2) in agreement with the mechanistic pathway reported by Brand-Williams and co-workers.[23]

The presence of such an equilibrium highlights a limitation with using this type of data to analyze antioxidant capacity (i.e. the number of radicals scavenged by one antiradical molecule) as it is not possible to measure quantitatively the amount of radical scavenged by each antioxidant. To achieve an improved understanding of how antioxidants may behave in a biofuel, the assay was repeated using ethanol as the solvent with the aim of moving closer to a typical fuel or automotive fluid medium. A calibration of DPPH was carried out and equimolar solutions of BHT (**1**), **6** and **7** were analysed for their radical scavenging properties. In addition, a typical lubricant antioxidant, Irganox L135 (**8**), was also investigated for an industrially relevant comparison. From the 'time-scavenging' profile, shown in Figure 5, a steady-state was observed after 3 hours and again a percentage of the DPPH remained at the end of the 3-hour test in all of the four compounds tested. It was observed that Irganox L135 (**8**), which possesses one phenolic hydroxyl, was scavenging radicals at a very similar rate to **6** which had two phenolic hydroxyls. In addition, Irganox L135 (**8**) scavenged radicals faster than the structurally similar mono-phenol, BHT (**1**). The longer alkyl chain and the ester functionality in Irganox L135 may contribute a greater stabilizing effect than the methyl moiety in BHT, therefore allowing a more efficient scavenging pathway.

Polyphenols can be described as having a higher antioxidant capacity than mono phenols on the basis that there are more phenolic hydroxyls (per molecule) available for hydrogen donation to a radical species.[29] It was therefore expected that **6** would show a greater radical scavenging ability than Irganox L135 (**8**), but this trend was not observed in the present study. The first-generation polyphenol **6** has a bulkier end group in the 2,2 bis(hydroxymethyl)propionic acid [bis-(MPA)] branching unit when compared to Irganox L135 (**8**) which had an unbranched alkyl chain. Furthermore, the additional *tert*-butyl groups surrounding the phenolic hydroxyls on **6** may have

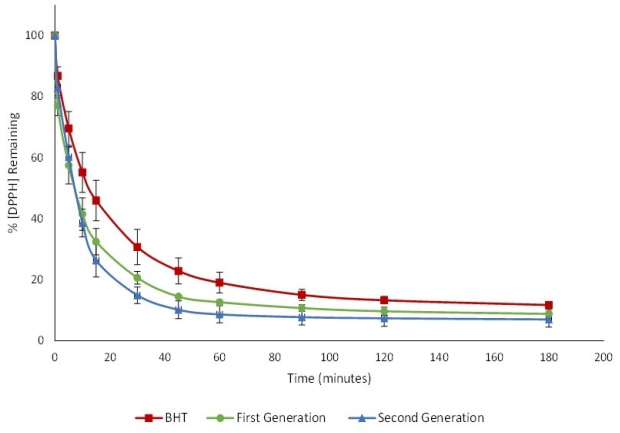
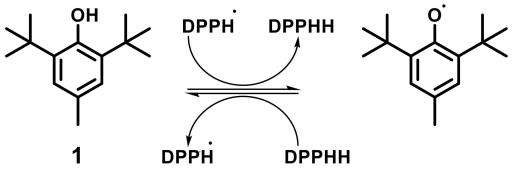


Figure 4. Radical scavenging analysis (25 °C) of equimolar solutions (6×10^{-4} mol dm^{-3} in methanol) of BHT (**1**) and the branched polyphenols **6** (green) and **7** (blue).



Scheme 2. Proposed equilibrium for radical scavenging between the antioxidant BHT (**1**) and the DPPH radical **4**.

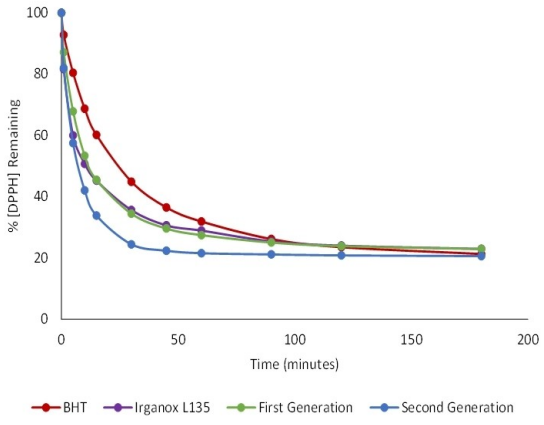


Figure 5. Radical scavenging analysis of equimolar solutions of BHT (**1**), the branched polyphenols **6** and **7** and Irganox L135 (**8**) in ethanol.

greater steric effect on the scavenging reaction by restricting access to the bulky DPPH radical.

To probe radical scavenging in a more typical hydrocarbon-based reaction medium in which the antioxidants would be utilised, BHT (**1**), Irganox L135 (**8**) and the branched polyphenols **6** and **7** were next analysed in 2,6,10,15,19,23-hexamethyltetracosane, commercially known as squalane. The analysis (Figure 6) revealed that BHT and Irganox L135 both followed the same 'intermediate' kinetic profile whereas **6** and **7** displayed a 'slow' kinetic profile similar to that observed in ethanol.

This data suggested that the cause of the differences could be ascribed to the solubility of the antioxidants and its intermediates, their ability to diffuse within the hydrocarbon matrix and also their capacity to react with a bulky radical such as DPPH (**4**). Compounds **6** and **7** are both bulky antioxidants and it would be sensible to suggest that diffusion within the oil would be reduced when compared to the smaller, more soluble Irganox L135 (**8**) and BHT (**1**).[32] From the 'time-scavenging' analysis in different solvents, it is clear that this type of analysis alone is not enough to determine antioxidant efficiency. There were significant differences in the results using different solvent systems, and the number of radicals scavenged could not be quantified as a result of complex mechanistic pathways attributed to each individual antioxidant and solvent. In an attempt to overcome these limitations and to standardize a method, an alternative procedure known as Efficient Concentration (EC_{50}) was investigated.

Efficient concentration (EC_{50}) analysis. Efficient concentration (EC_{50}) can be defined as the amount of antioxidant required to decrease the initial concentration of the DPPH radical by 50%. The advantage of using this method of analysis over the 'time-scavenging' procedure is that numerical values are obtained as a function of the molar ratio of antioxidant to radical, eliminating any issues with incomplete scavenging as a result of an equilibrium between the antioxidant and the radical. Initially, EC_{50} analysis was carried out for BHT (**1**), Irganox L135 (**8**) and the branched polyphenols **6** and **7** in ethanol. Ethanol was chosen as a result of the ease of handling, low cost and low toxicity. Solutions containing different molar ratios of antioxidant to DPPH radical, **4**, were prepared and allowed to stand in the dark at room temperature for 3 hours to ensure a steady state was reached. The absorbance of each solution was measured at 515 nm and converted to the percentage of DPPH radical remaining using Equations 1 and 2. The EC_{50} value was then determined graphically (SI, Figure S3) by finding the molar ratio when the remaining DPPH concentration was equal to 50%. The anti-radical power (ARP) was calculated as the inverse of EC_{50} whereby the larger the ARP the more efficient the antioxidant,[33] and the stoichiometry was calculated by multiplying the EC_{50} value by two giving the theoretical efficient concentration of antioxidant needed to reduce 100% of the DPPH radicals. The number of reduced DPPH radicals per mole of antioxidant was calculated subsequently by the inverse of the stoichiometry ($1/2 \times EC_{50}$). The EC_{50} value shown in Table 1 agreed well with the reported radical scavenging pathway (Figure 7), suggesting that BHT can scavenge 1.85 DPPH radicals per molecule. The pathway shown here suggests that a molecule of BHT should be able to scavenge a maximum of two radicals, but an additional pathway involving combination of two BHT radicals may account for the slightly lower value (1.85) observed experimentally.

Referring to the molecular structures of the polyphenols **1** and **2** and comparing to the radical scavenging pathway of BHT (see Figure S5), it might have been proposed that there are four and eight active scavenging sites for **6** and **7**, respectively. The results from the EC_{50} analysis revealed that this was true for the **7** but not for **6**, which showed a scavenging capacity of only two radicals per molecule, rather than the expected four. Initial considerations were that **7** was a much larger molecule when compared to **6** and it was surprising that the results for **7** revealed such a high scavenging efficiency (8.33) for a bulky radical like DPPH (**4**). However, computational analysis, (molecular mechanics, using Cerius2® modelling software[33]) suggested an immediate explanation for these observations, in that energy-minimization

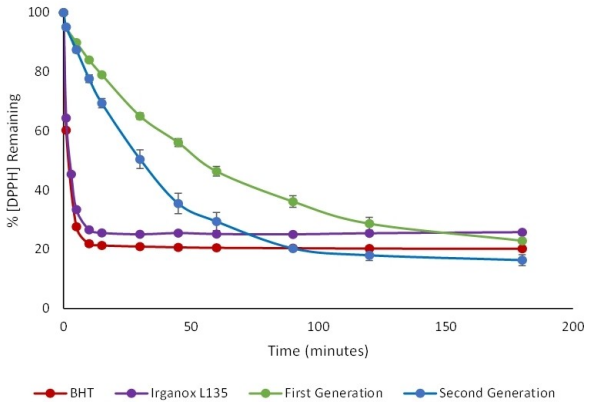


Figure 6. Radical scavenging analysis of equimolar solutions of BHT (**1**), Irganox L135 (**8**), branched polyphenols **6** and **7** in squalane.

Table 1. EC_{50} analysis of BHT (**1**), Irganox L135 (**8**), branched polyphenols **6** and **7**.

Compound	Number of OH groups	EC_{50}	ARP	Stoichiometric Value	Reduced DPPH radicals per molecule
BHT (**1**)	1	0.27	3.70	0.54	1.85
Irganox L135 (**8**)	1	0.49	2.05	0.98	1.02
6	2	0.22	4.55	0.44	2.27
7	4	0.06	16.67	0.12	8.33

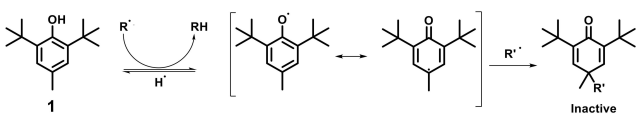


Figure 7. Radical scavenging pathway of BHT (**1**), for R′ = DPPH (**4**).

of **6** led to a structure in which one of the phenolic OH groups lies in close proximity to the para-position of aromatic ring of the other phenol (Figure 8a). Once the first phenol has been oxidized by radical scavenging, intramolecular hydrogen abstraction from the second phenolic OH can occur (Figure 8), thereby deactivating two of the four potential radical-scavenging sites in molecule **6**.

An alternative deactivation pathway, involving oxidation of both phenolic OH groups followed by intramolecular radical recombination at the associated *para*-positions, was also investigated by computational modelling (see SI, Figures S4 and S5). However, optimisation of a model for the resulting cyclic compound resulted in a relatively high-energy minimum, with significant levels of strain resulting from steric interactions between the bulky *t*-butyl groups on neighbouring 6-membered rings. Consequently, this potential deactivation pathway seems inherently less likely than that shown in Figure 8. In contrast, energy-minimization of **7** led to a much more open structure (Figure 9) in which there are no close contacts that would permit intramolecular deactivation. Consequently, the full potential of **7** for radical scavenging (8 radicals per molecule) should be retained, as is indeed observed experimentally.

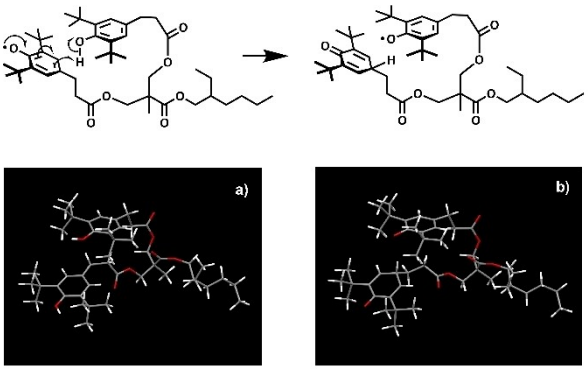


Figure 8. Energy-minimized computational models of (a) the first generation polyphenol **6** and (b) the same molecule after oxidation and intramolecular hydrogen-abstraction as shown in the scheme above.

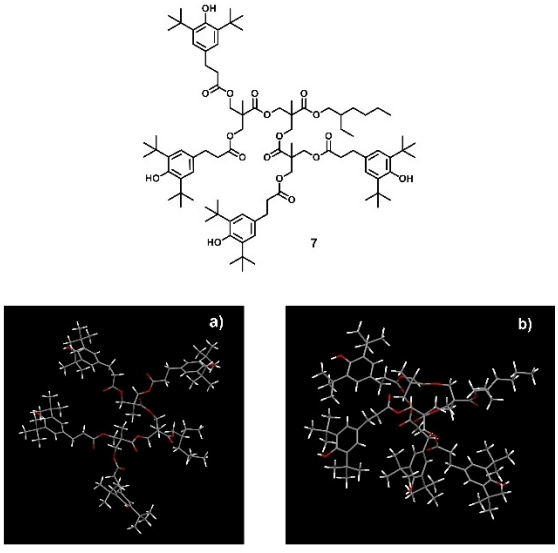


Figure 9. Computational models of the second generation polyphenol **7**. (a) Starting model. (b) energy-minimised structure showing a more "open" final conformation than that found for compound **6**, with no close intramolecular associations between phenolic OH groups and aromatic rings.

Conclusion

In summary, a series of antioxidants were analysed for their radical scavenging properties using the stable free radical DPPH (**4**). Using this assay, both the kinetic profiles and stoichiometry of the radical scavenging reactions were investigated. The radical scavenging reactions in ethanol revealed a 'slow' kinetic profile for BHT (**1**), Irganox L135 (**8**), and branched polyphenol antioxidants **6** and **7**, in agreement with data reported by Brand-Williams and co-workers. When the solvent system was changed from ethanol to squalane, significant shifts in the kinetic profiles of the mono-phenols BHT (**1**) and Irganox L135 (**8**) were observed whereby a steady state was achieved in less than 10 minutes when compared to over 60 minutes in ethanol. Additional factors were considered when analyzing the polyphenolic antioxidants such as increased bulkiness and steric hindrance surrounding the radical scavenging sites within the molecules. A quantitative analysis, termed EC_{50}, was carried out to determine the stoichiometry of the reaction between the antioxidants and the radicals. The results of this analysis (eight radicals scavenged per molecule of **7**, but only two radicals per molecule of **6**) were accounted for in terms of a low-energy conformation for **6** that promotes intramolecular deactivation of two of its four potential radical-scavenging sites.

Supporting Information Summary

Experimental details for radical scavenging studies and computational modelling; UV-vis spectroscopic analysis for reduction of DPPH (**4**); Calibration plot for the DPPH radical (**4**); Graphical analysis of the EC_{50} value for BHT (**1**). Computational modelling of an alternative deactivation pathway for compound **6**.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: antioxidant · DPPH · phenol · radical · scavenger

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