ORIGINAL ARTICLE

The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating antioxidant activity

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Abstract

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The use of the stable free radical diphenylpicrylhydrazyl (DPPH) to estimate the activity of antioxidants is reviewed. Current applications of the method are examined, particularly the use of the parameter EC_{50} (substrate concentration to produce 50% reduction of the DPPH). Some recommendations are made as to the most suitable ways of carrying out this assay and evaluating the data produced.

Key words : DPPH, diphenylpicrylhydrazyl, free radical, antioxidant activity

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There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources (Abdalla and Roozen, 1999). There is therefore a parallel increase in the use of methods for estimating the efficiency of such substances as antioxidants (Sánchez-Moreno, 2002; Schwarz, *et al*., 2001).

One such method that is currently popular is based upon the use of the stable free radical diphenylpicrylhydrazyl (DPPH). The purpose of this paper is to examine the basis of this method, and to further examine the use of the parameter "EC_{50}" (equivalent concentration to give 50% effect) which is currently used in the interpretation of experimental data from the method.

It should be noted that the present paper is not concerned with the correlation between the results of the DPPH method and the actual activity of the substance in autoxidation reactions (Schwarz, *et al*., 2001); neither is it concerned with the actual efficiency of these substances either as antioxidants or as "life-style enhancers" in humans (Wanjek, 2001).

Basis of the Method

1. DPPH - free radical and reduced form

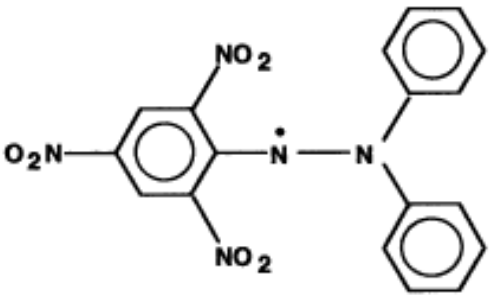
The molecule of 1,1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl; DPPH: **1**) is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to the deep violet colour, characterised by an absorption band in ethanol solution centred at about 520 nm.

When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (**2**) with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present). Representing the DPPH radical by $Z^{\bullet}$ and the donor molecule by AH, the primary reaction is

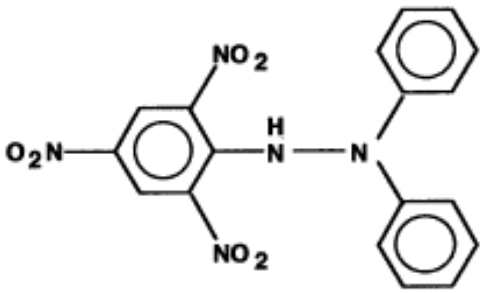
$$Z^{\bullet} + AH = ZH + A^{\bullet} \qquad [1]$$

where ZH is the reduced form and $A^{\bullet}$ is free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorised) by one molecule of the reductant.

The reaction [1] is therefore intended to provide the link with the reactions taking place in an oxidising system, such as the autoxidation of a lipid or other unsaturated substance; the DPPH molecule $Z^{\bullet}$ is thus intended to represent the free radicals formed in the system whose activity is to



1: Diphenylpicrylhydrazyl (free radical)



2: Diphenylpicrylhydrazine (nonradical)

be suppressed by the substance AH.

2. The original Blois method

The DPPH method as summarised above was evidently introduced nearly 50 years ago by Marsden Blois, working at Stanford University (Blois, 1958). Although this paper is short (a little over one page in the journal Nature), it provides a succinct and clear account of the method. He used as his model antioxidant the thiol-containing amino acid cysteine. Representing the DPPH radical by $Z^{\bullet}$ and the cysteine molecule by RSH, the initial reaction is then

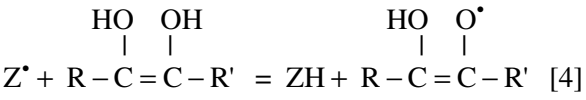
$$Z^{\bullet} + RSH = ZH + RS^{\bullet} \qquad [2]$$

The free radical $RS^{\bullet}$ evidently then reacts with another molecule of the same kind that was produced by a parallel reaction to [2]

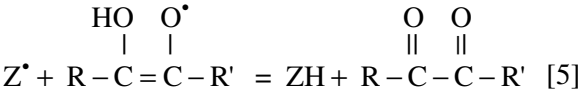
$$RS^{\bullet} + RS^{\bullet} = RS - SR \qquad [3]$$

This therefore leads to the observed reduction of two molecules of DPPH by two molecules of cysteine, that is, a 1:1 stoichiometry.

If however the molecule has two adjacent sites for hydrogen abstraction which are internally connected, as is the case with ascorbic acid (Vitamin C), then there may be a further hydrogen abstraction reaction after the first one:



$$Z^{\bullet} + R-C(HO)=C(OH)-R' = ZH + R-C(HO)=C(O^{\bullet})-R' \qquad [4]$$



$$Z^{\bullet} + R-C(HO)=C(O^{\bullet})-R' = ZH + R-C(=O)-C(=O)-R' \qquad [5]$$

This leads to a 2:1 stoichiometry, that is, two molecules of DPPH reduced by one molecule of ascorbic acid. The same stoichiometry is shown in the reaction with hydroquinone (1,4-dihydoxybenzene) that leads to the production of quinone (1,4-benzoquinone) by a similar two-step mechanism.

It was noted in the original paper that among other compounds active in this reaction are glutathione, aromatic amines (such as *p*-phenylene diamine and *p*-aminophenol), and α-tocopherol (Vitamin E - 2:1 stoichiometry) and polyhydroxy aromatic compounds (such as hydroquinone and pyrogallol). On the other hand, monohydric phenols (such as tyrosine), simple sugars (such as glucose), purines and pyrimidines, do not react, while proteins are precipitated. It was also noted that "inorganic ions in lower valence states may of course interfere and must be eliminated or determined separately" which presumably applies most importantly to ferrous iron (Blois, 1958).

In the original paper, a so-called "typical calibration curve" is presented; this however seems to have been constructed artificially from the original experimental data, since the absorbance values (there called by the previous name, "optical density") are round number values (0.6 down to 0.2), which have therefore evidently been calculated. The graph was also not been extended to allow the line to meet the axis as would be expected to give the end-point for the titration. When extended down to the x-axis, the end-point would correspond to 2.3×10^{-7} moles (230 nanomoles) of this substrate (cysteine hydrochloride). The presumed full titration if continued beyond the end point is shown in idealised form in Figure 1; this graph, however, takes no account either of any residual yellow colour from the reduced form, or of any absorbance contribution there may be from the added sample itself.

It should be evident that the method is a constant-volume colorimetric titration, although the slowness of the overall reaction (with mixtures having to be left for 30 minutes before the absorbance reading is taken) complicates the experimental procedure.

3. The current situation

The original Blois method has been followed by several recent workers (Kim *et al*., 2002; Zhu *et al*., 2002). The more recently introduced method of Brand-Williams and colleagues (Brand-Williams *et al*., 1995) has been used as a reference point by several groups of workers (Gó mez-Alonso *et al*.,

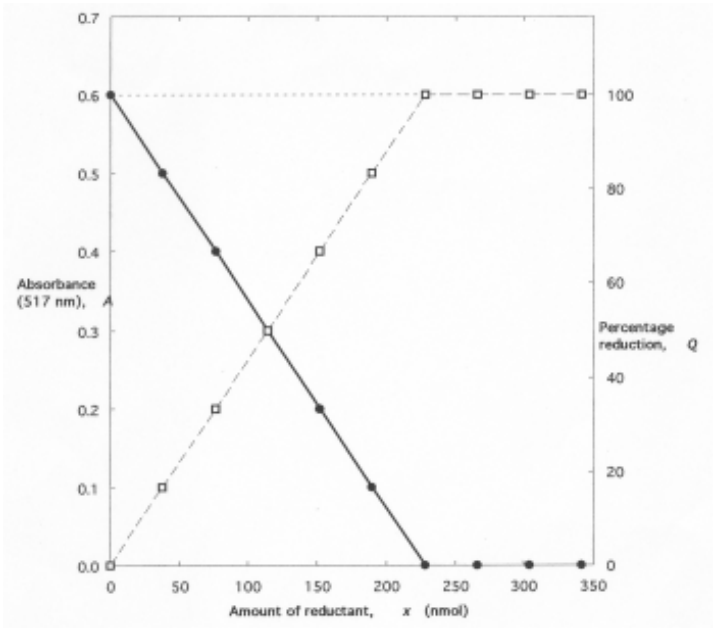


Figure 1. Idealised plots of absorbance *A* (left hand scale and filled circles), and percentage reduction *Q* (right hand scale and open squares), versus the amount of reductant added, for the constant-volume colorimetric titration of DPPH with cysteine hydrochloride; adapted from Blois (1958).

2003; Lebeau *et al*., 2000; Yepez *et al*., 2002). This more recent work has indicated that the picture originally suggested by Blois is somewhat over-simplified, and that because of the complexity of the reactions that follow the initial one [equation 1], the overall stoichiometry need not necessarily be a whole number (integer) such as 1 or 2. Furthermore, the initial step [equation 1] may be reversible, as can be demonstrated by adding the reduced form ZH at the end of the reaction (Bondet *et al*., 1997). Nevertheless, the Blois picture remains a useful one, and the original paper should be read by anyone proposing to use the DPPH method.

4. The parameter EC_{50} ("efficient concentration" value)

One parameter that has been introduced recently for the interpretation of the results from the DPPH method, is the "efficient concentration" or EC_{50} value (otherwise called the IC_{50} value). This is defined as the concentration of substrate that causes 50% loss of the DPPH activity (colour).

This parameter was apparently introduced by Brand-Williams and his colleagues (Brand-Williams *et al*., 1995; Bondet *et al*., 1997), and has been used subsequently by several groups of workers for presenting their results (Kim *et al*., 2002; Lebeau *et al*., 2000; Leitão *et al*., 2002; Lu and Foo, 2000; Sánchez-Moreno *et al*., 1998; Sánchez-Moreno *et al*., 1999). As a term, it was presumably introduced on analogy with "biological" parameters such as LD_{50}.

However, such terminology seems to obscure the true nature of the method, particularly when used alongside such terms as the "dose-response curve" to refer to the titration plot; for it gives the impression that this is in itself some test of biological activity, giving validation to the use of the substrate as an antioxidant in a biological system. Indeed, if anything it is the "EC_{100}" value that we are concerned with, that is, corresponding to the endpoint of the titration. It should be noted

that in all these cases, any residual (yellow) colour from the reduced form or any non-specific absorbance from the sample has to be taken into account in defining the "endpoint" of the titration, or the "50%" point.

This EC_{50} parameter also has the drawback that the higher the antioxidant activity, the lower is the value of EC_{50}. This is a disadvantage particularly when results are presented graphically as a bar chart (Sánchez-Moreno *et al*., 1999) even if the same data are also available in numerical form (Sánchez-Moreno *et al*., 1998).

Recommended Methods of Measurement and Interpretation

1. Introduction

In this section, some recommendations are made as to the methods to be used in the DPPH technique, and in the interpretation of the experimental data. They arise in part from trying to disentangle the method as used in a number of recent papers. It seems that the basis of the original Blois procedure has been lost sight of with the passage of time, and that some pitfalls have therefore appeared in the application of this apparently straightforward technique.

2. Reaction vessel

Assuming that the measurements are carried out using standard 1-cm pathlength spectrophotometer cuvettes, with a maximum working volume of 4 ml, then for the optimum analytical accuracy the mixtures should of 2 mL DPPH solution and 2 mL of the reductant, unless the amounts available preclude this. The common practice of using smaller volumes in either case (such as 0.1 mL plus 3.9 mL, or vice versa) reduces the accuracy of the relative volumes. Since the absorption is well into the visible region (§ 5) then it is possible to use cheap plastic "disposable" cuvettes, which are not attacked by the solvents most commonly used here (methanol or ethanol) (Bondet *et al*., 1997). Numbers of reactions, representing the points along the titration plot (Figure 1), can thus be carried out in parallel. This enables the progress of the reaction to be followed, and allows adequate time for the overall reaction to go to completion in each individual reaction mixture (§ 6).

3. Solvent and pH

Regarding the solvent to be used, the method seems to work equally well with methanol or ethanol, neither of which seems to interfere with the reaction. The use of other solvent systems, such as almost neat extracts in water or acetone, seems to give low values for the extent of reduction (Guo *et al*., 2001).

Regarding the pH level, in the original Blois paper it was suggested that the system should be maintained at a pH in the range 5.0 to 6.5 by using acetate buffers; however, this precaution seems to have been abandoned in current practice. Indeed there is great uncertainty in the meaning of pH values in these predominantly organic (methanol or ethanol) media.

4. Reagent concentration, and the use of standards

In accordance with the normal practice in spectrophotometry, the initial DPPH concentration in the cuvette should be chosen to give absorbance values less than 1.0 (which corresponds to the light intensity being reduced no more than tenfold in passing through the sample). This implies a concentration for the stock solution in the range 50 to 100 μM (§ 7). In the originating paper (Blois, 1958) it was noted that the stock solutions of this "stable free radical" do slowly deteriorate; it was therefore recommended to use an automatic burette with a nitrogen atmosphere and covered with aluminium foil, whereby the loss of free radical activity may be reduced about 2-4 per cent per week.

The substrate concentrations may initially be chosen over a wide range to scan the titration plot, but when the approximate end-point has been found then the values should be spaced evenly up to twice the end-point value to define the two linear sections of the plot (Figure 1).

It should also be noted that when the molar mass of the substrate is known, the practice of

working in terms of masses (grams, milligrams, etc) rather than molar units completely obscures the interpretation of the data on a molecular basis (Sánchez-Moreno *et al*., 1998; Sánchez-Moreno *et al*., 1999), and requires the results to be re-calculated using the relative molar mass M_r, for DPPH ($C_{18}H_{12}N_5O_6$: M_r = 394.33). The use of a only a single mass-in-volume concentration does not help to elucidate the structural basis of the antioxidant activity, since it provides at most only two points on the titration curve (Yepez *et al*., 2002). Likewise, to work in terms of numbers of free radicals (Schwarz *et al*., 2001) necessitates the use of the Avogadro number at some stage to bring the values on to a mole basis.

In the case of mixtures of defined substances, the end-point result will be the sum of that from the individual components. In the case of complex mixtures such as plant extracts, the results should be expressed as DPPH equivalents per gram of material; this would be similar to the expression of the capacity values for ion-exchange resins.

In all these titrations, it is good practice to use standards or "positive controls" alongside the main sample under study. Suitable standards that are widely used are ascorbic acid (Vitamin C) (Brand-Williams *et al*., 1995; Kim *et al*., 2002; Lu and Foo, 2000; Sánchez-Moreno *et al*., 1998; Sánchez-Moreno *et al*., 1999) and α-tocopherol (Vitamin E) (Guo *et al*., 2001; Lu and Foo, 2000; Sánchez-Moreno *et al*., 1998; Sánchez-Moreno *et al*., 1999). These serve to check that the procedures are working correctly. Thus, the fact that, in studies of the antioxidative activity of oolong tea extracts (Zhu *et al*., 2002), the titration plots (percent quenching Q versus sample concentration - see § 7 below) obtained with both with ascorbic acid and with the main samples do not pass through the origin, casts some doubt on the results obtained with the tea extracts themselves.

5. Absorbance measurements - wavelength and instrument used

The working wavelength of maximum absorbance, λ_{max}, to be used for the absorbance measurements is given variously as 515 nm (Bondet *et al*., 1997; Brand-Williams *et al*., 1995; Gomez-Alonso *et al*., 2003; Lebeau *et al*., 2000; Sánchez-Moreno *et al*., 1999), 516 nm (Schwarz *et al*., 2001), 517 nm (Blois, 1958; Lu and Foo, 2000; Zhu *et al*., 2002), 518 nm (Leitão *et al*., 2002), and 520 nm (Kim *et al*., 2002). However, in practice, given that the "peak" is a maximum, that is, round topped, and that the absolute absorbance values are not important, the wavelength can be set to that giving the maximum absorbance in the instrument that is used.

Similarly, although it is general practice to use a spectrophotometer to determine the absorbance, it should be possible to use a simpler and cheaper colorimeter with the filter chosen to give the maximum absorbance with DPPH solutions.

6. Reaction time

In the original method a reaction time of 30 minutes was recommended, and this has been followed in more recent work (Kim *et al*., 2002). Shorter times have also been used, such as 5 minutes (Lebeau *et al*., 2000), or 10 minutes (Schwarz, *et al*., 2001). However, in view of the fact that the rate of reaction varies widely among substrates (Brand-Williams *et al*., 1995; Bondet *et al*., 1997), the best practice seems to be to follow the reaction until it has gone to completion ("plateau") (Lu *et al*., 2000; Sánchez-Moreno *et al*., 1999; Yepez *et al*., 2002). The rate of reaction has also been proposed as a further parameter to characterise the antioxidant activity (Sánchez-Moreno *et al*., 1998; Sánchez-Moreno *et al*., 1999).

7. Plotting the data

The simplest approach in interpreting the data is to plot absorbance against substrate concentration, extending the concentration range beyond the end-point to define the subsequent section of the plot so that the intersection point may be defined most accurately (Figure 1); this would allow for any residual colour from the reduced DPPH, as well as any inherent absorbance from the substrate itself at the working wavelength. The substrate concentrations used should, for definiteness, be those that would be in the reaction

cuvette in the absence of any DPPH. Alternatively, the amount (moles) of substrate added to the reaction vessel may be used (Figure 1).

An alternative method that is commonly used is to work in terms of the percentage reduction of the DPPH, Q, sometime referred to as "inhibition" or "quenching", which is defined by

$$Q = 100\ (A_0 - A_c)/A_0 \qquad [6]$$

where A_0 is the initial absorbance and A_c is the value for added sample concentration c. This value of A_c should be that in the cuvette (or other mixing vessel) in the absence of any DPPH, and should take into account the dilution of the original sample solution by the added DPPH solution. Sometimes the A_0 value is referred to as that of the "control", that is, in the absence of any sample, such as may be used to confirm the stability of the measuring system. It is also presumed that the total concentration of DPPH is kept constant in the measurement sequence.

In some cases the results are presented in the form of residual concentrations of DPPH as obtained from a calibration curve. Strictly speaking, this is an unnecessary complication in view of the fact the DPPH obeys Beer's law in this concentration region (Blois, 1958), so that absorbances are accurately representative of concentrations in these comparative measurements. The combined Beer-Lambert relation takes the standard form

$$A = \varepsilon\, c\, L \qquad [7]$$

where ε is the extinction coefficient, c is the solute concentration, and L is the path length (conventionally, 1 cm). The value of ε for DPPH (in methanol or ethanol at 515 nm, with c in mol L^{-1}) is given variously in the literature as 1.09×10^4 (Lebeau *et al*., 2000), 1.16×10^4 (correcting an error by a factor of 100) (Sánchez-Moreno *et al*., 1998; Sánchez-Moreno *et al*., 1999), and 1.25×10^4 (Bondet *et al*, 1997; Brand-Williams *et al*., 1995).

It is also notable that, in the literature (Bondet *et al*, 1997; Brand-Williams *et al*., 1995; Lebeau *et al*., 2000; Sánchez-Moreno *et al*., 1999), an additive constant invariably creeps into the Beer's law relation (eqn [7]), which is thus presented in the form:

$$A = A_1 + \varepsilon\, c\, L \qquad [8]$$

where the value of the intercept A_1 lies in the range $\pm 1\text{-}3\times10^{-3}$ absorbance units. This presumably arises from a too literal interpretation of the results of a computer program for linear regression on the data. There should be a strict proportionality [equation 7] between A and c so long as, following standard practice, the instrument is zeroed with solvent in a matching cuvette for each sample reading. The origin ($c = 0, A = 0$) is thus a multiple experimental point (once for each sample absorbance reading); which justifies forcing the linear regression line to go through the origin, with $A_1 = 0$.

8. Presenting the results

Insofar as the reaction between the DPPH and the substrate may be expected to be stoichiometric, the end-point may then be represented in terms of n_{DPPH}, the number of DPPH molecules reduced by one molecule of the substrate. This form of notation also serves as a reminder that the result may be expected to depend on the nature of the scavenging molecule, whether this is DPPH or another similar molecule.

In cases where the substrate does not have a defined molar mass, as with plant extracts, the results may be presented in equivalences of DPPH per gram of the extract; this would be analogous to the manner in which the activities of ion-exchange resins are quoted.

Where, to conform with current practice the EC_{50} value is used, this value should represent the concentration of the substrate in the reaction vessel (cuvette) in the absence of DPPH, and the initial DPPH concentration should also be specified; while the stoichiometry value, n_{DPPH}, should also be quoted when the molar mass of the substrate is known.

9. Case study

The application of the notes above may be illustrated by studies on the antioxidative activity of tribromodihydroxybenzyl methyl ether (TDB) (Kim *et al*., 2002). The method used was stated to follow that of Blois (1958), using ascorbic acid as the standard, and mixing 1 mL of the DPPH solution with 4 mL of the substrate solution. The results were presented as values of IC_{50}, (that is, EC_{50}), giving 28.4 μM for ascorbic acid and 7.8 μM for TDB. The conclusions were drawn that "TDB has a higher antioxidant activity as compared with L-ascorbic acid" and that "TDB had strong ... DPPH radical scavenging activity".

It would be desirable to put this on a more quantitative basis, but this is complicated by the fact that stock DPPH solution was quoted as having a concentration of 1.5 M, which evidently in error (§ 4). In addition, the values quoted evidently relate to the sample concentration before dilution by the factor of 4/5 in the cuvette, which give the actual concentrations in the cuvette as 22.7 μM and 6.24 μM respectively.

For ascorbic acid, this corresponds to an end-point concentration of 45.4 μM in the cuvette, and with a 2:1 stoichiometry for this substrate this requires a corresponding DPPH starting concentration in the cuvette of 90.8 μM, and hence a stock DPPH concentration (diluted in the cuvette by a factor of 5) of 454 μM. On the basis of the extinction coefficient data already listed (§ 7), this cuvette concentration gives an initial absorbance value of about 1, which is a reasonable value (§ 4).

Regarding the TDB, the ratio of the IC_{50} values is 3.64, and with a 2:1 stoichiometry for ascorbic acid, this corresponds to that for TDB of 7.3. This is an anomalously high value, but at least it gives a quantitative result to augment the purely qualitative conclusions that were originally drawn by the authors (Kim *et al*., 2002).

Conclusions

The DPPH method has been widely applied for estimating antioxidant activity in recent years, but its applications should to be carried out bearing in mind the basis of the method, and the need wherever possible to establish the stoichiometry for the quenching reaction, so that the antioxidant activity may be related to the structure of the substrate molecule. Likewise, in the case of complex mixtures, at least the presumed presence of active sites in the material should be recognised by working in terms of equivalences of the DPPH molecule. Finally, the originating paper (Blois, 1958) should be consulted by all who use the method, but read in conjunction with the more recent work of Brand-Williams and colleagues (Bondet *et al*., 1997; Brand-Williams *et al*., 1995) which indicates that the situation may not always be as simple as that originally presented.

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