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A novel approach to quantitative structure-property relationships in antioxidants

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Abstract

The autoxidation of linoleic acid, in presence of varying concentrations of antioxidants, was followed by measuring the decoloration of β-carotene. The absorbance versus time curves were fitted to the following expression: $A=A_0\exp(m_i t)$, and the m_i obtained for each antioxidant were fitted to $m_i / m_0 = \text{alog}(1/C)^{\omega}$. When the values of ω were correlated to the ^{13}C NMR chemical shift (δ) of the *ipso*-carbon of the OH group and to the ionisation potential (Ip), the following expressions were obtained: $\omega = -70 + 700/Ip$ or $\omega = \exp(-12 + 120/Ip)$ and $\omega = -80 + 13000/\delta$ or $\omega = \exp(-13 + 23000/\delta)$. Highly significant statistical evidence was obtained in all cases. This methodology is proposed for studies of quantitative structure-property relationships in antioxidants. ω is proposed as a measure of the antioxidant property of a compound.

Key words: Antioxidants; QSPR; quantitative structure-property relationships.

Un nuevo enfoque a las relaciones cuantitativas actividad-estructura en antioxidantes

Resumen

Siguiendo la técnica de la decoloración del b-caroteno, se estudió la autoxidación del ácido linoleico, en presencia de concentraciones variables de antioxidantes. Las curvas del cambio de la absorbancia en función del tiempo fueron ajustadas estadísticamente a la expresión: $A=A_0\exp(m_i t)$, y los valores de allí derivados de m_i, para cada concentración de antioxidante fueron ajustados a la expresión $m_i / m_0 = \text{alog}(1/C)^{w}$. Cuando los valores de w de cada uno de los antioxidantes estudiados, se correlacionaron con el corrimiento químico de Resonancia Magnética Nuclear del ^{13}C ipso al grupo OH (d) y con el Potencial de Ionización de los compuestos (Ip), se obtuvieron las siguientes expresiones: w=-70+700/Ip ó w=exp(-12+120/Ip) y w=–80+13000/d o w= exp(013+23000/d. Para todas esas expresiones se obtuvo evidencia estadística altamente significativa. Los autores proponen el uso de esta metodología para el estudio de las relaciones cuantitativas entre la estructura y las propiedades de compuestos antioxidantes. Asimismo proponen el uso del parámetro w como una medida cuantitativa del poder antioxidante de un compuesto.

Palabras clave: Antioxidantes; relaciones cuantitativas actividad-estructura.

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Introduction

The inhibition of the propagation of free radicals by antioxidants (Eq. 1) has been measured following a variety of techniques, such as, oxygen consumption (1-8), stopped-flow spectrometry (8-10), ESR spectroscopy (11, 12), conjugated dienes formation by UV spectrometry (8, 13-15), polyamide fluorescence (16), peroxide value (17, 18), hexanal formation by static headspace GC (8, 10), hydroperoxide accumulation by HPLC (14). The reaction has been self-initiated or started by free radical initiators (6, 7, 11, 14, 15). The reaction media have been phospholipid bylayers (7), extracts of natural oils (8, 10, 18), styrene solutions (1, 11), fatty acid emulsions (4, 5, 15, 19).

$$ROO\bullet + ArOH \rightarrow ROOH + ArO\bullet$$

Most of the comparisons among antioxidants have been carried out by measuring k_1 (the velocity constant of the equation above) but, in most cases, results have been difficult to interpret due to the use of different methodologies, to the choice of varying end points, and to variations in the interfacial properties of oils and emulsions (8, 10, 20). For example, Huang *et al.* (8) showed that the antioxidant activity depends on the concentration of the substrate, on the length of the oxidation, on the method used to follow the reaction, and, when working with emulsions, on the interfacial affinities.

Barclay *et al.* (1) investigated steric and electronic effects in hindered phenols and hydroxychromans with a second fused ring. Takahashi *et al.* (21), working with phenols and aromatic amines (20), and Iwatsuki *et al.* (14) working with amino phenols and related compounds, did not make their comparison using k_1 but the ratio of the inhibited rate of oxidation to the uninhibited rate, R_{inh}/R_o.

The purpose of this paper is to present a different approach to the search for Quantitative Structure-Property Relationships (QSPR) among antioxidants. We started by adapting a technique developed by Marco (19) and modified by Miller (22), based on the decoloration of β-carotene in the presence of oxidising linoleic acid in an aqueous emulsion. This technique was chosen because it is simple, rapid and requires basic equipment and minimal quantities of reagents. The kinetic data is fitted to mathematical expressions from which a parameter, ω, is obtained. We propose that ω can be used to define the antioxidant property of a compound.

Experimental

Instrument

A Spectronic 3000 from Milton Roy (Rochester, NY, USA), equipped with an eight port thermostable multicell holder.

Materials

Linoleic acid, 2,6-*di*-*t*-butyl-*p*-cresol (BHT), polyoxyethilenesorbitan monolaureate (Tween 20) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); β-carotene was from BHD Chemical Ltd. (Poole, UK); 2-*t*-butyl-4-methoxyphenol (BHA), *o*-cresol and *p*-cresol were from E. Merck (Darmstadt, GE); 2,6-di-terbutylphenol, salicylaldehyde, 4-cyanophenol from Aldrich (Milwaukee, WI, USA); SEP-PAK silica cartridges were from Waters (Milford, MA, USA).

Handling of the linoleic acid

The content of a 10 g ampoule was distributed in 0.5 mL vials. The vials were kept under dry purified argon, sealed and stored at -15°C. Marco's criteria for linoleic acid purity, solidification at -15 °C in less than 16 hr[21], was replaced by using linoleic acid with an absorbance, at 234 nm, not higher than 0.1.

Preparation of the β-carotene solutions

1 g of β-carotene was recrystalized from ethanol, placed in a vial flushed with argon,

sealed and stored at -15°C. 20 mg of β-carotene were dissolved in 500 µL chloroform and taken, with petroleum ether, to a 10 mL volumetric flask. This solution, kept under dry purified argon at -15°C, can be used for several days.

Preparation of the β-carotene emulsion

300 µL of the β-carotene solution was passed through a SEP-PAK silica cartridge, previously flushed with petroleum ether, and eluted, into a 100 mL volumetric flask, with 6 mL of the same solvent. The solution was concentrated to 10% its original volume by flushing dry purified argon. 200 mg Tween 20 and 13.5 mmol linoleic acid was added. The remaining solvent was thoroughly evaporated. The content of the flask was taken up to 100 mL using de-ionised water, which had been saturated with dry purified argon. The flask was taken into an ultrasonic bath to degasify and to homogenise the emulsion. The resulting emulsion should be transparent. 30 mL of this emulsion were saturated with oxygen for 60 s. At this point, the emulsion should be used immediately.

Preparation of the antioxidant solutions

Ethanolic solutions of the antioxidants were prepared at concentrations selected as explained later.

Other recommendations

De-ionised water should have conductivity no greater than 4 µS, otherwise the emulsion will not be homogeneous; nor will the kinetic data be reproducible. After each use the SEP-PAK was washed with ethanol and with 10 mL petroleum ether, the cartridges were discarded when channels began to appear in the silica. *O*-cresol and *p*-cresol were freshly distilled. The preparation of the solutions, emulsions, and the oxidation reactions must be carried out under dim diffuse light.

Oxidation procedure

Preparation of the reaction cells: Cell 1 (reference cell, 100% T) contained 2 mL of the reference solution (200 mg Tween 20 in 100 mL de-ionised water). Cells 2 to 7 (reaction cells) 100 µL of the antioxidant solution in order of decreasing concentration. Cell 8 (control cell, no antioxidant) 100 µL ethanol. 2 mL of the β-carotene emulsion was added to all but the reference cell. The multiport cell holder was placed in the spectrophotometer, brought to a constant 50.0 ± 0.1°C, and the absorption was measured in the 455-465 nm range. Readings were automatically taken every 2 min during 90 min. All experiments were carried out in triplicate.

Experimental design

For every antioxidant, six different concentrations, plus the control, were evaluated simultaneously in each experiment. Readings of the absorbance were taken every two minutes. The experiments were repeated at least three times for each compound.

Calculations. The antioxidants ^{13}C NMR chemical shift, δ, were estimated using ChemWindows3 v. 3.0.2 (SoftShell Int, Grand Junction, CO, USA). CS Chem 3D Pro (CambridgeSoft, Mass, USA) was used to optimise the molecular geometry, first by the molecular mechanics method MM2 and then by the semiempirical method AM1, from this last result the Highest Occupied Molecular Orbital (HOMO) energy of the singlet molecule was obtained. The value of a variety of electronic, topological and lipophilicity indexes, for each compound, were also obtained from this software. Statgraphics Plus 6.0 (Manugistics Inc, Rockville, MD, USA) was used for the statistical analysis of the data.

Results and Discussion

The kinetic of the autoxidation of linoleic acid was followed by measuring the decrease in the absorbance due to the bleaching of β-carotene, as a function of time. Figure 1 shows an example of the family of

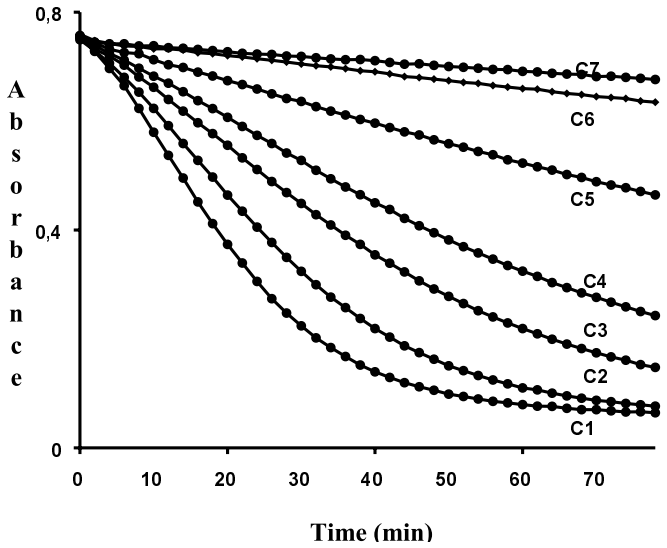


Figure 1. Typical absorbance-time behaviour of an antioxidant at different concentrations. The concentration increases from C_2 to C_7, C_1 is the control.

curves obtained when the effect of a range of concentrations of an antioxidant on the oxidation of linoleic acid are studied by this technique. The lowermost curve, C_1, corresponds to the control, that is, the autoxidation of the linoleic acid without any antioxidant added; while the uppermost curve,C_7, corresponds to the highest antioxidant concentration added to the reaction system. Obviously, the family of curves illustrates the smooth raising of the inhibitory effect when increasing the antioxidant concentration. In order to obtain those curves, the range of appropriate concentrations had to be first determined for each of the compounds studied, because above a certain value the concentration is so high that no reaction occurred, on the other hand too low a concentration will show no antioxidant effect. The best concentration ranges were: 1×10^{-5} to 2×10^{-7} M for BHA (a); 1×10^{-5} to 3×10^{-7} M for BHT (b); 1×10^{-5} to 1×10^{-7} M for 2,6 diterbutylphenol (c); 1×10^{-2} to 2×10^{-4} M for *p*-cresol (d); 1×10^{-2} to 8×10^{-4} M for *o*-cresol (e); 7×10^{-4} to 1×10^{-6} for 2, hydroxybenzaldehyde (f) and 1×10^{-2} to 2×10^{-3} M for 4, cyanophenol (g). In the figures, those compounds will be referred according to their accompanying letters.

The data depicted in Figure 1 is then fitted to the following expression:

$$A = A_0 \exp(m_i t) \qquad \text{[Eq. 1]}$$

where A is the absorbance at t minutes, A_0 is the absorbance a t = 0, m_i is the constant obtained for the ith concentration, and t is the elapsed time. For each concentration one gets a value of m_i, the one for the control (no antioxidant added) is referred to as m_0.

An example of this calculation is shown in Figure 2. The dots represent the experimental values, the smooth line is the regression curve. Similar graphs were obtained for each concentration of all the compounds. In every case, the results from the regressions are supported by highly significant statistical evidence. The correlation coefficients-squared, R^2, (which measures the relationship between the predicted and observed values of the dependent variable) were higher than 0.93, indicating a very strong relationship between the variables. The number of experimental points included in

the regression, n, were larger than 150. The standard error of the estimations by the model, s. e. e., (measures the amount of variability in the dependent variable which is not explained by the estimated model) were always smaller than 0.009. And the F values (the ratio between regression and residual variances) were always larger than 80000, which ensure a highly statistical significance of the model at the 99% confidence level.

Marco (19) and Miller (22) were capable of qualitatively comparing the efficiency of different antioxidants by measuring their absorbance *versus* time behaviour at the same antioxidant concentration. Using this technique we compared the antioxidants under study in this work and found the following qualitative order for their activities (Scheme 1).

Evidently, the better the antioxidant the slower (more inhibited) will be the velocity of the reaction, or in other words, the better antioxidants will have smaller (in absolute value) slopes in a graph like Figure 1. In this opportunity the terms C_1 to C_7 identify, not one antioxidant measured at seven different concentrations, but seven antioxidants assayed at the same concentration.

We are interested in QSPR calculations, for that we need a parameter that does not depend on the concentration. For that reason we related the ratio m_i / m_0 to the concentration, C, according to a multiplicative lineal regression:

$$m_i / m_0 = aX^{\omega} \quad \text{where } X = \ln 1/C \qquad \text{[Eq. 2]}$$

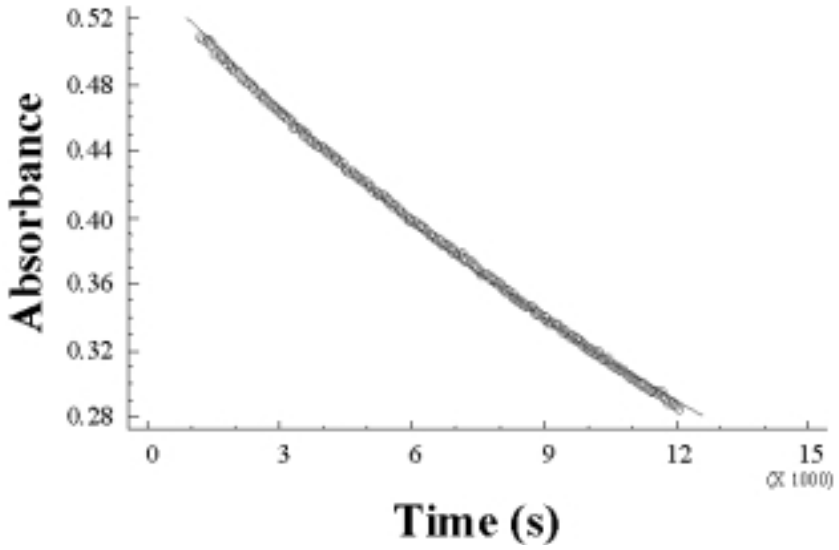
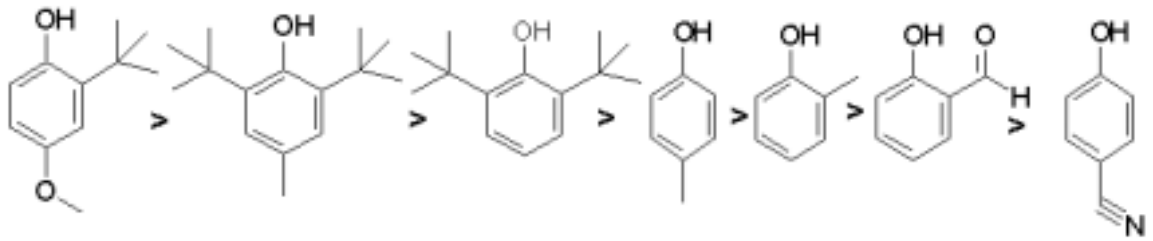


Figure 2. An example of the calculations of m_i, (Eq. 1).



Escheme 1.a > b > c > d > e > f > g

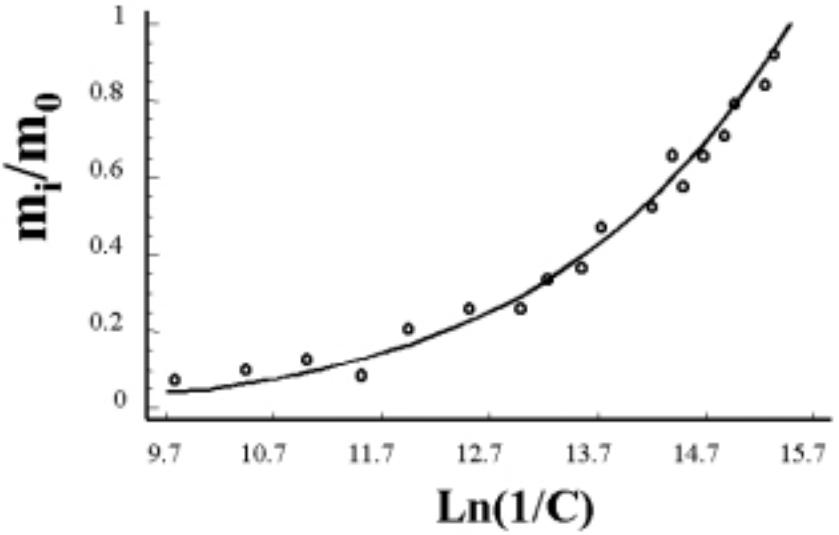


Figure 3. A typical result of the calculation of ω, (Eq. 2). m_i is the constant derived from eq. 2 for the ith concentration of the antioxidant and m_o is the constant when no antioxidant has been added.

Table 1
ω, ANOVA results, Ip and $\delta\ ^{13}C$ of the antioxidants studied

	$\omega \pm$ s.e.	F	R^2	s. e. e.	n	I_p	$\delta\ ^{13}C$
BHA	10.8 ± 0.4	739	96.4	0.2	30	196.0	146.3
BHT	8.7 ± 0.3	737	97.8	0.1	27	198.6	147.7
2,6-diterbutyl phenol	6.4 ± 0.2	263	94.6	0.1	29	203.0	150.7
o-cresol	4.3 ± 0.3	338	92.1	0.3	26	207.5	158.0
p-cresol	3.8 ± 0.3	331	95.7	0.2	20	204.8	154.3
Salicylaldehyde	3.1 ± 0.1	253	93.5	0.3	18	216.5	158.5
4-cianophenol	2.1 ± 0.1	211	94.1	0.3	20	219.3	161.6

All ω values are statistically significant at the 99% confidence level. s. e. is the standard error of ω. F is the F value of the regression. R^2 is correlation coefficient squared (%). s. e. e. is the standard error of the estimation by the model. n is the number of points in the regression. I_p and $\delta\ ^{13}C$ are the ionization potential (Kcal / mol) and the ^{13}C NMR chemical shift of the ipso-carbon of the OH group.

Figure 3 is an example of the calculations carried out to obtain ω. Table 1 shows the values of ω obtained for each of the compounds. From the parameters of the ANOVA: F, R^2, n and e.s.e., it is concluded that the regressions are all statistically significant at the 99% confidence level.

As a first result, the values of ω, Table 1, sort the studied compounds in exactly the same order as we had previously found experimentally. The sequence agrees with the order predicted by the influence of the groups in the ring, i.e., electron-donor groups increase the antioxidant activity, while electron-withdrawing groups decreases it. The antioxidant activity is also increased by the presence of bulky R groups in *orto* position. This predictive capability points toward the conclusion that ω can be used to quantitatively represent the antioxi-

dant efficiency of a compound, which is one of the primary aims of this work.

A variety of electronic, topological and lipophilicity indexes were evaluated for each compound (dipole moment, dispersion polarity, hydrogen bond acceptor, hydrogen bond donor, surface area, molecular volume, molecular length, molecular width, molecular depth, log P, hydrophilicity-lipophilicity balance, % hydrophilic surface, solubility parameter, parachor, water of hydration, connectivity and valence indexes, kappa shape index and substituent parameters: pi, sigma and MR). Statistically significant correlations with ω were only obtained for the HOMO energy and the ^{13}C NMR chemical shift of the *ipso*-carbon of the OH group (represented as $\delta\ ^{13}C$).

Figures 4 and 5 show the best two models obtained for the correlation of the ω values with the ionisation potential, Ip, which was estimated as the energy of the HOMO, expressed as Kcal/mol. Highly significant regressions at the 99% confidence level are obtained, the corresponding fitted models are:

$\omega = -70 + 700/Ip$ [Eq. 3, Fig. 4]
$R^2 = 83.4\%$, F = 25.1, n = 7, e.s.e = 1.6

$\omega = \exp(-12 + 120/Ip)$ [Eq. 4, Fig. 5]
$R^2 = 95.2\%$, F = 98.7, n = 7, e.s.e = 0.14

Matsushita (23) had suggested that Ip should correlate well with the ability of phenols to donate electrons and hence to the antioxidant activity, but the goodness of this correlation was later refuted (24). Our results support Matsushita's suggestion.

The following were the best models obtained for the relationship of ω with the ^{13}C NMR chemical shift of the *ipso*-carbon of the OH group ($\delta\ ^{13}C$). In both cases, statistical significance was obtained at the 99% confidence level:

$\omega = -80 + 13000/\delta$ [Eq. 5, Fig. 6]
$R^2 = 89.8\%$, F = 44.1, n = 7, e.s.e = 1.2

$\omega = \exp(-13 + 23000/\delta)$ [Eq. 6, Fig. 7]
$R^2 = 96.0\%$, F = 120.2, n = 7, e.s.e = 0.13

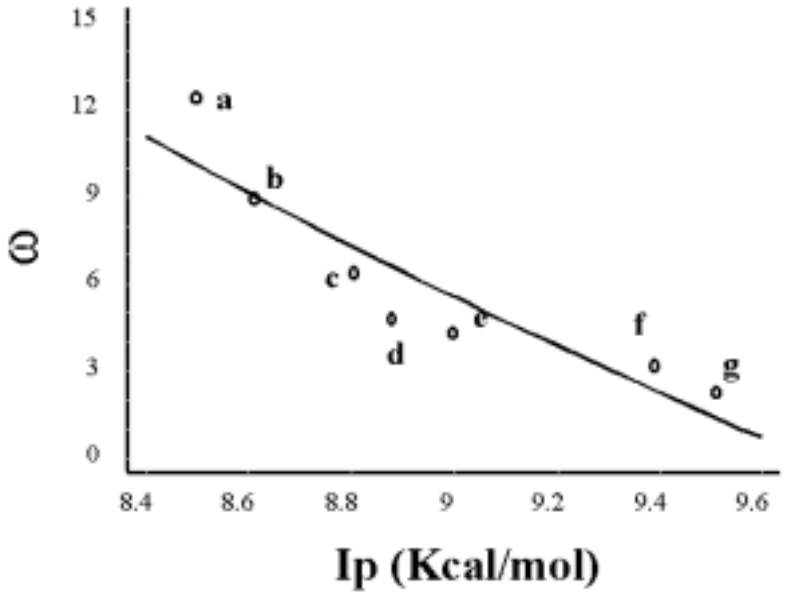


Figure 4. Regression of ω on 1/Ip, (Eq. 3). Letters denote the compound associated with the experimental point. a=BHA, b=BHT, c=2,6 diterbutylphenol, d=*p*-cresol, e=*o*-cresol, f=hydroxybenzaldehyde, g=cyanophenol.

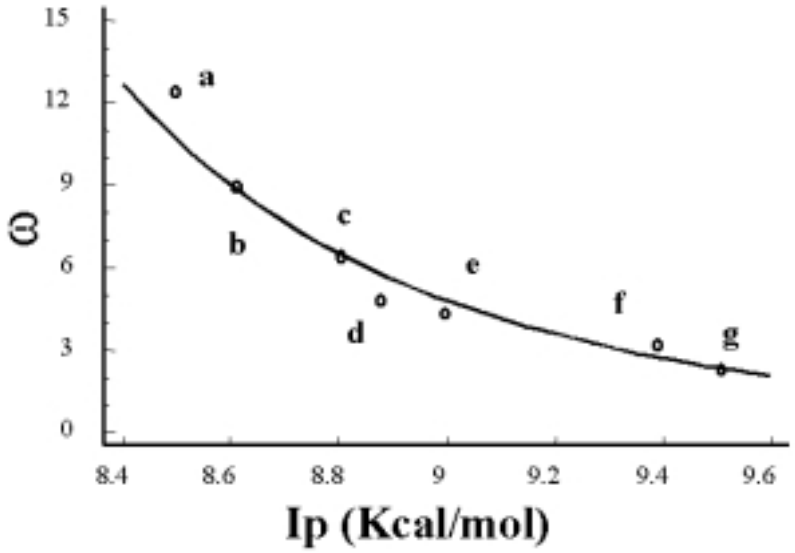


Figure 5. S-curve regression of ω on Ip, (Eq. 4). Letters denote the compound associated with the experimental point. a=BHA, b=BHT, c=2,6 diterbutylphenol, d=*p*-cresol, e=*o*-cresol, f=hydroxybenzaldehyde, g=cyanophenol.

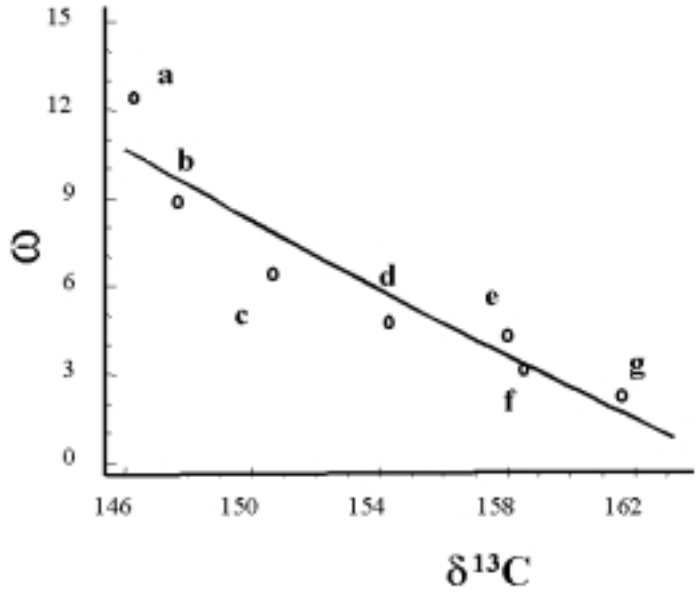


Figure 6. Regression of ω on $1/\delta\,^{13}C$, (Eq. 5). Letters denote the compound associated with the experimental point. a=BHA, b=BHT, c=2,6 diterbutylphenol, d=*p*-cresol, e=*o*-cresol, f=hydroxybenzaldehyde, g=cyanophenol.

It has been reported (25) that the rates of oxidation during the induction period correlate well with the ^{13}C NMR chemical shift of the *ipso*-carbon of the OH group. At this point it might be worth to consider a recent paper (26) where the methodology used to derive the rate of the inhibited reaction, based upon kinetic data obtained during the

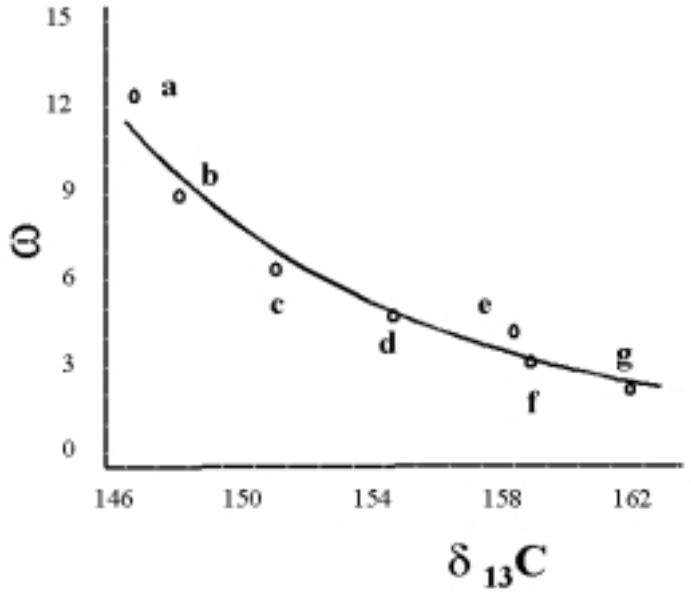


Figure 7. S-curve regression of ω on δ ^{13}C, (Eq. 6). Letters denote the compound associated with the experimental point. a=BHA, b=BHT, c=2,6 diterbutylphenol, d=*p*-cresol, e=*o*-cresol, f=hydroxybenzaldehyde, g=cyanophenol.

fully inhibited period (27), is challenged because the consumption of the antioxidant during the induction period is not taken into account.

Using an approach like the one we present in this paper, no assumptions regarding the mechanism of the reaction need to be made; which avoids obstacles such as the above mentioned criticism on the use of the induction period kinetic data, the eventual uncertainty in the number of radical trapped per antioxidant molecule, the assumption that production of labile radicals by the reaction of antioxidant radicals with the substrate -the chain transfer reactions- are negligible; or the need to express the antioxidant power only in relation to another compound chosen as standard.

We propose that ω can be used as a quantitative measure of the antioxidant property of a compounds and, based on the good correlations obtained in this work, we also propose that ω can be calculated from a combination of electronic, topological and lipophilicity indexes of the compound.

In our current research we are working with a large number of compounds in order to refine the regression models and to test for the necessity of other parameters to be included in the model.

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References

1. BARCLAY L.R.C., VINQVIST M.R., MUKAI M., ITOH S., MORIMOTO H. ***J Org Chem***, 58: 7416, 1993.
2. PRYOR W.A., STRICKLAND T., CHURCH D.F. ***J Am Chem Soc***, 110: 2224, 1988.

3. BURTON G.W., INGOLD K.U. ***J Am Chem Soc*** 103: 6472, 1981.
4. CASTLE L., PERKINS M.J. ***J Am Chem Soc*** 108: 6381, 1986.
5. PRYOR W.A., KAUFMAN M.J., CHURCH D.F. ***J Org Chem*** 50: 283, 1985.
6. COSGROVE J.P., CHURCH D.F., PRYOR W.A. ***Lipids***, 22: 299, 1987.
7. NIKI E., TSUCHIYA J., YOSHIKAWA Y., YAMAMOTO Y., KAMIYA Y. ***Bull Chem Soc Jpn***, 59: 497, 1986.
8. HUANG S.W., FRANKEL E.N., GERMAN J.B. ***J Agric Food Chem*** 42: 2108, 1994.
9. MUKAI K., FUKUDA K., TAJIMA K., ISHIZU K. ***J Org. Chem***, 53: 430, 1988.
10. ITOH S., NAGAOKA S.I., MUKAI K., IKESU S., KANEKO Y. ***Lipids*** 29: 799, 1994.
11. BURTON G.W., DOBA T., GABE E.J., HUGHES L., LEE F.L., PRASAD L., INGOLD K.U. ***J Am Chem Soc*** 107: 7053, 1985.
12. LUCARINI M., PEDULLI G.F. ***J Org Chem*** 59: 5063, 1994.
13. FRANKEL E.N., HUANG S.W., KANNER J., GERMAN J.B. ***J Agric Food Chem*** 42: 1054, 1994.
14. IWATSU M., KOMURO E., NIKI E. ***Bull Chem Soc Jpn*** 68: 620, 1995.
15. PRYOR W.A., CORNICELLI J.A., DEVALL L.J., TAIT B., TRIVEDI B.K., WITIAK D.T., WUM. ***J Org Chem*** 1993, 58, 3521.
16. PORTER W.L., BLACK E.D., DROLET A.M. ***J Agric Food Chem*** 37: 615, 1989.
17. YANISHLIEVA N. VI., MARINOVA E.M. ***Fat Sci Technol*** 94: 374, 1992.
18. MARINOVA E.M., YANISHLIEVA N. VI., KOSTOVA I.N. ***Food Chem*** 51: 125, 1994.
19. MARCO G.J. ***J Am Oil Chem Soc*** 45: 594, 1968.
20. FRANKEL E.N. ***Trends Food Sci Technol*** 4: 220, 1993.
21. TAKAHASHI M.J., TSUCHIYA J., NIKI E. ***Bull Chem Soc Jpn*** 62: 1880, 1989.
22. MILLER H. ***J Am Oil Chem Soc*** 48: 91, 1971.
23. MATSUSHITA S. in: ***Medical, biochemical and chemical aspects of free radicals,*** Vol. 1, eds. Hayaishi O., Niki E., Kondo M. and Yoshikawa T., Elsevier, New York (USA), p. 327, 1988.
24. VAN ACKER S.A.B.E., KOYMANS L.M.H., BAST A. ***Free Radical Biology & Medicine,*** 15: 311, 1993.
25. TOMIYAMA S., SAKAI S., NISHIYAMA T., YAMADA T. ***Bull Chem Soc Jpn*** 66: 299, 1993.
26. LISSI E.A. ***Bol Soc Chil Quim*** 40: 101, 1995.
27. NIKI E. ***Chem Phys Lipids*** 44: 227, 1987.