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Comparative evaluation of the antioxidant capacity of smoke flavouring phenols by crocin bleaching inhibition, DPPH radical scavenging and oxidation potential

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Abstract

The antioxidant capacity of the main phenolic compounds present in wood smoke and smoke flavourings used in the food industry was investigated by three methods, based on a kinetic and thermodynamic approach: the bleaching of the carotenoid crocin, the scavenging of the DPPH radical, and the determination of the oxidation potential. The reaction with the DPPH radical was evaluated calculating the effective concentration (EC_{50}) and the antiradical efficiency (AE). The compounds tested were 2-methoxyphenols (guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-vinylguaiacol, 4-propylguaiacol, eugenol, isoeugenol, vanillin, acetovanillone, 2-propiovanillone), 2,6-dimethoxyphenols (syringol, 4-methylsyringol, 4-allylsyringol, syringaldehyde, acetosyringone) and dihydroxybenzenes (catechol, 3-methylcatechol, 4-methylcatechol, 3-methoxycatechol and hydroquinone). The trend in antioxidant capacity was similar in all the three methods, with dihydroxybenzenes > 2,6-dimethoxyphenols > 2-methoxyphenols, although some discrepancies in the ranking within the groups were present. Considering the overall ranking, isoeugenol was amongst the most active compound, like dihydroxybenzenes, evidencing the role of a conjugated double bond at *para* position for the stabilization of the phenoxyl radical in the radical scavenging process.

Keywords: Antioxidant capacity; Crocin; DPPH radical; 2-Methoxyphenols; 2,6-Dimethoxyphenols; Dihydroxybenzenes; Oxidation potential; Smoke flavourings

1. Introduction

The use of wood smoke and smoke flavourings for the curing of food such as ham, bacon, sausages, fish and cheese is still nowadays widespread both for flavouring and preservation. Smoking affects the organoleptic properties of food in many ways, by imparting a characteristic flavour and taste, by modifying the texture and the color and by improving the shelf life by its antimicrobial and antioxidant activities (Toth & Potthast, 1984).

From the point of view of their chemical composition, both smoke and smoke flavourings are very complex mixture, which depend on the type of wood, the pyrolysis process conditions and the treatments of the smoke. The composition of smoke has been extensively studied in recent years and more than 2000 compounds were identified (Guillén, Manzanos, & Zabala, 1995; Guillén & Manzanos, 1996, 1997, 1999a, 1999b, 2002; Guillén & Ibargoitia, 1996a, 1996b; Guillén & Ibargoitia, 1999; Guillén, Manzanos, & Ibargoitia, 2001). These compounds belong to many different chemical classes: aldehydes; ketones; alcohols; acids; esters; furan and pyran derivatives; phenolic derivatives; hydrocarbons; nitrogen compounds. Among them, the phenolic fraction probably represents the most important one both from the qualitative and quantitative point of view. This fraction is constituted mainly by phenol, 2-methoxyphenol (guaiacol), 2,6-dimethoxyphenol (syringol) and their derivatives and by dihydroxybenzenes originated from the pyrolysis of lignin. Guillén and

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Ibargoitia (1996b) and Guillén and Manzanos (1999b) reported concentrations in the range 1300–2000, 500–1500 and 300–400 mg/l, respectively for guaicol derivatives, syringol derivatives and dihydroxybenzenes, in liquid smoke flavourings prepared in laboratory. The phenolic compounds, and in particular the methoxyphenols, have been considered the major contributors to smoke aroma. Moreover they are responsible for the antimicrobial and antioxidant effects in smoked foods (Estrada-Munoz, Boyle, & Marsden, 1998; Faith, Yousef, & Luchansky, 1992; Guillén & Ibargoitia, 1998; Suñen, Aristimuno, & Fernandez-Galian, 2003). Regarding this last aspect, some authors reported an increase of the oxidative stability in bacon and other smoked meat and fish products with respect to the corresponding non smoked products ascribing the antioxidant effect to the phenolic compounds present in the wood smoke (Coronado, Trout, Dunshea, & Shah, 2002; Espe, Nortvedt, Lie, & Hafsteinsson, 2002; Schwanke, Ikins, Kastner, & Brewew, 1996). Very few results are reported about the amount of smoke phenols in smoked foods. Recently Cardinal et al. (2004) determined by spectrophotometry the total phenols content in a large number of cold-smoked salmon samples reporting a concentration range from 0.04 mg/100 g to a maximum of 2.0 mg/100 g expressed as phenol. Barclay, Xi, and Norris (1997) studied the antioxidant properties of phenolic lignin model compounds (among them 4-propylguaiacol, eugenol, isoeugenol and 4-allyl-2,6-dimethoxyphenol), reporting a higher antioxidant capacity of these compounds with respect to the commercial 2,6-di-*tert*-butyl-4-methylphenol (BHT). The antioxidant activities of eugenol, guaiacol and 4-allyl-2,6-dimethoxyphenol were also reported by Ogata, Hoshi, Shimotohno, Urano, and Endo (1997). Kajiyama and Ohkatsu (2001) studied the effect of *para*-substituents of phenolic compounds determining the antioxidant activities of a group of *para*-substituted 2-methoxyphenols. Kjällstrand and Petersson (2001a, 2001b) discussed the phenolic composition of wood smoke from the point of view of the antioxidant properties.

In this work, the antioxidant capacity of the principal phenolic compounds (Fig. 1) of wood smoke and smoke flavourings used for food was evaluated in order to improve the knowledge about their effect on the oxidative stability of smoked foods. These compounds, due to their chemical structure, are moreover well suitable to study the relationship between antioxidant capacity and structure, in particular the effect of substituents at the *para* position of the guaiacyl and syringyl derivatives.

There are several analytical methods which are routinely used to evaluate the capacity of antioxidant compounds (Prior & Cao, 1999). Among them, the oxygen radical absorbance capacity (ORAC) assay, the trolox equivalent antioxidant capacity (TEAC) assay and the diphenyl-1-picrylhydrazyl radical (DPPH) assay are probably used most often. These methods have been used to test a large variety of plant extracts, such as rosemary, sage, oregano, thyme, olive, tea and grapes, which are of interest to the food industry, as alternatives to synthetic antioxidants or in the field of functional foods and nutritional supplements. The crocin bleaching inhibition assay (Bors, Michel, & Saran, 1984) was used by Tubaro, Micossi, and Ursini (1996), in a modified form, to analyze single compounds as well as complex mixtures like virgin olive oils, rosemary extract and Maillard reaction products. With this method, which is less frequently used, the ability of a compound to quench peroxyl radicals is measured by way of comparison of parallel reactions where the antioxidant and the carotenoid crocin compete for the peroxyl radicals formed by thermal decomposition of a diazo-compound. By changing the solvent, the method can be applied both to lipophilic as well to hydrophilic compounds and the antioxidant capacity is then calculated relatively to α-tocopherol or Trolox C.

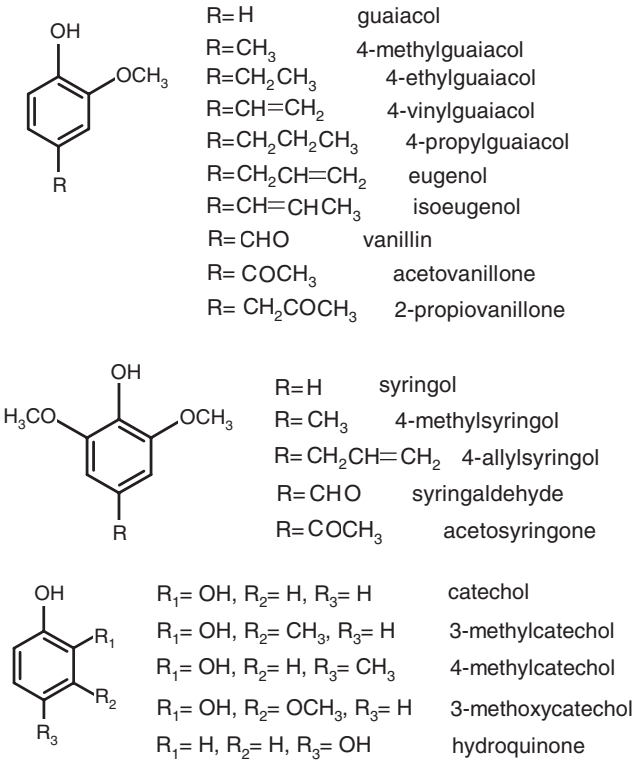

Fig. 1. Structures of studied phenolic compounds.

In recent years an approach involving electrochemical methods has been used by some authors, to evaluate the antioxidant capacity of wines, flavonoids, edible plant extracts and natural phenolic compounds (Buratti, Pellegrini, Brenna, & Mannino, 2001; Chevion, Roberts, & Chevion, 2000; Kilmartin, Zou, & Waterhouse, 2001; Mannino, Brenna, Buratti, & Cosio, 1998; Peyrat-Maillard, Bonnely, & Berset, 2000; Yang, Kotani, Arai, & Kusu, 2001). Kilmartin et al. (2001) ranked a group of wine phenolic acids, flavonoids and ascorbic acid on the basis of their oxidation potentials. The oxidation potential measures the ability of a compound to act as a reducing agent and thus to function as an antioxidant and depends on the chemical structure of the molecule. Contrary to the crocin method where the antioxidant effect is displayed by hydrogen transfer, the

voltammetric method involves a different pathway, based on electron transfer. Moreover, the measure of the oxidation potential does not require the intervention of other chemical species, like radicals or competing molecules. In this work, all three methods, the bleaching of the carotenoid crocin, the scavenging of the DPPH radical and the determinations of oxidation potentials, were used to evaluate the antioxidant capacity of smoke phenols. The results obtained were compared and discussed with regard to the ranking of each compound and the relationship between activity and structure.

2. Materials and methods

2.1. Chemicals

All solvents were of analytical grade. 2,2′-Azo- *bis*(2-amidinopropane) dihydrochloride (ABAP), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-vinylguaiacol, 4-propylguaiacol, eugenol, isoeugenol (mixture of *trans* and *cis* isomers), vanillin, 1-(4′-hydroxy-3′-methoxyphenyl)-ethanone (acetovanillone), 1-(4′-hydroxy-3′-methoxyphenyl)-2-propanone (2-propiovanillone), syringol, 4-methylsyringol, 4-allylsyringol, syringaldehyde, 1-(4′-hydroxy-3′,5′-dimethoxyphenyl)-ethanone (acetosyringone), catechol, 3-methylcatechol, 4-methylcatechol, 3-methoxycatechol, hydroquinone and Trolox C were purchased from Sigma–Aldrich (Milan, Italy).

2.2. Crocin bleaching inhibition method

Crocin bleaching was measured according to the method reported by Tubaro et al. (1996). Crocin was extracted from saffron by methyl alcohol and the concentration of the solution was measured at 443 nm ($\varepsilon = 1.33 \times 10^5\ M^{-1}\ cm^{-1}$). The phenolic compounds were dissolved in 10% (v/v) ethanol in water. To 1200 μl of 10 mM phosphate buffer pH 7.01, were added different aliquots of the sample, 50 μl of a 0.7 mM methanolic solution of crocin and the volume was adjusted to 2450 μl with 10% (v/v) ethanol in water. Final concentrations were in the ranges of 1–10, 10–100 and 100–1000 μM, depending on the activity of the compounds. The reaction was started by adding 50 μl of a fresh 0.25 M ABAP solution in water. Bleaching reaction rates (V) at five different concentrations of antioxidant (AH) and a blank (V_0), were tested at the same time, by following the absorbance decrease at 443 nm and at a temperature of 40 °C, by means of a UV–Visible spectrophotometer (Varian Cary 1E) equipped with a thermostable multicell block (Varian, Australia). Reference bleaching rate was determined using Trolox C under the same experimental conditions. The ratios V_0/V were plotted as a function of the concentration ratio, [antioxidant]/[crocin], and the slopes calculated by linear regression analysis. The antioxidant capacity of a compound, relative to the capacity of Trolox C, was obtained by dividing the slope of the compound by that of Trolox C.

2.3. DPPH radical scavenging method

The method of Brand-Williams, Cuvelier, and Berset (1995) was used for measuring the DPPH radical scavenging ability of the compounds. DPPH was dissolved in methanol at a final concentration of about 6×10^{-5} M. The exact concentration of DPPH was calculated from a calibration curve, $\varepsilon_{calc} = 11{,}870\ M^{-1}\ cm^{-1}$ at 515 nm, ($\varepsilon = 12{,}509$ (Brand-Williams et al., 1995)); ($\varepsilon = 11{,}240$ (Goupy, Dufour, Loonis, & Dangles, 2003)). Different aliquots of a methanolic solution of the phenolic compounds were added to 2450 μl of DPPH solution and the volume adjusted to a final value of 2500 μl with methanol. Five different concentrations were used for each assay. The decrease of the DPPH radical was followed at 515 nm, until the reaction reached a steady state. The thermally controlled multicell block was set at 25 °C. The final concentrations of DPPH at the steady state, corrected for the natural disappearance of the DPPH under the same conditions and after the same time intervals, were plotted as a function of the molar concentration ratio [AH]/[DPPH] to determine the Effective Concentration (EC_{50}). Moreover the time needed to reach the steady state for EC_{50} (T_{EC50}) and the Antiradical Efficiency defined as $AE = 1/(EC_{50} \times T_{EC50})$ were also calculated (Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998).

2.4. Electrochemical measurements

Cyclic voltammetric experiments were conducted at 20 ± 0.1 °C in a conventional three-electrode cell. In all voltammetric tests, the working electrode was a glassy carbon disk with an apparent geometric area of $7 \times 10^{-2}\ cm^2$, mirror-polished with graded alumina powder prior to each experiment. The counter electrode was a platinum sheet, while the reference electrode was an aqueous Ag/AgCl, Cl^-_{sat} electrode connected to the cell by a salt bridge, containing the same medium employed in the test solution. Voltammetric measurements were carried out using a PGSTAT model 30 (Ecochemie, Twente, Holland) controlled by GPES 3.2 software from Ecochemie, running on a PIII personal computer. All tests were run on nitrogen-purged solutions and the potential was scanned from −0.1 to 1.0 V at a sweep rate of 100 mV s^{-1}. Test solutions were prepared diluting suitable amounts of each phenolic derivative in aqueous 0.1 M phosphate buffer (pH 7.02), to obtain a final concentration of 10^{-3} M.

3. Results and discussion

3.1. Crocin method

By this method, the ability of a compound to react with peroxyl radical is measured by a competition kinetics procedure, where the antioxidant competes with the carotenoid crocin for the peroxyl radical, produced by thermal decomposition of a diazo-compound. The results are

expressed relative to the antioxidant Trolox C and they represent the concentration of the compound investigated that exhibits the same antioxidant capacity as a unit concentration of Trolox C. The antioxidant capacity of guaiacol, syringol and their derivatives and that of the dihydroxybenzenes, determined by this method, are reported in Table 1. Lower values correspond to higher antioxidant activities.

Considering the group of guaiacol and its derivatives, the results showed a clear relationship between antioxidant activity and chemical structure, in relation to the effect of the *para*-substituents. The role of a "chain-breaking antioxidant" is to interrupt the chain propagation, according to the inhibition reaction $ROO^{\cdot} + ArOH \rightarrow ROOH + ArO^{\cdot}$, where $ROO^{\cdot}$ represents a lipid peroxyl radical and ArOH a phenolic compound. The antioxidant property of a phenolic compound depends on the rate of hydrogen abstraction, which can be related to the bond dissociation enthalpy (BDE) of the phenolic-hydrogen bond. The guaiacyl derivatives with an alkyl group at the *para* position showed an antioxidant capacity higher than guaiacol. This can be explained by the electron-donating ability of the alkyl groups in the *para* position which stabilizes the phenoxyl radical to a higher extent than the phenoxyl radical of guaiacol, lowering the BDE of the OH bond, (Barclay et al., 1997; Kajiyama & Ohkatsu, 2001; Wright, Johnson, & Di Labio, 2001). Among the 4-alkyl derivatives, 4-methylguaiacol, 4-ethylguaiacol, 4-propylguaiacol, 2-propiovanillone and eugenol showed similar antioxidant activities, whereas in the case of isoeugenol and 4-vinylguaiacol there was an evident further increase. The presence in these two compounds of a double bond conjugated with the aromatic ring provides additional stabilization of the phenoxyl radical by extended conjugation (Barclay et al., 1997; Kajiyama & Ohkatsu, 2001; Wright et al., 2001). In fact, in the case of eugenol, whose double bond in the propenyl group is not conjugated, the antioxidant capacity was similar to that of 4-propylguaiacol where the alkyl group is saturated.

Table 1
Antioxidant capacity obtained by the crocin assay

Compounds	pH 7.01 (mol AH[a]/mol Trolox C)
Guaiacyl derivatives	
Isoeugenol	2.79 ± 0.03
4-Vinylguaiacol	7.5 ± 0.2
Vanillin	9.9 ± 0.5 (n.d.)[b,c]
Acetovanillone	10.4 ± 0.1 (1053 ± 67)[b]
2-Propriovanillone	20.3 ± 1.1
4-Propylguaiacol	20.9 ± 1.5
Eugenol	20.9 ± 1.9
4-Methylguaiacol	22.0 ± 0.5
4-Ethylguaiacol	22.3 ± 0.5
Guaiacol	34.5 ± 3.4 (244 ± 12)[b]
Syringyl derivatives	
Syringaldehyde	0.29 ± 0.03 (64 ± 2)[b]
Acetosyringone	0.56 ± 0.02 (86 ± 4)[b]
4-Allylsyringol	4.0 ± 0.4
4-Methylsyringol	4.2 ± 0.4
Syringol	7.8 ± 0.4 (30 ± 1.8)[b]
Dihydroxybenzenes	
3-Methoxycatechol	1.0 ± 0.02
3-Methylcatechol	1.17 ± 0.03
4-Methylcatechol	1.7 ± 0.1
Catechol	2.3 ± 0.1
Hydroquinone	3.5 ± 0.1

The values are the mean of three determinations ± standard deviation.
[a] Antioxidant.
[b] 10 mM phosphate buffer, pH 3.0.
[c] No antioxidant effect detected.

The effect of the *para*-alkyl substituents and of a conjugated double bond were reported by Barclay et al. (1997), by determining the inhibition rate constants for the inhibited peroxidation of styrene in chlorobenzene for 4-propylguaiacol, eugenol, isoeugenol, coniferyl alcohol, coniferyl aldehyde, 4-allyl-2,6-dimethoxyphenol and two dimeric compounds. Kajiyama and Ohkatsu (2001) reported the rate of oxygen absorption, under similar oxidation conditions, of a group of *para*-substituted 2-methoxyphenols, among them guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-vinylguaiacol, eugenol, isoeugenol, vanillin and acetovanillone. The ranking of the antioxidant activities obtained with the crocin test was generally in agreement with that reported by these authors for the same compounds, except for vanillin and acetovanillone. In fact, for these two compounds, Kajiyama and Ohkatsu (2001) did not report any improvement of the antioxidant capacity, with respect to guaiacol. Also Wright et al. (2001) predicted a higher BDE for the phenolic O–H bond in the case of a conjugated *p*-CHO group, relative to phenol.

In the ranking of the antioxidant capacity obtained by the crocin method at pH 7.01, (Table 1), vanillin and acetovanillone showed an antioxidant capacity higher than that of guaiacol, a behaviour opposite to that expected. The electron-attracting inductive effect of the α-carbonyl group in the *para* position should in fact destabilize the phenoxyl radical and reduce the radical scavenging ability of these compounds (Kajiyama & Ohkatsu, 2001; Wright et al., 2001). It was hypothesized that this behaviour could be due to the effect of the pH on the ionization of the phenolic hydroxyl. At pH 7.01 the phenolic hydroxyl group, having a pK_a of about 10, is not ionized. In the case of vanillin, the electron attracting effect of the α-carbonyl group lowers the pK_a to a value of 7.40 (Roses, Rived, & Bosch, 2000) and the hydroxyl group is therefore about half ionized in the reaction medium. The phenolate ion is a more active species as an electron donor, with respect to the unionized form, and the antioxidant capacity of vanillin is therefore increased. A similar behaviour should be expected also for acetovanillone (pK_a 7.81) (Ragnar, Linfgren, & Nilvebrant, 2000). The determinations were repeated at pH 3.0, and, as expected the ranking was inverted, with vanillin and acetovanillone showing an antioxidant capacity much lower than that of guaiacol (Table 1). However, changing the pH could have had other effects on the reactions involved in the assay. A decrease in the antioxidant capacity at lower pH was, observed also for guaiacol (pK_a 9.94; 9.93) (Ragnar et al., 2000; Roses et al., 2000), but in this

case the reduction was very low as compared with that of vanillin and acetovanillone.

The results obtained for syringol and its derivatives are also reported in Table 1. All the 2,6-dimethoxy compounds analyzed showed an antioxidant capacity higher than the corresponding guaiacyl derivatives, maintaining a similar ranking regarding the effect of the *para*-substituents. The syringol and its derivatives are characterized by a second methoxy group at the position 6 of the aromatic ring which would add an additional stabilization of the phenoxyl radical, enhancing the radical scavenging ability of these compounds with respect to the guaiacyl derivatives (Barclay et al., 1997; de Heer, Korth, & Mulder, 1999; Kjällstrand & Petersson, 2001a; Kjällstrand & Petersson, 2001b). The considerations about the effect of the pH on the antioxidant capacity of vanillin and acetovanillone can be applied also to the corresponding syringyl derivatives, syringaldehyde (pK_a 7.34) and acetosyringone (pK_a 7.88) (Ragnar et al., 2000). For these two compounds, the antioxidant capacity became lower than that of syringol, when changing the pH from 7.01 to 3.0 (Table 1). Although only a limited number of syringyl derivatives were analyzed, it is reasonable to hypothesize a similar behaviour between the corresponding compounds of the two groups.

The antioxidant activities of the dihydroxybenzenes (Table 1) were higher than that of the 2-methoxyphenols and 2,6-dimethoxyphenols. The presence of a second hydroxyl group in the *ortho* (catechol) or in the *para* (hydroquinone) position reduced, the BDE of the O–H bond to a higher extent, with respect to the alkyl or methoxy substituents (Pokorny, 1987; Wright et al., 2001). Among the dihydroxybenzenes tested, the substituted catechols, 3-methoxycatechol, 3-methylcatechol and 4-methylcatechol had the highest antioxidant capacity, followed by catechol and hydroquinone. When considering all compounds tested, only isoeugenol had a value comparable with that of catechol and hydroquinone, underlining the stabilizing effect of a conjugated double bond.

3.2. DPPH method

This method evaluates the radical scavenging ability of a compound by its reaction with the stable radical DPPH. The assays were carried out in methyl alcohol and the results expressed as EC_{50}, which represents the antioxidant concentration necessary to decrease the initial DPPH concentration by 50%. The EC_{50} values of the phenolic compounds are reported in Table 2. Lower values correspond to highest radical scavenging capacity.

Considering the group of guaiacol and its derivatives, the compounds with the higher radical scavenging capacity were 4-ethylguaiacol, 4-propylguaiacol, eugenol, 4-methylguaiacol and 2-propiovanillone with EC_{50} values between 0.24 and 0.37, followed by 4-vinylguaiacol, isoeugenol and guaiacol with practically the same values between 0.49 and 0.58, and finally vanillin and acetovanillone as the least active compounds. This ranking was different from that obtained by the crocin method for isoeugenol and 4-vinylguaiacol. These compounds which were the most active with the crocin method, showed with the DPPH method an activity comparable to that of guaiacol, as if there was no effect by the conjugated double bond in the side chain. The most active compounds were the guaiacyl derivatives with a saturated side chain and eugenol with an isolated double bond.

In order to investigate this discrepancy it is necessary to consider that for the determination of the EC_{50} value the compound is allowed to react with the DPPH radical, till the attainment of a steady state. The time needed to reach the steady state can be very different, depending on the compound, and on this basis, some authors classified antioxidants into fast, intermediate and slow (Brand-Williams et al., 1995; Sanchez-Moreno et al., 1998). Among the 2-methoxyphenols, isoeugenol and 4-vinylguaiacol were the fastest with times between 3 and 8.8 min, whereas the other compounds with lower EC_{50} values showed much longer times, between 100 and 170 min. This behaviour was shown by guaiacol and isoeugenol. These compounds had practically the same EC_{50} values, 0.58 ± 0.04 and 0.54 ± 0.04, respectively, but the time necessary for guaiacol to reach the steady state was about 160 min, whereas only 3 min were needed in the case of isoeugenol, denoting a completely different kinetic behaviour. The EC_{50} value does not therefore discriminate between these two compounds, even if they showed a very different antioxidant capacity when tested with the crocin method. Also the ranking of the isoeugenol and eugenol was inverted in the DPPH method, in comparison with the crocin method.

The ranking of the antioxidant activities obtained with the crocin method were in agreement with the results reported in literature (Barclay et al., 1997; Kajiyama & Ohkatsu, 2001) and based on the rate constant for the inhibition reaction between a peroxyl radical and the antioxidant. On the other hand, the EC_{50} value depends on the number of DPPH moles reduced by the antioxidant during the reaction. The DPPH radical can however be reduced by the initial antioxidant molecule or by other similar reactions involving chemical species like dimers originating from the reaction of two phenoxyl radicals of the antioxidant, which still retain antioxidant capacity. Kawabata, Okamoto, Kodama, Makimoto, and Kasai (2002) reported the formation of dimers from gallic and protocatechuic acids, after reaction with DPPH radical. Brand-Williams et al. (1995) and Bondet et al. (1997), studying the reaction mechanism of DPPH with BHT, eugenol and isoeugenol, proposed the intervention of a more complex reaction mechanism, eventually involving dimeric species, for those compounds with a slow kinetic behaviour like eugenol. A similar mechanism could also be applied to the other 2-methoxyphenol derivatives analyzed in this research. The formation of dimers by reaction of two phenoxyl radicals was also reported by Pokorny (1987) and Denisov and Khudyakov (1987).

Some authors, studying the antioxidant capacity of natural compounds with the DPPH assay and electrochemical methods, reported the presence of a good correlation

Table 2
Effective concentration (EC_{50}) and antiradical efficiency (AE) obtained with the DPPH assay

Compounds	EC_{50} (mol AH[a]/mol DPPH)	$AE \times 10^3$ (mol DPPH/(mol AH[a] × t))
Guaiacyl derivatives		
4-Ethylguaiacol	0.24 ± 0.02	39 ± 4
4-Propylguaiacol	0.25 ± 0.04 (0.22)[e]	37 ± 9
Eugenol	0.26 ± 0.01 (0.27)[b], (0.25)[e]	31 ± 2
4-Methylguaiacol	0.29 ± 0.01	30 ± 2
2-Propiovanillone	0.37 ± 0.01	15.7 ± 0.5
4-Vinylguaiacol	0.49 ± 0.05	229 ± 33
Isoeugenol	0.54 ± 0.04 (0.51)[b], (0.49)[e]	592 ± 45
Guaiacol	0.58 ± 0.04 (0.25)[b]	10.9 ± 0.9
Vanillin	2.1 ± 0.1 (20)[b]	2.2 ± 0.1
Acetovanillone	36 ± 5	0.14 ± 0.02
Syringyl derivatives		
4-Methylsyringol	0.15 ± 0.01	135 ± 6
4-Allylsyringol	0.32 ± 0.01	86 ± 11
Syringol	0.45 ± 0.02	43 ± 5
Syringaldehyde	1.17 ± 0.01	4.7 ± 0.1
Acetosyringone	19 ± 0.1	0.24 ± 0.01
Dihydroxybenzenes		
Catechol	0.09 ± 0.001 (0.14)[c], (0.22) [f]	91 ± 6
3-Methylcatechol	0.13 ± 0.01	72 ± 10
4-Methylcatechol	0.13 ± 0.01	65 ± 7
3-Methoxycatechol	0.16 ± 0.01	48 ± 6
Hydroquinone	0.22 ± 0.01 (0.19)[c], (0.37)[f]	469 ± 63
Trolox C	0.26 ± 0.01 (0.24)[d], (0.19)[c], (0.21)[e]	871 ± 74

The values are the mean of three determinations ± standard deviation; values in brackets are from the literature.
[a] Antioxidant.
[b] Brand-Williams et al. (1995).
[c] Ancerewicz et al. (1998).
[d] Gordon et al. (2001).
[e] Nenadis et al. (2003).
[f] Hotta et al. (2002).

between the EC_{50} values and the numbers of exchanged electrons, during experiments of column flow electrolysis, using slow flow (Hotta, Sakamoto, Nagano, Osakai, & Tsujino, 2001; Hotta et al., 2002). These authors observed that decreasing the flow resulted in an increased number of exchanged electrons and they attributed this effect to the oxidation of compounds formed by slow chemical reactions following the first oxidation process.

The EC_{50} values of compounds with long reaction times, can therefore reflect a radical-scavenging capacity, due not only to the contribution of the original antioxidant but also of other chemical species. Similar considerations were recently reported by Arts, Dallinga, Voss, Haenen, and Bast (2003) Arts, Haenen, Voss, and Bast (2004) about the TEAC assay. This method is based on the ability of a compound to scavenge the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical, with respect to Trolox C. Studying the reaction between ABTS and the flavonoid chrysin, these authors reported the formation of a product with an antioxidant capacity higher than that of the parent compound, chrysin, meaning that the TEAC assay does not necessarily reflect the antioxidant effect of only one chemical species.

Ancerewicz et al. (1998) introduced a new parameter, $\log Z$, derived from initial second-order rate constants and antioxidant/DPPH ratios, to differentiate the compounds according to their intrinsic reactivity. Also Goupy et al. (2003) used a kinetic approach to determine the rate constants of the first H atom abstraction by DPPH radical.

By using a different approach and considering that the EC_{50} value does not take into account the time needed to reach the steady state, Sanchez-Moreno et al. (1998) introduced the antiradical efficiency (AE), defined as $AE = 1/(EC_{50} \times T_{EC50})$ where T_{EC50} is the time needed to reach the steady state at the concentration corresponding to EC_{50}. They claimed that AE comprehends both the potency of the antioxidant, reflected by the inverse of EC_{50} ($1/EC_{50}$), as well as the reaction time (T_{EC50}), and was therefore more discriminatory than the EC_{50}.

The AE values calculated for the compounds investigated in this work are reported in Table 2. Based on the AE values, isoeugenol and 4-vinylguaiacol were found to be the most active 2-methoxyphenol derivatives due to their very short T_{EC50}, followed by the other *para*-alkyl substitutes with a similar behaviour, and then guaiacol, vanillin and acetovanillone. The AE was indeed able to discriminate between compounds with practically the same EC_{50}, but different T_{EC50}.

The EC_{50} values for the 2,6-dimethoxyphenols were lower than those of the corresponding 2-methoxyphenols, with the exception of 4-allylsyringol. However, the AE

values were all higher. The dihydroxybenzenes had the lowest EC_{50} values, among the compound tested, with the exception of hydroquinone, which had an EC_{50} value similar to those of 4-ethylguaiacol and 4-propylguaiacol. The T_{EC50} values were however inverted with hydroquinone (T_{EC50} values 10 min) rapid and catechol and its derivatives slow, with T_{EC50} values between 109 and 130 min. As a consequence, the AE of hydroquinone was high, like that of isoeugenol, whereas the AE of the other dihydroxybenzenes were similar to those of the 4-alkylsyringols. Nenadis, Boyle, Bakalbassis, and Tsimidou (2003) reported EC_{50} values of 0.18 and 0.11 for two compounds with a catechol group, caffeic acid and dihydrocaffeic acid, respectively. These values were similar to those found in this work for catechol and its derivatives. The T_{EC50} calculated from the AE data reported by the same authors (Nenadis et al., 2003) were 5 min for caffeic acid and 23 min for dihydrocaffeic acid. These values are lower than those of catechol, 3-methylcatechol, 4-methylcatechol and 3-methoxycatechol, probably due to the presence of a propenyl group with a conjugated double bond in caffeic acid and of a propyl group in dihydrocaffeic acid. Other authors (Gordon, Paiva-Martins, & Almeida, 2001) reported EC_{50} values, for hydroxytyrosol, of 0.19, 0.13 and 0.099, calculated after a reaction time of 15, 60 and 250 min, respectively. Although the authors did not report the T_{EC50}, this behaviour was indicative of a slow kinetic rate, similar to that of the catechol derivatives investigated in this work.

The considerations about the behavior of hydroquinone apply also to Trolox C, which in spite of its relatively high EC_{50}, has the highest AE among the compounds analyzed due to its low T_{EC50}.

The discrepancies observed between the crocin method and the EC_{50} for isoeugenol and 4-vinylguaiacol were overcome by using the AE values, which take into account the reaction time. The trend of the activities of the 2-methoxyphenols and 2,6-dimethoxyphenols, based on the AE values, were similar to those obtained with the crocin method. In the case of the dihydroxybenzenes, there were some differences, in particular for hydroquinone. This compound was the least active with the crocin method, but the most active, considering the AE value. Catechol and the substituted catechol, which were the most active with the crocin method, had, on the contrary, AE values similar to those of 4-allylsyringol and syringol.

The possible contributions of the reaction products to the EC_{50} and AE values of a compound limit the use of the DPPH method, to evaluate structure–activity relationships.

3.3. Oxidation potential

The oxidation potentials (E_{pa}) of the first anodic peak of the 2-methoxyphenols, 2,6-dimethoxyphenols and dihydroxybenzenes are reported in Table 3. Lower oxidation potentials correspond to a higher ability of a compound to donate an electron and therefore to act as an antioxidant. The electrochemical oxidation of a phenol can be describe by the reaction $AH \rightarrow A^{\bullet} + e^{-} + H^{+}$ where, similarly to hydrogen transfer reactions, the breaking of the same OH bond was involved (van Acker et al., 1996). Therfore, the same considerations about the effect of the substituents on the BDE of the phenolic hydroxyl, that apply to the scavenging process, can be used for the electrochemical process. It is important to underline that the oxidation potential, because of its thermodynamic nature, does not give any information about the reaction rate.

Table 3
Oxidation potential (E_{pa})

Compounds	E_{pa} (V)[a] (pH 7.02)
Guaiacyl derivatives	
Isoeugenol	0.293 ± 0.002
4-Vinylguaiacol	0.380 ± 0.003
4-Methylguaiacol	0.404 ± 0.005
4-Ethylguaiacol	0.415 ± 0.003
4-Propylguaiacol	0.418 ± 0.003
Guaiacol	0.454 ± 0.004
2-Propiovanillone	0.486 ± 0.003
Eugenol	0.520 ± 0.005
Acetovanillone	0.588 ± 0.003
Vanillin	0.620 ± 0.003
Syringyl derivatives	
4-Methylsyringol	0.308 ± 0.001
4-Allylsyringol	0.321 ± 0.002
Syringol	0.425 ± 0.003
Acetosyringone	0.517 ± 0.002
Syringaldehyde	0.530 ± 0.005
Dihydroxybenzenes	
Hydroquinone	0.153 ± 0.003 (0.207)[b]
3-Methoxycatechol	0.200 ± 0.003
3-Methylcatechol	0.209 ± 0.005
4-Methylcatechol	0.218 ± 0.005
Catechol	0.234 ± 0.005 (0.240)[b]
Trolox C	0.185 ± 0.003

The values are the mean of three determinations ± standard deviations; values in brackets are from the literature.
[a] E_{pa} (V vs. Ag/AgCl, Cl^{-}_{sat}).
[b] Hotta et al. (2002).

Considering the 2-methoxyphenols, isoeugenol and 4-vinylguaiacol were the compounds with the lowest E_{pa} (0.293 and 0.380 V, respectively), followed by 4-methylguaiacol, 4-ethylguaiacol and 4-propylguaiacol with E_{pa} between 0.404 and 0.418 V. All the guaiacyl derivatives with electron-donating *para*-alkyl substituents were oxidized more easily than guaiacol, except for eugenol. This exception can be accounted for by considering the higher degree of irreversibility that characterized the oxidation of this compound, which displayed a quite sluggish anodic wave. The effect of a conjugated double bond was, moreover, well evidenced, by the lowest oxidation potentials of isoeugenol and 4-vinylguaiacol.

In contrast to the ranking obtained by the crocin method, vanillin and acetovanillone, characterized by an electron attracting group, showed also at pH 7.01 an oxidation potential higher than that of guaiacol.

The oxidation potentials of the 2,6-dimethoxyphenols were below those of the corresponding 2-methoxyphenols,

maintaining the same ranking regarding the effect of the *para* substituents.

The dihydroxybenzenes characterized by a second hydroxyl group in the *ortho* or the *para* position showed the lowest oxidation potentials, with values in the range 0.153–0.234 V. Hydroquinone was the most active, followed by the 3-substituted catechols and catechol. Hotta et al. (2002) reported the oxidation potentials (E_{pa}, V vs. Ag/AgCl), of many natural compounds, measured at a carbon electrode in a 1:1 (v/v) water–ethanol mixture containing 50 mM KCl and 50 mM phosphate buffer (pH 7.0). Considering in particular ferulic acid (0.430 V), sinapic acid (0.314 V) and caffeic acid (0.212 V) whose chemical structures differ only by the presence of the 3-methoxy-4-hydroxy-, 3,5-dimethoxy-4-hydroxy- and 3,4-dihydroxy-substituents on the benzene ring, respectively, their oxidation potentials were similar to those of the compounds, with the same groups, analyzed in this work. The E_{pa} of Trolox C was 0.185 V similar, to that of dihydroxybenzenes confirming the good antioxidant property of this compound.

Considering the methoxyphenols, ranking of the antioxidant capacity by crocin and E_{pa} were similar, whereas in the case of dihydroxybenzenes there was a discrepancy for hydroquinone. The regression analysis between crocin results (pH 7.01) and E_{pa} gave a low correlation coefficient $r = 0.43$, which improved ($r = 0.81$) by eliminating compounds with pK_a near 7: vanillin; acetovanillone; syringaldehyde and acetosyringone.

The considerations about the ranking of isoeugenol and 4-vinylguaiacol made for the crocin and DPPH methods can also be valid for the voltammetric and DPPH methods.

Hotta et al. (2002) reported a low correlation ($r = 0.74$) between the oxidation potentials and the reciprocal of EC_{50} for 34 natural antioxidants. A similar correlation ($r = 0.73$) for all the compounds tested, between E_{pa} and $1/EC_{50}$, was also obtained in this work. An even lower correlation ($r = 0.56$) was obtained between E_{pa} and AE. Zhuang, Scholz, and Pragst (1999) and Hotta et al. (2002) reported a value of 0.340 V vs. Ag/AgCl at pH 7.0 for the formal potential of DPPH radical. Considering the oxidation potentials reported in Table 3, it can be seen that the compounds vanillin, acetovanillone, syringaldehyde and acetosyringone which had the highest Epa values, and were less prone to react with DPPH, had also the highest values of EC_{50}.

Mannino et al. (1998) claimed that the compounds with an $E_{pa} \leqslant 0.4$ V (vs. Ag/AgCl), typical of molecules characterized by the presence of an *ortho* or *para* diphenol group, are good antioxidants. In this respect, isoeugenol, 4-vinylguaiacol, the 4-alkylsyringols and the dihydroxybenzenes meet very well this requirement.

4. Conclusion

All the three methods used, although based on different approaches, showed the effect of the substituents on the antioxidant capacity of the phenolic compounds analyzed. The trend in the ranking of antioxidant capacity given by the three methods was similar with dihydroxybenzenes > 2,6-dimethoxyphenols > 2-methoxyphenols. Some discrepancies in the ranking within the groups were however present, probably due to the intrinsic differences of the methods. A better comprehension of these discrepancies could be obtained by investigating the mechanism involved, in particular the reaction between antioxidant and DPPH radical.

The effect of these compounds on the oxidative stability of smoked foods needs further investigation. We must, remember that the final antioxidant effect depends also on the solubility of the compounds, as the latter affects the distribution between, for example, fat and lean parts of a meat product.

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