Comparison of different methods for measuring antioxidant activity

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INTRODUCTION

Recent approaches for characterising antioxidant and/or pro-oxidant actions has led to the development of new experimental tools using free radical reactions. It is accepted that reactive oxygen species (ROS) such as hydroxyl radical $OH^{\bullet}$, superoxide radical $O_2^{\bullet-}$ and singlet oxygen $^1O_2$ are agents causing oxidative damage (Bamforth et al., 1993). However, in most cases, addition of other radicals is applied in the assay in order to increase the response.

This paper presents a comparison of different methods for measuring antioxidant activity applied on several potential antioxidant compounds. We shall attempt in such a way to understand and better define the mechanisms of actions of these different methods. Therefore, we suggest classifying these assays in two categories depending on the targeted measurement:

- **free radical scavenging activity only** (decolorization of the DPPH radical or inhibition of the AAPH-induced oxidation of an aqueous dispersion of linoleic acid);
- free radical generation risk and scavenging activity **(ESR – spin trapping detection of hydroxyl radicals generated during an aerobic forced ageing).**

RESULTS AND DISCUSSION

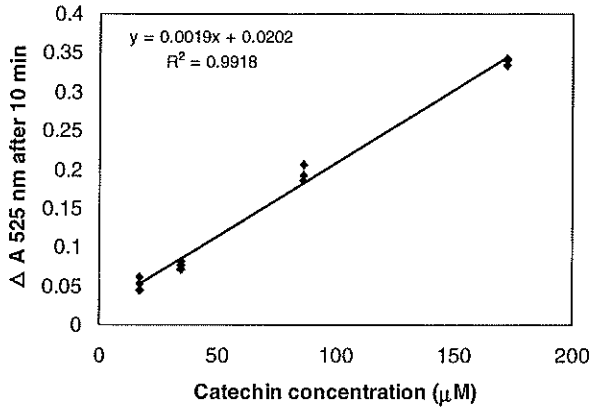
Measurement of the free radical scavenging activity (antioxidant potential)

1. $DPPH^{\bullet}$ - reducing activity

The radical, 1,1-diphenyl-2-picryl-hydrazyl radical ($DPPH^{\bullet}$), shows colour changes with its reduction. The decolorization of the DPPH radical is measured at 525 nm at room temperature as a function of time in presence of reducing substances (in an ethanolic/acetate buffer (pH 4.3) 2:1 (v/v) solution). The decreasing of the absorbance after 10 minutes is directly proportional to the antioxidant concentration (figure 1).

When applied to several potential antioxidants, it seems that DPPH$^{\bullet}$ – reducing activity correlates well with polyphenol compounds, though not with SO_2 (table 1).

Figure 1. Effect of catechin concentration on the DPPH$^{\bullet}$ – reducing activity



2. Inhibition of the AAPH-induced oxidation of an aqueous dispersion of linoleic acid

Liégeois et al. (2000) proposed the kinetic measurement of AAPH-induced oxidation of an aqueous dispersion of linoleic acid in the presence of antioxidants coming from malt or hops (Lermusieau, 2000). This sensitive method is more representative of what happens in a dispersed lipid matrix such as wort.

The oxidation of linoleic acid initiated by the water-soluble azo compound, AAPH [2,2'-azobis(2-amidinopropane) dihydrochloride], proceeds as follows: azo radical initiators generate free radicals by thermal decomposition and are able to initiate lipid peroxidation, even at 37°C (equations 1-3). As the lipid chosen is linoleic acid, products resulting from peroxidation are the conjugated diene hydroperoxides which absorb at 234 nm. In the absence of a radical initiator, the rate of spontaneous oxidation at 37°C can be considered as negligible. Addition of AAPH induces oxidation, which starts at a constant rate of conjugated diene formation. When an antioxidant is added, oxidation is delayed and the resulting inhibition period is observed.

$$A\text{-}N{=}N\text{-}A \rightarrow (1\text{-}e)\,A\text{-}A + 2\,e\,A^{\bullet} + N_2 \quad [1]$$

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet} \quad [2]$$

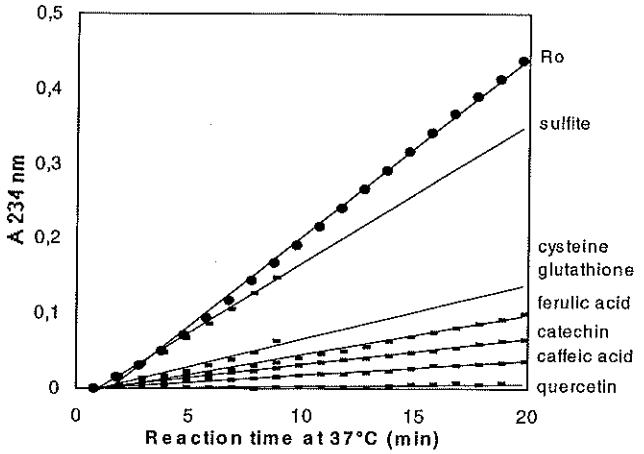
$$AOO^{\bullet} + LH \rightarrow AOOH + L^{\bullet} \quad [3]$$

with A = $-C(CH_3)_2C(NH_2){=}NH.HCl$ and e = efficiency of free radical production.

Figure 2 shows the results obtained at the beginning of the oxidation assay in the presence of several antioxidants (conc. 2 μM). During this inhibition period, phenolic compounds proved

to inhibit oxidation most efficiently, thiols to a lesser extent. Surprisingly, sulfites did not inhibit AAPH-induced oxidation of linoleic acid. We can assume that the antioxidant activity of sulfites detected by the ESR technique is mainly due to changes in hydroxyl radical concentrations, which are underestimated in the presence of AAPH. The ratio of the initial oxidation rate in the presence of antioxidants (Rinh) to the initial oxidation rate in their absence (Ro) provides a first antioxidant activity index (table 1). It teaches about how strong antioxidants inhibit lipid oxidation.

Figure 2. Effect of various antioxidants (2 μM) on AAPH-induced linoleic oxidation, as measured by kinetics of conjugated diene formation. Reprinted with permission from Liégeois et al., 2000. Copyright 2000 American Chemical Society.



A second antioxidant activity index is the inhibition time (Tinh: measured at the intersection between the tangents to the inhibition- and propagation-phase curves, under precise oxidation conditions) (figure 3a). The measured inhibition time is directly proportional to the antioxidant concentration (figure 3b) and can also be related to the stoechiometric number of peroxyl radicals trapped per molecule of antioxidant. When applied to several potential antioxidant compounds (table 1), Tinh is proportional to the concentration of the additive, except for sulphites, which did not inhibit AAPH-induced oxidation of linoleic acid. Among the pure antioxidants tested, phenolic compounds proved to be the most efficient in scavenging peroxyl radicals.

Figure 3. (a) Effect of Trolox concentration on AAPH-induced linoleic oxidation, as measured by kinetics of conjugated diene formation. (b) Inhibition time (Tinh) as a function of the Trolox concentration. Reprinted with permission from Liégeois et al., 2000. Copyright 2000 American Chemical Society.

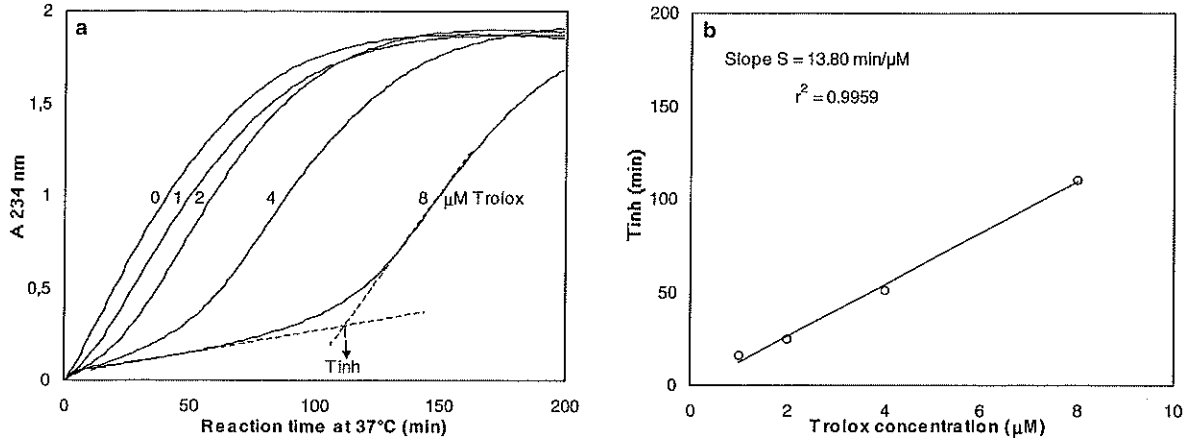


Table 1. Comparison of two methods for measuring a free radical scavenging activity

Compounds	Antioxidant activity as scavenger of		
	DDPH radical	peroxyl radical	
	Δ A525 nm /mM	Rinh/Ro	Tinh (min/µM)
Quercetin . 2 H_2O	2.91	0.04	59.24
Catechin . H_2O	1.89	0.15	42.26
Caffeic acid	1.71	0.09	34.97
Vitamin E	1.46	0.06	22.19
Ferulic acid	1.03	0.23	20.35
Glutathione	0.34	0.44	3.85
Ascorbic acid	1.71	0.12	3.65
Sulfite	0.29	0.87	0

Measurement of both free radical generation risk and scavenging activity

Scavenging of the hydroxyl radicals generated by aerobic forced ageing – ESR detection

This method has been developed by Uchida and Ono (1996) to assess the *endogenous anti-oxidant activity* (EA value) of beer. The generation of hydroxyl radicals is followed during an oxidative forcing test at 60°C. The hydroxyl radical generation is mainly dependent on the concentration of both antioxidants and pro-oxidants in beer. The highly reactive hydroxyl

radicals are trapped by *N*-tert-butyl-α-phenyl nitrone (PBN) and detected by ESR spectroscopy.

In the case of beer, a "lag phase" is first observed before the generation of hydroxyl radicals. According to the authors, this "lag phase" is an indicator for the *endogenous antioxidant activity (EA value)* of beer. Our results indicate that only sulfites are able to create this lag phase. Moreover, Forster and Back (1999) showed that the lag time presents an exponential correlation with the sulphur dioxide content during fermentation.

Therefore, although hydroxyl radicals should be also detected in wort, no lag time can be measured. In that case, the *hydroxyl radical generation activity* of wort after 2 hours of the oxidative forcing test will be determined (Takemura et al., 1997).

Three different answers of the ESR test in wort are depicted in figure 4:

- polyphenols and phenolic acids give an ESR signal decreasing with concentration. They act as scavenger of hydroxyl radicals;
- ascorbic acid presents an opposite answer; it increases the generation of hydroxyl radicals with increasing concentration;
- sulfites cause the appearance of a lag phase before the hydroxyl radicals generation.

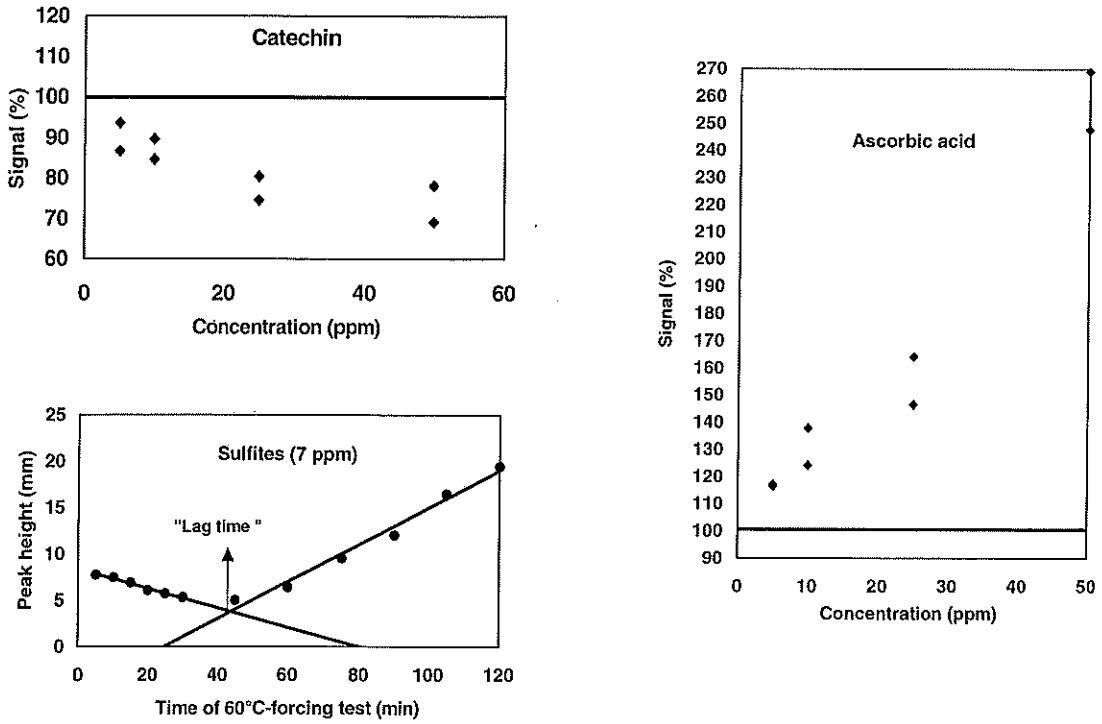


Figure 4. Effect of various antioxidants on the ESR test in wort

Comparison of the different methods

Figure 5 gives the free radical scavenging activity only, measured by different techniques. The peroxyl radical scavenging activity obtained with the AAPH-induced linoleic acid oxidation assay gives similar information compared to the DPPH$^{\bullet}$ – reducing activity when applied to phenolic compounds (very efficient scavengers) or sulfites (no scavenger activity). On the other hand, as expected, the high scavenging activity of ascorbic acid can only be measured with assays in which short periods are followed (e.g. Ro/Rinh or DPPH).

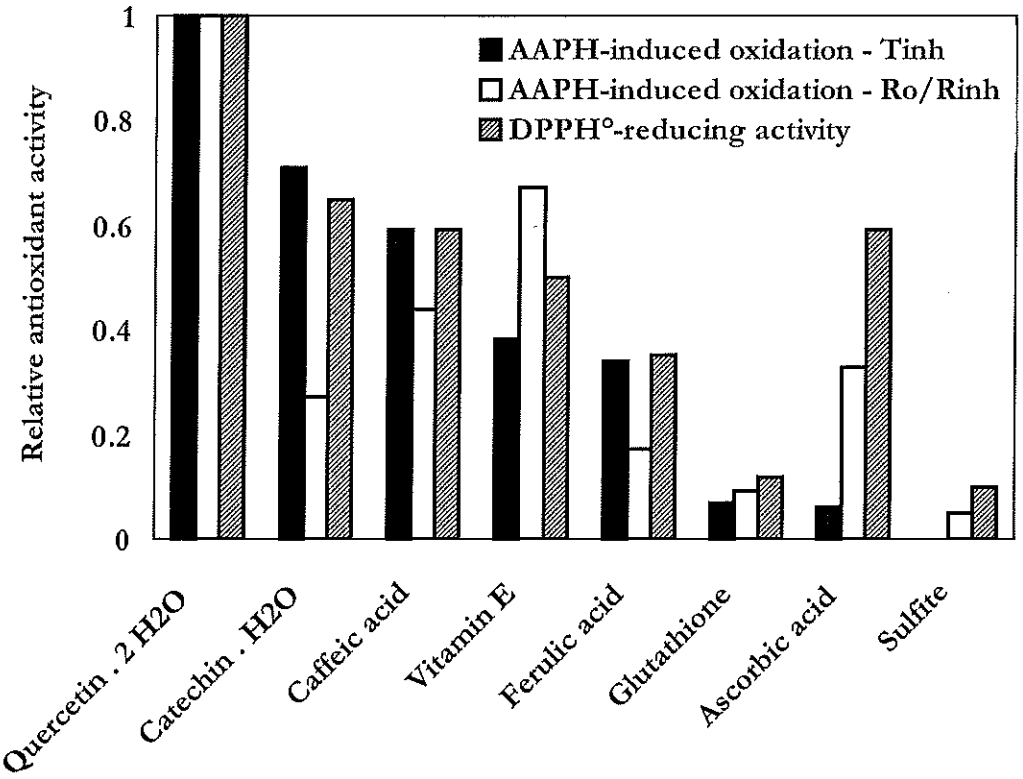


Figure 5. Free radical scavenging activity only of different compounds

As shown in table 2, higher differences appear when assays taking into account the free radical generation risk are compared to the former. In the ESR – spin trapping method, ascorbic acid logically appears as pro-oxidant due to its ability to regenerate iron ions for the Fenton reactions. Moreover, in assays where hydroxyl radicals are naturally produced in the medium from oxygen, sulfites are able to exhibit a low antioxidant activity.

Table 2. Free radical scavenging activity only (Tinh) or free radical generation risk and scavenging activity (relative ESR signal, concentration of 25 ppm in wort) for various antioxidants

Compounds	Tinh (min/ppm)	relative ESR signal*
Caffeic acid	194	0.90
Quercetin . 2 H_2O	175	0.87
Catechin . H_2O	146	0.75
Ferulic acid	105	0.97
BHT	78	0.92
Vitamin E	52	1.12
Ascorbic acid	21	1.55
Glutathione	13	0.98
Cysteine . HCl	10	0.85
Sulfite	0	0.95

* values under 1 indicate an antioxidant effect; values above 1 indicate a pro-oxidant effect.

CONCLUSIONS

A number of assays have been introduced in the literature for the measurement of antioxidant and/or pro-oxidant actions reflecting the increasing interest in this area. Recent approaches have led to the development of specific assays, for a particular mechanism. Depending on the specific targeted activity, assays can be classified in two categories: the measurement of free radical scavenging activity only, or the measurement of free radical generation risk and scavenging activity. The main difficulty is the complexity of food matrix, in which many potential oxidative mechanisms can take place. In order to take into account all possible chemical routes, several assays should be combined.

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