Measuring Antioxidant Efficiency of Wort, Malt, and Hops against the 2,2′-Azobis(2-amidinopropane) Dihydrochloride-Induced Oxidation of an Aqueous Dispersion of Linoleic Acid

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This paper presents a simple, convenient method for determining the efficiency of antioxidants in aqueous systems. Production of conjugated diene hydroperoxide by oxidation of linoleic acid in an aqueous dispersion is monitored at 234 nm. 2,2′-Azobis(2-amidinopropane) dihydrochloride is used as a free radical initiator. Among 12 antioxidants tested, phenolic compounds proved to be the most efficient, both kinetically and in terms of the inhibition time (T_{inh}). Applied to wort, malt, and hops, the method confirmed a significant antioxidant activity in such products, especially hops. This assay can be used to follow oxidative changes throughout the brewing process and to understand the contribution of each raw material.

Keywords: *Antioxidant; lipid oxidation; AAPH; beer*

INTRODUCTION

As lipid oxidation is the major pathway of alkenal formation, inhibiting lipid oxidation during the brewing process should provide brewers with a key to regulate alkenal production and hence beer staling (Collin et al., 1999; Lermusieau et al., 1999; Noël et al., 1999a). Enzymatic degradation of linoleic acid produces *trans*-2-nonenal and many other volatile aldehydes during mashing, but autoxidation during boiling has emerged from a recent work as the main source of this cardboard-like off-flavor in aged beer (Noël et al., 1999b). Inhibiting the oxidative deterioration of wort in the kettle will thus require protection of the intrinsic antioxidant potential of malt and hops and/or addition of exogenous antioxidants.

Initiation of lipid autoxidation, enhanced either by free radicals ($O_2^{\bullet -}$, $OH^{\bullet}$) or by singlet oxygen ($^1O_2$), can usually be prevented by singlet oxygen quenchers, free radical scavengers, or chelatants (Niki, 1987). The propagation chain reaction can also be broken with peroxy radical scavengers such as phenolic compounds (Torel et al., 1986; Gardner, 1989; Porter et al., 1995).

Over the past few years, the antioxidant activity of wort and beer has been investigated by various methods including (1) measuring the capacity to reduce the iron(II)−dipyridyl complex (Chapon, 1981), (2) measuring the ability to scavenge the radical cation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) in an aqueous phase (Araki et al., 1999), (3) measuring the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (Kaneda et al., 1995a,b), (4) measuring chemiluminescence, either directly or after reaction with the radical scavenger 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (CLA) (Kaneda et al., 1990a,b, 1991, 1994), (5) electron spin resonance (Kaneda et al., 1988, 1989; Uchida and Ono, 1996), (6) assaying 2-thiobarbituric acid reactive substances (Grigsby and Palamand, 1976), (7) electrochemical measurement of the redox potential (van Strien, 1987; Galic et al., 1994; Buckee et al., 1997), (8) measuring the capacity to delay methyl linoleate oxidation (in lipidic media and at high temperature) followed by gas chromatography (Boivin et al., 1993; Maillard and Berset, 1995), and (9) quantification of linoleic acid hydroperoxide in a Fenton-type reaction (Bright et al., 1998). Unfortunately, some of these assays are either time-consuming or specially designed for lipidic media. Moreover, it appears of great interest to optimize an antioxidant assay relevant to wort, which could also be applied to all raw materials.

Azo compounds generating free radicals through spontaneous thermal decomposition are useful for in vitro studies of lipid peroxidation (eqs 1−3) (Niki, 1987; Pryor et al., 1993; Ghiselli et al., 1998). The water-soluble azo compound 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) has been extensively used as a clean and controllable source of thermally produced alkylperoxyl free radicals (Niki, 1990). To date, however, only Fantozzi et al. (1998) have applied it in the brewery field to assess the effect of beer on the oxidation of human low-density lipoprotein (LDL).

$$A{-}N{=}N{-}A \xrightarrow{k_d} (1 - e)\ A{-}A + 2e\ A^{\bullet} + N_2 \quad (1)$$

$$A^{\bullet} + O_2 \rightarrow AO_2^{\bullet} \quad (2)$$

$$AO_2^{\bullet} + LH \rightarrow AOOH + L^{\bullet} \quad (3)$$

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

The aim of the present work was to use this hydrophilic azo compound to assess the oxidability of linoleic acid in an aqueous dispersion (resembling wort) in the presence of antioxidants from malt or hops. First, the efficiency of 12 recognized antioxidants, some naturally

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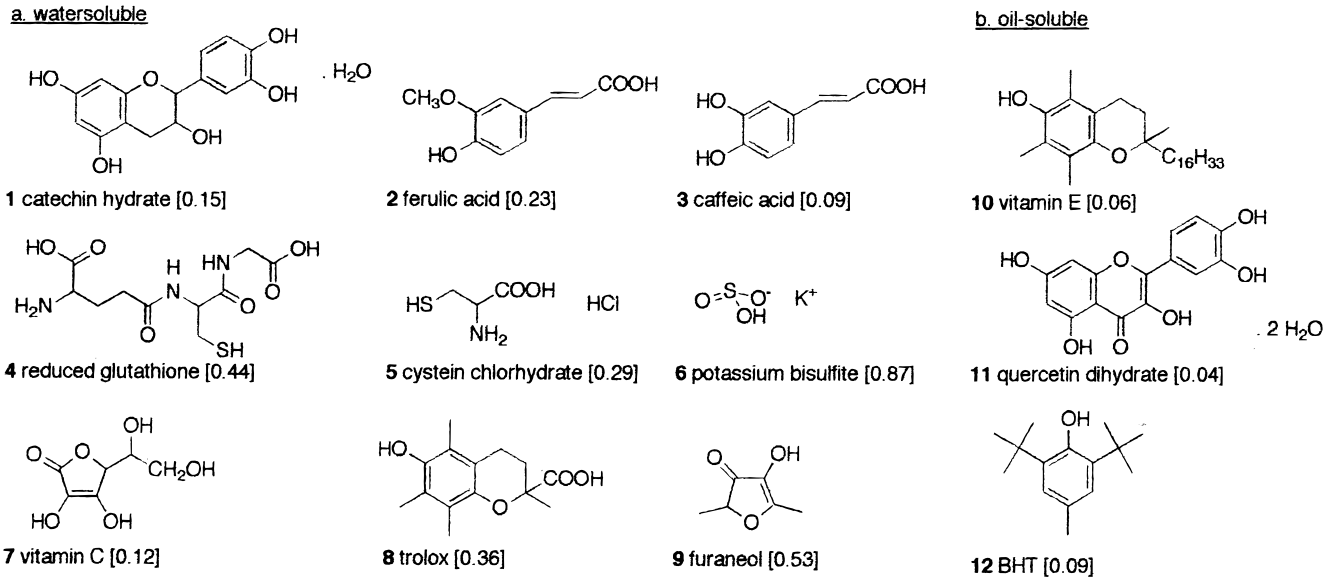

Figure 1. Antioxidants used in this study (a, water-soluble; b, oil-soluble). The numbers in brackets give the initial rates of oxidation in the presence of 2 μM antioxidants compared to the controls [R_{inh}/R_o].

occurring in beers, was investigated to determine the main contributors to the antioxidant activity of wort.

MATERIALS AND METHODS

Reagents. Twelve antioxidants have been compared (see Figure 1): catechin hydrate 98%, **1**; 4-hydroxy-3-methoxycinnamic acid 99% (ferulic acid), **2**; 3,4-dihydroxycinnamic acid (caffeic acid), **3**; reduced glutathione, **4**; L-cysteine chlorhydrate, **5**; potassium metabisulfite, **6**; L-(+)-ascorbic acid (vitamin C), **7**; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid 97% (Trolox), **8**; 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone 95% (Furaneol), **9**; (±)-α-tocopherol 95% (vitamin E), **10**; quercetin dihydrate, **11**; and butylated hydroxytoluene (BHT), **12**. Compounds **1**, **3**, **10**, **11**, and **12** were from Sigma Chemical Co. (St. Louis, MO); **2**, **4**, **7**, and **9** were from Acros Organics (Geel, Belgium); **5** was from Merck (Darmstadt, Germany); **6** was from Vel (Leuven, Belgium); and **8** was from Aldrich Chemical Co. (Milwaukee, WI).

Linoleic acid (99%) used as substrate was obtained from Sigma Chemical. The water-soluble radical initiator, AAPH, was obtained from Aldrich Chemical Co. Tween 20 (polyoxyethylenesorbitan monolaurate), used as a surfactant, and anhydrous dipotassium hydrogenphosphate p.a., potassium dihydrogen phosphate p.a., and boric acid p.a., used for buffer solutions, were purchased from Merck, and dimethylsulfoxide p.a. (DMSO) was from Fluka Chemie (Buchs, Switzerland). Methanol 205 super purity from Romil (Cambridge, U.K.) was used for solvent extraction.

All aqueous solutions and reagents were made using Milli-Q (Millipore, Bedford, MA) double-distilled water (resistance = 18 mΩ/cm^2).

Substrate. An aqueous solution of linoleic acid was prepared weekly according to the method of Surrey (1964), with minor modifications: under continuous stirring, linoleic acid (0.25 mL) was added dropwise to 5 mL of 0.05 M borate buffer, pH 9, containing 0.25 mL of Tween 20. The resulting dispersion was clarified by adding 1 mL of 1 N sodium hydroxide. The volume was adjusted to 50 mL with additional borate buffer. This ~16 mM linoleic acid solution was stored at 4 °C in the dark under argon until needed. Before use, the substrate was checked for autoxidation, and solutions exhibiting >3% autoxidation were discarded.

AAPH Solution. Forty millimolar AAPH was freshly prepared in 0.05 M phosphate buffer, pH 7.4.

Antioxidants. Stock solutions (0.01 M) of oil-soluble antioxidants were freshly prepared in pure methanol (preparation at 50 °C for antioxidant **11**). Stock solutions (0.01 M) of water-soluble antioxidants were prepared in 0.05 M phosphate buffer, pH 7.4 (preparation at 60 °C for antioxidants **1**−**3**) except for Trolox **8** solution, which was first prepared in DMSO. Appropriate dilutions were prepared in phosphate buffer (containing 0.05% v/v Tween 20 in the case of lipophilic antioxidants) just before the antioxidant assay.

Extraction of the Antioxidants from Malt and Hop. Malt was finely ground in a DLFU-mill from Bühler-Miag (Braunschweig, Germany); hop pellets were crushed in a mortar. Ground samples (1 g) were extracted four times under nitrogen with 7 mL of methanol by shaking for 15 min and sonicating for 5 min. After centrifugation (3500*g*, 10 min), the supernatant (25 mL) was collected. For the CO_2 hop extracts, an aliquot (1 g) was dissolved under nitrogen in 25 mL of methanol by sonicating for 10 min. Appropriate dilutions were prepared in pure methanol by taking into account a maximum methanol level of 0.33% (v/v) in the antioxidant assay.

Antioxidant Assay. Thirty microliters of the 16 mM linoleic acid dispersion described above was added to the UV cuvette containing 2.81 mL of 0.05 M phosphate buffer, pH 7.4, prethermostated at 40 °C. The oxidation reaction was initiated at 37 °C under air by the addition of 150 μL of 40 mM AAPH solution. Oxidation was carried out in the presence of aliquots (10 μL) of either wort samples (wort dilution factor in test = 700 to 300), methanolic malt extracts (final concentration in test = 133.3 mg/L), methanolic hop extracts (final concentration in test = 1.7−16.7 mg/L), or aqueous solutions of antioxidants (final concentration in test = 0−8 μM). In the assay without antioxidant (curve 0), lipid oxidation was measured in the presence of the same level of methanol (maximum = 0.33%). We carried out all of the assays without chelatants to assess overall antioxidant activity including prooxidant effects of transition metals.

The rate of oxidation at 37 °C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. A molar extinction coefficient of 28000 M^{-1} cm^{-1} was used in all calculations (Lulai and Baker, 1976). A Shimadzu UV−visible 240 spectrophotometer (Antwerp, Belgium) equipped with an automatic sample positioner allowed analysis of six samples every minute. In all cases, the measurements were run in duplicate against the buffer and compared with a separate AAPH-free control to check for any spontaneous oxidation. AAPH has a relatively high absorbance below 260 nm, which changes as the compound decomposes: the A_{234} of a 2 mM solution was ~0.9 at the start of the incubation, increasing to 1.3 after 4 h at 37 °C. Therefore, its absorbance measured in a separate cuvette in the absence of linoleic acid was subtracted from each experimental point.

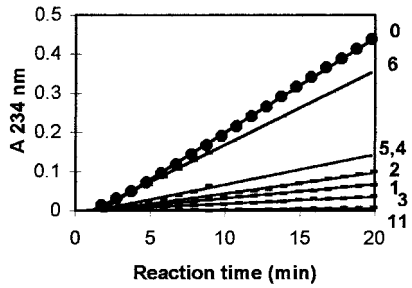

Figure 2. Effect of various antioxidants on AAPH-induced linoleic acid oxidation, as measured by kinetics of conjugated diene formation. Linoleic acid (0.16 mM) was incubated with 2 mM AAPH in 50 mM potassium phosphate buffer, pH 7.4, at 37 °C under air, in the absence (●) and presence of 2 μM antioxidant. The numbers give the antioxidant shown in Figure 1.

Table 1. Initial Rates of Oxidation (R_o) of Linoleic Acid (LH) in an Aqueous Dispersion at 37 °C

run	[LH], mM	[AAPH], mM	10^8 R_o, M dienes formed/s
1	0.1	2	0.95
2	0.16	1	1.05
3	0.16	2	1.47
4	0.16	4	1.62
5	0.2	2	1.86
6	0.4	2	3.31
7	0.5	2	3.88
8	0.8	2	5.11

The inhibition time (T_{inh}) was estimated with Microsoft Excel software as the point of intersection between the tangents to the inhibition—and propagation—phase curves, under precise oxidation conditions. The rate of radical generation from AAPH in an aqueous phase (R_i) was measured with Trolox, a water-soluble vitamin E analogue, as the radical scavenger (Niki et al., 1986).

RESULTS AND DISCUSSION

Oxidation of Linoleic Acid in an Aqueous Dispersion and Its Inhibition by Antioxidants. In the absence of a radical initiator, the rate of spontaneous oxidation at 37 °C can be considered negligible. Addition of AAPH induces oxidation, which starts at a constant rate of conjugated diene formation. As shown in Table 1, the concentrations of both linoleic acid and AAPH strongly influence the initial oxidation rate (R_o) in the aqueous dispersion. On the basis of this observation, the concentrations (0.16 and 2 mM for linoleic acid and AAPH, respectively) and temperature (37 °C) were chosen to obtain appropriate rates of oxidation.

Figure 2 shows the results obtained at the beginning of the oxidation assay in the presence of several antioxidants (2 μM). During this inhibition period, phenolic compounds (**11**, **3**, **1**, and **2**) proved to inhibit oxidation most efficiently, thiols (**5** and **4**) less so. Surprisingly, sulfites **6** did not inhibit AAPH-induced oxidation of linoleic acid. We can assume that the antioxidant activity of sulfites detected by ESR technique (Uchida and Ono, 1996) 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 (R_{inh}) to the initial oxidation rate in their absence (R_o) provides a first antioxidant activity index (Iwatsuki et al., 1995). The R_{inh}/R_o values for all of the antioxidants tested are shown in Figure 1 (see values in brackets).

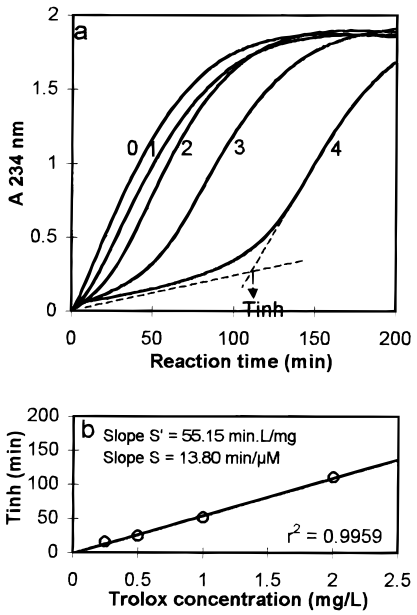

Figure 3. (a) Effect of Trolox concentration on AAPH-induced linoleic acid oxidation, as measured by kinetics of conjugated diene formation. Linoleic acid (0.16 mM) was incubated with 2 mM AAPH in 50 mM potassium phosphate buffer, pH 7.4, at 37 °C under air, in the presence of 0, 1, 2, 4, and 8 μM Trolox (curves 0, 1, 2, 3, and 4, respectively). (b) Inhibition time (T_{inh}) as a function of the Trolox concentration.

As shown in Figure 3a for Trolox **8**, an inhibition time (T_{inh}) can also be determined for each antioxidant concentration. In all cases, once this inhibition time is over, oxidation proceeds at the same rate as in the absence of inhibitor. The slope of the curve representing the T_{inh} versus the antioxidant concentration, shown in Figure 3b (S or S′), can be used as a second very interesting antioxidant activity index. Figure 4 shows the slopes obtained in the same way for the various tested antioxidants. The measured inhibition time was directly proportional to the concentration of the additive in the tested concentration range.

From these data, it is possible to calculate a stoichiometric coefficient n representing the number of peroxyl radicals trapped by each antioxidant molecule (see values in Figure 4). Using Trolox as a reference inhibitor removing two radicals per added molecule (Niki et al., 1986), it is easy to determine the rate of radical generation (R_i) under our assay conditions from the equation $R_i = n[\text{antioxidant}]/T_{inh}$.

Using our experimental R_i value [1.2075×10^{-6} [AAPH] (s^{-1})], to be compared with that obtained by Niki et al. (1986) in a similar medium [1.30×10^{-6} [AAPH] (s^{-1})], n for the other molecules can be deduced.

Caffeic acid **3**, catechin **1**, and quercetin **11** produced a longer inhibition period than vitamin E **10**, ferulic acid **2**, or BHT **12**, indicating that the first three can scavenge more radicals.

As recently observed by Koga et al. (1998) in emulsified methyl linoleate, Furaneol **9** proved less potent than vitamin C **7**.

Antioxidant Activity of Unboiled Wort. Although linoleic acid is the major fatty acid in wort (~50%, 1−100 mg/L), its concentration is negligible once the wort is diluted several hundred times. Therefore, addition of wort to the oxidative system elicited a behavior similar to that reported for pure antioxidants. A preliminary dose−response experiment, performed with

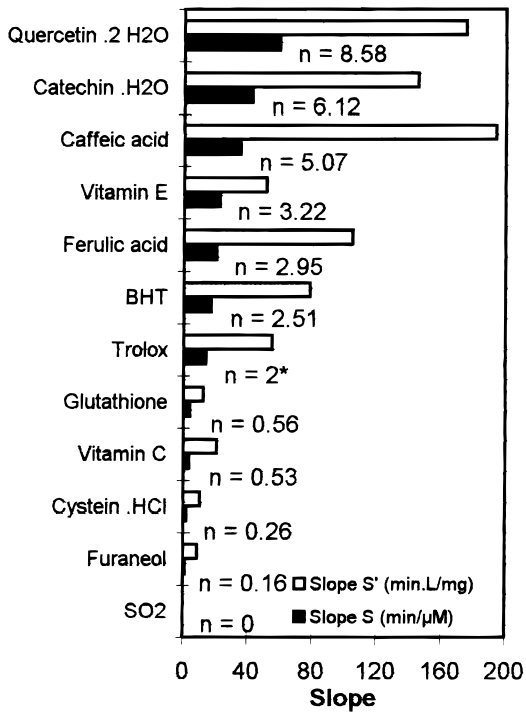

Figure 4. Comparison of the peroxyl radical scavenging activity of various antioxidants. *n* represents the stoichiometric number of peroxyl radicals trapped per molecule of additive (*, taken as reference; Niki et al., 1986). Experimental conditions were as in Figure 3.

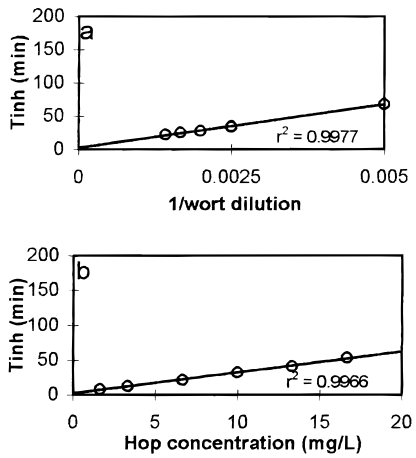

Figure 5. Inhibition time (T_{inh}) as a function of (a) the reciprocal of the final wort dilution in test and (b) the final hop concentration in test. Experimental conditions were as in Figure 3.

wort at different dilutions, showed a linear relationship between the inhibition time and the reciprocal of wort dilution (Figure 5a). On the basis of this first experiment, we chose an appropriate wort dilution: 400-fold dilution in phosphate buffer for an initial gravity of 12 °P. An inhibition time of 33.8 min was found with a variation coefficient of 2.2% (quadruplicate assays).

The antioxidant efficiency of wort is expressed in Table 2 in reference antioxidant equivalents according to the following equation:

$$\text{antiox activity (12 °P wort, antiox equiv in mg/L)} = [T_{inh}\text{ (diluted wort sample)} \times \text{dilution factor (400 in our case)}]/S'\text{(ref antiox)}$$

Table 2. Antioxidant Activity of an Industrial Wort (12 °P) and a 1 mg/L Methanolic Solution of Hop Pellets or Pale Malt Given in Reference Equivalents

mg/L equiv	wort	hop pellets	pale malt
Furaneol	1550	0.433	0.0141
cysteine·HCl	1312	0.367	0.0119
glutathione	1080	0.302	0.0098
vitamin C	653	0.183	0.0059
vitamin E	262	0.073	0.0024
Trolox	245	0.069	0.0022
BHT	172	0.048	0.0016
ferulic acid	129	0.036	0.0012
catechin·H_2O	93	0.026	0.0008
quercetin·$2H_2O$	77	0.022	0.0007
caffeic acid	70	0.019	0.0006

Table 3. Efficiency of Four Successive Extractions with 7 mL of Methanol Applied to 1 g of Pale Malt or Hop Pellets[a]

no. of extractions with 7 mL of methanol	T_{inh} (min)	
	(A) Pale Malt	
1	8.6[b]	8.5
	8.4[b]	
2	13.0[b]	13.0
	13.0[b]	
3	14.3[b]	14.2
	14.1[b]	
4	14.6[b]	15.7
	16.8[b]	
	(B) Hop Pellets	
1	28.6[c]	30.7
	32.7[c]	
2	39.3[c]	42.3
	45.3[c]	
3	45.1[c]	48.3
	51.5[c]	
4	49.1[c]	50.2
	51.3[c]	

[a] The final malt concentration in the assay is 133.3 mg/L; the final hop concentration in the assay is 13.3 mg/L. [b] Assay duplicates. [c] Extraction duplicates.

As estimated according to the method of Bishop (1972), the total polyphenol content of the studied wort was 107 mg/L. Compared to the antioxidant activity given in polyphenol equivalents in Table 2, polyphenols emerge here as the main contributors to the antioxidant activity of wort.

Antioxidant Activity of Malt and Hops. Methanol was chosen as a solvent for its capacity to dissolve both liposoluble and more hydrophilic compounds. As depicted in Table 3A, the malt extraction procedure was found to be fully satisfactory with four successive 7-mL methanol aliquots (variation coefficient of 3.2% based on triplicate extractions). Successive extractions were also found to be more efficient than one extraction with 28 mL of methanol. Linoleic acid oxidation in the presence of methanolic malt extracts confirms the presence of peroxyl radical scavengers in malt. Here again, the antioxidant activity was inversely proportional to the dilution ratio (data not shown).

The same method was further applied to methanolic hop extracts. Figure 5b presents results for methanolic solutions of CO_2 hop extracts. On hop pellets, best results were also obtained with four successive extractions with 7 mL of methanol [variation coefficient of 1.7% based on quadruplicate extractions (Table 3B].

As in the case of wort, the antioxidant activities of methanolic solutions of hop pellets (1 mg/L) or pale malt (1 mg/L) can be expressed in equivalents of various well-

known antioxidants (Table 2) according to the following equation:

$$\text{antiox activity (1 mg/L malt or hop, antiox equiv in mg/L)} = T_{\text{inh}}\,(133.3\ \text{mg/L malt or } 13.3\ \text{mg/L hop in the test})/[S'(\text{ref antiox}) \times \text{constant (133.3 for malt or 13.3 for hop)}]$$

In this assay hop appeared as an oxidation inhibitor 30 times more efficient than the tested malt. This suggests a promising means of optimizing the antioxidant activity in the kettle: selecting hop cultivars with the highest antioxidant activity. Further research is required, however, to identify the most active hop fractions.

Conclusion. We propose a new reliable, quick, low-cost spectrophotometric method for measuring the antioxidant activity of raw materials, worts, or beers. The method, based on the inhibition of lipid oxidation, provides a measure of how efficiently natural antioxidants protect against lipid oxidation in vitro. Oxidation of exogenous linoleic acid by a thermal free radical producer (AAPH) is followed by UV spectrophotometry in a highly diluted sample. The high initial velocity of the reaction makes it easy to estimate the extent to which oxidation is delayed in the presence of antioxidants. Our method emerges as very useful for exploring oxidative changes during the brewing process and assessing the contribution of all raw materials. Applied to wort, it confirms the predominant role of polyphenols. For the first time, hops were found to display much higher antioxidant activity than pale malt.

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