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Comparative study of antioxidant capacity of yellow birch twigs extracts at ambient and high temperatures

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Abstract

The antioxidant capacity of yellow birch (*Betula alleghaniensis* Britton) twigs aqueous acetone extract and its fractions was evaluated at ambient and at elevated temperatures. The crude extract, obtained by maceration in acetone, was further fractionated with *tert*-butyl-methyl ether and ethyl acetate. The antioxidant activity at room temperature was studied by methods based on 1,1-diphenyl-2-picrylhydrazyl (DPPH·). Differential scanning calorimetry (DSC) and thermogravimetry (TG) were employed at high temperatures. The commercial antioxidant 2,6-di-*tert*-butyl-4-methyl-phenol (BHT) was used as a reference. Among samples, the ethyl acetate and aqueous fractions exhibited the highest antioxidant capacity in DPPH· assays. The ether fraction and crude extract showed more antioxidant activity at high temperatures. The commercial antioxidant BHT exhibited the best antioxidant behaviour in most of the reported tests. The only exception was in the kinetic method with DPPH·. The results of this study confirm that antioxidant activity is a property that strongly depends on the oxidation conditions used in the particular oxidation test.

Keywords: *Betula alleghaniensis*; Twigs extracts; Antioxidant activity; DPPH; Lipid peroxidation; Thermoanalytical analysis

1. Introduction

The content of phenols in food and beverages is correlated with a reduced incidence of several diseases. There is considerable epidemiological evidence indicating a relationship between fruit-and vegetable-rich diets and a decreased risk of certain forms of cancer. The role of polyphenolic compounds from higher plants as antioxidants, antimutagenic, antiinflammatory and antimicrobial agents is widely recognized (Hatano et al., 2002; Lee, Howard, & Villalón, 1995). The health impact of antioxidants in foods and the hazardous effects of synthetic preservatives have led to active research in the field of natural antioxidants.

Our previous studies of lipophilic extracts from foliage and wood of yellow birch (*Betula alleghaniensis)* have proved the presence of bioactive molecules in high concentrations (Lavoie & Stevanovic, 2005; Lavoie & Stevanovic, 2006). Squalene, betulonic acid and acetyl methyl betulinate were identified as the most valuable compounds present in lipophilic extract from solid wood and industrial sawdust (Lavoie & Stevanovic, 2006). Other phenols, e.g. syringaldehyde, chlorogenic acid and salidroside, were also found in dichloromethane extract, but in lower concentrations. Keinänen, Julkunen-Tiitto, Rousi, and Tahvanainen (1999) compared the foliar phenolic compositions of several birch species. (+)-Catechin, 3-caffeoylquinic acid, 3-coumaroylquinic acid, quercetin-3-arabinopyranoside, kaempherol-3-ramnoside and apigenin derivatives were identified in yellow birch extracts. Phenolics and terpenoids were also screened in seedlings of several birch species (Julkunen-Tiitto et al., 1996). Salidroside and (+)-catechin were identified in methanolic extracts of *B. alleghaniensis*.

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These results demonstrate that the extracts of yellow birch are rich in phenolic compounds and, as such, could be used as sources of natural antioxidants.

There are several tests to measure antioxidant capacity. Usually these methods are based on the oxidation of a more or less complex substrate or on the reactivity towards reference free radicals (Huang, Ou, & Prior, 2005). Sanchez-Moreno and Larrauri (1998) classified antioxidant tests in food and biological system in two groups. The first group includes assays used to evaluate lipid peroxidation in lipoproteins or lipids in which the degree of oxidation inhibition under standard conditions is measured. The second group is formed by assays measuring the free radical-scavenging capacity.

The tests most commonly used to measure the free radical-scavenging capacity are those involving chromogen compounds of a radical nature. The presence of antioxidants leads to the disappearance of these radical chromogens. Generally, these methods are preferred for their sensitivity and speed. The free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$) is widely used for these purposes. It is a stable free radical, easy to manipulate. The antioxidant activity is generally reported as the concentration of antioxidant needed to decrease (by 50%) the initial DPPH$^{\cdot}$ concentration at a fixed time (Cuvelier, Richard, & Berset, 1992). Other methods measure the constant rate of the DPPH$^{\cdot}$ reaction in the presence of antioxidants (Diouf, Merlin, & Perrin, 2006; Rousseau-Richard, Auclair, Richard, & Martin, 1990). These methods are commonly performed at room temperature.

Lipid peroxidation at higher temperatures can be evaluated by accelerated oxidation stability tests. Such tests are often highly relevant if the conditions used in the oxidation tests are similar to those that the oils and fats encounter during processing or use (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002). The effect of natural and synthetic antioxidants is evaluated upon inhibition of lipid oxidation, by determination of peroxide concentrations or by measurement of rate of oxygen consumption.

Thermoanalytical methods have been used to characterize the antioxidant behaviour at high temperatures. Kowalski (1991) used differential scanning calorimetry (DSC) to evaluate the antioxidant activity of vegetable oils and lard by heating the samples to 360 °C. Two samples of highly rancid rapeseed and sunflower oils inhibited with BHT and propyl gallate were also studied. Litwinienko, Kasprzycka-Guttman, and Studzinski (1997) investigated the kinetic features of linoleic acid thermoxidation under DSC non-isothermal conditions (50–300 °C). The authors found that DSC was a relatively simple, convenient and fast method for screening the efficiency of phenol antioxidants. This technique was also used to measure the efficiency of various commercial hindered phenol antioxidants in squalene (Breese, Lamèthe, & DeArmitt, 2000).

In spite of being a well known method for studying oxygen absorption in the polymer industry, thermogravimetric analysis (TGA) has received limited attention for evaluating oxidative stability in the food industry. Nieschlag, Hagemann, and Rothfus (1974) estimated the oxidative stability of crambe oil samples by TGA. The weight change in an oxygen environment was monitored under controlled heating rates. An estimation of oil resistance to oxidation was obtained by measuring the percent weight gain due to oxidation. Gennaro, Piccioli-Boca, Modesti, Masella, and Coni (1998) used TGA to estimate the effect of antioxidants on oxidative stability of virgin olive oil. This technique was also used by Van-Aardt et al. (2004) to study the oxidative stability of various edible oils and triacylglycerides and the effect of α-tocopherol, ascorbic acid and synthetic antioxidants in trinolein. TGA showed high reproducibility and proved to be a valuable technique for evaluating oxidative differences between oils and fats with and without antioxidants. Rudnik, Szczucinska, Gwardiak, Szulc, and Winiarska (2001) studied the oxidative stability of linseed oil by the classical tests based on determination of peroxide value by Rancimat and thermoanalytical methods. The results obtained by TG and DSC matched those obtained using the peroxide value and Rancimat methods. Therefore they concluded that thermoanalytical methods can be suitable for the prediction of oxidative stability of vegetable oils and for evaluating the antioxidant efficiency of food additives.

Because most natural antioxidants and phytochemicals are multifunctional compounds, several methods, covering various oxidation conditions, should be tested to evaluate their antioxidant properties (Frankel & Meyer, 2000). That explains why the use of several techniques to measure the antioxidant capacity of natural products is becoming a common feature in recent publications (Mathew & Abraham, 2006; Senevirathne et al., 2006).

The aim of this paper is to analyse the antioxidant capacity of yellow birch twigs aqueous acetone extract and its fractions by several antioxidant methods and to correlate the results with the total phenol content of the studied extracts.

2. Materials and methods

2.1. Chemicals

Folin–Ciocalteu phenol reagent (FCR), gallic acid monohydrate, sodium carbonate, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,6-di-*tert*-butyl-4-methyl-phenol (BHT) were purchased from Sigma–Aldrich (St. Louis, MO). Azo-bis-isobutyronitrile (AIBN) from Merck and methyl linoleate (MeL) from Tokyo Kasei Kogyo Co. Ltd. were used. Petroleum ether, *tert*-butyl-methyl ether, ethyl acetate and acetone were obtained from Fisher Scientific Chemicals (Tustin, CA).

2.2. Extraction

Yellow birch twig meal (20 g, 40–60 mesh) was extracted 3 times with 70% aqueous acetone (200 ml), under continuous stirring (220 rpm) at room temperature for 24 h. At

the end of each of the first two extractions, stirring was stopped and the supernatant collected and retained. Afterwards, fresh 70% acetone was added to the solid precipitate and stirring was resumed. The final extract was filtered through Whatman No. 4 paper on a Büchner funnel by vacuum; the solids were washed with an additional 100 ml of 70% acetone. The combined extracts were rotary-evaporated under vacuum at 40 °C to remove acetone. The aqueous layer was freeze-dried to yield an aqueous acetone extract. The extract was suspended in water (1:50 w/v), defatted with petroleum ether and sequentially partitioned with *tert*-butyl-methyl ether (3 × 50 ml) and ethyl acetate (3 × 50 ml) to obtain three different fractions: *tert*-butyl-methyl ether fraction ($TBME_f$), ethyl acetate fraction (EA_f) and aqueous fraction (A_f).

2.3. Total phenols content

The total phenols content in crude extract and its fractions was determined by the Folin–Ciocalteu method (Singleton & Rossi, 1965). This method uses the Folin reagent diluted 10 times in water and a sodium carbonate solution (75 g l^{-1}). The samples (0.3 g l^{-1}) were mixed with Folin reagent and the sodium carbonate solution. The final solution was heated at 50 °C during 10 min, after which the absorbance at 765 nm was read. A standard curve generated with gallic acid was used for calibration. Total phenol content was expressed as milligrammes of gallic acid equivalents (GAE) per gramme of extract.

2.4. Evaluation of antioxidant capacity

2.4.1. General

The antioxidant capacity of extracts at ambient temperature was evaluated using two free radical (DPPH·) assay approaches. Tests at high temperatures were carried out by thermoanalytical methods.

2.4.2. Methods at ambient temperature

2.4.2.1. Kinetic DPPH· method. The kinetic DPPH· method is one of the most frequently used rapid kinetics techniques to measure antioxidant activity. Small volumes of solutions are transferred by high performance syringes to a high efficiency mixer. The rate constant is used to estimate the mobility of hydrogen atom from phenol (ArOH) and is used as an estimate of antioxidant capacity (Diouf et al., 2006). With an excess of ArOH, it is easy to measure the pseudo-first order rate constant of the reaction with DPPH·

$$\mathrm{ArOH} + \mathrm{DPPH}^{\cdot} \rightarrow \mathrm{ArO}^{\cdot} + \mathrm{H\text{–}DPPH}$$

Methanolic solutions of 2×10^{-4} M DPPH· and 2 g l^{-1} of extracts were mixed in the "Rapid Kinetics Accessory" SFA-11 (HI-TECH Scientific, SALISBURY, England) and the absorbance of DPPH· at 516 nm was monitored. The rate constant of the hydrogen atom transfer pseudo-first order reaction, k_a, was estimated using the kinetic software of a UV–visible spectrometer (Varian model Cary 50). These results were verified by the Guggenheim method (Ahmad & Hamer, 1964; Diouf et al., 2006). Higher rate constant values correspond to higher antioxidant capacities.

2.4.2.2. Equilibrium DPPH· method. The equilibrium DPPH· method is widely used to study the antioxidant activity and consists in measuring the concentration of an extract or compound necessary to reduce (by 50%) the initial concentration of DPPH· (C_{50}) (Diouf et al., 2006; Mathew & Abraham, 2006). All tests were carried out under equilibrium conditions. A solution of 2×10^{-4} M DPPH· in methanol was mixed with the studied samples. The reaction progress was monitored, following the absorbance at 516 nm for 30 min at 30 °C. The solution colour faded as the reaction progressed. The remaining percentage of DPPH· ($DPPH^{\cdot}_R$) was calculated as $(DPPH^{\cdot}_R)\ (\%) = (DPPH^{\cdot}_T)/(DPPH^{\cdot}_0) \times 100$ where $(DPPH^{\cdot}_T)$ is the concentration of DPPH· at 30 min and $(DPPH^{\cdot}_0)$ is the initial concentration. The percentage of DPPH· remaining was plotted versus different concentrations of crude extract and fractions in order to obtain the amount of antioxidant needed to decrease (by half) the initial DPPH· quantity. Lower values of C_{50} indicate higher antioxidant capacity.

2.4.3. Methods applied at high temperatures

2.4.3.1. General. The crude extract and its fractions were kept under vacuum/N_2 flow to dryness and the residue was dissolved in 0.3 ml of methanol to 30 g l^{-1} final concentration. Methanol was chosen because it is a good solvent for the polyphenols and it is miscible in methyl linoleate (MeL). The resulting solution was added to 3 g of MeL containing 10^{-2} M AIBN. Preliminary experiments showed that 0.3 ml of pure methanol does not have any effect on MeL oxidation. The equivalent amount of BHT in methanol was used in order to compare the results with those obtained for natural antioxidants.

2.4.3.2. DSC measurements. Differential scanning calorimetry (DSC) is commonly used to investigate processes which involve enthalpy changes. Lipid oxidation is an exothermic process, so DSC is a convenient method for recording the heat released. The temperature of the onset point (OIT^*_{DSC}) is determined as the temperature at which the oxidation process begins. For a constant value of heating rate the OIT^*_{DSC} is characteristic for the given substance.

A Mettler Toledo DSC 822^e was used to carry out the tests. All samples were studied at a heating rate of 2 °C min^{-1} from 25 to 170 °C under an oxygen flow of 50 ml min^{-1}; 5.5 ± 0.5 mg of each sample were analysed in a standard 40 µl aluminium pan. OIT^*_{DSC} can be determined by two procedures: the first one, using the tangents intersection point in the thermograms and the second one the second derivative of the first peak (Breese et al., 2000). Results obtained by the two procedures were very close, but the second derivative data were less scattered and therefore this method has been chosen for the

OIT^{*}_{DSC} determinations. Higher values of OIT^{*}_{DSC} correspond to samples with higher oxidation stability.

2.4.3.3. Thermogravimetric method. Thermogravimetric analysis (TGA) is also used to estimate the resistance of certain materials to oxidation. The weight gained by the sample as a result of oxygen uptake can be used as a good indicator of oxidation stability. This technique also allows measuring the onset temperature at which oxygen uptake starts (OIT^{*}_{TGA}).

The experimental conditions used to prepare the studied samples were similar to those employed in the DSC method. All tests were performed in the presence of oxygen and nitrogen at flow rates of 50 ml min^{-1} using a Mettler Toledo thermobalance (TGA 850, TGA/SDTA 851[e]). Measurements were performed using approximately 20 mg of samples at a heating rate of 2 °C min^{-1} from 25 to 170 °C. All tests were performed three times.

The mass gained due to oxygen uptake was calculated as follows: $\Delta_t = (TG_tO_2 - TG_tN_2)$ (mass %) where TG_tO_2 is the mass percent of sample in time t under O_2 environment and TG_tN_2 corresponds to mass percent of sample in time t but under N_2 environment. OIT^{*}_{TGA} represents the temperature at which the mass starts to increase and was determined from the tangents intersection point in the mass gain Δ (mass %) curve. Higher values of OIT^{*}_{TGA} and lower oxygen mass gains correspond to samples with more resistance to oxidation.

2.5. Statistical analysis

The experimental results are expressed as means ± SD of three measurements. The results were processed using Microsoft Excel 2003 and the data were analysed by an analysis of variance (ANOVA) ($p \leqslant 0.05$) and means separated by Duncan's test. Results were processed by SAS program 8.2 software (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Total phenols content

The total phenol contents in crude extract and in all fractions determined by the Folin–Ciocalteu method are presented in Table 1. This parameter was solvent-dependent. The more polar fractions (EA_f and A_f) exhibited the larger phenol contents, followed by the crude extract and $TBME_f$ respectively. Senevirathne et al. (2006) studied the antioxidant potential of different fractions of methanolic extract of *Ecklonia cava* and also reported the total phenols content by the Folin–Ciocalteu method. Among the organic solvent fractions, the ethyl acetate fraction also exhibited the highest level of total phenols. The organic chloroform fraction and methanolic extract also showed high contents of phenolic compounds.

It is clear that different solvents allow extraction of different kinds of phenolic compounds. Thus, the most polar fractions should contain higher amounts of hydrophilic phenols and condensed tannins while $TBME_f$ may include the hydrophobic phenolic compounds of low-molecular-weight. Phenolic compounds of both kinds are present in crude extract.

3.2. Antioxidant properties at ambient temperature

The results obtained for k_a and C_{50} determination by the DPPH· reaction in the presence of crude extract, its fractions and BHT are presented in Table 1. It can be observed that the antioxidant activities vary widely between the fractions. Results of the kinetic DPPH· method show that the aqueous fraction exhibited the most rapid reaction rate, followed by EA_f and BHT, which exhibited equivalent results. Crude extract and $TBME_f$ were less efficient. By the equilibrium method, the BHT and EA_f were the samples with the best antioxidant activity, followed by A_f, crude extract and $TBME_f$, respectively. In general, fractions with higher contents of phenols showed better antioxidant capacities by both of methods (Fig. 1). These results agree with those of Senevirathne et al. (2006) who found an increase in DPPH radical-scavenging capacity towards the ethyl acetate fraction with increasing solvent polarity and content of total phenols.

However, though the A_f had less than half phenols present in EA_f, its rate constant was higher. This behaviour can be explained by the nature of phenols in this fraction, allowing a higher mobility of hydrogen atoms from phenols. In fact, the nature of the phenols is also a factor influencing the antioxidant activity.

Table 1
Antioxidant capacities of crude extract, BHT and different fractions of *Betula alleganiensis* twigs

	Total phenols content (mg g^{-1})	Antioxidant capacity			
		DPPH· k_a (min^{-1})	DPPH· C_{50}(mg l^{-1})	OIT^{*}_{DSC} (°C)	OIT^{*}_{TGA} (°C)
Crude extract	$94.4^{} \pm 12.9$	$1.83^{c} \pm 0.002$	$57.5^{b} \pm 8.13$	$122^{b} \pm 1.32$	$94.6^{b} \pm 4.34$
$TBME_f$	$59.7^{} \pm 16.6$	$0.35^{d} \pm 0.011$	$141^{a} \pm 6.15$	$123^{b} \pm 0.608$	$96.8^{b} \pm 6.58$
EA_f	$227^{} \pm 2.40$	$2.05^{b} \pm 0.120$	$19.9^{d} \pm 0.109$	$121^{b,c} \pm 1.57$	$90.2^{b,c} \pm 0.762$
A^f	$99.9^{} \pm 5.65$	$2.27^{a} \pm 0.006$	$37.7^{c} \pm 2.51$	$119^{c,d} \pm 0.261$	$89.4^{b,c} \pm 0.779$
BHT	–	$1.97^{b,c} \pm 0.168$	$8.40^{e} \pm 0.435$	$140^{a} \pm 1.56$	$145^{a} \pm 6.72$
MeL	–	–	–	$118^{d} \pm 1.09$	$85.4^{c} \pm 4.59$

Each value in table represents the means ± SD for tree determinations. Different superscript letters mean significant differences ($p \leqslant 0.05$). $TBME_f$ = *tert*-butyl-methylether fraction; EA_f = ethyl acetate fraction; A_f = aqueous fraction; BHT = 2,6-di-*tert*-butyl-4-methyl-phenol; MeL = methyl linoleate.

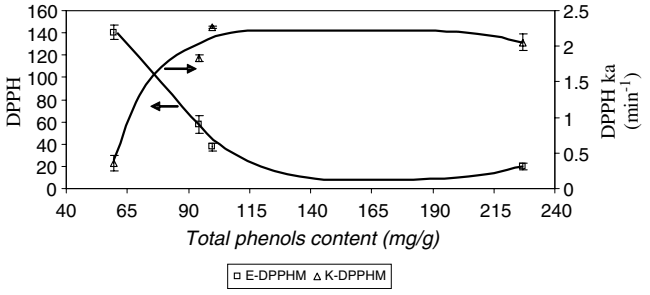

Fig. 1. Correlation between total phenols content and k_a obtained by kinetic DPPH· method (K-DPPHM) and C_{50} obtained by equilibrium DPPH· method (E-DPPHM).

Brand-Williams, Cuvelier, and Berset (1995) related the activity of a potential antioxidant with its structural characteristics. They suggested that, for certain phenolic compounds, a rapid reaction with DPPH· corresponded to the number of available hydroxyl groups. Goupy, Dufour, Loonis, and Dangles (2003) suggested that the ability of different dietary polyphenols to transfer labile H atoms to the DPPH radical was strongly related to their structural characteristics. They found that in some cases this ability was favoured by substitutions in the aromatic rings. The authors also confirmed that, for caffeic acid, chlorogenic acid and some oligomers, the rate constants for first H atom abstraction by DPPH· was sensitive to steric hindrance. The overall mechanism in the case of natural extracts is usually more complex, due to interaction of different compounds. Thus, the antioxidant capacity of natural extracts should be attributed to the total phenols content, the functional groups of these phenols and to the synergism between molecules.

The reaction mechanism between BHT and DPPH· has been described by Brand-Williams et al. (1995). This mechanism involves the donation of a second hydrogen atom following electron delocalization onto the *para*-substituted group. McGowan, Powell, and Raw (1959) also demonstrated that compounds with a hydroxyl group sterically hindered by a *tert*-butyl group present higher antioxidative efficiency.

The results obtained by the equilibrium DPPH· method show a better correlation with total phenol contents than those obtained by the kinetic DPPH· method (Fig. 1). The Folin–Ciocalteu assay is commonly used for the determination of the total phenols content but measures a sample's reducing capacity. This is not surprising if one considers the similarity of chemistry behind the equilibrium DPPH· method and the Folin–Ciocalteu assay. One can thus consider that the kinetic method evaluates antiradical activity, while the equilibrium method is more representative of the evaluation of antioxidant activity: the hydrogen atom transfer is a necessary but not sufficient condition to confer antioxidant effectiveness.

The kinetic DPPH· method can be used to evaluate the reactivity of antioxidants with DPPH·. Hence, it is more suitable for determining the antiradical activity. However, results of the equilibrium method are partly influenced by the involvement of oxidative transformation products of the antioxidants. In fact, it takes into account not only the reactions that lead to hydrogen atom transfer but also the effects of reversible reactions. Therefore this method seems to be better suited to the evaluation of the antioxidant capacity of samples under ambient conditions.

3.3. Methods at high temperatures

3.3.1. DSC measurements

In the DSC method, the MeL oxidation was observed as a sharp increase in the energy flow due to the exothermic nature of the oxidation reactions. Methyl linoleate oxidation is a radical process which proceeds via a chain reaction, including induction, propagation and termination steps (Fig. 2). Induction period is a preparatory stage in which the chemical compounds needed for the full development of oxidation are formed. This period is considered as a relative measure of oxidative stability. During the induction period, alkyl radicals are formed which undergo reaction with oxygen molecules to form hydroperoxides and peroxide radicals during the propagation phase. Termination proceeds via association of two radicals to form a stable adduct (Brand-Williams et al., 1995).

OIT^*_{DSC} values, determined by the DSC method, are presented in Table 1. Among samples, the commercial antioxidant offers the most important protection against thermal oxidation of MeL. Only the $TBME_f$ and EA_f as well as the crude extract exhibited significant antioxidant effects. These results agree with those obtained by Kulisic, Dragovic-Uzelac, and Milos (2006) who compared the antioxidant activities of natural extracts from oregano, thyme and wild thyme with BHT using several antioxidant methods. The authors also found an enhanced antioxidant capacity of BHT compared to natural samples by Rancimat assay at 100 °C. It is clear that, in the case of natural samples, the chemical complexity and the relative dilution of active substances in the presence of others, less active

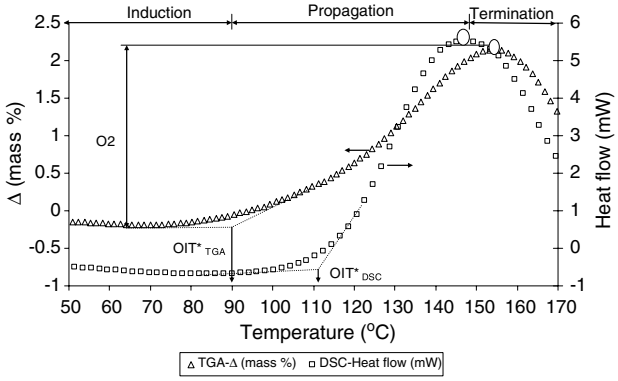

Fig. 2. Typical thermoanalytical curves obtained for methyl linoleate oxidation by DSC and TGA methods. OIT^*_{TGA} = onset temperature by TGA method; OIT^*_{DSC} = onset temperature by DSC method; ΔO_2 = maximum oxygen gain by TGA method.

or inactive, can influence the antioxidant activity. Additionally, the temperature effect on the integrity of molecules in the extracts, and the lower affinity of natural compounds for MeL when the solvent is evaporated, constitute other factors which could explain the lower antioxidant capacity of the studied extracts.

The results obtained by the DSC method differ from those obtained by DPPH· methods at room temperature but are similar to some results reported in the literature. Brand-Williams et al. (1995) compared the antiradical activities of various antioxidants, including isoascorbic and ascorbic acids by the DPPH· test. The authors also determined the activity of samples by an accelerated autoxidation test using MeL at 110 °C under an oxygen-saturated atmosphere. Ascorbic acid and isoascorbic acid reacted rapidly with DPPH·; however, they showed no or little antioxidant activity in the MeL test at high temperature. The authors explained these results as a consequence of the harsh conditions of the MeL test, combined with a poor affinity of the substrate for the solvent. In general, it is known that the protection predicted at high temperatures for an antioxidant will be usually less than that found at lower temperatures.

In spite of the complications of tests using elevated temperatures, they are important since food processing involves heat (e.g. deep-fat frying and microwave heating) and lipid oxidation is one of the major deteriorative reactions of heated oils, resulting in a significant loss of quality.

3.3.2. Thermogravimetry method

Fig. 2 represents a typical curve of MeL oxidation in which an induction period, characterized by a minimal mass change, is followed by a rapid mass increase due to oxygen uptake. A final temperature (T_f) at which the oxygen mass gained reaches a maximum (Δ mass, %) can be clearly identified. The maximum mass gained is known to be an important index of lipid autoxidation extent (Gennaro et al., 1998). The sample stops adsorbing oxygen at temperature beyond T_f.

Results of onset temperature determination are shown in Table 1. The OIT^*_{TGA} values obtained for A_f and EA_f are not significantly different from those obtained for MeL. Among samples, BHT also shows the most important protection against thermoxidation of MeL, followed by $TBME_f$ and crude extract. As the oxidation conditions are similar to those used in DSC measurements, these results can be explained using the same argument.

The results obtained by measuring weight gain percent as a function of oxygen uptake are presented in Fig. 3. It is interesting to note that, while the tested natural samples show less weight gain than does MeL, BHT reached a greater weight gain. Indeed, it was impossible to observe the point of maximum gain at the examined temperature intervals. It is supposed that samples containing BHT should continue gaining weight at higher temperatures. In fact, it can be noted that, even if the BHT addition significantly increased the induction oxidation temperature compared to MeL alone,

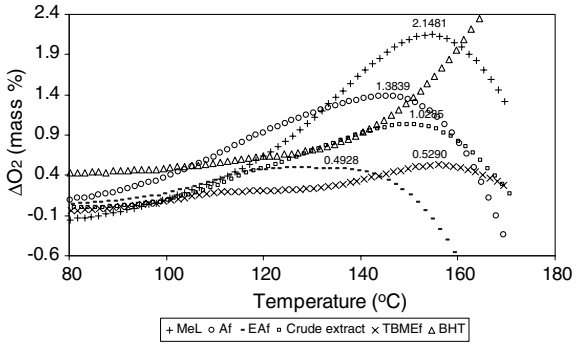

Fig. 3. Comparison of methyl linoleate oxidation with and without antioxidants. MeL = methyl linoleate; Af = aqueous fraction; EAf = ethyl acetate fraction; TBMEf = *tert*-butyl-methylether fraction; BHT = 2,6-di-*tert*-butyl-4-methyl-phenol.

it tended to lose its protective action in further steps of oxidation. This effect is contrary to expected results if one only analyses the onset of temperature. In fact, Gennaro et al. (1998) found similar results for BHT and caffeic acid in olive oil stability. These two antioxidants caused only an increase of onset temperature, showing in some cases an increase or no variations in Δ % values.

In spite of the lower antioxidant capacity of natural extracts than BHT, as determined by thermoanalytical methods, less polar fractions exhibited better antioxidant behaviours than did polar fractions. These results contrast with the "polar paradox" theory described by Porter (1993). According to this paradox, lipophilic antioxidants are more active in oil-in-water emulsions whereas polar antioxidants are more active in bulk oil systems. Other authors have found similar problems in evaluating their results according this theory. Torres de Pinedo, Penalver, and Morales (2007) investigated the structure–activity relationships of synthetic phenolic-based derivatives as potential antioxidants by the Rancimat method at 120 °C. Neither of these authors found a clear correlation between the polarity of the compounds and their radical-scavenging activity. Khan and Shahidi (2000) also reported that the behaviour of endogenous antioxidants in borage and evening primrose oil-in-water emulsions was very difficult to evaluate according to this theory. In fact, there is only a limited knowledge of how partitioning of natural extracts influence the antioxidant effectiveness in the bulk oils and heterophasic systems at high temperatures. Most investigations, taking into account this theory, use the temperatures below 60 °C and employ synthetic pure compounds or phenolic compounds isolated from natural extracts.

Results also show that there is no correlation between the OIT^*_{TGA} and OIT^*_{DSC} and the phenol content determined by the Folin–Ciocalteu method. In fact, among the natural samples, the ether fraction and the crude extract possess less phenols and yet their addition causes the greatest onset temperatures. These results suggest that not only is the amount of phenols in samples important for evaluating the antioxidant capacity under accelerated conditions,

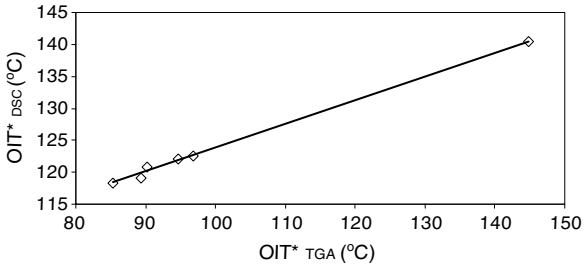

Fig. 4. Correlation between DSC and TGA methods.

but other factors, e.g. their susceptibility at high temperatures and their affinity towards substrate, have to be taken into account to explain the antioxidant capacity.

The tendencies of dynamic TG results are well correlated with those obtained by dynamic DSC measurements (Fig. 4). However, OIT^{*}_{TGA} values are generally lower than OIT^{*}_{DSC}values. DSC monitors changes in enthalpy while TGA measures the mass gain as a function of oxygen uptake. These processes are associated, but there is a certain delay between the absorption of oxygen, measured by TG, and the moment at which the oxidation reactions release energy, as measured by DSC. This delay is controlled by the rate of oxidation reactions and by the response time of DSC instruments.

Results of this study demonstrate that the TGA is a suitable test for estimating the antioxidant capacity in the induction, propagation and termination periods. So it can be used to study the protection of antioxidants during the whole oxidation.

4. Conclusions

Results of this work confirm that the antioxidant capacity depends on the method and test conditions used. It is clear that the activity and ranking of different antioxidants depend on whether they are tested at high or low temperatures. Oxidation mechanism can change at different temperatures.

Antioxidant activity is not necessarily correlated with high amounts of phenolic compounds. Total phenolic content, measured by the Folin–Ciocalteu procedure, does not give a full idea of the nature of the phenolic constituents in the extracts. Thus, the antioxidant capacity of an extract cannot be predicted solely on the basis of this determination.

On the basis of this study, polar fractions exhibited better antioxidant behaviours at low temperatures. By contrast, less polar fractions showed enhanced antioxidant capacities at high temperatures. BHT showed the best general antioxidant behaviour; however, regulations specify a limited maximum of its addition to fats and oils. Natural antioxidants are generally recognized as safe when used in accordance with good manufacturing practice, and therefore are not limited.

These results are only a first estimate of the possibilities of the crude extract and fractions of *B. alleghaniensis* Britton twigs extracts applications as antioxidants under certain conditions. Further studies are needed to confirm the antioxidant effects under different conditions. Before utilising crude extract and fractions as a source of natural antioxidants, further characterizations of the phenolic composition are required.

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