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Radical scavenging activity of lipophilic antioxidants and extra-virgin olive oil by isothermal calorimetry

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ABSTRACT

This work proposes an oxidative stress test based on the measurement of the heat generated during the reaction of AIBN (2,2′-azobis(2-methylpropionitrile)) with lipophilic antioxidants. Without antioxidants, AIBN generates an exothermic peak (induction period = 7.50 h, peak time = 8.55 h, area = 3.6 kJ mol^{-1} of AIBN, at 50 °C). In the presence of antioxidants, such peak is delayed. The extent of such delay provides a simple and direct estimate of the radical-scavenging activity of the sample. Standard solutions of well-known antioxidants lead to the following ranking (from high to low radical scavenging activity): ethoxyquin > > > (±)-α-tocopherol > butylated hydroxytoluene (BHT) > retinyl acetate, in close agreement with the 2,2-diphenyl-L-picrylhydrazyl (DPPH) assay. The proposed assay was applied to characterize the radical-scavenging activity of five extra-virgin olive oil samples. The results were in good agreement with the total phenol content of each sample (R^2 = 0.975).

1. Introduction

Lipophilic antioxidants are a very important class of chemical species that can increase the oxidative stability of food matrices, prevent rancidity of lipid fractions [1,2] or control the formation of free radicals in the organism [3]. Natural antioxidants such as tocopherol or synthetic antioxidants such as ethoxyquin and butylated hydroxytoluene are widely used by food, feed or pharma manufacturers to enhance the oxidative stability of their products. However, every manufacturer has been faced, at least once, to determine the best dose or the best selection of lipophilic antioxidants for a specific application, and the decision is often based on the results obtained from simple spectrometric assays, like 2,2-diphenyl-1-picrylhydrazyl (DPPH) [4], 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) [5] or oxygen radical absorbance capacity (ORAC) [6]. However, all these assays, although widespread, are rarely conclusive. The main limitation is that the results are typically expressed in terms of inhibitory concentration (IC50). IC50 is a concentration and, as such, cannot express the rate of reaction toward free radicals. Instead, the capacity of an antioxidant to work as a radical scavenger should be expressed in terms of a kinetic parameter, such as rate or induction time. In addition, most of the aforementioned assays make use of polar protic solvents, like methanol or aqueous buffers. Such solvents hinder the possibility to test many insoluble lipophilic antioxidants [7]. Last, all these assays are performed at temperatures that generally differ from the condition typically found in practical situations, i.e. during storage or processing [8].

To overcome such problems, the development of a new stress testing assay has been proposed [9]. Typically, such assays use aprotic solvents, like acetonitrile, where the lipophilic active ingredients are dissolved together with an oxidizing booster, such as peroxides [10,11] or radical initiators [12]. A recent work by Alsante showed that, among those boosters, the azo dye AIBN (2,2′-azobisisobutyronitrile) is the most used [13,14].

However, stress testing assays when applied to lipophilic antioxidants are still challenging without due regard to the many experimental variables that may influence the result. Typically, once the antioxidant has reacted with the oxidizing booster, the sample needs to be evaporated, filtered and re-dissolved into a suitable running buffer for chromatography. This impedes the real time monitoring of the reaction, allowing only a step–by–step view of the degradation process, with possible loss of important information.

This work had the aim to overcome such limitations by proposing an isothermal calorimetry (IC) adaptation of the classical stress testing with AIBN [15,16]. The aim was to determine the radical scavenging activity of lipophilic antioxidants without the need of extractions, purification or separation steps. In this respect, IC offers some advantages over chromatography-based assays as the reaction between antioxidants and AIBN can be followed directly, continuously and under ideal isothermal conditions. Also, with respect to the spectrometry-based assay, IC is more robust as it is not limited by the turbidity

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of the sample, absorbing interfering species or by the sample physical state (i.e. solid, liquid or emulsion). As such, the heat-flow measured by IC is a universal indicator of the rate of change, whose utility can become especially important when multi-component systems, such as natural extracts, must be analyzed [17].

The current work investigates the calorimetric response of AIBN in propylene carbonate solvent. The interference of methanol was discussed. An index of the radical scavenging activity of the samples is proposed and applied to important antioxidants for the food and pharmaceutical sectors, including BHT, ethoxyquin, tocopherol and retinyl acetate. Finally, the proposed approach is applied to extra virgin olive oil samples and the results compared with their total phenols content.

2. Material and methods

2.1. Chemicals

The antioxidants (±)-α-tocopherol (PubChem CID: 14985) (synthetic, ≥96%, HPLC), ethoxyquin (PubChem CID: 3293) (≥75%, Capillary GC) and BHT (PubChem CID: 31404) (≥99%, GC), were purchased from Sigma-Aldrich. Retinyl acetate (PubChem CID: 638034) was purchased from Dr. Ehrenstorfer (Germany, 99.4%). All these chemicals were used as received without any further purification.

Ethanol (PubChem CID: 702) (absolute, 99,97%) was purchased from VWR, propylene carbonate (PubChem CID: 7924) (99%, Reagent plus) from Sigma-Aldrich, acetonitrile and methanol (PubChem CID: 887) (LC–MS grade) were bought from VWR and Fischer Scientific.

The reagents DPPH (PubChem CID: 2735032), gallic acid (PubChem CID: 370) (97,5–102,5%; titration), AIBN (PubChem CID: 6547) (solution 0.2 M in toluene) and sodium carbonate (PubChem CID: 10340) (≥99%) were purchased from Sigma-Aldrich. Folin Ciocalteu reagent solution was purchased from Merck, trolox (PubChem CID: 40634) (97%) from Acros Organic.

2.2. Preparation of the samples

Stock solutions (2 mM) of each antioxidant were prepared in propylene carbonate (PC). Standard solutions were prepared by adding a known volume of each stock solution to a glass ampoule filled with propylene carbonate (2 mL) to a final concentration ranging from 25 to 150 μM. Before analysis, 80 μL of AIBN (0.2 M in toluene) was added into the ampoules to reach a final concentration of 8 mM.

Extra virgin olive oil samples were extracted with methanol/water (80:20). Each extract was dissolved in PC to a final concentration of 11, 22, 33, 44 and 66 μg/mL. 2 mL of these solutions (PC plus extra virgin olive oil extract) were placed in glass ampoules and finally mixed with 80 μL of AIBN (8 mmol/L, final concentration). The samples were analyzed calorimetrically for 24 h.

2.3. Isothermal calorimetry (IC)

A microcalorimeter (Thermal Activity Monitor, Model 421 TAM III, TA Instruments, Sollentuna, Sweden), equipped with 24 channels for 4 mL glass vials, was used to measure the heat rate. The oil in the thermostat was maintained at a constant temperature of 50, 60 or 70 °C, with an absolute accuracy of ± 0.0001 °C. Each channel of the instrument is a twin calorimeter where the two units are positioned above each other. The microcalorimeters are equipped with built-in metal reference specimens having a heat capacity approximately equal to that of a vial. IC runs were typically performed with 2 mL samples in 4-mL glass vials sealed with silicone septa. The heat rate was measured continuously over time. Following the manufacturer's instructions, a Joule effect calibration was applied to each channel prior to measurement: an electric impulse released a heat flux of 3 mW for 30 s yielding a total heat of 90 mJ.

A glass ampoule was prepared by adding a fresh PC solution of radical scavenger of a given concentration. After addition of the radical initiator (AIBN), the ampoule was quickly sealed and settled into the calorimetric space. After 15 min thermal equilibration in an upper location, the ampoule was pushed down and fixed in the measurement position.

2.4. Direct injection mass spectrometry (DIMS)

The direct injection mass spectrometry analysis of BHT was performed with a HPLC (1260 Infinity, Agilent Technology) equipped with auto-sampler, solvent degasser and auto-sampler (1290 Infinity, Agilent Technology). The LC system was combined with a quadruple precursor ion selection with an HR-AM Orbitrap instrument (Q-Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer, Thermo Scientific). The samples were injected with a loop of 5 μL volume at a constant 100 μL min^{-1} flow rate. The solvent was 0.1% ammonium acetate in acetonitrile/water 95:5. The H-ESI II ionization source was operated in negative ion mode. The optimized tune file parameters are briefly listed here: Full MS scan range 65–500 *m/z*, spray voltage = −3.5 kV, set resolution 70,000 FWHM at 200 *m/z*, AGC target = $1 \cdot 10^6$, maximum inject time 300 ms, sheath gas flow rate = 20u, auxiliary gas flow rate = 5u, auxiliary gas temperature = 150 °C, transfer line capillary temperature = 320 °C, S-lens RF value = 50u. For the MS2 experiments, the set parameters were: R = 17,500 FWHM, AGC target = $1 \cdot 10^5$, max. inj. time 75 ms, isolation window 4 *m/z*, isolation offset 1 *m/z*. Data were post-processed with Xcalibur (Thermo Scientific) software.

2.5. DPPH assay

The DPPH method was used according to Brand Williams [18] with minor modifications: briefly, 1.9 mL of a stock solution of 2,2-diphenyl-L-picrylhydrazyl (DPPH, 10 mg in 250 mL of ethanol) was mixed with 0.1 mL of the sample (solution containing radical scavenger). The absorbance, *A*, at 517 nm was measured with a spectrophotometer (Cary 100 UV-VIS, Agilent, Italy). The result were expressed as inhibition of DPPH (%):

$$\%\ \ Inhibition\ \ of\ DPPH \left[\frac{A_{control} - A_{sample}}{A_{sample}}\right] x100 \quad (1)$$

where $A_{control}$ and A_{sample} stand for absorbance before and 60 min after the sample addition (at room temperature), respectively. The parameter for the evaluation of DPPH method is the IC_{50} value (Inhibition concentration at 50%), which indicates the concentration of antioxidant that causes 50% loss of the DPPH activity. The analyses were performed in triplicate.

2.6. Determination of total phenolic content

The total content of phenolic compounds in olive oil extracts was determined with the Folin Ciocalteu reagent adapted from the colorimetric method described by Singleton and Rossi [19]. This method allows a colorimetric reaction of phenolic compounds with the Folin Ciocalteu reagent which can be measured in the visible light spectrum [20]. The Folin Ciocalteu assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 765 nm [21]. Gallic acid solutions were used for the calibration curve. Several dilutions were made to remain in the linear range of the spectrophotometer. Cuvettes were filled with 1.2 mL distilled water and 40 μL of sample or gallic acid solution. 300 μL of 20% sodium carbonate solution and 100 μL of Folin Ciocalteau reagent were added simultaneously and the cuvettes incubated for 1 h in the dark. The absorption was measured with a

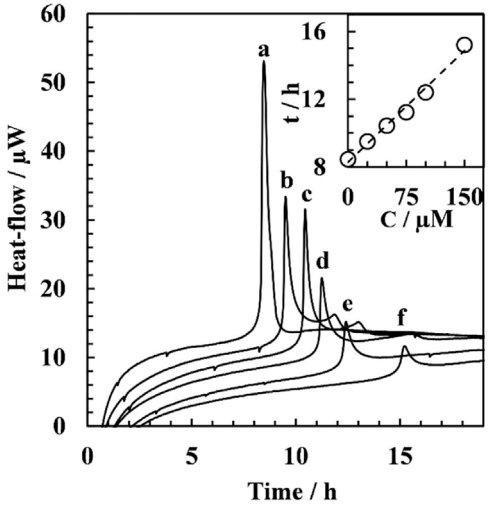

Fig. 1. Calorimetric signal for the reaction between AIBN (8 mM) and increasing concentrations of BHT: (a) 0 µM; (b) 25 µM; (c) 50 µM; (d) 75 µM; (e) 100 µM and (f) 150 µM. All experiments were performed at 50 °C in propylene carbonate. Inset: AIBN peak time as a function of increasing concentration of BHT.

spectrophotometer at 765 nm. The total phenolic content was expressed as milligram gallic acid equivalent per mL of extract (mg GAE/mL extract).

3. Results and discussion

3.1. Effect of BHT on the thermal degradation of AIBN

Fig. 1 shows the calorimetric signal of AIBN (8 mM) when mixed with increasing concentrations of BHT (0–150 µM). At 50 °C, in the absence of BHT, an exothermic peak appeared after about 8.55 h (induction period ~7.50 h), with a peak width of ~2 h and maximum intensity of 53.1 µW (curve *a*). When BHT (25 µM) was added, the peak shifted about 1 h (curve *b*). Both the time delay and the area beneath the AIBN peak (namely, the overall enthalpy of the process) were linearly correlated with the concentration of BHT (see inset of Fig. 1, $R^2 = 0.99$). The peak area, that was initially 3.6 kJ mol^{-1} for AIBN alone, decreased with increasing concentration of BHT. Accordingly, either the peak time, or the peak area can be conveniently used to express the radical scavenging activity of an antioxidant like BHT.

This behavior is expected because BHT quenches the early formation of radicals from AIBN, suppressing any further exothermic reaction. The same type of mechanism was previously observed in the ORAC assay, where the reactivity of AAPH, a water analogue of AIBN, in the presence of antioxidants such as BHT, is suppressed [22]. The time delay of the AIBN peak could be expressed either with an induction time (the initial slow phase of the reaction which later accelerates) or with the peak time [23]. In this work we expressed the delay time as a function of the peak time. As will become clear in later experiments, at higher temperatures or at higher concentrations of AIBN, the degradation of AIBN is very fast and the induction time cannot be easily measured. Accordingly, for coherence, the "peak time" was used.

3.2. Mechanism of AIBN degradation

The degradation of AIBN under isothermal elevated temperature implies a very complex mechanism, which is influenced by several parameters. One of the most important is the presence of oxygen. When oxygen is removed from the headspace of the ampoule (i.e. by purging with nitrogen), the resulting IC signal shows a flat trace, corresponding to a negligible heat-flow (~4 µW), without the presence of the previously observed peak. Accordingly, the presence of oxygen in the headspace of the ampoule is essential for the degradation of AIBN.

A second important parameter affecting the degradation process of AIBN is its initial concentration. Fig. 2-**A** shows that, at low AIBN concentration (2 mM), the heat rate is approximately zero and no peak was observed. An unreactive blank sample (i.e. only PC in the ampoule) shows a similar, but endothermic reaction. The subtraction of the two signals (AIBN and the blank sample) reveal the exothermic nature of the overall reaction. However, experiments dealing with higher AIBN concentrations reveal the complex nature of the thermal degradation process. At a concentrations of 5 mM, the IC trace showed a single sharp exothermic peak; for concentrations larger than 5 mM, additional peaks or shoulders appeared. This evidence is in line with the expectation of a series of consecutive reactions that govern the degradation process of AIBN, as described below (stoichiometric coefficients were omitted for simplicity) [24]:

$$AIBN \xrightarrow{T} R^* + N_2 \xrightarrow{O_2} ROO^* \xrightarrow{disp.} RO^* \xrightarrow{O_2} products \quad (2)$$

Briefly, AIBN undergoes homolysis under the influence of heating, leading to carbon centered radicals (R*) and nitrogen (N_2). R* may react with oxygen to form peroxy radicals (ROO*). When no antioxidant is available or when AIBN is used in molar excess over antioxidants, ROO* undergoes disproportionation, generating alkoxy radicals (RO*). Such species are highly reactive and may further decompose with the generation of heat [24].

The shape of the observed curves, including the peak, suggests the presence of an autocatalytic reaction following an induction period. This has often been seen in other oxidations of organic materials [25].

When AIBN is dissolved in propylene carbonate at 5 mM, the resulting calorimetric signal is as reported in Fig. 2-**B** (line *a*). As before,

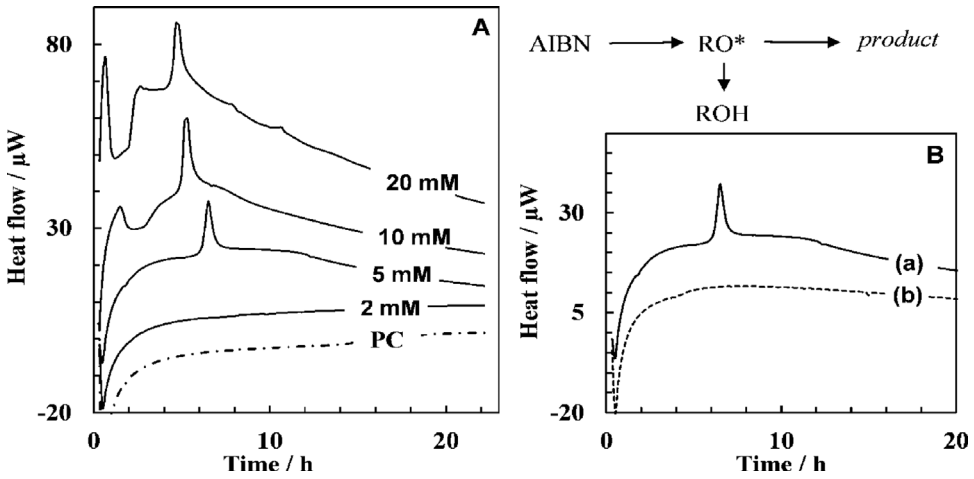

Fig. 2. Calorimetric trace of AIBN in PC (A) at different concentrations (from 2 to 20 mM). (B) Calorimetric trace of AIBN (5 mM) in the (a) absence and (b) presence of methanol (10%). Experiments performed at 55 °C.

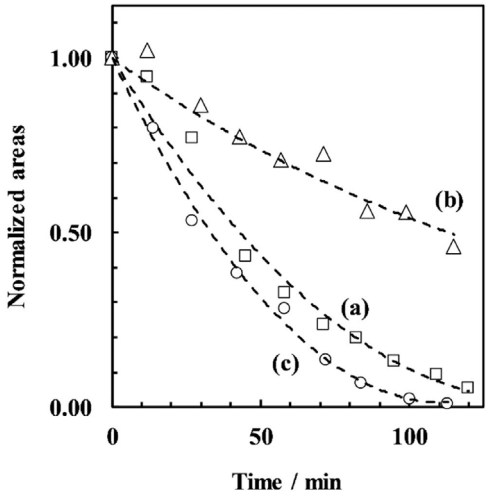

Fig. 3. DIMS analysis of BHT degradation. A) Degradation of BHT in PC, B) degradation of BHT in PC with 10% MeOH, C) degradation of BHT in acetonitrile. $[BHT]_{initial}$ = 0.5 mM, $[AIBN]_{initial}$ = 20 mM, 70 °C. Data expressed as normalized area (over the initial value) of 219.175 *m/z*. Calibration for BHT was performed with the same experimental and instrumental conditions.

the signal is typical of an overall exothermic reaction with a peak occurring at about 6.5 h. However, when AIBN is mixed with methanol (10%), the peak disappeared completely (Fig. 2-**B**, line *b*). This finding is similar to that reported by others, and is explained by considering that even small amounts of methanol (1%) can quench the alkoxy radicals (RO*) [26]. In our case, the presence of methanol completely suppresses the exothermic peak. As a consequence, this means the origin of the peak is due to the subsequent degradation of RO* and not to its formation or other earlier reactions.

The effect of methanol on the consumption of BHT was monitored by direct injection mass spectrometry (DIMS). Fig. 3 shows the evolution of normalized extracted ion chromatogram (XIC) areas for 219.175 *m/z* as function of time, performed either in PC (a), in acetonitrile (b), or in PC + 10% methanol (c). For AIBN solutions in PC or acetonitrile, the nearly complete depletion of BHT occurred within two-hours at 70 °C. In the presence of 10% methanol, the rate of BHT consumption halved. Methanol apparently decreased the activity of AIBN free radicals by quenching RO*. This results in a smaller radical scavenging activity for BHT [27].

3.3. Comparison of the radical scavenging activity of different radical scavengers

The calorimetric assay was used to compare the radical scavenging activity of different antioxidants. Fig. 4 shows the effect of increasing concentrations (0–150 µM) of (A) retinyl acetate, (B) BHT, (C) tocopherol and (D) ethoxyquin on the resulting AIBN peak at 60 °C. The selected antioxidants show very different behavior. Even small amounts of ethoxyquin (25 µM), a powerful radical scavenger used in feed manufacturing, produced more-than 1.5 h of delay in the AIBN peak. Tocopherol and BHT (25 µM), two well-known food radical scavengers, showed similar efficacy, namely, 0.67 h and 1.00 h delay, respectively. Finally, retinyl acetate, a vitamin with weak radical scavenging activity, produced a delay of about 0.67 h, but only at a concentration of 100 µM. These findings reflect previous reports that compared the radical scavenging activity of the above antioxidants with voltammetry investigations [28].

3.4. Radical scavenging activity index

The radical scavenging activity of the samples was summarized with an index originally proposed by Antolovich, *Aox*. This index defines the extent of the radical scavenging activity with reference to the delay of the AIBN peak [29]. For a fixed set of conditions (i.e. T = 50 °C, AIBN 8 mM, in propylene carbonate), Eq. (3) defines the *Aox* index as:

$$Aox = \frac{t - t_{REF}}{t_{REF}} \cdot \frac{1}{[AH]} \quad (3)$$

where t_{REF} and t stand for the peak time of AIBN, respectively, before and after the addition of the sample; [AH] is the concentration of the sample. The *Aox* index is zero if $t = t_{REF}$ (i.e. negligible radical scavenging activity) and becomes larger for $t > t_{REF}$ (i.e. higher radical scavenging activity).

Applying this index to the results of the present work, ethoxyquin showed the highest activity ($Aox \sim 29.10^3\ M^{-1}$), followed by tocopherol ($Aox \sim 7.10^3\ M^{-1}$), BHT ($Aox \sim 5.10^3\ M^{-1}$) and retinyl acetate ($Aox \sim 1.10^3\ M^{-1}$). By plotting the *Aox* index *vs* 1/[AH], a straight-line with zero or negligible slope was achieved for all the antioxidants. The same ranking was obtained with the DPPH assay. Again, the IC_{50} values indicate that EQ is the most powerful radical scavenger and that the scavenging effect decreases in the following order EQ (35 +/− 2) > TOCO (70 +/− 3) > BHT (160 +/− 2) > RA (186 +/− 5).

3.5. Analytical performance of the radical scavenging index

The precision of the proposed protocol was estimated by ten independent measurements (in different ampoules) at 50 °C. Briefly, each measurement consisted of dissolving 80 µL of AIBN solution in PC in an ampoule containing 2 mL of PC, resulting in 8 mM AIBN as final concentration. The maximum of the AIBN peak occurred at 8.50 ± 0.06 h, after the insertion of the ampoules into the sample holder of the calorimeter, resulting in a precision of the heat flow peak of about ± 1%.

The peak time of AIBN and the concentration of each bioactive (0–150 µM range) was linearly related with a R^2 ranging from 0.994 to 0.999 and sensitivity varying with the kind of radical scavenging, from a minimum of 11.4 h/mM for retinyl acetate to 252 h/mM for ethoxyquin.

3.6. Application to natural extracts

The calorimetric assay was applied to extra-virgin olive oil extracts (EVOO1; EVOO2; EVOO3; EVOO4 and EVOO5). Fig. 5 shows the calorimetric curves obtained for different concentrations of the samples, obtained by dilution with PC to reduce the radical scavenging capacity. The inset in Fig. 5 reports the *Aox* index, as defined in Eq. (3), for these five samples at different dilution ratios. Finally, Fig. 6 shows the correlation between the *Aox* indices of the extra-virgin olive oil extracts and their total phenol content as measured by the Folin Ciocalteu method. The relationship is linear (R^2 = 0.975), providing evidence that the radical scavenging activity of the samples is strictly correlated with their polyphenol content.

4. Conclusion

Isothermal calorimetry was used to estimate the radical scavenging activity of synthetic, as well as natural lipophilic antioxidants through the reaction with AIBN. A potential advantage of this system is that the process can be monitored in real time and applied directly to lipophilic compounds. The simplicity of the proposed method allows implementation in a range of commercially available calorimetric instruments based on heat conduction and power compensation. The typical drawbacks of the spectrophotometric AIBN assay were overcome. The calorimetric results of individual antioxidants were positively correlated with those obtained by the DPPH assay, and the results with extra virgin olive oil samples were positively correlated with the total phenol content.

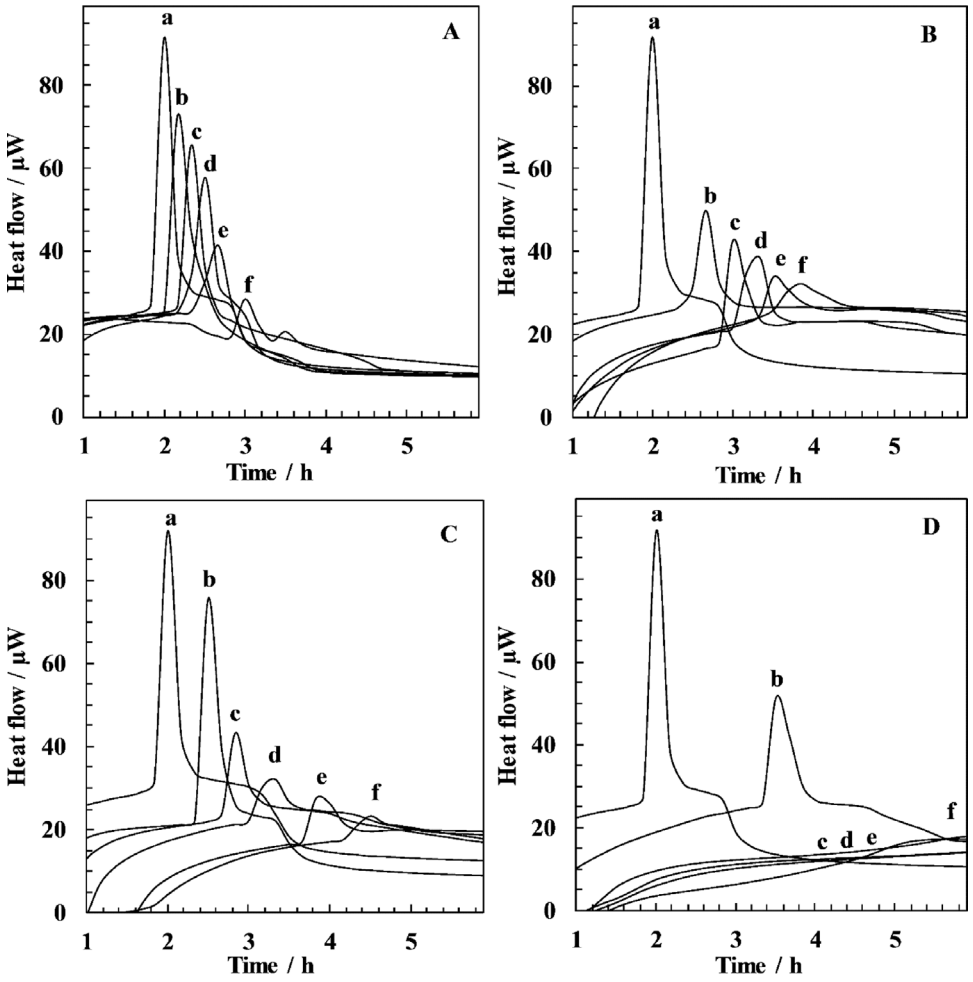

Fig. 4. Isothermal (T = 60 °C) calorimetric signal for the reaction between AIBN (8 mM) in PC and increasing concentrations of (A) retinyl acetate, (B) BHT, (C) tocopherol and (D) ethoxyquin: (a) 0 µM; (b) 25 µM; (c) 50 µM; (d) 75 µM; (e) 100 µM and (f) 150 µM.

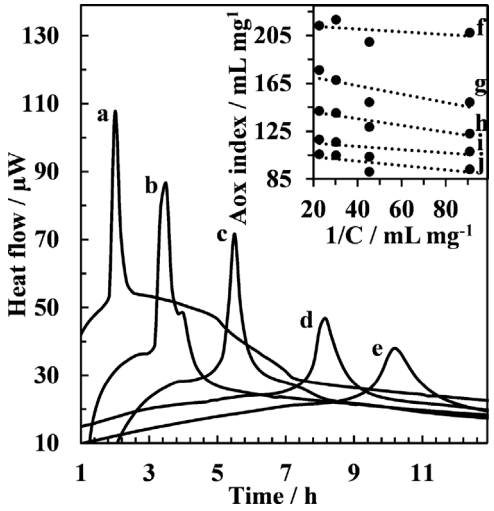

Fig. 5. Calorimetric signal for the reaction between AIBN (8 mM), PC and increasing concentrations of an extra virgin olive oil sample (EVOO5): (a) 0 µg mL^{-1}; (b) 11 µg mL^{-1}; (c) 22 µg mL^{-1}; (d) 33 µg mL^{-1} and (e) 44 µg mL^{-1}. All experiments were performed at 60 °C. Inset: *Aox* index applied on five extracts at increasing concentrations. Letters from (f) to (j) refer to the extracts obtained from different extra virgin olive oil samples.

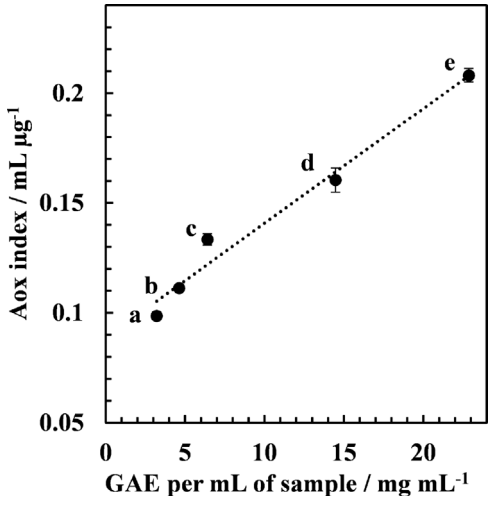

Fig. 6. Correlation plot between the *Aox* indices applied to five sample extracts and the total phenol content measured with the Folin Ciocalteu reagent expressed as gallic acid equivalent (GAE).

Conflict of interest

The authors declare no conflict of interests.

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References

[1] B. Halliwell, Antioxidant defence mechanisms: from the beginning to the end (of the beginning), Free Radic. Res. 31 (1999) 261–272, http://dx.doi.org/10.1080/10715769900300841.
[2] F. Shahidi, Antioxidants in food and food antioxidants, Nahrung/Food 44 (2000) 158–163.
[3] H.J. Forman, K.J.A. Davies, F. Ursini, How do nutritional antioxidants really work: {Nucleophilic} tone and para-hormesis versus free radical scavenging in vivo, Free Radic. Biol. Med. 66 (2014) 24–35, http://dx.doi.org/10.1016/j.freeradbiomed.2013.05.045.
[4] C.S.-M. Gonzalez, Methods used to evaluate the free radical scavenging activity in foods and biological systems, Food Sci. Technol. Int. 8 (2003) 121–137.
[5] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved {ABTS} radical cation decolorization assay, Free Radic Biol. Med. 26 (1999) 1231–1237, http://dx.doi.org/10.1016/S0891-5849(98)00315-3.
[6] K. Bentayeb, P. Vera, C. Rubio, C. Nerín, The additive properties of oxygen radical absorbance capacity (ORAC) assay: the case of essential oils, Food Chem. 148 (2014) 204–208, http://dx.doi.org/10.1016/j.foodchem.2013.10.037.
[7] M. Iwatsuki, J. Tsuchiya, E. Komuro, Y. Yamamoto, E. Niki, Effects of solvents and media on the antioxidant activity of α-tocopherol, Biochim. Biophys. Acta – Gen. Subj. 1200 (1994) 19–26, http://dx.doi.org/10.1016/0304-4165(94)90022-1.
[8] Y.Y. Thoo, S.K. Ho, J.Y. Liang, C.W. Ho, C.P. Tan, Effects of binary solvent extraction system, extraction time and extraction temperature on phenolic antioxidants and antioxidant capacity from mengkudu ({Morinda} citrifolia), Food Chem. 120 (2010) 290–295, http://dx.doi.org/10.1016/j.foodchem.2009.09.064.
[9] G.R. Buettner, B.A. Jurkiewicz, Ascorbate free radical as a marker of oxidative stress: an EPR study, Free Radic. Biol. Med. 14 (1993) 49–55, http://dx.doi.org/10.1016/0891-5849(93)90508-R.
[10] A. Corma, P. Esteve, A. Martínez, Solvent effects during the oxidation of olefins and alcohols with hydrogen peroxide on Ti-beta catalyst: the influence of the hydrophilicity–hydrophobicity of the zeolite, J. Catal. 161 (1996) 11–19, http://dx.doi.org/10.1006/jcat.1996.0157.
[11] A. Arnous, C. Petrakis, D.P. Makris, P. Kefalas, A peroxyoxalate chemiluminescence-based assay for the evaluation of hydrogen peroxide scavenging activity employing 9,10-diphenylanthracene as the fluorophore, J. Pharmacol. Toxicol. Methods 48 (2002) 171–177, http://dx.doi.org/10.1016/s1056-8719(03)00055-8.
[12] K. Miyauchi, T. Urakami, H. Abeta, H. Shi, N. Noguchi, E. Niki, Action of pyrroloquinolinequinol As an antioxidant against lipid peroxidation in solution, Antioxid. Redox Signal. 1 (1999) 547–554, http://dx.doi.org/10.1089/ars.1999.1.4-547.
[13] K.M. Alsante, L. Martin, S.W. Baertschi, Pharmaceutical technology, a stress test, Benchmarking Study 27 (2) (2003) 60–73.
[14] E. Moukhina, Thermal decomposition of AIBN part C: SADT calculation of AIBN based on DSC experiments, Thermochim. Acta 621 (2015) 25–35, http://dx.doi.org/10.1016/j.tca.2015.06.012.
[15] R. Amorati, J. Zotova, A. Baschieri, L. Valgimigli, Antioxidant activity of magnolol and honokiol: kinetic and mechanistic investigations of their reaction with peroxyl radicals, J. Org. Chem. 80 (2015) 10651–10659, http://dx.doi.org/10.1021/acs.joc.5b01772.
[16] L. Liu, Z. Wang, X. Fu, C.-H. Yan, Azobisisobutyronitrile initiated aerobic oxidative transformation of amines: coupling of primary amines and cyanation of tertiary amines, Org. Lett. 14 (2012) 5692–5695, http://dx.doi.org/10.1021/ol302708r.
[17] J.E. Ladbury, M.L. Doyle (Eds.), Biocalorimetry 2: Applications of Calorimetry in the Biological Sciences, Wiley, Chichester; Hoboken, NJ, 2004.
[18] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, LWT – Food Sci. Technol. 28 (1995) 25–30, http://dx.doi.org/10.1016/S0023-6438(95)80008-5.
[19] V. Lounds Singleton, Joseph A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, Am. J. Enol. Vitic. 16 (1965) 144–158.
[20] L.M. Magalhães, M.A. Segundo, S. Reis, J.L.F.C. Lima, A.O.S.S. Rangel, Automatic method for the determination of folin-ciocalteu reducing capacity in food products, J. Agric. Food Chem. 54 (2006) 5241–5246, http://dx.doi.org/10.1021/jf060324s.
[21] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventos, Analisys of total phenols and other oxidation sobstrates and antioxidants by means of Folin Ciocalteau reagent, Methods Enzymol. 299 (1999) 152–178 http://www.sciencedirect.com/science/article/pii/S0076687999990171.
[22] D. Huang, B. Ou, R.L. Prior, The {Chemistry} behind {Antioxidant} {Capacity} {Assays}, J. Agric. Food Chem. 53 (2005) 1841–1856, http://dx.doi.org/10.1021/jf030723c.
[23] L.D. Hansen, D.J. Eatough, E.A. Lewis, R.G. Bergstrom, D. Degraft-Johanson, K. Cassidy-Thompson, Shelf-like prediction from induction period calorimetric measurements on materials undergoing autocatalytic decomposition, Can. J. Chem. 68 (1990) 2111–2114.
[24] S.W. Baertschi, K.M. Alsante, R.A. Reed, Pharmaceutical Stress Testing: Predicting Drug Degradation, CRC Press, 2011.
[25] L.D. Hansen, E.A. Lewis, D.J. Eatough, R.G. Bergstrom, D. DeGraft-Johnson, Kinetics of drug decomposition by heat conduction calorimetry, Pharm. Res. 6 (1989) 20–27.
[26] E.D. Nelson, G.M. Thompson, Y. Yao, H.M. Flanagan, P.A. Harmon, Solvent effects on the AIBN forced degradation of cumene: implications for forced degradation practices, J. Pharm. Sci. 98 (2009) 959–969, http://dx.doi.org/10.1002/jps.21489.
[27] M.A. Watkins, S. Pitzenberger, P.A. Harmon, Direct evidence of 2-cyano-2-propoxy radical activity during AIBN-based oxidative stress testing in acetonitrile-water solvent systems, J. Pharm. Sci. 102 (2013) 1554–1568, http://dx.doi.org/10.1002/jps.23500.
[28] S.M. Lemma, M. Scampicchio, A. Bulbarello, M. Mason, L. Schweikert, Concerted determination of the hydrogen atom and electron transfer capacity of lipid soluble reducing agents, Electroanalysis 26 (2014) 1582–1587, http://dx.doi.org/10.1002/elan.201400096.
[29] M. Antolovich, P.D. Prenzler, E. Patsalides, S. McDonald, K. Robards, Methods for testing antioxidant activity, Analyst 127 (2002) 183–198, http://dx.doi.org/10.1002/1521-3803(20000501)44:3<158:AID-FOOD158>3.0.CO;2-L.