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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