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LABORATORY EXPERIMENT

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Studies on the Food Additive Propyl Gallate: Synthesis, Structural Characterization, and Evaluation of the Antioxidant Activity

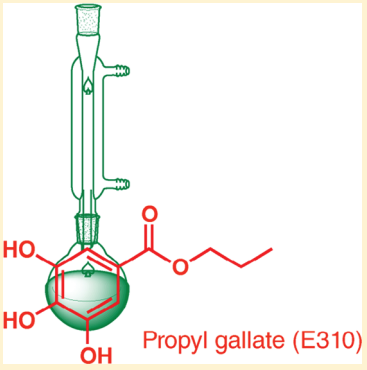
Jorge Garrido,[†] E. Manuela Garrido,[†] and Fernanda Borges*[,‡]

[†]Departamento de Engenharia Química, Instituto Superior de Engenharia do Porto, 4200-072 Porto, Portugal

[‡]CIQUP, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, 4169-007 Porto, Portugal

Supporting Information

ABSTRACT: Antioxidants are additives largely used in industry for delaying, retarding, or preventing the development of oxidative deterioration. Propyl gallate (E310) is a phenolic antioxidant extensively used in the food, cosmetics, and pharmaceutical industries. A series of lab experiments have been developed to teach students about the importance and significance of antioxidants in industry. In the first laboratory, the antioxidant propyl gallate is obtained and the structure identified. Students become acquainted with laboratory techniques such as extraction, crystallization, and thin-layer chromatography. In the second laboratory, spectroscopic data (IR, ^{1}H and ^{13}C NMR) is acquired and interpreted. Students become familiar with the basic concepts of organic compound identification. In the third laboratory, the antioxidant activity of the synthesized additive and gallic acid is evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay using trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as standard. Concepts such as free radical chemistry, preparation of analytical samples, calibration methods, and UV−vis spectrophotometry, are reviewed. This series of experiments can also be used to explore the effect of substituents on radical stability because structurally related compounds were found to have qualitatively different antioxidant profiles.



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Food additives have been used since ancient times to preserve or increase the quality of food products.[1] A food additive is defined as "any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods".[2] Additives play a key role in maintaining the food quality and characteristics that consumers demand, keeping food safe, wholesome, and appealing from farm to fork. Food additives are divided into 25 groups and include flavorings, colorings, stabilizers (gelling and thickening agents), aroma and taste enhancers, sweeteners, and a wider range of preservatives and antioxidants. Food additives are carefully regulated and the general criteria for their use is that they must implement a useful purpose, must be safe, and do not mislead the consumer. To regulate the additives and inform consumers, each compound is named and assigned with a unique number. Initially, these were the "E numbers" used in Europe for all approved additives. This numbering scheme has now been adopted and extended by the Codex Alimentarius Commission to internationally identify all additives.

ANTIOXIDANTS

Oxidation−reduction reactions (redox reactions) are complementary chemical processes that involve loss of electrons (oxidation) by one reactant and a corresponding gain of electrons (reduction) by another reactant. Although oxidation reactions are crucial for life, they can also be damaging. Oxidation reactions happen when chemicals in the food are exposed to oxygen in the air. In natural conditions, animal and plant tissues contain their own endogenous antioxidants, but in foods, these natural systems break down and oxidation is bound to follow. Oxidation of food is a destructive process, causing loss of nutritional value and changes in chemical composition. Oxidation of fats and oils leads to rancidity and in fruits such as apples it can result in the formation of compounds that discolor the fruit. Fats and oils, or foods containing them, are the most likely to have problems with oxidation. Fats react with oxygen and even if a food has a low fat content it may still need the addition of an antioxidant.

Lipid oxidation processes can produce free radicals that are able to start radical chain reactions. The homolytic bond cleavage can be initiated by the action of external radical initiators such as heat, light, singlet oxygen, ionizing radiation, or by transition-metal catalysis involving copper, iron, or manganese ions. Airtight packaging, using inert gases such as nitrogen, vacuum packing, and refrigeration can be used to delay the oxidation process. However, these processes can still be inefficient and

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adding antioxidants can be an effective way of extending the shelf life of a product.

Antioxidants are additives (classified from E300 to E385) defined as any substance, when present in low concentrations, that is capable of delaying, retarding, or preventing the development of deterioration due to oxidation. Antioxidants can inhibit or retard oxidation via two different mechanisms: by directly scavenging free radicals (primary antioxidant) or by a process that does not involve the direct scavenging of free radicals (secondary antioxidant).

Lipid oxidation reactions usually proceed by a three-stage free radical mechanism that includes initiation, propagation, and termination steps. In this process, primary radical-scavenging antioxidants (AH) can delay or inhibit the initiation step by neutralizing a lipid radical, $L^{\bullet}$ (eq 1), or inhibit the propagation step by reacting with a peroxyl radical, $LOO^{\bullet}$ (eq 2), or alkoxyl radical, $LO^{\bullet}$ (eq 3):

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet} \quad (1)$$

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet} \quad (2)$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet} \quad (3)$$

Termination reactions, in which free radicals combine to form molecules that do not feed the propagating reactions, will stop the self-propagating chain. Different products formed between the antioxidant free radical ($A^{\bullet}$) and lipidic radicals can also be obtained in the process:

$$A^{\bullet} + LOO^{\bullet} \rightarrow LOOA \quad (4)$$

$$A^{\bullet} + LO^{\bullet} \rightarrow LOA \quad (5)$$

If there is an increase in the A−H and L−H bond dissociation energies, the activation energy of the antioxidant reactions increases. Hence, the efficiency of the antioxidant increases with decreasing A−H bond strength. In other words, the weaker A−H bonds yields the more efficient antioxidants. Secondary antioxidants operate by a variety of mechanisms such as binding to transition-metal ions or deactivating singlet oxygen. However, some antioxidants operate by both ways. Synergism between antioxidants has also been noted, and many commercial antioxidant formulations contain several antioxidants.

Antioxidants are widely used in baked foods, cereals, fats, oils, soaps, medicines, and cosmetics. The major antioxidants of organic type are

- Tocopherols (E306−E309), BHA (butylhydroxyanisole, E320) and BHT (butylhydroxytoluene, E321); gallic acid derivatives (E310−E312) are regularly applied to protect edible fats, vegetable oils, and salad dressings from turning rancid.
- Ascorbic acid and derivatives (E300−E304) and citric acid (E330) are usually used to preserve the color of freshly cut fruits and vegetables.

Chemically, gallates are alkyl esters of the 3,4,5-trihydroxybenzoic acid (gallic acid) and differ from each other in their side chains. The variants usually employed are propyl gallate (PG), octyl gallate (OG), and dodecyl gallate (DG). They have been extensively used in the food, cosmetics, and pharmaceutical industries.[3] According to the European Directive on alimentary additives,[4] the maximum allowed dose is 200 mg/kg in fats and oils destined to the professional manufacture of heat-treated alimentary products and in a number of manufactured foods, with the exceptions of dehydrated potatoes (25 mg/kg), chewing gums (400 mg/kg), and dietary supplements (400 mg/kg).

ANTIOXIDANT CAPACITY ASSAYS

The potency of antioxidants under real circumstances can be investigated in an in vitro model system with relatively simple and controlled circumstances. Although in vitro methods provide a useful indication of antioxidant activities, data obtained by these methods are difficult to apply to biological systems. The most widely employed chemical tests have different assessment end points such as the radical scavenging capacity, the uptake of oxygen, the inhibition of induced lipid autoxidation, the reducing power, and the chelation of the transition metals. Unfortunately, none of these assays were regarded as universal.[5−11] Two types of analytical methods are currently used for evaluation of the antioxidant activity: (i) inhibition methods, in which the inhibition of oxidative damage of the target molecule is measured in the presence of antioxidants and (ii) methods based on direct measurement of stable free radicals scavenging by antioxidants present in the sample. One of the most popular and simple spectrophotometric methods to measure the ability of antioxidants to trap free radicals is the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.[5−11] It is a rapid, simple, inexpensive, and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity of foods. It can also be used to quantify antioxidants in complex biological systems for solid or liquid samples.[12]

EXPERIMENT OVERVIEW

A series of lab experiments have been developed to teach students about the importance and significance of antioxidants in industry. In the first laboratory, the antioxidant propyl gallate (E310) is obtained and the structure identified. Students become acquainted with laboratory techniques such as extraction, crystallization, and thin-layer chromatography (TLC). In the second laboratory, spectroscopic data (IR, ^{1}H NMR, and ^{13}C NMR) are acquired and interpreted. Students become familiar with the basic concepts of organic compound identification. In the third laboratory, the antioxidant activity of the synthesized additive and gallic acid is evaluated by DPPH assay using trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as standard.

These experiments were devised for chemistry-based curriculums, namely, chemistry, food chemistry, and chemical engineering courses. All the students had taken general chemistry, organic, and analytical chemistry disciplines. Students performed these experiments individually or in groups of two or three students. Each group of students or an individual student submits a lab report one week after completing the lab work.

Synthesis and Structural Characterization of Propyl Gallate (E310)

Phenolic antioxidants (ArOH) can act as free radical scavengers. The activity depends mainly on different structural features such as O−H bound dissociation energy, resonance delocalization of the phenoxyl radical ($ArO^{\bullet}$), and steric hindrance derived from the presence of bulky substituents in the aromatic ring. The chemical modification of phenolic acid antioxidants, for instance, by esterification with *n*-alkyl alcohols, has been suggested as a

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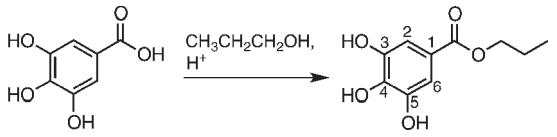
Scheme 1. Synthesis of Propyl Gallate by Fischer Esterification



gallic acid (E300) propyl gallate (E310)

suitable procedure to enhance their hydrophobicity and improve their antioxidant properties.

Esters are a common derivative of carboxylic acids and are widely distributed in both nature and industry. They represent important final products or synthetic intermediates for food, pharmaceutical, and cosmetic industries. A classic procedure to synthesize esters is the Fischer esterification wherein a carboxylic acid is treated with an alcohol in the presence of a mineral inorganic acid catalyst.[13] In this condensation reaction, the equilibrium may be influenced by either removing one product from the reaction mixture or by employing an excess of one reactant. The reaction proceeds by a nucleophilic acyl substitution mechanism. The reaction product is purified by multiple liquid−liquid extraction and crystallization with a mixture of solvents. The product is identified by the melting point and infrared spectra. Nuclear magnetic resonance spectra are also used for further practice in interpreting the data.

Evaluation of the Antioxidant Activity

The DPPH method is used in the quantification of free radical scavenging activity. The process involves a color change from violet to yellow that can be easily monitored at 515−520 nm. The DPPH changes color when the nitrogen atom in DPPH is reduced by a process in which a hydrogen atom from antioxidant compounds plays a part. The DPPH method is simple and only requires a UV−vis spectrophotometer: in the presence of a hydrogen or electron donor (free radical scavenging antioxidant), the absorption intensity decreases, and the radical solution is discolored according to the number of electrons captured. There are different methods of interpreting the results of the DPPH assay.[12] The majority of the studies express the results as the IC_{50} value defined as the quantity of antioxidant necessary to decrease the initial DPPH concentration by 50%. This value is calculated by plotting inhibition percentage against extract concentration. Other indexes express the results as the antioxidant activity power, namely, the antiradical power (ARP), that is defined as $1/EC_{50}$ (where EC is the efficiency concentration; the larger the ARP, the more efficient the antioxidant) or the antioxidant activity index (AAI) calculated as AAI = final DPPH concentration/IC_{50}.

A comparative study of propyl gallate, gallic acid, and trolox (the water-soluble vitamin E analogue) is carried out to evaluate the relative antioxidant activities. This assessment evaluates the influence of the alkyl side chain in the stabilization of the radical formed during oxidation and the influence of hydrophobicity in the improvement of the antioxidant properties.

EXPERIMENTAL PROCEDURE

First Class Period: Synthesis and Purification of Propyl Gallate (E310)

Propyl gallate is synthesized by a Fisher esterification reaction (Scheme 1). The analytical control is performed by TLC using the following system: silica gel, dichloromethane/methanol (8:2). The spots are visualized under UV detection (254 and 366 nm) and iodine vapor. The purification steps include a multiple liquid−liquid extraction and recrystallization.

Second Class Period: Structural Characterization Propyl Gallate

The melting point of propyl gallate is determined to assess its purity. Students obtain infrared spectra using potassium bromide disks and the most significant absorption bands are reported (υ_{max}, cm^{-1}). Students acquire the ^{1}H NMR and ^{13}C NMR spectra at room temperature with dimethylsulfoxide-d_6 as the solvent; the chemical shifts, expressed in δ (ppm) values, and coupling constants (J) are reported.

Third Class Period: DPPH Radical Scavenging Assay

The antioxidant activity of gallic acid (GA), propyl gallate (PG), and trolox are determined using the DPPH method. Solutions of DPPH and antioxidants at different concentrations are prepared in ethanol. After an incubation period at room temperature, in the dark, the absorbance of the solutions at 517 nm is measured using a calibrated UV−vis spectrophotometer. IC_{50}, antiradical power (ARP), and antioxidant activity index (AAI) are calculated and critically discussed.[5,9]

HAZARDS

Protective clothing, goggles, and gloves should be worn. Gallic acid is hazardous in case of eye contact and ingestion and slightly hazardous in the case of skin contact and inhalation. Propyl gallate may induce skin sensitization. DPPH is classified as harmful and a sensitizer. It can cause skin, mucous, and eye irritation. Dichloromethane is harmful if swallowed or inhaled; may be harmful by skin contact. Additional information regarding the potential hazards in handling these chemicals are available in the Supporting Information.

DISCUSSION

The main structural feature responsible for the antioxidant and free radical-scavenging activity of propyl gallate (PG) and gallic acid (GA) is the number and arrangement of phenolic groups in the aromatic nucleus. This type of antioxidant is able to donate the hydrogen atom of the phenolic group to free radicals, thus, stopping or minimizing the propagation radical chain during the oxidation process. The electron-withdrawing property of the carboxylic group has a negative influence on the H-donating ability of the hydroxybenzoic acids. To minimize this drawback, this group is often esterified with fatty alcohols and, in some cases, a positive impact on the resulting antioxidant activity is attained. Moreover, the relative low solubility of phenolic compounds in apolar media, seen as a disadvantage considering their use as antioxidants on organic media, could be overcome by increasing their hydrophobicity via reactions that increase lipophilicity (e.g., esterification).

DPPH assays constitute a widespread and easy-to-use protocol to study antiradical reactivity. Accordingly, the antioxidant activities of PG, GA, and the reference compound trolox are evaluated by their effects of scavenging the stable free radical, 1,1-diphenyl-2-picryl hydrazyl (DPPH). The data obtained show that GA has a lower DPPH radical-scavenging activity than its ester derivative, PG. The different indexes considered for the potency expression of the antioxidant activity, namely, the antiradical power (ARP) and the antioxidant activity index

(AAI), strengthen this trend. According to the AAI index, it is possible to conclude that all the compounds under study exert a noteworthy antioxidant activity. PG emerges as the most effective of the tested compounds and much more efficient than the trolox standard used as reference. This behavior is consistent with the antiradical activity described in literature for related phenolic systems of ester type.[10]

CONCLUSION

In addition to motivating class enthusiasm toward synthetic organic and analytical chemistry, this experiment encompasses educational practical and theoretical concepts. Students become reacquainted with laboratory techniques such as heating under reflux, extraction, evaporation, crystallization, vacuum filtration, and thin-layer chromatography. The experiment allows students to collect and interpret their own spectroscopic data (IR, ^{1}H NMR, and ^{13}C NMR) obtaining the basic concepts of identification of organic compounds. The knowledge gained by analyzing their own data allows students to evaluate their work. Of special interest is the ^{1}H NMR spectra that illustrates proton shielding−deshielding and spin−spin splitting. Purity evaluation was also evaluated by chromatographic and melting point measurements. The evaluation of the antioxidant activity of the propyl gallate is proposed as an opportunity for the students to criticize their own work checking the properties of the synthesized compound and also to review several concepts of the utmost importance in food chemistry.

ASSOCIATED CONTENT

Supporting Information

Instructions for the students; notes for the instructor; spectra of gallic acid and propyl gallate for using in classroom for those without direct student access to the apparatus. This material is available via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: fborges@fc.up.pt.

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