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Synergistic effect of 3,4-dihydroxyphenylglycol with hydroxytyrosol and α -tocopherol on the Rancimat oxidative stability of vegetable oils.

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ABSTRACT

The current work evaluated the ability of pure 3,4-dihydroxyphenylglycol (DHPG) and hydroxytyrosol (HT) in combination with a commercial phenolic extract from *Olea europaea* rich in HT to prevent the oxidation of edible vegetable oils. The commercial phenolic extract was highly soluble in all matrices and significantly improved the oxidation stability and delayed the onset of rancidity of oils compared to a control (with no antioxidant). The combinations of DHPG with other natural antioxidants, such as HT and α -tocopherol, showed that it can be a useful supplement to increase the shelf life of sunflower oil. Synergism was observed when DHPG and HT were added in combination. The synergistic effect was directly related to the concentration ratio between both antioxidants. The mixtures containing DHPG/ α -tocopherol and DHPG/HT/ α -tocopherol also showed effectiveness in delaying oxidation.

Keywords: Antioxidants, 3,4-dihydroxyphenylglycol, hydroxytyrosol, *Olea europaea* extracts, phenolic compounds, synergistic effect.

1. Introduction

Fat oxidation is an important form of food deterioration together with alterations caused by microorganisms and the thermal processing. Food manufacturers attempt to avoid oxidation through different methods, like vacuum-packed or opaque containers, as well as with the use of antioxidants that slow oxidative damage to foods. Most fatty foods contain their own natural antioxidants, although often these are lost during processing. A common example is the loss of tocopherols by evaporation during the industrial refining of vegetable oils to produce edible oils (Kreps, Vrbíková, & Schmidt, 2014), a loss which must be compensated for. Low-cost but highly efficient synthetic or artificial antioxidants have been used in the food industry for many years. However, the global trend is changing towards a reduction in the use of synthetic food antioxidants, which are often subject to important legal restrictions because they may have negative effects on health if they are consumed in excess (Williams, 1993). Special attention is being paid to antioxidant compounds of natural origin whose presence is frequent and abundant in plants. Moreover, the use of natural antioxidants as food additives is increasingly important due to their healthy properties and development as food supplements.

The antioxidant activities of phenolic compounds from *Olea europaea* (fruits, leaves or olive oil) have been well described. In particular, the antioxidant properties of hydroxytyrosol (HT), the major representative phenol in *Olea europaea*, have been extensively studied (Roche, Dufour, Mora & Dangles, 2005; Fernández-Mar, Mateos, García-Parrilla, Puertas & Cantos-Villar, 2012). HT's remarkable antioxidant activity

makes it a highly promising alternative to synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are still commonly used as food preservatives in spite of their confirmed toxicity (Babich, 1982). Notably, HT has been shown to contribute to the stability of olive oil against rancidity caused by oxidation, hence demonstrating its potential utilization as a natural and non-toxic food preservative (Deffieux, Gossart & Quideau, 2014). In this sense, 3,4-dihydroxyphenylglycol (DHPG), another olive phenolic compound, would also be a good candidate as a natural antioxidant. DHPG has been isolated and purified from the by-products of olive oil (Rodríguez, Lama, Trujillo, Espartero & Fernández-Bolaños, 2009) using a highly adapted and patented procedure (Fernández-Bolaños, Guillén, Jiménez, Rodríguez, Rodríguez, & Lama, 2011), which results in the extraction of the compound with a purity exceeding 90% dry weight. DHPG has the same ortho-diphenolic structure as HT but with an additional hydroxyl group in the β position. This compound might be of interest in the field of fatty foods due to its powerful antioxidant properties (Rodríguez, Rodríguez, Fernández-Bolaños, Guillén & Jiménez, 2007).

Following a request from the European Commission, the European Food Safety Authority gave a scientific opinion on a list of nutrition and health claims made on foods pursuant to article 13 of the Regulation (EC) No 1924/2006 in relation to polyphenols in olive (olive fruit, olive mill waste waters or olive oil, *Olea europaea* L. extract and leaf) and their beneficial physiological effects. In April 2011, the Panel on Dietetic Products, Nutrition and Allergies (NDA) concluded that “a cause and effect relationship has been established between the consumption of olive oil polyphenols (standardized by the content of HT and its derivatives) and protection of LDL particles from oxidative damage.” The Panel considers that: “in order to bear the claim, 5 mg of

HT and its derivatives (e.g. oleuropein complex and tyrosol) in olive oil should be consumed daily. These amounts, if provided by moderate amounts of olive oil, can be easily consumed in the context of a balanced diet” (EFSA, 2011). However, the concentrations of HT in some olive oils may be too low to reach this recommended consumption of polyphenols, and other oils, such as soybean and sunflower, contain no polyphenols because they are removed by the refining process. The recommended daily consumption of olive oil (20 g) means that the total polyphenol content of the oil should be 200-250 mg/kg to meet the EFSA requirement. Studies are lacking on the possible supplementation of refined oils with hydroxytyrosol extracts to produce a functional oil based on olive oil to help increase the dietary intake of biologically beneficial hydroxytyrosol. The supplementation of vegetable oils with HT to produce functional oils based on olive oil would increase the dietary intake of HT. However, clinical trials are required to confirm the biological benefits of HT supplementation before such an approach can be endorsed.

It is well known that synergism between certain mixtures of antioxidants can enhance the oxidative stability of edible fats and oils (Han, Yi & Shin, 1991; Jaswir, Che Man & Kitts, 2000). The aim of this work was to add antioxidant phenolic compounds to edible vegetable oils (palm, soybean, and sunflower oils) as well as to olive oil, using combinations of DHPG with other natural antioxidants such as HT and α -tocopherol (DHPG, DHPG/HT, DHPG/ α -tocopherol and DHPG/HT/ α -tocopherol) in order to test whether the combined use of these antioxidants had a synergistic effect on the prevention of oil oxidation.

We also compared the antioxidant activity of one commercially-available antioxidant extract from *Olea europaea* rich in HT, sold under registered trademark “Olivefen”, added to edible vegetable oils to make them more resistant to oxidation and to obtain

functional oils. This extract is already being used as an additive in animal feed. Palm, soybean and sunflower oils were selected for this study because they lead world production and consumption (Gunstone, 2011). A solubility study was performed by adding increasing amounts of Olivefen® to oils. The antioxidant activity of Olivefen® was evaluated by measuring the oxidative stability of the enriched oils compared with the unenriched oils using the Rancimat method. Because the refined olive, palm, soybean and sunflower oils are often used in frying, baking, and other types of cooking, their thermally sensitive constituents, such as tocopherols, must be protected by other antioxidants. In this sense, the use of Rancimat in which high temperatures are used could be useful for determining the antioxidant protection using natural phenols.

2. Materials and methods.

2.1. Materials and chemicals.

Olive, palm, soybean and sunflower oils were purchased from local markets. The antioxidants naturally present in oils were removed by purification through alumina according to the 'free solvent' procedure (Yoshida, Kondo & Kajimoto, 1992). Briefly, 200 g of oil were poured into a glass chromatography column packed with 100 g of alumina activated at 200 °C for 3 h, and the oil was drawn through the column by vacuum suction. Each purified lipid matrix was stored at -18 °C under nitrogen gas until its use. Olivefen® extract was supplied by the company *Subproductos Vegetales del Mediterráneo, S. L. (SVM)* (Seville, Spain) that has exploitation rights of a method for the extraction and purification of HT from by-products of the olive oil extraction process (Fernández-Bolaños et al., 2014). The Olivefen® extract has the appearance and

consistency of syrup and was used at concentrations found in olive oil to avoid any toxicity.

HT and DHPG were isolated and purified with a high degree of purity ($\geq 95\%$) from olive oil by-products (Fernández-Bolaños et al., 2014; Fernández-Bolaños et al., 2011). The chemical structure and purity of HT and DHPG were confirmed by ^1H -NMR and HPLC according to the procedure reported in Rodríguez et al., (2009).

HPLC-grade methanol was purchased from Merck (Darmstadt, Germany) and ultrapure water was obtained using a Milli-Q water system (Millipore, Milford, MA, USA). All other chemicals and solvents were of analytical grade and used as received from commercial sources. Dimethylformamide and hexane were supplied by Romil Ltd. (Waterbeach, Cambridge, UK). Syringic acid and α -tocopherol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Experimental procedure.

Increasing amounts of Olivefen® (from 8 to 80 mg) were added to 50 g of vegetable oil and mixed using a magnetic stirring at ambient temperature (except for palm oil which had to be completely liquefied at 28 °C) for 1 h, with protection from light and in absence of oxygen. The oil mixtures were filtered and any undissolved Olivefen® was washed with hexane and dried. The percentage of Olivefen® in oils was considered as the difference between the initial amount of extract added and the amount of undissolved extract.

2.2.1. Analysis of HT content solubilized in oils.

The concentration of individual phenols in the Olivefen® extract and the concentration of HT dissolved in the enriched oils were measured following the procedure reported by

Romero and Brenes (2012) with some modifications. Briefly, an oil aliquot (0.6 g) containing 20 µg syringic acid as internal standard was extracted three times with 0.6 mL of dimethylformamide (DMF). The DMF extract was then washed twice with 2 mL of hexane and nitrogen was bubbled through the extract for 10 min to remove residual hexane. Finally, the extract was filtered through a 0.22 µm pore size and 20 µL were injected into the HPLC system.

A Hewlett Packard HPLC system (Series 1100) equipped with a diode-array detector was used for the quantification of HT at 280 nm. A Tracer Extrasil ODS-B analytical column (5 µm, 25 x 0.46 cm.i.d., Teknokroma®, Barcelona, Spain) was used. The mobile phase was Milli-Q® water (pH adjusted to 3.0 ± 0.1 with H_3PO_4) and HPLC-grade methanol (solvent B) at 35 °C. The flow rate was maintained at 1 mL/min. Linear gradient conditions for separation were as follows: 10 to 30% B (0 to 10 min); 30% B (10 to 20 min); 30 to 40% B (20 to 30 min); 40% B (30 to 35 min); 40 to 50% B (35 to 40 min); 50% B (40 to 45 min); 50 to 60% B (45 to 50 min); 60 to 70% B (50 to 55 min); 70 to 100% B (55 to 60 min); 100 to 10% B (60 to 65 min); 10% B (65 to 75 min). The limit of detection (LOD) was 0.5 mg/L.

2.2.2. Determination of oxidative stability by the Rancimat method.

The 679 Rancimat instrument (Metrohm Ltd., Herisau, Switzerland) was used for the determination of oxidative stability of the enriched oils. An oil sample (2.50 ± 0.01 g) was weighed into the reaction vessel directly. The temperature of the heating block in the Rancimat instrument was set at 100 °C and air flow through the reaction vessel was adjusted at 10 L/h. In the case of DHPG/HT, DHPG/ α -tocopherol and DHPG/HT/ α -tocopherol mixtures, the samples were measured at 80 °C. The outlet airstream was collected in deionized water (65 mL) in which the conductivity was monitored

continuously at room temperature until induction was reached. The induction time (IT), which characterizes the oxidation stability of the oils, is the time until secondary oxidation products are detected, and was expressed in hours.

The antioxidant activity of DHPG and HT, and the possible synergistic effects of DHPG/HT, DHPG/ α -tocopherol and DHPG/HT/ α -tocopherol mixtures were studied in a lipid matrix without antioxidants. The lipid matrix was obtained from sunflower oil by purification through alumina as described above. Purified sunflower oil containing the compounds in different proportions was prepared by the addition of 0.1 M solutions of DHPG and HT and α -tocopherol in methanol. The samples were stirred for 10 min to mix the antioxidants with the oil, with heating at 50 °C in a vacuum to remove all of the methanol. The final concentration ranges of the compounds varied between 0.125-3 mmol/kg.

2.3. Statistical analysis.

All experiments were carried out in duplicate, and analysis were carried out in triplicate and the results were expressed as mean values $\pm$ standard deviation. The results obtained for solubility of Olivefen® and those obtained for IT values corresponding to the antioxidant effects of DHPG and its mixtures with HT and α -tocopherol were analyzed by analysis of variance (ANOVA) and with the Student's *t*-test, respectively, at a significance level of 0.05. The coefficient of determination (r^2) was calculated from the results for all relationships.

3. Results and discussion

3.1. Composition of Olivefen® extract.

The major component in Olivefen® was phenols, constituting over 70 % of the total extract (w/w), with a HT content of 36.75% (w/w), followed by tyrosol and low concentration of vanillin and vanillic acid (Olivefen®'s chemical composition is shown in the chromatogram at 208 nm, **Figure 1**). The rest of the total phenol must be condensed or polymeric phenolic fractions that don't absorb UV in the HPLC detection method used (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2013).

3.2. Solubility study of Olivefen® in the oils.

Edible vegetable oils naturally contain tocopherols that help to slow oxidation processes caused by light, oxygen and the presence of trace metals. However, in general, tocopherols have little antioxidant power against the high amounts of polyunsaturated fatty acids contained in these oils (Gutiérrez, 2000). Hence, there is growing interest in the enrichment of fats and oils, and other foods, with natural antioxidants as a method for protection against oxidation. Phenolic extracts from olive oil by-products are a good source of natural compounds with antioxidant activity, due to the presence of molecules with phenolic hydroxyl groups in the ortho-position. The solubility of natural antioxidants in aqueous solutions is high, but such a hydrophilic nature might be a constraint for practical applications in lipid media (Trujillo et al., 2006). One of the main prerequisites for the use of a compound, an extract or mixtures of compounds as antioxidant is that it is soluble in oily matrices with a homogenous distribution. The Olivefen® extract is soluble in water and ethanol.

With the aim of studying its behavior in oily systems, Olivefen® was directly added to four vegetable oils and its solubility was studied (**Table 1a**). The solubility of the

extract was very high: 92.7% of Olivefen® was solubilized in soybean oil. Using the olive, palm, and sunflower oils as lipid media, the maximum solubilisation of Olivefen® was 82.3, 88.8, and 81.6%, respectively. At the 95% level of confidence, two factor ANOVA indicated that there were significant differences in the percentages of Olivefen® solubilized in the four oils ($F = 28.74 > 3.86 = F_{crit}$; $p < 0.05$). However, there were no significant differences in the percentages for the four amounts of extract added (p -value > 0.05), suggesting that the extract's solubility limit was not reached. One important technological aspect to consider was that undissolved Olivefen® extract could be separated easily by sedimentation and filtration. On the other hand, the results indicated that HT concentrations obtained in oils (**Table 1b**) were also elevated. Around 90% of HT present in the extract dissolved in the palm, soybean, and sunflower oils. Several authors indicated the problem of reduced solubility in oils of natural antioxidants, and especially of many plant extracts (Škerget, Kotnik, Hadolin, Hraš, Simonič & Knez, 2005). Other authors even proposed the synthesis of more lipophilic antioxidants from their corresponding natural derivatives (Trujillo et al., 2006). However, the results of the solubility of Olivefen® showed that it has an ideal lipophilic character in several lipid media. Moreover, 40 mg of Olivefen® were enough to reach the required HT concentration for the protection of LDL particles from oxidative damage.

3.3. Antioxidant activity measurement of Olivefen®.

The Rancimat method, which measures the activity of a selected antioxidant to slow or delay lipid oxidation in fats and oils, was developed to evaluate the oxidative stability of fats and oils under accelerated conditions such as elevated temperature.

The activity of Olivefen® in preventing lipid oxidation was measured by comparing the induction times (IT) of the oils with or without the extract, with the results reported in hours beyond control (**Table 2**). The effectiveness of Olivefen® against rancidity in bulk oils was attributed to HT due to its major concentration in the extract, but other phenols and components may also be responsible for the activity, such as tyrosol, which is the other major phenolic antioxidant. The IT values for the controls, which contained no added antioxidants, were 30.3 h (olive), 47.7 h (palm), 12.9 h (soybean) and 7.3 h (sunflower). The increases compared to the extract-free control were significant in all cases (p -value <0.05), although the best results were achieved with olive oil, thus indicating that the effect of Olivefen® depends on the lipid matrix. At higher HT concentrations (500 ppm), stabilities over 110 h at 100 °C were obtained, which when compared to the control, resulted in an increase of 271%. The IT increases observed (109%, 84% and 105% for palm, soybean and sunflower oils, respectively) result in a longer conservation period of the oils as well as a delay of the main problem due to oxidation, namely the formation of volatile compounds with unpleasant odors typical of rancidity of fats or oils.

To compare the relative effectiveness of Olivefen® in the different oils, a protective index (PI) was determined by dividing the IT of antioxidant-enriched oil with that of the control, as described by Villanueva, Rodríguez, Aguirre, and Castro (2017). The PI was higher in olive oil than palm and sunflower oils, and lowest in soybean oil. The PI increased significantly in olive and palm oils (confidence intervals at 95% of the values do not overlap), although Olivefen® was more effective in the former. However, in soybean oil, only the higher HT concentration (500 ppm) produced a statistically significant difference in the PI. HT at concentrations of 250 and 500 ppm had the same

effect on the PI in sunflower oil but the PIs were significantly different for 50 and 100 ppm.

The activity of Olivefen® is higher than that reported for olive leaf extract or olive waste cake extract. 1500 ppm of olive leaf extract, rich in oleuropein, increased the olive oil stability up to 46% (Sahin, Sayim, & Bilgin, 2017), a value reached by the extract at lower concentrations (50-100 ppm). 100 and 200 ppm of olive waste cake extract, rich in HT, improved the sunflower oil stability up to 32 and 56% respectively, (Abd-ElGhany, Ammar, & Hegazy, 2010) versus the 60 and 78% using 100 and 250 ppm of Olivefen®.

In the case of olive oil (non-virgin olive oil), the commercial standard for olive oils (IOOC, 2009) only allows for the addition of α -tocopherol as an antioxidant to restore the natural loss during the refining process. However, the legislation of some Latin American countries, where the tradition of consuming other vegetable oils (mainly from seed) is more ingrained, allows the importation of olive oil enriched with phenolic compounds from natural extracts. The European Commission on Regulation (EU) No. 29/2012 establishes that Member States may prohibit the production in their territory of blends of olive oil and other vegetable oils for internal consumption, although they may neither prohibit the marketing in their territory of such blends coming from other countries nor the production in their territory of such blends for marketing in another Member State or for exportation. Therefore, enriched olive oil could be used to protect and functionalize refined vegetable oils for the European market.

3.4. Antioxidant effect of DHPG in bulk oil.

The antioxidant effect of pure DHPG and HT in purified sunflower oil at 80 °C was evaluated at various concentrations (**Figure2A**). The IT value for the control, containing

no antioxidant, was 3.40 ± 0.14 h. The oxidative stability of sunflower oil increased with increasing concentrations of both DHPG and HT in a linear dose-dependent response. However, DHPG was less effective than HT in the tested concentration range from 0.025 to 3 mmol/kg, with statistically significant differences above 0.5mmol/kg. These differences may be attributed to the different behaviour of DHPG and HT at elevated temperatures: DHPG is more thermally sensitive than HT (Rodríguez et al., 2009). To date no studies have determined the antioxidant activity of DHPG in oil. The activity of HT was previously described by the Rancimat method and was reported to have a similar antioxidant activity as HT 100 ppm (0.65 mmol/kg of oil) purified from solid thermally-treated olive oil waste, causing an increment of the olive oil stability of up to 71%, (Fernández-Bolaños, Rodríguez, Rodríguez, Heredia, Guillén, & Jiménez, 2002).

3.4.1. DHPG in combination with other antioxidants.

The use of mixtures of antioxidants with synergistic action is of great significance to improve the oxidative stability of vegetable oils (Velasco, Dobarganes & Márquez-Ruiz, 2010). The key aim of this study was to identify whether synergism occurred between HT, DHPG and α -tocopherol. The latter was selected because it is naturally present in all vegetable oils studied in this work. The antioxidant activity of HT, α -tocopherol and their mixtures have been evaluated by other authors (Mateos, Domínguez, Espartero & Cert, 2003). However, the antioxidant effect of DHPG has never been studied in vegetable oils in isolation or in combination with other antioxidants.

3.4.1.1. DHPG in combination with HT.

The synergistic effects of DHPG and HT on the preservation of sunflower oil were investigated by the Rancimat method at 80 °C (as this would reduce the individual levels needed to achieve the same effect). The antioxidant combination formed by pure DHPG and HT in a 50:50 proportion did not show a synergistic effect in the full range of concentrations studied (**Figure 2B**) since the combined impact of both antioxidants on the IT was equal to the sum of the individual IT values, i.e. the effect was purely additive. The IT values versus concentration followed a linear trend. However, when increasing DHPG concentrations, 0.025-2 mmol/kg (or 4-340 ppm), were combined with a constant concentration of HT (1 mmol/kg or 154 ppm), a clear synergistic effect was observed (**Figure 2C**) and the linear effect was lost. At different proportions of DHPG and HT, the IT was much higher than the sum of the effects of each single antioxidant, highlighting the potential application of this antioxidant combination. The synergistic increases in IT values compared to a hypothetical additive effect were obtained for the mixtures of HT/DHPG at 4:1 and 2:1. However, when the DHPG concentration reached the same proportion as HT (around 1 mmol/kg), the positive effect was inverted. Therefore, the Rancimat stability depends on the concentrations of both phenolic antioxidants and the ratio between them. It is worth highlighting that Olivefen® provided significantly lower oxidative stability compared to the DHPG/HT combination, with a lower amount of the antioxidant combination preserving sunflower oil, thus strengthening the idea that the activity of natural antioxidants is affected by multicomponent foods. Therefore, such combinations would likely be more applicable in oil-based foods than the Olivefen® extract.

3.4.1.2. DHPG in combination with α -tocopherol.

The antioxidant efficiency of DHPG was previously demonstrated in aqueous medium and shown to improve the effect of antioxidants such as ascorbic acid and HT (Rodríguez et al., 2007), specifically in lipid medium, preventing peroxidation to a comparable degree as α -tocopherol despite its high polarity. For this reason, such interaction may be of great interest to protect vegetable oils which naturally containing α -tocopherol.

For concentrations of DHPG <1.5 mmol/kg, the presence of α -tocopherol in increasing concentrations caused a positive effect on purified sunflower oil preservation, although never higher than the additive effect (**Figure 3A**). For concentrations of DHPG around 1.5 mmol/kg, the presence of α -tocopherol did not produce a significant improvement on stability at any concentration. However, when DHPG was combined with α -tocopherol at concentrations >1.5 mmol/kg, the presence of α -tocopherol showed an antagonistic effect. A similar trend was observed by Mateos et al., (2003) who studied the antioxidant effect of mixtures of hydroxytyrosol and α -tocopherol in virgin olive oil, and whose results are concordant with those of the present study, albeit in a different type of oily matrix. In the case of the HT/ α -tocopherol mixture, the antagonistic effect occurred at lower concentrations of HT (0.2 mmol/kg). This could offer an advantage of DHPG versus HT, since DHPG can be added at higher concentrations without experiencing the negative effect on oxidative stability. Other studies also reported a negative effect of α -tocopherol with different antioxidant extracts rich in polyphenols in sunflower oil (Becker, Ntouma & Skibsted, 2007).

3.4.1.3. DHPG in combination with HT and α -tocopherol.

Finally, the ITs were evaluated for the antioxidant combination of DHPG, HT and α -tocopherol (**Figure 3B**). Although no synergistic effects were observed at any

concentration assayed for combinations with α -tocopherol, significant positive effects were seen for a large range of concentrations. However, when the concentrations of phenolic antioxidants were high (2mmol/kg of the 50/50 mixture of DHPG and HT) the addition of α -tocopherol either had no effect (at α -tocopherol concentrations up to 0.5 mmol/kg) or an antagonistic effect (at α -tocopherol concentrations beginning from 0.75 mmol/kg).

4. Conclusions

Several fortified and enriched functional foods contain bioactive compounds extracted from plants. Olivefen®, rich in HT and DHPG extracted from a natural source (by-products of olive oil), could be an important addition to functional foods as an antioxidant in edible vegetable oils (palm, soybean, sunflower, etc.) to prevent their oxidation. In this study, Olivefen® and DHPG were tested for the first time as potential antioxidants added to oils. They possess some of the properties of an ideal natural antioxidants, namely to be effective at low concentrations, be readily available, be easy to handle and easy to use, possess versatility for use in a variety of oily matrices and water-soluble forms, and significantly prolong the shelf life of foodstuffs. Olivefen® showed a high solubility in lipid media and a greatly increased oxidative stability. DHPG also showed potential antioxidant properties, mainly in its synergistic protective effect when added in combination with HT. Synergism was observed in sunflower oil when DHPG was used in combination with HT and was directly related to the concentrations of both DHPG and HT. In addition, the interaction of DHPG with α -tocopherol should be taken into account because it may play a very important role on the preservative effect of DHPG and HT in vegetable oils.

More research is needed in various areas to increase the efficacy and improve the utilization of Olivefen® and DHPG as antioxidants in the food industry, among others: test there are no harmful physiological effects caused by consumption; test their addition has minimal effects on the color, aroma, and flavour of the oils; and optimize the synergistic effect of combining these antioxidants.

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Figure captions

Figure 1. Phenolic profile of the Olivefen® extract at 280 nm. The table lists the composition data and values of the constituents determined in Olivefen® extract (data are means $\pm$ standard deviations of triplicate experiments).

Figure 2. Antioxidant activity of DHPG and HT on the prevention of oxidation in purified sunflower oil heated at 80 °C. **(A)** Induction time (IT) values of sunflower oil with a 50/50 mixture of DHPG and HT and mixtures of both antioxidants in different proportions **(B)** compared with a hypothetical additive effect **(C)**.

Figure 3. **(A)** Induction times determined for combinations of DHPG and α -tocopherol added to bulk sunflower oil compared with single DHPG addition determined by the Rancimat method. **(B)** Induction times determined for combinations of DHPG, HT and α -tocopherol added to bulk sunflower oil compared with addition of a 50/50 mixture of DHPG and HT determined by the Rancimat method.

Table 1. Solubility data of Olivefen® in different vegetable oils (a) and results of dissolved HT from the extract (b).

a)

Oil	Added Olivefen® (mg/100mg of oil)			
	8	20	40	80
Olive	79.6 [*] a	79.2 a	82.3 a	75.2 a
Palm	85.7 b	88.8 bc	85.6 b	87.0 bc
Soybean	87.0 bc	92.7 c	90.7 c	91.6 c
Sunflower	80.2 a	81.6 a	80.4 a	78.6 a

^{*}Percentage of Olivefen® dissolved. Means with the same letter were not significantly different, $p < 0.05$.

b)

Oil	Added Olivefen® (mg/100mg of oil) Percentage of HT dissolved determined by HPLC (ppm of HT dissolved in brackets)			
	8	20	40	80
Olive**	79.4 (47) b	71.8 (106) a	70.9 (208) a	71.4 (420) a
Palm	85.6 (50) c	85.8 (126) c	89.5 (263) cd	85.0 (500) c
Soybean	87.5 (51) c	90.2 (133) cd	90.1 (265) d	87.3 (513) c
Sunflower	92.7 (54) d	90.9 (134) cd	90.3 (265) cd	89.1 (524) cd

** For olive oil, the HT values corresponding to the differences between total HT and that naturally present. Means with the same letter were not significantly different, $p < 0.05$.

Table 2. Increases of the induction time (ΔIT) expressed as percentage in the different oil matrices according to the concentrations of dissolved HT from Olivefen® extract.

Oil	Added HT (ppm)	Induction time (h)	ΔIT (%)	PI [*]
Olive	Control ^{**}	30.3 ± 0.8	-	-
	50	42.2 ± 2.6	39	1.39 a
	100	56.1 ± 2.2	85	1.85 a
	250	75.9 ± 4.7	150	2.50 cd
	500	112.5 ± 1.2	271	3.71 e
Palm	Control	47.7 ± 1.4	-	-
	50	60.4 ± 2.0	27	1.27 a
	100	72.6 ± 0.9	52	1.52 a
	250	86.8 ± 0.4	82	1.82 a
	500	99.8 ± 2.0	109	2.09 c
Soybean	Control	12.9 ± 0.3	-	-
	50	17.9 ± 2.6	39	1.39 a
	100	19.5 ± 1.6	51	1.51 a
	250	21.6 ± 0.4	67	1.67 a
	500	23.8 ± 0.5	84	1.84 b
Sunflower	Control	7.3 ± 0.1	-	-
	50	9.7 ± 0.9	33	1.33 b
	100	11.7 ± 1.3	60	1.60 b
	250	13.0 ± 0.9	78	1.78 bc
	500	15.0 ± 0.3	105	2.05 c

* PI was calculated by dividing the value for the induction time of antioxidant-enriched oil with that of the control. ** Oils which contained no added antioxidant. Means have at least one letter in common are not significantly different, $p < 0.05$.

Figures

Figure 1.

Figure 2.

Figure 3.