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Role of Catechins in the Antioxidant Capacity of an Active Film Containing Green Tea, Green Coffee, and Grapefruit Extracts

M. Colon and C. Nerin*

Department of Analytical Chemistry, Aragon Institute of Engineering Research I3A, CPS-University of Zaragoza, Torres Quevedo Building, María de Luna Street 3, E-50018 Zaragoza, Spain

ABSTRACT: The oxygen radical absorbance capacity (ORAC) method was used to characterize the antioxidant capacity of natural extracts of green tea, green coffee, and grapefruit. These natural extracts were incorporated into a plastic film layer, which was subsequently subjected to a free radical gas stream in order to determine the antioxidant capacity directly in the active film. The green tea extract (GTE) afforded the strongest antioxidant activity. To identify the active compounds in the extract, concentration of the diverse catechins in samples were determined by HPLC-UV analysis. The results showed that the content of catechins in the GTE is around 77% (w/w), the major components being (−)-epigallocatechin gallate, (−)-epicatechin gallate, and (−)-epicatechin. A variation in the concentration profile of catechins was detected during the oxidation process. The chromatographic study demonstrated that (−)-gallocatechin, (−)- epigallocatechin, (+)-catechin, and (−)-catechin gallate exhibited the most radical scavenging.

KEYWORDS: *antioxidant, catechins, film, ORAC, free radicals*

INTRODUCTION

Antioxidants in food were originally defined as "substrates that in small quantities are able to prevent or greatly retard the oxidation of easily oxidizable nutrients such as lipids."[1] Currently, antioxidants are known for their role in preventing oxidative damage to food during the processing and the storage of meals. Consequently, antioxidants can provide more healthy food containing fewer lipid and protein oxidation byproducts.[2] In fact, antioxidants have attracted considerable attention in recent years because of their potential health benefits. They may contribute to reducing the risk of chronic diseases, cancer, and aging-related disorders.[3]

Selecting the best antioxidant method for a particular food is not an easy task. Several available methods for the determination of antioxidant capacity (AOC) in food and dietary supplements have been reported. Depending upon the reactions involved, antioxidants can deactivate radicals by two mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET). The majority of HAT-based assays include oxygen radical absorbance capacity (ORAC) and the total radical-trapping antioxidant parameter (TRAP).[4] The ORAC assay, which is an in vitro test, measures the antioxidant inhibition of peroxyl radical induced oxidations and thus reflects the classical radical chain breaking antioxidant activity by hydrogen atom transfer. In the basic test, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product, which can be quantified by fluorescence. The reaction with peroxyl radicals is followed by loss of fluorescence over time.[5] The ORAC assay is based upon the early work of Glazer[6] and was further completed by Cao et al.[7,8]

Food can be also protected against oxidation by packaging. Antioxidants can be incorporated into packaging materials in order to protect foodstuffs against the oxidation process and to extend the shelf life of packaged food.[9−15] These packaging materials are known as active materials. The evaluation of the antioxidant properties of such materials can be carried out by an updated ORAC assay adapted by Bentayeb et al.[16] to measure the antioxidant capacity of an active film. Nevertheless, in this procedure the antioxidant needs to be dissolved in a solution to be measured. In 2006, Pezo et al.[17,18] developed an analytical procedure to directly measure the radical scavenging properties in the material of the packaging, whether paper, plastic, or some other material. The determination of the antioxidant capacity of commercial active packaging materials is very important for selecting the most appropriate packaging for each foodstuff. The analytical technique referred to is based on the generation of an atmosphere rich in radicals, which passes through the active material carried by an inert gas and arrives at a salicylic acid (SA) solution at a pH below 4.5. The SA reacts with radicals not trapped by the active material, producing 2,3-dihydroxybenzoic acid (2,3-DHB) and 2,5-dihydroxybenzoic acid (2,5-DHB) as well as traces of cathecol. The analysis of the 2,5-DHB, which is the major reaction product in the reaction and highly sensitive to fluorescence detection, and of the remaining SA is performed by high-performance liquid chromatography (HPLC) with a fluorescence detector. Thus, the quantification of scavenged radicals provides a quantitative measurement of antioxidant capacity.

Antioxidants can be classified into two categories depending on their nature: (1) synthetic antioxidants, which are synthesized compounds with phenolic structures such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT),[19] and (2) natural antioxidants, which are commonly found in vegetables, fruits, leaves, oilseeds, cereals, bark and roots, spices and herbs, and other plant materials rich in

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phenolic derivatives.[20] Synthetic compounds are subject to restrictions in the food context because of their carcinogenic activity.[21−23] For instance, BHA and BHT are limited by Codex Alimentarius (FAO/WHO Food Standards, 2005)[24] as well as European regulations (Directive 2006/52/EC, 2006)[25] and the FDA Food Additive Status List (US Food and Drug Administration, 2006).[26] However, the usage level of extracts derived from plants is generally established by the flavor threshold in each type of food and does not depend on the toxicity generated. Extracts from plants are also accepted as antioxidants in the Food Additives list from the EU legislation.[25]

Green tea (*Camellia sinensis*) is the second most popular beverage worldwide[27] and is a natural source of antioxidant compounds, especially polyphenols. The phenolic fraction of tea contains flavonols (myricetin, quercetin, and kampferol), flavanols (catechins), and other products of condensation. The most abundant phenols in tea leaves are (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-catechin gallate, (−)-epicatechin gallate, (−)-gallocatechin, (−)-gallocatechin gallate, and (−)-epigallocatechin gallate. These contribute to the antioxidant capacity and organoleptic properties of green tea.[28−31] Several analytical methodologies have been proposed to analyze phenolic compounds from natural extracts or essential oils. For example, liquid chromatography (LC) is a widely used method with satisfactory results for the separation of tea catechins.[32−39] Recently, the increased interest in the use of tea components in dietary supplement formulations has led to phenolic compounds being analyzed with standard reference materials, with measurements performed by the NIST and by collaborating laboratories.[40] Unfortunately, no reports have been found that demonstrate the antioxidant activity of tea catechins when they are incorporated into a plastic film and are further submitted to an oxidative reaction. For this reason, a study of the profile of tea polyphenols in the presence of oxygen reactive species is required in order to evaluate the antioxidant properties of these compounds and indeed to identify the individual compounds responsible for the antioxidant properties.

The aim of this work is to evaluate the antioxidant capacity of several natural extracts, including green tea, green coffee, and grapefruit, and to identify the individual compounds responsible for the antioxidant properties of green tea extract, which afforded the best antioxidant values. From this study, the strongest antioxidant will be selected and incorporated into a plastic film to produce an antioxidant packaging material (called active material) for food. The evaluation and performance study of this new active material will be the second objective of this article. For this purpose, a chromatographic method has been optimized to identify catechins, the major polyphenols present in green tea extract. The evolution of the catechin concentration values after the scavenger process is reported and discussed.

MATERIALS AND METHODS

Reagents and Stock Solutions. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; 98%, CAS 258-422-8); fluorescein (3,6′-dihydroxypirol[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3one; Standard Fluka, CAS 518-47-8); and APPH (2,2′-azobis(2-methylpropionamide)dihydrochloride; 97%, CAS 2997-92-4) were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Disodium hydrogen phosphate dehydrate (99.5%, CAS 100028-24-7) and sodium dihydrogen phosphate hydrate (99%, CAS 7558-80-7) were supplied by Merck (Madrid, Spain). Sodium hydroxide (98%, CAS 1310-73-2) and methanol (high-performance liquid chromatography (HPLC) grade) CAS 67-56-1 were provided by Scharlab (Mollet del Vallés, Spain). Ultrapure water was obtained from a Millipore Milli-Q_{PLUS} 185 system (Madrid, Spain).

Sodium salicylate (>99.5%, CAS 54-21-7) and 2,5-dihydroxybenzoic acid (>99%, CAS 490-79-9) were supplied by Sigma-Aldrich Química S.A. Hydrogen peroxide (>50%, CAS 7722-84-1), orthophosphoric acid (85% reagent grade, CAS 7664-38-2), and sodium hydroxide (0.01 mol L^{-1}, CAS 1310-73-2) were purchased from Scharlab.

Gallic acid (CAS 149-91-7); caffeine (58-08-2); (+)-catechin (>99.0% (HPLC), CAS 154-23-4) (C); (−)-epicatechin (>95.0% (HPLC), CAS 490-46-0) (EC); (−)-epicatechin gallate (>98% (HPLC), CAS 1257-08-5) (ECG); (−)-catechin gallate (>98% (HPLC), CAS 130405-40-2) (CG); (−)-epigallocatechin (>95.0% (HPLC), CAS 970-74-1) (EGC); (−)-gallocatechin (>98% (HPLC), CAS 3371-27-5) (GC); (−)-gallocatechin gallate (>98% (HPLC), CAS 4233-96-9) (GCG); (−)-epigallocatechin gallate (>95.0% (HPLC), CAS 989-51-5) (EGCG); and formic acid (>98%, CAS 64-18-6) were all supplied by Sigma-Aldrich Química S.A.

Working solutions for the ORAC test were prepared in 75-mM sodium phosphate buffer (pH 7.0). A 2.3 $\mu g\ g^{-1}$ fluorescein solution was prepared weekly, whereas an APPH solution (34.4 mg g^{-1}) was prepared daily. Salicylate solution was prepared by dissolving sodium salicylate (1.25×10^{-5} mol L^{-1}) in H_3PO_4 (250 μmol L^{-1}) and adjusting the pH to 4.50 with a Crison GLP 22 pH-meter (Barcelona, Spain) by adding NaOH (0.01 mol L^{-1}).

Essential Oil and Extracts. Coffee essential oil (*Coffea Arbica*, CAS 84650-00-0) was supplied by Argolide (Barcelona, Spain). Green tea 95% polyphenol and 65% catechin extract (*Camellia thea Link aut sintesis Kuntze*, CAS 84650-60-2); grapefruit 50% dry hydroalcoholic extract (*Citrus grandis* (L.) *Osbeck*, CAS 900045-43-5); and green coffee 45% dry hydroalcoholic extract (*Coffea arabica* L., CAS 84650-00-0) were provided by EPO Istituto Farmochimico Fitoterapico (Milan, Italy). Two types of green tea extracts were supplied by DANISCO (Copenhagen, Denmark). The first one was a green tea extract formulation 1 (GTE1) that contained around 20% total catechins (w/w, HPLC determination provided by the supplier company), and the second one was a green tea extract formulation 2 (GTE2) that contained around 75% total catechins (w/w, HPLC determination). The concentration of the samples prepared in methanol was 400 μg natural essential oil or natural extract per gram of methanolic extract.

Polymeric Active Films. The active packaging was manufactured and supplied by the Spanish company Artibal S.A. (Sabiñánigo, Spain). It consists of a coating layer with a constant concentration of extract or essential oil in a common plastic film of polyethylene terephthalate. The system is under the EU patent EP1477519-A1.[41] The coating operation is applied at room temperature and only requires a flash of hot air, of about 40 °C, to eliminate the solvent. The coating formulation, which contains natural extracts as active agents, is therefore anchored onto the material and cannot be removed. Loss of antioxidant capacity is not appreciated when the natural extract is incorporated into the plastic film due to the low temperature applied during the manufacturing process. In addition, the polyphenols present in coffee extract[42] or catechins from green tea[43] are nonvolatile compounds and thermally stable at 40 °C. At this temperature of manufacture, the antioxidant properties of these compounds are unaltered, and the loss of activity is negligible.

The plastic film is 23 μm thick with a density of 18.73 ± 0.02 g/m^2. The active films contained the active substance expressed as a percentage of weight active agent/weight active layer. They were prepared in different grammages, from the least concentration (film A) to the high concentration of green tea (film D), in the interval from 0.7 g active/m^2 film to 3.0 g active/m^2 film. Different coating grammages were evaluated in order to study the influence of the active component on the antioxidant capacity. Films without essential oil or extracts were used as blanks. All of the tests were performed in triplicate. Additional information about the coating formula and the process is confidential and cannot be revealed in this work.

Sample Treatment. For the ORAC test, the commercial extracts and essential oil were dissolved in methanol (400 $\mu g\ g^{-1}$), diluted with sodium phosphate buffer, and filtered using a 0.22 μm Nylon syringe filter (KX Syringe Filter, 25 mm, 0.22 μm Nylon, Kinesis, UK).

For the free radicals test, the samples were prepared according to the procedure described in the previous work by Pezo et al.[17,18] Eight square centimeters of active films were used to prepare plastic bags with internal dimensions of 15 cm ×15 cm by thermosealing with an impulse sealer (PFS-200, Zhejiang Dongfeng Packing Machine Co., Wenzhou, Zhejiang, China).

For the chromatographic study of the catechins, green tea extract was dissolved in methanol (2 mg g^{-1}) and diluted with an aqueous solution of formic acid (0.3%, v/v). The solution was filtered using a 0.22 μm Nylon syringe filter. The active films were extracted in methanol during 20 min before and after the oxidation process. The extract was diluted with aqueous solution of formic acid (0.3%, v/v) and filtered through a syringe filter of 0.22 μm pore size prior to injection.

ORAC Assay. The ORAC is based on the reaction between peroxyl radicals produced by the decomposition of APPH and a fluorescent probe (fluorescein) to form a nonfluorescent product. Hydrogen atom abstraction from the probe results in a decrease in intensity of the fluorescence, which is recorded until the fluorescein is completely destroyed. The ORAC assay was carried out following the adapted procedure developed by Bentayeb et al.,[16] which permitted the reaction time between APPH and fluorescein to be reduced to 1 h. A chromatographic system, the Alliance 2795 Separations Module (Waters, Milford, MA, USA) coupled to a 474 Scanning Fluorescence Detector (Waters, Milford, MA, USA), was used to measure the fluorescein decay. First, 800 μL of fluorescein solution was mixed with 100 μL of diluted extract. Then, 600 μL of the APPH solution was added to start the reaction. Finally, 20 μL of reaction mixture was injected every minute using a 0.5 mL/min water flow. A total of 50 injections were made each assay. The ORAC reaction took place in a thermostatted autosampler of the chromatographic system at 32 °C. Excitation and emission wavelengths were set at 540 and 565 nm, respectively. ORAC values are usually reported as Trolox equivalents, which are commonly expressed as grams of Trolox per gram of the compound under study.

Free Radicals Assay. In 2006, Pezo et al.[17,18] developed an experimental laboratory-made system to determine the antioxidant capacity directly in a plastic layer commonly used as packaging material. The experimental setup designed by Pezo et al. was used for all the tests performed here. An atmosphere enriched in free radicals, generated by UV-light irradiation over an aqueous H_2O_2 (0.29 mol L^{-1}) aerosol, was carried by a nitrogen current and passed through the plastic bag to be evaluated. After in situ reaction with eight parallel polymeric samples, the remaining free radicals, which were not trapped by the antioxidant polymer, reacted quantitatively with a salicylic acid solution at pH 4.5 (eight parallel solutions, each one corresponding to each polymer), giving 2,5-DHB, 2,3-DHB, and catechol. Hydroxylation periods of 24 and 48 h were measured, and 48 h was selected as optimum. At 24 h, the hydroxylation percentage of SA was similar to that of the blank, and it was necessary to increase the reaction time. The formation of 2,3-DHB or catechol was not observed at 48 h, which means that some radicals were scavenged by the antioxidant material. The antioxidant capacity was indirectly assessed by HPLC-fluorescence determination of the highly sensitive 2,5-DHB formed. Chromatographic analysis of the 2,5-DHB and residual salicylic acid was performed in an Alliance 2695 Separations Module (Waters, Milford, MA, USA) coupled to a 474 Scanning Fluorescence Detector (Waters, Milford, MA, USA). The chromatographic separation was achieved on a Waters reversed phase column (100 mm long, 4.6 mm i.d., 3 μm) Atlantis dC18. The isocratic mobile phase was a mixture of aqueous acetate buffer (35 mmol L^{-1}, pH 5.8, 1.0 mL min^{-1}) and methanol, 90:10 (v/v). The injection volume was 20 μL. Excitation and emission wavelengths were set at 324 and 448 nm, respectively.

Chromatographic Study of Green Tea Extract and Active Plastic Film. GTE methanolic extraction and the methanolic extraction of the film containing GTE were analyzed by HPLC-PDA with a UV detector (Waters 2695/2996, Mildford, MA, USA) in order to identify their major catechins. An Atlantis dC18 Waters reversed–phase column (100 mm long, 4.6 mm i.d., 3 μm) was used. The mobile phases consisted of 0.1% formic acid in water (eluent A) and 0.1% formic acid in methanol (eluent B). The flow rate was 0.5 mL/min, and the injection volume was 20 μL. The gradient system was 0–5 min, 10% B; 5–14 min, linear gradient from10 to 20% B; 14–20 min, linear gradient from 20 to 50% B; 20–22 min, linear gradient from 50 to 90% B; 22–26 min, 90% B; 26–30 min, linear gradient from 90 to 10% B. Post-run time was 10 min. The column temperature was set to 25 °C. The major absorbance of catechins, gallic acid, and caffeine were detected at 275 nm for quantitative purposes with a data acquisition rate of 64 Hz. Gallic acid, caffeine, C, EC, CG, GC, ECG, EGC, GCG, and EGCG were confirmed by comparing the retention time and spectral data of their pure standards. To evaluate the selectivity of this method, Empower chromatographic software was used for gathering data and processing the chromatograms. This software enables one to calculate peak purity and operate spectral data. The peak purity calculation includes two variables: peak purity angle and peak purity threshold. The criteria of peak purity are fulfilled when the peak purity angle value is lower than the value of the peak purity threshold.

RESULTS AND DISCUSSION

ORAC Assay. The ORAC assay is a method used to quantify the antioxidant capacity of a broad range of sample types, including fruit and vegetable extracts, plasma, and pure phytochemicals. The aim of this assay was to evaluate the antioxidant capacity of the natural extracts and the extracts obtained from the active plastic films containing phenolic compounds, which are responsible for the antioxidant effect. One natural coffee essential oil and five natural extracts of green coffee, green tea, and grapefruit have been tested to evaluate their antioxidant capacity, according to the ORAC assay. The results of the evaluation for the extracts studied here are shown in Figure 1, and the values are expressed as g Trolox per g of

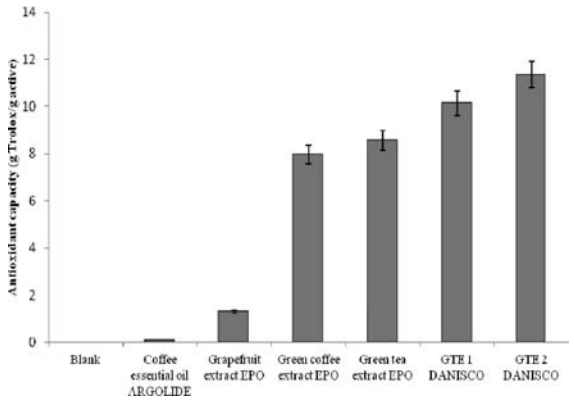

Figure 1. Antioxidant capacity of different natural extracts and one essential oil by the ORAC assay. Error bars corresponding to 95% confidence intervals, $n = 3$.

essential oil or extract. The antioxidant capacity increases in the following order: coffee essential oil (0.06), grapefruit extract (1.30), green coffee extract (7.96), green tea EPO extract (8.56), GTE1 (10.14), and GTE2 (11.34). Error bars represent a standard deviation between 2% and 9%. Green tea EPO extract, which contain 65% catechins showed less antioxidant capacity than GTE1, which contain 20% catechins. These results could be due to the solubility of both extracts in methanol. GTE1 exhibited higher solubility in methanol (1000 μg/g) than green tea EPO extract (2500 μg/g) and

consequently higher antioxidant capacity. GTE2 showed the most antioxidant capacity for the ORAC assay. This could be attributed to the greater concentration of catechins, as expected according to the % of catechins given by the supplying companies. In light of these results, GTE1 and GTE2 were selected for incorporation as antioxidants in the active film, and the resulting plastic films were evaluated. Green coffee EPO extract was also selected to study the differences between green tea and green coffee when they were incorporated as antioxidants into the plastic films. A wide range of food applications could be possible when different extracts were available.

Free Radicals Assay from Active Plastic Layers. The antioxidant activities of active films containing green coffee extract, GTE1, and GTE2 were evaluated by the free radical scavenger procedure developed by Pezo et al.[17,18] For this purpose, a set of plastic films with different grammages and with a common coating formula and concentration of active agent were prepared and supplied by Artibal S. A.

The active plastics were submitted to the oxidation atmosphere generated from an aqueous hydrogen peroxide solution. Figure 2 shows the results obtained at 48 h, expressed

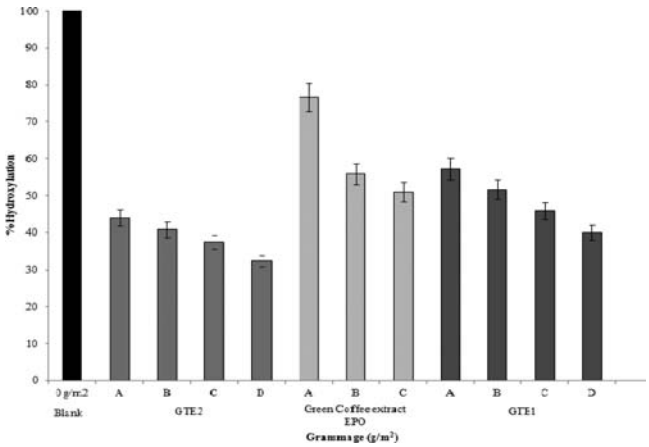

Figure 2. Antioxidant capacity of active film samples with different grammages subjected to hydroxylation after 48 h. The blank (plastic film without natural extract) is regarded as the 100% reference. Error bars corresponding to 95% confidence intervals, $n = 3$.

in percentage of hydroxylation (blank = 100%) together with the active film samples. As can be seen, the percentage decreases when the thickness layer increases for each sample. In fact, the grammage corresponding to film D provided the best results in all cases. The values obtained for GTE2 were 44% (grammage A); 41% (grammage B); 38% (grammage C); and 32% (grammage D). For green coffee extract EPO the values were 77% (grammage A); 56% (grammage B); and 51% (grammage C). Finally, the values observed for GTE1 were 57% (grammage A); 52% (grammage B); 46% (grammage C); and 40% (grammage D). The antioxidant scale obtained from the analysis of active films was as follows: GTE2 > GTE1 > green coffee extract EPO. Error bars were slightly higher than those values obtained in previous studies, with an average RSD of about 15%. These differences could be due to the low degree of homogeneity of the active layer during the coating process, and this could be also expected in industrial applications. The industrial application of coatings, even being very accurate and well done, does not provide a 100% of accuracy. The 15% RSD in the film coating is considered acceptable at the industrial level. The polymer containing GTE2 gave the highest antioxidant values. For film D, the hydroxylation of SA was 32%. Therefore, 68% of the radicals were scavenged by the active polymer, and approximately 32% of SA, which reacts with free radicals not trapped by the green tea extract, was converted into 2,5-DHB.

The free radicals assay indicated that the best antioxidant activity was provided by the plastic containing GTE2, in good agreement with the ORAC test applied to pure GTE2. Following these results, green tea extract GTE2 was selected in order to identify the compounds responsible for the antioxidant properties and to explain their behavior during the scavenging process.

Chromatographic Study of GTE2 and of Active Plastic Films Based on This Extract. Green tea is known for being an important source of antioxidant phenolic compounds, especially polyphenols. Catechins are the most abundant polyphenols in tea leaves.[44] Separation by HPLC followed by UV detection is the most widely used method for the analysis of tea catechins. Several studies have been reported determining simultaneously all the catechins in tea.[45,46] Following the procedure published by Zhou et al.,[47,48] a study of the catechins present in the GTE2 was carried out.

Although the antioxidant behavior of green tea extract has been demonstrated in this work, identifying the specific catechins responsible for this behavior remains a challenge. For this reason, the separation of each individual compound and the study of likely changes in both their concentration and chemical structure along with the oxidation process are important for understanding the role of each catechin. For this purpose, a mixture of standards from gallic acid, caffeine, and eight catechins in the concentration range of 1−75 $\mu g\ g^{-1}$ was prepared in methanol and then evaluated to establish first the analytical parameters of the method developed. The calibration curve, the linear dynamic range, the precision, the limits of detection, and the limits of quantification were calculated. The results showed that the responses for the standards were linear, reaching values over 0.997 for all the standards. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by a blank procedure at 275 nm. The limit of detection and limit of quantification were calculated according to Miller and Miller[49] using the following expressions: $LOD = 3_{sy/x}/m$ and $LOQ = 10_{sy/x}/m$, where $s_{y/x}$ is the standard deviation and m the slope of the analytical curve. The results obtained are expressed as ng of standards mixture per gram of methanolic solution. Very low limits of detection were obtained such as (−)-EGCG, (−)-GCG, (−)-ECG, and (−)-CG had detection limits bellow 0.9 ng g^{-1}. (−)-EC (11.1 ng g^{-1}), (−)-GC (11.3 ng g^{-1}), gallic acid (14.9 ng g^{-1}), (−)-EGC (45.9 ng g^{-1}), and (+)-C (117 ng g^{-1}) had higher limits of detection. The linear dynamic ranges obtained were calculated with at least seven calibration points, each one in triplicate, and the values are expressed as μg of standards mixture per gram of methanolic solution. Linear range varied from 0.001 to 75.1 $\mu g\ g^{-1}$ depending on the standards. This range was appropriate since it was not expected to have concentrations over these values in real samples. To calculate the reproducibility of the method, 3 solutions with the same concentration (around 50 $\mu g\ g^{-1}$) were analyzed on different days, and the results were compared. Reproducibility expressed as RSD in percentage was lower than 1% for all standards except for (−)-EGC and (−)-GCG, for which it was 3.51% and

4.34%, respectively. The recovery of the method was studied by comparing the results obtained from the methanolic extract of active plastic layers to those obtained when the equivalent solution of green tea extract in methanol was directly analyzed by HPLC-PDA. The average recovery rate of tea catechins was approximately 95%. This high percentage is due to the total solubility of the active coating in methanol and consequently the total solubility of tea catechins. As the active coating contains several ingredients including some resin, the influence of the matrix in the final analysis had to be studied. For this purpose, the methanolic extract of a blank film and the methanolic extract of an active plastic film containing green tea were analyzed. Both solutions were filtered prior to injection. Spectral data and peak purity calculations were used for identity confirmation. Chromatographic software Empower was used for gathering data and processing the chromatogram. To demonstrate method selectivity, spectral data were acquired at 275 nm, which is the UV maximum absorbance, and peak purity was calculated. Data are displayed in Table 1. For all

Table 1. Spectral Data and Peak Purity Calculations of Catechin Standards (λ_{max} = 275 nm)

catechins	t_R	peak purity angle	peak purity threshold
gallic acid	7.23	0.366	0.524
(−)-GC	9.03	0.201	0.356
(−)-EGC	12.01	0.233	0.34
(+)-C	12.27	0.346	0.434
(−)-EGCG	13.78	0.345	0.801
caffeine	14.10	0.285	3.687
(−)-EC	14.56	0.338	0.656
(−)-GCG	15.12	0.275	0.345
(−)-ECG	15.93	0.350	0.823
(−)-CG	16.62	0.125	0.302

catechin standards, the peaks were spectrally homogeneous because the purity angle was less than the purity peak. At the end of the chromatogram, several unknown peaks were found in all the methanolic extracts, both blank and active film. These were attributed to the composition of the plastic samples. The area of peaks and the retention times coincided in both cases. Thus, the absence of a matrix effect was confirmed.

Catechins Content in GTE2. Using this analytical method, the content of tea catechins together with gallic acid and caffeine in GTE2 was found to be approximately 82% of its weight (Table 2). The total quantity of polyphenols and caffeine was 485.27 μg active compounds per gram of methanolic extract. Eight catechins were determined, and

Table 2. % Tea Catechins in GTE 2

green tea compounds	% in GTE 2
(−)-GC	0.59 ± 0.01
(−)-EGC	1.67 ± 0.34
(+)-C	2.23 ± 0.07
(−)-EGCG	39.95 ± 0.69
(−)-EC	12.80 ± 0.38
(−)-GCG	6.56 ± 1.66
(−)-ECG	12.89 ± 0.47
(−)-CG	0.63 ± 0.04
gallic acid	0.65 ± 0.02
caffeine	4.88 ± 0.26
total	82.26 ± 0.39

(−)-EGCG (39.95%), (−)-ECG (12.87%), and (−)-EC (12.80%) were the three main components in the GTE2. These results of the total tea catechins (77.33%, without including % gallic acid and % caffeine) are comparable with the specifications provided by the producer in the product description data. A chromatogram of tea catechins obtained by HPLC-PDA is shown in Figure 3. Identification of

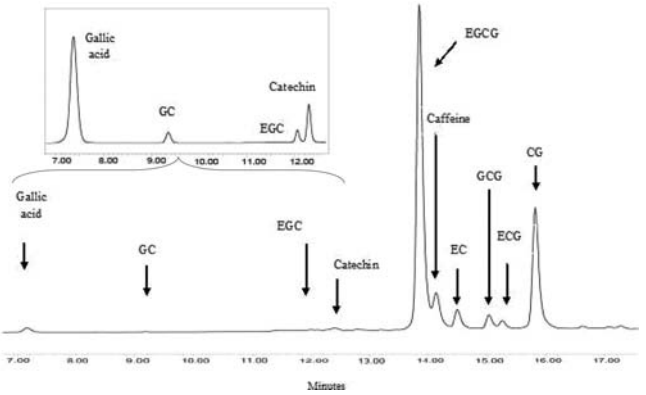

Figure 3. HPLC profile of tea catechins in GTE 2 and a zoom-view elution chromatogram (top) of gallic acid, GC, EGC, and catechin.

compounds by HPLC-PDA analysis was carried out by comparing retention times and UV spectra of the unknown peaks to those of the standards. Furthermore, deconvolution curves confirmed the purity of the chromatographic peaks and the absence of overlapped compounds.

Role of Catechins in the Oxidation Process. The purpose of the free radical assay was to evaluate the antioxidant capacity of green tea directly incorporated into the polymeric films. Additionally, the optimized chromatographic method permitted the tea catechins in the film to be quantified after the oxidative reaction. From the results obtained in the free radical assay, it may be assumed that green tea catechins were actively involved in the scavenging process of free radicals. The filtered methanolic extract of the active plastic films was analyzed by HPLC with UV detection before (0 h) and after (48 h) the radical scavenging process, in order to clarify the behavior of each compound in the presence of a radical enriched atmosphere. The active layers were films with the type D grammage, and they were analyzed in triplicate.

Table 3 shows the concentration value of each catechin at 0 and 48 h, expressed as μg of tea component per gram of methanolic extract, the reproducibility expressed as % RSD as well as the concentration of each catechin lost. In all cases, a change in the amount of tea components was observed. In general, the concentration of gallic acid, caffeine, and the eight catechins studied decreased but in different proportions. For instance in Table 3, (−)-EGCG and (−)-ECG, the major components in GTE2, showed less variation than the other compounds in the extract. However, (−)-GC, (−)-EGC, (+)-C, and (−)-CG exhibited an important change in their concentration values before and after the reaction with reactive species. In other words, (−)-GC, (−)-EGC, (+)-C, and (−)-CG present in GTE2 even at quite low concentrations provided better scavenging activity than (−)-EGCG and (−)-ECG which are the major components. This trend could be due to the affinity of (−)-GC, (−)-EGC, (+)-C, and (−)-CG for capturing free radicals more easily than the others. Moreover, the results suggest that (−)-EGC, (+)-C, (−)-GC,

Table 3. Concentration Values of Different Catechins before (0 h) and after (48 h) the Oxidation Process[a]

name	concentration (μg/g) t = 0 h	% RSD (n = 3) t = 0 h	concentration (μg/g) t = 48 h	% RSD (n = 3) t = 48 h	concentration loss (%)
gallic acid	1.28	1.91	0.95	2.99	25.67
(−)-GC	4.56	4.02	1.71	1.46	62.39
(−)-EGC	18.60	1.25	4.58	2.48	75.30
(+)-C	4.96	6.47	1.79	4.75	63.90
(−)-EGCG	95.70	2.82	85.9	0.39	10.18
caffeine	7.72	0.17	5.23	3.74	32.25
(−)-EC	20.10	4.31	10.90	1.15	45.59
(−)-GCG	18.90	4.73	15.70	2.87	17.40
(−)-ECG	38.20	1.74	26.60	0.33	30.29
(−)-CG	2.88	1.12	0.81	3.37	71.97

[a]Samples are the methanolic extract from the active plastic films.

and (−)-CG are the main components responsible for the antioxidant capacity in GTE2. It should be emphasized that the free radicals are in vapor phase, while the catechins are anchored on the solid plastic film. This means that the GTE2 incorporated into the film does not change its properties and composition compared to the pure extract. This fact emphasizes the idea of scavenging instead of release of the antioxidants. The anchoring of GTE2 in the plastic film does not affect their chemical structure, and thus, the scavenging properties remain unaltered.

Additionally, some new peaks were detected after the scavenging process, but these have yet to be identified. Many authors have reported that tea catechins could convert to their corresponding epimers in traditionally brewed tea infusions and canned tea drinks during manufacture, production, storage, or transport. The epimerization, i.e., the conversion of the tea catechins to their corresponding isomers, depends strongly on the pH and temperature.[50−52] However, these new compounds are presumably formed from the reaction of primary catechins with oxygen reactive species. They are unlikely to be epimers of catechins because the pH and temperature conditions are not modified during the free radicals assay. Figure 4 shows a comparison between the chromatograms of the methanolic extract before and after the scavenger process. In the bottom chromatogram, unknown new peaks appear. Each UV spectrum of these peaks was analyzed, but similarities with catechin spectra were not found. Unfortunately, no reports have been found which describe new compounds formed during the oxidative reaction in the presence of green tea catechins.

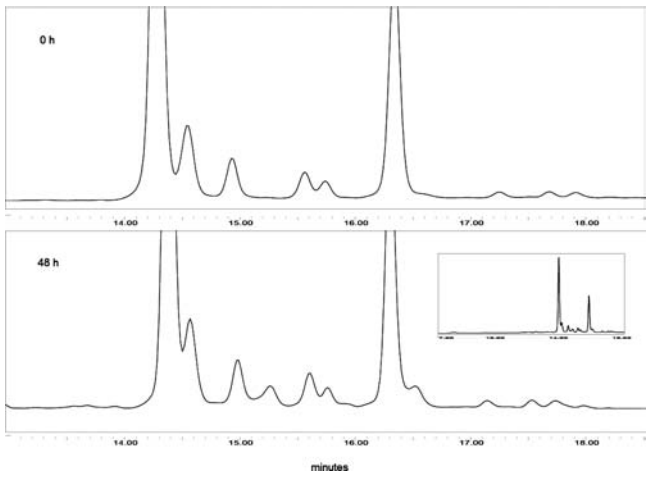

Figure 4. Comparison between a zoom-view chromatogram of the methanolic extract before the oxidative reaction (top) versus another zoom-view chromatogram of the same extract after the oxidative reaction (bottom).

Further work using UPLC-qTOF-MS is in progress in our laboratory to elucidate the identity of unknown compounds formed during the scavenger process as well as to clarify the mechanism of the behavior of tea catechins.

AUTHOR INFORMATION

Corresponding Author

*Tel: +34 976 761873. Fax: +34 976 762388. E-mail: cnerin@unizar.es.

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Notes

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