Adaptation of the ORAC assay to the common laboratory equipment and subsequent application to antioxidant plastic films

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Abstract

The oxygen radical absorbance capacity (ORAC) method has been adapted to the instrumental laboratory and optimized for the determination of the antioxidant capacity of a novel active packaging. As the ORAC assay requires the monitorization of a reaction at controlled temperature by means of the fluorescence signal decrease over time, specific instrumental is usually necessary. In this work, a common liquid chromatographic device has been adapted to perform the ORAC assay, leaving it accessible to any laboratory. Using this adaptation, five different essential oils have been determined resulting in the following antioxidant order: clove (2.66 g Trolox per gram of essential oil), oregano (2.25), cinnamon (1.93), rosemary (1.66), and ginger (1.47). After incorporating the essential oils to the film, its antioxidant capacity has also been checked and related to the concentration of essential oil as well as the thickness of the active film. The results point out that for the same amount of essential oil incorporated measured as grams per square meter, thicker films have more antioxidant capacity than the thinner and more concentrated ones. Furthermore, the antioxidant capacity found in the films was always higher than expected taking into account the amount of essential oil incorporated. Some likely explanations have been proposed, leading to the improvement of the antioxidant film under development

Keywords ORAC . Antioxidant film . Essential oil . Active packaging

Introduction

The active packaging and among them the antioxidant ones are becoming more and more important, as it is a good way to preserve the food against the oxidation process which limits the shelf life of the packaged food. However, when a new material is obtained, its characteristics have to be measured in an objective manner, so that the new material can be referred to a standard or compared with others. It could be argued that knowing the performance of the antioxidant agents used to prepare the active packaging should be enough to demonstrate the antioxidant behavior of the expected material. But this is not true, as, once in the material, the behavior of the active agents does not always correspond exactly to the prediction made. Previous
research carried out in this area drove us to develop a new system to measure the antioxidant capacity of the new plastic films containing essential oils as antioxidant agents [1, 2]. This system was based in the hydroxylation of salicylic acid by the OH radicals and the decrease of the hydroxylated products in the presence of the new antioxidant plastic was quantitatively measured. Thus, the mechanism of action as radical scavenger of the new active plastic was demonstrated. Nonetheless, the test requires more than 10 h is not very practical for daily control in industry. On the other hand, the developed system is mainly based on hydroxyl radicals and the radical scavenger in fact acts on all kind of radicals. Futhermore, the antioxidant capacity of the product depends not only on the film but also on the packaging shape, the free volume, the atmosphere inside the packaging during the product shelf life, and, of course, on the natural features of the food. This fact involves the optimization of the antioxidant tailor-made packaging.

When developing a new antioxidant active packaging material, it is important to be able to evaluate its final antioxidant capacity as the traceability requirements demand the control of every lot of plastic material produced. Furthermore, in order to assure the commercial features during the storage period of the packaging, its antioxidant properties should be also measured periodically. The antioxidant capacity of the natural essential oils depends on the supplier, as the composition of the natural essential oil may vary in function of the plant variety, the type of processing and drying, and the climate. For this reason, they usually have a wide lot-to-lot variability. Thus, the antioxidant properties of the final packaging produced have to be controlled.

Scavenger of radicals is one of the main antioxidant mechanisms and different procedures have been proposed based on producing and measuring such radicals to evaluate the antioxidant properties in liquid phase. However, none of them has been applied before to packaging materials. The oxygen radical absorbance capacity (ORAC) assay is a widely used method to characterize the antioxidant capacity of different materials such as biological fluids, essential oils, spices, foods, dietary supplements, or cosmetic products [3, 4]. The ORAC assay is based upon the early work of Glazer [5] and was further completed by Cao et al. [6, 7]. In the basic assay, a peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product; therefore, the reaction can be easily quantified by fluorescence. The peroxyl radical used is 2,2′-azobis(2-amidinepropane) dihydrochloride (AAPH), which reacts with fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]-xanthen]- 3-one) that is the fluorescent probe. The ORAC reaction is performed at 37°C and it is temperature sensitive, so close temperature control throughout the experiment is essential. The calculation of protective effects of an antioxidant is carried out from the net integrated areas under the fluorescence decay curves and accounts for lag time, initial rate, and total extent of inhibition in a single value. ORAC values are usually reported as Trolox equivalents [3]. The ORAC assay has been used to study the antioxidant capacity of many compounds and food samples [8–12]. The industry has accepted the method to the point that some nutraceutical manufacturers are beginning to include ORAC values on product labels [13, 14]. In this paper, the method ORAC has been adapted to check the antioxidant capacity of an active film and has been used to control the antioxidant film production and storage. Antioxidants are extracted and blended with the ORAC test reagents. Volumes, concentrations, and reaction times have been optimized in order to maximize the sensitivity of the analytical method. According to the results obtained, the antioxidant capacity has been correlated with both the nature and concentration of the essential oil and the film thickness, leading to deepen in the subsequent antioxidant behavior of the active film.

Materials and methods

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; 98%, CAS 258-422-8); fluorescein (3,6′-dihydroxypiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one; Standard Fluka, CAS 518-47-8) and AAPH (2,2′-azobis(2-methylpropionamidine) dihydrochloride; 97%, CAS 2997-92-4) were purchased from Sigma-Aldrich Química S. A. (Madrid, Spain). Acetone (high-performance liquid chromatography (HPLC) grade), disodium hydrogen phosphate dihydrate (99.5%, CAS 10028-24-7), and sodium dihydrogen phosphate hydrate (99%, CAS 7558-80-7) were supplied by Merck (Madrid, Spain). Sodium hydroxide (98%, CAS 1310-73-2) was provided by Scharlab (Mollet del Vallés, Spain). Ultrapure water was obtained from a Millipore Milli-QPLUS 185 system

(Madrid, Spain). All the essential oils were supplied by ARGOLIDE (Barcelona, Spain). Oils from the following plant species were used: cinnamon (Cinnamomum zeylanicum, CAS 8015-91-6), clove (Syzygium aromaticum, CAS 8000-34- 6), oregano (Origanum vulgare, CAS 8007-11-2), and ginger (Zingiber officinalis, CAS 8007-08-7). Amexol, a commercially available natural extract of rosemary (Rosmarinus officinalis, CAS not available) registered as spice for meat products was provided by Laboratorios Amerex (Madrid, Spain). Cinnamon essential oil is fortified with cinnamaldehyde in order to increase its antimicrobial properties [15].

Polymeric active samples

The active packaging was manufactured and supplied by the Spanish company ARTIBAL (Sabiñanigo, Spain) S.A. It consists of a multilayer with a known concentration of essential oils in a polypropylene film. The polypropylene film is of 20-μm thickness and a density of 18.93±0.02 g/m2. Active films under study have two different grammages in the interval from 1 to 3 g/dm2 and contain different concentrations of the essential oils (namely 0%, 2%, 4%, 6%, and 8%, weight of essential oil/weight active layer). Films without essential oil (0%) were used as blanks for the ORAC assay. Once produced, each individual film was then wrapped with aluminum foil in order to isolate them from the environment and stored at 4°C for a maximum period of a week. All the tests were performed in triplicate

Stock solutions

Working solutions were prepared in 75-mM sodium phosphate buffer (pH 7.0). A 2.3-μg/g fluorescein solution was prepared weekly, whereas AAPH solution (34.4 mg/g) was prepared daily. Both were stored at 4°C until used. To carry out the external calibration, a stock solution of Trolox (700 μg/g) was prepared when necessary.

Sample treatment

The extraction step was optimized in a previous work [16]. One square decimeter of the film was cut into four pieces and extracted with 6 mL of acetone in an ultrasonic bath at room temperature for 15 min. Then, the active compounds were quantitatively extracted. The extract was diluted nine
times with sodium phosphate buffer and filtered using a 0.2 μm Nylon® syringe filter. Essential oils were dissolved in acetone (450 μg/g), diluted with sodium phosphate buffer, and filtered by a 0.2-μm Nylon® syringe filter. As some essential oils had less antioxidant capacity than others, dilutions were performed with the aim of having a reaction time of 1 h.

ORAC assay

ORAC assay was performed according to the previous work of Ou et al. [4], but it was adapted to the available devices and, subsequently, optimized in order to have a reaction time of 1 h. The ORAC reaction was undergone in the thermostated autosampler of a chromatographic system Alliance 2795 Separations Module (Waters, Milford, MA, USA) at 32°C. Reagent solutions were preincubated for 15 min in the autosampler before mixing them to carry out the reaction. First, 800 μL of fluorescein solution was mixed with 100 μL of diluted extract of the sample. After that, 600 μL of the AAPH solution was added making the reaction starts. Then, 20 μL of the reaction mixture was injected every minute and taken, by a 0.5-mL/min water flow, to the online detector 474 Scanning Fluorescence Detector (Waters, Milford, MA, USA). Excitation and emission wavelengths were set up at 540 and 565 nm, respectively. A total of 50 injections were

made each assay, describing the fluorescein decay. The area under the curve (AUC) was calculated as

$$\text{AUC(area under curve)} = \left(\frac{f_1}{f_0} + \frac{f_2}{f_0} \cdots + \frac{f_i}{f_0} + \cdots\right) \times \Delta t$$

Where f0 is the area of the first peak observed; fi is the area of the peak i; and Δt is the time interval between consecutive peaks. The net AUC was obtained by subtracting the AUC of the blank from that of the sample. In order to obtain the calibration curve, Trolox solutions of up to 250 μg/g were analyzed by the same procedure as the samples. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net AUC. The results were expressed as Trolox equivalents as microgram per square meter of active film. Finally, five consecutive injections of the fluorescein solution were performed at the beginning of the day, in order to check the stability of the fluidic system. The system was considered suitable when the relative standard deviation of the five areas obtained was lower than 2%.

Results and discussion

ORAC assay has been thoroughly applied to spices, fruits, vegetables, and juices in order to find out their antioxidant properties [3, 9–12]. Nevertheless, in this occasion, the ORAC assay has been applied to a polymeric active film intended for food contact. The purpose of this method is to help in developing a film as much antioxidant as possible by incorporating essential oil into its composition. Apart from economical reasons, it is interesting to have in the packaging as little as possible of essential oil in order to avoid any alteration of the organoleptic features of the foodstuff.

ORAC assay

The adaptation here presented uses an online fluorescence detector, leading to the use of the chromatographic autosampler as a reaction chamber. The temperature of the autosampler can be set up from 4 to 40°C, which is enough for the ORAC reaction. The aim of the adaptation has been to maximize the signal observed that means to maximize the net AUC of the antioxidant extract obtained after the film treatment. Another interesting feature of the adapted method is to draw the fluorescein decay curve as much reproducible as possible, leading to distinguishing the films with similar antioxidant capacity. The more injections are performed in each assay, the more exact would be the representation of the decay curve. For practical purposes, 1 h was fixed for the assay run time. As the time between injections could not be reduced to less than about a minute, the number of points that represent the curve was set up to 50. The reaction volume is limited to the capacity of the vials used in the autosampler (1.8 mL) so that the reaction volume used for each injection was 30 μL in order to achieve a good reproducibility. In the HPLC equipment, the full-loop injection mode was chosen, so that the injection volume was set up to 20 μL (since the autosampler overfills the loop in the full-loop injection mode). As explained in the experimental section, the injections of the reaction mixture were taken to the detector by a water flow of 0.5 mL/min. Lower flow rates produce wider (and more reproducible) peaks, so that it is interesting to work at the lowest possible flow. However, it was noticed that, using a water flow lower than 0.5 mL/min, the system pressure was not high enough to assure a constant flow, leading to misshapen peaks. Besides, the ORAC assay reaction was optimized with the aim ofmaximizing the net AUC maintaining a run time of 1 h. It was performed as follows: reagent solutions were prepared according to literature [8], and different volumes of each solution were mixed. It was imposed that the AAPH and the fluorescein solution volumes should sum 1.4 mL, whereas

the extract antioxidant solution varied from 10 to 100 μL, so that the reaction volume was always constant at 1.5 mL. Furthermore, the assay temperature was also studied. The optimization was achieved by using the program MODDE v 6.0, applying a Plackett–Burman design with three replicates in the central point, which results in 11 experiments. For each experiment, a blank assay was also performed to find out the corresponding net AUC. The optimum reaction conditions which gave the best net AUC were 600 μL of AAPH solution, 800 μL of fluorescein solution, 100 μL of antioxidant extract, and a temperature of 32°C.

Analytical parameters

Once the procedure was optimized, the analytical parameters were calculated in order to confirm the goodness of the adapted method. The calibration curve, the intraday and interday precision, and the limits of detection and quantification were found out (Table 1). The calibration curve was determined by applying the adapted ORAC assay to the active films and was expressed as grams of Trolox per square meter of film versus the percentage of essential oil incorporated. Figure 1 shows the calibration curve of oregano films (2.8 g/m2) as an example. The limit of detection was found out as follows: it was considered that the minimum AUC determined to assert an antioxidant sample to be different from a non-antioxidant one is the essential oil percentage that corresponds to three times the standard deviation of the measurement of three replicates of the AUC of blank film samples. In the same way, the limit of quantification corresponds to ten times this standard deviation. The values obtained were lower than 0.65% and 2.27%, respectively. It must be pointed out that this limit of detection depends on the restriction of having a reaction time of 1 h since the extract was diluted previous to the reaction performance. It means that lower detection limits could be achieved by diluting less the extract, but longer reaction times would be subsequently observed with highconcentration samples.

Finally, the intraday precision was calculated from the determination of five samples of active film of oregano, 2.8 g/m2 and containing 4% of essential oil (w/w); all of them were carried out the same day. The interday precision was obtained from repeating the calibration curves three times in three different days (see Table 1).

Table 1 Analytical parameters of the oregano antioxidant films

		Oregano		Cinnamon		Clove	
		2.8g/m^2	1.5g/m^2	2.6g/m^2	1.5g/m^2	2.5g/m^2	1.3g/m^2
Calibration curve[a]		$y=-0.005x^2+0.1346x+0.0038$ $R^2=0.9995$	$y=-0.0003x^2+0.0611x+0.0011$ $R^2=0.9987$	$y=-0.0024x^2+0.1388x+0.0027$ $R^2=0.9994$	$y=-0.001x^2+0.0614x+0.0025$ $R^2=0.9981$	$y=-0.01x^2+0.1724x+0.0089$ $R^2=0.9981$	$y=-0.0037x^2+0.0818x-0.0009$ $R^2=0.9981$
Limit of detection[b]		0.53		0.65		0.45	
Limit of quantification[b]		1.95		2.27		1.78	
Intraday precision (%, $n=5$)		10.8%[c]		–		–	
Interday precision (%, $n=3$)	2%[d]	10.1	14.4	14.5	4.6	6.1	17.6
	4%[d]	7.1	5.0	5.7	9.4	5.6	8.3
	6%[d]	6.5	18.0	8.5	10.5	4.4	11.1
	8%[d]	12.0	16.3	3.3	5.2	1.5	8.9

[a] Expressed as grams of Trolox per square meter as a function of the percentage of essential oil (weight of essential oil/weight of active film)

[b] Expressed as percentage of essential oil (weight of essential oil/weight of active film)

[c] It was found out for oregano, 2.8 g/m^2 and 8% films

[d] Percentage corresponds to percentages of essential oil (*w/w*)

Essential oils

Before applying the adapted ORAC method to antioxidant films, the determination of the natural essential oils used was achieved. Ginger, cinnamon, oregano, clove, and rosemary essential oils were tested in order to find out their antioxidant capacities (according to ORAC assay). The concentration of the essential oil prepared in acetone was 444 μg/g, which is the same with what it is supposed to have the antioxidant extract obtained from the more concentrated film samples (2.8 g/m2 and 8%). The results obtained were expressed as grams of Trolox per gram of essential oil. As can be seen in Fig. 2, the essential oils tested can be ordered according to their antioxidant capacity as follows: clove, oregano, cinnamon, ginger, and rosemary. Error bars represent a standard deviation lower than 11%, which is similar to that found out in the analytical parameters section. From these results, it was decided to study the antioxidant capacity of active films of clove, oregano, and cinnamon.

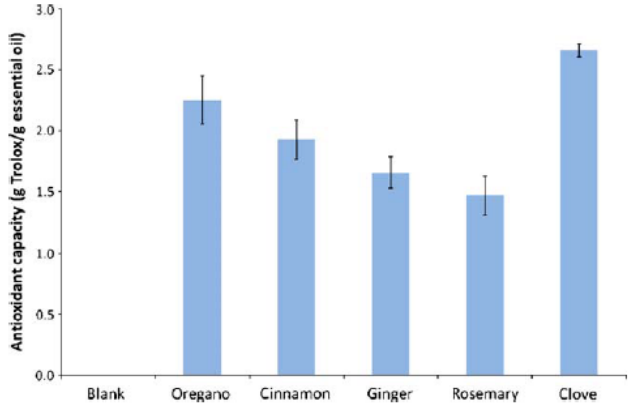

Fig. 2 Antioxidant capacity of different essential oils (n=3)

Antioxidant film samples

It is interesting to understand how the manufacturing procedure affects the antioxidant properties of the active films. As different active layer thicknesses can be produced, its influence was studied in the interval from 1 to 2.5 g/m2, by manufacturing films with 1 and 2.5 g/m2. On the other hand, nominal concentrations of 0%, 2%, 4%, 6%, and 8% (w/w) were studied. Figure 3a–c represents the antioxidant values obtained in these assays.

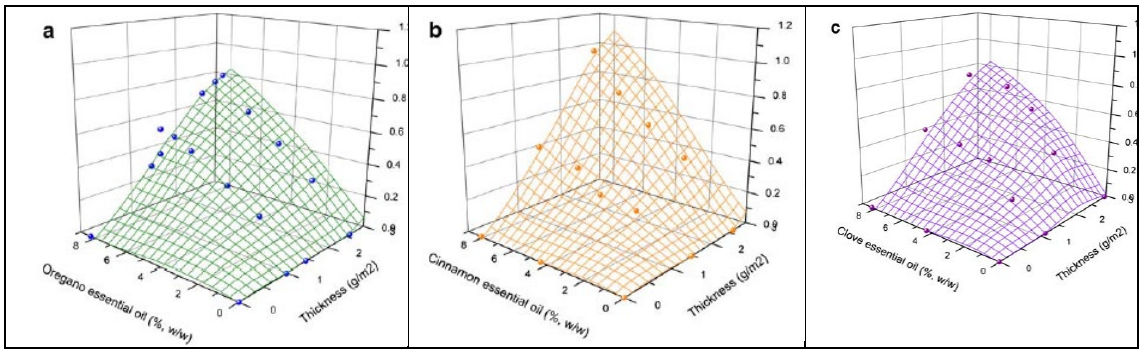

Fig. 3 a Antioxidant capacity surface obtained from oregano active films (n=3). (b). Antioxidant capacity surface obtained from cinnamon active films (n=3). c Antioxidant capacity surface obtained from clove active films (n=3)

It is observed that, by increasing the amount of essential oil incorporated, the antioxidant capacity raises but not in a linear way. At high concentration of essential oil, the slope seems to get smoother. This fact can be attributed to either an interaction between the antioxidant compounds of the essential oil and the film components or a decrease in the essential oil composition when manufacturing the active films. Taking this into account, it is worth to represent the antioxidant capacity of the films versus the total amount of essential oil added per square meter, including both the thickness and the nominal percentage incorporated. Figure 4a–c shows these relationships.

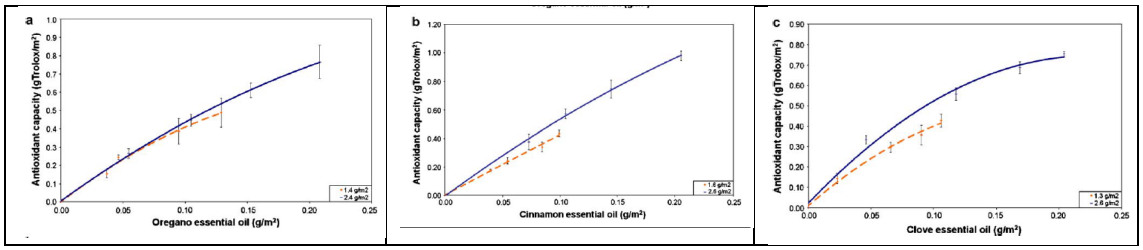

Fig. 4 a Antioxidant capacity versus total oregano essential oil (n=3). b Antioxidant capacity versus total cinnamon essential oil (n=3). c Antioxidant capacity versus total clove essential oil (n=3)

Despite not having significant statistically differences between low and high thicknesses, it seems that the thicker ones (lower density of essential oil) maintain the antioxidant capacity better than the thinner ones. Another point of view could be that the manufacturing process affects less to films with more essential oil incorporated. From the results obtained, it can be inferred that the differences between the films with different essential oil are not enough to distinguish them only by determining their antioxidant capacity, except for the high concentration of essential oil. This fact leads to a selection of the essential oil according to the organoleptic resemblance between it and the foodstuff. On the other hand, when taking into account the antioxidant capacity of the films obtained, as well as the antioxidant capacity of the essential oils, the essential oil concentration in the films could be inferred. However, when comparing the calculated essential oil concentration with the theoretical concentration in the film (the concentration initially added to the film), significant differences were observed. In Table 2, these differences are shown in detail for the three kinds of films (oregano, cinnamon, and clove).

Table 2 Comparison between antioxidant capacity of essential oils and active films

Essential oil	Antioxidant capacity of the essential oil (gram Trolox per gram essential oil)	Antioxidant capacity of the active film, 2.8g/m^2 and 8% (gram Trolox per square meter)	Essential oil calculated in the film, 2.8g/m^2 and 8% (g/m^2)	Essential oil nominal concentration, 2.8g/m^2 and 8% (g/m^2)	Excess of essential oil (%)
Oregano	2.26	0.83	0.37	0.22	65
Cinnamon	1.93	0.97	0.52	0.20	132
Clove	2.66	0.66	0.25	0.21	11

Films containing cinnamon showed an antioxidant capacity 2.3 times higher than the expected, whereas oregano and clove films showed an over antioxidant properties of 65% and 11%, respectively. This fact could be explained by assuming that the concentration of the antioxidant essential oil gets higher value when manufacturing the active film because of a likely evaporation of the solvents used for making it. The differences found

between the essential oils could be explained by taking into account that the most volatile compounds that contribute to the antioxidant properties could also have disappeared when manufacturing the film in a different way depending on the essential oil. Anyway, the final—and useful— concentration of the antioxidant compounds in the film is different from that expected. Besides, the antioxidant capacity scale obtained from the analysis of the essential oils (clove > oregano > cinnamon) is entirely different from that of the films (cinnamon > oregano ≈ clove). As a consequence, films with other essential oils such as ginger and rosemary could have more antioxidant capacity than the tested ones.

Conclusions

The ORAC method has been adapted to the instrumental laboratory. In this paper, any device intended only for performing the ORAC method has been used, but it has been carried out by coupling a chromatographic system and a fluorescent detector as well as by using the thermostated autosampler as the reaction chamber. The method was optimized for the determination of the antioxidant capacity of a novel antioxidant packaging. Five different essential oils have been studied resulting in clove, oregano, and cinnamon being the three ones selected to be incorporated into the active film for subsequent studies. The antioxidant films were studied according to the thickness and the formula concentration of essential oil. The results indicate that the most antioxidant films are those made with cinnamon, with the maximum amount available (8% and 2.8 g/m2). If less quantity of essential oil has to be incorporated (in order to minimize organoleptic effects), the study suggests to manufacture films with higher thickness and low concentration of essential oil. Finally, it has been also observed that the antioxidant capacity of the film was always higher than that expected according to the amount of essential oil incorporated, leading to a likely increase in the concentration when manufacturing the active films.

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