The additive properties of Oxygen Radical Absorbance Capacity (ORAC) assay: The case of essential oils

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Abstract

The ORAC assay is applied to measure the antioxidant capacity of foods or dietary supplements. Sometimes, the manufacturers claim antioxidant capacities that may not correspond to the constituents of the product. These statements are sheltered by the general understanding that antioxidants might exhibit synergistic properties, but this is not necessarily true when dealing with ORAC assay values. This contribution applies the ORAC assay to measure the antioxidant capacity of ten essential oils typically added to foodstuffs: citronella, dill, basil, red thyme, thyme, rosemary, oregano, clove and cinnamon. The major components of these essential oils were twenty-one chemicals in total. After a preliminary discrimination, the antioxidant capacity of eugenol, carvacrol, thymol, a-pinene, limonene and linalool was determined. The results showed that 72–115% of the antioxidant capacity of the essential oils corresponded to the addition of the antioxidant capacity of their constituents. Thus, the ORAC assay showed additive properties

Keywords:
ORAC, Antioxidant capacity, Antioxidant. Essential oil, Oregano, Thyme, Additive properties

1. Introduction

The Oxygen Radical Absorbance Capacity (ORAC) assay measures the antioxidant capacity of pure compounds or mixtures such as biological fluids, foods, dietary supplements or cosmetic products (Ou, Hampsch-Woodill, & Prior, 2001; Prior, Wu, & Schaich, 2005). The ORAC assay was developed by Cao, Alessio, and Cutler (1993) and Cao, Verdon, Wu,Wang, and Prior (1995), in turn based on a previous work (Glazer, 1990). In this assay, 2,20-azobis(2-amidinepropane) dihydrochloride (AAPH) is used to produce peroxyl radicals that react with fluorescein (30,60-dihydroxy-3H-spiro[2-benzofuran-1,90-xanthen]-3-one) giving non-fluorescent compounds. The addition of an antioxidant delays the fluorescence decay. The quantification of the antioxidant capacity is carried out from the net integrated areas under the fluorescence decay curves, and accounts lag time, initial rate and total extent of inhibition in a single value. The results are compared to those of a reference antioxidant, usually Trolox. The radical reaction is temperature sensitive. Cao et al. designed the ORAC assay to simulate the oxidative processes that occur in human cell degeneration, so the assay is typically performed at 37 _C. The ORAC assay has been widely used to characterise the antioxidant capacity of pure compounds and complex mixtures (e.g., essential oils) (Wang, Cao, & Prior, 1996; Wu et al., 2004). The industry has adopted the method to the extent that some food manufacturers include ORAC values on product labels (Bank,
2004; Wright, 2004). Nevertheless, the value printed on the label could be misleading, by exaggerating the antioxidant properties derived from the food components. Knowing the composition of the foodstuff (regarding antioxidants), it is expected to infer the

antioxidant properties of the whole product. The question that arises now is if the ORAC response of the whole product should correspond to the simple addition of the ORAC values of the components or, by contrast, there could be synergistic or antagonist effects between the antioxidant components. On the one hand, it is generally understood that antioxidants may behave in a synergistic way by enhancing their antioxidant power further than the mere addition of their individual antioxidant capacities. Indeed, the food industry works with mixtures of preservatives that act in synergistic combination on the foodstuffs (Decker, McClements, & Elias, 2010). On the other hand, the ORAC assay is based on a reaction much simpler than the oxidation processes that foodstuffs undergo. Indeed, the ORAC assay mainly accounts the capacity to donate a proton to the AAPH radical by the antioxidant under study. As this reaction is fast and spontaneous, it could be expected that a mixture of antioxidants would produce a set of parallel reactions with no cross-interaction (Bentayeb, Rubio, & Nerín, 2012), giving antioxidant values that would correspond to the addition of the individual antioxidant values. To the best of our knowledge, very few manuscripts deal with these issues. This contribution aims to better understand the additive properties of the ORAC assay

2. Materials and methods

2.1. Chemicals

The following reactants were used. Trolox (6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid) (purity 98%, CAS 53188-07-1); fluorescein (30,60-dihydroxy-3H-spiro[2-benzofuran- 1,90-xanthen]-3-one) (98.5%, CAS 518-47-8) and AAPH (2,20-azobis(2-methylpropionamidine)dihydrochloride) (97%, CAS 2997-92-4) were purchased from Sigma–Aldrich Química S. A. (Madrid, Spain). 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). Acetone (HPLC grade, CAS 67-64-1) and sodium hydroxide (98%, CAS 1310-73-2) were provided by Scharlab (Mollet del Vallès, Spain). Ultrapure water was obtained from a Millipore Milli-QPLUS 185 system (Madrid, Spain). Table 1 contains the essential oils studied in this contribution. All of them were supplied by Argolide Química S.L. (Barcelona, Spain). Finally, the antioxidant capacity of the following species was tested: eugenol (99%, 97-5-0), carvacrol (97%, CAS 499-75-2), thymol (99.5%, CAS 89-83-8), linalool (97%, CAS 78-70-6), R-(+)- limonene (97%, CAS 5989-27-5) and a-pinene (98%, CAS 80-56- 8), all purchased from Sigma–Aldrich Química S. A. (Madrid, Spain).

2.2. Stock solutions

Working solutions were prepared in 75 mM sodium phosphate buffer (pH 7.0). A solution of fluorescein of 2.3 lg/g was prepared weekly, whereas AAPH solution (34.4 mg/g) was prepared daily. Both solutions were stored in the dark at 4 _C until use. A stock solution of Trolox (700 lg/g) was prepared when necessary, to carry out the external calibration. Stock solutions of pure standards were prepared in acetone (1000 lg/g), followed by dilution with phosphate buffer solution until 100 lg/g. Essential oils were also dissolved in acetone (450 lg/g) and diluted with sodium phosphate buffer. All solutions were filtered through a 0.22 lm Nylon_ syringe filter from Análisis Vínicos (Tomelloso, Spain) to prevent clogging in the chromatograph autosampler. As some essential oils/pure compounds had less antioxidant capacity than others, appropriate dilutions were performed with the aim of having a reaction time of one hour.

2.3. ORAC assay

The ORAC assay was performed in a chromatographic system Alliance 2795 Separations Module (Waters, Milford, MA) according to a previously reported adaptation of this assay (Bentayeb, Vera, Rubio, & Nerín, 2009). Briefly, 800 lL of fluorescein solution were mixed with 100 lL of antioxidant solution. After that, 600 lL of the AAPH solution were added triggering the radical reaction. Fluorescence was monitored by a 474 Scanning Fluorescence Detector (Waters, Milford, MA), where excitation and emission wavelengths were 540 and 565 nm, respectively. The area under the curve (AUC) was calculated and the corresponding value of the blank (without antioxidant) was subtracted to obtain the net AUC. The AUC was calculated as:

$$\text{AUC(Area Under Curve)} = \left(\frac{f_1}{f_0} + \frac{f_2}{f_0} + e\frac{f_i}{f_0} + \right)eat$$

where f0 is the area of the first peak observed, fi is the area of the peak i, and Dt is the time interval between consecutive peaks. Trolox solutions of a concentration up to 250 lg/g were analysed by the same procedure, so that the antioxidant capacity of the samples was expressed as grams of Trolox per gram of essential oil or pure compound. 2.4. Additive/non-additive response of the essential oil components The additive/non-additive response of the essential oils components was determined by applying the ORAC assay to both essential oils (Table 1) and their major components (Table 2).

Table 1: Essential oils studied in this contribution.

Essential oil	Species	CAS number	Origin
Basil	*Ocimum basillicum*	8015-73-4	Vietnam
Cinnamon (leaves)	*Cinnamomum zeylanicum*	8015-91-6	Indonesia
Citronella grass	*Cymbopogon winterianus*	8000-29-1	China
Clove (leaves)	*Syzygium aromaticum*	8000-34-6	Indonesia
Dill (seeds)	*Anethum graveolens*	8006-75-5	Hungary
Oregano A	*Origanum vulgaris*	8007-11-2	Morocco
Oregano B	*O. vulgaris*	8007-11-2	Balkans
Red thyme	*Thymus vulgaris ct. thymol*	8007-46-3	Spain
Rosemary	*Rosmarinus officinalis*	8000-25-7	Morocco
Thyme	*T. vulgaris*	8007-46-3	Morocco

Table 2: Major components of selected essential oils (%, w/w).

Compound	CAS number	Basil	Citronella grass	Cinnamon(leaves)	Clove(leaves)	Dill(seeds)	Oregano A	Oregano B	Red thyme	Rosemary	Thyme
Eugenol	97-53-0			80–85	85–90						
Linalool	78-70-6	0–5	0–5	0–5		0–5	15–20	5–10	5–10	0–5	5–10
Benzyl benzoate	120-51-4			0–5							
Cinnamaldehyde	104-55-2			0–5							
Safrole	94-59-7			0–5							
Carvacrol	499-75-2						75–80	75–80	0–5		5–10
Thymol	89-83-8						0–5	5–10	50–55		10–15
R-(+)-Limonene	5989-27-5	0–5	5–10			50–55	0–5	0–5	5–10	0–5	0–5
β-Pinene	127-91-3									10–15	
Camphor	76-22-2									10–15	
Camphene	79-92-5									5–10	
α-Terpineol	98-55-5									0–5	
α-Pinene	80-56-8								5–10		5–10
Estragole	140-67-0	85–90									
Methyl eugenol	93-15-2	0–5									
R-(−)-Carvone	6485-40-1					50–55					
p-Cymene	99-87-6					0–5					
Geraniol	106-24-1		25–30								
Citronellol	106-22-9		15–20								
Citronellal	106-23-0		35–40								
Farnesol	4602-84-0		0–5								
Not determined					10–15				10–35	45–75	50–75
Total		85–105	80–110	80–105	85–90	100–120	90–110	85–105	65–90	25–55	25–50

A careful examination of the molecular formula shows that most of them would not have antioxidant properties (absence of phenolic structures or delocalized electrons) (Pokorný, Yanishlieva, & Gordon, 2006). For practical reasons, we decided to measure only the ones more likely to exhibit antioxidant behaviour. In order to choose the target components, their antioxidant capacity was first estimated from the antioxidant capacity of the essential oils (see Section 3) and by assuming additive properties. A system of linear equations was constituted from the data shown in Fig. 1 and Table 2:

$$AOXC_{ai}[ai] + AOXC_{aj}[aj] + \ldots + AOXC_{an}[an] = AOXC_a$$
$$AOXC_{bi}[bi] + AOXC_{bj}[bj] + \ldots + AOXC_{bn}[bn] = AOXC_b$$
$$\ldots$$

where AOXCai corresponds to the antioxidant capacity of the compound i belonging to essential oil a; [ai] is the concentration of the compound expressed in weight fraction, and AOXCa refers to the antioxidant capacity of the essential oil a. The antioxidant capacity of the essential oils and the concentration of their components were 'known' in this system of equations. These data constituted a system of 10 equations (10 essential oils) with 21 unknowns (21 compounds), which is an unknown system and, therefore, insolvable. Then, the number of unknowns was diminished manually. To this end, some compounds belonging to only one essential oil were pooled to calculate the contribution of all of them in a single value, resulting in a system of 11 equations with 13 unknowns. An approximate solution was found by using the Solver tool from Microsoft Excel as follows. A target cell was defined as the sum of the relative errors between predicted values and measured values of the antioxidant capacity of the essential oils. Subsequently, the value of the target cell was minimised by modifying the antioxidant capacities of the components, and including the restriction that all were greater than or equal to zero. According to the preliminary adjustment of the system of equations, four compounds showed much higher antioxidant capacity than the rest, eugenol, carvacrol, thymol and a-pinene. Eugenol, carvacrol and thymol are phenolic compounds and are supposedly antioxidants. However, a-pinene does not have this type of structure. Accordingly, the antioxidant capacity of these four compounds was measured, as well as R-(+)- limonene and linalool, because these compounds were present in many essential oils. Finally, the combined value of antioxidant capacity was calculated as the weighted sum of the individual values of antioxidant capacity. The combined value was divided by the value obtained from the measurement of the essential oil. Precision errors (three replicates) were carefully manipulated to calculate the resulting ratio error (Miller & Miller, 2005).

3. Results and discussion

Fig. 1 shows the antioxidant capacity of the studied essential oils expressed as g Trolox/g essential oil (gT/gEO). Oregano B (2.62 gT/gEO), clove (2.43 gT/gEO), oregano A (2.24 gT/gEO) and cinnamon (2.11 gT/gEO) showed the highest antioxidant capacity values according to ORAC assay, followed by red thyme (1.24 gT/ gEO) and thyme (0.79 gT/gEO). The rest of essential oils showed little or no antioxidant capacity.

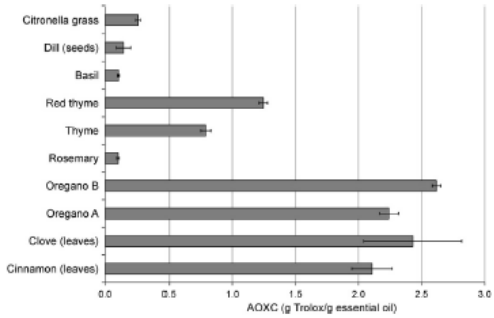

Fig. 1. Antioxidant capacity of the studied essential oils (g Trolox/g essential oil). Error bars correspond to the standard deviation of three replicates.

These results confirmed those previously found by Pezo et al. by in situ gas-phase hydroxyl radical generation and high-performance liquid chromatography- fluorescence

detection (Pezo, Salafranca, & Nerín, 2006, 2008). We wondered if the antioxidant capacity as measured by the ORAC assay is an additive property or, conversely, shows synergistic or antagonistic effects. Addressing this issue, we proceeded to measure the antioxidant capacity of the individual components. Table 2 shows the major components (above 5% w/w) of the essential oils provided by the manufacturer. Twenty-one chemicals in the ten essential oils under study are listed; with some chemicals belonging to more than one essential oil (e.g., linalool was present in all essential oils but clove). Most of them were terpenes and terpenoids. A mathematical approach was used to discriminate some of them, and the rest were chosen to be measured by the ORAC assay. The values obtained are shown in Table 3.

Table 3: Antioxidant capacity of the selected components (g Trolox/g pure compound, n = 3) according to the ORAC assay.

Compound	Antioxidant capacity(g Trolox/g pure compound, $\mu \pm \sigma$)
Eugenol	1.94 ± 0.17
Carvacrol	2.25 ± 0.05
Thymol	2.29 ± 0.08
α-Pinene	0.08 ± 0.03
R-(+)-Limonene	0.09 ± 0.03
Linalool	0.45 ± 0.02

Finally, the AOXC of the essential oils was calculated from the AOXC of the pure chemicals and the composition supplied by the manufacturer (Table 2). The results are shown in Fig. 2. As can be seen, 72–115% of the AOXC of the most antioxidant essential oils (above 0.50 gT/gEO) could be explained from their major components. In most cases, the predicted AOXC gave lower values than the measured AOXC of the essential oil. Nevertheless, red thyme showed higher predicted AOXC than measured AOXC. The differences between predicted and measured AOXC values could be explained in different ways: (1) there may be minor components with important antioxidant capacity, (2) the composition of the essential oils could differ from the composition provided by the manufacturer and (3) there are some synergistic effects that increase the ORAC assay response. That is to say, the sum of the antioxidant capacity of the components of a mixture is not equal to the antioxidant capacity of the mixture. After considering the precision of the measurements and the approximations performed during the previous discussion, the authors concluded that the AOXC of the essential oils can be roughly explained from their chemical composition when applying the ORAC assay. Near additive properties were also observed by Parker et al. with rutin, pcoumaric acid, abscisic acid, a sugar solution and ascorbic acid combinations (Parker, Miller, Myers, Miguez, & Engeseth, 2010). This performance is also illustrated in Fig. 3, which compares the decay curves of fluorescein in the ORAC assay of both the essential oil and the major constituents for cinnamon and oregano essential oils. As can be seen, the curves of cinnamon and its major component, eugenol, share the same shape. The same happens with the curves of oregano and carvacrol and thymol, which are clearly different from the ones of cinnamon or eugenol. This means that, if there is any difference between the AOXC of the essential oil and the AOXC of the major component, this is not reflected in the shape of the fluorescein decay curve. Therefore, the slight differences found between predicted and measured AOXC values were likely aused by the incertitude of the measurements or the composition of the essential oils. In conclusion, the ORAC assay showed near additive properties. This statement provides additional criterion when purchasing essential oils from different suppliers, or when considering the consumption/purchase of food products whose label shows ORAC data. It is also worth to emphasise that the ORAC assay is a simplification, as it reduces the complex antioxidant mechanisms to a reaction between the radicals produced by AAPH and the

studied antioxidant. The combined actuation of a mixture of antioxidants on different paths of the food oxidation processes, effectively acts in a synergistic way, providing extra protection higher than the addition of the effects of the single antioxidant components (Pokorny ´ et al., 2006).

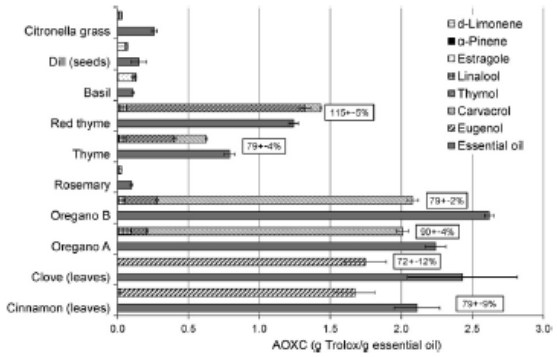

Fig. 2. Antioxidant capacity of the studied essential oils (g Trolox/g essential oil). Solid bars (below): measured antioxidant capacity of the essential oils. Pattern bars (above): weighted sum of the measured antioxidant capacity of the components. Error bars correspond to the standard deviation of three replicates.

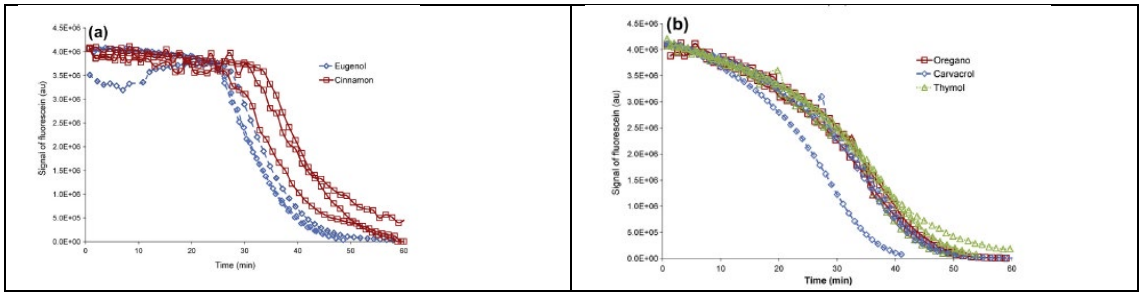

Fig. 3. Decay of fluorescein in its reaction with AAPH in the presence of: (a) cinnamon essential oil (solid line, squares), eugenol (dashed line, diamonds), and (b) oregano essential oil (solid line, squares), carvacrol (dashed line, diamonds), and thymol (dotted line, triangles).

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