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The peroxyl scavenger activity of the vegetal product *NATURAL SOD* measured using ORAC method

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Abstract

We report results regarding the stability of the peroxyl radicals scavenger activity of a green barley extract, studied by Oxygen Radical Absorbance Capacity (ORAC) assay. The tested product was developed and patented in Romania as Natural SOD. To investigate the stability of the mentioned product regarding its peroxyl radicals scavenging capacity, with respect to time, temperature, plant culture conditions and phases of the technological process, an adapted ORAC assay has been designed and validated. The results showed a time-dependent decrease of the scavenger activity. Increasing product storage temperature had a detrimental influence, ORAC values obtained at 25℃ and 37℃ being lower than those at 4℃. ORAC values also differed from one batch to another, depending on the culture conditions, while no significant involvement of the technological process was found. We showed that the adapted ORAC assay represented a valuable quality control method for the Natural SOD extract. Systematic product testing using ORAC technique could offer relevant information regarding the stability of the peroxyl radicals scavenger activity, as well as its quality and economic matters associated with Natural SOD production.

Key words : vegetal extracts, green barley, peroxyl radicals scavenger, ORAC

Introduction

Free oxygen radicals, or equivalently-reactive oxygen species (ROS), are oxygen containing single or multi-atom structures, possesing one or more unpaired electrons (Halliwell & Gutteridge, 1984 [1]). These systems involve very unstable configurations, leading to extremely high chemical reactivities and consequent quick reactions with other radicals or molecules in their surroundings. In a controlled environment, these reactions need to be avoided, ROS can be inactivated by addition of specific scavenger products (ROS scavengers) that preferentially bind oxygen radicals, making them unavailable for other unwanted reactions.

Among the commonly studied ROS (•HO, O_2^-•, R-OO•) the peroxyl radicals (R-OO•) play a special role due to their importance in biological systems (Niki, 1987 [2]; Kohen & Nyska, 2002 [3]; Goldstein & Samuni, 2007 [4]), in atmospheric reactions (Atkinson & Arey, 2003 [5]), as well as in industry (Bonifačić et al, 1991 [6], Alfassi et al, 1989 [7], Nandi et al, 2002 [8], Flyunt et al, 2003 [9]). The peroxyl radicals react with inorganic and organic compounds, either by electron transfer, hydrogen abstraction or addition, the determination of their reaction rate constants making the subject of a significant number of published works (Neta et al, 1990 [10]). Since peroxyl radicals generally appear as end-products of O_2 reactions with alkyl radicals (R•), which may be generated by different pathways in the presence of organic sources (Neta et al, 1990 [10]), their action induces detrimental effects

especially in biological systems (Spiteller, 2006 [11]). Therefore, the search for products with appropriate R-OO• scavenger properties is of actual interest.

In our study we have used an adapted Oxygen Radical Absorbance Capacity (ORAC) method (Cao & Prior, 1998 [12]), to evaluate the preservation/constancy/stability/ of the peroxyl radical-scavenger properties of the green barley extract "*Natural SOD*", patented in Romania (OSIM patent No. 142622, 1989) by „Cantacuzino" National Institute of Research and Development for Microbiology and Immunology (Szegli et al, 1989 [13]).

ORAC is one of the best standardized methods to determine the scavenger activity of a compound/product against oxygen radicals (Honzel et al, 2008 [14]), the R-OO• peroxyl radical being considered the standard radical of the method (Awika et al, 2003 [15]). The assay quantifies the capacity of a tested product to block the action of peroxyl radicals upon a fluorescent substrate (2′,7′-Dichlorodihydrofluorescein diacetate), which is degraded and loses its fluorescence when reacts with peroxyl radicals. In ORAC technique, these radicals are generated by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). The ORAC assay is a very sensitive method, it is inexpensive, no special equipment is needed, except the microplate reader, and it allows high sample throughput. A further advantage of the ORAC assay is its reaction conditions, which reflect a physiological milieu with a pH value of 7.4.

The development of *Natural SOD* product by "Cantacuzino" National Institute of Research and Development for Microbiology and Immunology in Romania, was motivated by the reports of Hagiwara (1985), who conducted studies on 150 different plants and revealed the multitude of nutrients, chlorophyll, enzymes and oxygen radical-scavengers contained by the green barley extract obtained from young plants (maximum 20-25 cm height) (Hagiwara, 1985 [16]).

The biochemical characterization of *Natural SOD* product revealed a rich content of low molecular weight compounds, such as vitamins (C, E, A), glucides, flavonoids, seven essential aminoacids and essential oligoelements (K, Na, Zn, Mg, Ca, Mn, P) (Cremer et al., 1998 [17]). Designed to be administered *per os* only, *Natural SOD* is well tolerated by the human body, inducing no significant side effects (except for celiac patients who should not use the product because of its gluten content). *Natural SOD* possesses anti-inflammatory properties, mainly given by its micromolecular components prone to scavenge oxygen radicals and to down-regulate TNF production, two types of pro-inflammatory mediators produced by specialised cells (Cremer et al., 1996 [18], Cremer et al., 1998 [17]).

In this study we aim to assess the adequacy of the ORAC protocol described in detail below, to be used as an internal quality control method to test the stability of *Natural SOD* product, with respect to its peroxyl radical-scavenger capacity.

Materials and Methods

1. The manufacturing process for obtaining the green barley extract „Natural SOD"

According to the patented technology (Szegli et al., 1989 [13]), the obtaining of the green barley extract *Natural SOD* consists in several operations, as described in Fig.1. Briefly, the raw material was pressed by using a hydraulic press (600 atm) for removing the cellulose part of the plant. The obtained mixture was centrifuged to separate the liquid phase of interest. Finally, the extract was sterile filtered through Millipore nitrocellulose membrane (0.22 μm).

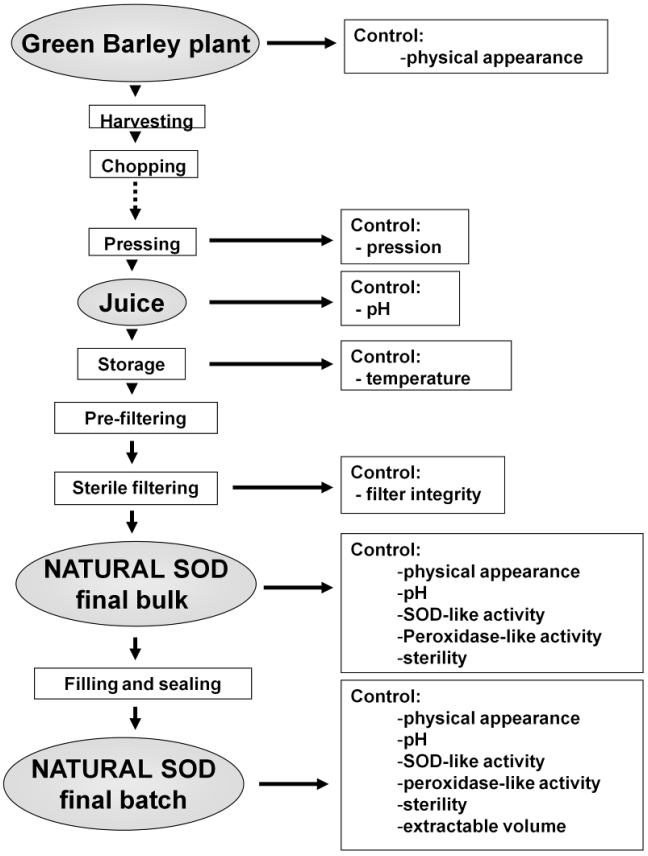

Figure 1. Schedule of the technological process for obtaining *Natural SOD*

One can presume that the majority of metal ions in the green barley extract are found as kelates (in complex with aminoacids or with glycopeptides). These findings sustained the fact that we obtained a green barley juice rich in essential oligoelements, comparable to Hagiwara's "Green Barley Essence" extract. Even if *Natural SOD* has a lower content of Ca and Mg, it contains more Na and P (Cremer et al., 1998 [17]), the late element beeing particularly important for its quality as good energizer.

2. Peroxyl Radicals Scavenger Capacity

a. Samples selection for each particular study

All the tested *Natural SOD* samples were randomly selected and they were during the period of validity, as indicated by the manufacturer. Preparation of samples for all experiments was made at room temperature (25ºC±2ºC).

b. ORAC assay for measuring peroxyl radicals scavenger activity of hydrosoluble products

Briefly, in order to quantify the peroxyl scavenger activity of *Natural SOD*, we validated a hydrophylic ORAC technique adapted for a Fluorimeter/Chemiluminometer (Fluoroskan FL, Thermo) equipment. The method had two phases: a) the manual phase (20-25^{0}C); b) the automatic phase (37^{0}C).

Controls (blank samples) and the tested products were measured simultaneously, containing equal amounts of AAPH and fluorescein. The oxygen radical-scavenger capacity was evaluated with respect to the reference standard (S)-(-)-6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid = Trolox, the synthetic analogue of the hydrosoluble E vitamin.

Thus, in a 96 wells flat-bottom plate, 150 µl of 4 nM Fluorescein Sodium Salt solution in phosphate buffer pH=7.4 were added to each well. To avoid the margin errors, in the outskirts wells, the Fluorescein volume was 175 µl and the corresponding results were not taken into consideration. Then, the following reagents were added: (1) 25µl phosphate buffer saline pH=7.4 for „blank" wells; (2) 25µl of the corresponding solutions for Trolox ((S)-(-)-6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) standard curve (in phosphate buffer saline pH=7.4): 100µM, 50µM, 25µM, 12.5µM, 6.25µM, and 3.125µM; (3) 25 µl sample (4 wells / sample).

For the automatic phase, we used a Fluorimeter/Chemiluminometer (Fluoroskan FL, Thermo) equipment, with a dispenser as endowment and connected to a PC with a dedicated Ascent software. The protocol consisted of several successive steps, as follows: strong stirring (10s, 1200rpm, d=1mm); incubation (30 min, 37ºC); fluorimetric reading (ex. 485nm and em. 523nm) to detect autofluorescence (Integration time 20ms, Lag time 0s, Beam – normal, Unit – Trolox Eq); automatic dispensing to each well of 25 µl AAPH (2,2'-azobis-2-amidino-propane dihydrochloride), 0.25M in phosphate buffer saline, pH=7.4 (prepared prior to use); strong stirring (10s, 1200rpm, d=1mm); fluorimetric reading (485nm and 523nm, 50 complete cycles, Δtime between readings = 1 min); data processing.

All chemicals were purchased from Fluka.

With the aid of the fluorimeter dedicated Ascent software, results were calculated by substracting the blank area from the net area under the fluorescence decreasing curve in the presence of the sample and they were related to the net area corresponding to 1 µM Trolox. The obtained values were multiplied with the dilution factor. The measurements were performed three times (4 wells/sample/experiment). Final data were expressed as Trolox equivalents/liter (TE/l); 1 TE = 1 µM Trolox.

In every ORAC assay, Gallic Acid (50µM), as a known peroxyl radicals scavenger (Prior et al., 2005 [19]), has been run along with *Natural SOD* samples.

c. Peroxyl radicals scavenger activity measured by ORAC method on a significant number of Natural SOD batches

By using the ORAC method, we measured the peroxyl radicals scavenger activity of 14 randomly selected batches of *Natural SOD*, as follows: 3 batches produced in 2010 (06-10; 07-11; 15-10; 39-10), 3 batches produced in 2011 (08-11; 11-11; 49-11) and 7 batches produced in 2012 (03-12; 04-12; 05-12; 13-12; 16-12; 21-12; 24-12).

d. ORAC method applied for Natural SOD samples taken at different time-points of the technological process

The peroxyl radicals scavenger activity of three randomly selected batches of *Natural SOD* was measured by ORAC assay. Samples were taken at three important time-points of the technological process (after pressing – juice; after sterile filtering – final bulk; after filling and sealing – final batch).

e. Time-depending stability of Natural SOD at 25°C and 37°C

The antioxidant capacity against peroxyl radicals of the tested vegetal extract was tested by ORAC assay (Prior et al, 2005 [19], Ou et al, 2002 [20], Prior et al., 2003 [21], Hernandez-Ledesma, 2007 [22]), performed every 30 days, for a period of 1 year.

A 12 months study regarding the stability of *Natural SOD* at room temperature (25ºC±2ºC) and 37ºC±2ºC was also performed. Parameters like macroscopic appearance (According to E.P. 7.0 / 01 / 2011 / 2.2.1, 2.2.2) and pH (According to E.P. 7.0. / 01 / 2011 / 2.2.3) of all the tested *Natural SOD* samples were within the limits of acceptability and they did not have any influence on the working conditions for ORAC assay. The mixture between *Natural SOD* and phosphate buffer used for samples dilution had no influence on pH value of the reaction mixture used in ORAC assay (no significant errors could be generated).

ORAC assay was performed each month for *Natural SOD* samples stored at 25ºC±2°C and at 37ºC±2ºC. In conjuction with these samples, *Natural SOD* samples stored at 4°C±2°C C were also tested each month.

f. ORAC assay applied for Natural SOD subject to temperature forced increase

In order to avoid the potential loss of the scavenger activity of *Natural SOD*, if the product should be accidentally exposed to increased temperatures, we have tested *Natural SOD* subject to temperature forced increase (between 25ºC si 90ºC). Measurement of peroxyl radicals scavenger activity was made by ORAC technique.

In these experiements we used *Natural SOD* vials coming from batches 05-10; 08-10; 25-10; before testing, the vials were stored at 4ºC±2ºC. Using a thermo block TDB-120 (Biosan), each sample has been subjected to temperature forced increase for 30 minutes, after which the antioxidant activity has been measured by ORAC assay.

3. *Statistics and ORAC assay performance parameters*

In all experimental models, the measurements were performed three times (4 wells /sample /experiment). The results were given as mean ± standard deviation (SD). For verification purpose, in every assay, solutions corresponding to 2 points in the calibration curve (10μM and 75μM Trolox) were also run. The accepted coefficient of variation (CV) for every sample was ≤10%.

For the ORAC assay, the following performance parameters were calculated: repeatability, reproducibility, linearity (correlation coefficient), limit of quantification (LOQ), limit of detection (LOD).

Results and Discussions

Peroxyl radicals scavenger activity measured by ORAC method on a significant number of Natural SOD batches

By using the ORAC validated method, we measured the peroxyl radicals scavenger activity of 14 randomly selected batches of *Natural SOD* (Fig. 2).

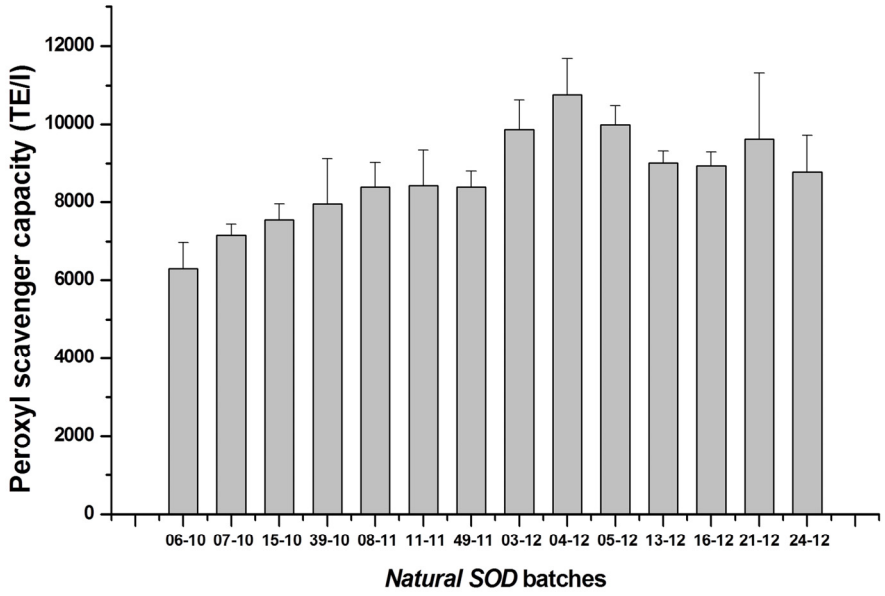

Figure 2. Peroxyl radicals scavenger activity measured by ORAC method for 14 batches of *Natural SOD*. The results are given as mean ± standard deviation (SD).

The obtained values showed a significant variability of peroxyl radicals scavenging capacity corresponding to samples from different batches of *Natural SOD*. These differences could be due to:

- plant quality, as a result of culture conditions (climatic conditions, abundance of nutrients in the soil), time of harvesting, plant storage before its processing for extract obtaining;
- vegetal extract quality - this is unlikely in view of the fact that the processing for obtaining the final batch and the storage of *Natural SOD* were made in compliance with a series of specific procedures which equally influenced all the tested samples.

ORAC method applied for Natural SOD samples taken at different time-points of the technological process

Results obtained for ORAC assay applied for Natural SOD samples taken a three important time-points of the technological process are presented in Fig.3.

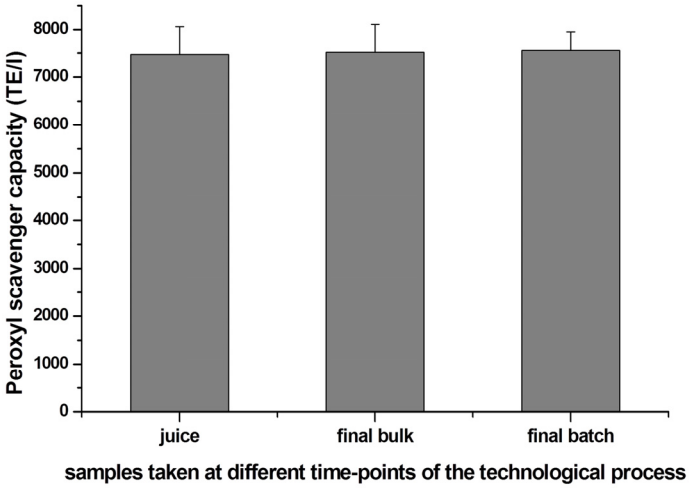

Figure 3. Peroxyl radicals scavenger capacity of *Natural SOD* samples taken at different time-points of the technological process. The results are given as mean ± standard deviation (SD).

The obtained values were almost equal: 7473.500 ±591.154 TE/l for juice, 7519.500 ±594.792 for final bulk and 7555.520 ± 398.960 TE/l. The above mentioned hypothesis that the observed variations of the peroxyl radicals scavenger activity of *Natural SOD* depended mostly on the plant culture conditions, rather than product manufacturing phases, was also supported by the fact that no significant involvement of the technological process was detected.

Any detection of a low peroxyl scavenging capacity in different time-points of the technological process should exempt costs incurred in later phases, in order to obtain a final batch. In addition, the low variability of peroxyl scavenging capacity, between the first and the last time-point, confirms the fact that the technological process does not influence the antioxidant properties of the product and does not decrease its quality.

Time-depending stability of Natural SOD at 25°C and 37°C

Results obtained for the peroxyl scavenger activity of *Natural SOD* samples, stored at 25ºC±2°C and 37ºC±2ºC for 12 months, measured by ORAC technique are presented in Fig.4, by comparing with samples stored at 4°C±2°C.

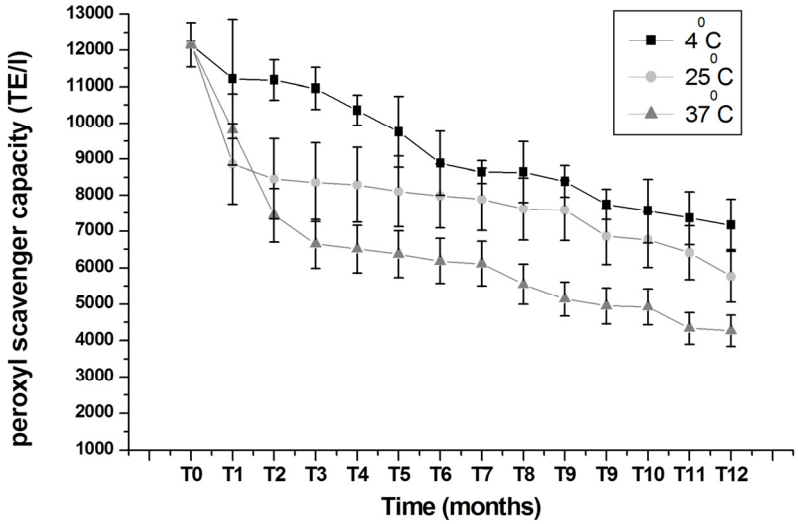

Figure 4. Variation of peroxyl radicals scavenger activity measured by ORAC for Natural SOD stored for 12 months at 4°C, 25°C, 37°C; results represent average values ± Standard Deviation (SD) for 3 batches of *Natural SOD*

This graph showed a time-dependent decrease of peroxyl scavenger capacity for all the tested temperatures (4°C±2°C, 25°C±2°C and 37°C±2°C). Average values obtained for 25°C±2°C were lower than those obtained for 4°C±2°C, while the lowest amounts corresponded to samples stored at 37°C±2°C. These data support the hypothesis that the temperature increase has a negative influence on peroxyl radicals scavenging capacity.

The results displayed in Fig.4 revealed sudden drops of the mean ORAC values during the first 3 months of storage at 25°C±2°C and 37°C±2°C. While for these two temperatures the obtained average values were 31.209% and 45.266% respectively, lower than To, for the samples stored at 4°C±2°C, where only a 9.888% decrease was detected. For longer storage times (3-12 months), the decrease rates of the ORAC values for the three tested temperatures were in aproximately the same range. The final values (after 12 months) were with 41.039% , 52.504% and 64.866% lower than the initial ones for 4°C±2°C, 25°C±2°C and 37°C±2°C respectively.

The above results indicate and support the adequacy of the described ORAC assay to detect variations of the peroxyl radical scavenging capacity of the *Natural SOD* extract, with respect to its storage temperature and time.

ORAC assay applied for Natural SOD subject to temperature forced increase

The peroxyl radicals scavenger activity of three randomly selected batches of *Natural SOD*, subject to temperature forced increase (05-10; 08-10; 25-10), was measured by ORAC assay (Fig.5)

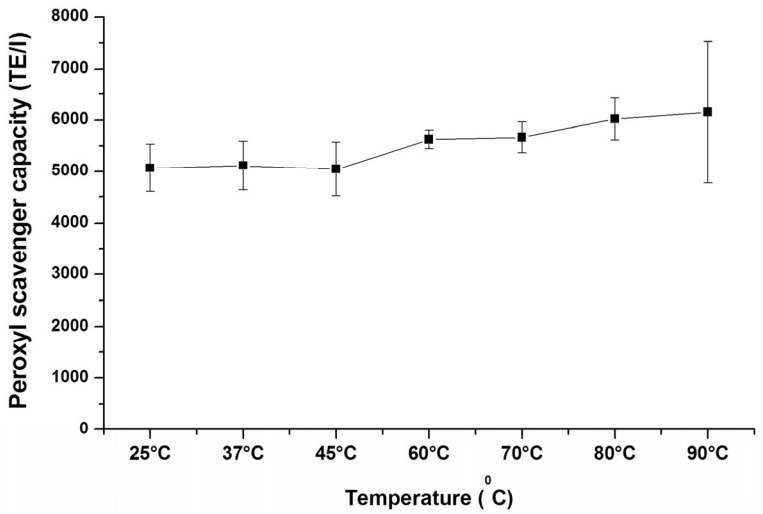

Figure 5. Peroxyl radicals scavenger capacity measured by ORAC method for Natural SOD subject to gradual increase of temperature. Results represent average values ± Standard Deviation (SD) for 3 batches of *Natural SOD*

Data presented in Fig. 5 showed that *Natural SOD* maintained the ability to scavenge peroxyl radicals, even after an exposure for 30 minutes at 45°C. Results obtained for higher values of temperature did not have a clear biological significance, and may be experimental artifacts due to thermal degradation of various components of the product.

This statement was also sustained by the increased values of standard deviation at 90°C. We mention that only the product to be tested has been heat-treated, the ORAC assay beeing made in accordance with the conditions already validated. In these circumstances, changes obtained were due strictly to the product quality change, a coherent and well-founded explanation requiring additional testing.

In every ORAC assay, a known peroxyl scavenger, Gallic Acid 50μM (Sigma), has been run along with *Natural SOD*. For Gallic acid, ORAC obtained value from all experiments was 1.096 ± 0.024 TE/μmol. This result was in accordance with literature data (Prior et al, 2003[21]) that reported ORAC value 1.120 ±0.071 TE/μmol for 50μM gallic acid).

Performance parameters of ORAC assay

We obtained very good performance parameters for the ORAC assay. Repeatability: ±10%. Reproducibility: ±9.68%. Linearity: Correlation coefficient (R^2) were >0.950, with values between 0.959-1.000, showing comparable results with other papers performing the ORAC assay (Price et al. 2006 [23]) where R^2 was between 0.940-0.999.

Limit of quantification (LOQ), defined as the lowest concentration on the calibration curve, was 3.125□M, whereas limit of detection (LOD), defined as the lowest amount of peroxyl radicals scavenger that can be detected was 1□M. Values cited in the literature for the ORAC method are 6.25□M for LOQ and 5.0□M for LOD (Huang et al., 2002 [24]). Expanded uncertainty was 7.91%, for the coverage factor k=2 and the confidence level of 95%.

Conclusions

In the present study, the ORAC assay, which provided a simple, but very accepted method for the assessment of the peroxyl scavenger activity, was adapted to Fluoroskan FL fluorimeter/chemiluminometer (Thermo), and then it was validated on this equipment. The method validated by our group is high sensitive, with a running time of 50 minutes and a sample throughput of 8 samples per each run.

The results obtained by testing 14 batches of *Natural SOD* have demonstrated the need to apply the current ORAC assay for measuring peroxyl radicals scavenging capacity of this product. The peroxyl radicals scavenging capacity of the green barley extract *Natural SOD* can vary significantly, depending on the culture conditions. Taking into account that, using plants of poor inappropriate quality, could lead to the obtaining of an end-product of bad quality, it is advisable to apply the ORAC assay for samples taken at different time-points of the technological process; the results obtained could provide important information, either for the product quality, or for the economic matters associated to *Natural SOD* production.

Test performed on samples taken at different time-points of the technological process revealed almost equal values for the three types of samples (juice, final bulk and final batch), which proved the technological process efficiency, in the sense that peroxyl radicals scavenging capacity was not affected by different operations performed during the manufacturing process. In addition, identification of a low scavenging activity in the first time-points of the technological process exempts costs incurred in later operations and avoids getting a poor quality product.

The peroxyl radical-scavenger capacity of *Natural SOD* showed a time-dependent decrease for samples stored at 4°C±2°C, 25°C±2°C and 37°C±2°C. Moreover, the temperature increase had a negative influence on the average ORAC values, found to be significantly lower for 25°C±2°C and 37°C±2°C, in comparison to the corresponding samples stored at 4°C±2°C. The lowest scavenger activities corresponded to samples stored at 37°C±2°C.

To investigate the potential loss of the scavenger activity of *Natural SOD*, assuming its accidental exposure to temperatures higher than its recommended storage temperature (4°C±2°C), we have tested samples thermally treated at 25ºC, 37ºC, 45ºC, 60ºC, 70ºC, 80ºC and 90ºC. *Natural SOD* maintained its ability to neutralize peroxyl radicals, even after a exposure of 30 minutes at 45°C.

The above results indicate and support the adequacy of the described ORAC assay to detect variations of the peroxyl radical scavenging capacity of *Natural SOD* extract, with respect to different parameters: temperature (storage temperature or acute variation of temperature), time, green barley culture conditions or phases of the technological process.

In conclusion, the ORAC assay, as validated above, could be introduced as a control method during the technological process for obtaining *Natural SOD* vegetal extract. ORAC method, developed and validated in our group, is adequate for this purpose.

Acknowledgments

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