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Long-Wavelength Fluorimetric Determination of Food Antioxidant Capacity Using Nile Blue as Reagent

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ABSTRACT: A method for the determination of the antioxidant capacity using long-wavelength fluorescence measurements is described for the first time. This method is a modification of the conventional oxygen radical absorbance capacity (ORAC) method that uses fluorescein or phycoerythrin and the generator of peroxyl radicals, 2,2′-azo-bis-(2-methylpropionamidine) dihydrochloride (AAPH). The long-wavelength fluorophor nile blue is proposed as an analytical reagent alternative to these conventional fluorophores. Kinetic curves have been obtained by monitoring the fluorescence variation (λ_{ex}, 620; λ_{em}, 680 nm) with time, using the 96-well microplate format. The vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) has been chosen as the model analyte, and the normalized area under the decay curve has been used as the analytical parameter. The dynamic range of the calibration curve is 0.8−8.0 μM, and the detection limit is 0.45 μM. The precision of the method, expressed as relative standard deviation and assayed using 1 and 5 μM Trolox concentrations, was 5.6 and 2.9%, respectively. The method has been applied to the analysis of fruit juices and wines, obtaining results that did not differ significantly from those provided using the ORAC method with fluorescein as reagent.

KEYWORDS: Nile blue, antioxidant capacity, long-wavelength fluorimetry, 96-well plate

INTRODUCTION

Oxidation processes involving free radicals contribute to the development of many types of illnesses, such as, e.g., cardiovascular disease, Alzheimer's disorder, and cancer. This redox phenomenon is produced by reactive oxygen species (ROS), which damage cell membranes, with this effect being reduced by the ingest of antioxidant compounds. The antioxidant capacity of foods is mainly given by polyphenols, such as flavonoids, and vitamins C and E, among others.[1] From an analytical point of view, there are two types of methods for assessing this parameter. The first group involves a single electron-transfer reaction, which can be followed by a change in the color as the oxidant species is reduced. The second involves the use of hydrogen atom transfer reactions, in which the antioxidant and the substrate compete for the free radicals generated. The oxygen radical absorbance capacity (ORAC) assay is an example of the latter methods. This assay involves the use of 2,2′-azo-bis-(2-methylpropionamidine) dihydrochloride (AAPH) to give rise to peroxyl radicals that directly attack absorbing or fluorescent probes, leading to the quenching of their absorbance or fluorescence, respectively. The dyes β-phycoerythrin[2−4] and fluorescein (FL)[1,5−14] have been by far the most used fluorescent probes, although Pyrogallol Red has also been described to develop photometric approaches for the determination of the antioxidant capacity in berry extracts[15] and human blood plasma and urine.[16] This assay relies on the performance of photometric measurements of Pyrogallol Red bleaching by action of the peroxyl radical generator, AAPH. It has been reported that this dye allows for the separate estimation of the contribution of ascorbic acid and some polyphenols to the total antioxidant capacity of fruits with high ascorbic acid concentration, thus allowing for the determination of ascorbic acid in complex samples. The ORAC assay has also been used to assess, e.g., the antioxidant capacity of human milk.[14]

Despite the high sensitivity that the above-mentioned fluorophores offer, these compounds feature a relatively short Stokes shift (about 27 nm), which favors scattering phenomena. Also, β-phycoerythrin is relatively expensive and suffers from photobleaching, with this fact being the main one responsible for the almost widespread use of FL as a probe for the ORAC assay. However, this fluorophor shows a limitation common to organic conventional fluorophores that emit in the 400−550 nm range, which is an increased influence of static background signals from the sample matrix. The usefulness of long-wavelength dyes as fluorescent probes to overcome this limitation has been previously demonstrated in several analytical methods,[17] but their potential application to the determination of the antioxidant capacity has not been studied up to now.

Nile blue (NB) is a dye from the oxazine group (Figure 1), which emits light in the red region of the spectrum and features a Stokes shift of about 60 nm. This reagent has been previously used for the fluorimetric determination of the synthetic antioxidant butylhydroxyanisole (BHA)[18] and the photometric determination of cerium(IV).[19] Other recent applications of this fluorophor have been its use in energy- and electron-transfer reactions in covalently functionalized carbon nanotubes[20] and to describe a hydrogen peroxide biosensor from its immobilization, together with horseradish peroxidase.[21]

The work described here involves a modification of the ORAC assay using NB as the fluorescent probe and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as the reference standard for antioxidant capacity. The method has achieved its

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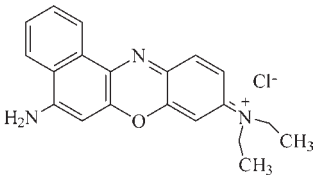

Figure 1. Chemical structure of NB chloride.

automation using a microplate reader, which monitored the variation of the fluorescence intensity with time using 620 and 680 nm as excitation and emission wavelengths, respectively. The proposed method has been applied to the analysis of fruit juice and wine samples, and the results obtained have been compared to those provided by the ORAC approach using FL as the reference method, with no statistically relevant differences between the results given by both methods. Also, a recovery study was carried out to validate the usefulness of the proposed method to the analysis of real samples, which has been scarcely reported in the ORAC methods described up to date. These results confirm the usefulness of NB as an analytical reagent for antioxidant capacity determination with better spectral selectivity than the ORAC−FL assay, owing to the use of long-wavelength fluorescence measurements.

MATERIALS AND METHODS

Instrumentation. A 1420 multilabel counter Victor ^{3} V microplate reader (Perkin-Elmer and Analytical Sciences, Wallac Oy, Turku, Finland) was used to perform fluorescence measurements. Different filters (nominal wavelength/passband) were used to select the excitation (485/15 nm for FL and 620/8 nm for NB) and emission (535/25 nm for FL and 680/10 nm for NB) wavelengths used to monitor ORAC−FL and ORAC−NB systems, respectively. Each measurement was obtained in 0.5 s, and the subsequent measurements for each well with time were made in 1 min intervals.

Reagents. All reagents used were of analytical grade. NB chloride was supplied by Sigma (St. Louis, MO), and Trolox and AAPH were obtained from Aldrich (Milwaukee, WI, and Steinheim, Germany). Dipotassium hydrogen phosphate was purchased from Merck (Darmstadt, Germany). Phosphate buffer solutions (0.085 M at pH 6.9 and 0.075 M at pH 7.5) were prepared by dissolving appropriate amounts of dipotassium hydrogen salt and adjusting the pH values with hydrochloric acid to develop ORAC−NB and ORAC−FL methods, respectively. A NB stock solution (29.4 μM) was prepared by dissolving the appropriate amount of the dye in distilled water by magnetic stirring for 24 h and stored at room temperature. Working standard solutions of 89 nM NB were prepared daily by diluting the appropriate volume of the stock solution in phosphate buffer solution (0.085 M at pH 6.9). A stock 4.5×10^{-4} M FL solution was prepared by dissolving the appropriate amount of sodium fluorescein in distilled water, and subsequent dilutions were made daily in phosphate buffer (0.075 M at pH 7.5) to prepare working 70 nM FL solutions. A 0.012 M AAPH solution was also prepared daily in phosphate buffer solution (0.085 M at pH 6.9) and kept at room temperature protected from light to prevent its degradation.

Procedures. *Determination of the Antioxidant Capacity by the ORAC−NB Method.* A volume (20 μL) of standard (0.8−8 μM Trolox), wine or juice diluted sample, or blank (0.085 M phosphate buffer at pH 6.9) solutions was added to each well together with 120 μL of 89 nM NB. This mixture was preincubated in sealed plates at 37 °C for 15 min, and then, 60 μL of 0.012 M AAPH was added to each well using an eight-channel electronic micropipet to achieve the simultaneous addition of this reagent. Immediately, the plate was inserted into the microplate reader, and the variation of the fluorescence intensity with time was monitored at 37 °C for 60 min, using the instrumental conditions indicated above. Each measurement was performed in triplicate, and a blank triplicate was recorded at the beginning of each series to control potential changes that may occur, owing to the lack of stability of AAPH, which was kept protected from light at room temperature to ensure identical thermal conditions at time 0 of the reaction. The decay curves were integrated using Origin software, and then, the normalized net area under the curve (AUC) was calculated by subtracting the blank signal from the signal obtained in the presence of the standard or sample and dividing the result by the blank signal.

Determination of the Antioxidant Capacity Using the ORAC−FL Method. The ORAC−FL measurements were performed according to the instructions described elsewhere,[8] which involve the use of a microplate reader and FL as reagent. Briefly, 20 μL of standard or diluted sample solutions were mixed with 120 μL of 70 nM FL in phosphate buffer (0.075 M at pH 7.4) for 15 min at 37 °C, and then, 60 μL of 0.012 M AAPH was added. The variation of the fluorescence intensity with time was monitored for 60 min using 485 and 535 nm filters to select excitation and emission wavelengths, respectively. The curves obtained were processed in the same way as that described for the ORAC−NB method.

Determination of the Antioxidant Capacity in Commercial Wine and Fruit Juice Samples. Several wines (white, semi-dry, and red) and fruit juices (peach, pineapple, and apple) were bought in a local market and analyzed immediately after they were opened according to the following procedure: A volume (4.5 mL) of sample was treated with 300 μL of 1 M NaOH to increase the pH to neutral values, and it was raised to 5 mL with phosphate buffer. Then, an adequate dilution (1:500−1:10 000 dilutions) with the same phosphate buffer was performed to match the dynamic ranges of the calibration curves of either the ORAC−FL or ORAC−NB method. A volume (20 μL) of the diluted sample was treated according to the procedures indicated above for both methods.

RESULTS AND DISCUSSION

Selection of the Long-Wavelength Fluorophor. ROS, such as peroxyl radicals (ROO•), hydroxyl radicals (OH•), superoxide ion ($O_2^{-\bullet}$), and singlet oxygen ($^1O_2$) are involved in the physiology of some diseases. Radical chain-breaking antioxidants convert reactive free radicals into stable and non-aggressive molecules by mechanisms in which AAPH radicals formed in air-saturated solutions react rapidly with molecular oxygen to give rise to peroxyl radicals, ROO•, as described elsewhere.[6] The presence of antioxidant compounds gives rise to the formation of a hydroperoxide and a stable antioxidant radical that breaks the action of peroxide radicals. Although a wide variety of antioxidant compounds can be used as reference standards in ORAC assays,[13] such as gallic, caffeic, and ascorbic acids, the vitamin E analogue, Trolox, has been chosen to develop the ORAC−NB method presented here.

The reactions involving the formation of free radicals can be followed by the decrease in the inhibition of the fluorescence from some organic molecules, such as phycoerythrin and FL, in the presence of samples with antioxidant capacity.[1−14] However, the short Stokes shift of these compounds can give rise to light-scattering phenomena that could affect the performance of fluorescence measurements. Also, β-phycoerythrin shows a low photostability, and its price is relatively high. With the aim of studying the potential use of fluorescent dyes that emit in the red region of the spectrum as alternative reagents for this purpose, several oxazine and thiazine dyes, namely, NB, azure A, and azure B, were assessed. Figure 2 shows the curves obtained for each fluorophor, in the presence and absence of Trolox, which was used as a standard. Although it was possible to measure the

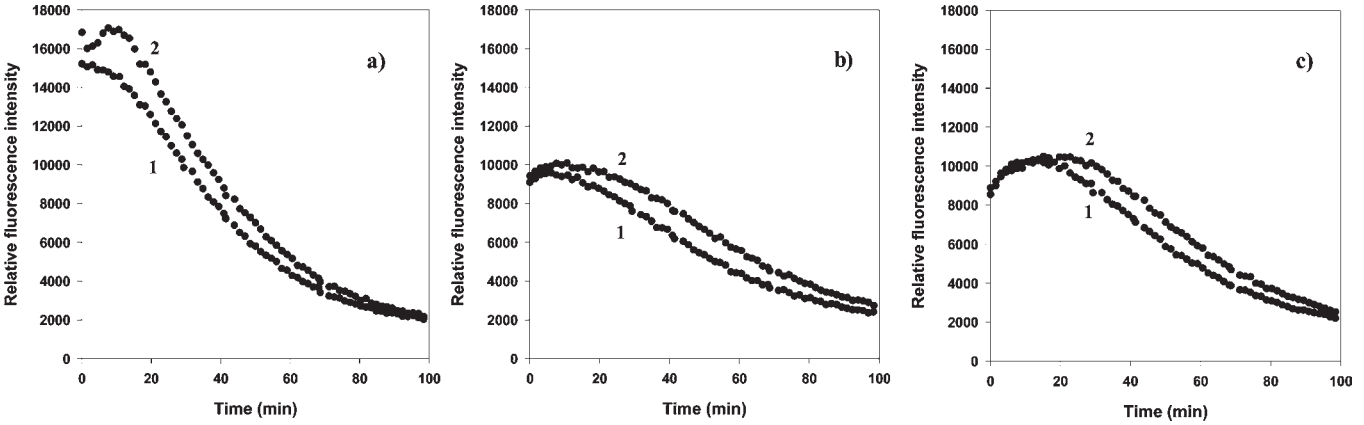

Figure 2. Curves obtained for the ORAC assay using (a) NB, (b) azure A, and (c) azure B fluorophores: (1) blank and (2) 2 μM Trolox.

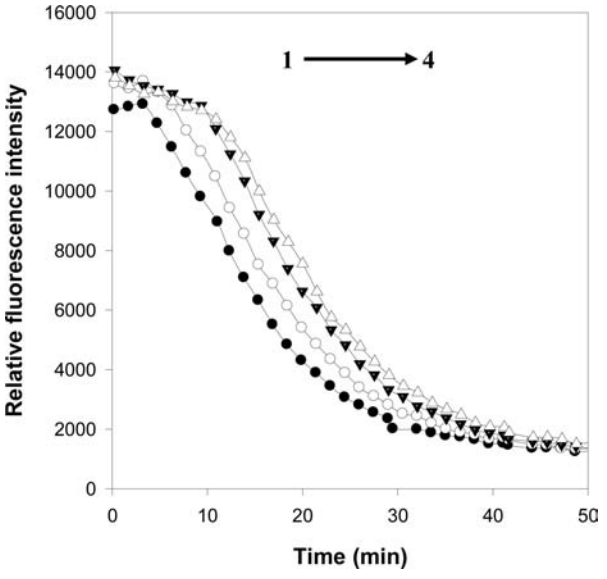

Figure 3. Antioxidant capacity curves obtained at different Trolox concentrations: (1) 0 μM, (2) 1 μM, (3) 2 μM, and (4) 4 μM. Experimental conditions: [NB], 89 nM; [AAPH], 0.012 M; pH, 6.9; [phosphate], 0.085; and temperature, 37 °C.

difference between the blank and standard with all of the fluorophors assayed, NB gave the best results. Also, NB was chosen because it has a wider Stokes shift that the other two reagents, which avoids potential light-scattering signals. The decay curves obtained in the presence of NB, AAPH, and different Trolox concentrations are shown in Figure 3, in which the AUC increased with the Trolox concentration.

Optimization of the ORAC−NB Method. The variables affecting the system were optimized by the univariate method. Each result was the average of three measurements. The analytical parameter used to optimize the system was the net AUC, calculated as indicated above in the procedure of the ORAC−NB method.

The influence of the pH was investigated in the range of 4−11, using 0.075 M acetate, phosphate, borate, and carbonate buffer solutions to keep the pH constant in the buffering region of each solution. As Figure 4A shows, there was not appreciable net AUC at pH values below 5.8 and above 8. The behavior of the system at low pH values could be ascribed to the fact that the pK_a value of Trolox is about 3.89, showing a low solubility at this pH.[22] The solubility increases with the pH, which improves the value of the net AUC, obtaining the best results in the pH range of 6.0−7.0. A pH of 6.9 was chosen, which is slightly lower than that required to develop the ORAC−FL method (pH 7.4). The influence of the buffer concentration was studied by assaying phosphate concentrations in the range of 0.02−0.15 M, finding that a 0.085 M phosphate buffer solution gave the best signal.

NB and AAPH concentrations are two critical variables that are interrelated. The influence of the fluorophor concentration was studied by adding a fixed volume (120 μL) of solutions with NB concentrations ranging from 60 to 370 nM (Figure 4B). The system was practically independent of this variable in the range of 80−130 nM, choosing 89 nM NB for the development of the method. The influence of the peroxyl radical generator, AAPH, was evaluated in the range of 0.006−0.024 M, finding that the AUC signals of both analyte and blank solutions decrease, with the difference also decreasing between both signals, when the AAPH concentration increases. However, the curves obtained at low AAPH concentrations were less defined and showed a low reproducibility. Thus, a 0.012 M AAPH concentration was chosen.

The temperature is an important variable in kinetic studies, and also, it has a remarkable effect on the decomposition of AAPH,[7] mainly at values close to 37 °C. The influence of this variable on the system was studied by mixing NB with Trolox for 15 min at 37 °C, and after the addition of 60 μL of 0.012 M AAPH, the mixture was kept at different temperatures, in the range of 25−40 °C. The kinetic curves obtained at 25 °C were relatively slow, providing a net AUC value of 1.5 times lower than that obtained at 37 °C. This temperature was chosen to develop the method because the curves obtained were also more defined, which could probably be ascribed to the fact that the decomposition reaction of AAPH was not the limiting step. The method using FL as reagent[8] is also developed at 37 °C.

Analytical Features. The kinetic curves were obtained under optimum conditions, using λ_{ex} of 620 nm and λ_{em} of 680 nm to monitor the variation of the fluorescence intensity with time for 60 min and the net AUC as the analytical parameter. The dynamic range of the calibration graph was 0.8−8 μM Trolox. The regression equation was AUC = $(0.02 \pm 0.01) + (0.074 \pm 0.006)X$, where X was the Trolox concentration expressed in micromolar. The regression coefficient (R) is 0.993, which is indicative of a good linearity of the calibration curve. The detection limit, calculated following International Union of Pure and Applied Chemistry (IUPAC) recommendations,[23] was 0.45 μM, which is lower[6,7] or similar[8] to those obtained in other

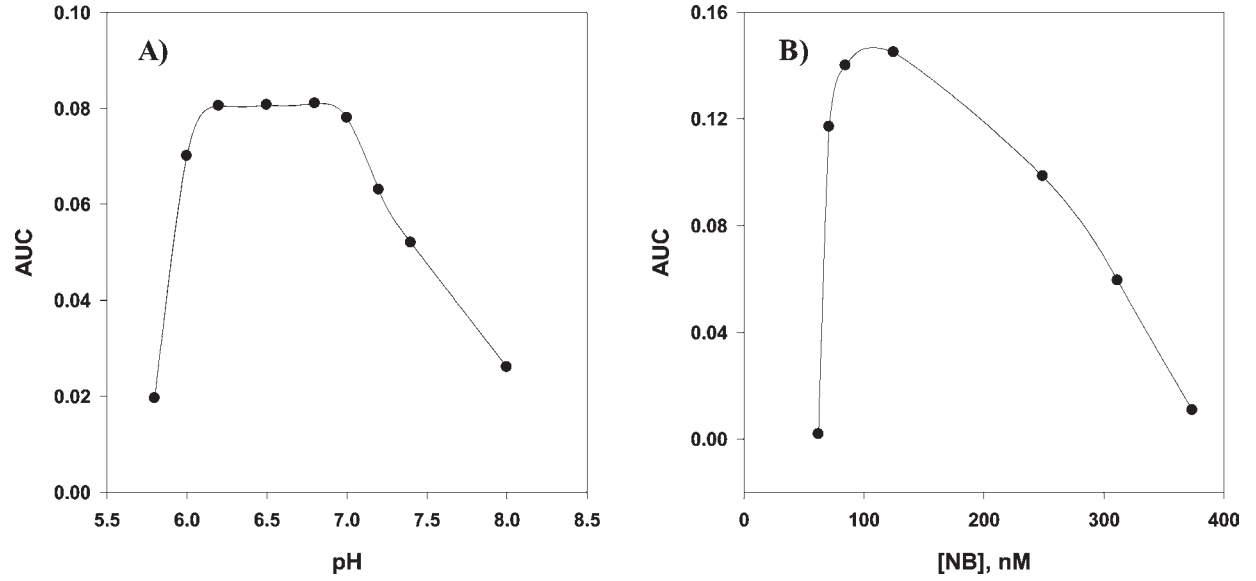

Figure 4. Influence of (A) pH and (B) NB concentration on the ORAC−NB method. In panel A, [NB], 120 nM; [phosphate], 0.075 M; [AAPH], 0.024 M; [Trolox], 2 μM; and temperature, 27 °C. In panel B, [AAPH], 0.012 M; [Trolox], 2 μM; pH, 6.9; [phosphate], 0.085 M; and temperature, 37 °C.

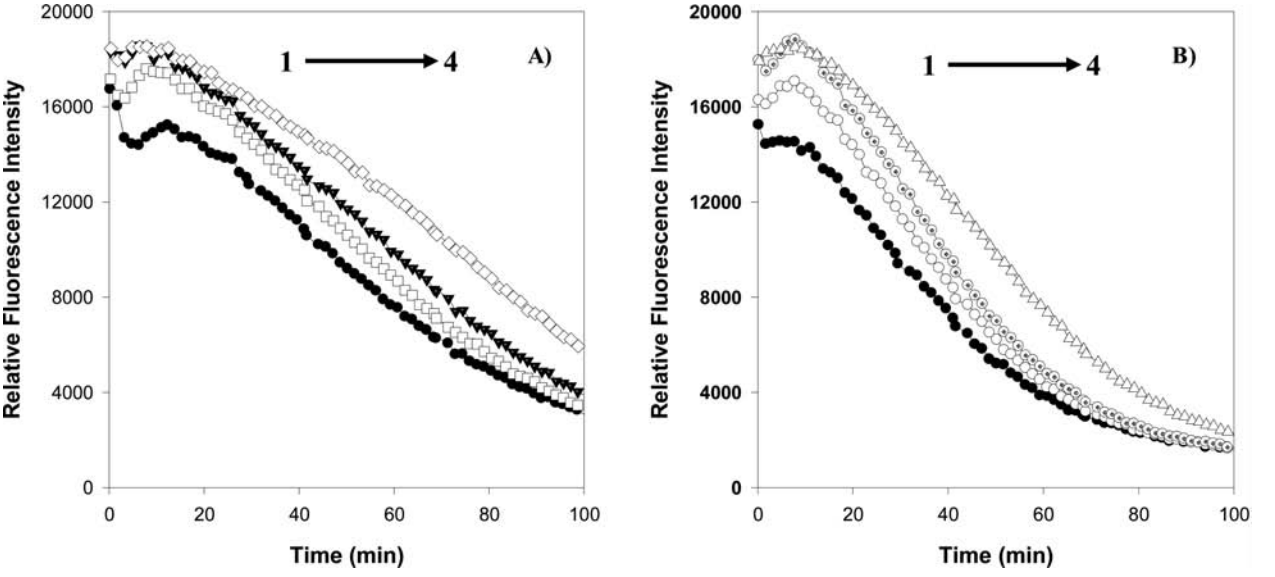

Figure 5. Antioxidant capacity curves obtained in the presence of (A) semi-dry white wine and (B) pineapple juice samples at different sample dilutions. Experimental conditions are as follows: [NB], 89 nM; [AAPH], 0.024 M, pH, 6.9; [phosphate], 0.085 M; and temperature, 27 °C. In panel A, (1) blank and (2, 3, and 4) 1/4000, 1/2000, and 1/1000 dilutions of the white wine sample, respectively. In panel B, (1) blank and (2, 3, and 4) 1/2000, 1/1000, and 1/500 dilutions of the pineapple juice sample, respectively.

ORAC methods involving FL. The precision of the method was assessed at two different Trolox concentrations, 1 and 5 μM, and expressed as the percentage of relative standard deviation, giving 5.6 and 2.9%, respectively.

Applications. The proposed method was applied to the analysis of wines, namely, white, semi-dry and red, and commercial fruit juices, namely, pineapple, peach, and apple. These samples were analyzed by both ORAC−FL and ORAC−NB methods with a simple sample treatment, which consisted in the adjustment of the pH to neutral values, because the samples featured acidic pH values. Then, the samples were diluted to accommodate the antioxidant content to the dynamic range of the calibration curve. Figure 5 shows the curves obtained for the blank and those obtained at different sample dilutions for (A) white wine and (B) pineapple juice samples. It can be seen from these curves that the area proportionally increased as the sample dilution decreased. The net AUC of the samples was measured as $(AUC_{sample} - AUC_{blank})/AUC_{blank}$, and this result was interpolated in the calibration curve obtained for Trolox standards. After the application of the corresponding dilution factors, the antioxidant content of each analyzed sample, expressed as millimolar Trolox equivalents of sample, was calculated. Table 1 lists the content found in the samples using both ORAC−NB and ORAC−FL methods. The paired *t* test was applied to the results at a 95% significance level, and it was found that there were not significant differences in the results provided by both methods, which confirms the practical

Table 1. Antioxidant Capacity (Expressed as Millimolar Trolox Equivalents of Sample) of Food Samples Analyzed by ORAC−NB and ORAC−FL Methods

sample	ORAC−NB[a]	ORAC−FL[a]
white wine (Manzanilla)	1.50 ± 0.09	1.3 ± 0.2
semi-dry wine	7.7 ± 0.9	4.4 ± 0.4
red wine	15.3 ± 0.4	10.4 ± 0.9
peach juice	2.222 ± 0.001	1.93 ± 0.09
pineapple juice	2.7 ± 0.3	2.399 ± 0.006
apple juice	2.7 ± 0.3	3.5 ± 0.4

[a] Mean ± standard deviation (SD) (n = 3).

Table 2. Recovery Values Obtained for the Different Samples Analyzed

	recovery study		
sample	added[a]	found[a,b]	recovery (%)
white wine	2.2	2.0 ± 0.2	92.0
	8.9	6.7 ± 0.9	75.2
	11.1	9.2 ± 0.8	82.9
semi-dry wine	4.4	3.6 ± 0.2	81.8
	8.8	8.1 ± 0.3	92.1
	13.2	12.9 ± 0.6	97.0
red wine	17.8	18 ± 2	101.1
	35.6	38 ± 4	106.7
	44.4	44 ± 4	99.1
peach juice	2.2	2.1 ± 0.1	95.5
	4.4	3.5 ± 0.4	79.5
	5.5	4.3 ± 0.2	78.2
pineapple juice	2.2	2.5 ± 0.2	113.6
	4.4	4.5 ± 0.3	102.3
	5.5	5.8 ± 0.4	105.5
apple juice	4.4	3.2 ± 0.2	72.7
	8.8	7.9 ± 0.5	89.6
	11.1	11 ± 1	99.1

[a] Units in millimolar Trolox equivalents of sample. [b] Mean ± SD (n = 3).

utility of the proposed ORAC−NB method to the analysis of these food samples. Also, the values found for both types of samples agree with values reported in the literature.[9,10] A recovery study was also carried out to validate the method. It was performed by adding three different amounts of Trolox to each sample and subtracting the results obtained from similarly treated unspiked samples. Table 2 shows the recovery percentages, which ranged from 72.7 to 113.6%. The mean recovery values obtained were 92.0 and 92.9% for wine and juice samples, respectively. This internal validation also confirms the usefulness of the developed ORAC−NB method for the analysis of real samples.

The results obtained show the feasibility of long wavelength fluorimetry for the determination of the antioxidant capacity in foods using the fluorophor NB for the first time as an analytical reagent. The use of this reagent instead of other fluorophores previously proposed for this purpose, such as FL or β-phycoerythrin, is a useful alternative to avoid potential background signals from the sample matrix, which can be a limitation when the above-mentioned fluorophores are used. Finally, the probability of photobleaching processes for NB is lower than that for β-phycoerythrin, as well as its cost.

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ABBREVIATIONS USED

AAPH, 2,2′-azo-bis-(2-methylpropionamidine) dihydrochloride; AUC, area under the curve; BHA, butylhydroxyanisole; FL, fluorescein; NB, nile blue; ORAC, oxygen radical absorbance capacity; ROS, reactive oxygen species.

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