Biotechnology and Bioprocess Engineering **2004**, 9: 514-518

Development of New Method for Antioxidant Capacity with ORP-pH System

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Abstract Many methods are used in the measurement of antioxidative capacity. These methods require very complex procedures and pretreatment. Our suggestions for research will be simple and accurate methods for obtaining many kinds of samples, especially colored samples such as natural product extracts for measuring antioxidative capacities. For oxidation-reduction potential (ORP) system value, we examined the relationships between the ORP-pH system and the ORAC, FRAP methods. To evaluate ORP System value, we calculated the absolute slope/intercept from the linear regression of each standard material at different concentrations and ORP-pH system, and compared the correlations with ORAC and FRAP values.

Keywords: ORP system value, ORP, ORAC method, FRAP method, ORP-pH system

INTRODUCTION

Oxidative stress is one of the most important factors in human health, causing many diseases due to the presence of free radicals [1,2]. Generally, triplet oxygen is useful for breathing, but oxygen is turned into free radicals by physical, chemical and environmental factors with enzyme system, reduction metabolism, chemical substances, pollutants and phytochemical reactions [3,4]. The free radicals formed include superoxide radical ($O_2^{\cdot-}$), hydroxyl radical (·HO), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). These reactive oxygen species (ROS) mainly attack unsaturated fatty acids in the brain, liver and WBC, and decrease immune system activity [5]. Therefore, antioxidants are required in our body for the purpose of scavenging these free radicals. Natural antioxidative products can act as good antioxidants according to the properties of their own pharmaceutical characters. Some simple, accurate methods for measuring antioxidative capacity are necessitated in this case. Current methods used for measuring antioxidative capacity include: DPPH method [6], ORAC method [7,8], FRAP method [9,10], D.O. analysis method [11], Hydroxy radical scavenging method [12-14], and measurement of aromatic and phenolic compounds [15].

But, the defects of all these methods except D.O. analysis method are inaccurate for colored sample when using spectrophotometer, and complexity because these methods are required so many kinds of reagents for the reaction. D.O. analysis method is the only method for using colored sample, but linoleic acid is so expensive. ORAC method is so time-consuming and FRAP method has a weak point at unstable color after reaction. DPPH method is required for pretreatment of the samples like heating. Shortly, all these methods are complex, time-consuming, and require pretreatment of the sample. We want to suggest a new, modified ORP (oxidation reduction potential)-pH system, and compare this to the oxygen radical absorbance capacity (ORAC) and the ferric reducing ability of plasma (FRAP) methods. Because ORP value represents an activity level of electrons [16,17], it is applicable to the determination of the degree of oxidation or reduction. Since it also represents the concentration of electrons, it is possible to measure the antioxidative capacity owed to evaluate the degree of oxidation and the reduction of materials [18-24].

For establishing the new method, standard materials were needed. Ascorbic acid was used as a water soluble standard; butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), and α-tocopherol were utilized as fat soluble standard materials. Each standard material was prepared as 100, 50, 25, 10, 5, and 1 mM solution. We calculated the ORP system value, which can be obtained (absolute value of slope/intercept) from the linear regression of the ORP value *vs.* pH. For the purpose of generalization, we have established the correlationship between ORP-system value and ORAC value, and between ORP-system value and FRAP value. From these relationships, we attempted to calculate more accurate antioxidative capacity values for colored samples, especially natural products, for which we cannot obtain antioxidative capacity values from a spectrophotometer, due to their natural color.

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MATERIALS AND METHODS

Reagents and Apparatus

ORP values were measured with an ORP meter (pH/ISE 750P; Istek, Seoul, Korea). For measuring ORAC and FRAP values, fluorescence spectrophotometer (RF 5301, Shimazu, Tokyo, Japan) and UV-spectrophotometer (UV-2101PC, Shimazu, Tokyo, Japan) were used, respectively. Ascorbic acid, BHT, BHA, and α-tocopherol, obtained from Sigma chemical company, were used as standard substances.

Preparation of Standard Substances

Ascorbic acid was dissolved in water. BHT, BHA and α-tocopherol were dissolved in ethyl alcohol. Six concentrations for each standard substance were prepared for ORP values: 100, 50, 25, 10, 5, and 1 mM.

Measurement of ORP Values

ORP values were measured at pHs ranging from 1.0 to 14. 0.5 M, 1.0 M, 10 mM, and 1 mM of NaOH and HCl were used for changing pH [18].

ORAC Method

Area under curve (AUC) was evaluated using 6-hydroxy-2,5,7,8-tetramethylchloroman-2-carboxylic acid (Trolox) as a standard solution, β-phycoerythrine (β-PE), and a indicator protein, H_2O_2 + Fe^+ as free radical generator, with fluorescence spectrophotometer values from 565 nm (emission) to 540 nm (excitation) for every 5 min until absorbance approached 0 (zero) [7,8]. ORAC value was calculated using the formula:

$$\text{ORAC value} = k(S_{sample}-S_{blank})/(S_{trolox}-S_{blank})$$

k: dilution factor, S_{sample}: AUC for Sample, S_{blank}: AUC for Blank, S_{Trolox}: AUC for Trolox

FRAP Method

FRAP solution was prepared with TPTZ (2,4,6-tri(2-[pyridyl]-s-triazine), ferric chloride ($FeCl_3 \cdot 6H_2O$), acetate buffer and water, and was mixed with the sample. Absorbance was measured at 593 nm with the spectrophotometer. A standard curve was drawn with $FeSO_4 \cdot 7H_2O$ and the FRAP value was calculated according to [9,10].

Relationships Among ORP System Values, ORAC Values, and FRAP Values

We defined ORP system value is defined to express strength of antioxidative capacity by the absolute slope value/intercept value from the linear regression of ORP values against the varying pH with each concentration of standard solution, such as ascorbic acid, BHA, BHT and α-tocopherol. The correlation between ORP system values and ORAC values, and between the ORP system values and the FRAP values at different standard concentration was examined.

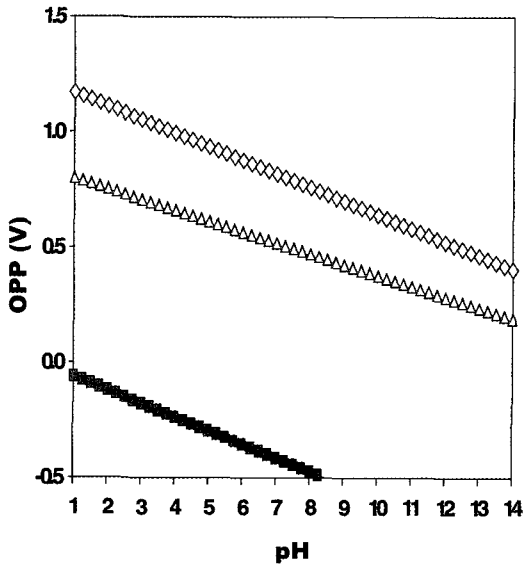

Fig. 1. Relationship between pH and ORP values (◆: boundary of oxidizing decompositions of water (ORP = 1.23~0.059 pH), ■: boundary of reducing decompositions of water (ORP = -0.059 pH), ▲: pure water (ORP = 0.84~0.047 pH)).

RESULTS AND DISCUSSION

ORP Values of Standard Substances with Each Concentration

ORP values of standard substances with each concentration were located within the reduced system boundaries. With increasing concentration, ORP values shifted for the reduced system boundaries (Fig. 1). Absolute slope value, intercept values, and ORP system values from the linear regression of pH-ORP system are shown in Table 1, according to each concentration of standard substances. Fig. 2 shows the correlation between ORP system value and the concentration of each standard substance. Since the correlations were more than r^2 = 0.97, ORP system values can be used to express antioxidative capacity using the pH-ORP system.

Relationship Between ORAC Values and ORP System Values

The relationship between ORAC values and ORP system values from the evaluation of ORAC value with varying concentrations of standard substances is shown in Fig. 3a. Because the correlation coefficient between ORAC values and ORP system values was more than 0.97 for each standard substance from Fig. 3b, ORP system values were assumed to express ORAC values.

Table 1. Absolute values of slope (a), intercept (b), and ORP system values from linear regression ($y = ax + b$) of each standard material, applied pH-ORP system

Standard materials Concentration (mM)	Ascorbic acid			BHT			BHA			α-tocopherol		
	A*	B*	C*	a	b	c	a	b	c	a	b	c
100	0.1683	0.2035	0.8275	0.0495	0.3683	0.1344	0.0493	0.345	0.1429	0.0542	0.4424	0.1344
50	0.1071	0.2307	0.4642	0.0487	0.3811	0.1278	0.0472	0.3584	0.1317	0.0525	0.4272	0.1284
25	0.0710	0.2497	0.2825	0.0476	0.3826	0.1244	0.0456	0.3622	0.1259	0.0501	0.4053	0.1253
10	0.0327	0.1895	0.1728	0.0464	0.3791	0.1224	0.0382	0.3116	0.1226	0.0497	0.3966	0.1236
5	0.0298	0.2159	0.1380	0.0441	0.3624	0.1217	0.0361	0.2974	0.1214	0.0483	0.3762	0.1229
1	0.0222	0.2018	0.1100	0.0424	0.3498	0.1212	0.0357	0.2963	0.1205	0.0472	0.3512	0.1225

A*: absolute value of slope, B*: intercept, C*: ORP system values (A/B)

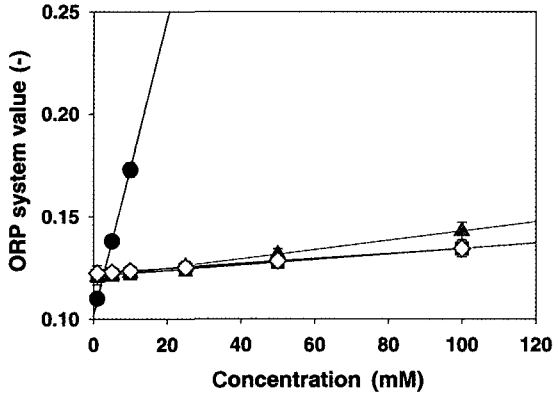

Fig. 2. Correlationship between ORP system value and concentration of each standard material (●: ascorbic acid (r^2 = 0.97), ■: BHT (r^2 = 0.98), ▲: BHA (r^2 = 0.97), and ◆: α-tocopherol (r^2 = 0.97)).

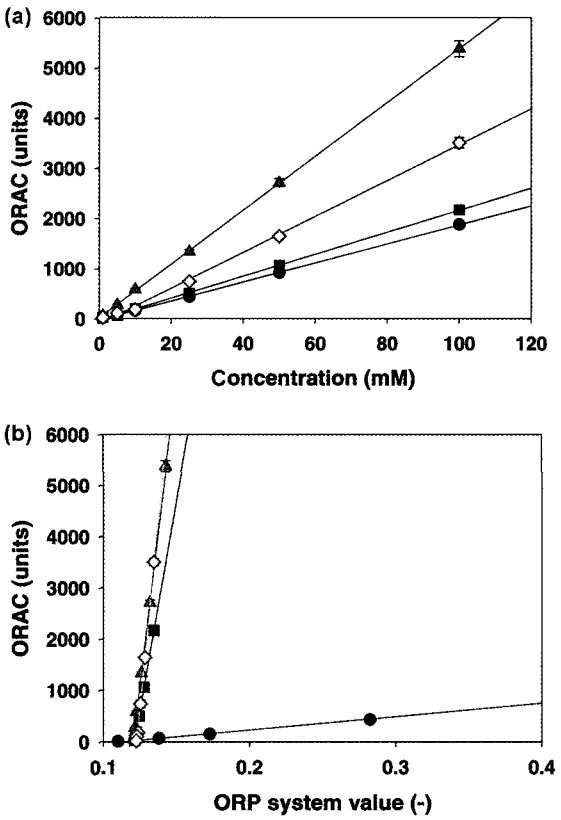

Fig. 3. Correlationship between ORAC value and concentration (a) and ORAC value and ORP system value (b) by linear regression of each standard material (●: ascorbic acid (r^2 = 0.97) ■: BHT (r^2 = 0.98), ▲: BHA (r^2 = 0.97), and ◆: α-tocopherol (r^2 = 0.97)).

Relationship Between FRAP Values and ORP System Values

The relationship between FRAP values and ORP system values from the evaluation of FRAP value with varying concentrations of standard substances is shown in Fig. 4a. Since the correlation coefficients between FRAP values and ORP system values was high (r^2 = 0.97) for each standard substance from Fig. 4b, ORP system values can be expressed as ORAC values.

Relationship Between ORAC Values and FRAP Values Based on ORP System Values

The relationships between the FRAP values and the ORAC values can be examined based on the ORP system value (Fig. 5). From these relationships, it is possible to compare the antioxidative capacities of different measuring methods, including the FRAP or ORAC methods.

We also suggested a new method of measuring the antioxidative capacities for colored samples from natural products, which are impossible to measure with spectrophotometer, due to their natural color.

A more convenient method to obtain from this information is to transfer the ORAC values, or FRAP values from ORP system values.

When the absolute slope and intercept from the linear regression of ORP values at various pHs are known, ORP system value can be calculated. The proper molar concentration of standard substances, such as ascorbic acid,

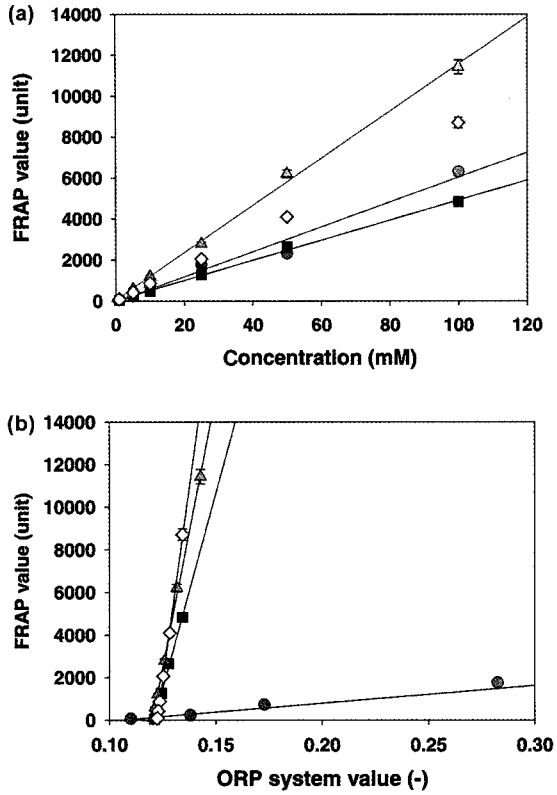

Fig. 4. Correlationship between FRAP value and concentration (a) and FRAP value and ORP system value (b) of each standard material (●: ascorbic acid (r^2 = 0.97), ■: BHT (r^2 = 0.98), ▲: BHA (r^2 = 0.97), and ◆: α-tocopherol (r^2 = 0.97)).

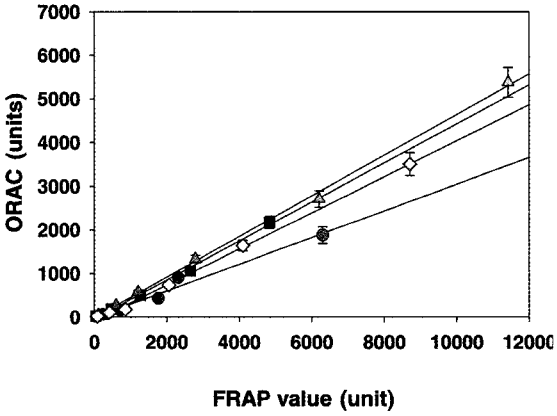

Fig. 5. Correlationship between FRAP value and ORAC value of each standard material (●: ascorbic acid (r^2 = 0.97), ■: BHT (r^2 = 0.98), ▲: BHA (r^2 = 0.97), and ◆: α-tocopherol (r^2 = 0.97)).

BHA, or BHT, α-tocopherol, can then be calculated from the linear regression of ORP system values against each concentration of standard substance based on water or fat soluble antioxidants.

The proper value of the ORAC method or the FRAP method may be chosen from the ORP system value.

CONCLUSION

Since the relationships between the ORAC values and the ORP system values, and between the FRAP values and the ORP system values, have high correlation coefficients (r^2 = 0.97) (Figs. 4 and 5), it is possible to measure antioxidative capacity with simplicity and correctness. It is possible to measure the antioxidative capacity of water soluble materials through the use of a standard linear regression of ORP system values versus concentrations of ascorbic acid.

The capacity of fat soluble antioxidants can also be measured using the standard curve of linear regression from ORP system values against concentrations of BHA, or BHT, α-tocopherol. A new, simpler, more accurate measuring method, which is without pretreatments and complexity, has been introduced. The antioxidative capacity of colored sample from natural products, which are impossible to measure with spectrophotometer due to their natural color, can be measured using this method.

It is anticipated that this new measuring method could be used to measure the antioxidative capacity of various samples.

REFERENCES

[1] Lee, H. J. and S. Y. Oh (1994) Interrelationship between environmental stresses, reactive oxygen species, and stress-ethylene. *J. Kor. Soc. Eco.* 17: 91-100.

[2] Kim, S. W. and S. D. Kim (1996) Influences of the duration of diabetes on the impairment in the relaxative responses and on the sensitivity to oxygen-free radical induced damage in streptozotocin-diabetic rat aortas. *J. Kor. Assoc. Intern. Med.* 50: 19-26.

[3] Masao, H. (2002) *A Functional Food and Health*. pp. 85-105. Academic press, NY, USA.

[4] Kobayashi, M. (2003) Astaxanthin biosynthesis enhanced by reactive oxygen species in the green alga *Haematococcus pluvialis*. *Biotechnol. Bioprocess Eng.* 8: 322-330.

[5] Satoh, M. S., C. J. Jones, R. D. Wood, and T. D. Lindahl (1993) DNA excision-repair defect of xeroderma pigmentosum prevents removal of a class of oxygen free radical-induced base lesions. *Proc. Natl. Acad. Sci. USA* 90: 6335-6339.

[6] Lu, L. C., Y. W. Chen, and C. C. Chou (2003) Antibacterial and DPPH free radical-scavenging activities of the ethanol extract of propolis collected in Taiwan. *J. Food Drug Anal.* 11: 277-282.

[7] Helaine, M. A. and G. C. Richard (1993) Oxygen-radical absorbance capacity assay for antioxidants. *Med. Biol. Med.* 14: 303-311.

[8] Robert, J. D. and N. G. Alexander (1989) Phycoerythrin fluorescence-based assay for peroxy radicals: A screen for biologically relevant protective agents. a screen for biologically relevant protective agents. *Anal. Biochem.* 177: 300-306.

[9] Reka, S. and l. S. Varga (2002) Total antioxidant power in some species of labiatae (adaptation of FRAP method). *Proceedings of the 7th Hungarian Congress on Plant Physiology.* S2-P24, 46: 125-127.
[10] Griffin, S. P. and R. Bhagooli (2004) Measuring antioxidant potential in corals using the FRAP assay. *J. Exp. Mar. Biol. Ecol.* 302: 201-211.
[11] Outa, S. (1987) *Food and Antioxidant*. pp. 179-188. Syokuhinzairyokenkyukai, Tokyo, Japan.
[12] Packer, L and N. G. Alexander (1990) Determination of aldehydic lipid peroxidation products. pp. 407-431. In : B. Ames, R. J. Mehlhorn, A. Anthony, P. William, L. Ernster, S. Helmut, F. Irwin and F. S. Trevor (eds.). *Methods in Enzymology. Part B: Oxygen Radicals and Antioxidants.* Academic Press, San Diego, CA, USA.
[13] Rimbach, G., S. De Pascual-Teresa, B. A. Ewins, S. Matsugo, Y. Uchida, A. M. Minihane, R. Turner, K. VafeiAdou, and P. D. Weinberg (2003) Antioxidant and free radical scavenging activity of isoflavone metabolites. *Xenobiotica* 33: 913-925.
[14] Kim, J. Y., S. I. Choi, and T. R. Hoe (2003) Screening of antioxidative activity of bifidobacterium species isolated from Korean infant feces and their identification. *Biotechnol. Bioprocess Eng.* 8: 199-204.
[15] Nalin, S., K. W. Lee, S. H. Kim, J. W. Ha, and Y. J. Jeon (2003) Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. *Food. Sci. Tech. Int.* 9: 339-348.
[16] Peng, Y. Z., J. F. Gao, S. Y Wang, and M. H. Sui (2001) Use pH and ORP as fuzzy control parameters of denitrification in SBR process-wastewater treatment and sludge management. *Water Sci. Technol.* 46:131-137.
[17] Khanal, S. K., C. Shang, and J. C. Huang (2002) Use of ORP (oxidation-reduction potential) to control oxygen dosing for online sulfide oxidation in anaerobic treatment of high sulfate wastewater. *Water Sci. Technol.* 47: 183-189.
[18] Okouchi, S., M. Suzuki, K. Sugano, and S. Ikeda (2002) Water desirable for the human body in terms of oxidation-reduction potential (ORP) to pH relationship. *J. Food Sci.* 67: 1594-1598.
[19] Yang, G. H. and S. Y. Choi (1982) Toxicological studies of antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). *J. Kor. Soc. Food Sci.* 14: 283-288.
[20] Elad, Y. (1992) The use of antioxidants (free radical scavengers) to control grey mould (*Botrytis cinerea*) and white mould (*Sclerotinia sclerotiorum*) in various crops. *Plant Pathol.* 41: 417-426.
[21] Jung, S. E., Y. G. Yun, Y. G. Jung, G. J. Seo, G. J. Choi, and J. S. Jung (2000) The antioxidant effect of vitamin C and deferoxamine on paraquat induced lipid peroxidation in rats. *J. Kor. Soc. Emerg. Med.* 11: 421-436.
[22] Panganamala R. V. and D. G. Cornwell (1982) The effect of vitamin E on arachidonic acid metabolism. *Ann. NY Acad. Aci.* 393: 376-393.
[23] Urons S., H. H. Midori, N. Tochihi, M. matsuo, M. Shiraki, and H. Ito (1991) Vitamin E and the susceptibility of erythrocytes and reconstituted liposomes to oxidative stress in aged diabetics. *Lipid* 26: 58-61.
[24] Terai, J. (1989) Antioxidant activity of β-carotene related carotenoids in solution. *Lipid* 24: 657-660.

[Received October 20, 2004; accepted September 25, 2004]