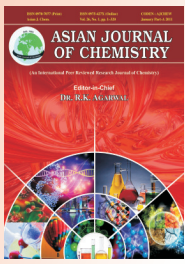
Asian Journal of Chemistry; Vol. 26, No. 16 (2014), 5171-5173

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.16622



# Determination of Synergistic Effects of Catalase and Natural Antioxidants in Scavenging •OH by Capillary Electrophoresis

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*Received*: 1 November 2013; *Accepted*: 14 January 2014; *Published online*: 28 July 2014; AJC-15655

A capillary electrophoresis method for the determination of serum •OH is established. The ascorbic acid/$Fe^{2+}$ system was used to generate the •OH and the benzoic acid was used to trap the •OH. The optimal conditions such as the SDS concentration, buffer pH and separation voltage were investigated under the optimal condition, the impacts of substrate concentrations and reaction time towards the production of •OH were studied. All the substances could be well separated basically; the linear range of the reaction product (*m*-hydroxylbenzoic acid) was $5 \times 10^{-6}$ to $3 \times 10^{-3}$ mol/L and the limit of detection was $1.0 \times 10^{-6}$ mol/L. Then the method was applied to determine the $IC_{50}$ of catalase, catechin and quercetin towards the serum •OH. The method was stable and quick and could be specifically applied into the research of the synergistic effects of catalase and natural antioxidants in scavenging the serum •OH.

**Keywords: Capillary electrophoresis, Catalase, Catechin, Quercetin, Hydroxyl radical, Synergistic antioxidant.**

## INTRODUCTION

The antioxidants of natural materials refer to the substances which are extracted from the natural flora and fauna or their metabolites, and could be used to scavenge the oxygen free radical or reactive oxygen, thus inhibiting or slowing down the oxidation reaction. In nature, many kinds of chemical substances, including flavonoids, phenolic acids, alkaloids, polysaccharides and glycosides, *etc.*[1], could exhibit the abilities of reacting with the free radicals and thus reducing the oxidative damages[2,3]. Catechin (Cate), main component of tea, belongs to one kind of reducible polyhydric phenols and widely exists in nature. It could be easily oxidized in the air and its antioxidative activity is even higher than $V_E$, therefore it could be used as the antioxidant to clean the free radicals and slow the aging. At the same time, it also possesses the bioactivities of antibacterial, preventing decayed tooth and inhibiting the blood pressure and blood sugar, *etc.*[4-6]; quercetin (Que), is also widely distributed in the natural products, possessing the bioactivities of anticancer, inhibiting the release of 5-HT and the platelet aggregation, *etc.*[7-9].

In the mechanism researches, whether the natural antioxidants could synergistically scavenge the oxygen free radicals with the antioxidase or not has gradually become the research hotspot. In the metabolic processes of the organisms, a variety of reactive oxygen species, including $O_2$, $H_2O_2$, •OH, NO• and alkoxyl radicals[4], could be produced, among which the oxidative activity of •OH was the strongest, with the standard electrode potential reaching 2.80 V and could kill the red blood cells, degrade the AND, cell membranes and polysaccharides[10]. Some biomolecules could be non-specifically oxidized by •OH, such as lipids, nucleic acids, proteins and thus leading to the oxidative damages and many diseases such as arthritis, atherosclerosis, cirrhosis, diabetes, senile dementia, cancer and aging, *etc.*[11-13]. Catalase (Cat) is a peroxisomal marker enzyme, widely present in biological peroxides, accounting for approximately 40 % of total enzymes and found in all known animal organizations, particularly highly concentrated in livers, having the ability of catalytically oxidizing the metabolic intermediate ($H_2O_2$) *in vivo* to $H_2O$ and $O_2$, thus preventing the infringement of $H_2O_2$ towards the organs, at the same time, catalase also has the clear effect towards •OH[14].

A •OH generating system and the determination method should firstly be established in the determination of scavenging effects of the enzyme or natural antioxidants towards •OH, •OH is very active, transitory and rare[15]. Normally, there are 4

methods to generate the •OH: 1. Fenton reaction; 2. under-catalyzer non-Fenton reaction; 3. self-oxidation reduction of certain sulfur-containing compounds; 4. UV radiation. After considering several methods[16-19], the ascorbic acid/$Fe^{2+}$ was accepted as the •OH generating system and benzoic acid was used as the •OH scavenger, through quantitatively calculating the target product, *m*-hydroxybenzoic acid, the generated •OH in the system could be measured. When the system exists the •OH scavenger, the quantity of the *m*-hydroxybenzoic acid would be reduced, therefore the clearance rate of the •OH scavenger could be determined. In this study, the electrophoresis method was established to isolate the overlapping absorption spectra of benzoic acid and *m*-hydroxybenzoic acid and applied into the study of the synergistic effects of catalse, catechin and quercetin scavenging the serum •OH.

## EXPERIMENTAL

The CL1030A capillary electrophoresis, equipped with the N2000 data acquisition workstations and VWD detector (Huayang Limin Instrument Co., Ltd., Beijing, China); uncoated quartz capillary column (60 cm × 75 μm id, with the effective length as 50 cm)was the product of Handan Ruifeng Chromatographic Co., Ltd.; 1200 series HPLCwas the product of the Agilent instrument Co., Ltd., with the solid phase as Chromasil RP-C18 (250 × 4.6 mm, 5 mm, Agilent Co., Ltd., USA); WH-B electronic balance was purchased from the Hengwei scientific instrument Co., Ltd. China; MTEG8200 pH meter was the product of Germany MTEG instrument Analysis Co., Ltd., Germany.

The catalase (enzyme activity = 2000 U/mg), catechin (HPLC = 98 %) and quercetin (HPLC = 98 %) were purchased from Shanghai Yuanye Biotechnology Co., Ltd.; Gibco newborn calf serum was purchased from the Invitrogen Corporation; other reagents such as the boric acid, borax, SDS, EDTA-$Na_2$, $FeSO_4 \cdot 7H_2O$, benzoic acid, *m*-hydroxybenzoic acid, L-ascorbic acid, dehydrated alcohol and triphenylphosphine were all of the analytical grade and purchased from the Shanghai Sinopharm Chemical Reagent Co. Ltd.; the experimental water was the deionized water.

**Electrophoresis:** Before the first use, the capillary should be washed with the following procedure: methanol for 5 min, water for 5 min, 1 mol/L HCl for 5 min, water for 5 min, 1 mol/L NaOH for 10 min, water for 5 min, 0.1 mol/L NaOH for 10 min, water for 5 min and buffer for 5 min. The inter-sample washing used the 0.1 mol/L NaOH, water and buffer for 3 min, respectively.

Referring to the literature, the running buffer was the mixture solution of 35 mmol/L SDS and 10 mmol/L sodium borate (pH 9.5), with the separation voltage as 19 kV, the pressure as 3.5 kPa, the injection time as 5 s and the separation column temperature as 25 °C. The detection wavelength was 230 nm.

**Experimental methods:** 20 mL catalase-inactivated serum, 50 mL 0.2 mol/L phosphate buffer solution (pH 7.4), •OH scavengers (catalase, catechin and quercetin with different concentrations), 0.5 mL 10 mmol/L $Fe^{2+}$-EDTA and 0.1 mL 10 mmol/L benzoic acid were added into a 1.5 mL clean electrophoresis tube in turn and finally added 10 mL 0.1 mol/L ascorbic acid to start the reaction; then quickly added 1 mL distilled water into the system, mixed and put into the constant-temperature water bath at 37 °C for 2 h reaction in the dark situation; then the reaction products were performed the capillary electrophoresis.

## RESULTS AND DISCUSSION

**Identification and optimization of •OH generating system:** As for the impacts of the actual sample matrix, the catalase-inactivated serum sample matrix was used in this study. In order to produce the largest amount of •OH, after the investigation of the impacts of the concentrations of benzoic acid, the radical scavenger, it was found that the target product, *m*-hydroxybenzoic acid, would be strongly affected by the concentration of benzoic acid, in the range of 0.15 -1.05 mmol/L, when the concentration of benzoic acid increased to 0.75 mmol/L, the product concentration reached the maximum, while continued to increase the concentration of benzoic acid, the amount of the product slightly decreased, which probably was because the generated •OH could also react with the ethanol, resulting in a slight decline in the amount of the product (Fig. 1).

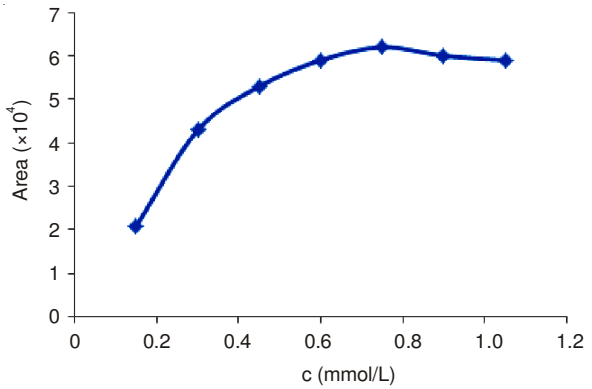


Fig. 1. Effect of benzoic acid concentration on the peak area of the target product

The reaction time also affected the formation of the target product. Fig. 2 showed that the target product amount increased with the reaction time gradually and exhibited the saturation at 2 h, the extension of the reaction time would not significantly increase the product. This might because the ascorbic acid had been reacted completely, the $H_2O_2$-derived •OH therefore reached the saturation. Therefore, the reaction time was selected as 2 h.

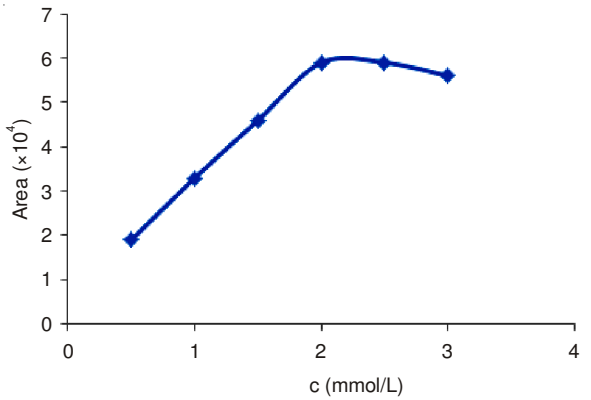


Fig. 2. Effect of reaction time on the peak area of the target product

At the same time, in order to accurately determining the clearance rate of •OH scavenger, the scavengers should be ensuredto be able to adequately react with •OH, the benzoic acid should not interfere with the reaction. In this study, the benzoic acid was added at different time intervals 0-100 min after the addition of catalase, catechin and quercetin and it was proved there was no impact of the adding time of benzoic acid towards the amount of product formation, *i.e*. the reaction rate of •OH with the scavenger was much greater than that of •OH with benzoic acid, therefore the •OH scavenger might be added simultaneously with the benzoic acid.

**Electrophoresis optimization:** The SDS concentration could change the retention factor of each analyte and thus affect the resolution. In the study, it was found that when increased the SDS concentration, the retention time of the materials in the micelles enhanced and the migration time gradually extended, resulting in the better separation. While too high SDS concentration would totally retain the materials, so the SDS concentration was decided as 35 mmol/L, in which the peak height was the maximum.

The pH of the buffer would affect the separation results of the analytes through affecting the ionization degrees of wall silanols and components. In this study, the pH of the buffer was tested in the range of 5-10. When pH was < 8, the baseline separation of EDTA and the reaction product, namely *m*-hydroxybenzonic acid, could not be obtained. When pH was in the range of 8-10, the separation was better; when pH was > 10, the higher pH would affect the capillary wall and thus cause the instability of the electrical flow. To protect the capillary and maintain the separation effect and migration time, pH 9.5 was finally decided.

The separation voltage was also investigated in the range of 14-26 kV, the results revealed that the higher separation voltage the faster migration time, while when the voltage exceeded 20 kV, the baseline noise would increase sharply, therefore the separation voltage was set as 19 kV.

The peak area of *m*-hydroxybenzoic acid was the quantitative basis and the clearance rate was calculated as $I = (A_0-A)/A_0 \times 100$ %, among which $A_0$ was the peak area determined without •OH scavenger, while A was the peak area with •OH scavenger. In this research, the regression equation wasy = $4.51 \times 10^8 x + 3472.11$ ($R^2 = 0.997\ 3$, n = 5), in which y was the peak area and x was the concentration of *m*-hydroxybenzoic acid (mol/L); the linear range was $5 \times 10^{-6}$ mol/L to $3 \times 10^{-3}$ mol/L, with the LOD as (S/N = 3) $1.6 \times 10^{-6}$ mol/L.

**Reproducibility and recovery:** The reaction mixture was repeatedly injected into the HPLC 5 times, the RSDs of the product peak area and migration time were 3.6 and 3.3 %. After the reaction, 5 mL 1 mmol/L *m*-hydroxybenzoic acid was added into the reaction system for the recovery determination. The average recovery of the target product 5 times was 93.1 %, indicating that the recovery was suitable for the further research.

**4•OH scavenging activity of catalase, catechin and quercetin:** The concentration series of catalase, catechin and quercetin were added into the reaction mixture to determine their clearance rates of •OH, respectively, with 5 repeated injections of each concentration for the average value. The linear regression equation was drawn with the scavenging rate Y (%) *vs.* concentration X (mg/L), respectively (Table-1). Thus the half scavenging rate ($IC_{50}$) of each substance would be obtained through the equation in the background of 20 µL catalase-inactivated serum matrix (Table-1). Catechin and quercetin were the polyhydric phenols, therefore the possible mechanism of •OH scavenging might be the reaction of •OH with the phenolic hydroxy groups[20], or the active H directly connected with the C ring participated the oxidation process[21].

TABLE-1
REGRESSION EQUATION AND $IC_{50}$ OF DIFFERENT •OH SCAVENGER (n = 5)

| •OH scavenger | Regression equation | $R^2$ | $IC_{50}$ (mg/L) |
|---|---|---|---|
| Catalase | Y = 23.31X + 4.72 | 0.998 1 | 2.31 |
| Catechin | Y = 51.77X + 3.21 | 0.997 2 | 7.88 |
| Quercetin | Y = 10.98X + 2.28 | 0.998 3 | 13.09 |

## REFERENCES


1. L. Zhang, G. Chen, R. Fang, L. Yin, J. Shao, J. Xiao, Y. Yu and X. Tao, *Chinese J. Anal. Chem*., **41**, 1571 (2013).
2. K. Wirginia, *Open Cardiovasc. Med. J*., **5**, 179 (2011).
3. A. Gumieniczek, B. Owczarek and B. Pawlikowska, *Exp. Diabetes Res*., **2012**, 1 (2012).
4. S. Kaushik, H.P. Bais, M.L. Biedrzycki and L. Venkatachalam, *Plant Signal. Behav*., **5**, 1088 (2010).
5. V. Bolduc, E. Baraghis, N. Duquette, N. Thorin-Trescases, J. Lambert, F. Lesage and E. Thorin, *Am. J. Physiol. Heart Circ. Physiol*., **302**, H1330 (2012).
6. A.A. Alshatwi, *J. Exp. Clin. Cancer Res*., **29**, 167 (2010).
7. Y.-C. Chan, C. Roos, M. Inoue-Murayama, E. Inoue, C.-C. Shih, K.J.-C. Pei and L. Vigilant, *PLoS ONE*, **8**, e542 (2013).
8. A.J. Larson, J.D. Symons and T. Jalili, *Adv.Nutr.*, **3**, 39 (2012).
9. J. Jakubowicz-Gil, E. Langner, D. Badziul, I. Wertel and W. Rzeski, *Tumour Biol*., **34**, 2367 (2013).
10. T.-T. Ching, W.-C. Chiang, C.-S. Chen and A.-L. Hsu, *Aging Cell*, **10**, 506 (2011).
11. X. Chen, X. Zhao, Y. Xiong, J. Liu and C.-G. Zhan, *J. Phys. Chem. B*, **115**, 12208 (2011).
12. J. Nguyen, Y. Ma, T. Luo, R.G. Bristow, D.A. Jaffray and Q.-B. Lu, *Proc. Natl. Acad. Sci. USA*, **108**, 11778 (2011).
13. E. Mayaan, A. Moser, A.D. MacKerell Jr. and D.M. York, *J. Comput. Chem*., **28**, 495 (2007).
14. C. Sim and D.L. Denlinger, *J. Insect Physiol*., **57**, 628 (2011).
15. S.T. Roberts, P.B. Petersen, K. Ramasesha, A. Tokmakoff, I.S. Ufimtsev and T.J. Martinez, *Proc. Natl. Acad. Sci. USA*, **106**, 15154 (2009).
16. S.S. Karayalcin, C.W. Sturbaum, J.T. Wachsman, J.H. Cha and D.W. Powell, *J. Clin. Invest*., **86**, 60 (1990).
17. L. Tartier, V. Michalik, M. Spotheim-Maurizot, A.R. Rahmouni, R. Sabattier and M. Charlier, *Nucleic Acids Res*., **22**, 5565 (1994).
18. A. Natarajan, C. Chun, J.J. Hickman and P. Molnar, *J. Biomater. Sci. Polym. Ed*., **19**, 1319 (2008).
19. T. Zögg, M. Sponring, S. Schindler, M. Koll, R. Schneider, H. Brandstetter and B. Auer, *Structure*, **21**, 929 (2013).
20. J. Wang, J. Lou, C. Luo, L. Zhou, M. Wang and L. Wang, *Int. J. Mol. Sci*., **13**, 11349 (2012).
21. S. Bagheri, H. Ahmadvand, A. Khosrowbeygi, F. Ghazanfari, N. Jafari, H. Nazem and R.H. Hosseini, *Asian Pac. J. Trop. Biomed*., **3**, 22 (2013).