ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 9, Issue, 09, pp.58110-58113, September, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

APPLICATION OF A NEW AREA UNDER CURVE METHOD FOR ANTIOXIDANT CAPACITY ASSAYS OF SOME THIOSEMICARBAZONE COMPOUNDS

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ARTICLE INFO

Article History:
Received 29th June, 2017
Received in revised form
17th July, 2017
Accepted 09th August, 2017
Published online 30th September, 2017

Key words:

Area under curve aproach,
Gallic acid equivalent ,
Ferric thiocyanate method,
5-Methoxyisatin-3-(N-4chlorophenyl) thiosemicarbazone, 5-Methoxyisatin-3-(N-phenyl) thiosemicarbazone.

ABSTRACT

Aims&Objectives: The area under curve (AUC) method is the first quantitative method measuring antioxidant protection against lipid oxidation, with a slightly different order of antioxidative effectiveness from reductive assays because of interfacial effects. In this study, peroxidation of copper (II) -induced linoleic acid (LA) emulsion was investigated using a new method of 'area under curve' (AUC) approximation based on iron thiocyanate colorimetry.

Materials and Methods: The formation of hydroperoxides in the linoleic acid emulsion was carried out at 37 ° C and in an aerated incubation at pH 7. The peroxidation of LA followed by pseudo first order kinetics, and the increases of absorbance obtained as a function of the incubation period gave sigmoidal curves. When the maximum absorbance of the oxidation products is close to 1.0, the area under the kinetic curve (AUC) and the net AUC, i.e. ($AUC_{sample} - AUC_{blank}$) calculations, the calibration curve plotted with the net AUC versus the concentration for gallic acid.

Results: Gallic acid equivalents (GAE) for 5-Methoxyisatin-3-(N-4chlorophenyl) thiosemicarbazone ($H_2$5MI3ClPT) and 5-Methoxyisatin-3-(N-phenyl) thiosemicarbazone ($H_2$5MI3PT) compounds were calculated using standard curves. As a result, the gallic acid equivalents (GAE) of antioxidant compounds followed the order of BHT (3,5-Di-tert-4-butylhydroxytoluene) > ($H_2$5MI3PT) > ($H_2$5MI3ClPT) with the use of this new AUC approach.

Citation: Temel kan BAKIR, İnci Sönmezoğlu, Fatma Kandemirli and Reşat APAK. 2017. "Application of a New Area Under Curve Method for Antioxidant Capacity Assays of Some Thiosemicarbazone Compounds.", *International Journal of Current Research*, 9, (09), 58110-58113.

INTRODUCTION

Thiosemicarbazones have attracted significant attention with a broad spectrum of biological activity with the ability to form stable complexes with transition metal ions. They are also known to have DNA binding, antibacterial and antiviral (Garoufis *et al.*, 2009, Ramachandran *et al.*, 2009), anticancer (Sîrbu *et al.*, 2017) **,** antifungal (Parrilha *et al.*, 2011)**,** antioxidant (Sathiyaraj *et al.*, 2014), activities. Chain breaker antioxidant compounds can inhibit lipid peroxidation and prevent cell damage in biological systems. (Lobo *et al.*,2010)Therefore, it is very important to examine the capacity of antioxidants and many methods can be used for these investigations.The 'Under the Curve' (AUC) method is one of the methods used to determine the antioxidant capacity that can be applied to methods based on hydrogen atom transfer (HAT) such as the ORAC assay.

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(Magalhaes *et al.*, 2008, Ching *et al.*, 2006,Apak *et al.*, 2016) The advantage of determining the antioxidant activity of the AUC approach is that it is equally well applied to antioxidant samples exhibiting different delay phases. As the maximum absorbance of the oxidation products approached 1 [*i.e.* $A_{max} \sim 1$], the following parameters could be found: (i) area under the kinetic curve (AUC) and 'net AUC', [*i.e.* $\Delta AUC = (AUC_{blank} - AUC_{sample})$]; (ii) standard calibration line by plotting ΔAUC against the concentration of the test compound (or gallic acid as reference compound); (iii) gallic acid-equivalent antioxidant capacity (GAEAC) of the test compound by comparing the slopes of the relevant calibration lines.(Apak *et al.*,2013, Huang *et al.*, 2005). In this study, the peroxidation of LA in the presence of Cu(II) ion alone and with antioxidants was investigated in aerated and incubated emulsions at 37°C and pH 7. The TAC values of 5-Methoxyisatin-3-(N-4chlorophenyl) thiosemicarbazone ($H_2$5MI3ClPT) and 5-Methoxyisatin-3-(N-phenyl) thiosemicarbazone ($H_2$5MI3PT) compoundswere quantified Fe(III)-SCN method adapted to an 'area under curve (AUC)' approach.

MATERIALS AND METHODS

Chemical substances and instruments

All chemicals which were analytical grade provided from Sigma-Aldrich Co. LLC. In each stage deionized purity water was used. Absorbents was measured using a SHIMADZU the UVM-1240 UV-Visible spectrophotometer (Shimadzu Corp., Kyoto, Japan manufactures) with a pair of identical quartz cuvette of 1 cm thickness.Cu(II)-catalyzed autoxidation of LAwere investigated in 80 mM phosphate buffer at pH 7. The pH measurements were made using a Metrohm 632 Digital pH-meter (Metrohm AG, CH-9100 Herisau, Switzerland) with a combined glass electrode. All the experiments were carried out at 37 ◦C by means of a thermostated system (±0.5 ◦C) which contained an immersion circulator (Thermomix 1419 B. Braun Melsungen AG, Melsungen, Germany).

Experimental design

Linoleicacid oxidation processwas investigated by spectrophotometric measurements for 4 hours.Thechange ofabsorbancewithincubation periodduetotheformationofferric thiocyanate showedsigmoidal curves.

Analysis of hydroperoxides (primary oxidation products)

The ferric thiocyanate method was used to detect the production of hydroperoxides, the product of the primary oxidation in the linoleic acid emulsion system. The primary oxidation grade wasmeasured by sequentially adding ethanol (4.7 mL, 75% v/v), ammonium thiocyanate (0.1 mL, 30% w/v), sample solution (0.1 mL), and ferrous chloride (0.1 mL of 0.02 M in 3.5% v/v HCl). The Fe (III) -SCN complex was determined at 500 nm andperoxide concentration was analysed by reading against a blank containing identical components without LA.(Yıldoğan-Beker *et al.,*2011)

Preparation ofstandard and samplesolutions

Linoleic acid emulsions and antioxidant solutions were prepared freshly every day and all other solutions stored at degree of 4 ^{o}C. The stock LA emulsion was prepared by mixing with a same gram (0.2804 g) of LA and Tween 20 , and then 50 mL of phosphate buffer (0.2 mol L^{-1}, pH 7) was added to the mixture. A stock solution of cupric nitrate was prepared at a Cu(II) concentration of 0.05 mol L^{-1}.(Bakır *et al.*2014)

Standard system solution , {linoleic acid+Cu(II)}

The standard {LA+Cu(II)} solution was prepared as 25 mL (stock 0.02 mol L^{-1}) LA + 20 mL phosphate buffer (pH 7) + x mL (stock 0.05 mol L^{-1}) Cu(II) + (5–x) mL absolute ethanol or DMSO (dimethyl sulfoxide) in a total reaction volume of 50 mL.

Sample (linoleic acid + Cu(II) +antioxidant) system solution

The sample solution was prepared as 25 mL (stock 0.02 mol L^{-1}) LA + 20 mL phosphate buffer (pH 7) + x mL (stock 0.05 mol L^{-1}) Cu(II) + y mL antioxidant+ [5–(x + y)] mL absolute ethanol or DMSO (dimethyl sulfoxide) in a total reaction mixture volume of 50 mL.

Statistical analysis

All experiments were performed in triplicate, and the results for (Fe(III)-SCN) method were expressed as {mean ± standard deviation}. Descriptive statistical analyses were performed using MICROCAL ORIGIN 8.5.1 (Origin Lab Corp., Northampton, MA, USA) for calculating the final AUC values. The same program was used to find the relationship with the net area under curve (AUC) and concentration of antioxidant.

RESULTS

In this study, oxidation was monitored by recording absorbance *versus* time, where the total concentrations of hydroperoxides were proportional to the formation of Fe(III)–thiocyanate (recorded as $A_{500\ nm}$). Changes of absorbance measured by Fe(III)-SCN assay as a function of incubation time exhibited sigmoidal curves, as shown for some antioxidants (Fig. 1 for Gallic acid , Fig. 2 for BHT, Fig. 3 for ($H_2$5MI3ClPT) , Fig. 4 for ($H_2$5MI3PT). (Watanabe *et al.*, 2005, Bakır *et al.*, 2013)

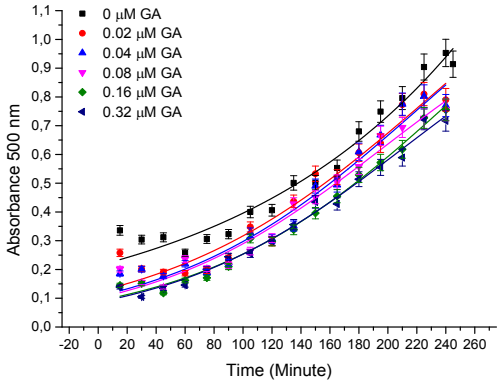

Figure 1.Change in absorbance over time in linoleic acid oxidation system for gallic acid (GA)

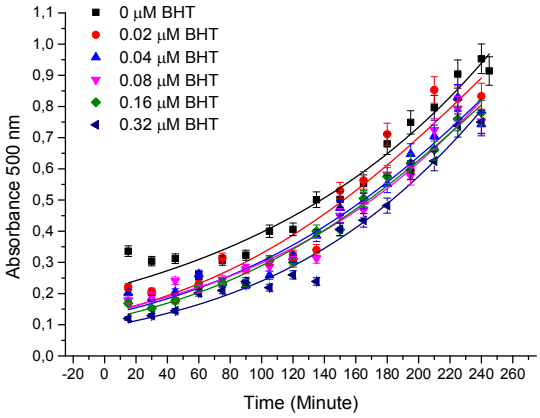

Figure 2.Change in absorbance over time in linoleic acid oxidation system for 3,5-Di-tert-4-butylhydroxytoluene (BHT).

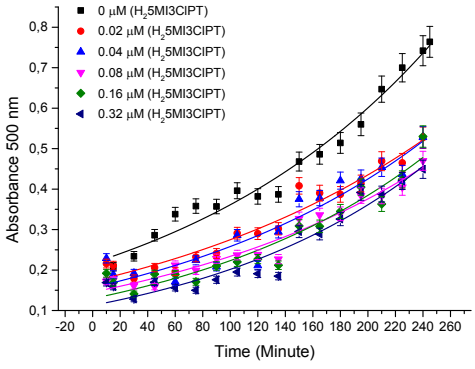

Figure 3.Change in absorbance over time in linoleic acid oxidation system for 5-Methoxyisatin-3-(N-4chlorophenyl) thiosemicarbazone ($H_2$5MI3ClPT)

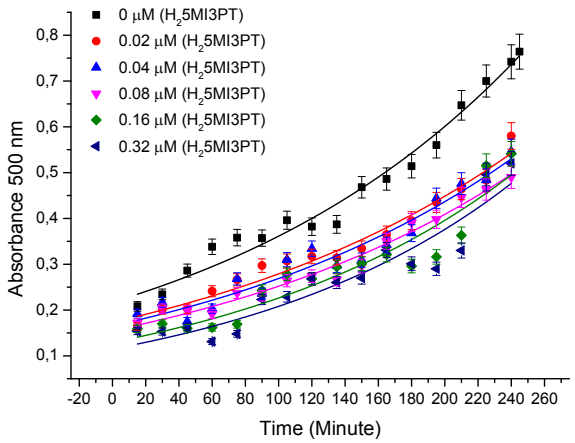

Figure 4. Change in absorbance over time in linoleic acid oxidation system for 5-Methoxyisatin-3-(N-phenyl) thiosemicarbazone ($H_2$5MI3PT).

From the absorbance/time curves, AUC values of antioxidants as a function of antioxidant concentration were found.Thus the net AUC (net area under curve) was calculated by comparative analysis of (AUC_{blank}-AUC_{sample}). Standard calibration lines were obtained by plotting net AUC against the concentration of tested compounds (or gallic acid as reference compound) (net AUC= ax ± b , where b is the intercept, a is the slope, and x is the concentration of antioxidant) **(Fig. 5)**.

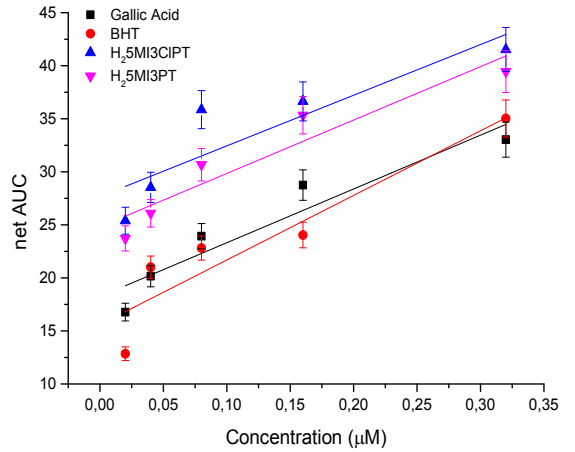

Figure 5. Net AUC *versus* concentration of gallic acid (GA) in linoleic acid oxidation measured by Fe (III)-SCN assay

The gallic acid-equivalent antioxidant capacity (GAEAC) was found by comparing the slopes of the calibration lines. **(Table 1).** To determine the GAEAC coefficient of each compound, the ratio of the slope **(*m*)** of the linear regression curve of the tested compound to that of gallic acid was used:

$$GAEAC = m_{compound} / m_{Gallic\ acid}$$

Table 1. Calculated of GAEAC (gallic acid equivalent antioxidant capacity) values of antioxidants in the linoleic acid oxidation system measured by Fe(III)-SCN method

Compound (AOX)	Calibration equation Net AUC: y=ax ± b	R^2	GAEAC(unitless) c=(2.5–4.0) x10^{-4} M
GA	y=50.711 x 10^6c +18.243	0.870	-
BHT	y=60.905 x 10^6c + 15.598	0.832	1.201
($H_2$5MI3ClPT)	y=47.761 x 10^6c + 27.669	0.730	0.941
($H_2$5MI3PT)	y=50.339 x 10^6c + 24.814	0.868	0.992

*The correlation between antioxidants for the Fe(III)-SCN test obtained at p<0.05 .

DISCUSSION

Antioxidant assays of different thiosemicarbozone derivatives have been measured in a number of previous studies. (Subhashree *et al.*, 2017, Elsayed *et al.*, 2017, Hosseini-Yazdi *et al.*, 2017)To the best of our knowledge, however, studies investigating antioxidant behavior in the lipid peroxidation environment are rather limited.(Karatas *et al.*, 2006) The hydroperoxides resulting from lipid peroxidation may raise Fe (II) to Fe (III). Thus, the iron thiocyanate method based on the formation of a red complex can be used to measure the antioxidant behavior of thiosemicarbazone derivatives with similar structure and redox potentials. Previously, many studies using the AUC approach were performed in antioxidant design calculations. However, these studies are limited by the TBARS (thiobarbituric acid reactive substances) method based antioxidant capacity determinations.(Schisterman *et al.*, 2001, Kerrihard *et al.*, 2016, Hernandez-Salinas *et al.*, , 2015)The AUC method can be used to measure both the kinetic lag time as a measure of the area under the absorbance time curves and the thermodynamically fixed times as a coincidence.(Huang *et al.,*2005) Concentrations of substance with protective antioxidant activity against lipid peroxidation can be calculated by taking the curve integrals corresponding to the AUC values.

The AUC method, a measure of the area under the absorbance time curves, is an indication of the simultaneous measurement of thermodynamic quantities such as fixed time and kinetic as well as lag time.Concentrations of substance with protective antioxidant activity against lipid peroxidation can be calculated by taking the curve integrals corresponding to the AUC values.Monitoring of lipid oxidation products requires highly sophisticated but costly techniques.Although the rate constants for lipid peroxidation are calculated in the presence and absence of antioxidants, the quantification of the antioxidant capacities of thiosemicarbazone derivatives has not been investigated.For this reason, AUC-based approach suggested by Bakir *et al*., (2017). In this study, GAEAC values calculated with the help of iron thiocyanate colorimetric method was used for the determination of total antioxidant capacity. Thus, calculated galic acid equivalents were used for comparative analysis of antioxidant capacity of various thiosemicarbazone compounds tested in the LA oxidation system. As a result of this new AUC approach, the GAEAC coefficients of antioxidants in a LA emulsion with respect to the Fe(III)-SCN method were: BHT >($H_2$5MI3ClPT) >($H_2$5MI3PT).

Conclusion

This study is important for the first time application of the AUC approach to thiosemicarbazone compounds. Thus, a simple and understandable method has been demonstrated to be able to conveniently use thiosemicarbazone complexes and similar molecules as conventional methods in cellular and extracellular antioxidant or prooxidant studies in the lipid environment. It is believed that the AUC method will be an important method for the investigation of lipid oxidation studies and prevention by antioxidants.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgement

This work was supported by Kastamonu University, Research Fund, KÜBAP Project No : KÜ-BAP01/2016-2. Dr. Bakır would like to thank Kastamonu University, Research Fund for these support.

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