THE DEVELOPMENT OF IODATE BASED ANTIOXIDANT CAPACITY DETERMINATION METHOD

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ABSTRACT

In the last decade, free radicals have attracted great attention, as they are a natural product of many biological reactions. Accumulation of the free radicals leads to oxidative stress which plays the essential role of the cell ageing leading to serious diseases. But the cell can defend itself by natural compounds called antioxidants. These defender compounds may be naturally occurred in the cell or can be provided by outside supplements. Antioxidant capacity is determined by several determination methods but they are expensive and complicated. This increased the need to develop an easy, reliable and inexpensive method to determine the antioxidant capacity and that is the goal of this study. In this study the capacity of antioxidant will be determined by the reaction between iodate and hydroxyl amine to release the nitrite which will form a pink colour.

Keywords: free radical, oxidative stress, antioxidants, antioxidants capacity.

1. INTRODUCTION

Antioxidant is a group of substances that reverse the effect of Oxygen, which is harmful for human body as about 1-3 % of Oxygen we breathe in goes to produce a (O2-) one-electron reduction product and H2O2 the two-electron reduction product as a products of reactive oxygen species (ROS).(Halliwell, B., Zentella, A., Gomez, E. O., & Kershenobich, D.,1997).

Mechanistic definitions of antioxidants are focusing on the ability to be a hydrogen donor or an electron donor. Capacity of the antioxidant is a measure of the amount (in moles) of a given free radical removed by a sample. Measurements of antioxidant capacity yield the amount of a heterogeneous mix of antioxidants that react together to produce the total removing ability of the sample. The antioxidant capacity of each individual component is not measured (Guevara, I., Iwanejko, J., Dembińska-Kieć, A., Pankiewicz, J., Wanat, A., Anna, P., ... & Szczudlik, A., 1998)

Antioxidant capacity can be classified as either hydrogen transfer assays or single electron transfer reaction based assays. These assays measure the radical removing capacity or the reducing ability, respectively, not the total antioxidant capacity of the sample.

One of the simple methods to determine the antioxidant capacity is Griess reaction. Since some of pathological processes are accompanied by the release of nitric oxide (NO), there was a need to monitor the concentration of (NO2-, NO3-).

Griess reaction involves the reduction of nitrates to nitrites in the presence of NADPH-sensitive reductase, can be successfully applied for measurement of NOx levels in human body fluids (Granger, D. L., Taintor, R. R., Boockvar, K. S., & Hibbs Jr, J. B., 1996).

An imbalance between oxidants and antioxidants in favour of the oxidants, usually leads to damage, and called “oxidative stress”. Usually happens when Oxidant level as a normal product of aerobic metabolism produced at elevated rates. Under certain pathophysiological

conditions, Antioxidant defence involves several strategies, both enzymatic and non-enzymatic (Sies, H., 1997)

2. LITARATURE BACKGROUND

Free radicals are chemical species that have an unpaired electron that is considered to be as fragments of molecules and which are very reactive species. They are produced always in cells either as accidental by-products of metabolism or during, phagocytosis. The most important reactants in free radical biochemistry in aerobic cells are oxygen and its radical derivatives (superoxide and hydroxyl radical), hydrogen peroxide and transition metals. Cells have developed a comprehensive array of antioxidant defence system to prevent free radical formation or decreasing their damaging effects.

These include enzymes to break peroxides, proteins to sequester transition metals and a range of compounds to 'scavenge' free radicals. Reactive free radicals formed within cells can oxidize biomolecules like proteins, lipids, and DNA and lead to cell death and tissue injury. (Cheeseman, K. H., & Slater, T. F., 1993)

In the last decades an interest in the role of free radicals has been increased, to understand its role in the pathogenesis of human disease. These interests led to an increased need for techniques to measure free radicals and their reactions in vivo. Free radicals are extremely reactive species with short life time. Consequently, free radicals are not amenable to direct assay and free radical activity is usually assessed by indirect methods such as measurement of the various end products of reactions with lipids, proteins and DNA. Measuring of these end products needed a great development in measuring technique as the only samples available for measuring are blood, urine and expired breath. Lipid peroxidation is the most intensively studied process and provides a number of possibilities for assays. Protein and nucleic acid oxidation are attracting increasing attention at the present time.

The techniques currently available, however, are limited to semi-quantitative assays of damage to broad classes of biomolecules and there is an urgent need for more specific and informative methods. (Holley, A. E., & Cheeseman, K. H., 1993)

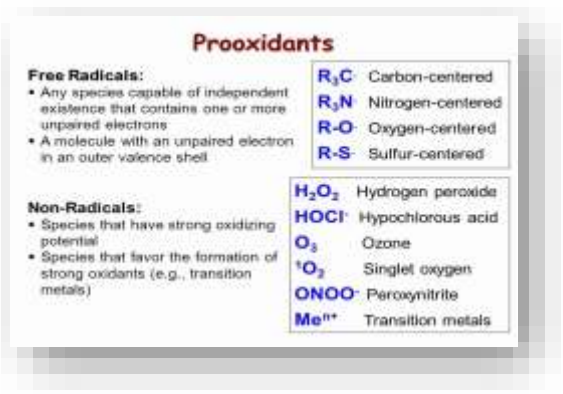

Figure (1) Types of reactive oxygen species

- **Oxidative stress**

It happens due to the imbalance between oxidants levels and antioxidant levels in human body which usually leads to damage. Under pathophysiological conditions, oxygen is released as a product of aerobic metabolism. (Sies, H., 1991), it is released as Oxygen free radicals which are called reactive oxygen species (ROS) or as Nitrogen oxides which are called reactive nitrogen species (RNS). Both species generated by regulated enzymes Like NO synthase and NAD (P) H oxidase isoforms. Both reactive species play as a double face species, at low and moderate levels they play an important role in defence against infections, and induction of mitogenic response and many other physiological processes.

Many of the ROS protect the cells against oxidative stress and restore "redox homeostasis" (expression. Dröge, W., 2002) At high levels of (ROS & RNS) or when they accumulate in the human body they become harmful resulting in oxidative stress which could result in the

damage of the cell structure, lipids, proteins, amino acids and DNA. (Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J., 2007)

Oxidative stress leads to redox imbalance which is accompanied with cancer cells rather than normal cells. This imbalance may lead to DNA mutation which was noted as a DNA Lesions (8-OH-G) in many kinds of tumours (Fang, Y. Z., Yang, S., & Wu, G., 2002)

- **Antioxidants**

Halliwell & Gutteridge (1989) defined Antioxidants as any substance that when present at low concentrations compared with that of an oxidizable substrate significantly delays or inhibits oxidation of that substrate. This includes compounds of a non-enzymatic as well as an enzymatic nature. Clearly, the diversity of anti-oxidants matches that of pro-oxidants (Sies, 1993), (Anderson, D. Y. T. W., Yu, T. W., Phillips, B. J., & Schmezer, P., 1994).

An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property. (Lobo, V., Patil, A., Phatak, A., & Chandra, N., 2010)

- **Types of antioxidants:**

Multiple internal defence mechanisms are activated due to the exposure of cells, tissues and the extracellular matrix to the harmful effects of free radicals which activate large number of reactions. This defence mechanism eliminates free radicals and their derivatives. These mechanisms are: are considered to be the first line of defence, preventing reactions of free radicals and their derivatives with biological substances in the body, it works on repairing by preventing into a radical oxidation reaction from completing its pathway and inactivating the products of free radical reaction and their derivatives, by repairing or eliminating structural damage.

Antioxidants are reducing agents such as thiols, ascorbic acid, or polyphenols molecules that inhibit the oxidation of other molecules by being oxidized. Plants and animals have complex systems of different types of antioxidants such as glutathione, vitamin C, vitamin A and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Antioxidants are investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness, to be used in dietary supplements. Initial studies suggested that antioxidant supplements may decrease the possibilities of degenerative diseases and improve health, on the other hand large clinical trials with a limited number of antioxidants detected no benefit and even suggested that excess supplementation with certain putative antioxidants may be harmful. From the literature review we may conclude that the diets high in antioxidants (fruits and vegetables) are nearly almost beneficial, but this is not the case for diet supplementations. The possible explanation is that, in the diet, there is a mix of antioxidants and it is well recognized that they work as a continuous chain, while supplementation is usually given using one or two substances.

Therefore, the antioxidant chain is not completely available. In this regard, it is well-known that after scavenging free radicals, if an antioxidant is not restored by the following antioxidant in the chain, it begins to be a pro-oxidant. In this situation, the final effect of such supplementations would be no effect or a damaging effect. Therefore, in antioxidant therapy complimentary antioxidants cannot always substitute the fruits and vegetables high in antioxidant (Rafieian-Kopaei, M., Baradaran, A., & Rafieian, M., 2013)

Type of ROS	Neutralizing Antioxidants
Hydroxyl radical	vitamin C, glutathione, flavonoids, lipoic acid
Superoxide radical	vitamin C, glutathione, flavonoids, SOD
Hydrogen peroxide	vitamin C, glutathione, beta carotene, vitamin E, CoQ10,flavonoids, lipoic acid

Table (1) Types of antioxidants

In their definition of the term, Halliwell & Gutteridge (1989) state that an antioxidant is 'any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate'. This definition includes compounds of a non-enzymatic as well as an enzymatic nature. Clearly, the diversity of antioxidants matches that of pro-oxidants. (Gutteridge, J. M., & Halliwell, B., 1989)

Antioxidants are the first line of defence against reactive oxygen species. This defence includes several strategies and interactions between the reactive species and the various types of antioxidants. These strategies include prevention, diversion, interception and repair. .

- **Antioxidant capacity**

Total antioxidant capacity (TAC) is a term originated at first in chemistry and then was applied to biology and medicine (Sies, H., 2007) Antioxidant capacity is defined as a measure of the amount (in moles) of a given free radical removed by a sample. Measurements of antioxidant capacity yield the amount of a heterogeneous mix of antioxidants that react together to produce the total removing ability of the sample. The antioxidant capacity of each individual component is not measured (Guevara, I., Iwanejko, J., Dembińska-Kieć, A., Pankiewicz, J., Wanat, A., Anna, P., ... & Szczudlik, A. ,1998)

Total antioxidant capacity (TAC) is the measure of the amount of free radicals scavenged by a test solution (Ghiselli, A., Serafini, M., Natella, F., & Scaccini, C. (2001)., being used to evaluate the antioxidant capacity of biological samples (Marques, S. S., Magalhães, L. M., Tóth, I. V., & Segundo, M. A. ,2014) (Bartosz, G.,2010). (Pinchuk, I., Shoval, H., Dotan, Y., & Lichtenberg, D., 2012).

- **Methods of TAC determination**

Several methods have been developed to measure the total antioxidant capacity of a biological sample. The use of peroxyl or hydroxyl radicals as prooxidants in the oxygen radical absorbance capacity (ORAC) assay makes it different and unique from the assays that involve oxidants that are not necessarily prooxidants. An improvement in quantitation is achieved in the ORAC assay by taking the reaction between substrate and free radicals to completion and using an area-under-curve technique for quantitation compared to the assays that measure a lag phase. The interpretation of the changes in plasma or serum antioxidant capacity becomes complicated by the different methods used in detecting these changes.

Determination methods include: (1) Spectrometric techniques that includes ABTS method, ORAC assay, HORAC assay, and CUPRAC method. (2) Electrochemical techniques that include amperometric method, biamperometric method, and biosensor method. (3) Chromatographic methods that include gas chromatography. (Jayaprakasha, G. K., & Rao, L. J., 2000)

- **Materials and Methods**

The idea of the experiment of this study is working on the reaction of Antioxidants with Iodine and comparing it with the results obtained from the reactions of the same antioxidants with CUPRAC and ABTS methods. In this study the capacity of antioxidant will be determined by the reaction between iodate and hydroxyl amine to release the nitrite which will form a pink colour. In the reaction between the potassium iodate with the hydroxyl amine in the presence of water, nitrite ion will be release forming a pink colour. The intensity of pink colour will be decreases when it react with the antioxidant to reduce the iodate ion which has (– 5) oxidation number to Iodine with (0) oxidation number. The decrease in the intensity of the pink colour is proportional to the antioxidant capacity. The

intensity of the pink colour will be measured using the UV- Visible Spectrophotometer against reagent blank at 540 nm. The difference between the intensity of the sample and the blank reagent will resemble the antioxidant capacity.

In this study, the Total antioxidant capacity (TAC) of 8 antioxidants has been studied using iodate method and the results were compared with the results of TAC using CUPRAC and ABTS methods. Those antioxidants were Gallic acid (GA), Caffeic acid (CFA), Glutathione (GSH), cysteine (CYS), Homocysteine (HCYS), N-Acetyl-Cysteine (NAC), Trolox (TR) and Catechin (CAT).

Three Mixes of Antioxidants has been tested with the same methods. The mixes were Gallic acid and Caffeic acid, Caffeic acid and Trolox while the last mix was Glutathione and Cysteine.

At the end of the experiment a real sampled in the Green tea was tested.

- **Results**

All tested antioxidants gave stright line with correlation coeffecient near to 1.0 in the Iodate method as those obtained from the two other determination methods. İn Iodate method the used volume ranged from 20 to 1000 (µl) while the concentartion ranged from $5* 10^{-3}$ M to 0.1 M.

The determination coefficient values were all near to 1

İn the following tables, the linear equation correlating ΔA to antioxidant, molar concentration (c), linear concentration ranges and determination coeffecient (R^2) of tested Antioxidants

Values of tested antioxidants in Iodate method was demnostrated in (Table 2) method (Table 4).

AOX	Sample Volume (µl)	Final conc.	Linear equation for calibrating AOX	Determination coefficient (R2)
GA	20 - 100	0.1M	ΔA = 319C + 0.104	R^2= 0.9280
CFA	20 - 100	0.1M	ΔA = 222C + 0.090	R^2= 0.9930
GSH	30 - 150	10^{-3} M	ΔA = 2825C - 0.071	R^2= 0.9960
CYS	20 - 100	10^{-2} M	ΔA = 4676C - 0.071	R^2= 0.9430
HCYS	200 - 600	$5*10^{-3}$ M	ΔA = 1584C - 0.198	R^2= 0.9530
NAC	200 - 1000	10^{-3} M	ΔA = 3535C + 0.020	R^2= 0.9970
TR	20 - 100	10^{-2} M	ΔA = 3876 C - 0.025	R^2= 0.9420
CAT	20 - 100	$2*10^{-2}$ M	ΔA = 1398C + 0.503	R^2= 0.8770

Table (2) values of tested antioxidants in Iodate method

İn CUPRAC method the sample volume ranged from 0.1to 1.0 (µl) while the final concentration ranged from $5*10^{-4}$ to 10^{-3} M. The determination coefficient of all tested antioxidants gives values near to 1.0

AOX	Sample Volume (µl)	Final conc.	Linear equation for calibrating AOX	Determination coefficient (R2)
GA	0.2 - 0.6	10^{-4} M	Δ = 44685 C + 0.032	R^2= 0.9960
CFA	0.2 - 0.6	10^{-4} M	Δ = 41380 C - 0.005	R^2= 0.9980
GSH	0.1 - 0.5	10^{-3} M	Δ = 7632 C - 0.012	R^2= 0.9990
CYS	0.2 - 1.0	$5*10^{-4}$ M	Δ = 8583 C - 0.007	R2= 0.9990
HCYS	0.1 - 0.5	10^{-3} M	Δ = 4782 C + 0.008	R^2= 0.9980
NAC	0.1 - 0.5	10^{-3} M	Δ = 7201 C + 0.007	R^2= 0.9990
TR	0.1 - 0.5	$5*10^{-4}$ M	Δ = 17107 C - 0.017	R^2= 0.9990
CAT	0.1- 0.5	10^{-4} M	Δ = 68965 C - 0.007	R^2= 0.9990

Table (3) values of tested antioxidants in CUPRAC method

In ABTS method, the volume of the samples ranged from 0.1 to 1.0 (µl) while the final concentration ranged from $5*10^{-5}$ to 10^{-4} M, the determination coefficient gives values near to 1.0

AOX	Sample Volume (µl)	Final conc.	Linear equation for calibrating AOX	Determination coefficient (R2)
GA	0.1 - 0.6	$5*10^{-5}$ M	Δ = 99653 C + 0.629	R^2= 0.9870
CFA	0.1 - 0.6	10^{-4} M	Δ = 33858 C + 0.017	R^2= 0.9960
GSH	0.1 - 0.6	10^{-4} M	Δ = 21575 C + 0.124	R^2= 0.9200
CYS	0.2 - 1.0	10^{-4} M	Δ = 17493 C + 0.017	R2= 0.9970
HCYS	0.1 - 1.0	$5*10^{-5}$ M	Δ = 33154 C + 0.009	R^2= 0.9950
NAC	0.1 - 1.0	$5*10^{-5}$ M	Δ = 52169 C - 0.010	R^2= 0.9930
TR	0.1 - 0.6	10^{-4} M	Δ = 32310 C - 0.011	R^2= 0.9960
CAT	0.1 - 0.6	$5*10^{-5}$ M	Δ = 27523 C + 0.264	R^2= 0.9160

Table (4) values of tested antioxidants in ABTS method

As an example on tested antioxidants, the values of the Gallic acid in iodate method were plotted to demnostrate the relation between concentartion and absorbance in figure (2), the values gave straight line with R^2 equal to 0.928 near to 1.0

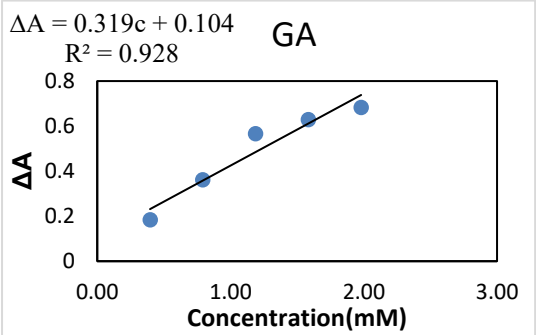

Figure (2) Gallic acid graph in Iodate method

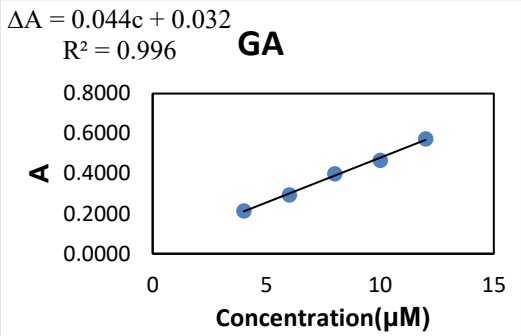

Figure (3) Gallic acid graph in CUPRAC method.

The graph of Gallic acid in CUPRAC method gave the straight line like the graph of Gallic acid in Iodate method with R^2 equal to 0.996 near to 1.0. Also the graph of gallic acid in ABTS method gave the R^2 equal to0.987 near to 1.0

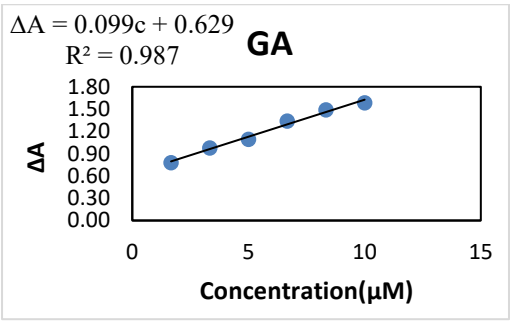

Figure (3) Gallic acid graph in CUPRAC method.

Another example on the tested antioxidants is caffeic acid which gave R^2 in Iodate method equal to 0.9993 while in CUPRAC method it gave R^2 equal to 0.998 and in ABTS method it gave R^2 equal to 0.996.

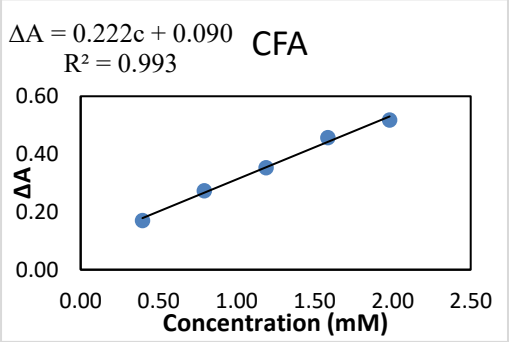

Figure (4) caffeic acid in IODATE method

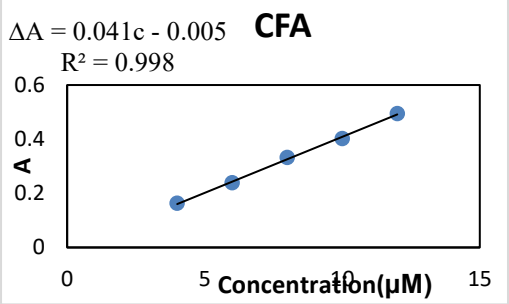

Figure (5) caffeic acid in CUPRAC method

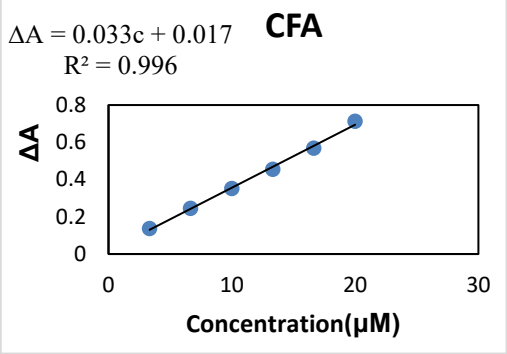

Figure (6) caffeic acid graph in ABTS method.

Almost the same values obtained from other antioxidants tested with the three methods as shown in tables (2), (3) and (4).

Testing a mix of antioxidants with iodate method gave an accebtable percentage error values ranged from 11.1473 eto 22.5707 while in cuprac method it gave a percentage error ranged from 2.8500 to 14.5192. In ABTS method the percentage error ranged from 3.3737 to 0.9994. the values are demnostrated in table (5), (6), and (7).

AOX	A	A (AOX-)	ΔA	Percentage error
GA	0.7284	0.9501	0.2217	
CFA	0.7224		0.2277	
GA+CFA	0.5525		0.3976	-11.5265
CFA	0.9690	1.1536	0.1846	
TR	0.8216		0.3320	
CFA+TR	0.7536		0.4000	-22.5707
GSH	0.7987	0.9423	0.1436	
CYS	0.4113		0.5310	
GSH+CYS	0.3429		0.5994	-11.1473

Table (5) perccentage errors of mix of antioxidants in iodate method

AOX	A	Percentage error
GA	0.1996	
CFA	0.1675	
GA+CFA	0.4204	14.5192
CFA	0.1608	
TR	0.3199	
CFA+TR	0.4944	2.8500
GSH	0.3152	
CYS	0.1559	
GSH+CYS	0.5104	8.3421

Table (6) perccentage errors of mix of antioxidants in CUPRAC method

AOX	A	Percentage error
GA	0.1996	
CFA	0.1675	
GA+CFA	0.4204	14.5192
CFA	0.1608	
TR	0.3199	
CFA+TR	0.4944	2.8500
GSH	0.3152	
CYS	0.1559	
GSH+CYS	0.5104	8.3421

Table (7) perccentage errors of mix of antioxidants in CUPRAC method.

Working on Trolox antioxidant in Green Tea as a real sample for determining the total antioxidant capacity, the values were obtained using the three methods of determination are demonstrated in table (8).

Antioxidant capacity of real sample			
Sample	Iodate	CUPRAC	ABTS
Green Tea	1.69 ± 0.05	1.07 ± 0.03	0.66 ± 0.3

Table (8) values of TAC in the three methods for the green tea as real sample

From the table we can see that the TR equivalent TAC of green tea was close to each other for Iodate, CUPRAC, and ABTS method, which indicate the efficiency of Iodate method in determining TAC.

- **Discussion**

The previous results indicate that using antioxidants led to an oxidation which appears in the form of decreasing the pink colour and a difference in the intensity of the colour that led to a difference in the absorbance of the samples that was referred to as (ΔA).

As the volume of the used antioxidant increases the intensity of pink colour increases which means more oxidation occurs. ΔA is directly proportional to the volume of antioxidant.

Which means that; as the antioxidant concentration increases, the oxidation capacity increases and the antioxidant capacity increases. The antioxidant capacity was measured using the three methods of determination, which were CUPRAC method, ABTS method and finally the IODATE method which proved its ability to determine the antioxidant capacity as well as the previous methods.

The graphs that were plotted using the values obtained from the experiments formed a straight line with fixed slope and a correlation coefficient near to 1.0 which proves the direct proportional relation.

From the figures we find that correlation coefficient (R^2) is near to 1.0 which indicates the effectiveness of the antioxidant in oxidizing the nitrite resulting in the formation of pink colour.

All these factors proved the effectiveness of iodate method in determining the antioxidant capacity

- **Conclusion**

There is a deep need in the scientific field to recognize and determine the total antioxidant capacity to understand the nature of free radicals and how it lead to serious diseases to the human body. TAC could be measured by several techniques such as spectrophotometric techniques, electrochemical techniques and chromatographic techniques.

Each technique used to determine the TAC of specific groups of compounds. Although there are many determination techniques for determining TAC but still there is a need to develop a simple, reliable, inexpensive techniques which will help developing more studies and discovering more antioxidants in order to manage control on the human diseases and help improve the man health. By measuring the antioxidant capacity by Iodate reaction, which depends on the Griess reaction, it was clear that antioxidants have the ability to oxidize the nitrite into nitrate resulting in decreasing the intensity of the formed pink color. The values of the tested antioxidant was plotted and yielded straight-line graphs with a fixed slopes and correlation coefficient near to 1.0 which indicates the effectiveness of Iodate method in determining the (TAC). Iodate method was proved to be an effective un-expensive way to determine the antioxidant capacity.

From the previous points, Antioxidants are representing the first defense compounds that the ability to remove ROS and RNS. Doing more research on the antioxidants is a very important matter as they are a very important defense compound against ROS and RNS.

In addition, conducting more investigations for determining the total antioxidant capacity (TAC) and on the content of antioxidant in different food kinds is highly recommended. In addition, we recommend that any person should take a daily share of food that contains a needed content of antioxidants to protect himself from aging. From the results demonstrated in the results section, it is preferred to use Iodate method in future researches to investigate the total antioxidant capacity (TAC), as it is a simple, reliable and un-expensive method.

- **Acknowledgment**

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