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M.Sc. THESIS

A METHOD DEVELOPMENT FOR DETERMINATION OF IODATE BASED ANTIOXIDANT CAPACITY

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FOREWORD

After giving praise to Allah, who gave me the strength to go on this research study and complete it by granting me the opportunity and the ability to proceed till the end.

I'd like to thank my supervisor Professor Dr. Sema DEMİRCİ ÇEKİÇ for her patience, continuous effective guidance and support during this study.

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AGOUB ABDULHAFITH YOUNES MUSSA

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	Explanation
ROS	: Reactive oxygen species
RNS	: Reactive nitrogen species
TAC	: Total Antioxidant capacity
GSH	: Glutathione reduced
GSSG	: Glutathione disulphide
AD	: Alzheimer's disease
SOD	: Superoxide dismutase
LOO•	: Peroxyl radicals
ORAC	: Oxygen radical absorbance capacity
DPPH•	: (2,2-diphenyl-1- picrylhydrazyl) radical
ABTS •	: (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical
FRAP	: Ferric reducing antioxidant power method
TPTZ	: (2,4,6-tri(2-pyridyl)- 1,3,5-triazine) ferric in complex
AAPH	: 2,2'-azobis-(2-amidino-propane) dihydrochloride
HORAC	: Hydroxyl radical averting capacity assay
TRAP	: Total peroxyl radical trapping antioxidant parameter assay
CL	: Chemiluminescence
TBARS	: Thiobarbituric acid-reactive substances
PFRAP	: Potassium ferricyanide reducing power method
GC	: Gas chromatography
CTL	: Catalase
GPx	: Glutathione peroxidase
HAT	: Hydrogen Atom transfer
ET	: Electron transfer
nm	: nano meter
Aox	: Antioxidant
µL	: Micro litter
GA	: Gallic Acid (GA)
CFA	: Caffeic acid
CYS	: Cysteine
HCYS	: Homocysteine
NAC	: N-Acetyl-Cysteine

TR	: TROLOX
CAT	: Catechin
A	: Absorbance
O_2^{-}•	: Super oxide radical
KIO_3	: Potassium Iodate
HA	: Hydroxyl ammonium chloride
SA	: Sulfonamide
NED	: N-1-naphthyl ethylene diamine dihydrochloride
NC	: Neocuproine
ETOH	: Ethanol

ÖZET

YÜKSEK LİSANS TEZİ

İyodat Esaslı Antioksidan Kapasitesi Tayini İçin Bir Yöntem Geliştirilmesi

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Oksijen içeren serbest radikaller ve bazı belli radikal olmayan oksijen bileşikleri "reaktif oksijen türleri" (ROS) olarak adlandırılırlar ve insan vücudunda hem iç hem dış kaynaklar yoluyla oluşurlar. Birçok biyolojik reaksiyonda ROS doğal olarak oluşur. İnsan vücudunun ROS karşısında kendi savunma mekanizması mevcuttur. Bu sistem enzimatik yada enzimatik olmayan antioksidanlardan (AOx) oluşmaktadır. Ayrıca, antioksidanca zengin besinlerin, özellikle polifenol içeren sebze, meyve ve bitki çaylarının tüketilmesi AOx savunması adına yararlıdır. ROS, AOx tarafından dengelenemediğinde, serbest radikal akımı oksidatif strese yol açar ki bu da ciddi hastalıklara yol açan hücre yaşlanmasınında önemli bir role sahiptir. Son yirmi otuz yıldırhem ROS hem de toplam antioksidan kapasite (TAC) belirlenmesi yönünde artan bir ilgi söz konusudur. Bu da kolay, güvenilir ve düşük maliyetli metodların geliştirilmesi konusunda bir ihtiyaç meydana getirir. Bu aynı zamanda bu tezin amacını da oluşturmaktadır. Çalışmada yeni bir TAC yöntemi geliştirilmiştir. Burada sunulan yöntem temel olarak nitrit tayininde en çok kullanılan prosedürlerden biri olan Griess reaksiyonuna dayanmaktadır. Çalışmada, nitrit iyonu iyodat (IO_3^-) ve hidroksilamin hidroklorür arasındaki reaksiyon sonucunda oluşturuldu. Sonrasında oluşan nitrit ve sülfanilamit arasında asidik ortamda gelişen reaksiyon sonucu bir diazonium tuzu meydana gelir. Ve N-(1-naftil)etilendiamin varlığında pembe renkli bir azo kromofor ortaya çıkar. Renk şiddeti nitrit konsantrasyonuyla orantılıdır. Reaksiyon ortamında AOx bileşiklerin var olamsı durumunda IO_3^- bunlar tarafından tüketilir ve bu da nitrit miktarının ve bunun sonucunda gelişen pembe rengin şiddetinde azalmaya yol açar. Pembe rengin şiddetindeki azalma AOx konsantrasyonuyla orantılıdır. Pembe rengin şiddeti 540 nm de AOx varlığında ve yokluğunda ölçüldü ve denenen herbir AOx için konsatrasyon ve absorbans farkı arasında kalibrasyon grafikleri çizildi. Geliştirilen yöntem AOx karışımlarına ve gerçek örneğe (yeşil

çay ve beyaz çay) uygulandı. Tez çalışmasında standart karşılaştırma yöntemleri olarak CUPRAC ve ABTS/TEAC kullanıldı.

Haziran 2019, 67 sayfa.

Anahtar kelimeler: İyodat, Griess reaksiyonu, serbest radikal, oksidatif stres, antioksidanlar, toplam antioksidan kapasite

SUMMARY

M.Sc. THESIS

A METHOD DEVELOPMENT FOR DETERMINATION OF IODATE BASED ANTIOXIDANT CAPACITY

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Oxygen containing free radicals and some certain non-radical oxygen compounds are known as “reactive oxygen species” (ROS) and they can be produced inside or outside the cells of the the human body. ROS may generate naturaly and they can be produced both externally and internally in human body during biological reactions. ROS may generate naturally. Against the ROS Human body has its own defense system against ROS which consists of enzymatic and non-enzymatic antioxidants (AOx). Additionally, consuming AOx rich foodstuff especially polyphenol containing fruits, vegetables and herbal teas are useful for AOx defense. When ROS cannot be balanced by AOx, accumulation of the free radicals leads to oxidative stress which plays the essential role of the cell ageing that leads to serious diseases. In recent decades, there has been an increasing interest as to the determination of TAC and the need for the development of easy, reliable and inexpensive methods which is also the goal of this thesis.

In this study a new TAC determination method was developed. The presented assay basically bases on Griess reaction which is one of the most common procedures for nitrite

determination. In the study, nitrite ion was produced by the reaction between iodate and hydroxylamine hydrochloride. Then as a result of the reaction between generated nitrite and sulfanilamide in the acidic medium, a diazoniumsalt is produced. And in the presence of N-(1-naphthyl) ethylendiamine a pink colored azo chromofore was produced. The color intensity is related to nitrite concentration. In the presence of AOx compounds in the reaction medium IO_3^- is consumed by them and it reduces the resulting nitrite and leads to decrease in the pink color. The decrease in the intensity of the pink color is proportional to the AOx concentration. The intensity of the pink color was measured at 540 nm against reagent blank twice, first in the presence of AO_x, and the second time in the absence of AOx compounds and calibration graphs were drawn between absorbance difference and molar concentration for each AOx tested. The proposed method was applied to AOx mixtures and real sample (green tea and white tea). In the thesis CUPRAC and ABTS/TEAC methods were used as standard comparison methods.

June 2019, 67 pages.

Keywords: Iodate, Griess reaction, free radical, oxidative stress, antioxidants, total antioxidant capacity

1. INTRODUCTION

As an aerobic organism oxygen methabolism is an inseparable part of human body. During this mechanism different type of oxygen species may generate and some of them are defined as "Reactive Oxygen Species" or briefly they known as ROS. One of ROS is superoxide anion radical ($\bullet O^{2-}$) and it formed from about 1-3 % of oxygen that we use to breathe, this anion is one-electron reduction product and hydrogen peroxide (H_2O_2) [1]. ROS can be at different reactivities and can be play usefull roles but the amout of them should be controlled by the substances called antioxidants. These are substances that reverse the harmful effect of oxygen. The mechanistic definitions of antioxidants are a group of substances which reverse the effect of harmful oxygen species in human body. Antioxidants are the compounds that can donate hydrogen and/or electron. The capacity of a given antioxidant is depend on its ability of removing free radicals from the sample [2].

There are different types of systems for classifation of antioxidant capacity measurements. Maybe the most common of it to calssify total antioxidant capacity determinations as depending singlet electron transfer or hydrogen ion transfer.

In the study Griess reaction was applied to total antioxidant capacity determination. Since some of pathological processes are accompanied by the generation of nitric oxide (NO), there was a need to monitor the concentration of (NO_2^-, NO_3^-)

Griess's method involves the producing of nitrites from nitrates in the presence of NADPH as reduction agent. Griess reaction is a common and useful way of NOx level determination in body fluids such as serum and urine [3].

In the human body there are an equilibrium between oxidants and antioxidants. When oxidant species can not be balanced by antioxidants this situation define as "Oxidative stress". It may harm certain biomacromolecules and causes a series of serious disease. The damage that usually happens when oxidants which they are side products come from the come out from the aerobic metabolic reactions which are produced at increased rates under certain physiological conditions. Enzymatic and non-Enzymatic mechanisms are the ways that antioxidant use to overcome oxidative stress [4]

The lack of Antioxidant in human body could cause serious problems such as Cancer, heart diseases, and aging.

In the thesis study it was aimed to introduce an a low-cost and reliable assay for determination of the total Antioxidant capacity (TAC). In this study, the interaction between iodine and hydroxylamine is determined by the nitrite reaction resulting from Griess mechanism. The intermediate antioxidants may join and then reduces the iodate by the antioxidant and the actual oxidation reaction between the iodate free medium causes the decrease of the Griess nitrite. As a result of this reaction, a pink colored reaction product occures, the method depends on the measurement of this pink colored product. In the presence of antioxidants the formation of this decreases and it causes a decrease in the absorbance coming from this pink color. The absorbance values were measured both with and without antioxidants. The absorbance values obtained and the difference of the (A) were calculated. ΔA will be plotted against the antioxidant concentration to obtain the calibration diagrams. Subsequent treatments were applied using reference TAC determination methods namely CUPRAC and ABTS/TEAC.

Free radicals are an important class of ROS containing one or more unpaired electrons that is considered to be as very reactive parts of molecules, always produced as side products of metabolic reactions or phagocytosis. Superoxides and hydroxyl radicals are the oxygen derivatives that react in aerobic condition in the reaction of hydrogen peroxide and transition metals. Cells have their own defence mechanisms to prevent the formation of free radicals and decreasing its harmful effect. Peroxides and proteins are broken by enzymes to transition metals to overcome excess free radicals. The danger of free radicals is that it oxidize cell components like proteins, lipids and DNA leading to cell damage [5].

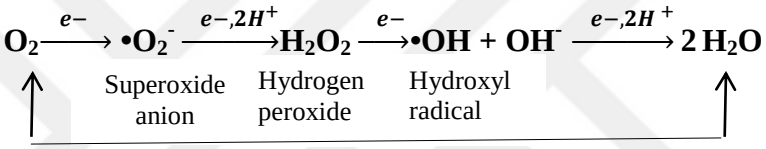
The reaction of free radicals are not easy to be detected, so measuring its reactivity takes place indirectly by measuring the end products of its reaction with other components like lipids, proteins, and DNA.

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. The most studied reaction is lipid peroxidation, as it provide different assays. Protein and nucleic acid oxidation are attracting increased attention. The available techniques that are used to measure those end products are limited, so there is a deep need to develop new techniques [6].

1.1. FREE RADICALS AND ITS DISEASE-CAUSING EFFECTS

Free radicals are can be molecules or molecular fragments which differentiate than other biological products in having unpaired electrons in their outer orbitals (e.g. HO•). This makes them highly reactive molecules and available to combine by its unpaired electron with any other biological components. It include lipids, proteins, cell membrane, and DNA to induce its oxidation. This can cause disturbance or damage in cell membrane, DNA mutations [7]. Oxygen radicals considered the most important group of free radicals [8, 9].

Formation of free radicals takes place enzymatically or non-enzymatically in human body. Oxidation-reduction reactions can take place in the mitochondria and endoplasmic reticulum [10].



Complete O_2 Reduction

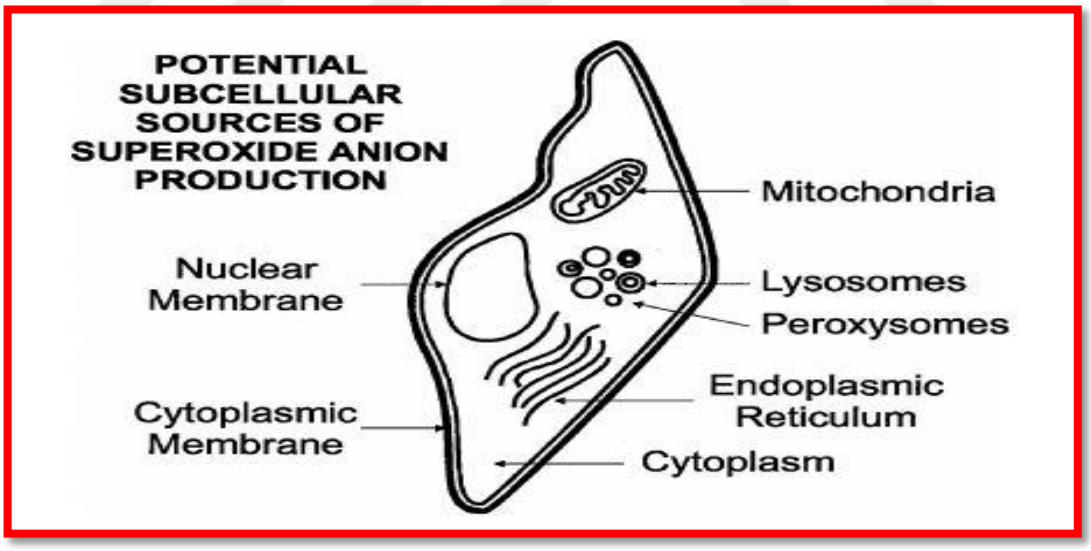


Figure 1.1: Shows cellular sites that produce oxygen free radicals.

Mitochondria is known energy production center of cell. This energy generation and respiration mechanism called "electron transport chain". It is also called "the powerhouses of the cell" [11]. In the certain conditions, some of the electrons react with Oxygen can escape

from the electron transport chain to produce free radicals of oxygen as about 1-3% of mitochondrial oxygen.

The most affected part by oxygen free radicals in mitochondria is the mitochondrial DNA (mt DNA). Most of damages to nuclear DNA could be fixed except that happens to (MT DNA). That damage accumulates causing death to cells and aging of the organism [10].

There are different reactions generating free radicals as intermediate products. The most known system is covalent bond cleavage either thermally or photochemically another pathway is the reduction-oxidation reaction which occur in biological system. Molecule-molecule interaction that results in formation of free radicals is unusual reaction. Substituting an atom in a neutral molecule, or adding a group to multiple bonds, and fragment rearrange are some of reaction that free radicals may undergo. Free radicals react with a substrate to produce a new radical, which reacts with the first one to give a product. That is the chain reaction, which continues until removing the radicals from the biological system. In oxidative processes, free radicals can undergo variety of reactions instead of substrate reactions producing many different compounds [12].
Here are two equations showing free radical reaction:

$$RO\text{-}OR \rightarrow 2RO\bullet$$

$$R\text{-}N{=}N\text{-}R \rightarrow 2R\bullet + N2$$

And here is an example on chain reaction:

$$Rad\bullet \text{ (from peroxide)} + AB \rightarrow Rad\ B + A\bullet$$

$$A\bullet + X{=}Y \rightarrow AXY\bullet$$

$$AXY\bullet + AB \rightarrow AXYB + A\bullet$$

The last two steps are called a Chain Reaction [12].

$$ROOH + M^{n+} \rightarrow RO\bullet + OH^{-} + M^{(n+1)+}$$

Many evidences ensured that free radicals take a part in various human diseases [13]. Direct evidences are rare as most of evidences based on animal modules. There are some evidence in the literature abnormal production of free radicals may cause or accelerate of generation of DNA mutation and some diseses such as certain types of cancer or diabetes [14].

1.2.TYPES OF REACTIVE OXYGEN SPECIES

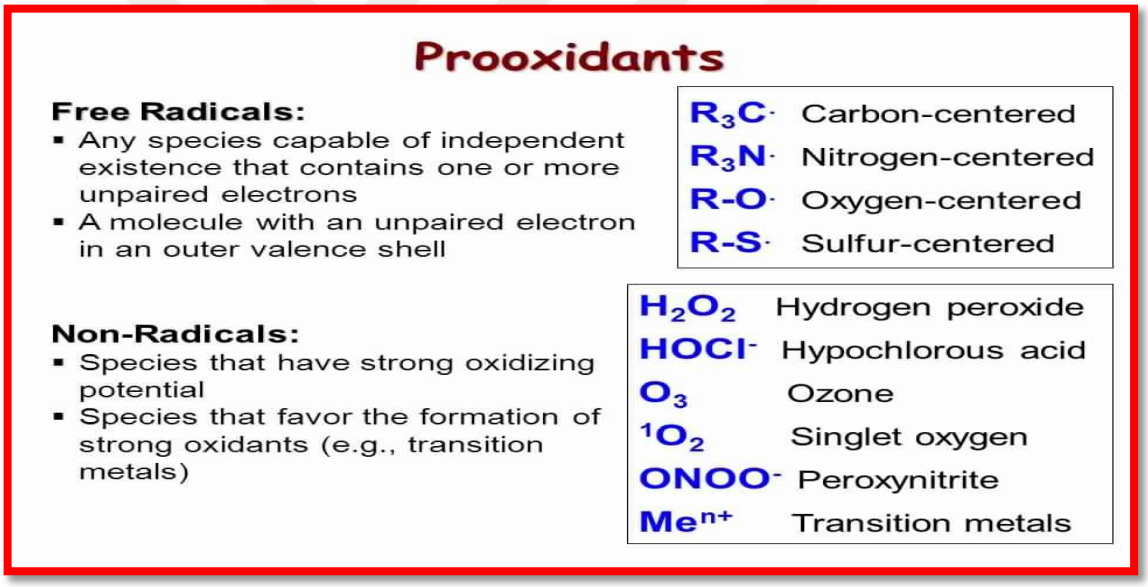


Figure 1.2: Types of Oxidants including radical and non-radical molecules.

1.3. OXIDATIVE STRESS

An imbalance between oxidants' levels and antioxidants levels in human body resulting in oxidative stress that usually leads to cellular damage. Under pathophysiological conditions, oxygen is released as a product of aerobic metabolism [15], it is released as Oxygen free radicals or as Nitrogen oxides. Both species generated by enzymatic activities among these enzymes NO synthase and NAD (P) H oxidase isoforms can be mentioned. The reactive species have double faces, at low and moderate levels they defend the cell against infections,

and induction of mitogenic response and many other physiological processes such as restoring "redox homeostasis" [16]. 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 [17].Oxidative stress leads to redox imbalance which is accompanied with cancerous 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 tumors) [18].

1.4. TYPES OF ANTIOXIDANTS

There are a multiple internal defense mechanism in human body, large mumber of reactions ocur in extra cellular matrix and tissues to eliminate free radicals. These mechanisms are considered to be the premiere defense mechanisms. It works on preventing the reaction of free radicals with biological molecules repairing the cell by interfering with the reaction pathway to inactivate the free radicals and stopping it. Besides endogenous ones we can get antioxidants exogenously mostly thanks to foodstuff. Especially vegetables, fruits, grains are quite rich in polyphenols and they are very usefull antioxidant sources.

1.4.1. Endogenous Antioxidants

Certain enzymes are the biggest part of the endogenous antioxidants additionally there are some non-enzymatic naturally occuring ones. One of the most known enzym acting as an antioxidant is superoxide dismutase (SOD). In the body superoxide anion turns to H2O2 and molecular oxygen with a certain reaction and SOD catalyses the reaction. Another group of enzymatic antioxidants that play as a first line soldier are catalase (CTL), glutathione reductase (GR), peroxiredoxins (Prxs) and glutathione peroxidase (GPx). The non-enzymatic antioxidants that play an important part in defense which are preventive antioxidants are found in plasma, they are albumin, transferrin, ferritin, and ceruloplasmin. It prevent the formation of new ROS and RNS by reacting with ions of transition metals (e.g. iron and copper) [19].

The molecules that have the ability to combine with the reactive oxygen species are considered the non- enzymatic second line of defense against ROS. The third line of defense system is represented by repairing assays that mechanisms that repair the ROS damage.

Enzymatic antioxidants provide this kind of defence, that repair damaged DNA and proteins, intervene with oxidized lipids, stop peroxyl lipid radicals from completing the chain growth, and rebuilt cell membranes. Vitamin E, vitamin C, carotenoids, and some minerals like Zn, Mn, flavonoids, phenolic acids, stilbenes, and lignans are diatery antioxidants that may have an effect on the endogenous antioxidants activity. The two types of antioxidant may work together to restore the balanced level of ROS.

1.4.2. Exogenous Antioxidants

Exogenous antioxidants are present in fruits with in effective amounts, also found in vegetables, beverages like tea, coffee, nuts and juices. Antioxidants that are found in food products can affect the aging process and may also decrease the effect of some diseases complications like diabetes, and cardiovascular diseases.

According to the mechanisms of the endogenous antioxidants it could be true scavengers or they may act as metal ion chelators. These are prevent ROS generation with Fenton lile reactions by chelating metals [20].

1.5. THE REACTION OF FREE RADICALS WITH ANTIOXIDANTS

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

1.5.1. Prevention

Prevention of oxygen species formation is the first step in the human's protection against ROS, which is one among many in the defense strategies against ROS. Some of the enzymes that are responsible for generating free radicals are designed ingeniously. One of the most active enzymes that carry out most of reduction reactions is cytochrome oxidase. Although this reaction has Cu and Fe, it can't release Superoxides or any other radicals. As the same is ribonuclotide reductase the 3- dimensional enzyme that forms an appropriate "cage" to keep the radical features of tyrosyl functions in the B subunit from to get into the environment. Binding metal ions -like copper and iron ions-is kind of defense that prevent the initiation of chain reactions. Fragmentation of DNA and lipid peroxidation is inhibited by metal chelation.

The metal binding proteins such as ferritin, transferrin and coeruloplasmin play an important role in this situaion [21]

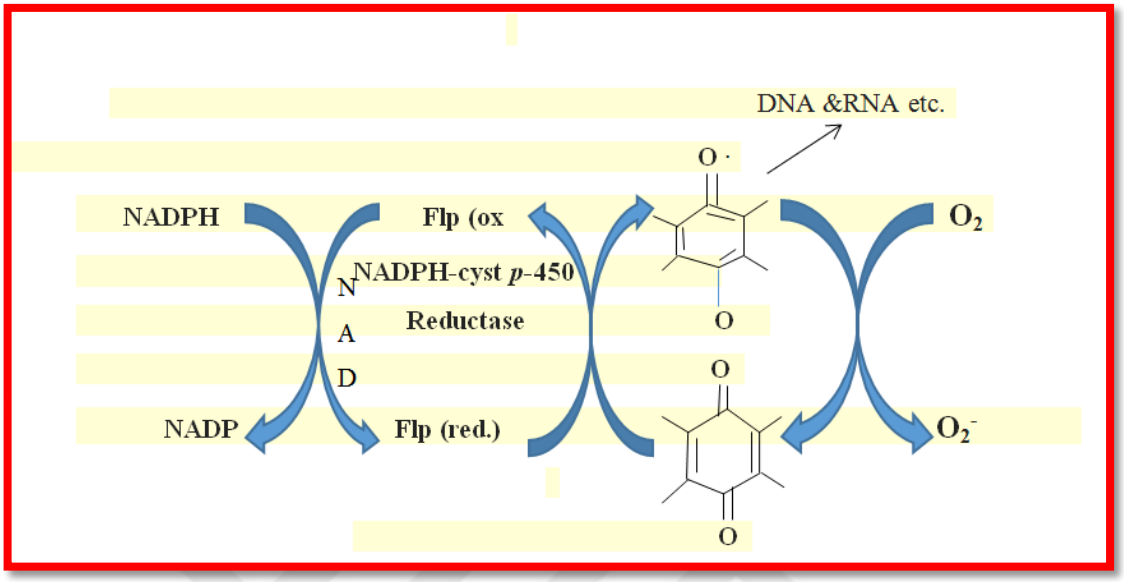


Figure 1.3: The reaction of NADPH with Quinone oxidoreductase.

1.5.2. Diversion

It is one of the preventative anti-oxidation strategies. This could be happened by converting the oxidizing species into less harmful products hence damage risk can be lowered. An example on this concept which go extremely far when it involve whole cells is the intestinal mucosal cells which are exposed to a many kinds of reactive intermediate species and xenobiotics with high rate of accumulation of oxidative products, the matter which leads to elimination of whole cells to prevent spreading more reactive species.

1.5.3. Interception

1.5.3.1. Non- Enzymatic Mechansim of Antioxidants

Intercept the damaging reactive species to prevent it from making further deleterious reactions as a form of deactivation process. Deactivation of free radicals takes place by the formation of non-reactive end products. Free radicals by nature undergo a chain reaction as follow; the

radical compound will react with another compound to switch the place of the unpaired electron ('radicals beget radicals').

Another strategy to intercept the reactive species represented in transferring the radical species away from the sensitive targets to less sensitive sites in which oxidative reactions would be less harmful, this may occur by converting the hydrophobic compounds into hydrophilic compound or by changing the phase it occur in [22, 23]

Carotenoids and oxy-carotenoids are the only compounds that may intercept with highly efficient polyene quenchers. It provides a good protection against ROS, regardless to the short lifetime and high reactivity [24, 25]. As much as the concentrations of the carotenoids increase, the efficiency of the quenching increases.

1.5.3.2. Hydrogen Atom Transfer (HAT) Defense Mechanism

HAT is usually a common step in the important biological and environmental reactions, the following equation represents the principal reaction

$$A\text{-}H + B \longrightarrow A + H\text{-}B$$

At the point when change metal buildings oxidize substrates by evacuating H• (e− + H+), regularly the electron exchanges to the metal and the proton to a ligand. This phenomenon are examined on different cases with imidazolinate, vanadium-oxo, and numerous different edifices [26]

1.5.3.3. Electron Transfer Defense Mechanism

Electron-transfer forms are crucial components of vitality transduction pathways in living cells. An exceptional collaboration amongst hypothesis and trial has created a highly vital understanding of the elements that direct this 'current of life'. Investigations of Ru-modified proteins have given bits of knowledge into the distance- and driving-force conditions of intra-protein electron exchange rates. [27]

Generally, ET active elements of the substances are more positive than -0.5 V. ET, ROS, and OS have been progressively embroiled in the method of activity of medications and poisons, e.g., anti-infective operators [28], anticancer medications [29], cancer-causing agents [30],

regenerative poisons [31], nephrotoxins, hepato-toxins, cardiovascular poisons [32], nerve poisons [33], mitochondrial poisons, manhandled drugs, and different classifications of medications and poisons, including human diseases [34, 35].

1.5.3.4. Enzymatic Defense Mechanism of Antioxidants

There are strongly effective antioxidative enzymes in the cell of the eukaryotic organisms. The three main groups of enzymes showing antioxidant activity are Superoxide dismutases, Catalases and Glutathione (GSH) peroxidases.

Many specialized antioxidants enzymes react with ROS to prevent it from causing more harm. The secondary function of antioxidants are removing and substraction of reactive substances.

Flavoprotein GSSG reductase act to convert glutathione disulphide (GSSG) to GSH, it is an example of the backup function. The example on the transport and elimination mechanism are glutathione S-transferases and glutathione S-conjugates [36].

In human methabolism only natural defence systems is not effective 100% against ROS. Hence additive antioxdants are needed most of times. Accumulation of products of damage which continuously formed in low concentrations causes DNA damage. The damage may occur in the bases, in one strand or the two strands. On the other hand, there are certain enzyme systems to repair DNA [37].

1.6. SAMPLES OF REPAIR SYSTEM

1.6.1. The Reaction Between Free Radicals And Vitamin E

Tocotrienols which generally known as Vitain E, tocopherols, ascorbic acid (vitamin C), and the carotenoids usually gives reaction react with peroxyl radicals and singlet molecular oxygen ($^1O_2$). RRR-a-Tocopherol is also one of the most effective peroxyl radical scavenger in lipophylic systems such as membranes or low-density lipoproteins [38].

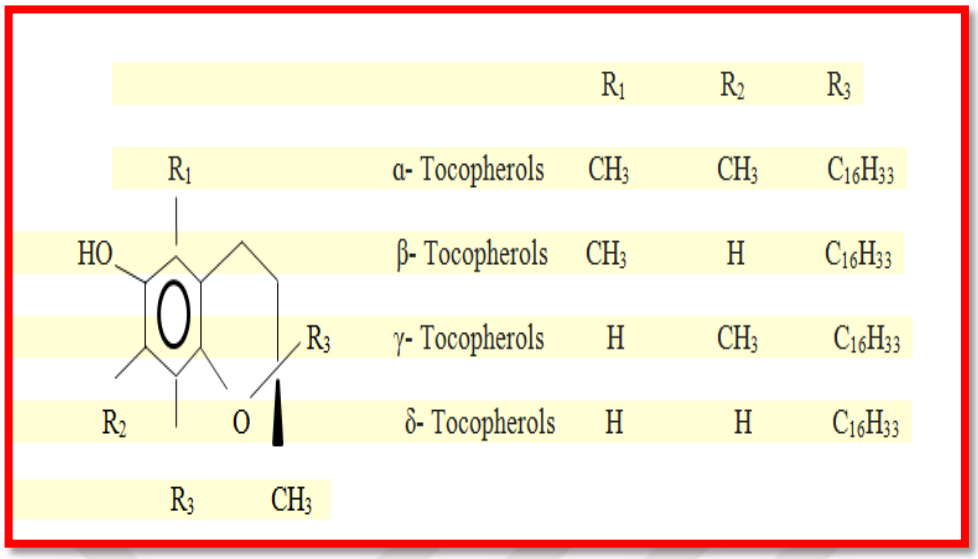


Figure 1.4: Different Homologs of vitamin E.

Another natural antioxidative vitamin is Ascorbic acid and it is found in cytosol, plasma, and other body fluids and it can reduce the tocopheroxyl radicals. On the other hand, it has several metabolic important cofactors function in enzyme reactions, especially hydroxylations. Another important lipophilic natural antioxidant class is carotenoids, which can remove $^1O_2$ or free radicals [24].

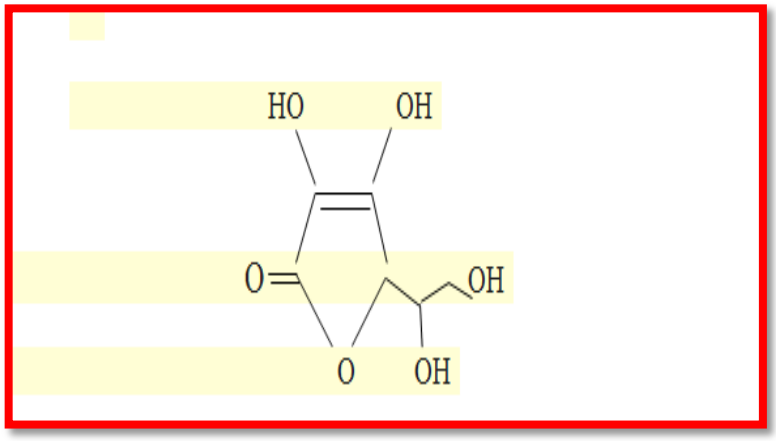


Figure 1.5: Chemical structure of vitamin C.

The term vitamin E represents four homologs, RRR-a-tocopherol is the most abundant one in the human. There is a reaction occurs between vitamin E and organic peroxyl radicals and a

reduction in the chromane ring of vitamin E during the reaction. It acts as endogenous antioxidant that protect the tissue lipids from oxidation. Vitamin E is a lipid soluble compound and it gives reaction with lipid peroxyl radicals to protect lipids from oxidation.

Lipid peroxyl radicals are turned into lipid hydroperoxides, which are relatively more stable, by the reaction with thocopherols. The radical chain reaction is interrupted by the tocopheryl radical and a protection against lipid peroxidation is achieved. Vitamin E is the major content of plasma and red blood cells that can protect lipids against oxidation [39, 40]. The concentration of vitamin E determines the degree of protection against oxidation of microsomes, low density lipoproteins, hepatocytes, or whole organs [39-46]

Studied a case in which phosphatidyl choline liposomes was taking as a model membrane, they found that peroxyl radical which is soluble in water initiate lipid peroxidation while α-tocopherol protects liposomes from the perioxidation till the full consumption. Even at low concentrations Vitamin E can protect the liposomes from peroxidation as when the hepatic microsomes were treated with water soluble peroxyl radical, the peroxidation process started when 30 % of Vitamin E in microsomes remained [47]

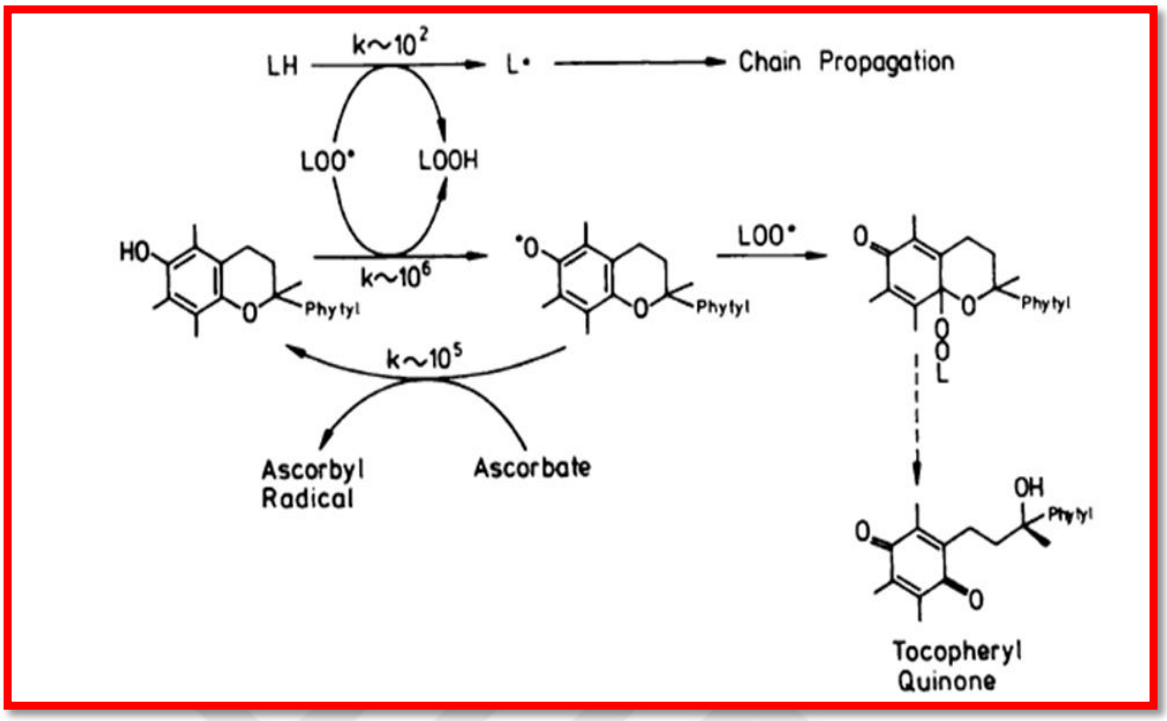


Figure 1.6: Illustration of the chemical equation of the reaction betweenVitamin E and peroxyl radical

1.7. METHODS USED TO DETERMINE TOTAL ANTIOXIDANT CAPACITY

There are different antioxidant capacity or activity determinations in the literature and these assays are classified by using differenet approaches. The methods can be sorted by analytical techniques or reaction mechanisms. In the presented study the methods were classified by analytical techniques.

The increase in TAC in the serum or the plasma is not necessarily reflecting a good condition it may be a response for high level of oxidative stress (e.g. increasing uric acid concentration may be measured in TAC but it may be the reflection of the destruction of cells). Also, the decrease in the TAC is not necessarily a bad condition. Maybe the most important one is to keep the equilibrium between oxidants and antioxidants and to keep the changes in a single person's conditions. So to determine TAC is not a simple issue [48].

1.7.1. Spectrometric Techniques For Antioxidant Capacity Determination

It depends on the generation of colorful species with a specific reaction or they may measure the decrease in the color intensity generated in the reaction medium. These methods may depend on electron transfer (ET), hydrogen atom transfer (HAT) or both.

1.7.1.1. The CUPRAC (CUPric Ion Reducing Antioxidant Capacity) Assay

The method basicly an example of assays depending electron transfer. In the CUPRAC method a stable chelat complex generates between the main reactives namely Cu(II) and 2,9-Dimethyl-1,10-phenanthroline (neocuproine, Nc) in the presence of pH 7 buffer (1 M ammonium acetate). This blue colored product turns to Cu(I)-Nc complex in the presence of antioxidants. The color of Cu(I)-Nc is yellow and the absorbance is read at 450 nm after 30 min incubation at room temperature [49].

1.7.1.2. The ABTS/TEAC Method

The method is accepted as having a mix mechanism depending on ET and HAT together. During the assay a stable, bluish-green colored cationic 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) is formed. During the reaction ABTS is usually oxidized by potassium persulphate. In the presence of radical quenching antioxidants in the reaction medium the $ABTS^{\bullet+}$ is scavenged and the initial color of the solution decreases. This decrease is proportional with the antioxidant concentration. The absorbance generated from $ABTS^{\bullet+}$ in the solution is read at 734 nm and the results are given as trolox equivalent [50].

1.7.1.3. The Ferric Reducing Aniioxidant Power (FRAP) Method

This is the anothet ET based assay. According to the method Fe(III) is reduced to Fe(II) by antioxidants in the presence of 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) ligand and a navy-blue colored Fe(II)-TPTZ complex generates [51]

1.7.1.4. Oxygen Radical Absorpiion Capacity (ORAC) Assay

The method depends on the measurement of peroxyl radical scavenging activity of antioxidants. During the assay the radical is produced from 2, 2'-azobis-(2-amidino-propane) dihydrochloride (AAPH) at $37^{o}C$ and also fluorene is used as the reagent in the fluorimetric

method. The reaction between antioxidants and the peroxyl radical causes a decrease in the fluorescence

Prooxidants in ORAC assay such as peroxyl or hydroxyl radicals, differentiate it than other assays that involve other oxidants differ than prooxidants. The reaction between the substrate and the free radicals has witnessed an improvement due to the using of area-under-curve technique for evaluation and compare it with the assays that measure a lag phase [52].

1.7.1.5. The Hydroxyl Radical Averting Capacity (HORAC) Assay

It is an assay, which measures the metal-chelating ability of antioxidants. The chelation of netal ions prevents the hydroxyl radical formation in the Fenton-like systems. In the method Co(II) complex is used and the fluorometric reagent is fluorescein similar to ORAC method. During the analysis, fluorescein is incubated with the sample and Fenton reaction mixture is added into it. [53].

1.7.1.6. Total Peroxyl Radical Trapping Antioxidant Parameter (TRAP)Assay

The reaction of peroxyl radical is monitored with the luminal-enhanced chemiluminescence (CL). The thermal degradation of AAPH results in the formation of CL signal accompaine with the formation of Luminal derived radicals. The time in which the sample use to absorb the chemiluminescence signal is the TRAP value and it refelects the concentration of the present antioxidant.

1.7.1.7. The Lipid Peroxidation Inhibition Assay

In the study a Fenton-like system ($Co(II)+H_2O_2$) is used to induce fatty acids and fatty acid peroxidation occurs. A model substrate is mixed with the sample in the presence of reagents of Fenton-like reaction. The measurement of lipid peroxidation index is measured using the concentration of thiobarbituric acid-reactive substances (TBARS) [54].

1.7.1.8. Potassium Ferricyanide Reducing Power (PFRAP) Method

The method depends on the readuction of potassium ferricyanide to potassium ferrocyanide in the presence of antioxidant compounds. The producing potassium ferrocyanide then reacts with $FeCl_3$ and the absorbance of typical blue color of the complex is measured at 700 nm.

1.7.1.9. 2, 2-diphenyl-1- picrylhydrazyl (DPPH) Method

The delocalization of the spare electron makes 2, 2-diphenyl-1- picrylhydrazyl free radical (DPPH•) rather stable. The stability prevents free radical from dimerization. The solution containing DPPH• has a purple color. The reaction between DPPH• and a hydrogen donor compounds DPPH• is reduced to molecular DPPH and the formation of this molecule causes a decrease in the intensity of purple color [55].

1.7.2. Electrochemical Techniques

Because of the redox active feature of the antioxidants electrochemical techniques can be used for TAC determination. Among the most used electrochemical methods are Cyclic voltammetry and biamperometry can be mentioned.

1.7.2.1. Cyclic Voltammetry

Cyclic voltammetry is a potentiodynamic electrochemical measurement method. In which the potential of the anode is disposed straightly against time. The capability of the anode is directly examined from an underlying incentive to a last esteem; at this time the current power is recorded wihle the cathode current is ploted versus the connected voltage, this result in formation of cyclic voltammogram.

On account of a reversible framework, the estimations of the forces of the cathodic and anodic pinnacles are equivalent. For irreversible framework, just the nearness of one pinnacle is observable on the voltammogram.

1.7.2.2. The Amperometric Method

Takes place by measuring the flowing current between workingand reference electrodes at a definite potential. Oxidation/reduction of an electro active analyte generates the current.

All the experiments were performed in a electrochemical cell composed of three electrodes at definite potential, definite concentration of the solutions and definite PH. This method is used to determine antioxidant capacity of antioxidant compounds that are soluble in water or ethanol, also it is used with other samples like wine and tea and other beverages [56].

1.7.3. Chromatographic Methods

Chromatographic methods are used to antioxidant separation and detection. The spectrophotometric and fluorimetric assays are very usefull tools to determine TAC. It can be an advantage to evaluate all synergistic and antagonistic effects between the antioxidant compounds in the sample but the detection of antioxidant compound individually is not possible. Chromatographic techniques provides identication of the antioxidants individually.

Both high performance liquid chromatography (HPLC) and gas chromatography (GC) are used frequently for antioxidant determination. Most of the time the chrpmatographic techniques are combined with mass spectrometry (MS). Using MS may improve the identification of substances.

To use GC analyzing compounds can be vaporized without decomposition. The flame ionization and thermal conductivity detectors are used with GC generally. This method used to measure the TAC of Turmeric oil and its fractions previously [57].

1.8. THE NEED TO DEVELOP A SIMPLE, INEXPENSIVE, RELIABLE METHOD TO DETERMINE TAC

As mentioned previously the most important phenomenon in the redox biology of human is keeping the balance between oxidant and prooxidants. Because today it is known very well neither oxidants nor antioxidants are solely good or bad. For human fluids such as serum, plasma, urine antioxidants are together with a numerous different compound and there are considerable interactions between them. It is also same for the foodstuffs. On the other hand as summarized above, there are many different TAC determination techniques depending different mechanisms in the literature.

The determination of TAC in serum or plasma may be an indicator to evaluate certain diseases. However, it should be noted that the TAC level show big differences for each individual. As the most important source of exogenous antioxidants determination of TAC in the vegetables, fruits, grains, beverages are also important.

The availability of a simple, easy, inexpensive method to determine TAC will help improving the accuracy of the determination process and will enhance the scientist ability to determine

the TAC for more kinds of antioxidants. This will participate in decreasing the rate of diseases and improve human health.

In this thesis we applied one of the most known Griess reaction to TAC determination. According to this nitrite generation is realized with the reaction between iodate and hydroxyl amine and this nitrite is used for diazotization of sulfanil amide and in the presence of N-(1-naphthyl)ethylenediamine a pink colored azo chromophore occurs. The absorbance of pink end product is read at 540 nm. We utilized this basic concept in the presence of antioxidant compounds NO_2^- generation decreased because of the reductive effect of antioxidants and so the intencity of resulting pink color decreased. The degree of this resuction is proportional the antioxidant concentartion.

2. MATERIALS AND METHODS

2.1. INSTRUMENTS AND CHEMICALS USED

2.1.1 Instruments

Radwag AS 220/C/2 analytical balance was used to weigh chemicals and samples. Elmasonic ultrasonic bath was used to dissolve the solid materials to prepare the related solutions. VELP Scientifica ZX3 vortex was used for mixing the solutions. To measure pH during the experiments HANNA HI 221 pH meter was used. GFL 2008 water distiller was used to produce distilled water for the experiments. And finally Varian CARRY 100 UV-Vis spectrophotometer was used for absorbance measurements. The measurements were carried out in matched HELLMA quartz cuvettes with a light path in 1.0 cm.

2.1.2. Chemical Used

Potassium iodate (KIO_3), gallic acid (GA), ethanol (ETOH), potassium persulfate, N-Acetyl Cysteine (NAC), L-glutathione (GSH), L-cysteine (CYS) and H_3PO_4 were purchased from Sigma–Aldrich (St. Louis, MO, USA), trolox (R)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was supplied from Sigma–Aldrich (St. Louis, MO, USA), hydroxyl ammonium chloride ($NH_2OH.HCl$), sulfanilamide ($H_2NSO_2NH_2$) and copper(II) chloride dihydrate ($CuCl_2.2H_2O$) were bought from Merck (Darmstadt, Germany). N-1-naphthyl ethylene diamine dihydrochloride ($C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$), (NED) neocuproine (2,9-dimethyl-1,10-phenantroline) hydrochloride, 2,2'-Azino-bis(3-ethylbenzithiazoline-6-sulfonic acid) diammonium salt (ABTS), caffeic acid (CFA), catechine hydrate (CAT), DL-homocysteine were purchased from Sigma, St. Louis, MO, USA ammonium acetate (NH_4Ac) was bought from Riedel de Haen (Steinheim, Germany).

2.2. REAGENTS AND SOLUTIONS

2.2.1. Solutions Of The Proposed Method

Sulfanilamide (SA) solution at 4.0% (w/v): 1 gram of sulfanilamide was dissolved in 2.9 mL of concentrated (14.83 M) H_3PO_4 and the volume was completed to 25 mL with distilled water.

N-1-naphthyl ethylene diamine dihydrochloride (NED) solution at 0.2% (w/v): 0.05 g solid material was dissolved in 25 mL distilled water.

Griess Reagent Mixture: SA and NED solutions were mixed 1:1 just before use to get reagent mixture.

KIO_3 solution at 1.1×10^{-3} M concentration: 0.0240 g solid material was dissolved in 100 mL distilled water.

Hydroxyl ammonium chloride (HA) solution at 0.2 M concentration: 0.6949 g of solid material was dissolved in 50 mL distilled water.

2.2.2. CUPRAC Method Solutions

To prepare 1.0×10^{-2} M $CuCl_2.2H_2O$ and 1.0 M ammonium acetate (NH_4Ac) the suitable amounts of solid materials were dissolved in distilled water and to prepare 7.5×10^{-3} M neocuproine (Nc) suitable amounts of solid was dissolved in ethanol.

2.2.3. ABTS Method Solutions

For ABTS/TEAC method, potassium persulfate and ABTS were dissolved in distilled water separately and they mixed to obtain a stock $ABTS^{+}$• solution. The final concentrations of ABTS and potassium persulfate in the mixture were 2.45 mM (mL) and 7.0 mM (mL) respectively. The stock $ABTS^{+}$• solution was diluted for 40-fold with ethanol just before use.

2.2.4. Antioxidant solutions

The stock solutions of antioxidants were prepared as mentioned below:

GA, CFA at 0.1 M; TR at 1.0×10^{-2} M; CAT at 2.0×10^{-2} M were prepared by dissolving suitable amounts of solids in ethanol. GSH at 1.0×10^{-2} M and NAC at 1.0×10^{-3} M were prepared by dissolving suitable amounts of solids in distilled water. And finally to prepare CYS at 1.0×10^{-2} M and HCYS at 5.0×10^{-3} M, suitable amounts of solids were dissolved in 0.5 mL of 1.0 M HCl and the volume was completed to 25 mL with distilled water. Stock solutions were diluted to prepare related working solutions for each method if necessary.

2.2.5. Preparation of the real samples

Green tea and white tea (ready-to-use bag) were purchased from a local market. One bag of green tea was weighed as 1.1333 g and the white tea was weighed as 1.6395 g. They were prepared as described by Apak et al. earlier (Apak et al, 2006). The tea bags were dipped into and pulled out to 250 mL of boiling water for 2 min in a beaker then it was let to stand for 3 min in the boiling water. The infused tea solutions were cooled to the room temperature and filtered by the aid of a black band Whatman quantitative filter paper. The prepared sample solution was used without any dilution for proposed method and they were diluted 10 times for CUPRAC and ABTS/TEAC methods.

2.3. PROCEDURES

2.3.1. The Proposed Method

The NO_2^- ion was produced by the reaction between IO_3^- and hydroxylamine. And the produced nitrite was colorimetrically detected with the Griess Reaction. Antioxidants caused a decrement in absorbance.

3.4 mL distilled water, 0.5 mL of 1.0×10^{-3} M KIO_3, (100-x) μL ETOH, x μL AOx, 1.0 mL of 0.2 M hydroxyl amine were mixed in this order. The mixture was allowed to stand at room temperature for 5 min. Then 50 μL reagent mixture was added and the generated absorbance was read at 540 nm after 15 min.

2.3.2. CUPRAC Method

The original CUPRAC method was developed by Apak et. al. in 2004 [49]. According to the method 1.0 mL of 1.0×10^{-2} M $CuCl_2.2H_2O$ + 1.0 mL of 7.5×10^{-3} M Nc + 1.0 mL of pH 7.0 buffer (1.0 M NH_4Ac) + (2.0 - x) mL distilled water/or EtOH (according to solution of the sample) + x ml sample (individual or binary mixtures of AOx compounds or real sample-green tea and White tea) were mixed in this order and the resulting absorbance is read at 450 nm against reagent blank after 30 min. incubation at room temperature.

2.3.3. ABTS/TEAC Method

The method was developed by Re at al. at 1999 [50]. (R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, *Free Radic. Biol. Med*.,1999, 26, 1231–1237) According to the method (1-x) mL ethanol + x mL sample solution (individual or binary mixtures of AOx compounds or real sample-green tea) and 2 mL $ABTS^{+}$• solution are added in this order and the mixture was alowed to stand for 6 minutes at room temperature. Then the absorbance was read against ethanol at 734 nm.

2.3.4. Optimization Of The Proposed Method

2.3.4.1. Optimization Of IODATE Concentration

For the determination of optimum KIO_3 concentration, experiments were conducted as described below: In a test tube (4.0-x) mL of water, x mL of KIO_3 at 5.0×10^{-4} M concentration, 1.0 mL of HA at 0.2 M concentration were added in this order, and the mixture was incubated for 5 minutes at room temperature. Finally 50 μL reagent mixture was added and after 15 minutes absorbance was read at 540 nm. The tested volumes of KIO_3 were between 0.1 and 2.0 mL.

2.3.4.2. Optimization Of HA Concentration

For this purpose, (4.5–x) mL water, 0.5 mL of 1.0×10^{-3} M KIO_3, x mL 0.1 M HA were mixed in this order and after incubation for 5 min. at room temperature Finally 50 μL reagent mixture was added and after 15 minutes absorbance was read at 540 nm. x was between 0.3-4.0 mL.

2.3.4.3. Optiımization Of Reagent Mixture Concentration

As described at Reagents and Solutions section the mixture consisted of equal volumes of SA at 4.0% concentration and NED at 0.2% were named as reagent mixture and to optimize the volume of reaction mixture experiments were operated as follows: In a test tube 3.4 mL water, 0.5 mL of 1.0×10^{-3} M KIO_3, 1.0 mL of HA at 0.2 M concentration were added in this order, and the mixture was incubated for 5 minutes at room temperature. Finally (100-x) μL 5 % H_3PO_4 and finally x μL reagent mixture were added. After 15 minutes absorbance was read at 540 nm. When concentration was increased, absorbance was increased.

2.3.4.4. The Application Of The Proposed IODATE Method To The Selected Individual Antioxidants.

Different phenolic and thiol type AOx compounds were tested by the proposed method. These AOx compounds were two phenolic acids gallic acid (GA), cafeic acid (CFA), one flavanol catechine (CAT), thiols glutathione (GSH), N-acetyl cysteine (NAC), cysteine (CYS), homocysteine (HCYS) and also trolox (TR). For this purpose 0.1 M of GA and CFA; 1.0×10^{-2} M GSH, CYS, TR; 5.0×10^{-3} M HCYS, 1.0×10^{-3} M NAC; 2.0×10^{-2} M CAT were used. A volume range between 20-100 μL were taken from GA, CFA, TR and CYS; the tested volume range for GSH was 30-150 μL, 30-100 μL for CAT and 0.2 – 1.0 mL for NAC, 0.2 – 0.6 mL for HCYS. The method was applied as mentioned earlier. The absorbance due to the Griess reaction of nitrite was decreased in the presence of antioxidants. Each absorbance value recorded as A_i and also the method was applied in the absence of any AOx, this value was recorded as A_0 and absorbance difference was calculated by using $A_0 - A_i$ formula and it was named as ΔA.

2.3.4.5. The Application Of CUPRAC Method To The Selected Individual Antioxidants

For this purpose a volume range between 0.2 – 0.6 mL were taken from 1.0×10^{-4} M GA and CFA; 0.1 – 0.5 mL from 1.0×10^{-3} M GSH, HCYS and NAC; 0.2-1.0 mL from CYS at 5.0×10^{-4} M concentration; 0.1 – 0.5 mL were taken from TR at 5.0×10^{-4} M; samples at 0.1 – 0.5 mL were taken from CAT at 1.0×10^{-4} M concentration. The method was applied as stated before.

2.3.4.6. The Application Of ABTS/TEAC Method To The Selected Individual Antioxidants

For this purpose a volume range between 0.1 – 0.6 mL were taken from 5.0×10^{-5} M GA; 0.1 – 0.6 mL from 1.0×10^{-4} M concentrations GSH, CFA both; 0.2 -1.0 mL from CYS; 0.1 – 0.1 mL from HCYS and NAC at 5.0×10^{-5} M concentration both; 0.1 - 0.6 mL from TR at 1.0×10^{-4} M ; 0.2 - 0.7 mL from CAT at 5.0×10^{-5} M. The method was applied as mentioned earlier. Each absorbance value recorded as A_i and also the method was applied in the absence of any AOx, this value was recorded as A_0 and absorbance difference was calculated by using $A_0 - A_i$ formula and it was named as ΔA.

2.4. DETERMINATION OF AOX MIXTURES

To examine the additivity of the tested methods different binary mixture were prapared. The constituent of the mixtures were tested individually also. For Iodate and ABTS/TEAC methods the procedures were prepared without any AOx as well. The differens between the absorbance in the presence and absence of individual AOx and mixtures were calculated and recorded as ΔA and percentage errors were calculated. For this purpose to calculate $\Delta A_{theoretical}$ the ΔA values of tested AOx constituing binary mixture were added. The absorbance of the mixture were read and the absorbance differens were calculated by subtracting $A_{mixture}$ from A value taken for in the absence of any AOx and this value was recorded as $\Delta A_{experimental}$. Finally the formula shown at the below was used.

$$\text{Sum of } \Delta A_{theoretical} \,\&\, \Delta A_{experimental} \longrightarrow (1)$$

$$\frac{\Delta A_{experimental} - \Delta A_{theoretical}}{\Delta A_{theoretical}} \times 100 \longrightarrow (2)$$

2.4.1. IODATE Method

Three different binary mixtures were tested, these were 20 μL 0.1 M GA + 20 μL 0.1 M

CFA, 40 μL 0.01 M TR + 40 μL 0.1 M CFA, 40 μL 0.1 M 0.01 GSH + 40 μL 0.01 M CYS. The method was applied as stated earlier.

2.4.2. CUPRAC Method

Three different binary mixtures were tested, these were 0.2 mL 1.0×10^{-4} M GA + 0.2 mL 1.0×10^{-4} M CFA, 0.2 mL 5.0×10^{-4} M TR + 0.2 mL 1.0×10^{-4} M CFA, 0.2 mL 1.0×10^{-3} M GSH + 0.2 mL 5.0×10^{-4} M CYS. The method was applied as stated earlier.

2.4.3. ABTS/TEAC Method

Three different binary mixtures were tested, these were 0.3 mL 5.0×10^{-5} M GA + 0.3 mL 1.0×10^{-4} M CFA, 0.3 mL 1.0×10^{-4} M TR + 0.3 mL 1.0×10^{-4} M CFA, 0.3 mL 1.0×10^{-4} M GSH + 0.3 mL 1.0×10^{-4} M CYS.The method was applied as stated earlier.

2.4.4. Application Of The Methods To The Real Sample

0.2 mL of green tea and 0.3 mL of white tea samples were taken separately from working solutions of tea samples. On the other hand 0.3 mL of green tea and 0.2 mL of white tea samples were taken separately for CUPRAC method and 0.2 mL of green tea and 0.1 mL of white tea samples were taken separately for ABTS/TEAC method.

3. RESULTS

3.1. OPTIMIZATION OF PROPOSED METHOD

3.1.1. Optimization of Iodate Concentration

The method for determination of the best iodate concentration was applied as stated in Material and Method section. The tested KIO_3 volumes which were taken from the solution at 5.0×10^{-4} M concentration, the obtained results were shown at the figure 3.1.

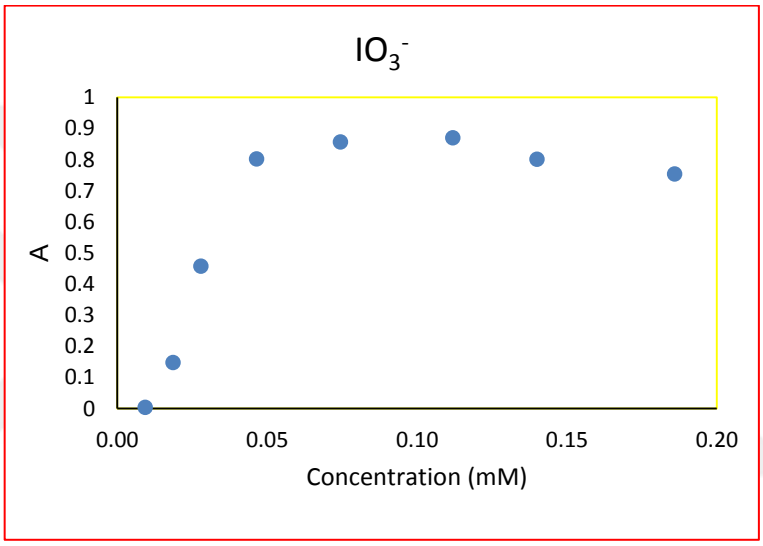


Figure 3.1: Optimization of Iodate Concentration.

As can be seen from the figure above; there were a linear increase in the absorbance with the increasing concentrations for the first four concentrations that have been tested, then absorbances were almost constant or show a small decrease. The best value was determined as 1.0×10^{-4} M for the final concentartion of IO_3^- and the further experiments were conducted by using this value. To get this final value 0.5 mL of 1.0×10^{-3} M IO_3^- was added into the reaction mixture for this proposed method.

3.1.2. Optimization Of HA Concentration

The method for determination of the best HA concentration was applied as stated in Material and Method section. As mentioned earlier the volume range between 0.3 and 4.0 were taken

from a HA solution at 0.1 M and the method were applied each of the volumes. The obtained results were shown in the figure 3.2.

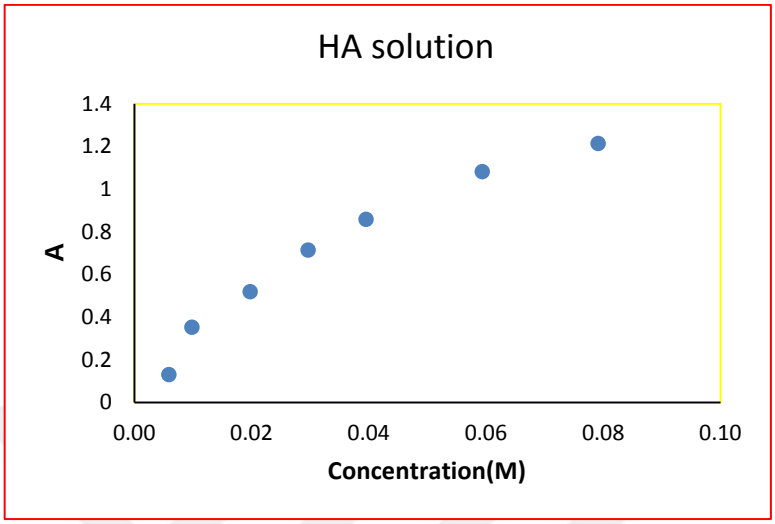


Figure 3.2: Optimization of Hydroxylamine Concentration.

As can be seen from the figure, there was a constant increase with the increasing volumes of the HA solution. After getting absorbance value above 1.2 the experiments were finished. According to the Lambert-Beer law, the absorbance values over the 1.0 are more open to the deviations therefore the final HA concentration that gives an absorbance value approximately 0.8 was chosen as optimal concentration and 0.04 M HA final concentration was used for the further experiments. For this purpose 1.0 mL of 0.2 M HA was added during the application of the proposed method.

3.1.3. Optimization of the Reagent Mixture Concentration

As mentioned earlier SA and NED were mixed and used as a reagent mixture. The method was applied as described in material and method section. The tested volumes were between 10 and 100 μL. The absorbance values were increased between the addition of 10-50 μL reaction mixture but for the higher valumes absorbance was almost constant so 50 μL reagent mixture was chosen as optimal volume.

3.1.4. Investigating the effect of ETOH

Since a number of antioxidant are dissolved in EtOH the effect of ethanol was investigated. For this purpose. the proposed method was applied as stated at section materials and methods

in the presence or absence of EtOH without using any AOx. In the addition of 100 µL EtOH a slight increase in absorbance was observed so the EtOH volume was restricted to 100 µL.

Additionally as explained in Materials and Method section before the addition of reagent mixture (SA+NED) the reaction mixture was allowed to stand for 5 minutes. This time provided an increase in the absorbance but above incubation time longer than 5 min. absorbance values were constant. And finally the stability of generated absorbance was examined. For this purpose the absorbance values were read with 5 min. intervals. In first 15 min. an increase was observed with the time but the absorbance values were constant after 15 min. and before reading the generated absorbance the reaction mixture was allowed to stand for 15 min at room temperature.

3.1.5. The Application Of The Proposed Method To The Selected Individual Antioxidants

The proposed method was applied as stated at Material and Method section (2.3.1.) and the related graphs were drawn between concentration and ΔA. The graphs obtained for individual AOx samples were shown at the below:

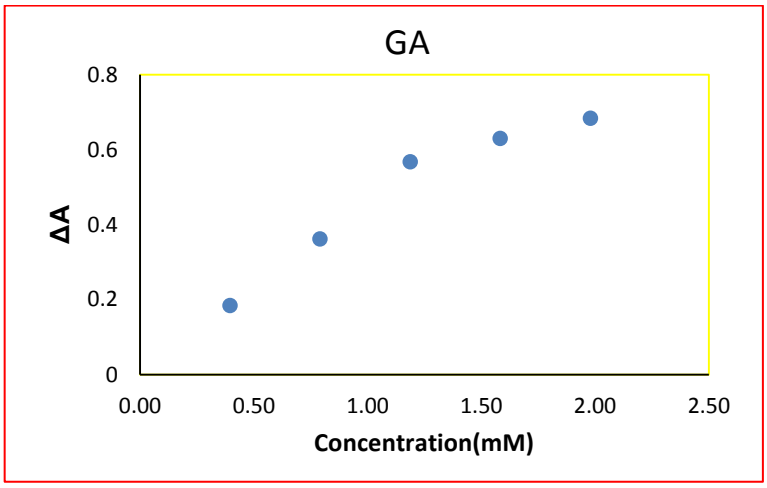


Figure 3.3: Demonstrate Gallic acid ΔA at different Concentration by iodate method.

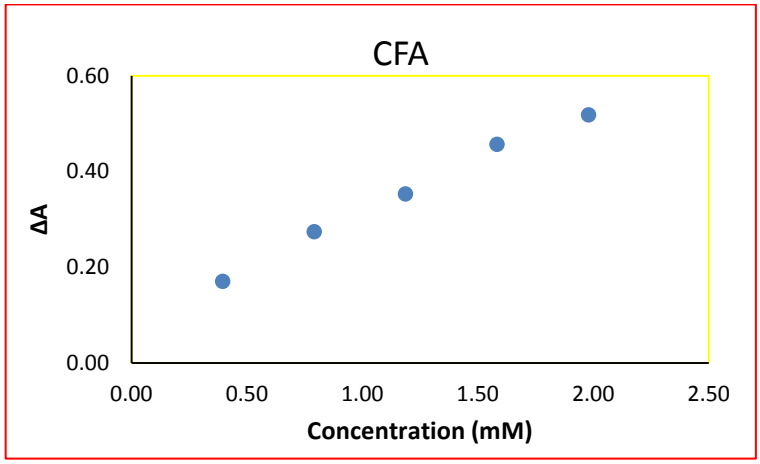


Figure 3.4: A graph demonstrates Caffeic acid results in Iodate method.

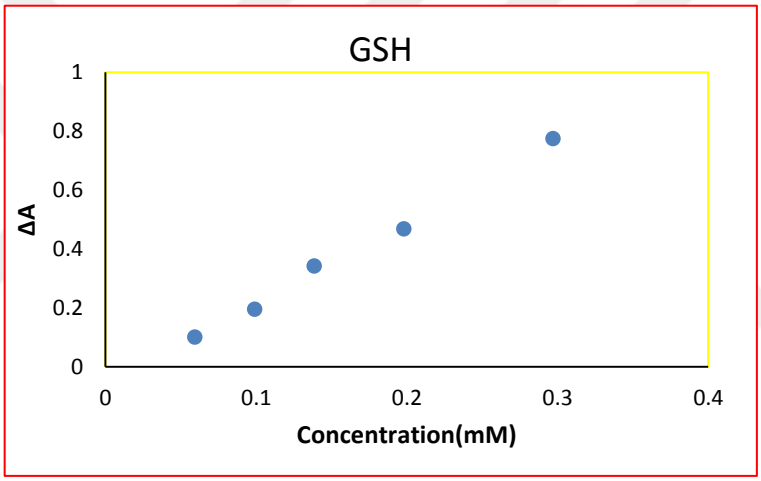


Figure 3.5: Demonstrates the linear graph of Glutathione in iodate method.

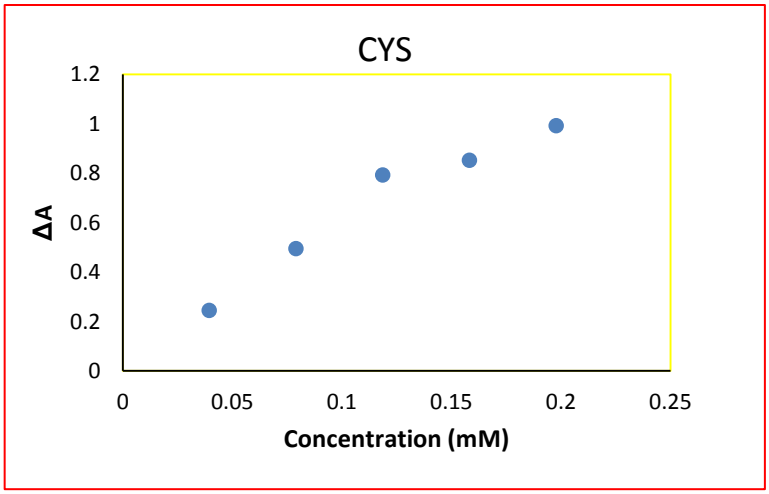


Figure 3.6: Show the linear graph of Cysteine in Iodate method.

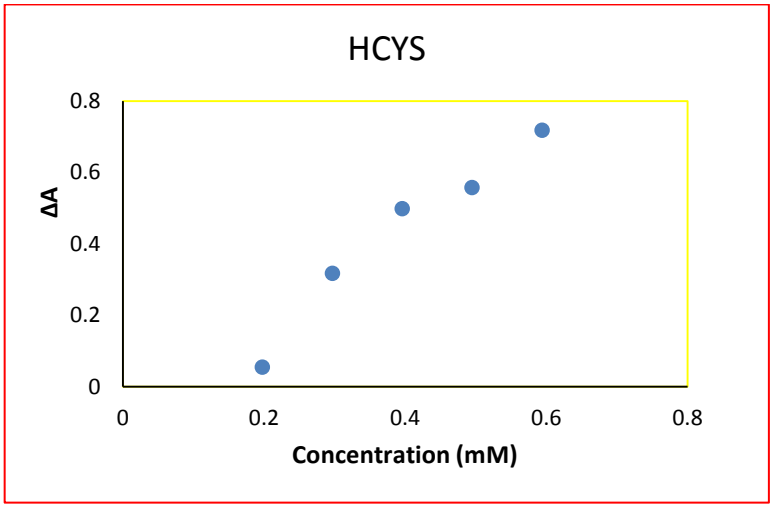


Figure 3.7: Linear graph of Homocysteine in iodate method.

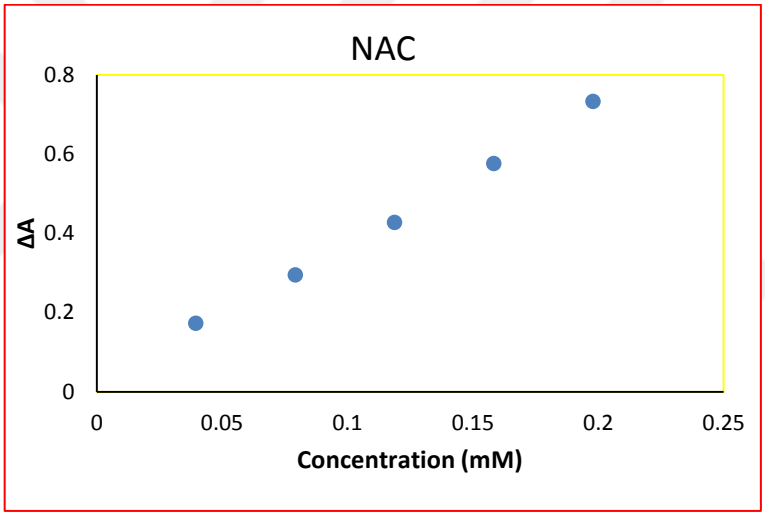


Figure 3.8: Linear graph of N-Acetyl-Cysteine in Iodate method.

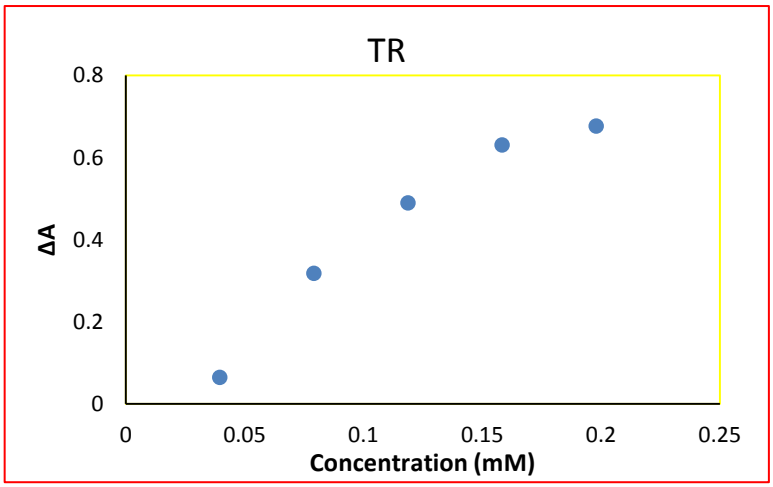


Figure 3.9: Linear graph of Trolox in Iodate method.

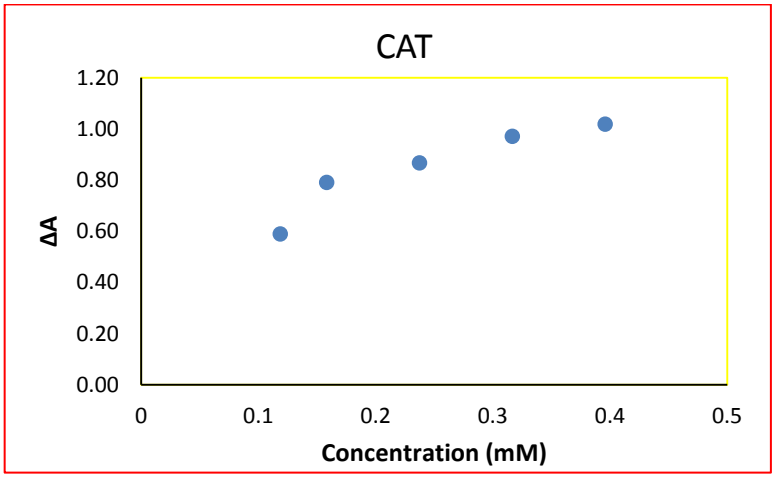


Figure 3.10: Linear graph of Catechin in Iodate method.

The linear calibration equations and determination coefficient (R^2) values were calculated and tabulated at Table (3.1)

Table 3.1: The equations of linear calibration graph with determination coefficient for tested AOx compounds.

AOX	Sample Volume (µL)	Final Conc. (mol L^{-1})	Linear calibration equation for AOX	Determination coefficient (R^2)
GA	20-100	0.1	$\Delta A=3.2\times10^{2}C+0.104$	$R^2 = 0.9280$
CFA	20-100	0.1	$\Delta A=2.2\times10^{2}C+0.090$	$R^2 = 0.9930$
GSH	30-150	1.0×10^{-2}	$\Delta A=2.8\times10^{3}C+0.071$	$R^2 = 0.9960$
CYS	20-100	1.0×10^{-2}	$\Delta A=4.7\times10^{3}C+0.071$	$R^2 = 0.9430$
HCYS	200-600	5.0×10^{-3}	$\Delta A=1.6\times10^{3}C+0.198$	$R^2 = 0.9530$
NAC	200-1000	1.0×10^{-3}	$\Delta A=3.5\times10^{3}C+0.020$	$R^2 = 0.9970$
TR	20-100	1.0×10^{-2}	$\Delta A=3.9\times10^{3}C+0.025$	$R^2 = 0.9420$
CAT	20-100	2.0×10^{-2}	$\Delta A=1.4\times10^{3}C+0.503$	$R^2 = 0.8770$

3.1.6. The Application of the CUPRAC Method to the Selected Individual Antioxidants

The method was applied as stated in the Materials and Method section (2.3.2.) The graphs for the individual AOxs were drawn between concentration and absorbance values.

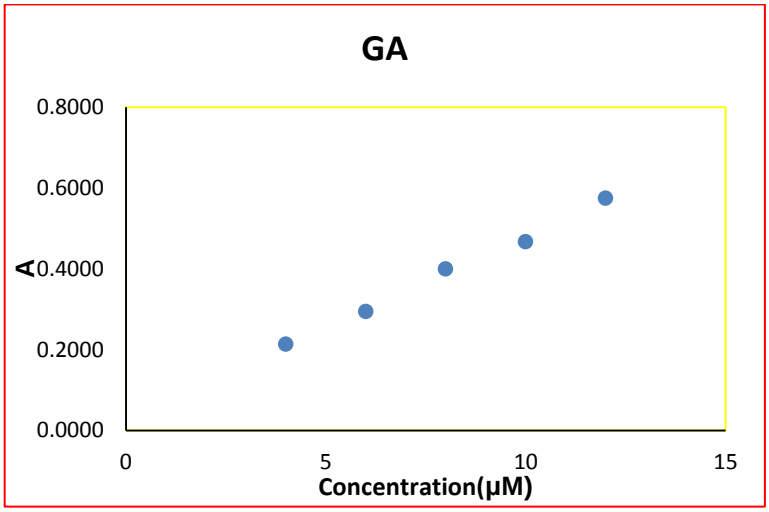


Figure 3.11: Shows the linear graph obtained from plotting the values obtained from CUPRAC method of Gallic acid.

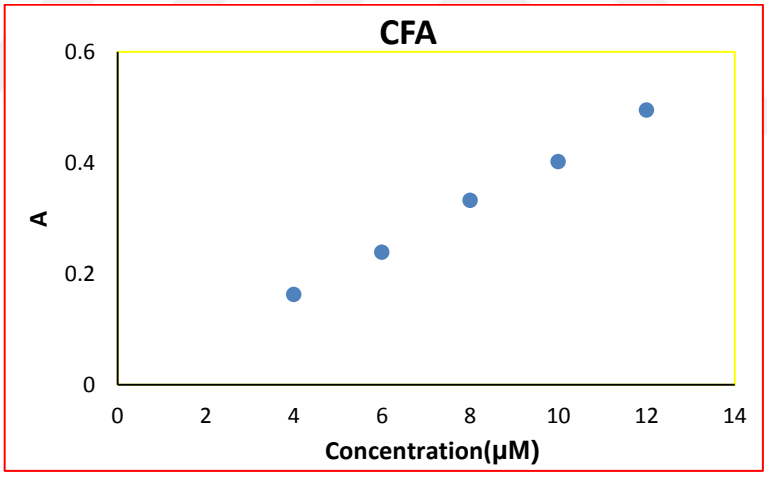


Figure 3.12: A graph demonstrates Caffeic acid results in CUPRAC method.

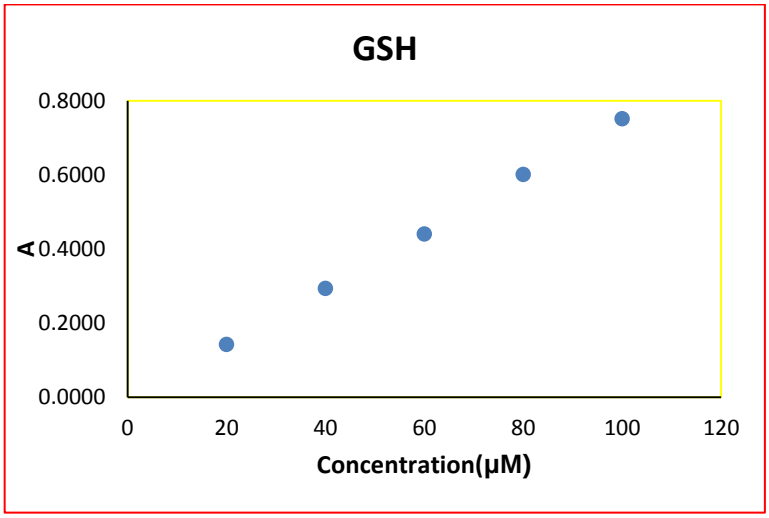


Figure 3.13: Demonstrates the linear graph of Glutathione in CUPRAC method.

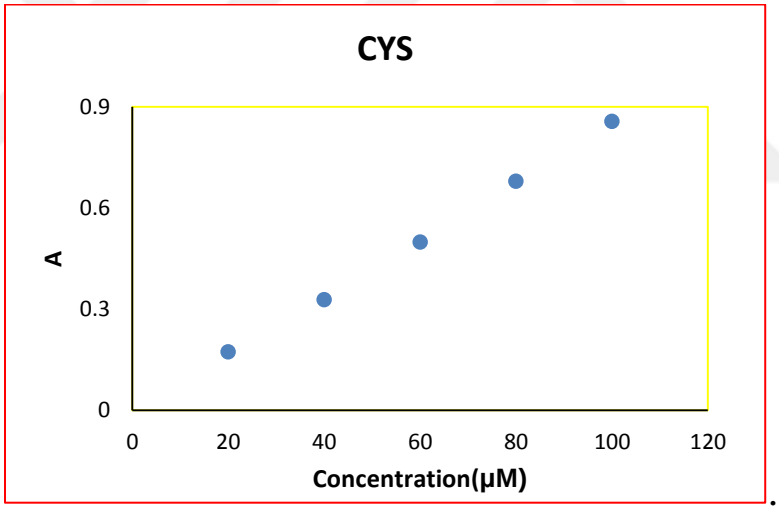


Figure 3.14: Show the linear graph of L- cysteine CUPRAC in method.

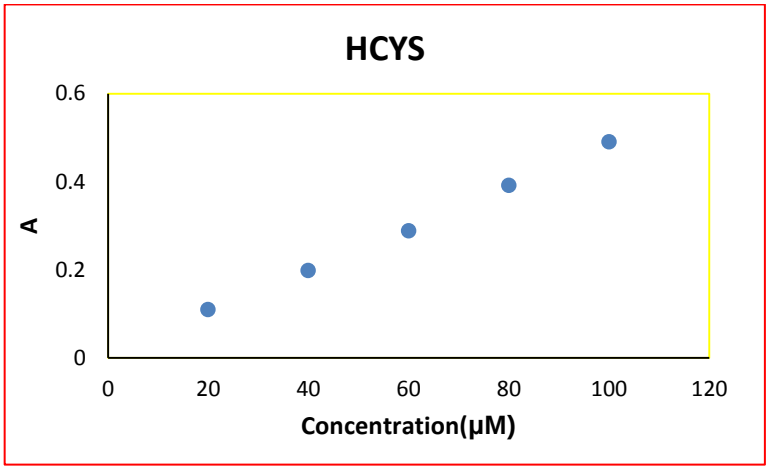


Figure 3.15: Linear graph of Homocysteine in CUPRAC method.

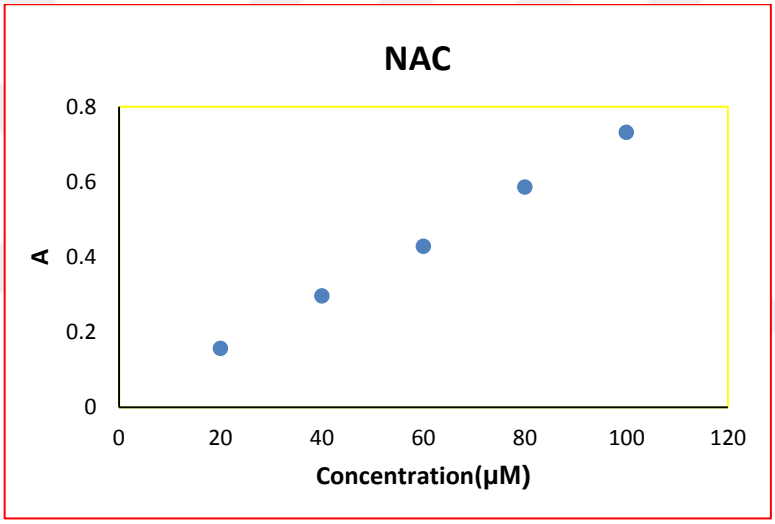


Figure 3.16: Linear graph of N-Acetyl-Cysteine in CUPRAC method.

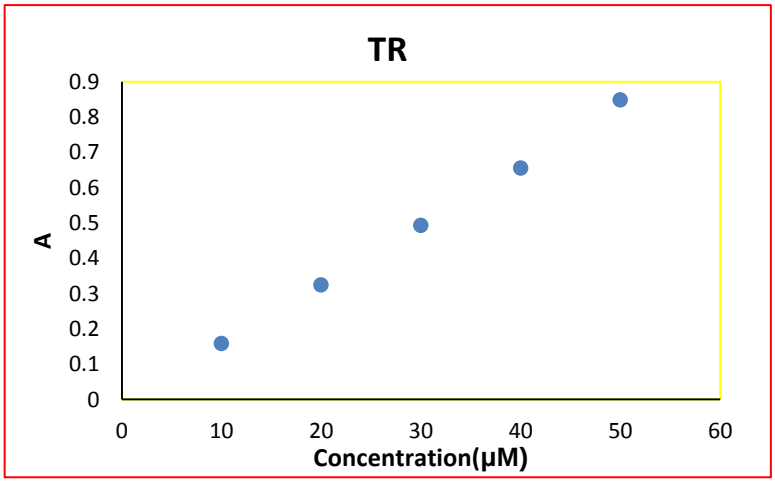


Figure 3.17: Linear graph of Trolox in CUPRAC method.

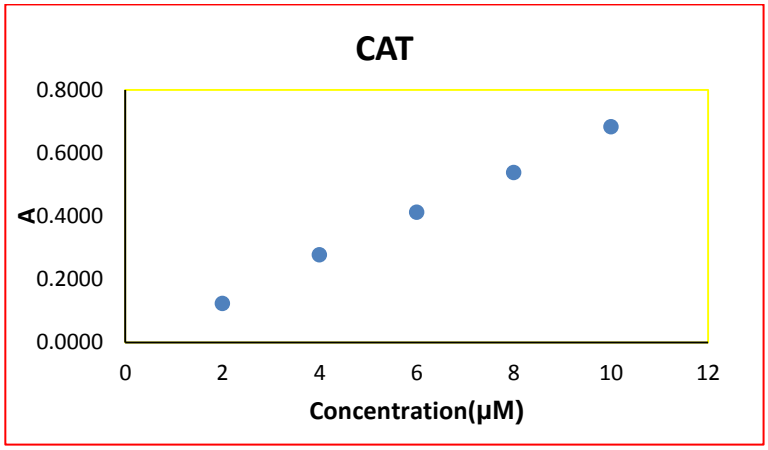


Figure 3.18: Linear graph of Catechin in CUPRAC method.

The linear calibration equations and coefficient of determination (R^2) values were calculated and tabulated at Table (3.2).

Table 3.2: The equations of linear calibration graph with determination coefficient for tested AOx compounds.

AOX	Sample Volume (mL)	Final Conc. (mol L^{-1})	Linear calibration equation for AOX	Determination coefficient (R^2)
GA	0.2-0.6	1.0×10^{-4}	$A=4.5\times10^{3}C+0.032$	$R^2 = 0.9960$
CFA	0.2-0.6	1.0×10^{-4}	$A=4.1\times10^{3}C+0.005$	$R^2 = 0.9980$
GSH	0.1-0.5	1.0×10^{-3}	$A=7.6\times10^{2}C+0.012$	$R^2 = 0.9990$
CYS	0.2-1.0	5.0×10^{-4}	$A=8.5\times10^{2}C+0.007$	$R^2 = 0.9990$
HCYS	0.1-0.5	1.0×10^{-3}	$A=4.7\times10^{2}C+0.008$	$R^2 = 0.9980$
NAC	0.1-0.5	1.0×10^{-3}	$A=7.2\times10^{2}C+0.007$	$R^2 = 0.9990$
TR	0.1-0.5	5.0×10^{-4}	$A=1.7\times10^{3}C+0.017$	$R^2 = 0.9990$
CAT	0.1-0.5	1.0×10^{-4}	$A=6.8\times10^{3}C+0.007$	$R^2 = 0.9990$

3.1.7. The Application of ABTS/TEAC method to the Selected Individual Antioxidants

The proposed method was applied as stated in Materials and Method section (2.3.3.) and the related graphs were drawn between concentration and ΔA. The graphs obtained for individual AOx samples were shown are below:

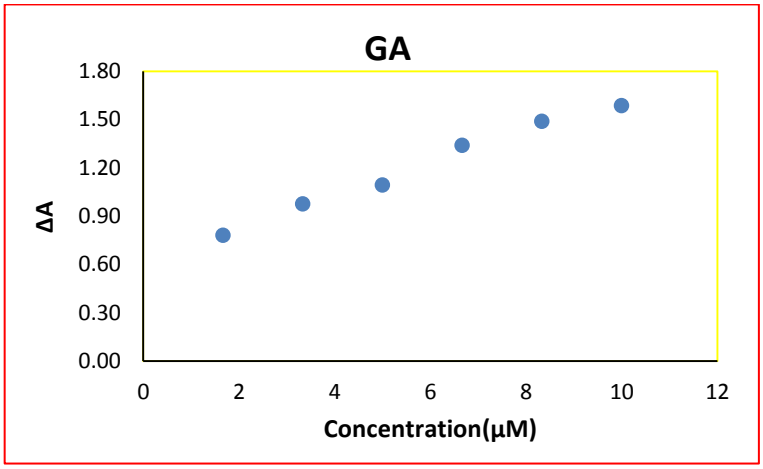


Figure 3.19: Shows the linear graph obtained from plotting the values obtained from ABTS method of Gallic acid.

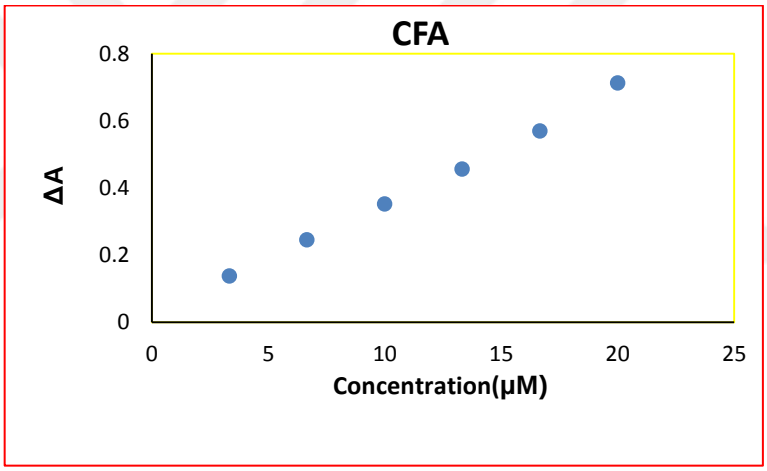


Figure 3.20: A graph demonstrates Caffeic acid results in ABTS method.

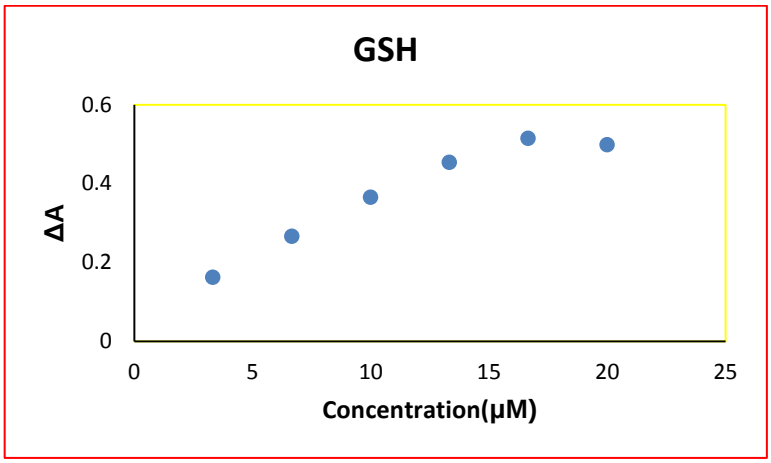


Figure 3.21: Illustration for the graph of Glutathione in ABTS method.

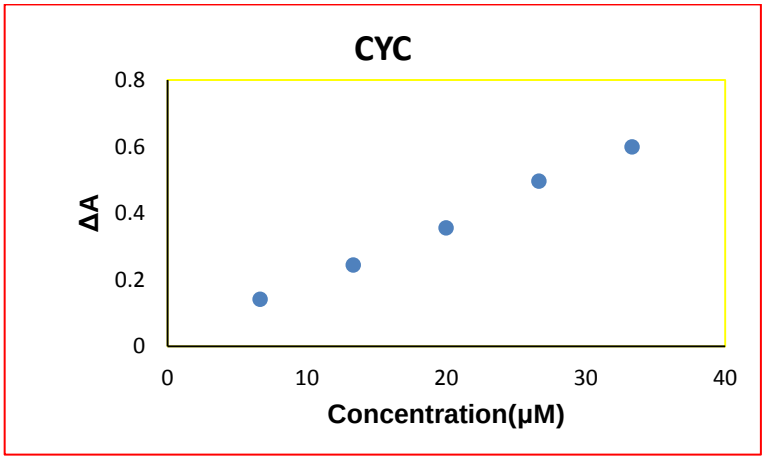


Figure 3.22: Show the linear graph of cysteine in ABTS method.

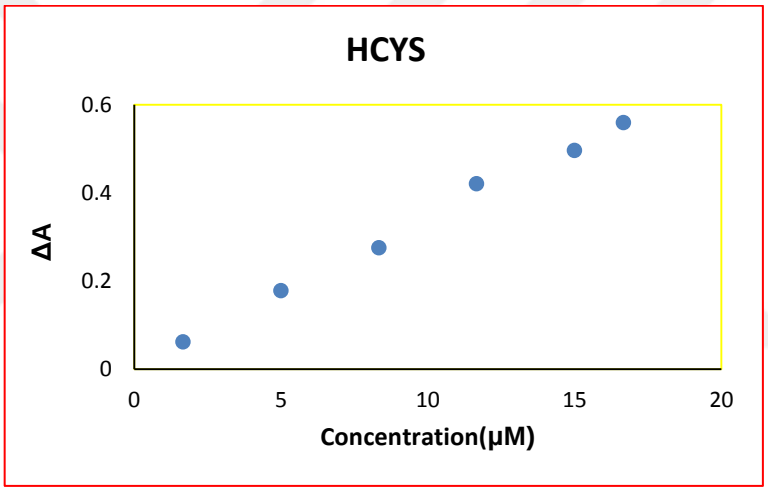


Figure 3.23: Linear graph of Homocysteine in ABTS method.

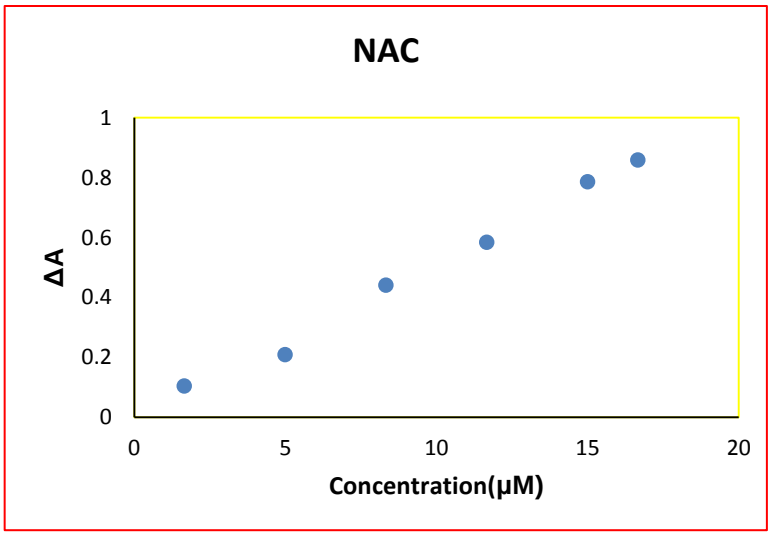


Figure 3.24: Linear graph of N-Acetyl-Cysteine in ABTS method.

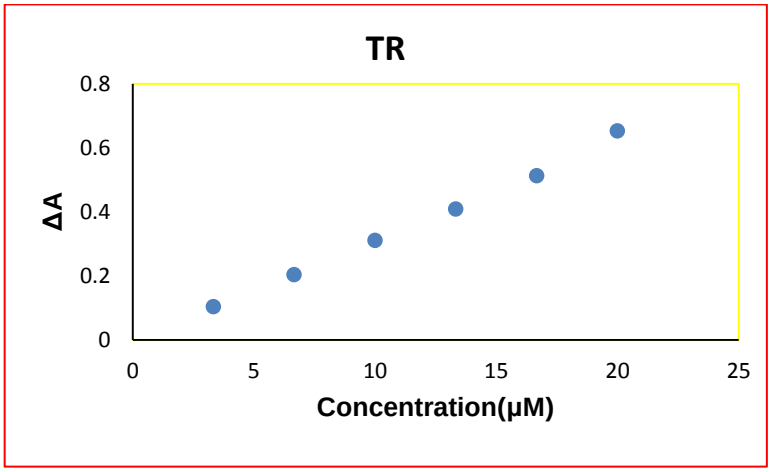


Figure 3.25: Linear graph of Trolox in ABTS method.

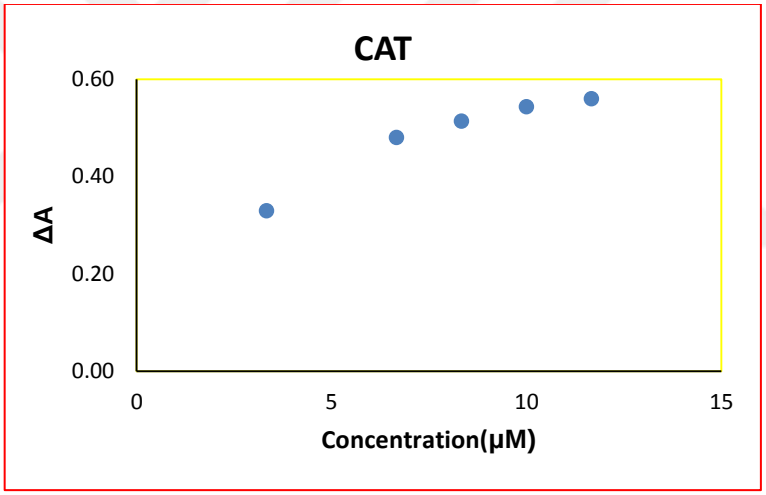


Figure 3.26: Linear graph of Catechin in ABTS method.

The linear calibration equations and coefficient of determination (R^2) values were calculated and tabulated at Table (3.3).

Table 3.3: The linear calibration equations and coefficient of determination (R^2) values for ABTS/TEAC method.

AOX	Sample Volume (mL)	Final Conc. (mol L^{-1})	Linear calibration equation for AOX	Determination coefficient (R^2)
GA	0.1-0.6	5.0×10^{-5}	$\Delta A=9.9\times10^{4}C+0.629$	$R^2 = 0.9870$
CFA	0.1-0.6	1.0×10^{-4}	$\Delta A=3.3\times10^{4}C+0.090$	$R^2 = 0.9960$
GSH	0.1-0.6	1.0×10^{-4}	$\Delta A=2.1\times10^{4}C+0.071$	$R^2 = 0.9200$
CYS	0.2-1.0	1.0×10^{-4}	$\Delta A=1.1\times10^{4}C+0.071$	$R^2 = 0.9970$
HCYS	0.1-1.0	5.0×10^{-5}	$\Delta A=3.3\times10^{4}C+0.009$	$R^2 = 0.9950$
NAC	0.1-1.0	5.0×10^{-5}	$\Delta A=5.2\times10^{4}C+0.010$	$R^2 = 0.9930$
TR	0.1-0.6	1.0×10^{-4}	$\Delta A=3.2\times10^{4}C+0.011$	$R^2 = 0.9960$
CAT	0.1-0.6	5.0×10^{-5}	$\Delta A=2.7\times10^{4}C+0.264$	$R^2 = 0.9160$

3.1.8. Determination of AOx Mixtures by the proposed Iodate method

To check the additivity of the proposed Iodate method different binary mixture were prepared from the tested AOxs. The details of these mixtures were given in the Material and method section (2.4.1.). The results obtained and the percentage error values calculated for the mixtures were tabulated.

Table 3.4: Absorbance values for the individual AOx compounds and binary mixtures with percentage errors for Iodate method.

AOX	A_i	A_0 (without AOX)	ΔA (A_0-A_i)	Percentage error
		0.9501		
GA	0.7284		0.2217	
CFA	0.7224		0.2277	
GA+CFA	0.5525		0.3976	-11.5265
		1.1536		
CFA	0.9690		0.1846	
TR	0.8216		0.3320	
CFA+TR	0.7536		0.4000	-22.5707
		0.9423		
GSH	0.7987		0.1436	
CYS	0.4113		0.5310	
GSH+CYS	0.3429		0.5994	-11.1473

To compare the additivity results of the proposed method with the standard methods tested namely CUPRAC and ABTS/TEAC, the same binary AOx mixtures were prepared and the methods were applied as mentioned earlier. The experimental details for the standard method applications for binary mixtures were explained in the Material and methods section (2.4.2., 2.4.3.). The results for CUPRAC are shown in the table below Tables for CUPRAC method and ABTS/TEAC methods respectively**.**

Table 3.5: Absorbance values for the individual AOx compounds and binary mixtures with percentage errors for CUPRAC method.

AOX	A	Percentage error
GA	0.1996	
CFA	0.1675	
GA+CFA	0.4204	14.5
CFA	0.1608	
TR	0.3199	
CFA+TR	0.4944	2.8
GSH	0.3152	
CYS	0.1559	
GSH+CYS	0.5104	8.3

Table 3.6: Absorbance values for the individual AOx compounds and binary mixtures with percentage errors for ABTS/TEAC method.

AOX	A_i	A_0 (Without AOX)	ΔA (A_0-A_i)	Percentage error
		1.0308		
GA	0.5771		0.4537	
CFA	0.7641		0.2667	
GA+CFA	0.3032		0.7276	1.0
		1.3892		
CFA	1.0590		0.3302	
TR	1.0301		0.3591	
CFA+TR	0.7412		0.6480	-6.0
		1.5231		
GSH	0.7861		0.7370	
CYS	1.1219		0.4012	
GSH+CYS	0.4233		1.0998	-3.4

3.1.9. Applications of the Methods to the real sample

The proposed Iodate method was applied to real samples. Different types of teas are consumed commonly in Turkey, two kinds of tea (green and white ones) were chosen as real

sample. Iodate method and the standard methods (CUPRAC and ABTS/TEAC) were applied as stated earlier and the results were calculated as mmol Trolox equivalent and the results were shown at the table below:

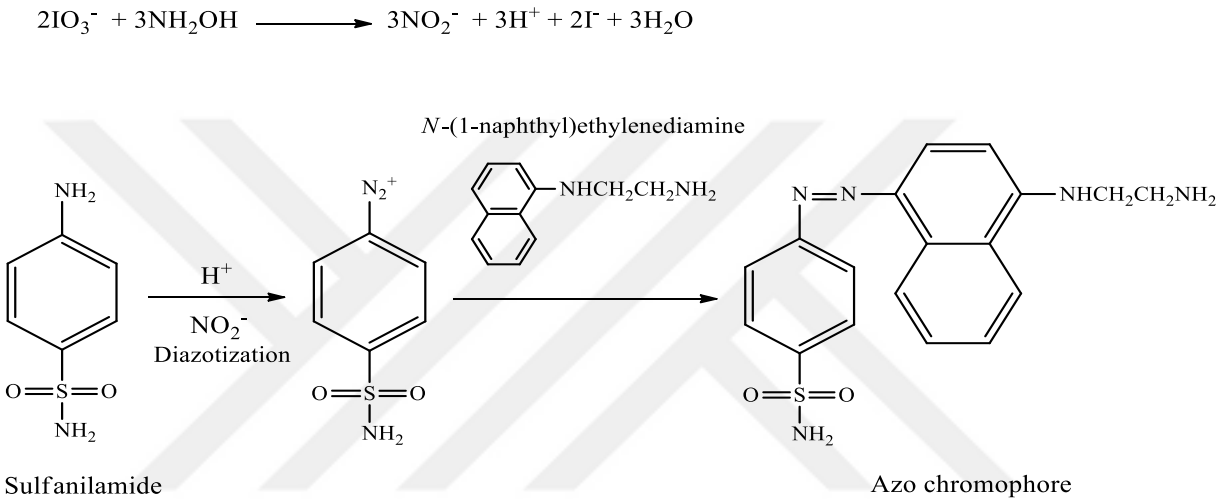
Table 3.7: TR equivalent TAC of real samples (Green Tea and White Tea).

Total Antioxidant Capacity of real sample (green tea) in mmol TR equivalent g^{-1}			
Sample	Iodate	CUPRAC	ABTS
Green Tea	0.6202±0.057	1.0402±0.038	0.5983±0.005
White Tea	0.3973±0.019	0.9382±0.029	0.5680±0.049

From the table 3.7. we can see that the TR equivalent TAC of green tea was close to each other for Iodate, CUPRAC methods. Only ABTS/TEAC method gave lower value. On the other hand, although Iodate and ABTS/TEAC methods closer each other's than CUPRAC method. ABTS/TEAC result is slightly higher than Iodate method's result. It can be explained by the mechanism of the methods. While Iodate and CUPRAC methods depend on redox reactions ABTS/TEAC depends on radical scavenging.

4. DISCUSSION

In the proposed study a new TAC determination method was developed. According to the method due to the reaction between iodate and hydroxyl amine, nitrite is generated. And then a typical Griess reaction takes places by the aid of sulfanilamide and naphthyl ethylene diamine according to the reaction shown below. The resulting azo chromophore is pink colored with a λ_{max}= 540 nm.



To optimize the developed method firstly the parameters such as concentration of iodate, hydroxyl ammonium chloride (HA) were investigated. As mentioned in the results 0.5 mL of 1.0×10^{-3} M iodate determined as optimal volume and concentration. For tested volumes the maximum absorbance gets at that volume and concentration. And for the HA we choose 1.0 mL of 0.2 M. In the study since the decrease in the absorbance was used, it was tried to get an initial absorbance about 1.0, according to Lambert-Beer Law. Although we observed the increase of the absorbance continued with the increasing volume of the HA, the experiment finished at the absorbance value at 1.2.

The other parameter is the concentration of reagent mixture. As stated in the previous sections, Sulfonanide (SA) and N-1-naphthyl ethylene diamine dihydrochloride (NED) were mixed and the mixture was added to the reaction medium. For this purpose, the concentrations of the regents were 4.0% and 0.2% respectively. The reagents mixed at 1:1 volume ratio and the volumes 10-100 µL were tested and the optimal volume was selected as 50 µL in the

study. Between the volumes 10-50 μL absorbance increased but for the higher volumes it was seen that the absorbance values were constant. According to this finding we chose that volume as optimal amount for the reagent mixture.

The other parameter investigated is effect of ethanol. In the study a number of antioxidants were tested. It is known alcohol (both ethanol and methanol) is the most used solvent in antioxidant studies. It is also known that some of the antioxidants are not water soluble. For this purpose, we investigated effect of alcohol in the absence of any antioxidant. Only ethanol at different volumes were added in the reaction mixture. Since we prepared a reagent black it is important to detect effect of alcohol even in the absence of antioxidants. For the addition ethanol above the 100 μL a slight increase was observed. Hence, the volume of ethanol was restricted with that volume. Not to exceed this volume, antioxidant solutions were prepared at a range of 1.0×10^{-2} - 1.0×10^{-3} M and the volumes between 30-100 μL were used in the experiments. Only for water soluble antioxidants, higher volumes up to 1.0 mL were used.

As stated earlier in the proposed study, determination of the antioxidants depends on the decrease of the absorbance obtained via the Griess reaction. During the reaction iodate is reduced to iodide, while hydroxyl ammonium is oxidized to nitrite. In the presence of AOx compounds in the KIO_3, NH_2OH reaction mixture they reduce the resulting NO_2^- concentration. It means a decrease in the intensity of pink color and the absorbance difference was calculated as ΔA in the presence and absence of AOx. As can be seen at the graphs in the results section there is a linear relationship between AOx concentration and ΔA. The related linear calibration equations were calculated and tabulated. According to these equations the effectiveness of them can be sorted as CYS > TR > NAC >GSH > HCYS > CAT > GA > CFA. Also, the binary mixtures were tested to see additivity and the percentage errors were calculated and these were founded as approximately -11% and -22%. Finally, the method was applied to green and white tea as real sample and CUPRAC and ABTS/TEAC methods were applied as standard methods. While calculated TAC values of tea samples relatively close each other with CUPRAC and Iodate methods results obtained by ABTS/TEAC method was lower from the others. The reason of these different results could be explained by the different slope values of TR for the methods tested.

5. CONCLUSION AND RECOMMENDATIONS

Certain metabolic reactions in the biological system result in releasing of reactive species. An unbalance in favor of ROS/RNS in the cell lead to oxidative stress. This reactive species can be radical like ($^{\bullet}OH$, $O_2^{-\bullet}$, etc….) or non-radical like (H_2O_2, HOCl, O_3 etc….) free radicals have unpaired electron in their outer orbital and they are highly reactive compounds. These reactive species react with the other biological components by its unpaired electron resulting in damage at the level of cell, tissue and organ. Accumulation of that damage may lead to several diseases such as cancer, Alzheimer (AD), heart diseases and aging that lead finally to death. The cell has a defense mechanism represented in the presence of antioxidants. Antioxidants delay or inhibit oxidation of the substrate. These compounds can be in enzymatic or non-enzymatic feature. The Antioxidants have different defense mechanisms by which it can control the activity of ROS / RNS and prevent its accumulation in the cell. These mechanisms are prevention, diversion, interception and repair. Each mechanism has its own condition and ROS/RNS can be removed out from the biological system by this mechanism. Each antioxidant can react with several types of oxidants and free radicals.

There is a deep need in the scientific field to recognize and determine the total antioxidant capacity, so as to understand the nature of free radicals and how it leads 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.

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