

# **WORKSHOP ON ANTIOXIDANT MEASUREMENT ASSAY METHODS**

## **BOOK OF ABSTRACTS**

21<sup>st</sup> April, 2010  
Istanbul, TURKEY



## **FOREWORD**

Dear Colleague,

It is a pleasure to invite you to the “International Workshop on Antioxidant Capacity/Activity Assay Methods”. ‘Mid-term meeting’ of expert speakers from Germany, Israel, Turkey and USA, the workshop will take place on 21<sup>st</sup> April 2010, in Istanbul, Turkey.

This special workshop is associated with the IUPAC (International Union of Pure & Applied Chemists) Project (Project No: 2008-031-1-500 Apak) entitled ‘Methods of measurement and evaluation of natural antioxidant capacity/activity’. The workshop will provide a forum among antioxidant researchers from all fields: food analytical chemistry, food technology, biochemistry and medicinal chemistry, and is expected to enable sharing the achievements of the IUPAC project with other participants. The meeting will take place in the historical building of Istanbul University at Beyazit Campus.

Prof. Dr. Reşat APAK

Task Group Chairman of the Project and Workshop

April 2010, Istanbul, Turkey



## IUPAC PROJECT INFO

**Project name:** Methods of Measurement and Evaluation of Natural Antioxidant Capacity/Activity

**Project No:** 2008-031-1-500

**Aim:** To bring in terms of definitions or definition-like characterization and classification the chemical and biochemical methods of antioxidant assays as well as related antioxidants chemistry and to provide analytical, food chemical, biomedical/clinical and environmental communities with critical evaluation on this topic.

**Description:** The chemical diversity of natural antioxidants makes it difficult to separate, detect and quantify individual antioxidants from the complex matrix. Moreover, the total antioxidant power is often more meaningful to evaluate health beneficial effects because of the cooperative action of individual antioxidant species. Currently there is no single antioxidant assay for food labeling because of the lack of standard quantification methods. Antioxidant assays may be broadly classified as the electron transfer (ET) - and hydrogen atom transfer (HAT)-based assays. The results obtained are usually hardly comparable because of the different mechanisms, redox potentials, pH- and solvent- dependencies, etc., in various assays. This project will aid to identify and quantify of properties and mutual effects of antioxidants, to bring a more rational basis to the classification of antioxidant and antioxidant assays and to make the results more comparable and understandable. In this regard, the task group members are experienced in various methods of antioxidants assay. The team chairperson has developed a novel CUPRAC (cupric reducing antioxidant capacity) method [1] which has been successfully applied to antioxidants assay in food plants, human serum, and to hydroxyl radical scavengers [2].

### References:

- [1] R. Apak, K. Güçlü, M. Özyürek, S. E. Karademir, J. Agric. Food Chem. 52 (2004) 7970-7981.
- [2] M. Özyürek, B. Bektaşoğlu, K. Güçlü, R. Apak, Anal. Chim. Acta, 616 (2008) 196-206.



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**TABLE OF CONTENT**

<b>FOREWORD</b>	Page 1
<b>IUPAC PROJECT INFO</b>	2
<b>COMITTEES</b>	3
<b>TABLE OF CONTENT</b>	5
<b>ABSTRACTS</b>	9

**PLENARY LECTURES**

	Page
ANTIOXIDANT CAPACITY/ACTIVITY ASSAYS APPLIED TO FRUITS, VEGETABLES, AND CEREALS <b>Shela GORINSTEIN</b>	12
CRITICAL CONSIDERATIONS IN ORAC, TRAP, ABTS/TEAC, AND DPPH ASSAYS OF ANTIRADICAL ACTION <b>Karen M. SCHAICH</b>	13
DETERMINATION OF HYDROPHILIC AND LIPOPHILIC ANTIOXIDANT CAPACITY - COMMENTS AND RESULTS <b>Volker BÖHM</b>	14
CUPRAC (CUPRIC REDUCING ANTIOXIDANT CAPACITY) ASSAY AS A NOVEL ELECTRON TRANSFER-BASED ANTIOXIDANT CAPACITY ASSAY, AND ITS VARIOUS MODIFICATIONS FOR CAPACITY/ACTIVITY MEASUREMENT IN DIVERSE MATRICES (INCLUDING BIOLOGICAL FLUIDS) <b>Reşat APAK</b>	15

## INVITED LECTURES

	Page
<b>OP-01</b> COMBINED HPLC-CUPRAC ASSAY: SINGLE ASSAY FOR EVALUATION OF FOOD REGARDING BOTH INDIVIDUAL ANTIOXIDANT CONSTITUENTS AND TOTAL ANTIOXIDANT CAPACITY Esma TÜTEM	18
<b>OP-02</b> DIRECT MEASUREMENT OF THE TOTAL ANTIOXIDANT CAPACITY OF FOODS: THE QUENCHER APPROACH Vural GÖKMEN	19
<b>OP-03</b> QUALITY CONTROL OF EDIBLE OIL AND OTHER NATURAL PRODUCTS BY CHEMILUMINESCENCE <u>Anthony C. CALOKERINOS</u> , Anastasios ECONOMOU, Dionysis CHRISTODOULEAS, Thalia TSAKA, Panagiotis KEFALAS and K. PAPADOPOULOS	20
<b>OP-04</b> SALVIA DITERPENOIDS AS A NATURAL ANTIOXIDANT SOURCE Gülaçtı TOPÇU	21
<b>OP-05</b> ANTIOXIDANT PROFILES OF THREE CHERRY TYPES GROWN IN TURKEY Esra ÇAPANOĞLU, Jules BEEKWILDER, <u>Dilek BOYACIOĞLU</u> , Ric C.H. de VOS, Robert D. HALL	22
<b>OP-06</b> PREPARATION AND CHARACTERIZATION OF ANTIOXIDANT LIPOSOMES CONTAINING WHEY PROTEINS <u>A. Suha YALCIN</u> , Ahmet KILINÇ, Gökhan BİÇİM	23
<b>OP-07</b> PROTOCOLS FOR CONDUCTING AND REPORTING SCIENTIFIC RESEARCH ON ANTIOXIDANT ACTIVITY Artemis KARAALI	24
<b>OP-08</b> NATURAL ANTIOXIDANTS: ACTIVITY-STRUCTURE INSIGHT İlhami GÜLÇİN	25
<b>OP-09</b> CHANGES IN ANTIOXIDANT AND METABOLITE PROFILES DURING PRODUCTION OF TOMATO PASTE <u>Esra CAPANOGLU</u> , <u>Dilek BOYACIOGLU</u> , Jules BEEKWILDER, Ric De VOS, Robert HALL	26

## POSTER PRESENTATIONS

	Page
<b>PP-01</b> ANTIOXIDANT ACTIVITIES, GS / MS ANALYSIS AND PHENOLIC PROFILES OF <i>Achillea biserrata</i> AND <i>Hysopus officinalis</i> EXTRACTS <u>Gönül HATİPOĞLU</u> , Münevver SÖKMEN, Atalay SÖKMEN, Ersan BEKTAŞ	28
<b>PP-02</b> CHANGES IN TOTAL PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY IN RESPONSE TO LOW TEMPERATURES IN OLIVE ( <i>Olea europaea</i> L. "GEMLIK") <u>Asuman CANSEY</u> , Hatice GÜLEN, Güler ÇELİK, Atilla ERİŞ	29
<b>PP-03</b> ANTIOXIDANT ACTIVITIES AND TOTAL PHENOLIC CONTENTS OF SOME AROMATIC EDIBLE PLANTS <u>Yasemin SAHAN</u> , Güler ÇELİK, Sibel TAŞKESEN	30
<b>PP-04</b> CHARACTERIZATION AND ANALYSIS OF THE ANTIOXIDANT CAPACITY OF FUNCTIONAL PHENOLICS OXIDIZED BY <i>Scytalidium thermophilum</i> CATALASE PHENOL OXIDASE (CATPO) <u>Betül SÖYLER</u> , İlkay ŞENSOY, Zümrüt B. ÖGEL	31
<b>PP-05</b> EXTRACTION OPTIMIZATION FOR ANTIOXIDANT ACTIVITY OF A POTENTIAL BIOACTIVE SOURCE: FEIJOA ( <i>Feijoa sellowiana</i> ) <u>N. Barış TUNCEL</u> , Neşe YILMAZ, Nesrin KURTAR BOZBIYIK	32
<b>PP-06</b> COMPARISON OF TWO COMMON ASSAYS FOR QUANTIFYING TOTAL FLAVONOIDS <u>Sevinç ENCU</u> , Türkan VURAL, Olcay ÇETİN, Nagihan KÖKYAR, Kevser SÖZGEN BAŞKAN, Esmâ TÜTEM, Reşat APAK	33
<b>PP-07</b> TEAC (TROLOX EQUIVALENT ANTIOXIDANT CAPACITY) COEFFICIENTS OF SOME ANTHOCYANINS ACCORDING TO CUPRAC AND ABTS METHODS <u>Sevda KARAMAN</u> , Çınar KÜÇÜKÇOBAN, Esmâ TÜTEM, Kevser SÖZGEN BAŞKAN, Reşat APAK	34
<b>PP-08</b> TOTAL ANTIOXIDANT CAPACITIES AND PHENOLIC CONTENTS OF SOME SELECTED HERBAL PLANTS <u>Şevda KARAMAN</u> , Esmâ TÜTEM, Kevser SÖZGEN BAŞKAN, Reşat APAK, Cevdet NERGİZ	35
<b>PP-09</b> EFFECTS OF MINIMAL PROCESSING AND REFRIGERATED STORAGE ON PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF ROCKET SALAD ( <i>Eruca sativa</i> , <i>Brassicaceae</i> ) <u>Gamze TOYDEMİR OTKUN</u> , Zeynep TACER CABA, Esra ÇAPANOĞLU GÜVEN, Dilek BOYACIOĞLU, Nalan DEMİR	36
<b>PP-10</b> ANTIOXIDANT ACTIVITIES OF SOME NEW 3-ALKYL-4-[3-(4-NITROBENZOXY)BENZYLIDENAMINO]-4,5-DIHYDRO-1H-1,2,4-TRIAZOL-5-ONES <u>Haydar YÜKSEK</u> , Özlem GÜRSOY-KOL, Mustafa CALAPOĞLU	37
<b>PP-11</b> SYNTHESIS AND ANTIOXIDANT ACTIVITIES OF SOME NOVEL 3-ALKYL-4-(3-HYDROXYBENZYLIDENAMINO)-4,5-DIHYDRO-1H-1,2,4-TRIAZOL-5-ONES <u>Haydar YÜKSEK</u> , Özlem GÜRSOY-KOL	38
<b>PP-12</b> ANTIOXIDANT PROTECTIVE EFFECT OF FLAVONOIDS ON THE LIPID PEROXIDATION INDUCED BY COPPER / ASCORBIC ACID SYSTEM Bilge YILDOĞAN, <u>İnci SÖNMEZOĞLU</u> , Filiz İMER, Reşat APAK	39



- PP-13** EFFECT OF INVASIVE *CAULERPA RACEMOSA* VAR. *CYLINDRACEA* 40  
EXTRACTS ON THE GROWTH AND BIOCHEMICAL CONSTITUENTS  
OF *ZEA MAYS* (L.)  
Levent CAVAS - Mehmet Ulas DURAL - Deniz CAPARKAYA - Teoman  
KESERCIOGLU
- PP-14** ARGUMENTS FOR THE ESTABLISHMENT OF CROCIN BLEACHING 41  
ASSAY AS A RELIABLE TOOL FOR ANTIOXIDANT ACTIVITY  
ASSESSMENT  
Stella A. ORDOUDI, Maria Z. TSIMIDOU
- PP-15** THEORETICAL METHODS IN RADICAL SCAVENGING ACTIVITY 42  
STUDIES OF PHENOLIC ANTIOXIDANTS: POTENTIAL AND  
LIMITATIONS  
Nikolaos NENADIS
- PP-16** DENSITY FUNCTIONAL STUDY ON THE STRUCTURAL 43  
CONFORMATIONS AND INTRAMOLECULAR CHARGE TRANSFER  
FROM THE VIBRATIONAL SPECTRA OF 6-AMINOFLAVONE  
Yusuf ERDOGDU, Ozan UNSALAN
- PP-17** EXPERIMENTAL (FT-RAMAN AND FT-IR) AND THEORETICAL (DFT) 44  
STUDIES ON 7-AMINOFLAVONE AND ITS INTRAMOLECULAR  
CHARGE TRANSFER MECHANISM  
Ozan UNSALAN, Yusuf ERDOGDU
- PP-18** PREDICTION OF TOTAL ANTIOXIDANT ACTIVITY FROM 45  
CHROMATOGRAMS BY NET ANALYTE SIGNAL AND ORTHOGONAL  
SIGNAL CORRECTION PARTIAL LEAST SQUARES REGRESSION  
Salih SAHİN, Esra SARIBURUN, Önder AYBASTIER, Cevdet DEMİR
- PP-19** CUPRAC (CUPRIC REDUCING ANTIOXIDANT CAPACITY) ASSAY OF 46  
SERUM INCORPORATING PROTEINS  
Nilay KARA, Sema D. ÇEKİÇ, Kevser S. BAŞKAN, Esma TÜTEM, Reşat APAK
- PP-20** ANTIOXIDANT ACTIVITY AND PHENOLICS COMPOUNDS OF EDIBLE 47  
WILD MUSHROOMS FROM TRABZON, TURKEY  
Huseyin SAHİN, Sevgi KOLAYLI, Rezzan ALIYAZICIOGLU, Ertugrul SESLI
- PP-21** ANTIOXIDANT ACTIVITIES OF AQUEOUS EXTRACT FROM 48  
ASPHODELUS AESTIVUS BROT. (LILIACEAE)  
Aysegül PEKSEL, Sema İMAMOĞLU
- PP-22** DPPH RADICAL SCAVENGING MAY NOT HAVE BEEN DETERMINED 49  
CORRECTLY JUST BASED ON MONITORING ABSORBANCE AT 517  
NM  
Murat KÜÇÜK, Zeynep İSKEFİYELİ, Nesibe A. BURNAZ, Nimet BALTAŞ, Ayça  
AKTAŞ
- PP-23** REDOX COLORIMETRIC SOLID-PHASE EXTRACTION COUPLED 50  
WITH FIBER OPTIC REFLECTANCE SPECTROSCOPY FOR  
DETERMINATION OF ASCORBIC ACID IN PHARMACEUTICAL  
FORMULATIONS  
Hayati FİLİK, Duygu AKSU, Derya GİRAY and Reşat APAK
- PP-24** TOTAL ANTIOXIDANT CAPACITY ASSAY USING 51  
OPTIMIZED FERRICYANIDE / PRUSSIAN BLUE METHOD  
K. Işıl BERKER, İzzet TOR, Birsen DEMİRATA, Reşat APAK
- PP-25** EFFECT OF MICROWAVE AND OVEN HEATING ON THE 52  
ANTIOXIDANT CONTENT OF ROSEHIP INFUSION (HERBAL TEA)  
Birsen DEMİRATA, Dilek ÖZYURT

*Workshop on Antioxidant Measurement Assay Methods*

<b>PP-26</b>	TOTAL ANTIOXIDANT CAPACITY OF COMMERCIAL GRAPE, GRAPE JUICES AND WINE EVALUATED BY SPECTROFLUORIMETRIC METHOD <b><u>Dilek ÖZYURT</u>, Birsen DEMİRATA, Reşat APAK</b>	53
<b>PP-27</b>	INVESTIGATION OF TOTAL ANTIOXIDANT CONTENTS BY SPECTROPHOTOMETRIC METHODS OF HEAT-PROCESSED DIETARY ONION PLANTS IN TURKEY <b>Birsen DEMİRATA, <u>Dilek ÖZYURT</u>, Binnur GÖÇ, Reşat APAK</b>	54
<b>PP-28</b>	MODIFIED CERIUM(IV)-BASED SPECTROPHOTOMETRIC ANTIOXIDANT CAPACITY MEASUREMENT IN SULFURIC ACID-SODIUM SULFATE MEDIUM WITH SELECTIVITY OVER CITRIC ACID AND SIMPLE SUGARS <b><u>Dilek ÖZYURT</u>, Birsen DEMİRATA, Reşat APAK</b>	55
<b>PP-29</b>	TOTAL ANTIOXIDANT CAPACITY OF POMEGRANATE (PUNICA GRANATUM) USING SPECTROPHOTOMETRIC METHOD (CERAC) <b><u>Dilek ÖZYURT</u>, Birsen DEMİRATA, Reşat APAK</b>	56



## **ABSTRACTS**



## **PLENARY LECTURES**

## ANTIOXIDANT CAPACITY/ACTIVITY ASSAYS APPLIED TO FRUITS, VEGETABLES, AND CEREALS

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KEY WORDS: Antioxidant Capacity/Activity, Fruits, Vegetables, Cereals, Antioxidant assays

Fruits, vegetables and cereals were analyzed for the composition of the major individual phenolic acids, ascorbic acid, flavonoids, flavanols and anthocyanins [1, 2]. The antioxidant capacities of aqueous/methanol, acetone and hexane extracts were comparatively assessed using the CUPRAC (CUPric Reducing Antioxidant Capacity), the TEAC (Trolox Equivalent Antioxidant Capacity by ABTS), the FRAP (Ferric Reducing Ability of Plasma), the DPPH (1, 1-Diphenyl-2-picrylhydrazyl method), the  $\beta$ -Carotene ( $\beta$ -Carotene Linoleate Model System), and the NO test (Scavenging activity against nitric oxide). Raw and processed vegetables were compared. It was found that raw natural products contain higher quantities of bioactive compounds and possess higher antioxidant activity than processed and the degree of the differences is directly connected to the processing conditions. The antioxidant capacity/activity values for each extract were well-correlated with the content of polyphenols ( $R^2=0.99$ ). The antioxidant activity of the studied vegetables according to all used antioxidant assays were in the following order: red onion > white onion = yellow onion > red pepper > garlic = green pepper > white cabbage; of the common fruits - apples > peaches > pears, of the citrus fruits - red grapefruits > oranges = lemons > sweetie and exotic fruits - durian > snake fruit > mangosteen, of cereals - buckwheat > oat > amaranth > quinoa > rice [1-3]. In conclusion, these antioxidant assays can be recommended for assessment of the bioactivity of different natural products.

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## **CRITICAL CONSIDERATIONS IN ORAC, TRAP, ABTS/TEAC, AND DPPH ASSAYS OF ANTIRADICAL ACTION**

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KEY WORDS: Total Antioxidant Capacity Assays, ORAC, TRAP, ABTS/TEAC, DPPH

A wide range of assays have been developed to screen antiradical actions of individual compounds and extracts from natural materials. Values from the most common of these -- ORAC, TRAP (a variant of ORAC), ABTS/TEAC, and DPPH assays – are now being used to recommend food choices and market food products and supplements. This paper addresses both technical and conceptual problems with each of these assays that need to be addressed before any standard procedures are adopted and must be considered when deciding appropriate design and uses of the assays.

As technical problems, ORAC and TRAP assays suffer limitations particularly in the chemistry of the probes currently used, temperature and oxygen control, proportions of reactants, and interactions of probes with antioxidants. TEAC and DPPH assays as currently performed use a single antioxidant concentration and measure reaction only at a single time point that varies from four minutes to 48 hours. These procedures provide skewed pictures of reactions and miss the most important aspects of the reaction chemistry. Both ABTS and DPPH radicals are nitrogen-centered and hindered, so molecular size plays a critical role in antioxidant reactions. In fact, detailed studies show that ABTS reactions are controlled almost entirely by molecular size rather than innate chemical properties, so should probably be abandoned as an assay. DPPH reactions are affected by molecular size, but inductive effects of ring adducts are more important. Although theoretically, both ABTS and DPPH react by both HAT and SET mechanisms, reactions in different solvents reveal that electron transfer is very fast and the dominant mechanism, while hydrogen transfer is very slow and uncompetitive. In addition, none of these methods has an adequate standardized method for reporting antiradical action.

As conceptual problems, it is becoming increasingly clear that negligible polyphenol absorption eliminates radical quenching as an important mechanism for dietary antioxidants *in vivo*, so the chemistry of current antioxidant assays is irrelevant. However, radical quenching is exactly how antioxidants act in foods and other materials. Recommendations will thus be made for changing the language of antioxidant assays, modifying procedures to elucidate antioxidant reaction kinetics and mechanisms as well as synergisms and antagonisms, and application of this new information to identify foods that can be productively combined to provide antioxidant protection and components in unused food waste such as peels and outer leaves that can potentially replace BHA and BHT in foods, cosmetics, and medical devices.

## **DETERMINATION OF HYDROPHILIC AND LIPOPHILIC ANTIOXIDANT CAPACITY – COMMENTS AND RESULTS**

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Phytochemicals with their antioxidative potential are regarded as protective food components. During the last decade, many analytical methods were developed to determine the antioxidant activity/capacity in vitro, measuring the ability to reduce prooxidants or to scavenge free radicals. These methods use different radicals or metal ions as oxidants. Seven common methods to determine the hydrophilic antioxidant activity are presented – five using radicals (TEAC assay: three versions, TRAP assay, DPPH assay, DMPD assay, PCL assay) and two using metal ions (LDL oxidation assay, FRAP assay) – and compared looking at four standard antioxidants used (ascorbic acid, gallic acid, Trolox<sup>®</sup> and uric acid). The results were not comparable. They can, however, give an idea of the protective potential of phytochemicals and plant food. For an adequate assessment of antioxidant power in biological systems the use of more than one method is necessary.

Analysing twelve food additives and six secondary plant products on their antioxidant activity by using three different test systems (TEAC assay, PCL assay, FRAP assay) resulted in differences depending on the assay. All the food additives showed antioxidant activities comparable to the calibration substance Trolox. In contrast, the secondary plant products had an up to 16 times higher antioxidant potential. This might present a good reason for the food industry to use natural antioxidants instead of synthetic ones to get storage stability for processed food items – which, according to recent surveys, is in the interest of consumers.

To look for the influence of the sample concentration on the measured hydrophilic antioxidant capacity, pure substances (ascorbic acid, gallic acid, Trolox, uric acid) and food extracts (strawberry nectar, tomato extract, white tea) were analysed using seven common antioxidant capacity assays (three versions of TEAC assay, FRAP assay, PCL assay, ORAC assay and total phenolics assay: Folin-Ciocalteu). For all applied pure substances and in most of the assays effects of the sample concentration on the measured antioxidant capacity were observed. Since it remains speculative how sample concentration does affect the measured antioxidant capacity exactly, it is strongly recommended to use at least three sample concentrations for analysis to detect and to discuss concentration-dependent effects.

Investigating the lipophilic antioxidant activity, often solutions of analytes in methanol or ethanol are used. However, these two alcohols are not able to dissolve hydrocarbons as e.g. the carotenes  $\beta$ -carotene and lycopene. Thus, common test systems to determine the hydrophilic antioxidant activity were modified. Analysing several carotenoids in four test systems (DPPH assay,  $\alpha$ TEAC assay, FRAP assay, chemiluminescence assay) showed lipophilic antioxidant activities being dependent on the number of conjugated double bonds, presence of  $\beta$ -ionone ring, number of hydroxyl groups and number of keto groups.

Concluding the presented investigations, determination of hydrophilic and lipophilic antioxidant activity/capacity is affected by various parameters. Until now, results can only be used to rank different compounds or food extracts in one laboratory within one assay. Thus, future interlaboratory tests are one possibility to make antioxidant potential results comparable between different publications.

**CUPRAC (CUPRIC REDUCING ANTIOXIDANT CAPACITY) ASSAY AS A NOVEL ELECTRON TRANSFER-BASED ANTIOXIDANT CAPACITY ASSAY, AND ITS VARIOUS MODIFICATIONS FOR CAPACITY/ACTIVITY MEASUREMENT IN DIVERSE MATRICES (INCLUDING BIOLOGICAL FLUIDS)**

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KEY WORDS: Antioxidant Capacity/Activity, Food and Serum Antioxidants, Flavonoids, ·OH Scavenging, XO Inhibition, H<sub>2</sub>O<sub>2</sub> Scavenging, Original CUPRAC Method

The accumulation of reactive oxygen species (ROS) in the organism, unless counterbalanced by antioxidants intaken through diet, may give rise under 'oxidative stress' conditions to several diseases like cancer, coronary disease, cellular ageing, mutagenism, immune system diseases, and oxidative damage to DNA and cellular membrane. The CUPRAC method of antioxidant measurement, introduced by our research group to world literature [1], is based on the absorbance measurement of the CUPRAC chromophore, Cu(I)-neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, Cu(II)-neocuproine, where absorbance is recorded at the maximal light absorption wavelength of 450 nm. The CUPRAC method of total antioxidant capacity (TAC) assay has been successfully applied to antioxidants in food plants, human serum, and to hydroxyl radical scavengers. In the assay of human serum antioxidants, hydrophilic antioxidants were measured after precipitation of proteins with HClO<sub>4</sub>, while lipophilic ones like  $\alpha$ -tocopherol and  $\beta$ -carotene were determined by n-hexane extraction, evaporation, followed by color development in dichloromethane of the Cu(I)-Nc chelate formed from their CUPRAC reaction [2]. In a miniaturized CUPRAC method without preliminary separation of phases, serum samples were centrifuged after 10% TCA precipitation, and CUPRAC was directly applied to the supernate. In measuring the hydroxyl radical scavenging activity of certain water-soluble compounds (metabisulfite, thiourea, glucose, lycine, etc.), the probes of *p*-aminobenzoate, 2,4-dimethoxybenzoate, and 3,5-dimethoxybenzoate were used to detect hydroxyl radicals, and the ·OH scavenging rate constants of these compounds were determined by competition kinetics [3]. In another similar method, salicylate probe was used to measure ·OH scavenging rate constants of certain antioxidants, and the method was validated against HPLC [4]. In the measurement of hydroxyl radical scavenging activities of polyphenolics, special measures were taken so as to prevent the redox cycling of phenolic compounds. In this newly developed method, the Fenton reaction was stopped at the end of the tenth minute with the addition of catalase to degrade hydrogen peroxide and cease ·OH production, and the dihydroxybenzoates formed from the salicylate probe under hydroxyl radical attack were measured with the CUPRAC method, rate constants being calculated with competition kinetics [5]. Lipophilic and hydrophilic antioxidants (e.g.,  $\beta$ -carotene,  $\alpha$ -tocopherol, ascorbic acid, quercetin, etc.) could be simultaneously assayed with a modified CUPRAC method in the same solvent medium of acetone-water (9:1, v/v) with the aid of their inclusion complexes formed with 2% methyl- $\beta$ -cyclodextrin [6]. In another modified CUPRAC method, the superoxide anion radical was formed with xanthine-xanthine oxidase, and the inhibition of the enzyme was measured upon addition of polyphenolics to the



system [7]. The hydrogen peroxide scavenging activity of the polyphenolics were measured in the presence of Cu(II) catalyzed with the HPS-CUPRAC method [8]. CUPRAC in urea buffer also responded to thiol-containing proteins in food [9]. In conclusion, the CUPRAC methodology is evolving into an “antioxidant measurement package” in biochemistry and food chemistry comprising many assays, and the results are in accordance with those of independent reference methods, having distinct advantages over certain established methods.

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## **INVITED LECTURES**

## COMBINED HPLC-CUPRAC ASSAY: SINGLE ASSAY FOR EVALUATION OF FOOD REGARDING BOTH INDIVIDUAL ANTIOXIDANT CONSTITUENTS AND TOTAL ANTIOXIDANT CAPACITY

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KEY WORDS: Combined HPLC-CUPRAC Assay, Total Antioxidant Capacity, Antioxidants, Phenolics

HPLC-CUPRAC assay named for the first time by our research group is based on separation, identification and quantification of individual antioxidants in the sample, multiplication of the HPLC-determined concentration of each antioxidant with its TEAC (trolox-equivalent antioxidant capacity, defined as the mM trolox equivalent concentration of 1 mM solution of the tested antioxidant) coefficient and summation of these products to yield the theoretical total antioxidant capacity (TAC) value by virtue of the additivity of absorbances of constituents in a mixture. Thus, the theoretical TAC of the investigated material could be estimated using the equation:

$$\text{TAC}_{\text{theoretical}} = \sum_{i=1}^n c_i (\text{TEAC})_i$$

where  $c_i$  is the concentration of antioxidant constituent  $i$  found with the help of HPLC, and  $(\text{TEAC})_i$  is the TEAC coefficient of constituent  $i$  with respect to CUPRAC (cupric ion reducing antioxidant capacity) [1].

The proposed HPLC-CUPRAC assay enables a realistic comparison of antioxidant constituents of complex samples by HPLC analysis, and of their calculated TAC values (without performing the actual antioxidant assay) in trolox equivalents. This comparison is certainly more meaningful than that of total phenolic contents made on a mass basis [2].

For synthetic mixtures, the experimental CUPRAC and theoretical HPLC-CUPRAC results for TAC were very close to each other. This finding confirms that if all the antioxidants in a complex mixture were identified and quantified with the help of HPLC techniques, their contribution to the overall capacity could be envisaged, and the experimentally found TAC values (with the use of the spectrophotometric CUPRAC antioxidant assay) could be correctly estimated by theoretically calculating the TAC using the principle of additivity of individual antioxidant capacities by HPLC-CUPRAC assay.

HPLC-CUPRAC assay was successfully applied to methanolic extracts of nettle, parsley and celery leaves [3], plums, and apple juices [4] by our research group.

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## DIRECT MEASUREMENT OF THE TOTAL ANTIOXIDANT CAPACITY OF FOODS: THE QUENCHER APPROACH

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The determination of total antioxidant capacity has gained a growing interest as tool for exploring the putative role of antioxidant-rich products in the prevention of degenerative diseases and for the selection of variety having high health potential. However, antioxidants in plants are difficult to extract since solubility ranges from water-soluble to lipid-soluble and many are covalently bound to cell wall. To date, several methodologies have been used to extract free soluble antioxidant compounds. Various solvents such as water, ethanol, methanol, and acetone have been used individually or in mixture to maximize the extraction yield. In spite of using different extraction procedures, total antioxidant capacities of cereals might have been underestimated due to difficulties on rendering bound phenolic compounds soluble prior to measurement. In the evaluation of the total antioxidant capacity of cereals, alkali, acid or enzymatic treatments of residue have been applied to complete the extraction of bound phenolic compounds. We have recently shown that antioxidants bound to insoluble matrice of various cereals, nuts and seeds have high antioxidant capacity (Serpen et al., 2007; Serpen et al., 2008; Açar et al., 2009). However, common procedures used to extract food antioxidants based on extraction/hydrolysis present several limitations. Many attempts using solvent mixtures or physical treatments have been made to increase solubility of food components in order to assess their antioxidant activity. Despite these efforts, food items stil have insoluble components that cannot be solubilized without altering their molecular nature. In this presentation, a direct procedure, so called the QUENCHER procedure for the determination of the total antioxidant capacity of foods is described (Gökmen et al., 2009). This procedure avoids time-consuming sample treatments such as solid/liquid extraction and chemical/enzymatic hydrolysis prior to antioxidant capacity measurement.

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## QUALITY CONTROL OF EDIBLE OIL AND OTHER NATURAL PRODUCTS BY CHEMILUMINESCENCE

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Edible oil is a complicated sample and analytical methods employed for quality control are often empirical and extensively dependant on experimental conditions. Chemiluminescence (CL) is an analytical method capable of measuring reactive oxygen species, radicals and other reactive molecules. The procedure involves mixing chemiluminogenic compounds such as luminol or lucigenin with hydrogen peroxide or other oxidants. Hence, the methodology can be easily applied to the estimation of antioxidant activity of edible oils and other natural products.

Initially, luminol CL with hydrogen peroxide in the presence of various catalysts has been investigated for the measurement of antioxidant activity [1] in olive oil and the method was successfully applied to the monitoring of olive oil wastewater after treatment by various refining procedures [2]. Antioxidant activity by luminol CL has also been applied to other natural products such as red wine [3]. Lucigenin with hydrogen peroxide has been proposed for the estimation of antioxidant activity of hydrophilic and hydrophobic antioxidants [4] and the method has been validated for the quality control of edible oils [5].

Measurement of peroxide value is also under investigation. The energy-transfer reaction of bis(2,4,6-(trichlorophenyl)oxalate (TCPO) with hydrogen peroxide or total peroxides in the presence of Mn(II) as catalyst and 9,10-dimethylanthracene as fluorophore has been applied to olive oil samples [6]. Luminol CL has also been evaluated in aqueous solutions in the presence of various catalysts [1] but it is further investigated in the presence of organic solvents [7].

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## SALVIA DITERPENOIDS AS A NATURAL ANTIOXIDANT SOURCE

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KEY WORDS: Antioxidant Activity, Salvia, Terpenoids, Flavonoids and Phenolics

*Salvia* (sage) species have been used as medicinal plants since ancient times in the treatment of cold, sore throat, tuberculosis, angina pectoris, stomach ache and menstrual disorders as well as sedative and memory enhancer [1]. *Salvia* species (Lamiaceae family) are rich in phenolics including phenolic abietane diterpenes and flavonoids and other phenolics which have antioxidant, antibacterial, antiviral, cytotoxic and antitumor properties [1, 2]. Recently, due to carcinogenic effects of some synthetic antioxidants, studies are concentrated in finding new natural antioxidants. In continuation of our studies on *Salvia* species [2, 4], we have now targeted to study potential *Salvia* extracts to discover new antioxidant agents, and investigated a series *Salvia* species for their antioxidant properties by several methods [3], namely lipid peroxidation inhibitory activity, DPPH free radical scavenging, ABTS cation radical scavenging, superoxide anion radical scavenging, CUPRAC and metal chelating activity assays. The activity directed isolation and structure elucidation studies were carried out by chromatographic (column, prep. TLC, flash chromatography, and HPLC) and spectroscopic methods (by 1D- and 2D NMR, mass, IR and UV spectroscopic techniques), respectively. Besides flavonoids and some phenolics, abietane diterpenoids were found to be main responsible constituents in the investigated *Salvia* species. In this presentation, antioxidant activity results of the active abietane diterpenoids ferruginol, taxodione, horminone and 7-acetylhorminone which were commonly found in Turkish *Salvia* species will be presented with the context of antiaging properties.

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## ANTIOXIDANT PROFILES OF THREE CHERRY TYPES GROWN IN TURKEY

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Sour cherry, cherry laurel, and cornelian cherry fruits are widely consumed in Turkey and have been used for their beneficial health effects for many years by the public; however, there is not enough investigation on their antioxidant potential. In this study, the antioxidant profiles of these three cherry types grown in Turkey were evaluated. Analyses of anthocyanins, total catechin, and total epicatechin were performed by HPLC. Total phenolics and total flavonoid contents were measured by spectrophotometric methods. The antioxidant capacity of the fruits were tested using; 2,2-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazil (DPPH), ferric reducing antioxidant power (FRAP) and copper reducing antioxidant capacity (CUPRAC) assays. According to the results, the highest total flavonoid content was found in cherry laurel (952.5 mg CE/100 g DW), whereas the total phenolic content was highest in cornelian cherry samples (4918.8 mg GAE/100 g DW). For all the samples, highest antioxidant capacity values were obtained by the CUPRAC method which was followed by the ABTS method. The total epicatechin content was much higher than the total catechin content in all cherry types. The total epicatechin content of cherry laurel was 3 to 30 times more than sour cherry and cornelian cherry, respectively. HPLC results showed that the 3-rutinoside and 3-glucoside of cyanidin were the major anthocyanins in all cherry types.

## PREPARATION AND CHARACTERIZATION OF ANTIOXIDANT LIPOSOMES CONTAINING WHEY PROTEINS

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KEY WORDS: Antioxidant activity, Gel chromatography, Liposome, Whey proteins.

It has been shown that whey and its components have a number of health promoting effects. In this study we aimed to isolate fractions containing whey proteins using liquid chromatography techniques and then prepared antioxidant liposomes suitable for cosmetic preparations. Fractionation of whey proteins was achieved by extraction, filtration and centrifugation followed by gel chromatography. Antioxidant activities of the fractions were determined by different methods: their copper ion reducing capacity (CUPRAC), Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging activity and H<sub>2</sub>O<sub>2</sub>-luminol chemiluminescence. Gel electrophoresis was used to characterize isolated proteins. Liposomes were prepared by thin film hydration and ultrasonification methods. Using Sephadex G-50 chromatography two different fractions were obtained. The first fraction contained major whey proteins, while the second fraction had small peptides. We have then determined the antioxidant activities of the fractions. The first fraction had the highest antioxidant activity. We have then prepared liposomes containing whey protein fractions and analyzed their sizes. We have also investigated the liposome structures under light, electron and atomic force microscope. Finally, we have prepared a cosmetic formula from liposomes containing the whey fractions. We believe that preparing antioxidant liposomes containing whey proteins will be an important contribution to pharmaceutical and dermal applications of different cosmetic formulas.



## PROTOCOLS FOR CONDUCTING AND REPORTING SCIENTIFIC RESEARCH ON ANTIOXIDANT ACTIVITY

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KEY WORDS: Antioxidants; Antioxidant Activity; In-vitro Antioxidant Assays; Foods; Protocols

In parallel with the considerable momentum gained over the last decade by research on antioxidants in plants and food ingredients, the market for both functional foods and for dietary supplements is increasingly being flooded by novel products claiming antioxidant power. There is now significant scientific agreement that bioactive molecules in foods need to be judged on the basis of many complex interactions arising from physiological metabolites and from cellular responses as well as on the basis of balanced/non-balanced diets; so it is not an easy task to scientifically assess and substantiate the “antioxidant power” claims made by the industry on the labels of such products. The validation of “health claims” in general has been the subject of extensive worldwide research and has led to some numerous “codes of practise” at both national and international regulatory levels. The struggle of the scientific community to establish some basic protocols for conducting and reporting research on antioxidant activity is also noteworthy in this respect.

This lecture will first cover the complications posed by the different mechanistic bases of analytical antioxidant studies[1], especially when trying to predict and control antioxidant activity in model food systems, since there will always be interactions and/or synergy with other food constituents. Furthermore, in complex food matrices, a choice of several techniques is deemed necessary to determine the different but complementary aspects of oxidation, using multidimensional and sometimes even multidisciplinary in-vivo approaches. In this context, a brief summary of the new competitive kinetic approaches for measuring formation of oxidation products as well as monitoring oxidisable substrate losses[2] will also be presented.

The lecture will conclude with a synopsis of the requirements set forth by the scientific community[3,4] for conducting and reporting research on antioxidants (hypothesis-driven sound study designs, valid testing systems, correct number and validity of test variables, repeatability and reproducibility of experiments, and sound statistical planning to substantiate the relevance of the findings) .

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## NATURAL ANTIOXIDANTS: ACTIVITY-STRUCTURE INSIGHT

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In terms of effects in human body an antioxidant can be defined as “a substance in foods that significantly decreases the adverse effects of reactive oxygen and nitrogen species, on normal physiological functions in humans”. In terms of foods, an antioxidant is compound able of delaying, retarding or preventing autooxidation processes.<sup>1,2</sup>

Natural antioxidants are present in fruits and vegetables, such as polyphenols, flavonoids and isoflavanones. On the other hand, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propylgallate (PG) are used in foods to retard lipid oxidation. However, their usage is regulated because of their possible health risks associated with many diseases. Besides, BHA and BHT are restricted by legislative rules because of doubts over their toxic and carcinogenic effects. For this reason, interest in finding natural antioxidants that represent an alternative to synthetic ones is high. Nowadays, there is a growing interest in natural and safer antioxidants. Phenolic compounds can trap the free radicals directly because of their structure. It is known that the compounds with structures containing two or more of the following functional groups: –OH, –SH, –COOH, –PO<sub>3</sub>H<sub>2</sub>, C=O, –NR<sub>2</sub>, –S– and –O– in a favorable structure-function configuration, can show antioxidant activity especially metal chelating activity.<sup>3,4</sup>

In this study, we clarified the antioxidant and radical scavenging and metal chelating mechanisms of some natural phenolic compounds such as caffeic acid, eugenol, resveratrol, adrenalin and curcumin.

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## **CHANGES IN ANTIOXIDANT AND METABOLITE PROFILES DURING PRODUCTION OF TOMATO PASTE**

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Tomato products and especially concentrated tomato paste are important sources of antioxidants in the Mediterranean diet. Tomato fruit contain well-known antioxidants such as vitamin C, carotenoids, flavonoids, and hydroxycinnamic acids. The industrial processing of tomato into tomato paste involves several treatments that potentially affect the final profile of antioxidants and other metabolites in the commercial product. In this study, both biochemical and metabolomic techniques to assess the effect of each separate step in the industrial production chain starting from fresh fruit to the final tomato paste were used. Material was collected from five independent tomato paste production events spread over two successive years. Samples comprised the intact ripe fruits and semifinished products after fruit-breaking, separation of the pulp from skin and seeds, evaporation, and finally after canning and pasteurization.

The effect of each processing step was determined by different types of analysis. First, the total antioxidant capacities using 2,2-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazil (DPPH), ferric reducing antioxidant power (FRAP) and copper reducing antioxidant capacity (CUPRAC) methods, as well as the total phenolic and total flavonoid contents were determined. Second, individual antioxidants in the extracts were identified and compared using an HPLC with online antioxidant detection. Third, in each sample the levels of the major individual antioxidants present, i.e., vitamin C, phenolic compounds (such as rutin and chlorogenic acid), tocopherols, and carotenoids, were quantified. Fourth, an untargeted metabolomic approach using LC-QTOF-MS was used to identify those production steps that have the largest impact on the overall metabolic profile in the final paste as compared to the original fruits.

The results showed that among all the total antioxidant capacity methods, CUPRAC method was found to be the best method capable of both determining the antioxidant capacity of hydrophilic and lipophilic phases of processed tomato. Although an overall increase of 6% in total phenolic content was observed by processing into paste, changes occurred in each step of processing were not statistically significant ( $p < 0.05$ ). Total flavonoid analysis showed that in the first step of processing (breaker), an increase by 31% compared to the fruit was observed. This multifaceted approach has also revealed that each processing step induces specific alterations in the metabolic profile, as determined by the different analysis procedures, and that in particular the fruit-breaking step and the removal of seed and skin significantly affect the levels of antioxidants and many other metabolites present in commercial tomato paste.



## **POSTER PRESENTATIONS**

## ANTIOXIDANT ACTIVITIES, GS / MS ANALYSIS AND PHENOLIC PROFILES OF *Achillea biserrata* AND *Hysopus officinalis* EXTRACTS

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**KEY WORDS:** Antioxidant Activity, DPPH,  $\beta$ -carotene-Linoleic Acid Method, Total Phenolic Content

In this study, hexane, chloroform and water extracts were prepared by making consecutive extraction of *Achillea biserrata* from the family *Asteraceae* and *Hysopus officinalis* from the family *Lamiaceae*. Volatile oil of the same types was obtained by water distillation that was continued 4 hours in the Clevenger fixture and dried by adding anhydrous sodium sulfate. After GS/MS analysis, volatile oil of the same types was kept at 4 °C in the refrigerator and dark until the work activity was performed. Volatile oil yields that were obtained from mixture of all dried flowers and plant leaves were determined for *Achillea biserrata* 0.4%(v/w) and *Hysopus officinalis* 1.3%(v/w). The antioxidant activities of extracts, total phenol, flavonoid, proanthocyanidin and anthocyanin amounts were determined. Two different tests were performed for measurements of antioxidant activities [1]. Especially, for the purpose of meeting the general properties of good additives' free radical cleaning activity was done by DPPH method and the inhibition of oxidation activity was done by with  $\beta$ -carotene method. In both methods, BHT was used as a positive control. The findings obtained were presented in Table 1. The total phenol, flavonoid amounts of extracts were presented in Table 2. [2]

**Table 1.**

Extract	<i>Achillea biserrata</i>		<i>Hysopus officinalis</i>	
	DPPH IC <sub>50</sub> (mg/mL)	% BAA	DPPH IC <sub>50</sub> (mg/mL)	% BAA
Hexane	Inactive	121,43±0,001	Inactive	230,95 ± 0,001
Chloroform	0,114±0,22	208,73±0,4	0,25±0,13	165,08±0,44
Water	0,0196±0,57	20,63±0,02	0,0188±0,82	15,07±0,001
Methanol-Water	0,0379±0,4	5,55±0,001	0,0288±0,18	12,30 ± 0,005
BHT	19.80±0.50	99.0±1	19.80±0.50	99.0 ± 1

**Table 2.**

Extract	<i>Achillea biserrata</i>		<i>Hysopus officinalis</i>	
	Total phenol	Flavonoid	Total phenol	Flavonoid
Chloroform	0,4646±0,06	0,0140 ±0,07	0,4963±0,003	0,0172±0,05
Water	0,2858±0,02	0,0106 ±0,02	0,5082±0,04	0,0085±0,002
Methanol-Water	0,3105±0,004	0,0110±0,002	0,3886±0,07	0,0125±0,001

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## CHANGES IN TOTAL PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY IN RESPONSE TO LOW TEMPERATURES IN OLIVE (*Olea europaea* L. "GEMLIK")

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KEY WORDS: Antioxidant Capacity, Phenolic Content, Olive, Low Temperature Stress

In many plant species several physiological and biochemical changes occur during low temperature-induced cold-acclimation [1]. Our previous studies in olive (*Olea europaea* L. cv. Gemlik) demonstrated a correlation between the level of accumulation of certain leaf proteins besides antioxidative enzyme activities and cold-hardiness [2, 3]. In this study, total phenolic content and antioxidant capacities were determined in olive leaves cv. Gemlik that were subjected to artificial low temperature tests (4°C, -5°C, and -20°C) in cold-acclimated (CA, in January) and non-acclimated (NA, in July) stages. Total phenolic content was estimated using the Folin-Ciocalteu colorimetric method and antioxidant capacity was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity. In addition, three different extraction methods using water, methanol (80 %) or acetone (80%; containing 0.2 % formic acid) were employed to analyze total phenolics and antioxidant capacity.

Cold acclimation induced accumulation of total phenolics, and this was positively related with antioxidant capacity. While higher phenolic content occurring especially after low temperature treatment of the samples resulted in a greater antioxidant capacity in CA stages, phenolic content and antioxidant activity were increased to some extent in NA stage with 4°C treatment but declined below control level following -5°C and -20°C treatments. Although low temperature treatments caused significant alterations in total phenolic content, changes in antioxidant capacity were less pronounced. Thus, we suggest using a different method for assaying antioxidant capacity other than DPPH method in olive tissues. On the other hand using methanol or acetone extraction method yielded higher results than water extraction.

Our results indicate that olive plant gains cold hardiness by secondary metabolite production and, in parallel, by enhancement of antioxidant capacity, during cold acclimation.

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## ANTIOXIDANT ACTIVITIES AND TOTAL PHENOLIC CONTENTS OF SOME AROMATIC EDIBLE PLANTS

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KEY WORDS: Antioxidant Capacity, Phenolic Content, Aromatic Edible Plants

Natural antioxidants occur in all parts of plants. These antioxidants include carotenoids, vitamins, phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), glutathione, and endogenous metabolites. These compounds have been shown to function as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers, enzyme inhibitors, and synergists [1]. Dietary antioxidants can increase cellular defenses and help to prevent oxidative damage to cellular components [2]. Superoxide radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical (OH), and singlet oxygen ( $^1O_2$ ) are extremely reactive compounds and caused oxidative damage in human body. Damage, resulting from the imbalance between reactive oxygen species generating and scavenging systems (oxidative stress), has been implicated in the pathology of a number of disorders, such as atherosclerosis, ischemia-reperfusion injury, cancer, malaria, diabetes, inflammatory joint disease, asthma, cardiovascular diseases, cataracts, immune system decline, and could play a role in neurodegenerative diseases and aging processes [3]. A way to control of these diseases is to increase the dietary intake of fruits and vegetables, many of which are rich sources of antioxidants.

The aromatic edible plants are consumed raw in salad and some are added to sauces. The plants have an important role in traditional diets in our country. The total phenolic contents was analyzed Folin-Ciocalteu assay and antioxidant capacity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging of parsley, mint, dill, cress, arugula and coriander. The assays of total phenolics and DPPH are used three different extraction methods which are including water, methanol (80%) and acetone (80%; containing 0.2% formic acid). Extraction methods containing aqueous acetone and aqueous MeOH are better than water extraction method. The total phenolic content of aqueous, acetone and methanolic extracts of the investigated plant species ranged from 2.45 to 5.99, from 3.81 to 5.71 and from 3.45 to 5.10 mg gallic acid equivalents  $g^{-1}$  fresh mass, respectively. The highest antioxidant capacity as  $\mu mol$  Trolox equivalents of 28,39  $g^{-1}$  fresh mass acetone extracts were obtained from mint. We suggested that the need to perform more than one type of antioxidant activity assay method to take into account the various mechanisms of antioxidant action for aromatic edible plant.

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**CHARACTERIZATION AND ANALYSIS OF THE ANTIOXIDANT CAPACITY OF  
FUNCTIONAL PHENOLICS OXIDIZED BY *Scytalidium thermophilum*  
CATALASE PHENOL OXIDASE (CATPO)**

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*Scytalidium thermophilum* is a thermophilic fungus effectively producing the enzyme catalase which is bifunctional with a phenol oxidase activity (CATPO) (1,2). CATPO is capable of oxidizing o-diphenols such as catechol, caffeic acid and L-Dopa. The aim of this study is to characterize the oxidation products of phenolic compounds oxidized by CATPO and to compare the antioxidant capacities of the phenolic compounds and their oxidation products. The phenolic compounds are selected according to their different chemistry and functionality. 15 phenolic substances were analyzed using both the supernatant and pure enzyme. The oxidation products of 5 of these compounds were further characterized (3). According to the findings; CATPO could oxidize catechol, hydroquinone, catechin, quercetin, chlorogenic acid and caffeic acid. The products of these phenolic substance reactions were analyzed by FTIR. The results indicate that the polymerization mainly takes place through C-C and C-O-C bonds. Dimer, trimer and tetramer structures were determined in another study by HPLC. The presence of multiple products and the progression of polymerization by time were also observed. The biological role of CATPO, and its possible contribution to the antioxidant mechanism of *S. thermophilum* is of interest. This is important because an additional phenol oxidase activity of catalases appears to be more wide spread than expected. The findings of our research laboratory deserve further investigations and are in progress still. Of particular interest are the difference between the antioxidant capacities of oxidation products and their unoxidized forms.

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**EXTRACTION OPTIMIZATION FOR ANTIOXIDANT ACTIVITY OF A POTENTIAL BIOACTIVE SOURCE: FEIJOA (*Feijoa sellowiana*)**

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KEY WORDS: Feijoa (*Feijoa sellowiana*), Antioxidant Activity, Extraction Optimization, TEAC, DPPH

The feijoa is a small tree, which originates from eastern regions of South America. It is native to Brazil but it also grows in parts of the USA and is grown widely in New Zealand for its highly aromatic fruit. Feijoa fruit is also commonly known as pineapple guava or guavasteen. It is a new bioactive source and it was reported that it has antimicrobial, anti-cancer, anti-inflammatory and antioxidant activity in very recent studies. The aim of the present study was to investigate the optimum extraction conditions for this antioxidant source both in skin and the pulp. Solvent to sample ratio (20:1, 40:1, 60:1 and 80:1), extraction temperature (25 and 40°C) and extraction time (0.5, 1 and 2 h) were applied one at a time to identify the optimum conditions. After selecting the optimum condition for extraction, six solvents (water, methanol, acetone, methanol:water (50:50, v/v), acetone:water (50:50, v/v), methanol:acetone (50:50, v/v) were used to identify the proper solvent for extraction. The TEAC (Trolox equivalent antioxidant capacity) and DPPH (2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity) assays were carried out to determine the antioxidant capacity of feijoa skin and pulp extracts.

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## COMPARISON OF TWO COMMON ASSAYS FOR QUANTIFYING TOTAL FLAVONOIDS

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KEY WORDS: Total Flavonoid Content,  $\text{AlCl}_3/\text{KAc}$  Assay,  $\text{AlCl}_3/\text{NaNO}_2$  Assay, Flavonoids, Phenolics

Flavonoids, which are phenolic substances widely existing in fruits, vegetables, whole grains, and other plants, with over 8000 individual compounds known, have been reported to have many health benefits including antioxidant activity, anti-inflammation, antibacterial and antiviral effects, antiallergenic reaction, and antimutagenic and anticancer properties. Most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge reactive species. Therefore, studies on plant foods generally deal with the determination of total flavonoid contents of these materials. Currently, there are two commonly used assays for the determination of total flavonoid content with the same main reagent,  $\text{AlCl}_3$  [1,2]. One of these methods uses potassium acetate as the second reagent. In this method ( $\text{AlCl}_3/\text{KAc}$ ), absorbance measured at 427 nm (modified from 415 nm used in ref [1]) after 30 minute-incubation at room temperature has been used for determining total flavonoid content of the studied sample. The other common method ( $\text{AlCl}_3/\text{NaNO}_2$ ) for total flavonoid content uses sodium nitrite reagent after  $\text{AlCl}_3$  addition [2]. There are incubation steps after each reagent addition in this method. Calculation of total flavonoid content is based on the absorbance measurement at 510 nm.

In this study, luteolin (flavone), kaempferol, quercetin and myricetin (flavonols), catechin (flavanol), hesperidin and naringin (flavonone glycosides) were studied as representatives of flavonoid classes and chlorogenic and caffeic acids (hydroxycinnamic acids) as potential interferences. Results demonstrate that  $\text{AlCl}_3/\text{KAc}$  assay determined total flavon and flavonol content, and  $\text{AlCl}_3/\text{NaNO}_2$  assay determined total flavon, flavonol and flavanol content as total flavonoids. Both assays do not respond to flavonone glycosides while  $\text{AlCl}_3/\text{KAc}$  assay additionally to flavanols, probably as a result of deficiency in  $\text{Al(III)}$  chelation. Besides, hydroxycinnamic acids that are phenolics but not flavonoids give positive interference to  $\text{AlCl}_3/\text{NaNO}_2$  assay. So, the two assays cannot truly evaluate the total flavonoid content of many plant foods including flavanols or flavonone glycosides as major flavonoids, possibly because the key to such assays is efficient  $\text{Al(III)}$  chelation.

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## TEAC (TROLOX EQUIVALENT ANTIOXIDANT CAPACITY) COEFFICIENTS OF SOME ANTHOCYANINS ACCORDING TO CUPRAC AND ABTS METHODS

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KEY WORDS: TEAC, Combined HPLC-CUPRAC Assay, ABTS Method, Anthocyanins, Anthocyanidins

Anthocyanins (ACNs) are pigments that give rise to the red to blue colours observed in many soft fruits and flowers. There is an increased interest in anthocyanins because of the various potential health benefits that include a reduced risk of coronary heart disease, visual improvement and anti-carcinogenic, anti-mutagenic and anti-inflammatory effects. Many of these health benefits have been attributed to the antioxidant property of anthocyanins. Anthocyanidins are also aglycons of anthocyanins.

It was aimed in this study to determine the TEAC (trolox-equivalent antioxidant capacity, defined as the mM trolox equivalent concentration of 1 mM solution of the tested antioxidant) coefficients of anthocyanidins comprising cyanidin chloride and peonidin chloride, and anthocyanins comprising kuromanin chloride (cyanidin-3-O-glucoside chloride), keracyanin chloride (cyanidin-3-O-rutinoside chloride), idaein chloride (cyanidin-3-O-galactoside chloride) and peonidin-3-O-glucoside for multipurpose usage such as evaluation of antioxidant power of anthocyanins and anthocyanidins or calculation of TAC (total antioxidant capacity) of mixture samples by the combined CUPRAC-HPLC assay [1].

Experimental studies were performed by determining the TEAC values of anthocyanin standards using two spectrophotometric methods, firstly the simple and low-cost CUPRAC (Cupric Ion Reducing Antioxidant Capacity) method [2] developed in our laboratory, and secondly the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) reference method [3] accepted in international literature for antioxidant assays.

The order of TEAC values determined with the CUPRAC method were: cyanidin chloride (5.84) > peonidin chloride (4.81)  $\cong$  keracyanin chloride (4.80) > kuromanin chloride (4.73) > idaein chloride (4.70) > peonidin-3-O-glucoside (3.41). The order of TEAC values determined with the ABTS method were: cyanidin chloride (5.10) > kuromanin chloride (4.68) > keracyanin chloride (4.50) > peonidin-3-O-glucoside (3.67) > idaein chloride (3.48) > peonidin chloride (2.36).

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## TOTAL ANTIOXIDANT CAPACITIES AND PHENOLIC CONTENTS OF SOME SELECTED HERBAL PLANTS

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**KEY WORDS:** Antioxidant Capacity, Herbal Plants, CUPRAC Method, ABTS Method, Folin-Ciocalteu Method

Polyphenolic compounds are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects, including antioxidant activity. As a part of alternative disease prevention strategies, folk medicinal plants have become important for the preservation of human health. These plants are low-cost, readily available, and can be used in their simple forms or preparates for health beneficial purposes.

In this study, the total antioxidant capacities (TAC) and the total phenolic contents of 70% methanolic extracts of some medicinal herbs consisting of hawthorn leaves (*Crataegus monogyna*), hops (*Humulus lupulus*), horsetails (*Equisetum arvense*) and mistletoe leaves (*Viscum album*) were investigated. Experimental studies were performed by determining the total antioxidant capacities of these herbal extracts arising from antioxidant compounds using two spectrophotometric methods, firstly the simple and low-cost CUPRAC (Cupric Ion Reducing Antioxidant Capacity) method [1] developed in our laboratory, and secondly the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) reference method [2] accepted in international literature for antioxidant assays. The total phenolic contents were found with the aid of Folin-Ciocalteu assay [3]. The TAC values of the extracts ranged from 0.053 to 0.312 mmol Trolox/g dw (dry weight), from 0.013 to 0.206 mmol Trolox/g dw for CUPRAC and ABTS methods, respectively. The total phenolic contents ranged from 0.001 to 0.354 mmol gallic acid/g dw. The order of total antioxidant capacities (mmol trolox/g dw) and total phenolic contents (mmol gallic acid/g dw) of these extracts determined with the CUPRAC, ABTS and Folin-Ciocalteu methods were: Hawthorn leaves (*Crataegus monogyna*) > Horsetails (*Equisetum arvense*) > Hops (*Humulus lupulus*) > Mistletoe leaves (*Viscum album*). It was seen that the Hawthorn leaves (*Crataegus monogyna*) extracts showed the highest antioxidant capacity and total phenolic content.

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**EFFECTS OF MINIMAL PROCESSING AND REFRIGERATED STORAGE ON PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF ROCKET SALAD (*Eruca sativa*, *Brassicaceae*)**

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Fruits and vegetables are proven to be rich sources of phytochemicals which are secondary products of plant metabolism and take part in the prevention and/or cure of several chronic diseases. However, postharvest storage and processing can greatly affect the content of these health promoting phytonutrients. Rocket salad is among the vegetables that are widely used as minimally processed products in various recipes so far. On the other hand, there are only a limited number of studies in the literature focusing on the changes in phenolic content and antioxidant capacity of rocket salad during processing and storage conditions.

The main objective of the study was to evaluate the effects of minimal processing and refrigerated storage on phenolic content and antioxidant activities of rocket salad samples.

Rocket salad leaves, obtained from a retail store, were separated into two groups as intact leaves and fresh-cut leaves, and each group was stored at 4°C for 7 days. At days 0, 1, 4, and 7 of storage, samples were taken from each group of both intact and fresh-cut leaves. These samples were freeze-dried before the extraction procedure and then their total phenolic contents, antioxidant activities and phenolic profiles were analyzed using Folin-Ciocalteu method, ABTS radical scavenging method, and RP-HPLC, respectively. Differences, originated from minimal processing and storage, were evaluated by one-way ANOVA ( $P < 0.05$ ).

The results of the present study revealed that total phenolic content of the intact leaves decreased from 1584,7 to 1289,1 mg Catechin/100 g sample, while for the fresh-cut leaves it changed from 1601,9 to 894,8 mg Catechin/100 g sample during the designated time of storage. At the end of day 1, the level of total phenolics was found to be higher than the initial content (day 0) for both of the intact and fresh-cut samples. Then, the total phenolic content showed the same tendency for both of the intact and fresh-cut leaves while decreasing significantly ( $P < 0.05$ ) at the end of day 4. On the other hand, it was observed that the total phenolic content continued to decrease for the fresh-cut samples whereas an increase was detected in the intact leaves in between days 4 and 7, although these changes were not statistically significant ( $P > 0.05$ ). The results of ABTS assay were found to be changing in the same manner with total phenolics; the antioxidant activity decreased from 3150,0 to 1866,4 mg TEAC/100 g sample for the fresh-cut leaves and the activity of intact leaves changed from 3241,7 to 2711,2 mg TEAC/100 g sample. There was a significant correlation between total phenolics and ABTS assay results (0,9889 and 0,9805 for the fresh-cut and intact leaves, respectively). In addition, there was a significant difference in the change of the antioxidant activities between intact and fresh-cut leaves. The decrease in antioxidant activity of intact leaves was statistically significant at the end of day 4 ( $P < 0.05$ ); while for the fresh-cut leaves the decrease was statistically significant ( $P < 0,05$ ) at the end of day 7. The main phenolic compound in rocket leaf samples was found to be kaempferol according to the HPLC analysis.

The current data presented in this study revealed that the processing and storage conditions have significant effects on the overall total phenolics and antioxidant activity of rocket salad, ensuing and differing particularly after the 4<sup>th</sup> day of storage.

**ANTIOXIDANT ACTIVITIES OF SOME NEW 3-ALKYL-4-[3-(4-NITROBENZOXY)BENZYLIDENAMINO]-4,5-DIHYDRO-1H-1,2,4-TRIAZOL-5-ONES**

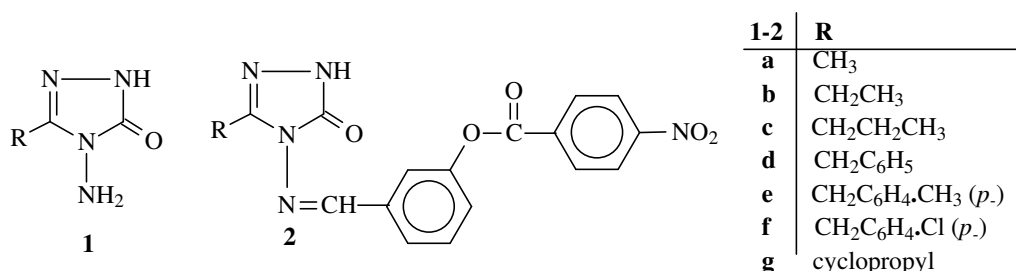
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Antioxidants are extensively studied for their capacity to protect organism and cell from damage that is induced by the oxidative stress. A great deal of research has been devoted to the study of different types of natural and synthetic antioxidant. A large number of heterocyclic compounds, containing the 1,2,4-triazole ring, are associated with diverse biological properties such as antioxidant, anti-inflammatory, antimicrobial and antiviral activity. Exogenous chemicals and endogenous metabolic processes in human body or in food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules by resulting in cell death and tissue damage. Oxidative damages play a significantly pathological role in human diseases.

In this study, new 3-alkyl(aryl)-4-[3-(4-nitrobenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-ones (**2**) were synthesized by the reactions of 3-alkyl(aryl)-4-amino-4,5-dihydro-1H-1,2,4-triazol-5-ones (**1**) with 3-(4-nitrobenzoxy)benzaldehyde, which were synthesized by the reaction of 3-hydroxybenzaldehyde with 4-nitrobenzoyl chloride by using triethylamine. The structures of seven new compounds are established from the spectral data. Then the antioxidant properties of the compounds were studied and evaluated using different three antioxidant assays, including reducing power, free radical scavenging and metal chelating activity. For the measurement of the reductive ability, Fe<sup>3+</sup> - Fe<sup>2+</sup> transformation was investigated in the presence of compound using by the method of Oyaizu [1]. The hydrogen atoms or electrons donation ability of the synthesized compound was measured by DPPH<sup>•</sup> using the method of Blois [2]. The chelating effect of ferrous ions by the compound was determined according to the method of Dinis et al [3]. BHT, BHA and  $\alpha$ -tocopherol were used as reference antioxidant compounds. In this study, results showed that the synthesized compounds displayed weak scavenging free radical activities, but newly synthesized compounds did not show any reductive activities. Metal chelating capacity was significant, since it reduced the concentrations of the catalyzing transition metal. Chelating agents, which form  $\sigma$ -bonds with a metal, are effective as secondary antioxidants, because they reduce the redox potential thereby stabilizing the oxidized form of metal ion. Future studies will be necessary to determine their possible role in mitigating the deleterious effect of ROS in different biological systems.



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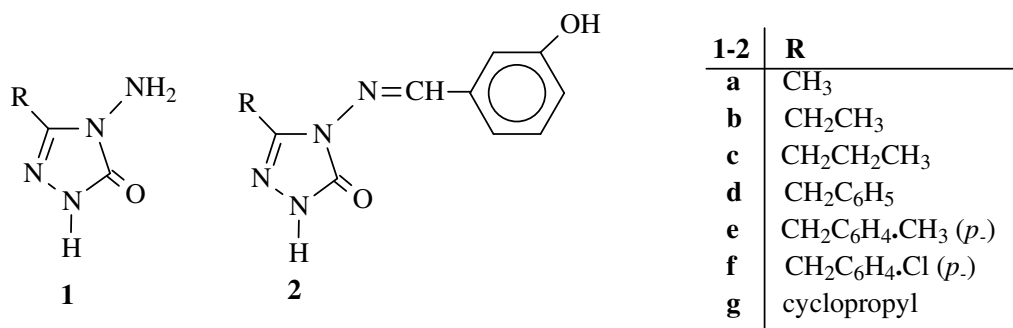
(This study was supported by the Scientific and Technological Council of Turkey (Project Number: TBAG 107T247))

**SYNTHESIS AND ANTIOXIDANT ACTIVITIES OF SOME NOVEL 3-ALKYL-4-(3-HYDROXYBENZYLIDENAMINO)-4,5-DIHYDRO-1H-1,2,4-TRIAZOL-5-ONES**

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1,2,4-Triazole derivatives have drawn considerable attention for the past few decades due to their diverse biological properties. Many 1,2,4-triazole derivatives are found to be potent antioxidant, anti-inflammatory, antimicrobial and antiviral agents. The identification of triazoles and determination of their antioxidant activities are of considerable interest because of the role they play in pharmacological actions. Seven novel 3-alkyl(aryl)-4-(3-hydroxybenzylidenamino)-4,5-dihydro-1H-1,2,4-triazol-5-ones (**2**) having 4,5-dihydro-1H-1,2,4-triazol-5-one ring were synthesized by the reactions of 3-alkyl(aryl)-4-amino-4,5-dihydro-1H-1,2,4-triazol-5-ones (**1**) with 3-hydroxybenzaldehyde and characterized by elemental analyses and IR, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR and UV spectral data. The synthesized compounds were analyzed for their in vitro potential antioxidant activities in three different methods, including 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging, reducing activity by Fe<sup>+3</sup> –Fe<sup>+2</sup> transformation and ferrous metal (Fe<sup>+2</sup>) chelating activities. Butylated hydroxytoluene (BHT) and α-tocopherol were used as reference antioxidant compounds.



In this study, results showed that the synthesized compounds displayed weak scavenging free radical activities, but newly synthesized compounds did not show any reductive activities. Metal chelating capacity was significant, since it reduced the concentrations of the catalyzing transition metal. Chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants, because they reduce the redox potential thereby stabilizing the oxidized form of metal ion. Future studies will be necessary to determine their possible role in mitigating the deleterious effect of ROS in different biological systems.

(This study was supported by the Scientific and Technological Council of Turkey (Project Number: TBAG 107T247))

## ANTIOXIDANT PROTECTIVE EFFECT OF FLAVONOIDS ON THE LIPID PEROXIDATION INDUCED BY COPPER / ASCORBIC ACID SYSTEM

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KEY WORDS: Lipid Peroxidation, Cumene Hydroperoxides, Antioxidant Protection, Fe(III)-thiocyanate Method

Lipid oxidation occurs when oxygen reacts with lipids in a series of free radical chain reactions that lead to complex chemical changes. Oxidation of lipids in foods causes quality losses. In vivo, lipid oxidation may play a role in coronary heart disease, atherosclerosis, cancer, and the aging process. Antioxidants are compounds that can delay or inhibit lipid oxidation. When added to foods, antioxidants minimize rancidity, retard the formation of toxic oxidation products, maintain nutritional quality, and increase the shelf life [1].

The peroxidation of linoleic acid (LA) in the absence and presence of Cu(II) ions was investigated in aerated and incubated emulsions at 37°C and pH 7. Additionally, the peroxidation of LA was studied in the presence of Cu(II) and ascorbic acid (AA) together. The effects of three different flavonoids of similar structure (quercetin, morin and catechin) as potential antioxidant protectors of AA were studied in the (copper+AA)-induced LA peroxidation system in which individual estimation of AA was possible using a modified CUPRAC (cupric reducing antioxidant capacity) assay.

If oxygen is passed through the LA emulsion system by adding copper(II) salt, cumene hydroperoxides are formed in the course of oxidation, causing rancidity of lipid foods. Through reaction with acidic Fe(II)-thiocyanate, these peroxides can be detected by formation of Fe(III)-thiocyanate which is bloody-red colored. The antioxidant activity of protector compounds added to the medium is inversely proportional to the amount of Fe(III)-thiocyanate complex formed per unit time. Peroxide production in a LA system assayed by ferric thiocyanate method was used to determine antioxidant and prooxidant activities initiated by a metal catalyst (Cu<sup>2+</sup>)[2]. The kinetic profile of peroxidation is characterized by three major parameters: the lag time preceding rapid oxidation, the maximal rate of oxidation (V<sub>max</sub>) and the maximal accumulation of oxidation products (OD<sub>max</sub>)[3]. Change of absorbance due to ferric thiocyanate as a function of incubation time exhibited curves of a sigmoidal pattern [4-5].

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## EFFECT OF INVASIVE *CAULERPA RACEMOSA* VAR. *CYLINDRACEA* EXTRACTS ON THE GROWTH AND BIOCHEMICAL CONSTITUENTS OF *ZEA MAYS* (L.)

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KEY WORDS: *Caulerpa racemosa* var. *cylindracea*, Macro Algae, Organic Agriculture, Seaweed Fertilizer, Antioxidants

Turkey has very long coasts where many sea plants and algae are found in this sea ecosystem. *Caulerpa racemosa* var. *cylindracea* is a green marine macroalgae and one of the well-known invasive species in the Mediterranean Sea. Until 1990 *Caulerpa racemosa* was not thought as an invasive species. This species showed invasive character and observed in 13 Mediterranean countries after 1991 [1]. Its high invasion tendency observed in the Turkish coasts has motivated us to transform this biomass into a seaweed based fertilizer. Seaweed fertilizer cocktails are prepared according to Sivasankari et al (2006) [2]. Our previous study showed that extracts from *Caulerpa racemosa* collected in autumn season stimulated the growth parameters and also antioxidant system parameters in *Vigna sinensis* and *Phaseolus vulgaris* [3]. In the present study we observed positive effects of *Caulerpa racemosa* var. *cylindracea* extracts which collected in spring season on growth parameters and antioxidant systems on *Zea mays* (L.) seeds. Higher growth rates, maximum CAT, APX activity and protein levels and minimum MDA levels of root, shoot and leaves of *Zea mays* (L.) were observed in extract supplemented group. Our results showed that *Caulerpa racemosa* var. *cylindracea* extracts supplementation gives better results than that of control group. Because of this promising presented results, *Caulerpa racemosa* var. *cylindracea* extracts can be used as a seaweed fertilizer in organic agriculture. The study was financially supported by TUBITAK (108O234).

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## ARGUMENTS FOR THE ESTABLISHMENT OF CROCIN BLEACHING ASSAY AS A RELIABLE TOOL FOR ANTIOXIDANT ACTIVITY ASSESSMENT

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KEY WORDS: Competitive Kinetics, Peroxyl Radical Scavengers, Antioxidant Activity, Saffron

The Crocin Bleaching Assay (CBA) developed more than three decades ago [1], employs a water-soluble carotenoid derivative, *crocin* as a probe for monitoring reactions of test antioxidants (AHs) with peroxyl radicals ( $\text{ROO}^\bullet$ ), according to a simple competition kinetic model. The rate of crocin bleaching in the presence or absence of AHs is monitored at 440 nm and then used to calculate either relative rate constants (kinetic approach) or  $\text{IC}_{50}$  values (Total Antioxidant Capacity, TAC approach) [2]. The information obtained by both approaches seems to be quite valuable in comparison with that offered by common in vitro assays ( $\text{DPPH}^\bullet$ ,  $\text{ABTS}^{\bullet+}$ , etc) indicating that CBA has a distinctive potential for application to pure molecules, plant extracts or biological fluids. Our presentation focuses on CBA data produced in our laboratory over the last few years for a large number of simple and complex phenolic compounds, polar plant extracts and human plasma samples [e.g. 3-5]. In each case study CBA data are discussed together with those produced by the same analyst using other in vitro assays (e.g. DPPH, ABTS, ORAC). Our arguments in favour of CBA point out its contribution in (a) building structure-activity relationships among structurally related phenols, (b) complementing data for redox activity of test compounds and (c) obtaining information about the prooxidant/antioxidant balance of biological fluids.

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## THEORETICAL METHODS IN RADICAL SCAVENGING ACTIVITY STUDIES OF PHENOLIC ANTIOXIDANTS: POTENTIAL AND LIMITATIONS

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KEY WORDS: Bond Dissociation Enthalpy, Computational Methods, DFT, Ionization Potential, Phenolic Antioxidants, Radical Scavenging

Theoretical methods have been recognized nowadays as a useful approach in antioxidant activity studies of phenolic compounds (PhOH) via the computation of suitable molecular descriptors for an optimum (lowest in energy) molecular structure. Among various descriptors, the most popular ones are the phenolic O-H bond dissociation enthalpy (BDE) and ionization potential (IP) values, which may well characterize the ability of PhOH to scavenge free radicals by hydrogen atom or electron donation, respectively.

So far in literature the values reported for PhOH have been computed with a diversity of methods, as there is no consensus on one suitable method. Nevertheless, method selection is the most critical point as it may have an impact on the cost of calculation and the accuracy of the derived results. The approaches employed can be divided into three categories [1]: (a) semiempirical methods (e.g. Austin Model 1, AM1), (b) advanced methods (e.g. *ab initio* and density functional theory, DFT usually employing the Becke 3 term with Lee, Yang, Parr exchange) and (c) their combination (e.g. DFT/AM1).

The potential and limitations of the three approaches are presented using characteristic examples of compounds for which there are published computational data. Computational time depends strongly on molecular size and approach adopted so that BDE value calculation to last few minutes, hours or days. In general, it can be accepted that BDE and IP values assist explanation of order of activity of a series of antioxidants under various experimental conditions though this cannot be taken as a rule [2]. Prediction of antioxidant behaviour in heterophasic systems cannot be supported by the above mentioned molecular descriptors.

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## DENSITY FUNCTIONAL STUDY ON THE STRUCTURAL CONFORMATIONS AND INTRAMOLECULAR CHARGE TRANSFER FROM THE VIBRATIONAL SPECTRA OF 6-AMINOFLAVONE

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KEY WORDS: FT-Raman, FT-IR, DFT, Intramolecular Charge Transfer, 7-aminoflavone

The FT-IR and FT-Raman spectra of 6-aminoflavone (6AF) have been recorded together for the first time between 4000 - 400 cm<sup>-1</sup> and 3500 - 5 cm<sup>-1</sup> regions, respectively. The molecular geometry and vibrational wavenumbers of the compound have been also calculated in its ground state by using *ab initio*, DFT/B3LYP functional with 6-311++G(d,p) basis set. The calculations were utilized to the C<sub>1</sub> symmetry of the compound. All calculations were performed with “Gaussian 03” software [1]. Normal coordinate analysis and Potential Energy Distribution (PED) analysis were studied using “VEDA 4” program [2] and its visualization interface in order to obtain a more complete description of the molecular motions involved in the fundamental vibrational modes of 6AF. Besides, C=O and NH<sub>2</sub> groups examined for their possible responsibility of bioactivity. Intramolecular charge transfer (ICT) properties were also determined. The main natural orbital interactions were analyzed with the “NBO 5.0” program [3]. Several other types of valuable data, such as hybridization, and partial charges, have been carried out from the NBO results. The biological properties of 6AF were evaluated using the software ‘pass-inet’.

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## EXPERIMENTAL (FT-RAMAN AND FT-IR) AND THEORETICAL (DFT) STUDIES ON 7-AMINOFLAVONE AND ITS INTRAMOLECULAR CHARGE TRANSFER MECHANISM

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KEY WORDS: FT-Raman, FT-IR, DFT, Intramolecular Charge Transfer, 7-aminoflavone

The FT-IR and FT-Raman spectra of 7-aminoflavone (7AF) have been recorded together for the first time between 4000 - 400 cm<sup>-1</sup> and 3500 - 5 cm<sup>-1</sup> regions, respectively. The molecular geometry and vibrational wavenumbers of the compound have been also calculated in its ground state by using *ab initio*, DFT/B3LYP functional with 6-31++G(d,p) basis set. The calculations were utilized to the C<sub>1</sub> symmetry of the compound. All calculations were performed with “Gaussian 03” software [1]. Normal coordinate analysis and Potential Energy Distribution (PED) analysis were studied using “VEDA 4” program [2] and its visualization interface in order to obtain a more complete description of the molecular motions involved in the fundamental vibrational modes of 7AF. Besides, C=O and NH<sub>2</sub> groups examined for their possible responsibility of bioactivity. Intramolecular charge transfer (ICT) properties were also determined. The main natural orbital interactions were analyzed with the “NBO 5.0” program [3]. Several other types of valuable data, such as hybridization, and partial charges, have been carried out from the NBO results. The biological properties of 7AF were evaluated using the software ‘pass-inet’.

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## PREDICTION OF TOTAL ANTIOXIDANT ACTIVITY FROM CHROMATOGRAMS BY NET ANALYTE SIGNAL AND ORTHOGONAL SIGNAL CORRECTION PARTIAL LEAST SQUARES REGRESSION

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KEY WORDS: *Prunella L.*, Total Antioxidant Activity, Net Analyte Signal, Orthogonal Signal Correction, Partial Least Squares Regression

*Prunella L.*, belonging to the *Lamiaceae* family, is known to contain large amounts of phenolic compounds [1] that exhibit high antioxidant potentials. The consumption of *Prunella L.* is therefore associated with protective effects against free radicals. Free radicals can damage cells, and may play a role in heart disease, cancer and other diseases. As a consequence, the antioxidant compounds may play an important role in the prevention of certain diseases. Multivariate chemometric methods such as partial least squares (PLS) allow to extract analytical information from the full-spectra/chromatogram, providing so to use simultaneously an elevated number of signals. Moreover, these techniques allow a rapid analytical response with minimum sample preparation, reasonable accuracy and precision and without a preliminary separation step in complex matrices [2]. Recently, comparative studies about advantageous and limitations of net analyte signal (NAS) [3] and orthogonal signal correction (OSC) [4] based methods and PLS calibration in complex matrices have been performed. The use of signal filtering algorithms such as NAS and OSC may help simplify calibration models and construct models with an adequate predictive ability. Preprocessing of chromatographic data is necessary for natural samples and chemometrics offers many tools well-suited to handling this task. However, it should be emphasized that a powerful application of multivariate calibration methods requires a careful preprocessing of the chromatograms. In the present study, NAS-PLS and OSC-PLS multivariate calibration methods were applied to the prediction of total antioxidant activity of *Prunella L.* extracts. The total antioxidant activity of *Prunella L.* extracts is determined by ABTS [2,2-azino-di-(3-ethylbenzothiazolone-sulphonic acid)] method. Simultaneously, these extracts were chromatographed with HPLC method. Multivariate calibration methods relating the chromatographic profiles with total antioxidant activity of *Prunella L.* extract were constructed. Smoothing, normalization, correlation optimized warping and column centering are applied on analytical signals and compared for preprocessing data. Root means square errors were calculated for calibration and validation set as comparison criteria. It was found that NAS-PLS, OSC-PLS and the validation set results were compatible. The results also demonstrate that NAS-PLS and OSC-PLS multivariate calibration methods can be applied successfully to predict total antioxidant activity of *Prunella L.* extracts.

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## CUPRAC (CUPRIC REDUCING ANTIOXIDANT CAPACITY) ASSAY OF SERUM INCORPORATING PROTEINS

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KEY WORDS: Modified CUPRAC Method, Serum Antioxidants, Proteins, Thiols, Ellman Method

Antioxidant activity/capacity assay methods existing in literature based on the measurement of radical scavenging activity of antioxidant compounds suffer from the difficulties encountered in the formation and stability of colored radicals such as ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2'-diphenyl-1-picrylhydrazyl). Although several methods have been developed to measure the total antioxidant capacity (TAC) of biological fluids such as human serum or plasma, none incorporate proteins. Apak *et al.* developed the Cupric Reducing Antioxidant Capacity (CUPRAC) spectrophotometric method and applied it to a complete series of plasma antioxidants for the assay of TAC of serum. As a distinct advantage over other electron-transfer based assays (*e.g.* Folin, FRAP, ABTS, DPPH), CUPRAC is superior in regard to its realistic pH (close to that of physiological pH), favourable redox potential, accessibility and stability of reagents, and applicability to lipophilic antioxidants as well as hydrophilic ones [1]. An important gap in literature is the unknown antioxidative contribution of proteins to the measured TAC, because due to inadequacies of analytical methodology in most TAC assays, proteins are initially separated by precipitation from the main matrix and their capacities are not at all considered. A modified CUPRAC method and selected standard TAC determination methods were applied to precipitated and redissolved serum proteins. In this way, some buried thiol and amino acid groups were exposed, and apparently gained the ability to react with the assay reagent. The results have shown that the contribution of proteins, especially thiol-containing proteins, to the observed TAC is by no means negligible. In the adaptation of the CUPRAC method to the measurement of antioxidant capacities of thiol-containing proteins [2], the classical pH 7 ammonium acetate (1 M) buffer was replaced with 8 M urea buffer of which the pH was adjusted to 7 by adding 6 M HCl. The function of urea is to make the embedded thiol groups of proteins open to oxidative attack so that they could be more easily oxidized by the antioxidant assay reagent. The aim of this work is to measure TAC of serum with standard reference methods (such as ABTS, FRAP, and Ellman thiols assay) along with the modified CUPRAC method, and to identify the contribution of serum proteins, especially thiol-containing proteins, to the measured TAC. As opposed to the Ellman method capable of determining only thiol compounds but not other common antioxidants, CUPRAC is most advantageous in regard to its strong response to both common antioxidants and thiol compounds.

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## ANTIOXIDANT ACTIVITY AND PHENOLICS COMPOUNDS OF EDIBLE WILD MUSHROOMS FROM TRABZON, TURKEY

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KEY WORDS: Antioxidant, mushroom, phenolic compound, FRAP method

In this study, we investigated and compared some phenolic compounds and *in vitro* antioxidant activities of three different types of Trabzon, Turkey edible wild mushroom. Taxonomic identification followed those done by Dr. Ertugrul SESLİ and representative voucher specimens were deposited at the herbarium of the Department of Biology, Faculty of Education, Karadeniz Technical University, Turkey. The species of mushrooms, (*Clitocybe geotropa* (Bull. ex DC.) Quél., *Pleurotus cornucopiae* (Paulet) Rolland and *Pleurotus ostreatus* (Jacq.) P. Kumm.), collected from the east of Black Sea Region. Ultrasound-assisted extraction was used for the determination of phenolic compounds present in the mushrooms. 17 different phenolic constituents were measured by reverse phase-high performance liquid chromatography (RP-HPLC) (1). Total phenolic compound and ferric reducing antioxidant power (FRAP) were used as antioxidant capacity determinants (2). All mushrooms showed moderate antioxidant activity contained large amounts of antioxidant compounds. Protocatechuic acid, catechin and cinnamic acid were detected in three mushroom samples at different amount while many phenolic acid and flavonoids such as gallic acid, chlorogenic acid, caffeic acid, epicatechin, rutin, ferulic and benzoic acid and quercetin were not detected in any of them. Total phenolic contents were found to be 2.74-7.07 mg gallic acid /g of methanolic propolis extract by using Folin-Ciocalteu method. Total antioxidant powers were found to be 44.11-193.54  $\mu$ mol Trolox (100) g samples by using FRAP method. Among the three different food samples investigated here, *Clitocybe geotropa* showed higher FRAP values related to their total phenolic compound.

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## ANTIOXIDANT ACTIVITIES OF AQUEOUS EXTRACT FROM ASPHODELUS AESTIVUS BROT. (LILIACEAE)

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KEY WORDS: Antioxidant Activity, *Asphodelus aestivus* Brot., Aqueous Extract, Free Radicals, Radical Scavengers

Free radicals can be generated from metabolic pathways within body tissues, and they can also be introduced from external sources such as drugs, food, UV radiation, smoke and environmental pollution. Free radicals have been implicated in the cause of several diseases such as liver cirrhosis, atherosclerosis, cancer, and diabetes and they play an important role in ageing. Oxidative stress can also contribute to the development of neuro-degenerative disorders, such as Alzheimer's and Parkinson's as well as other diseases. Antioxidants are capable of scavenging free radicals and effectively reducing the extent of oxidation. A great number of plant worldwide show a strong antioxidant activity and a powerful scavenger activity against free radicals. This antioxidant capacity can be explored in food industry by using plants as a source of low-cost antioxidants that can replace synthetic additives. Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties. The prevention of cancer, neurodegenerative and cardiovascular diseases has been associated with the ingestion of fresh fruits, vegetables or plants rich in natural antioxidants (1-2).

*Asphodelus aestivus* Brot., is a common spring-flowering geophyte encountered on the Marmara, Aegean and Mediterranean coasts of Turkey, has been utilized traditionally for culinary and medicinal purposes. The leaves of *A. aestivus* Brot. is commonly consumed cooked as a vegetable dish in Turkey, where it is known as "çiriş otu". In traditional medicine, the tuber and roots of this plant is used against, hemorrhoids, nephritis, burns and wounds (3-4).

In this study, we examined the antioxidant capacity of aqueous extract of leaves from *A. aestivus* Brot. using several test; scavenging capacity against the radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid (ABTS  $\cdot^+$ ), superoxide, hydroxyl, nitric oxide and hydrogen peroxide scavenging ability and inhibition of linoleic acid peroxidation. Those various antioxidant activities were compared to standard synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trolox, ascorbic acid and  $\alpha$ -tocopherol. We also determined proline, anthocyanin, total flavonoid, carotenoid and chlorophyll content. The extract exhibited antioxidant activity in all tests, and the extract could be considered as a source of natural antioxidants.

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## DPPH RADICAL SCAVENGING MAY NOT HAVE BEEN DETERMINED CORRECTLY JUST BASED ON MONITORING ABSORBANCE AT 517 NM

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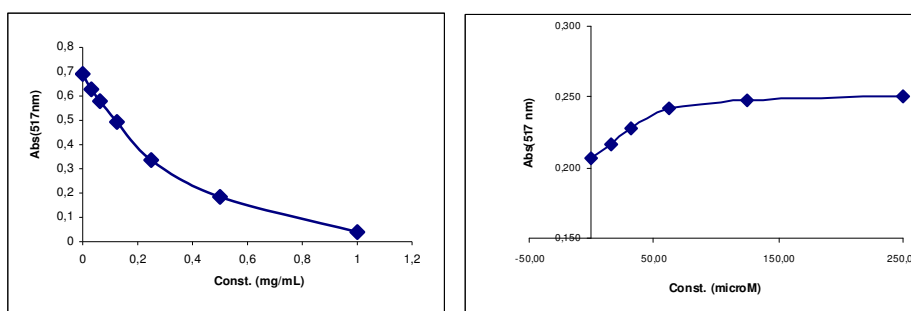
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KEY WORDS: Antioxidant Activity, DPPH Scavenging, IC<sub>50</sub>

Antioxidants, both endogenous and exogenous, are important in human health and in preventing and overcoming many pathological states. Determining accurately the antioxidant activity of individual compounds, either chemically synthesized or isolated from natural sources, and mixtures such as plant extracts have, thus, a very important effect on understanding of their potential as antioxidants as well as on their utilization in many areas of industry, including food, cosmetics, health, etc.

Amongst the many methods of antioxidant activity that have high rates of acceptance in scientific literature, DPPH radical scavenging activity measurements, with its various ways of application including end point and kinetic methods, leads the way, as evidenced from Web of Science hits. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical is commercially available, and the DPPH methods in the literature are relatively the easiest ones among the antioxidant tests available. There are other radical scavenging antioxidant methods, though their applications are more complicated in general.

DPPH method determines the antioxidant (radical scavenging) activity of the sample content based on the absorbance reduction at 517 nm, resulting from the consumption of DPPH radical itself that has absorbance maximum around that wavelength. Its ease of application, however, do not let DPPH methods to be error free or make the results perfectly explainable. Here in this presentation, the focus is on the unexpected rises (Figures) of absorbances at 517 nm especially at higher sample concentrations or even from the beginning in the graphs used to determine IC<sub>50</sub> values, the concentrations at which 50% of DPPH is scavenged.



Normally, the decrease in absorbance could cease and make a plateau, like in the graph on the left, though in some cases with both synthetic compounds and plant extracts, the graphs have either minima and the absorbance starts rising, and even going higher than the original absorbance of DPPH blank, or showing an increasing pace from the beginning, like in the graph on the right. These findings have been encountered in many different studies in our laboratory, and in fact attracted our attention on the species responsible for the increasing absorbances especially at higher sample concentrations. We are in the process of tackling with the issue with the help of HPLC methodologies.

## REDOX COLORIMETRIC SOLID-PHASE EXTRACTION COUPLED WITH FIBER OPTIC REFLECTANCE SPECTROSCOPY FOR DETERMINATION OF ASCORBIC ACID IN PHARMACEUTICAL FORMULATIONS

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KEY WORDS: Ascorbic Acid Sensing, Solid-phase Extraction, 2,2'-dipyridyl, Iron Complex, Reflectance Spectrometry.

Ascorbic acid (AA) is an important vitamin which occurs in different concentrations in a variety of natural samples. It is added to several pharmaceutical products as an essential ingredient, a stabilizer for vitamin B complex, and as an antioxidant [1,2]. Analyses by C-SPE are very quick, require only the simplest equipment, and can be performed almost anywhere. C-SPE is a novel sorption spectrophotometric technique that combines colorimetric chemistry with SPE to determine target analyte concentration by measuring the color change of single-use SPE cartridges, membranes and disks [3,4]. The extracted analyte, which is typically complexed with a colorimetric reagent, is then quantified directly on the solid surface by using a fiber optic reflectance spectrophotometer. A redox colorimetric solid-phase extraction (C-SPE) procedure for the determination of ascorbic acid (AA) in pharmaceutical formulations was proposed. Iron (III)-2,2'-dipyridyl (Fe(III)-Bpy) reagent solution was used as a coloring reagent for AA and the immobilisation of the redox product onto Amberlite XAD-16 resin was achieved. The analyte in the sample reacted with a solid sorbent loaded with the colorimetric reagent (Fe(III)-Bpy) and then quantified directly on the sorbent surface by using a fiber optic reflectance spectrometer (FORS). The amount of AA was reflectometrically determined in a few seconds with a total sample workup and readout time of ~ 10 min using only 10-mL sample volumes. The linear dynamic range of AA was found within the concentration range of 0.352–8.8 mg L<sup>-1</sup> with an LOD of 0.18 mg L<sup>-1</sup>. The C-SPE from different extractions (n = 5) gave a R.S.D. of 2.9 % at 5.28 mg L<sup>-1</sup> AA.

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## TOTAL ANTIOXIDANT CAPACITY ASSAY USING OPTIMIZED FERRICYANIDE / PRUSSIAN BLUE METHOD

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KEYWORDS: Total antioxidant capacity, Fe(III) reducing power, Ferricyanide / Prussian blue assay, Polyphenols, Flavonoids

Antioxidants are health beneficial compounds that protect biological macromolecules from undesired oxidation reactions. Thus, the requirements for simple, versatile and cost-effective methods of antioxidant capacity assay are on the rise. This work aims to optimize the existing ferricyanide/Prussian blue assay of reducing capacity measurement so as to obtain a more reproducible, linear and additive response from antioxidants with respect to concentration. In the conventional ferricyanide assay, the main oxidant, ferricyanide, oxidizes antioxidants and is itself reduced to ferrocyanide which eventually reacts with FeCl<sub>3</sub> to yield Prussian blue showing maximum absorbance at 750 nm. Our modification of this method involves the simultaneous use of ferricyanide and ferric ions as chromogenic oxidants so as to regulate more favourable redox conditions for a greater variety of antioxidants. Prussian blue precipitation was hindered with the addition of sodium dodecyl sulfate (SDS) to the medium, and the optimal pH was adjusted to 1.7 to maintain the redox activity of ferric ion while preventing its hydrolysis. Incubation of the reaction mixture (*i.e.*, Fe(III) + Fe(CN)<sub>6</sub><sup>3-</sup> + antioxidant) at room temperature for 30 min enabled more complete oxidations than observed in the conventional ferricyanide method. The order of trolox-equivalent antioxidant capacities was: quercetin > rosmarinic acid > gallic acid > ferulic acid ≥ catechin > caffeic acid ≥ rutin ≥ ascorbic acid ≈ trolox. The proposed assay proved to be non-responsive to simple sugars and citric acid (which are not true antioxidants) but responsive to biologically important thiols which are not oxidized by other Fe(III)-based antioxidant assays. The proposed (SDS-modified and pH-optimized ferricyanide) method was validated against conventional ferricyanide, CUPRAC [2], FRAP, and Folin methods by comparing the TAC results from medicinal plant extracts and infusions.

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## **EFFECT OF MICROWAVE AND OVEN HEATING ON THE ANTIOXIDANT CONTENT OF ROSEHIP INFUSION (HERBAL TEA)**

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KEY WORDS: Antioxidant Capacity, CUPRAC Method, CERAC Method, Rosehip, thermal processing

Oxidative processes in organisms can produce free radicals which can easily react with different molecules and damage cells. An antioxidant is a molecule capable of slowing or preventing the oxidation of lipids, proteins, and similar molecules. Antioxidants can terminate chain reactions initiated by free radicals *via* removing radical intermediates. The protective effect of fruits and vegetables against chronic diseases is attributed to their phytochemical content and corresponding antioxidant activity. The objective of this study is to evaluate the effect of thermal processing on the antioxidant activity of herbal (such as rosehip) tea by total antioxidant capacity measurement of the raw and thermally processed rosehip. CERAC (Ce(IV)-reducing) and CUPRAC (Cu(II)-reducing) antioxidant capacity methods were used to measure the antioxidant properties of thermally processed products[1,2].

The results showed that unheated and microwave-heated rosehip fruit showed similar antioxidant capacity. On the other hand, oven heating did show a decrease in this property. The samples heat-treated for the longer time of 24 h at 85 °C showed the greatest decrease in ascorbic acid content. Oven heating had much higher adverse effect on the antioxidant capacity of rosehip than microwave treatment.

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## TOTAL ANTIOXIDANT CAPACITY OF COMMERCIAL GRAPE, GRAPE JUICES AND WINE EVALUATED BY SPECTROFLUORIMETRIC METHOD

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KEY WORDS: Grape juice; wine; cerium(IV); cerium(III); antioxidant capacity; phenolic antioxidants

Polyphenolic compounds are found in vegetables, fruits, chocolate, tea, coffee, wine, grape juice at different concentrations. They are believed to be responsible for the healthy effects of moderate wine consumption. These dietary components have antioxidant properties because they can quench free radicals, and therefore, minimize oxidative stress damage. Many published articles show an inverse relationship between the intake of food rich in phenolic compounds (e.g., flavonoids) and the mortality rates due to coronary heart and chronic diseases. *Vitis vinifera* fruits show a high concentration and variety of phenolic compounds. Thus, grape juice and wine are a rich source of flavonoids and other phenolics in the human diet. The present work was performed to determine the antioxidant capacity of commercial black and white grape juices and red and white wine by a novel spectrofluorimetric method. The proposed method is based on the oxidation of antioxidant compounds with Ce(IV) sulphate in acidic medium and measuring the emission of the reduction product: Ce(III) at 360 nm. Since Ce(IV) is a non-fluorescent oxidizing agent, total antioxidant capacity (TAC) assay is possible simply by measuring the fluorescence of the Ce(III) produced. Quercetin and gallic acid were used as flavonoid and phenolic standard compounds, respectively. The procedure was applied to the TAC assay of white and black grapes, grape juices, and wine. Red and white wines made of different types of grapes from different regions of Turkey such as Diyarbakir- Elazig (Bogazkere), Nevsehir-Kapadokya (Emir), Tokat (Narince), Elazig (Kalecik Karasi), and Denizli-Pamukkale (Kalecik Karasi) were analyzed. The extracting solvents were water, 1:1 (v/v) ethanol-water mixture, and pure ethanol for black and white grapes. The highest value of antioxidant capacity was found to be  $3.1 \times 10^{-2}$  mmol gallic acid equivalent / g grape for black grapes in 1:1 (v/v) ethanol-water medium. The antioxidant capacity of black grape juice was found as  $2.15 \times 10^{-3}$  mmol galic acid equivalent / g grape juice in the analysis of black and white grape juices. For the analysis of different wine types, the highest TAC content was found in Bogazkere. Differences in the antioxidant capacities of grape, grape juice, and wines could be attributed to their different phenolic contents as well as to the presence of non-phenolic antioxidants in these samples.

**INVESTIGATION OF TOTAL ANTIOXIDANT CONTENTS BY  
SPECTROPHOTOMETRIC METHODS OF HEAT-PROCESSED DIETARY ONION  
PLANTS IN TURKEY**

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KEY WORDS: Antioxidant capacity assay, cerium(IV), heat processed, onion

Foods, drugs, cosmetics, smoke and other environmental/chemical factors introduced by modern life result in the production of prooxidants and free radicals in the organism. An antioxidant is a molecule capable of slowing or preventing the undesired oxidation of biological macromolecules like lipid, protein, and DNA. The protective effect of fruits and vegetables against chronic diseases is attributed to their phytochemical content and corresponding antioxidant activity. Onions possess a high level of antioxidant activity, which is attributed to the flavonoid content. Flavonoid consumption has been associated with a reduced risk of cancer, heart disease, and diabetes. The chemical diversity of antioxidants makes it difficult to separate and quantify antioxidants from the vegetable matrix. Therefore, it is desirable to measure the total antioxidant capacity (TAC) levels rather to determine individual antioxidant compounds directly from vegetable extracts.

In this study, the largely consumed dietary onions plants (*Allium* species) in Turkey were extracted with water and ethanol, and their TAC were determined with different methods such as CERAC (cerium(IV) reducing AC), CUPRAC (cupric reducing AC) and FOLIN total phenols assays. In general, CERAC and FOLIN methods yielded higher TAC values than CUPRAC, because their redox potentials were higher (*i.e.*, capable of oxidizing a greater variety of substrates). The TAC order of extracts greatly varied with respect to the methods of extraction and measurement. Onion species, namely yellow, red, white, fresh green leaf and fresh green root, were heated in a drying oven (at 50 °C, or 1-4 h) and in a microwave oven (at 90 W, for 1-4 min). The changes of TAC were noted using all three assays. Of the heat-processed onions, the highest CERAC and FOLIN TAC values were obtained for red onions, while the highest CUPRAC value was for green onion leaves. All three assays marked white onion as the lowest TAC content of heat-processed products. Although both heating processes caused a decrease in TAC, this change was not drastic. Moreover, onion fresh green leaves essentially maintained its TAC level after 4 min microwave- or 4 h drying oven- heating. Thus it was concluded that food processing by heat does not cause a drastic antioxidant loss in onions.

**MODIFIED CERIUM(IV)-BASED SPECTROPHOTOMETRIC ANTIOXIDANT CAPACITY MEASUREMENT IN SULFURIC ACID-SODIUM SULFATE MEDIUM WITH SELECTIVITY OVER CITRIC ACID AND SIMPLE SUGARS**

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KEY WORDS: Antioxidant capacity assay, cerium(IV), oxidation potential, sulfuric acid, spectrophotometry

Antioxidants are health beneficial compounds through their combat with reactive oxygen species and free radicals that may eventually give rise to various diseases. It is important to measure the total antioxidant capacity (TAC) of food material and human plasma for food quality estimation and for diagnosis and treatment of diseases, respectively. The authors have recently developed a Ce(IV)-based reducing capacity assay for this purpose. The aim of this work is to modify the existing cerium-based spectrophotometric assay so that Ce(IV) would selectively oxidize antioxidant compounds but not citric acid and reducing sugars. The redox potential of the Ce(IV) oxidant was fine tuned in 0.3 M H<sub>2</sub>SO<sub>4</sub> + 0.7 M Na<sub>2</sub>SO<sub>4</sub> aqueous medium so as to selectively oxidize true antioxidants but not citric acid, simple sugars, and other pharmaceutical ingredients. The trolox equivalent antioxidant capacity (TEAC) values in the order of quercetin > rutin > gallic acid > catechin > caffeic acid ≥ ferulic acid > naringenin ≥ naringin > trolox ≥ ascorbic acid were established with the proposed method, and were found to be compatible to those found with other antioxidant assays. It is noteworthy that naringin and rutin were also hydrolyzed in the acidic medium of the method so as to exert their full antioxidant capacity not measured by other TAC assays. The modified Ce(IV)-based method tolerated citric acid and reducing sugars without affecting the TAC measurement of quercetin. The simultaneous hydrolysis and oxidation of naringin is another advantage over other similar assays. The proposed TAC assay with Ce(IV) is simple, low-cost, rapid, and can be easily applied to modestly equipped conventional laboratories.



**TOTAL ANTIOXIDANT CAPACITY OF POMEGRANATE (PUNICA GRANATUM)  
USING SPECTROPHOTOMETRIC METHOD (CERAC)**

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KEY WORDS: Antioxidant capacity assay, cerium(IV), spectrophotometry, Pomegranate ( *punica granatum*)

The human diet contains a great variety of natural antioxidants, such as fibres, polyphenolic compounds, flavonoids, isoflavones, tocoferols, ascorbic acid etc. Antioxidants may act through the generation of reactive oxygen species (ROS). The generation of ROS is associated with environmental pollution, UV radiation and several normal metabolic processes. The role of ROS in various human diseases is becoming increasingly recognised, such as DNA damage and mutation (and promotion), that may be related to cancer, heart disease and aging. Besides the endogenous defences, the consumption of dietary antioxidants, such as tocoferol, ascorbic acid, carotenoids, phenolic compounds, play a vital role in protecting against ROS. In this study, we have recently reported a simple, flexible, reproducible, and low-cost spectrophotometric method for total antioxidant capacity assay of dietary polyphenols, flavonoids and ascorbic acid in plant extracts. This method, called the ceric ion reducing antioxidant capacity (CERAC) assay, is based on the room temperature - oxidation of antioxidant compounds with Ce(IV) sulfate in dilute sulfuric acid solution, and measuring the absorbance of unreacted Ce(IV) at 320 nm. Pomegranate (*punica granatum*) contains substantial amounts of polyphenols, flavonoids and ascorbic acid. The objective of the present study was to determine antioxidant capacity of pomegranate peel, seed and a byproduct of juice. Antioxidant-rich fractions were extracted from pomegranate (*Punica granatum*) peels and seeds using water, 1:1 (v/v) ethanol-water mixture, and pure ethanol. The extracts were screened for their potential as antioxidants capacity using various methods, such as CERAC, and CUPRAC. All the pomegranate extracts exhibited marked antioxidant capacity, but the pure ethanol extract was the lowest. Among these solvents, 1: 1 (v/v) ethanol-water extract gave the highest CERAC yield for the dried peels, fresh peels, fresh seeds, and juice of pomegranate. Pure EtOH gave the lowest antioxidant yield for all samples. The data also indicated that both the varieties of pomegranate and the parts could significantly influence the antioxidant activity.

*Workshop on Antioxidant Measurement Assay Methods*



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