Chapter 11
Determination of Antioxidant Biomarkers in Biological Fluids

Magdalena Jeszka-Skowron, Tomasz Podgórski, and Beata Czarczyńska-Goślińska

Abstract The presence of various bioactive markers in the human body reflects its health condition, physical endurance, and also the intake of some valuable compounds from food. Analytical methods for their determination are becoming a useful tool for gaining information on the levels of these physiologically essential substances. Antioxidants that can prevent or slow the harmful action of free radicals belong to one of the most significant biomarkers. Reactive oxygen species/reactive nitrogen species produced under oxidative stress act together and cause damage to all cellular biomolecules. Therefore excessive levels of such reactive species pose a threat to human organisms contributing to inflammatory responses. For measurements of oxidative stress or damage indicators, both the reactive species are analyzed, and also different markers considered useful indexes of the level of the phenomenon are determined. Biological samples usually include whole blood derivatives (serum and plasma), urine, and saliva. The fluorescence methods are most commonly applied for the determination of oxygen radicals. At the same time, markers of cellular oxidative damage are most often tested in body fluids and tissue homogenates using enzyme-linked immunoassay kits, high-pressure liquid chromatography, or even gas chromatography, both combined with mass spectrometry. For the assay of protein carbonyls in biological matrices, the derivatization of the carbonyl group, usually with 2,4-dinitrophenylhydrazine, is performed and followed

M. Jeszka-Skowron (✉)
Institute of Chemistry and Electrochemical Chemistry, Poznań University of Technology, Poznań, Poland
e-mail: magdalena.jeszka-skowron@put.poznan.pl

T. Podgórski
Department of Physiology and Biochemistry, Poznań University of Physical Education, Poznań, Poland
e-mail: podgorski@awf.poznan.pl

B. Czarczyńska-Goślińska
Department of Pharmaceutical Technology, Poznan University of Medical Sciences, Poznań, Poland
e-mail: bgoslinska@ump.edu.pl

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by the detection of various types, for example, using anti-DNP antibodies in immunoblotting.

Similarly, glutathione and antioxidant enzymes are determined by immunological assays and chromatographic and spectrophotometric techniques. For phenolic content quantification, different kinds of detection such as an electrochemical coulometric array, on-line connected photodiode-array and electro-array, chemical reaction detection techniques, mass spectrometry, and nuclear magnetic resonance can be used. Modern methods for assessing the levels of vitamins A, C, D, and E as well as total antioxidant capacity assays are also described.

Introduction

Most of the life processes lead directly or indirectly to the formation of oxidizing compounds. Some of these substances are transformed into radical species, molecules that have a deficit or surplus of electrons on the valence shell. It results in the appearance of very reactive agents, which cause damage to cell structures in the body. In the organism, the most sensitive structures to the effects of free radicals are nucleic acids (DNA and RNA as examples of polynucleotides) and low density lipoproteins (LDL) as well as proteins and carbohydrates. The impact of free radicals may contribute to the development of civilization diseases, including cancer or cardiovascular diseases. Also, during intensive physical activity, substances of acidic properties such as pyruvic and lactic acid are secreted into the bloodstream. They lower the pH value of blood, which in turn leads to the release of iron(III) ions (Fe^{3+}) associated with the transferrin and their excessive transformation into iron(II) ions (Fe^{2+}) (Cooper et al. 2002; Yamaji et al. 2004). These ions are involved in the processes responsible for the formation of free radicals in the body, which in the case of athletes, may adversely affect their endurance (Sjödin et al. 1990). It is also assumed that 5–10% of the oxygen involved in the oxidative phosphorylation process (respiratory chain) is converted into free radicals (Raha and Robinson 2000).

Small amounts of free radicals (reactive oxygen species (ROS)/reactive nitrogen species (RNS)) and other reactive species are found in the human body, but when the balance between formation of species and their neutralization is not maintained, the oxidative stress occurs. Oxidative stress in an organism caused by contamination, stress, and others factors can develop and progress various systemic disorders like Parkinson's disease, Alzheimer's disease, myocardial infarction, depression, diabetes, cancers and autism, and ADHD (attention-deficit/hyperactivity disorder) in children, among others (Fig. 11.1). The aging of the organism also results from oxidative stress, additionally connected with impaired antioxidant defense system (Goni and Hernández-Galiot 2019).

Both preventive and chain-breaking antioxidants are mainly divided into enzymatic and non-enzymatic systems, which represent prevalently cellular or non-cellular mechanisms of action (Fig. 11.2) (Ialongo 2017). To the first group belong

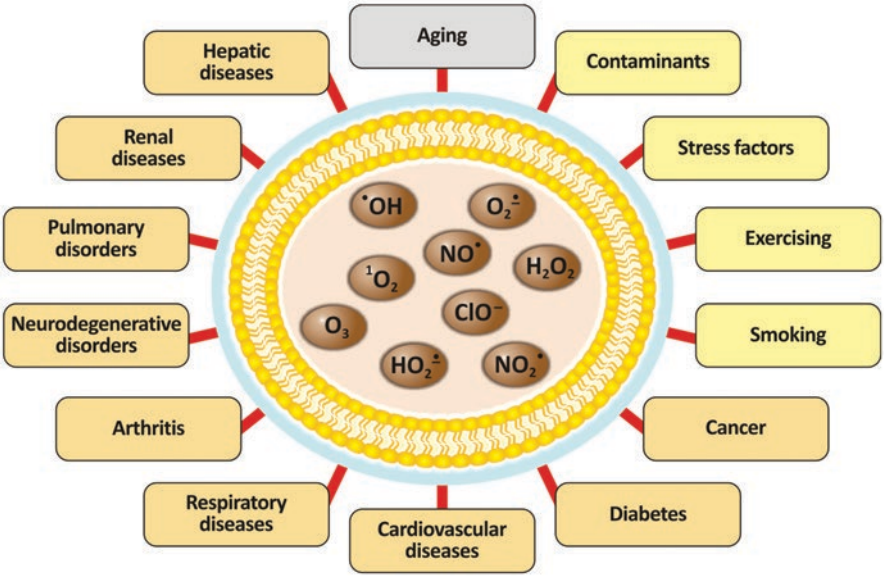

Fig. 11.1 Formation of reactive oxygen and nitrogen species in the cell by the exogenous and endogenous factors

the superoxide dismutases (SOD), the catalases (CAT), the peroxiredoxins (Prx), the thioredoxin reductase (TR), the reduced glutathione peroxidase (GPx), and the oxidized glutathione reductase (GR). The compounds of five major groups are the following:

(a) Endogenous low-molecular-weight antioxidants: free reduced glutathione (GSH), uric acid (UA), α-lipoic acid (ALA), coenzyme Q_{10} (CoQ_{10}), bilirubin, methionine, and cysteine,
(b) Exogenous low-molecular-weight antioxidants: ascorbic acid (vitamin C), the tocopherols (vitamin E), the carotenoids (e.g., β-carotene), and vitamin A,
(c) Proteins: albumin and cysteine-rich proteins,
(d) Polypeptidic antioxidants: thioredoxins, glutaredoxins, and sulfiredoxins,
(e) Metal-binding proteins: ceruloplasmin and metallothioneins are classes of the non-enzymatic system.

Exogenous and endogenous antioxidants interact with each other (e.g., urates →ascorbic acid → tocopherols) and inhibit the oxidative stress and the action of pro-oxidants (Ialongo 2017).

Plant food contains the compounds of high antioxidant activity. Proper intake of phenolics and antioxidant vitamins from food could help the antioxidant defense in the organism. Therefore, the measurement of phenolic compounds intake with dietary assessment (estimation of dietary intakes – questionnaire, diary, and 24-hour recall) and nutritional status are widely used (Jenab et al. 2009; Pico et al. 2019). However, these estimation methods are not sufficient and the specific biomarkers of

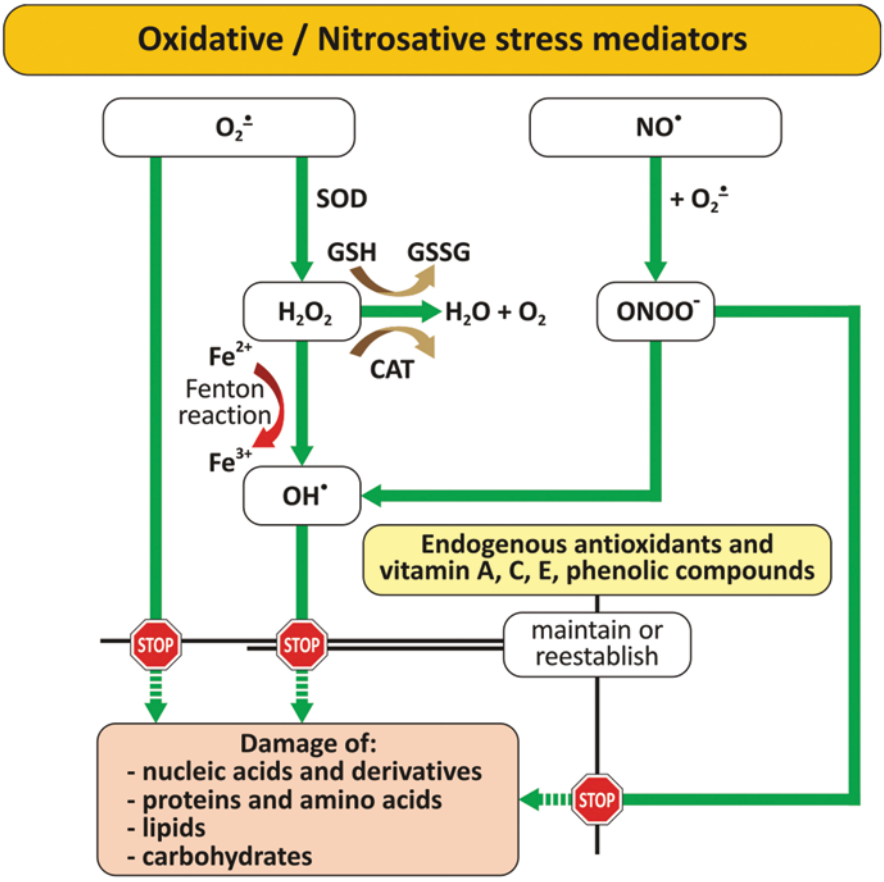

Fig. 11.2 The possible paths of reactive oxygen and nitrogen species in the organism. (SOD superoxide dismutase, GSH glutathione, GSSG glutathione disulfide, and CAT catalase)

oxidative damage/stress, as well as antioxidant status in the biological fluids, are becoming now more and more investigated for the medical and physiological research purposes (Frijhoff et al. 2015; Marrocco et al. 2017).

The World Health Organization (WHO) has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (WHO 2001). In the field of nutrition, the biomarkers are also used to reflect the intake, exposure, or status of some food or nutrient in the body. Also, the determination of the biomarkers in biological fluids in the future epidemiologic studies should be important to show the relations between dietary exposure, food composition, and risk of major chronic disorders such as cancer, cardiovascular diseases, or diabetes (Edmands et al. 2015; Stalmach et al. 2009).

Therefore, the biomarkers of oxidative stress or damage and antioxidant activities can be divided into the four following groups:

1. Reactive oxygen and nitrogen species
2. Markers of cellular structure oxidative damage
   (a) Oxidative damage of cell membrane (lipid fraction)
   - thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA),
   - 4-hydroxynonenal (4-HNE),
   - hexanal,
   - 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$).

   (b) Oxidative damage of nucleic acids and derivatives
   - 8-hydroxy-2′-deoxyguanosine (8-OHdG),
   - allantoin.

   (c) Oxidative damage of proteins and amino acids
   - protein carbonyl groups,
   - *o,o′*-dityrosine, *ortho*-tyrosine, *meta*-tyrosine,
   - homocysteine.
3. Glutathione and antioxidant enzymes.
   - glutathione (GSH),
   - glutathione peroxidase (GPx),
   - glutathione reductase (GR),
   - glutathione S-transferase (GST),
   - superoxide dismutase (SOD),
   - catalase (CAT).
4. Non-enzymatic markers – antioxidants and total antioxidant capacity (TAC) assays
   - phenolic compounds,
   - vitamins C, A, and E and in some cases vitamin D,
   - bilirubin,
   - ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay,
   - DPPH (2,2-diphenyl-1-picrylhydrazyl) assay,
   - FRAP (Ferric Reducing Ability of Plasma) assay,
   - ORAC (Oxygen Radical Absorbance Capacity) assay.

The evaluation of oxidative stress/damage remains a significant challenge for analysts. The biomarkers are mainly tested in the following biological samples: whole blood derivatives (serum and plasma), urine as well as in saliva, among others. Examples of the clinical or animal studies of the determination of antioxidant markers, also in the organism with oxidative stress/damage, are presented in Table 11.1.

Table 11.1 Clinical or animal studies on the determination of antioxidant markers

Experimental system	Tested compounds/ material/food	Antioxidant marker/s	Reference
Clinical studies			
A randomized clinical trial (healthy older adults)	LBP-standardized *L. barbarum* fruit juice	SOD, GPx, and MDA compared to pre-intervention and placebo	Amagase et al. (2009)
Antidepressant drug – patients with oxidative stress (total 132 patients in every five groups)	Vitamin C, α-lipoic acid, Vitamin C + α-lipoic acid	Vitamin C status, TAC (ABTS test), plasma cholinesterase levels compared to placebo	Kadar Ali and Raja (2019)
20 patients with autism spectrum disorder (ASD) (6–22 years old) and 12 controls (5–21 years old)	No special diet or vitamin – normal diet (TAC for ASD group was higher)	Urinary TAC – higher in controls Urinary levels of 8-OHdG and plasma SOD – not changed	Yui et al. (2017)
Chronic hemolytic anemic children (60 patients: 6–18 years old) and 30 healthy children	No special diet or vitamin supplementation	Serum levels of vitamin E and selenium – higher in control patients	Hamdy et al. (2015)
24 healthy men (20–22 years old) before and after 7, 14, 21, and 28 days (two charcoal-broiled beefburgers per day)	Diet with PAH	CAT, GPx, SOD, TBARS in erythrocytes; Plasma vitamin A, C and E	Elhassaneen (2004)
68 patients with acute ischemic stroke and 41 healthy controls	No special diet	Plasma concentrations of α and β-carotene are lower in patients with acute ischemic stroke than in healthy controls and negatively correlated with hs-CRP level and neurologic deficits	Chang et al. (2005)
14 healthy volunteers (11 men and 3 women) (0, 4, and 8 weeks)	Hydroxytyrosol supplementation	Serum TAC, MDA, vitamin A, D, & E – not changed; Serum vitamin C level higher after supplementation	Lopez-Huertas and Fonolla (2017)
25 Behcet's disease patients (13 men and 12 women) and 25 controls (6 weeks)	Vitamin E supplementation	Plasma vitamins A, E and β-carotene CAT, GPx, and MDA HNE-lipid adducts GSSG	Kokcam and Naziroglu (2002)
50 PCOS patients and 14 healthy controls	No special diet	Vitamin C B complex (eight vitamins)	Szczuko et al. (2020)

(continued)

Table 11.1 (continued)

Experimental system	Tested compounds/ material/food	Antioxidant marker/s	Reference
10 younger (18–40 years old) and 10 older (55–70 years old) healthy adults	Nitrate, vitamin C, and in combination	Vitamin C, nitrate, nitrite, c-GMP, tetrahydrobiopterin (BH4) and 3-NT	Ashor et al. (2020)
67 patients with vascular parkinsonism and 39 controls	No special diet	Vitamins A, C, E – not changed	Paraskevas et al. (2003)
Meta-analysis of 12 randomized controlled trials, the effects on mental health, biomarkers of inflammation and oxidative stress in patients with psychiatric disorders were assessed	Probiotic supplementation	CRP, IL-10, and MDA; TAC, NO, GSH – not changed	Amirani et al. (2020)
Meta-analysis of 23 trials (dietary anthocyanins more useful for unhealthy subjects)	Dietary anthocyanins	MDA, ox-LDL, isoprostane, TAC level, SOD, and GPx activities	Fallah et al. (2020)
Meta-analysis of randomized controlled trials patients with psychiatric disorders	Vitamin D supplementation	GSH, TAC, and CRP levels	Jamilian et al. (2019)
7 randomized controlled trials with 317 participants	Garlic supplementation	TAC and MDA	Moosavian et al. (2020)
Meta-analysis of randomized controlled trials in the pathophysiology of cystic fibrosis	No special diet	PC, 8-iso-prostaglandin F2α, MDA, vitamins A and E and β-carotene in plasma or serum	Causer et al. (2020)
Alzheimer's disease and brain aging: a meta-analysis	No special diet	MDA (slight difference); 4-hydroxynonenal, 8-hydroxyguanine, PC level, antioxidant enzymes activities, vitamin C, and α-tocopherol, all not changed	Zabel et al. (2018)
112 patients with chronic schizophrenia (18–65 years old)	Clozapine or risperidone or perphenazine	SOD, GSH – higher in patients with clozapine treatment, other antipsychotic agents not; protein carbonyl and MDA level not changed	Hendouei et al. (2018)

(continued)

Table 11.1 (continued)

Experimental system	Tested compounds/ material/food	Antioxidant marker/s	Reference
13 (8 male and 5 female) patients with ankylosing spondylitis and 13 (8 male and 5 female) controls	No special diet	Vitamins A, C, E, and β-carotene in plasma, GSH and GPx values in erythrocyte -lower in patients and TBARS higher	Naziroglu et al. (2011)
Systemic review of markers in depressive disorders	No special diet, vitamins C, A, or retinol palmitate	8-OHdG, 3-NT, PC, F2-ISO, 4-HNE, 8-iso-prostaglandin, MDA, SOD, CAT, GPx, and vitamins A and C	Barbosa et al. (2020)
10 healthy adults (0 and after 2, 4,5, 6, and 8 hours)	α-tocopherol and tocotrienol fractions	MDA-only FRAP, TEAC-ABTS, SOD, GSH – not changed	Fairus et al. (2019)
83 patients with T2DM 81 controls	No special diet	SOD, CAT, vitamin C, TAC, Serum NO, ROS, and MDA; GSH not changed	Mandal et al. (2019)
30 young patients with acute myocardial infarction	Folic acid and folic acid + vitamin E	Homocysteine, serum TAC [Cu(II) – Cu(I)]	Assanelli et al. (2004)
29 chronic lymphocytic leukemia patients in stage A, 21 patients in stages B and C, and 31 healthy volunteers	No special diet	CAT, MDA; GPx, H_2O_2 – not changed	Zelen et al. (2010)
28 cement workers and 30 volunteers	No special diet	MDA, GSH, vitamin C and E, total bilirubin; SOD and GPx in erythrocytes	Aydin et al. (2004)
116 lactating women during 3 months	No special diet	Serum vitamin A and E – lower during lactation	da Silva et al. (2019)
Animal studies			
Hypercholesterolemic Wistar rats (6 weeks)	High-fat diet with mulberry leaves extract in the diet	Plasma FRAP, total phenolics, and TBARS levels compared to control	Jeszka-Skowron et al. (2017)
Diabetic Wistar rats (4 weeks)	High-fat diet with mulberry leaves extract in the diet	Plasma FRAP, total phenolics, TBARS levels compared to control	Jeszka-Skowron et al. (2014)
Diabetic hindlimb ischemia rats (6 weeks)	Supplementation of simvastatin, vitamins E, and C, simvastatin + vitamin E, and simvastatin + vitamin C	Nitrite in plasma and tissue, MDA in plasma, SOD, CAT, and GSH in tissue	El-Azab et al. (2012)

(continued)

Table 11.1 (continued)

Experimental system	Tested compounds/ material/food	Antioxidant marker/s	Reference
Mice treated with mixture of heavy metals (Pb, Hg, Cd, and Cu) (7 weeks)	Heavy metals and vitamin E Heavy metals Vitamin E	Plasma urea Plasma uric acid Kidney SOD and GSH Testis SOD and GSH	Al-Attar (2011)
Cadmium induced lipid peroxidation in Wistar rats	CoQ_{10}, vitamin E, and olive oil	Tissue SOD, CAT, GPx, GR and GST, vitamins C and E	Ognjanović et al. (2010)
Hypercholesterolemic hamsters (10 weeks)	Vitamin E compared with black tea	ox-LDL LDL-α-Tocopherol LDL-γ- Tocopherol	Nicolosi et al. (1999)
Hepatocellular carcinoma Wistar rats (4 weeks)	Ginger extract	MDA and GSH	Vipin et al. (2017)
Parkinson's disease in rats (3 weeks)	Edaravone-caffeine combination	3-NT and MDA	Bandookwala et al. (2019)
Acute and chronic immobilization stress in swiss albino mice	*Celastrus paniculatus* seed oil (Jyothismati oil)	GPx, GST, and GR in brain tissues	Lekha et al. (2010)

Control group – without antioxidant material/vitamins and/or healthy
3-NT 3-nitrotyrosine, *4-HNE* 4-hydroxynonenal, *8-OHdG* 8-oxo-2′-deoxyguanosine, *CAT* catalase, *c-GMP* cyclic guanosine monophosphate, *CoQ_{10}* coenzyme Q10, *F2-ISO* F2-Isoprostanes, *FRAP* Ferric Reducing Ability of Plasma, *GR* glutathione reductase, *GSH* reduced glutathione, *GPx* glutathione peroxidase, *GSSG* glutathione disulfide, *GST* glutathione *S*-transferase, *IL-10* interleukin 10, *MDA* malondialdehyde, *PAH* polycyclic aromatic hydrocarbons, *PC* protein carbonyls, *T2DM* diabetes type 2, *TAC* total antioxidant capacity, and *TBARS* thiobarbituric acid reactive substances

Qualitative and Quantitative Analysis of Free Radicals and Other Reactive Species

The main free radicals and other reactive species in the organism are the following:

- hydrogen peroxide H_2O_2
- singlet oxygen $^1O_2$
- ozone O_3
- hydroperoxyl radical $HO_2^{\bullet -}$
- superoxide anion radical $O_2^{\bullet -}$
- hydroxyl radical $OH^{\bullet}$
- nitric oxide radical $NO^{\bullet}$
- nitric dioxide radical $NO_2^{\bullet}$
- hypochlorite anion ClO^-

Free radicals have in vivo a short half-life of 10^{-6} s ($O_2^{\bullet}$) to 10^{-9} s ($OH^{\bullet}$). Most free radicals have specific, usually enzymatic neutralization mechanisms. Hydroxyl radical ($OH^{\bullet}$) has the shortest life span and it is the most reactive species among

radicals. In its utilization, the body uses antioxidants derived from food, that is antioxidant vitamins or/and polyphenolic compounds. On the other hand, free radicals and other reactive species are a part of the human body defense system against microbes.

Hydrogen Peroxide (H_2O_2)

Amplex Red reagent, a colorless and non-fluorescent derivative of resorufin, can be oxidized by H_2O_2, in the presence of horseradish peroxidase (HRPO), producing a highly fluorescent product. H_2O_2 production is calculated by measuring the specific fluorescence of oxidized Amplex Red molecule (extinction = 350 nm, emission = 399 nm) according to a modification of the method of Zhou et al. (1997).

The buffered phenol red solution (PRS) contains: 140 mmol L^{-1} NaCl, 10 mmol L^{-1} potassium phosphate buffer, pH 7.0, 5.5 mmol L^{-1} dextrose, 0.28 mmol L^{-1} (0.1 g L^{-1}) phenol red, and 8.5 U mL^{-1} (50 μg mL^{-1}) of HRPO. PRS and HRPO were added to the buffer just before the start of the experiment. To 1 mL of PRS, 10 μL of H_2O_2 solution was added, to result in final concentrations of H_2O_2 from 0.1 to 100 pmol L^{-1}. The tubes were incubated for 5 min at room temperature (25 °C) and brought to pH 12.5 by the addition of 10 μL (1 mol L^{-1}) NaOH. The absorbance was read at 610 nm in a spectrophotometer against a blank sample containing 1 mL PRS, 10 μL H_2O_2, and 10 μL (1 mol L^{-1}) NaOH. There was a linear relationship between absorbance at 610 nm and H_2O_2 concentration in the 1–60 μmol L^{-1} range, corresponding to 1–60 nmol H_2O_2 per mL (Pick and Keisari 1980).

Superoxide ($O_2^{\bullet-}$)

For superoxide anion radical ($O_2^{\bullet-}$) determination of a novel fluorescence method has recently been developed (Han et al. 2017). The researchers have studied CdTe quantum dots (QDs) modified by L-cysteine Schiff base and characterized them with the use of Fourier transform infrared (FT-IR), thermal gravimetric analysis (TGA), ultraviolet-visible (UV-Vis) and fluorescent methods. X-ray powder diffraction pattern (XRD) and transmission electron microscope (TEM) were applied to confirm the particle size of CdTe QDs (3.1 nm) in the presence of β-cysteamine. The fluorescence intensity of CdTe QDs decreased with a modified Schiff base at 305 nm and enhanced at 610 nm with the addition of $O_2^{\bullet-}$. The method was characterized by the high sensitivity fluorescence for detecting the concentration of $O_2^{\bullet-}$ with high selectivity toward $O_2^{\bullet-}$.

Hydroxyl Radical ($OH^{\bullet}$)

The principle of the method (Korotkova et al. 2011) is based on the selective reaction of the hydroxyl radical with non-fluorescent terephthalic acid (TA), resulting in the formation of a fluorescent compound 2-hydroxy-terephthalic acid (TA-OH).

For the initial detection of fluorescence, a reaction solution (10 mL) is prepared. The following solutions are successively added: 2 mL of terephthalate solution (0.01 mol L^{-1}); 30 μL of standard Mohr salt solution (0.002 mol L^{-1}); 0.03 μL of standard hydrogen peroxide solution (0.001 mol L^{-1}); and 2 mL of phosphate buffer (0.025 mol L^{-1} equimolar mixture of Na_2PO_4 and KH_2PO_4 and 2% KCl; pH = 6.86). The remaining volume is made up with distilled water (V = 6.4 mL). The mixture is incubated at room temperature for 6 min.

The concentration of hydroxyl radicals in blood serum is determined by mixing 2 mL of standard TA solution (0.01 mol L^{-1}), 2 mL of phosphate buffer (0.025 mol L^{-1}; pH = 6.86), and 0.1 mL of serum. Distilled water is added to make up to a volume of a total 10 mL. TA-OH fluorescence is read at 327 nm. The intensity of light emission is directly proportional to the concentration of hydroxyl radicals in the biological material. The serum concentration of hydroxyl radicals in healthy people is from 270 to 410 μmol L^{-1}.

Nitric Oxide ($NO^{\bullet}$)

There are at least two methods for the determination of nitric oxide. One of them is the electrochemical analysis in which the electrode reacts by changing the voltage in contact with $NO^{\bullet}$. The difficulty of the method is the short half-life of this free radical in body fluids and tissues (Nagano and Yoshimura 2002). It is possible to bind $NO^{\bullet}$ using iron-dithiocarbamate complexes, whose product (mono-nitrosyl-iron complex) is measured by electron paramagnetic resonance (EPR) (Vanin et al. 2002). Total nitric oxide, as well as nitrate/nitrite, may be determined using enzyme-linked immunosorbent assay (ELISA) kit in serum, plasma, cell culture supernates, and urine. The reference values of these parameters in serum and urine are 13–97 and 369–2684 μmol L^{-1}, respectively.

Markers of Structural Oxidative Damage

Determination of TBARS and MDA

The direct effects of reactive oxygen species are damage to lipid cellular structures and lipid damage in blood. The LDL fraction is very sensitive to those compounds (Steinberg and Chait 1998). The impact of free radicals of aerobic origin on these

structures leads to the formation of a low-molecular compound – malondialdehyde (MDA), the concentration of which is directly proportional to the quantity of ROS in the organism and indirectly determines the body exposure to them (Kanter et al. 1993). Under the influence of peroxidation, the four-carbon segments of higher fatty acids are then disconnected. They are precursors of the produced adducts with thiobarbituric acid (TBARS), such as MDA, 4-HNE (4-hydroxynonenal), hexanal, and others (Janero 1990; Suarez-Pinzon et al. 1996; Zhou et al. 2005). The determination of MDA concentration with the use of the high-pressure liquid chromatography (HPLC) method, similarly to TBARS (determined by the spectrophotometric method), can be considered as an indicator of oxidative stress, that is the degree of body exposure to free radicals.

The concentration of TBARS is also modulated by physical activity. These changes depend on the individual variety of athletes, the intensity and duration of the activity, and the method of regeneration, as well as on blood sampling to post-effort analysis. A higher level of MDA is observed in smokers (Lykkesfeldt et al. 2004), in the conditions diagnosed as asthma (Hanta et al. 2003), cancer (Carbonneau et al. 1991), cirrhosis (Wasowicz et al. 1993), and diabetes (Arshad et al. 1991; Wasowicz et al. 1993), as well as in people on dialysis (Carbonneau et al. 1991; Wasowicz et al. 1993).

TBARS measurement is based on the methodology proposed by Buege and Aust (1978). Plasma (50 μL) was mixed with 50 μL of 0.01% butylated hydroxytoluene, 300 μL of 20% acetic acid, and 300 μL of 0.8% TBA and placed in 2 mL polypropylene screw-cap microcentrifuge tubes. After shaking, the tubes were placed into a water bath for 60 min at a temperature of 100 °C. Subsequently, all samples were brought to room temperature, vigorously shaken, and centrifuged for 10 min at 4000 g at 4 °C. The supernatant (200 μL) was pipetted onto an enzyme-linked immunoassay (ELISA) plate. Measurements were performed at 532 nm with a multimode microplate reader. The standard curve is created from stoichiometrically diluted 1,1,3,3-tetramethoxypropane (TMP) solutions. TBARS concentration is expressed in μmol L^{-1} of plasma. The modification of this method is proposed by Ohkawa et al. (1979).

8-Iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) and 8-Hydroxy-2-deoxyguanosine (8-OHdG)

8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is an isoform of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). It is characterized by higher stability than MDA in blood plasma (Roberts and Morrow 2000). 8-iso-$PGF_{2\alpha}$ is formed as a result of non-enzymatic free radical peroxidation of arachidonic acid and is excreted by the kidneys. Therefore, their concentration in the urine may be much higher than in the blood.

The serum or urine determination of 8-OHdG is an indicator of oxidative DNA damage and a measure of mutagenicity or carcinogenicity. The body exposure to

harmful agents, such as cigarette smoke, smog, mutagenic substances, as well as ultraviolet and ionizing radiation, increases the concentration of 8-OHdG. Usually, the cells have abilities that allow 8-OHdG to be converted back to deoxyguanosine. Sometimes such change does not occur, and 8-OHdG appears in the DNA chain, which can lead to carcinogenesis (Yasui et al. 2014). Furthermore, the presence of 8-OHdG may cause defective gene expression or induce subsequent mutations alone (Valavanidis et al. 2013).

Currently, both substances are most often determined in body fluids (blood and urine) and tissue homogenates using commercially available ELISA kits (Table 11.2). However, greater detection accuracy is possible due to liquid (LC) and gas chromatography (GC), especially with the use of mass spectrometers (Milne et al. 2007).

Allantoin

Allantoin, a heterocyclic compound formed in most mammals, except for the higher primates and man, by the oxidation of uric acid and by the catabolism of purine is also taken into account in the characterization of free radicals (Young et al. 1944). In the physiological conditions, this compound is not determined in blood because in the human body the enzyme, which converts the uric acid to allantoin, is not produced (Hellsten et al. 1997). Only the oxidation of uric acid by free radicals leads to the appearance of this substance in the organism. Therefore, allantoin is a marker of the intensity of free radical reactions in the body. It is sometimes considered to be a more sensitive indicator of these reactions because due to its production under both aerobic and anaerobic conditions, the concentration of this compound in the blood increases faster than that of TBARS (Mikami et al. 2000).

It has been revealed that allantoin possesses the features of a substance accelerating wound healing (Robinson 1935), regeneration of skin (Ho et al. 2006), and nerves (Loots et al. 1979). Moreover, it has anti-inflammatory properties and may alleviate the negative impact of pro-inflammatory factors, which are cytokines, secreted, among others, by leukocytes during physical activity.

Allantoin is determined in 250 μL of deproteinized serum, following the methodology developed by Grootveld and Halliwell (1987). After that, the samples are centrifuged (4000 g, 10 min, 4 °C), 250 μL of the supernatant is transferred to a

Table 11.2 Biochemical parameters of lipids and DNA degradation by ROS and other reactive species

Biochemical parameter	Reference values	References
8-iso-prostaglandin $F_{2\alpha}$ [pg mL^{-1}]	31.8±5.5	Bielecki et al. (2012)
8-hydroxy-2′-deoxyguanosine [ng mL^{-1}]	0.3–5.9 0.4–8.0	El-Zein et al. (2010) and Hamurcu et al. (2010) Kono et al. (2006) and Pan et al. (2007)

glass test tube, and 250 μL of 0.12 M NaOH is added. Next, the samples are boiled for 20 min in a water bath at 100 °C and mixed with 250 μL of 1 M HCl and 50 μL of 2,4-dinitrophenol and warmed up again for 5 min to the same temperature. The mobile phase consists of trisodium citrate and sodium acetate (pH 4.75). The measurements are performed using HPLC with UV detection at 360 nm. The standard curve is created from subsequent dilutions of the allantoin solution. Allantoin concentration is expressed in mmol L^{-1} of serum.

Oxidative Damage of Proteins and Amino Acids

Protein oxidation is both a part of cellular regulatory mechanisms and a consequence of damage to tissues as proteins are major targets for radicals, two-electron oxidants, and glycation reactions. Protein oxidation is believed to play a crucial role in the pathophysiology of diseases (atherosclerosis, neurodegenerative disorders, diabetes, pulmonary fibrosis, end-stage renal disease) and the aging process (Vivekanandan-Giri et al. 2011; Weber et al. 2015).

Oxidative damage to proteins may lead to the modification of sulfur-containing aromatic and aliphatic amino acids. Various reactive oxygen species present in blood, tissues, and cells generate protein carbonyl groups, which are used as a marker of global protein oxidation. Protein carbonyls are an irreversible and relatively stable form of protein modification. As a result of the impact of different oxidants on amino acids, both protein-bound and released carbonyl groups are produced.

The standard methods for determining protein carbonyls in biological samples (plasma, cellular extracts, erythrocytes, or isolated proteins) are based on the derivatization of the carbonyl group, most often with 2,4-dinitrophenylhydrazine (2,4-DNPH) to obtain the stable 2,4-dinitrophenylhydrazone (2,4-DNP) of an absorption maximum at 370 nm (Purdel et al. 2014). Derivatization is usually followed by a detection either by spectrophotometric methods, by an HPLC-based technique or using anti-DNP antibodies in immunoblotting or ELISA (Weber et al. 2015).

Levine et al. (1990) described methods relying on the modification of the carbonyl group (1) to alcohol with tritiated borohydride; (2) with 2,4-DNPH to form the 2,4-DNP; (3) with fluorescein thiosemicarbazide to form the thiosemicarbazone; and (4) with fluorescein amine to form a Schiff base followed by reduction to the secondary amine with cyanoborohydride. To avoid contamination of nucleic acids, they should be precipitated with streptomycin sulfate. If utilizing the borotritide or 2,4-DNPH methods, the protein should be concentrated by precipitation with trichloroacetic acid. According to Levine, the most sensitive procedure for carbonyl determination in purified proteins, homopolymers, extracts of cells and tissues is the method with tritiated borohydride, suitable for the assay of total protein content not exceeding 1 mg. This method requires less sample than that with

2,4-DNPH (Levine et al. 1990). However, nowadays, the most widespread procedure for the analysis of protein carbonyls is their derivatization with 2,4-DNPH, followed by various techniques of detection (Weber et al. 2015).

One of them can be the Western blot immunoassay. Shacter and co-workers (1994) evaluated the susceptibility of the major plasma proteins to oxidative modification based on immunoblotting. As protein oxidation leads to the formation of carbonyl groups (aldehydes and ketones) on some amino acids, these were derivatized with 2,4-DNPH, separated by sodium dodecyl sulfate-gel electrophoresis, and analyzed for carbonyl content by immunoassay with anti-DNP antibodies. This approach is very useful for measuring protein carbonyls as markers of oxidative modification in biological samples. The scientists concluded that 1 pmol of protein-associated carbonyls could be detected, and plasma fibrinogen turned out to be more susceptible to oxidative modification compared to the other major plasma proteins, albumin, immunoglobulins, and transferrin (Shacter et al. 1994).

Buss and co-workers (1997) carried out an ELISA and a colorimetric analysis for measuring protein carbonyls in plasma and lung aspirate samples to assess the oxidative injury. Protein samples were subjected to reaction with DNP and adsorbed to wells of an ELISA plate. After probing with a commercial biotinylated anti-DNP antibody, the reaction with streptavidin-linked horseradish peroxidase was performed to obtain a product for quantification. In the colorimetric assay, a proper amount of protein in phosphate buffer saline was subjected to reaction with DNP in 2 mol L^{-1} hydrochloric acid for 45 min with occasional mixing. The trichloroacid precipitates were washed three times with the solution ethanol/ethyl acetate (1:1). Pellets were sonicated, finally dissolved in 6 mol L^{-1} guanidine hydrochloride, 0.5 mol L^{-1} potassium phosphate, and the absorbance at 375 nm resulting from the presence of carbonyl groups was colorimetrically assessed. A blank with the protein was subjected to reaction with HCl without DNP and its absorbance was subtracted after adjusting for the protein loss that occurred with this method. Protein concentrations were determined for final samples (for albumin) by diluting and measuring absorbance at 280 nm or, for biological samples, by the simple colorimetric assay called BioRad (Buss et al. 1997).

The scientists compared immunohistochemistry and dihydroethidium fluorescence with mass spectroscopy in studies on amino acid oxidation markers, which served as molecular fingerprints of specific oxidative pathways. The firstly mentioned technique, despite its high sensitivity, appeared nonspecific and semiquantitative. In contrast, mass spectrometry (MS), in combination with GC, turned out to be a powerful, highly sensitive, and specific method that should be recommended for the determination of specific markers. MS detection enables identification of a target biomolecule based on its unique fragmentation pattern. The quantification is performed by adding a stable, isotopically labeled internal standard, identical to the target analyte except for the heavy isotope, which is introduced to the mixture. Due to specific ionization processes, such as electron capture negative-ion chemical ionization, even low femtomole levels of biomarkers can be determined. Additionally, the researchers confirmed the hypothesis that unique oxidants are generated in regions vulnerable to diabetic damage, and the role of antioxidant therapies seems

to be of great importance in preventing microvascular and macrovascular disease in diabetic patients (Vivekanadan-Giri et al. 2008).

Agarwal and Sohal (1995) separated protein fractions based on their molecular weight by gel filtration, and for determination of carbonyl proteins, they developed the HPLC method with diode array detection (at 370 nm). They noted that the gel filtration HPLC is more sensitive for the quantitation of oxidized purified proteins than electrophoresis but proteins of similar molecular weights cannot be separated entirely.

The Spanish scientists presented a very interesting approach with fluorescent hydrazides to the detection of carbonylated proteins (Tamarit et al. 2012). They carried out derivatization with fluorescent Bodipy-hydrazide of low molecular weight and no net charge. This enabled by two-dimensional gel electrophoresis. Bodipy means boron dipyrromethene and its analogs, whereas fluorescent Bodipy-hydrazide is a green-fluorescent dye used for the detection of carbonyl groups. Their reaction results in forming a Schiff base that can be later stabilized by reduction. Derivatization with Bodipy-hydrazide allowed easy matching of the spots of target proteins and those obtained by general fluorescent protein staining methods. Due to the cyanine hydrazide derivatization (Cy3-Hz and Cy5-Hz) analysis of two samples in the same gel was possible.

Measurement of Tyrosine Derivatives

Reactive oxygen species cause damage to proteins and amino acids. One of the structures particularly sensitive to the effects of free radicals are cross-linked two tyrosine molecules. They are present in many proteins (elastin and collagen) of vertebrates, including humans, and are sensitive to hydrogen peroxide (H_2O_2), resulting in proteolysis. Heinecke (2002) has shown that the product of the oxidative conversion of dityrosine to *o,o′*-dityrosine is 100 times higher in LDL fraction in patients with atherosclerotic symptoms compared to healthy people. Also, the hydroxyl radical increases the concentration of the modified forms of tyrosine, namely *ortho*-tyrosine and *meta*-tyrosine. These substances appear to be useful markers of protein and amino acid damage by free radicals. The concentration of these compounds is determined using a GC-MS method with isotope dilution. Research material may be: LDL isolated from blood serum (Leeuwenburgh et al. 1997) and urine (Bhattacharjee et al. 2001). Orhan et al. (2005) showed that the isotope dilution reversed-phase liquid chromatography-atmospheric pressure chemical ionization-ion-trap-tandem mass spectrometry (LC-APCI-MS/MS) with a triple quadrupole instrument is 2.5 times more sensitive than the ion-trap instrument. The concentration of *o,o′*-dityrosine in the urine of smokers is on average 0.08 ± 0.01 μmol L^{-1} or calculated as 10.1 ± 0.4 μmol mL^{-1} of creatinine.

Pennathur et al. (2001) identified amino acid oxidation markers and utilized highly sensitive and specific GC-MS in *Cynomolgus* monkeys. The animals were hyperglycemic due to streptozotocin (STZ) induced diabetes. Samples from seven

controls and eight diabetic monkeys were analyzed. *Ortho*-tyrosine, *meta*-tyrosine, and *o,o′*-dityrosine levels were higher in aortic proteins from diabetic monkeys than in those from control animals, whereas 3-nitrotyrosine levels remained unchanged. These proportions of oxidized amino acids can lead to the conclusion that a hydroxyl radical-like oxidant promotes aortic damage in hyperglycemic animals. The scientists also searched for the relationship between the level of glycemic control (measured as serum glycated hemoglobin) and levels of amino acid oxidation products in aortic tissue in control and diabetic *Cynomolgus* monkeys. They found a strong correlation between levels of both *ortho*-tyrosine and *meta*-tyrosine and glycated hemoglobin.

Homocysteine

Homocysteine is a product of methionine demethylation. The increased concentration of this amino acid is directly related to cardiovascular diseases (lifestyle diseases).

The homocysteine concentration is determined in serum or plasma using commercially available kits. About 80% of circulating homocysteine in the blood is bound to proteins. To determine the total concentration, the disulfide bridges must be reduced to release free homocysteine. Due to a specific enzyme cutting the homocysteine molecule, an intermediate product is created, which in combination with the appropriate reagent, leads to the formation of a stable fluorophore emitting far-red spectrum light (extinction = 625 nm, emission = 708 nm). The test is not influenced by the physiological concentrations of other biological thiols (such as glutathione, cysteine, and methionine) and it can be quickly adjusted (Kar et al. 2019). Also, the LC-MS method can be used to determine the homocysteine level in blood samples, even in 30 μL of a sample (Oosterink et al. 2015).

The concentration of homocysteine is usually higher in men than in women samples. Also, elevated levels were observed for the elderly people and those with a deficiency of B vitamins (B_6, B_9, B_{12}) (Oosterink et al. 2015). Reference values for this compound: 5–15 μmol L^{-1} (Maron and Loscalzo 2009).

Glutathione and Antioxidant Enzymes

Glutathione Analysis

Glutathione is a tripeptide built of three amino acids: glutamic acid, cysteine, and glycine. A unique role is played by cysteine, containing a thiol group (-SH), which has strong reducing properties. Therefore, glutathione has a high antioxidant potential, acting as a so-called free radical scavenger. This compound and antioxidant

enzymes arise in significant amounts during physical exercise, especially along with endurance characteristics.

Glutathione removes from erythrocytes hydrogen peroxide, which is a natural product formed during its metabolism. H_2O_2 significantly reduces the survival of red blood cells (erythrocytes) from 90 to 120 days and increases the rate of oxidation of hemoglobin to methemoglobin (metHgb). MetHgb loses the ability for oxygen transport, which reduces the physical performance of the athlete. NADPH (nicotinamide adenine dinucleotide phosphate – reduced form), which is formed in the pentose phosphate pathway, causes a reduction of glutathione, that is its re-activation (Fig. 11.3). Glutathione is also able to neutralize singlet oxygen ($^1O_2$) and other electrophile molecules (Lafleur et al. 1994).

Both glutathione and antioxidant enzymes are currently determined by ELISA. Most often, antioxidant enzyme activity is determined in erythrocyte hemolysate. The method is based on the isolation of erythrocytes by washing the red blood cells three times with saline in a 1:1 ratio. After each addition of physiological saline and thorough (gently) mixing, the sample is centrifuged, and the supernatant removed. Red blood cells are then frozen to induce hemolysis. GSH concentration is calculated per 1 g of hemoglobin. The concentration of hemoglobin is measured using a hematological analyzer.

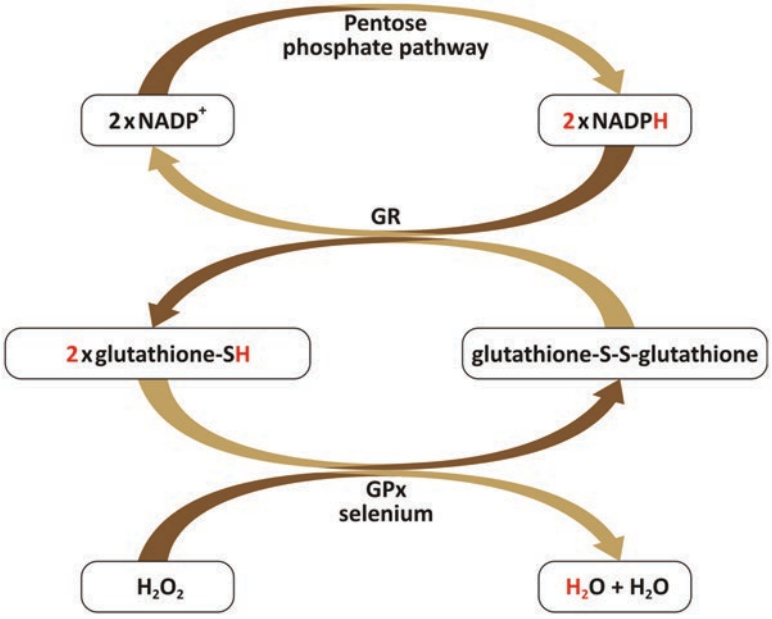

Fig. 11.3 Neutralization of hydrogen peroxide (H_2O_2) to the neutral molecule of water (H_2O) by reduced glutathione (glutathione-SH), which has previously been reduced by NADPH formed in the pentose phosphate pathway. The hydrogen atom, disconnected from NADPH, reduces glutathione, which in turn transfers it to H_2O_2 and transforms it into two water-neutral molecules

The concentration of the reduced and oxidized forms of glutathione in blood hemolysate presented in the literature:

- GSH – 8342 ± 769 nmol per gram Hb; glutathione disulfide (GSSG) – 23.3 ± 5.06 nmol per gram Hb (Rossi et al. 2002),
- GSH – 8460 ± 1750 nmol per gram Hb; GSSG – 13.2 ± 4.0 nmol per gram Hb (Khazim et al. 2013).

Also, HPLC and spectrophotometric techniques are used to determine glutathione and glutathione disulfide concentrations in blood and other tissues. Giustarini et al. (2013) have compared these two methods. The GSH recycling assay is a specific method with minimal or without interference from other thiols and disulfides for the determination of total GSH, and the limit of detection is 0.1 μmol L^{-1} in most tissues. GSH measurement was carried out using HPLC with UV detection at 265 nm in supernatants obtained from NEM-treated (N-ethylmaleimide) blood after acidification. For the preparation of human or animal blood samples, tripotassium ethylenediaminetetraacetic (EDTA) acid is needed and 100 μL of NEM per mL of blood.

Antioxidant enzymes neutralize two toxic reactive oxygen species, that is the superoxide radical and hydrogen peroxide to a water molecule.

Superoxide dismutase in humans (SOD; E.C. 1.15.1.1) is an enzyme that has a coenzyme in the form of a copper and zinc metal atom (SOD-1 and SOD-3) or manganese (SOD-2). This enzyme is involved in the utilization of the superoxide radical ($O_2^{\bullet -}$). In this way, it protects intracellular structures from oxidation and the generation of other free radicals, for example, nitric oxide ($NO^{\bullet}$). Liver cells show the highest SOD activity.

$$Cu^{2+} - SOD + O_2^- \rightarrow Cu^+ - SOD + O_2$$

$$Cu^+ - SOD + O_2^- + 2H^+ \rightarrow Cu^{2+} - SOD + H_2O_2$$

SOD triggers the production of hydrogen peroxide, which in turn is deactivated by the catalase enzyme (CAT; EC 1.11.1.6). It is an enzyme that contains iron (Fe^{3+}) in its structure. Its high activity is observed in the cytosol, peroxisomes, and erythrocytes.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Hydrogen peroxide (H_2O_2) is neutralized by another enzyme – glutathione peroxidase (GPx; EC 1.11.1.9). Also, it can reduce lipid radicals to alcohols. GPx has eight isoenzymes that contain a selenium atom (Fig. 11.4).

$$2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$$

Currently, antioxidant enzymes are determined using commercial 96-well plate kits basing on the ELISA method. As mentioned earlier, erythrocyte hemolysate is most often analyzed, although it is possible to determine the analytes in blood serum

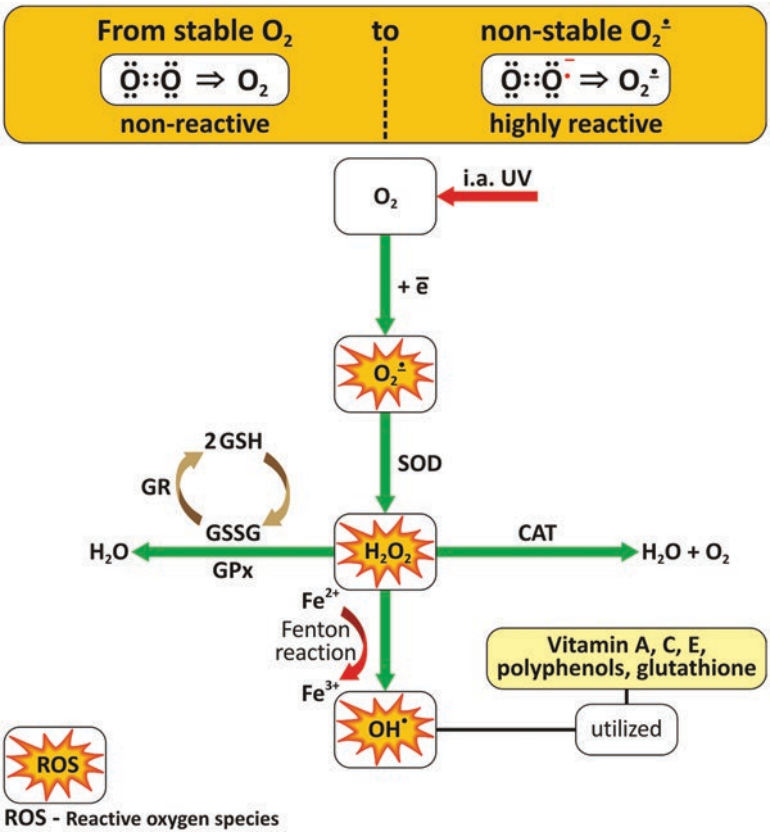

Fig. 11.4 The relationship between antioxidant enzymes in the utilization of free radicals. (SOD superoxide dismutase, GSH glutathione, GSSG glutathione disulfide, CAT catalase, GPx glutathione peroxidase, and GR reduced glutathione)

Table 11.3 The activity of antioxidant enzymes in human erythrocytes and plasma

	Enzyme activity	
Enzyme	Erythrocytes (U 10^{10} mL^{-1})	Plasma (U L^{-1})
Glutathione peroxidase	7.8–10.6	0.4
Glutathione reductase	2.7–3.7	0.03
Superoxide dismutase	550–800	5–20
Catalase	3800–5400	46–52

or tissue biopsies, for example, liver. Weydert and Cullen (2010) also demonstrated the method for determining the above mentioned enzymes in tissues and cell cultures using active gels. It takes 24–48 hours to carry out this type of determination. The activity of antioxidant enzymes in human erythrocytes and plasma can be found in Table 11.3.

Non-enzymatic Markers of Antioxidant Capacity Assessment and Antioxidants

Total Phenolic Compounds and Determination of Phenolic Compounds and Antioxidant Vitamins with the Use of Modern Techniques

Phenolic compounds (mainly flavonoids and phenolic acids) are the group of over 8000 plant origin substances. They also belong to natural plant dyes, the role of which is to protect against UV radiation, insects, and parasites. Their concentration in blood is an effect of proper nutrition and dietary supply of these compounds (Wiseman 1999). It has been revealed that a higher concentration of phenolic compounds in the blood protects the organism from civilization diseases, especially atherosclerosis and some cancers. Particularly symptomatic are the results of studies carried out in France, where the habit of drinking red wine for meals, despite a high-fat diet, leads to a lower number of deaths caused by cardiovascular disorders compared to other populations of European countries. Therefore, the phenomenon is named the French Paradox (Ferrières 2004).

Phenolic compounds possess potent antioxidant properties, often of greater strength than vitamins C, A, or E. The proper levels of these substances in human organisms indirectly protect antioxidant vitamins from oxidation. Phenolics, especially from fruit and vegetables, turned out to modulate the cellular concentration of natural antioxidants, as well as glutathione synthesized in the organism through gene regulation (Moskaug et al. 2005).

During physical exercises, there may be an increase in the production of free radicals, particularly of aerobic origin (ROS), which results in oxidative stress. Polyphenols are therefore considered to be one of the essential exogenous substances that protect the body from the negative effects of this condition in sports practice. It is known from the literature that high concentrations of antioxidants in the body prevent damage and premature muscle fatigue. However, a clear interpretation of this issue is contrary to research results, which indicate an increase in the total antioxidant potential during a long-term exercise – marathon (Liu et al. 1999) or half-marathon (Child et al. 1998), while moderate exercises have not led to its changes so far (Karolkiewicz et al. 2002).

For these compounds, various methods were used (for the determination of total phenolics – spectrophotometric method, and high-performance liquid chromatography (HPLC), or LC-MS/MS where the selectivity was needed). In the routine work, the method should be easy, repeatable, and when it is possible inexpensive. Phenolics are mainly detected in UV/VIS and UV-fluorescence regions. Besides, electrochemical coulometric array detection, on-line connected photodiode-array, and electroarray detection, chemical reaction detection techniques, mass spectrometric, and nuclear magnetic resonance (NMR) detection are used. It is known that phenol compounds with the intrinsic existence of conjugated double and aromatic bonds

exhibit a higher or lower absorption in the UV or UV/VIS region. For benzoic acids, the maximum is in the 200–290 nm range, excluding gentisic acid, which extends the absorbance to 355 nm. The cinnamate carbon framework derivatives, because of the additional conjugation, show a wide-ranging absorbance band from 270 to 360 nm. In the case of flavonoid aglycones, they possess at least one aromatic ring and, consequently, efficiently absorb UV light. There are two maxima, and the first is found in the 240–285 nm range due to the A-ring. The second maximum in the range: 300–550 nm is attributed to the substitution pattern and conjugation of the C-ring (Mabry et al. 1970).

Determination of phenolic compounds (also named as total polyphenols) in plasma (after preparation) is based on a method developed by Singleton and Rossi (1965), which uses the ability to oxidize phenolic groups by the Folin-Ciocalteu reagent. The resulting substances are complexed to form a blue compound. The color of the solution is measured using a spectrophotometer or a multimode microplate reader at $\lambda = 765$ nm. The standard curve is created from standard solutions of gallic acid. Total polyphenols concentration is expressed as gallic acid equivalent in g L^{-1} of plasma.

Some substances present in biological material (blood, urine, and saliva) may affect the polyphenol concentration obtained by the Folin-Ciocalteu method. To eliminate protein interference, mainly aromatic amino acids (tyrosine and tryptophan) (Sánchez-Rangel et al. 2013), deproteinization of the sample should be performed by protein precipitation, for example, with methanol. For this purpose, the serum is mixed with 80% methanol (1:1) and then centrifuged for 5 min at 14,000 g to separate proteins from a residual fluid (Lee et al. 2017). Also, the effect of uric acid can be eliminated from the sample, especially in people consuming products with a high content of polyphenols, for example, apple juice. Uric acid reacts with the Folin-Ciocalteu reagent, overstating the result of polyphenols content in the blood (Godycki-Cwirko et al. 2010).

When determining the concentration of polyphenols in urine, compounds dissolved in water should be removed (Singleton and Rossi 1965). For this purpose, the urine is acidified and then applied to the active cartridge for the extraction of biological material. The resulting filtrate containing polyphenols is eluted with methanol and formic acid solution (Roura et al. 2006).

The extraction and isolation methods of phenolics start with the protein precipitation with acetonitrile (Miniati 2007). In the chlorogenic acid analysis, liquid-liquid extraction (LLE) for preparing the blood and/or urine samples was also used (Han et al. 2020; Zhang et al. 2010). An internal standard (puerarin or tinidazole) was added with hydrochloric acid, methanol, and ethyl acetate. After vortexing and centrifugation, the clear supernatant was separated and evaporated to dryness under a gentle stream of nitrogen at room temperature. Then it was reconstituted with 100 μL of 0.5% formic acid in 50% methanol/water (with centrifugation 12,000 rpm in 5 min). Finally, an aliquot of 2 μL was injected into the UPLC-MS/MS system.

Another novel method was developed to analyze the markers of green coffee bean extract consumption (rich in chlorogenic acids) in the urine of healthy volunteers (Peron et al. 2018). Urine samples were centrifuged at 13,000 rpm for 10 min

and then 1 μL was directly injected into the UPLC-QTOF with C18 stationary phase. For better separation of the polar and smaller molecules, the researchers used LC-ESI-MS and C-3 stationary phase. They monitored the metabolites related to polyphenol administration such as hippuric acid, benzoic acid derivatives, dihydroferulic and dihydrosinapic acid sulfate, as well as carnitine derivatives and dicarboxylic acids. Stalmach et al. (2009) revealed that the usage of LC-PDA-MS^n to identify and quantify total 21 metabolites circulating in the bloodstream and being excreted in urine after the acute consumption of coffee by healthy human subjects was efficient to show the bioavailability and metabolic fate of dietary phenolics and their nutritional value. Other markers of coffee and other product consumption with the techniques of analysis can be found in Table 11.4.

Yang et al. (2019) proposed a method for the determination of 22 urinary polyphenol biomarkers with the use of LC-ESI-MS/MS. The urine samples were extracted and purified with Plexa PCX (polymeric cation-exchange resin) solid extraction cartridges, and 10 μL of the reconstituted solution was injected into LC with the QTRAP tandem mass spectrometer (triple quadrupole linear ion trap spectrometer). In another study of Yang et al. (2013), plasma with internal standard (ginsenoside Rc) and acetic acid was vortexed and centrifuged at 12,000 g for 5 min at 4 °C, and then the solution was applied to the SPE (solid phase extraction) cartridge (SEP-PAK C_{18}, 100 mg). The 5 μL of residues after methanol eluate evaporation was injected into LC-MS. The chlorogenic acid, kaempferol-7-*O*-β-d-glucoside, and ilexgenin A were determined after oral administration of *Ilex hainanensis* leaves extract in normal and non-alcoholic fatty liver disease (NAFLD) rats.

It was also revealed that after oral administration of the *Flos Lonicera Japonica* extract in rats, 68 CGA (chlorogenic acids) metabolites were found (Wang et al. 2018). UHPLC-LTQ-Orbitrap (LTQ – linear trap quadrupole) MS was used to identify the major-to-trace in vivo metabolites of CGAs in the urine and plasma samples of Sprague–Dawley rats. This method showed the profile of CGA metabolites, which can be useful in understanding the in vivo metabolic fate, effective forms, both pharmacological and toxic actions of CGA.

For the biological samples, the proper quality control has been used (Yang et al. 2013; Yang et al. 2019). Apart from linearity, plasma calibration curves, precision and accuracy, recoveries, the stability of samples have also been investigated at different concentrations.

Vitamin C Analysis

Vitamin C (L-ascorbic acid) is a water-soluble vitamin present mainly in fruit and vegetables. This vitamin is not absorbed in the human organism, and therefore it should be ingested with the diet. As an antioxidant, it protects neurons from oxidative damage also by restoring the reduced form of vitamin E (Grunewald 1993; Niki 1991). The ascorbic acid is essential for collagen formation, necessary as a cofactor in the biosynthesis of catecholamines, amino acids, and peptide hormones, in the

Table 11.4 Determination of selected markers of chlorogenic acids and other phenolic acids from food consumption in healthy adults plasma and/or urine samples

Analytes (after food consumption)	Samples of healthy adults (amount)	Sample pretreatment and extraction	Stationary phase and mobile phase	Technique	Reference
Caffeic, ferulic, and chlorogenic acids (after 100 g prune consumption)	Human plasma, urine (3)	Incubated in sulfatase and glucuronidase; acidified with H_3PO_4, extracted with methylene chloride, vortexed, centrifuged. (urine) mixed with sodium acetate buffer (pH 5.5) and $CaCl_2$ solution; incubated in a manner similar to that of the plasma samples; acidified with 6 mol L^{-1} HCl; extracted with ethyl acetate, vortexed, and centrifuged	Hamilton PRP-1 column/ A: 1% acetic acid in water; B: ACN; gradient: 20% B, 4 min; 50% B, 6 min, 100% B, 15 min	LC-ESI-MS	Cremin et al. (2001)
3-,4-, and 5-CQA, 3-diCQA, and caffeic, ferulic, isoferulic, and *p*-coumaric acids in plasma; 4-CQA, 5-CQA, and sinapic, *p*-hydroxybenzoic, gallic, vanillic, dihydrocaffeic, caffeic, ferulic, isoferulic, and *p*-coumaric acids in urine (after hydroalcoholic decaffeinated green coffee extract from *C. canephora* cv. Pierre beans 170 mg of CGA)	Human plasma, urine (10)	Plasma and urine aliquots + HCl	Magic C30 HPLC column (5 μm, 150 × 20 mm, 100 Å)/aqueous solution of 0.3% formic acid, and methanol	HPLC–DAD (confirmation by LC-MS)	Farah et al. (2008)

Analytes (after food consumption)	Samples of healthy adults (amount)	Sample pretreatment and extraction	Stationary phase and mobile phase	Technique	Reference
Chlorogenic acids and metabolites Six CGA (3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) and 16 other phenolic compounds: caffeic, vanillic, ferulic, isoferulic, *p*-coumaric, gallic, 4-hydroxybenzoic, dihydrocaffeic, syringic, sinapic, 2,4-dihydroxybenzoic, hippuric, 3,4-dihydroxyphenylacetic, 3-(4′-hydroxyphenyl)propionic, trans-3-hydroxycinnamic, and benzoic acids (after consumption of water, coffee, or coffee with milk)	Human urine (5)	Helix pomatia (Sigma-Aldrich) extract containing β-glucuronidase and sulfatase activities for deconjugation of glucuronic acid conjugates and sulfated forms	C18 Kromasil guard column and column/a gradient with methanol and 0.3% formic acid	HPLC–DAD (confirmation by LC-MS)	Duarte and Farah (2011)

(continued)

Table 11.4 (continued)

Analytes (after food consumption)	Samples of healthy adults (amount)	Sample pretreatment and extraction	Stationary phase and mobile phase	Technique	Reference
3-Caffeoylquinic acid lactone-O-sulfate 4-Caffeoylquinic acid lactone-O-sulfate Caffeic acid-3′-O-sulfate 3-Feruloylquinic acid 4-Feruloylquinic acid 5-Feruloylquinic acid 5-Caffeoylquinic acid Ferulic acid-4′-O-sulfate Dihydrocaffeic acid Dihydrocaffeic acid-3′-O-sulfate Dihydroferulic acid Dihydroferulic acid-4′-O-sulfate (after instant coffee consumption)	Human plasma (11)	Plasma (450 μL), acidified with 13.5 μL of 50% aqueous formic acid, was added drop-wise to an 1125 μL of acetonitrile to which had been added 50 μL of 10% (m/v) ascorbic acid containing 0.5 μmol L^{-1} EDTA, (and 1 μg of sinapic acid as an internal standard). Samples were vortexed for 30 s every 2 min over a 10 min and centrifuged at 1500 g for 20 min at 4 °C. The supernatant was decanted and the pellet re-extracted with 1125 μL of methanol and after centrifugation the two supernatants were combined and reduced to dryness under a stream of nitrogen at 35 °C. The dried samples were then re-suspended in 250 μL of 0.1% aqueous formic acid containing 10% methanol, and centrifuged at 4 °C for 20 min at 16,110 g in a 0.2 μm filter	SYNERGI POLAR-RP (4 μm, 4.6 mm × 250 mm)/5–16% gradient of acetonitrile in 0.5% aqueous acetic acid	LC-PDA-MS^n	Stalmach et al. (2014)

Analytes (after food consumption)	Samples of healthy adults (amount)	Sample pretreatment and extraction	Stationary phase and mobile phase	Technique	Reference
Chlorogenic acids and other metabolites: caffeic acid, ferulic acid, isoferulic acid, gallic acid, dihydrocaffeic acid, vanillic acid, benzoic acid, *p*-hydroxybenzoic acid, syringic acid, sinapic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, trans-3-hydroxycinnamic acid, 3-(4-hydroxyphenyl) propionic acid and 2,4-dihydroxybenzoic acid (after consumption of soy, coffee, and beverages)	Human urine (6)	Helix pomatia (Sigma-Aldrich) extract containing β-glucuronidase and sulfatase activities for deconjugation of glucuronic acid conjugates and sulfated forms. The pH 5.0 and in water bath at 37 °C for 2 hours. Than acidified with HCl and methanol aqueous solution (40%, v:v) was added with subsequent vortexing. Eppendorf tube with a cellulose filter (Microcon YM-10, Millipore) and centrifuged at 17,000 g, for 30 min, at 4 °C	C18 Kromasil column (5 μm, 4.6 mm × 250 mm)/ gradient with 0.3% formic acid solution and methanol	HPLC–DAD (confirmation by LC-MS)	Felberg et al. (2015)
Benzoic acid, hippuric acid, 4-hydroxybenzoic acid, quinic acid and dihydrocaffeic acid sulfate (after 400 mg of dried green coffee bean extract consumption [GCBE, 9% w/w CGA and 0.5% w/w caffeine] each day for a month)	Human urine (10)	Urine was centrifuged at 5000 rpm for 10 min	Zorbax Rapid Resolution High Definition (RRHD) SB-C18 column (1.8 μm, 2.1 mm × 50 mm)/solvent A (acetonitrile with 0.1% formic acid) and solvent B (water with 0.1% formic acid)	UPLC-QTOF (LC-ESI-MS with C-3 stationary phase)	Peron et al. (2018)

prevention of bleeding and for reduction of toxic effects of most xenobiotics (Daud et al. 2016; Uchendu et al. 2012). There is some evidence that this vitamin may be a useful biomarker in overall health and nutritional status, especially in regular measurements (Chung et al. 2001; Szeto et al. 2004). The levels of ascorbic acid in serum or plasma are affected by dietary intake, age, and gender as well as circadian rhythm, and therefore leukocyte and lymphocyte vitamin C levels are also analyzed (Emadi-Konjin et al. 2005). Nowadays, this biomarker shows the antioxidant activity in cancer prevention, also antioxidant/anti-inflammatory, antihypertensive properties, and lipid-lowering effects in cardiovascular diseases and antioxidant activities in cataract and ocular diseases (Daud et al. 2016; Szeto et al. 2004). It also possesses antioxidant activity in critically ill patients with sepsis. However, in high doses, it demonstrates prooxidant activities, the ability to generate reactive oxygen species, cytotoxicity, also in stimulating apoptotic way of the tumor cells (Carr and Frei 1999). The study based on the European Prospective Investigation into Cancer and Nutrition (EPIC) showed that higher concentration of vitamin C in plasma, but not the level of dietary vitamin C intake assessed by country-specific questionnaires, is linked to decreasing risk of gastric cancer (Jenab et al. 2006, 2009). It has been lately revealed that ascorbic acid in proper concentration and as supportive intervention could be used in oncologic care (Klimant et al. 2018).

After a recent systematic review of literature, researchers have found that cancer risk prevention consequent to the reduction of oxidative damage has been observed in some cases at low doses of retinol palmitate – a derivative of vitamin A and/or vitamin C in contrary to its higher doses (de Carvalho Melo-Cavalcante et al. 2019).

In terms of the determination of vitamin C due to its susceptibility to the environmental conditions, sample collection and stabilization have to be adequately performed. There are protocols that include the precipitation of proteins, usually with meta-phosphoric or trichloroacetic acid, depending on the method used (Salminen and Alfthan 2008; Vuilleumier and Keck 1989). Chung et al. (2001) proposed heparin as an anticoagulant apart from meta-phosphoric acid and then ascorbic acid, which is stable for up to 10 days at −70 °C.

The best option for the determination of ascorbic acid in plasma is HPLC with ultraviolet or electrochemical detection and mass spectrometry as well (Emadi-Konjin et al. 2005; Ross 1994). For lymphocyte separation and lymphocyte vitamin C extractions, these processes should be completed no later than 3 hours after blood drawing. The whole lymphocyte preparation/extraction process is prepared on ice, the lysate is protected from light and then the lymphocyte vitamin C extracts are stored at −80 °C before HPLC analysis (Emadi-Konjin et al. 2005).

The problem in the ascorbic acid analysis in serum consists mainly in DHAA (dehydroascorbic acid) reconversion to ascorbic acid, and it may lead to incorrect results (Margolis and Duewer 1996). In HPLC-UV methods, dithioerythritol (DTE) and 1,4-dithiothreitol (DTT) are used to determine total ascorbic acid in samples, but they are not used in clinical settings due to the unknown contribution of DHAA in patients samples (Card 2019). Therefore, the dual-wavelength UV detector can be employed (Washko et al. 1992). The injection volume should be 20–50 μL, and the samples in the autosampler should be protected from light and refrigerated to

4 °C. Typical C18 columns, 5 μm, and 250 × 4.6 mm, with methanol or acetonitrile in acidic pH, are used.

The FRASC method is the modification of the ferric reducing antioxidant power (FRAP) assay, which allows determining ascorbic acid at the same time as the assay (Benzie and Strain 1996; Benzie and Strain 1999). This method has also been validated and compared with the reference HPLC method (Chung et al. 2001). The addition of enzyme – ascorbate oxidase – is necessary.

Another example of methods used to quantify ascorbic acid in plasma is its oxidation with Cu^{2+} to a dehydroascorbic acid that reacts with acidic 2,4-dinitrophenylhydrazine to form a red bis-hydrazone. This color compound is measured spectrophotometrically with the use of a 2,4-dinitrophenylhydrazine method. There are modern spectrophotometry methods used with microvolume amounts at 265 nm (Witmer et al. 2016). In the spectrophotometric techniques, which are relatively cheaper, the results could be higher than in HPLC procedures because of lower selectivity. The normal levels of vitamins A, C, D, and E can be found in Table 11.5.

Simultaneous Determination of vitamin A, Carotenoids, Vitamin D, and Vitamin E

Vitamin A refers mainly to retinol in the organism but retinal, retinoic acid, and retinyl acetate are also known as retinoids (Blomhoff and Blomhoff 2006). The role of this vitamin in the organism is to participate in vision and many different aspects of mammalian physiology, including embryonic development, growth and development, immunity, and the maintenance of epithelial barriers (Dawson 2000). The retinol in the blood is linked to the specific retinol-binding protein (RBP), important for the determination of this vitamin. Its provitamins are specific carotenoids also considered as potent antioxidants in food and then in the human organism. In the case of vitamin E, α-tocopherol is another lipid-soluble antioxidant that is found in all cellular membranes and protects cells from lipid peroxidation (Sies and Stahl 1995). What is essential, the chain-breaking antioxidant prevents the chain initiation and propagation of free radical reaction and lipid peroxidation in cellular membranes. It also influences the cellular response to oxidative stress through the modulation of the signal-transduction pathway (Azzi et al. 1992; Kamal-Eldin and

Table 11.5 Normal levels of selected vitamins

Vitamin level	Plasma/serum	Leukocyte
Vitamin A	>70 μmol L^{-1}	
Vitamin C	23–85 μmol L^{-1} (5–15 mg L^{-1})	8.73 ± 4.13 μg per 10^8 cells
Vitamin D	50–75 nmol L^{-1}	
Vitamin E	>16.2 μmol L^{-1}	

Appelqvist 1996). Nevertheless, high doses (greater than or equal to 400 IU daily) of vitamin E supplements may increase the mortality (Miller et al. 2005).

It is worth mentioning that healthy humans have no detectable quantities of retinol in urine samples and it can be determined in patients with kidney pathologies and diabetes; a higher level of vitamin E in poorly controlled diabetic children was revealed, and it could also be a marker of oxidative stress (Gavrilov et al. 2006, 2012). What is more, the increased levels of lipid-soluble antioxidant vitamins in plasma could be beneficial to treat childhood obesity (Guerendiain et al. 2017).

The preparation of biological fluid samples is the most crucial step in analyzing fat-soluble vitamins and antioxidants. The blood can be extracted and left to spontaneous coagulation, centrifuged at 3000 g for 10 min (Rodríguez-Delgado et al. 2002). The previous methods were not "green," and chemicals were also expensive (Albahrani et al. 2016; Guerendiain et al. 2017) with the use of hexane and dichloromethane in methanol to prepare the samples. Lazzarino et al. (2017) have described the single-step, efficient, simple, rapid, and low-cost method to extract the following fat-soluble vitamins and antioxidants: all-trans-retinoic acid, all-trans-retinol, vitamin D: 25-hydroxycholecalciferol, carotenoids: astaxanthin, lutein, zeaxanthin, trans-β-apo-8′-carotenal, β-cryptoxanthin, phylloquinone, lycopene, α-carotene, β-carotene, vitamin E: α-tocopherol, γ-tocopherol, and coenzyme Q_{10} in biological fluids (serum, plasma, seminal plasma, seminal fluid, urine, cerebrospinal fluid, saliva, and synovial fluid). 250 μL of serum or seminal plasma sample was mixed with 500 μL of acetonitrile for 60 s, incubated at 37 °C for 1 hour in a water bath under agitation (extraction of lipid-soluble compounds); centrifuged at 20,690 g for 15 min at 4 °C to precipitate proteins. During every procedure, samples must be protected from light to avoid degradation of photo-sensitive compounds. Also, the SPE method for 100 μL of the sample has been recently developed (Wills et al. 2019).

The determination method of retinol and α-tocopherol should be fast, selective, and economical in routine use. HPLC is usually used to determine these vitamins (and also other compounds such as carotenoids) in few minutes with methanol on the C18 columns (Guerendiain et al. 2017; Khan et al. 2010; Rodríguez-Delgado et al. 2002) as well as methanol and acetonitrile (Lazzarino et al. 2017). Fluorometric, electrochemical, and MS detectors are also used. Detection of the vitamins is carried out at 292 nm for α-tocopherol and 325 nm for retinol. Standards are dissolved in a mixture of hexane–dichloromethane or dichloromethane and methanol with the addition of butylated hydroxytoluene (BHT) to protect vitamins from oxidation processes.

In 2004 the automated HPLC method not only for vitamins A and E but also for vitamin D (24,25-$(OH)_2$ Vitamin D_3 and 25-(OH) Vitamin D_3) determination was described (Quesada et al. 2004).

The validation of methods is also necessary. Mata-Granados et al. (2008) have used standard reference material (SRM 968c) which provides certified concentration values for all-trans retinol (vitamin A), α-tocopherol (vitamin E) and reference concentration values for 25-hydroxyvitamin D_3. They have also found a low level of vitamin D and a high level of vitamin A in the serum of healthy adults, which

reflects a problem for the public health of chronic pathologies. The high concentration of vitamin E in the Spanish population may be used as a cardiovascular risk marker.

Bilirubin

Bilirubin is the product of the degradation of heme, the hemoglobin component in erythrocytes. Bilirubin concentration indicates indirectly the quality of liver function, which is responsible for its metabolizing. Some people, mostly men, have increased bilirubin levels. In the disorder, called Gilbert's syndrome, there is an impaired conjugation of this metabolite in the liver. Gilbert's syndrome is most often asymptomatic, and yellowing of the skin or conjunctival layers of the eye can be observed during the consumption of large amounts of alcohol, fatigue, or intense exercise. The study conducted by Floreani et al. (1993) has not indicated any influence of elevated blood bilirubin concentration on sports performance. Due to the weak solubility of bilirubin in water, it is bound in blood with glucuronate or possibly albumin. It protects water spaces as well as cell membranes. It is also responsible for the protection from the peroxidation of unsaturated fatty acids, including linoleic and linolenic acids (Stocker et al. 1987). As an antioxidant, it also participates in singlet oxygen ($^1O_2$) neutralization.

Due to the light sensitivity of bilirubin, blood samples should not be exposed to sunlight. This may result in false under-estimation. In serum, bilirubin bound (conjugated) can be calorimetrically determined with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde; DMAB). Preparation of the reagent is the following: 1 g of DMAB is dissolved in 50 mL of 95% ethanol and 50 mL of concentrated HCl. The absorbance of samples is measured at 650 nm wavelength (Suzuki 1997). After the addition of methanol to the serum sample, free bilirubin is bound so that the concentration of total bilirubin can be determined. The concentration of free bilirubin can be estimated by calculating the difference between total and bound bilirubin.

In the whole heparinized blood, it is possible to determine the concentration of bilirubin by reading absorption at two wavelengths, 455 and 575 nm. This method can be used, among others, in gasometric analyzers (Grohmann et al. 2006).

Bilirubin present in the blood occurs in four fractions (α, β, γ, and δ). The concentration of individual fractions is determined by using HPLC (Table 11.6).

Table 11.6 The analysis of bilirubin fractions

Fraction	Bilirubin form	Wavelength (nm)
α (Bα or Bu)	Unconjugated	459
β (BMG)	Monoglucuronide	422
γ (BDG)	Diglucuronide	422
δ (Bδ)	Albumin-bound bilirubin	433

Also, Adachi et al. (1988) divided bilirubin present in the blood serum of people with hyperbilirubinemia into five fractions, additionally secreting the β′ fraction. The authors fractionated serum bilirubin by newly developed HPLC method.

A healthy person's urine does not contain bilirubin (below limit of detection, <LOD).

Total Antioxidant Capacity (TAC)

The antioxidant capacity is the term defined as "the moles of oxidants neutralized by one liter of body fluids; the ability of chemical compounds to prevent or delay the oxidation of various substances, such as lipids, proteins or DNA, necessary for the proper functioning of the body" (Lettieri-Barbato et al. 2013; Serafini et al. 2006). Delayed oxidation or antioxidation occurs in living organisms (plants and animals), but also in food products.

Plasma non-enzymatic antioxidants include endogenous substances (e.g., uric acid, bilirubin, and thiols) and nutritional/exogenous substances (e.g., tocopherols, ascorbic acid, carotenoids, and phenolic compounds) which cannot be determined directly because this is an activity of all compounds found in the biological fluid (Lettieri-Barbato et al. 2013; Serafini et al. 2006). TAC concerns "the cumulative action of all the antioxidants present in the matrix (plasma, saliva, food extracts, tissues, etc.), providing an integrated parameter rather than the simple sum of measurable antioxidants and giving an insight into the assessment of the antioxidant network" (Serafini et al. 2006). This value consists of the sum of concentrations of vitamins C, E, and A, trace elements, for example, selenium, proteins with thiol groups (–SH), bilirubin, metal ions affecting the antioxidant potential (Fe^{2+} and Cu^{2+}), and compounds of plant origin including polyphenols (Rice-Evans and Miller 1994). The in vivo antioxidant level has been found essential for a health condition, therefore measuring antioxidant capacity in biological fluids can enable the prediction of not only nutritional health condition but also the diseases. It is worth mentioning that the TAC parameter does not reflect the full capacity of the organism to deal with oxidative stress but it helps to compare the results of different biological samples (from patients, healthy subjects, etc.) (Bartosz 2010).

Some methods have been incorporated from food to biological fluids and/or vice versa, especially ABTS, DPPH, and FRAP assays are commonly used to measure antioxidant activity of food and beverages (Cano and Arnao 2018; Jeszka-Skowron and Zgoła-Grześkowiak 2017). These easy and fast spectrophotometric methods are based on the radical-scavenging activity (stable radical scavenger) and redox potential of antioxidants. Among other methods, ORAC (Oxygen Radical Absorbance Capacity) is a procedure based on the hydrogen atom transfer (HAT) in contrast to the single-electron transfer SET-based methods (ABTS, DPPH, or FRAP) (Ialongo 2017).

The preparation of plasma could involve isolation from ethylenediaminetetraacetic acid-treated blood samples by centrifugation at 3000 g for 20 min at 4 °C (Fairus

et al. 2019). Plasma samples can also be diluted with methanol and centrifuged for 5 min at 14,000 g to remove precipitated proteins (Lee et al. 2017).

Trolox equivalent antioxidant capacity (TEAC) assay with the use of stable cation radical ABTS was firstly reported by Miller et al. (1993). Trolox is a structural hydrosoluble analog of vitamin E (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and it is used as a standard for calibration curve not only in ABTS but also in DPPH or FRAP assays. Other standards, such as vitamin C, uric acid, or glutathione, are also used (Bartosz 2010; Janaszewska and Bartosz 2002; Lee et al. 2017). Reference values of TBARS, allantoin, total polyphenols, bilirubin, and antioxidant status parameters in human blood are presented in Table 11.7.

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) Assay

A blue/green $ABTS^{\bullet+}$ chromophore is generated in the chemical reaction between ABTS and potassium persulfate. This compound possesses absorption maxima at wavelengths 645 nm, 734 nm, and 815 nm. When it is mixed with antioxidants, the pre-formed radical cation is reduced (Re et al. 1999). The reduction of the radical cation $ABTS^{\bullet+}$ (green-blue color) to the decolored form takes several seconds except for GSH (hours) (Janaszewska and Bartosz 2002).

To form the ABTS radical cation ($ABTS^{\bullet+}$) it is necessary to mix 7 mmol L^{-1} ABTS with 2.45 mmol L^{-1} potassium persulfate at a ratio of 2:1. Then the stock solution is incubated in the dark for 12–16 hours at room temperature before storage at 2 °C. The TEAC working reagent is prepared by mixing 1 mL of $ABTS^{\bullet+}$ and 19 mL of phosphate-buffered saline stock solution. A 200 μL volume of the reagent

Table 11.7 Reference values of TBARS, allantoin, total polyphenols, bilirubin, and antioxidant status parameters in blood

Biochemical parameter	Reference values	Unit
TBARS	1.0–5.0	μmol L^{-1}
Allantoin	3.1–36.4	μmol L^{-1}
Total polyphenols	2.8–4.0	g L^{-1}
Bilirubin	5.1–17.1	μmol L^{-1}
ABTS	1300–1600	μmol L^{-1}
DPPH[a]	7.9–27.1	%
FRAP	400–1600	μmol L^{-1}
ORAC[a]	1500–3100	μmol L^{-1}

ABTS 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), *DPPH* 2,2-diphenyl-1-picrylhydrazyl, *TBARS* thiobarbituric acid reactive substances, *FRAP* ferric reducing ability of plasma, *ORAC* Oxygen Radical Absorbance Capacity
[a]Deproteinized serum

is continuously mixed with 20 μL of the sample, incubated at 37 °C, and after 6 min measured at 734 nm. The TEAC values of plasma samples are expressed as mmol L^{-1} of Trolox equivalent (TE) (Fairus et al. 2019; Re et al. 1999). The assay has a precision lower than 3% (Erel 2004; Lee et al. 2017).

DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

DPPH antioxidant status of plasma is measured by the method described by Brand-Williams et al. (1995) with the use of a stable free radical – DPPH. This compound possesses the spare electron over the whole molecule (molecule does not dimerize) with the delocalization in deep violet color. The absorption band in methanol/ethanol solution is centered at 515–520 nm. After the reaction of the DPPH solution with the antioxidants (substances that can donate a hydrogen atom), the reduced form of DPPH remains in the form of the picryl group and changes the color to pale yellow.

Fresh DPPH radical solution is usually prepared by dissolving 1 mol L^{-1} of DPPH in 200 mL of methanol. 5 μL of plasma is subjected to reaction with DPPH working solution (245 μL) for 30 min in the dark and the absorbance is measured at 517 nm (Lee et al. 2017). The TEAC values of plasma samples are expressed as mmol L^{-1} of TE.

FRAP Assay

The determination of FRAP is based on the methodology elaborated by Benzie and Strain (1996) and modified by Janaszewska and Bartosz (2002). Plasma (10 μL) is mixed with 30 μL of deionized water on an ELISA plate. Then, 300 μL of the reagent (37 °C), consisting of 250 μL of 300 mmol L^{-1} acetate buffer (pH 3.6), 25 μL of 10 mmol L^{-1} 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol L^{-1} HCl, and 25 μL of 20 mmol L^{-1} $FeCl_3 \times 6H_2O$ solutions, is added to all wells and mixed carefully. After 6 min of incubation, the color of the solutions is read on a multimode microplate reader at 593 nm. The standard curve is determined with a stoichiometrically diluted solution of iron(II) sulfate(VI) ($FeSO_4 \times 7H_2O$). FRAP is expressed in μmol L^{-1} of plasma.

ORAC Assay

The ORAC assay aims to measure the antioxidant capacity of plasma by the determination of the oxidative degradation of a fluorescent molecule (fluorescein), which has been previously mixed and heated with AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride). Plasma samples react with the ORAC working solution (150 μL). After adding AAPH for peroxyl radical generation, the fluorescence is measured for 80 min to determine the area under the curve (AUC) at 485 nm as excitation and 520 nm as emission. TAC is expressed as mg L^{-1} of TE (Cao et al. 1993; Huang et al. 2002; Lee et al. 2017).

Conclusions

There is a growing need to find markers of oxidative stress in the organism. In the literature reviews, researchers found out that damage to lipids in samples of adults was lower (level of oxidized proteins in plasma), and superoxide anion levels in neutrophils were also lower than in samples of the elderly individuals (Belenguer-Varea et al. 2019). They also observed that antioxidant biomarkers such as superoxide dismutase were lower and glutathione reductase activities were higher, as well as higher levels of vitamins A and E, lower level of coenzyme Q_{10} in comparison to elderly controls. Another meta-analysis on patients with cystic fibrosis revealed some markers of antioxidant status and oxidative stress such as protein carbonyl groups (DNPH assay), total F2-isoprostane, 8-iso-prostaglandin $F_{2\alpha}$, MDA, vitamins A and E, β-carotene (Causer et al. 2020). Whalley et al. (2003) have concluded that the general factors believed to raise the risk of vascular disease such as higher homocysteine levels, and also lower vitamin C levels can increase the likelihood of brain shrinkage or brain cell loss. Moreover, metabolomics can identify the biomarkers of dietary patterns and the influence of nutrition on breast cancer risk (Noh et al. 2017; Playdon et al. 2017a, b). It is also essential that there should be more research on biomarker levels in patients because it is necessary to use them for medical purposes.

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References

Adachi, Y., H. Inufusa, M. Yamashita, A. Kambe, K. Yamazaki, Y. Sawada, and T. Yamamoto. 1988. Human serum bilirubin fractionation in various hepatobiliary diseases by the newly developed high performance liquid chromatography. *Gastroenterologia Japonica* 23: 268–272.

Agarwal, S., and R.S. Sohal. 1995. Differential oxidative damage to mitochondrial proteins during aging. *Mechanisms of Ageing and Development* 85: 55–63.

Al-Attar, A.M. 2011. Antioxidant effect of vitamin E treatment on some heavy metals-induced renal and testicular injuries in male mice. *Saudi Journal of Biological Sciences* 18: 63–72.

Albahrani, A.A., V. Rotarou, P.J. Roche, and R.F. Greaves. 2016. A simultaneous quantitative method for vitamins A, D and E in human serum using liquid chromatography-tandem mass spectrometry. *The Journal of Steroid Biochemistry and Molecular Biology* 159: 41–53.

Amagase, H., B. Sun, and C. Borek. 2009. *Lycium barbarum* (goji) juice improves *in vivo* antioxidant biomarkers in serum of healthy adults. *Nutrition Research* 29: 19–25.

Amirani, E., A. Milajerdi, H. Mirzaei, H. Jamilian, M.A. Mansournia, J. Hallajzadeh, and A. Ghaderi. 2020. The effects of probiotic supplementation on mental health, biomarkers of inflammation and oxidative stress in patients with psychiatric disorders: A systematic review and meta-analysis of randomized controlled trials. *Complementary Therapies in Medicine* 49: 102361.

Arshad, M.A., S. Bhadra, R.M. Cohen, and M.T. Subbiah. 1991. Plasma lipoprotein peroxidation potential: A test to evaluate individual susceptibility to peroxidation. *Clinical Chemistry* 37: 1756–1758.

Ashor, A.W., O.M. Shannon, A.D. Werner, F. Scialo, C.N. Gilliard, K.S. Cassel, C.J. Seal, D. Zheng, J.C. Mathers, and M. Siervo. 2020. Effects of inorganic nitrate and vitamin C co-supplementation on blood pressure and vascular function in younger and older healthy adults: A randomised double-blind crossover trial. *Clinical Nutrition* 39: 708–717.

Assanelli, D., A. Bonanome, A. Pezzini, F. Albertini, P. Maccalli, M. Grassi, S. Archetti, R. Negrini, and F. Visioli. 2004. Folic acid and vitamin E supplementation effects on homocysteinemia, endothelial function and plasma antioxidant capacity in young myocardial-infarction patients. *Pharmacological Research* 49: 79–84.

Aydin, S., I. Aral, N. Kilic, I. Bakan, S. Aydin, and F. Erman. 2004. The level of antioxidant enzymes, plasma vitamins C and E in cement plant workers. *Clinica Chimica Acta* 341: 193–198.

Azzi, A., D. Boscoboinik, and C. Hensey. 1992. The protein kinase C family. *European Journal of Biochemistry* 208: 547–557.

Bandookwala, M., A.K. Sahu, D. Thakkar, M. Sharma, A. Khairnar, and P. Sengupta. 2019. Edaravone-caffeine combination for the effective management of rotenone induced Parkinson's disease in rats: An evidence based affirmative from a comparative analysis of behavior and biomarker expression. *Neuroscience Letters* 711: 134438.

Barbosa, M.L., A.-A.P.M. de Meneses, R.P.S. de Aguiar, J.M. de Castro e Sousa, A.A. de Carvalho Melo Cavalcante, and S.W. Maluf. 2020. Oxidative stress, antioxidant defense and depressive disorders: A systematic review of biochemical and molecular markers. *Neurology, Psychiatry and Brain Research* 36: 65–72.

Bartosz, G. 2010. Non-enzymatic antioxidant capacity assays: Limitations of use in biomedicine. *Free Radical Research* 44: 711–720.

Belenguer-Varea, Á., F.J. Tarazona-Santabalbina, J.A. Avellana-Zaragoza, M. Inglés, C. Mas-Bargues, and M. Martínez-Reig. 2019. Oxidative stress and exceptional human longevity: Systematic review. *Free Radical Biology and Medicine* 149: 51–63.

Benzie, I.F.F., and J.J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry* 239: 70–76.

Benzie, I.F.F., and J.J. Strain. 1999. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology* 299: 15–27.

Bhattacharjee, S., S. Pennathur, J. Byun, J. Crowley, D. Mueller, J. Gischler, R.S. Hotchkiss, and J.W. Heinecke. 2001. NADPH oxidase of neutrophils elevates o,o'-dityrosine cross-links in proteins and urine during inflammation. *Archives of Biochemistry and Biophysics* 395: 69–77.

Bielecki, A., G. Saravanabhavan, E. Blais, R. Vincent, and P. Kumarathasan. 2012. An efficient sample preparation method for high-throughput analysis of 15(S)-8-iso-PGF2α in plasma and urine by enzyme immunoassay. *Journal of Analytical Toxicology* 36: 595–600.

Blomhoff, R., and H.K. Blomhoff. 2006. Overview of retinoid metabolism and function. *Journal of Neurobiology* 66: 606–630.

Brand-Williams, W., M.E. Cuvelier, and C. Berset. 1995. Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft & Technologie* 28: 25–30.

Buege, J.A., and S.D. Aust. 1978. Microsomal lipid peroxidation. *Methods in Enzymology* 52: 302–310.

Buss, H., T.P. Chan, K.B. Sluis, N.M. Domigan, and C.C. Winterbourn. 1997. Protein carbonyl measurement by a sensitive ELISA method. *Free Radical Biology and Medicine* 23: 361–366.

Cano, A., and M.B. Arnao. 2018. ABTS/TEAC (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox-Equivalent Antioxidant Capacity) radical scavenging mixed-mode assay. In *Measurement of antioxidant activity and capacity: Recent trends and applications*, ed. R. Apak, E. Capanoglu, and F. Shahidi, 117–140. Hoboken: Wiley.

Cao, G., H.M. Alessio, and R.G. Cutler. 1993. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology & Medicine* 14: 303–311.

Carbonneau, M.A., E. Peuchant, D. Sess, P. Canioni, and M. Clerc. 1991. Free and bound malondialdehyde measured as thiobarbituric acid adduct by HPLC in serum and plasma. *Clinical Chemistry* 37: 1423–1429.

Card, D.J. 2019. Chapter 13 – Methods for assessment of vitamin C. In *Laboratory assessment of vitamin status*, 301–316. London: Academic Press.

Carr, A., and B. Frei. 1999. Does vitamin C act as a pro-oxidant under physiological conditions? *The FASEB Journal* 13: 1007–1024.

Causer, A.J., J.K. Shute, M.H. Cummings, A.I. Shepherd, M. Gruet, J.T. Costello, S. Bailey, M. Lindley, C. Pearson, G. Connett, M.I. Allenby, M.P. Carroll, T. Daniels, and Z.L. Saynor. 2020. Circulating biomarkers of antioxidant status and oxidative stress in people with cystic fibrosis: A systematic review and meta-analysis. *Redox Biology* 32: 101436.

Chang, C.-Y., J.-Y. Chen, D. Ke, and M.-L. Hu. 2005. Plasma levels of lipophilic antioxidant vitamins in acute ischemic stroke patients: Correlation to inflammation markers and neurological deficits. *Nutrition* 21: 987–993.

Child, R.B., D.M. Wilkinson, J.L. Fallowfield, and A.E. Donnelly. 1998. Elevated serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half-marathon run. *Medicine and Science in Sports and Exercise* 30: 1603–1607.

Chung, W.Y., J.K.O. Chung, Y.T. Szeto, B. Tomlinson, and I.F. Benzie. 2001. Plasma ascorbic acid: Measurement, stability and clinical utility revisited. *Clinical Biochemistry* 34: 623–627.

Cooper, C.E., N.B. Vollaard, T. Choueiri, and M.T. Wilson. 2002. Exercise, free radicals and oxidative stress. *Biochemical Society Transactions* 30: 280–285.

Cremin, P., S. Kasim-Karakas, and A.L. Waterhouse. 2001. LC/ES−MS detection of hydroxycinnamates in human plasma and urine. *Journal of Agriculture and Food Chemistry* 49: 1747–1750.

da Silva, A., A. de Sousa Rebouças, B. Mendonça, D. Silva, R. Dimenstein, and K. Ribeiro. 2019. Relationship between the dietary intake, serum, and breast milk concentrations of vitamin A and vitamin E in a cohort of women over the course of lactation. *Maternal & Child Nutrition* 15: e12772.

Daud, Z.A.M., A. Ismail, and B. Sarmadi. 2016. Ascorbic acid: Physiology and health effects. In *Encyclopedia of food and health*, 266–274. Cambridge, MA: Academic Press.

Dawson, M.I. 2000. The importance of vitamin a in nutrition. *Current Pharmaceutical Design* 6: 311–325.

de Carvalho Melo-Cavalcante, A.A., L. da Rocha Sousa, M. Alencar, J.V. de Oliveira Santos, A. da Mata, M. Paz, R.M. de Carvalho, N. Nunes, M.T. Islam, A.N. Mendes, J. Gonçalves, F. da Silva, P. Ferreira, and J.M. de Castro e Sousaa. 2019. Retinol palmitate and ascorbic acid: Role in oncological prevention and therapy. *Biomedecine & Pharmacotherapie* 109: 1394–1405.

Duarte, G.S., and A. Farah. 2011. Effect of simultaneous consumption of milk and coffee on chlorogenic acids' bioavailability in humans. *Journal of Agricultural and Food Chemistry* 59: 7925–7931.

Edmands, W.M., P. Ferrari, J.A. Rothwell, S. Rinaldi, N. Slimani, D.K. Barupal, C. Biessy, M. Jenab, F. Clavel-Chapelon, G. Fagherazzi, M.C. Boutron-Ruault, V.A. Katzke, T. Kühn, H. Boeing, A. Trichopoulou, P. Lagiou, D. Trichopoulos, D. Palli, S. Grioni, R. Tumino, P. Vineis, A. Mattiello, I. Romieu, and A. Scalbert. 2015. Polyphenol metabolome in human urine and its association with intake of polyphenol-rich foods across European countries. *The American Journal of Clinical Nutrition* 102: 905–913.

El-Azab, M.F., R.M. Hazem, and Y.M. Moustafa. 2012. Role of simvastatin and/or antioxidant vitamins in therapeutic angiogenesis in experimental diabetic hindlimb ischemia: Effects on capillary density, angiogenesis markers, and oxidative stress. *European Journal of Pharmacology* 690: 31–41.

Elhassaneen, Y.A. 2004. The effects of charcoal-broiled meat consumption on antioxidant defense system of erythrocytes and antioxidant vitamins in plasma. *Nutrition Research* 24: 435–446.

El-Zein, R.A., C.M. Monroy, A. Cortes, M.R. Spitz, A. Greisinger, and C.J. Etzel. 2010. Rapid method for determination of DNA repair capacity in human peripheral blood lymphocytes amongst smokers. *BMC Cancer* 10: 439.

Emadi-Konjin, P., Z. Verjee, A.V. Levin, and K. Adeli. 2005. Measurement of intracellular vitamin C levels in human lymphocytes by reverse phase high performance liquid chromatography (HPLC). *Clinical Biochemistry* 38: 450–456.

Erel, O. 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry* 37: 277–285.

Fairus, S., H. Ming Cheng, and K. Sundram. 2019. Antioxidant status following postprandial challenge of two different doses of tocopherols and tocotrienols. *Journal of Integrative Medicine* 18: 68–79.

Fallah, Aziz A., E. Sarmast, and T. Jafari. 2020. Effect of dietary anthocyanins on biomarkers of oxidative stress and antioxidative capacity: A systematic review and meta-analysis of randomized controlled trials. *Journal of Functional Foods* 68: 103912.

Farah, A., M. Monteiro, C.M. Donangelo, and S. Lafay. 2008. Chlorogenic acids from green coffee extract are highly bioavailable in humans. *Journal of Nutrition* 138: 2309–2315.

Felberg, I., A. Farah, M.C. Monteiro, R.L.O. Godoy, S. Pacheco, V. Calado, and C.M.J. Donangelo. 2015. Effect of simultaneous consumption of soymilk and coffee on the urinary excretion of isoflavones, chlorogenic acids and metabolites in healthy adults. *Journal of Functional Foods* 19: 688–699.

Ferrières, J. 2004. The French paradox: Lessons for other countries. *Heart* 90: 107–111.

Floreani, A., N. Corsi, D. Martines, M. Varnier, and R. Naccarato. 1993. No effect of endurance exercise on serum bilirubin in healthy athletes and with congenital hyperbilirubinemia (Gilbert's syndrome). *The Journal of Sports Medicine and Physical Fitness* 33 (1): 79–82.

Frijhoff, J., P.G. Winyard, N. Zarkovic, S.S. Davies, R. Stocker, D. Cheng, A.R. Knight, E.L. Taylor, J. Oettrich, T. Ruskovska, A.C. Gasparovic, A. Cuadrado, D. Weber, H.E. Poulsen, T. Grune, H.H. Schmidt, and P. Ghezzi. 2015. Clinical relevance of biomarkers of oxidative stress. *Antioxidants & Redox Signaling* 23: 1144–1170.

Gavrilov, V., T. Yermiahu, and R. Gorodischer. 2006. Renal pathology and retinol status in multiple myeloma patients. *Kidney International* 69: 173–177.

Gavrilov, V., I. Harman-Boehm, D. Amichay, G. Tessler, T. Shuster, M. Friger, and R. Gorodischer. 2012. Kidney function and retinol status in type 2 diabetes mellitus patients. *Acta Diabetologica* 49: 137–143.

Giustarini, D., I. Dalle-Donne, A. Milzani, P. Fanti, and R. Rossi. 2013. Analysis of GSH and GSSG after derivatization with N-ethylmaleimide. *Nature Protocols* 8: 1660–1669.

Godycki-Cwirko, M., M. Krol, B. Krol, A. Zwolinska, K. Kolodziejczyk, M. Kasielski, G. Padula, J. Grebowski, P. Kazmierska, M. Miatkowski, J. Markowski, and D. Nowak. 2010. Uric acid but

not apple polyphenols is responsible for the rise of plasma antioxidant activity after apple juice consumption in healthy subjects. *Journal of the American College of Nutrition* 29: 397–406.

Goni, I., and A. Hernández-Galiot. 2019. Intake of nutrient and non-nutrient dietary antioxidants. Contribution of macromolecular antioxidant polyphenols in an elderly Mediterranean population. *Nutrients* 11: 2165.

Grohmann, K., M. Roser, B. Rolinski, I. Kadow, C. Müller, A. Goerlach-Graw, M. Nauck, and H. Küster. 2006. Bilirubin measurement for neonates: Comparison of 9 frequently used methods. *Pediatrics* 117: 1174–1183.

Grootveld, M., and B. Halliwell. 1987. Measurement of allantoin and uric acid in human body fluids. A potential index of free-radical reactions *in vivo*? *The Biochemical Journal* 243: 803–808.

Grunewald, R.A. 1993. Ascorbic acid in the brain. *Brain Research Reviews* 18: 123–133.

Guerendiain, M., J. Mayneris-Perxachs, R. Montes, G. López-Belmonte, M. Martín-Matillas, A.I. Castellote, E. Martín-Bautista, A. Martí, J.A. Martínez, L. Moreno, J.M. Garagorri, J. Wärnberg, J. Caballero, A. Marcos, M.C. López-Sabater, C. Campoy, and EVASYON Study Group. 2017. Relation between plasma antioxidant vitamin levels, adiposity and cardiometabolic profile in adolescents: Effects of a multidisciplinary obesity programme. *Clinical Nutrition (Edinburgh, Scotland)* 36: 209–217.

Hamdy, M.M., D.S. Mosallam, A.M. Jamal, and W.A. Rabie. 2015. Selenium and vitamin E as antioxidants in chronic hemolytic anemia: Are they deficient? A case-control study in a group of Egyptian children. *Journal of Advanced Research* 6: 1071–1077.

Hamurcu, Z., F. Bayram, G. Kahriman, H. Dönmez-Altuntas, and G. Baskol. 2010. Micronucleus frequency in lymphocytes and 8-hydroxydeoxyguanosine level in plasma of women with polycystic ovary syndrome. *Gynecological Endocrinology* 26: 590–595.

Han, J., Z. Liu, Y. Guo, G.C. Han, W. Li, S. Chen, and S. Zhang. 2017. Determination of superoxide anion radical by modified CdTe quantum dots. *Journal of Photochemistry and Photobiology A: Chemistry* 349: 1–6.

Han, X., N. Li, S. Liu, S. Yang, Y. Song, and Y. Shi. 2020. A new method of ultra-performance liquid chromatography/tandem mass spectrometry for determination of chlorogenic acid in human plasma and urine. *Rapid Communication of Mass Spectrometry* 34: e8603.

Hanta, I., S. Kuleci, N. Canacankatan, and A. Kocabas. 2003. The oxidant-antioxidant balance in mild asthmatic patients. *Lung* 181: 347–352.

Heinecke, J.W. 2002. Oxidized amino acids: Culprits in human atherosclerosis and indicators of oxidative stress. *Free Radical Biology & Medicine* 32: 1090–1101.

Hellsten, Y., P.C. Tullson, E.A. Richter, and J. Bangsbo. 1997. Oxidation of urate in human skeletal muscle during exercise. *Free Radical Biology & Medicine* 22: 169–174.

Hendouei, N., S. Farnia, F. Mohseni, A. Salehi, M. Bagheri, F. Shadfar, F. Barzegar, S.D. Hoseini, J.Y. Charati, and F. Shaki. 2018. Alterations in oxidative stress markers and its correlation with clinical findings in schizophrenic patients consuming perphenazine, clozapine and risperidone. *Biomedecine and Pharmacotherapie* 103: 965–972.

Ho, W.S., S.Y. Ying, P.C. Chan, and H.H. Chan. 2006. Use of onion extract, heparin, allantoin gel in prevention of scarring in Chinese patients having laser removal of tattoos: A prospective randomized controlled trial. *Dermatologic Surgery* 32: 891–896.

Huang, D., B. Ou, M. Hampsch-Woodill, J.A. Flanagan, and R.L. Prior. 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry* 50: 4437–4444.

Ialongo, C. 2017. Preanalytic of total antioxidant capacity assays performed in serum, plasma, urine and saliva. *Clinical Biochemistry* 50: 356–363.

Jamilian, H., E. Amirani, A. Milajerdi, F. Kolahdooz, H. Mirzaei, M. Zaroudi, A. Ghaderi, and Z. Asemi. 2019. The effects of vitamin D supplementation on mental health, and biomarkers of inflammation and oxidative stress in patients with psychiatric disorders: A systematic review and meta-analysis of randomized controlled trials. *Progress in Neuropsychopharmacology and Biological Psychiatry* 94: 109651.

Janaszewska, A., and G. Bartosz. 2002. Assay of total antioxidant capacity: Comparison of four methods as applied to human blood plasma. *Scandinavian Journal of Clinical and Laboratory Investigation* 62: 231–236.

Janero, D.R. 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biology & Medicine* 9: 515–540.

Jenab, M., E. Riboli, P. Ferrari, J. Sabate, N. Slimani, T. Norat, M. Friesen, A. Tjønneland, A. Olsen, K. Overvad, M.C. Boutron-Ruault, F. Clavel-Chapelon, M. Touvier, H. Boeing, M. Schulz, J. Linseisen, G. Nagel, A. Trichopoulou, A. Naska, E. Oikonomou, V. Krogh, S. Panico, G. Masala, C. Sacerdote, R. Tumino, P.H. Peeters, M.E. Numans, H.B. Bueno-de-Mesquita, F.L. Buchner, E. Lund, G. Pera, C.N. Sanchez, M.J. Sanchez, L. Arriola, A. Barricarte, J.R. Quiros, G. Hallmans, R. Stenling, G. Berglund, S. Bingham, K.T. Khaw, T. Key, N. Allen, F. Carneiro, U. Mahlke, G.G. Del, D. Palli, R. Kaaks, and C.A. Gonzalez. 2006. Plasma and dietary vitamin C levels and risk of gastric cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST). *Carcinogenesis* 27: 2250–2257.

Jenab, M., N. Slimani, M. Bictash, P. Ferrari, and S.A. Bingham. 2009. Biomarkers in nutritional epidemiology: Applications, needs and new horizons. *Human Genetics* 125: 507–525.

Jeszka-Skowron, M., and A. Zgoła-Grześkowiak. 2017. Usage of capillary isotachophoresis and antioxidant capacity measurement in analysis of changes in coffee properties after roasting, steaming and decaffeination. *Food Analytical Methods* 10: 1245–1251.

Jeszka-Skowron, M., E. Flaczyk, J. Jeszka, Z. Krejpcio, E. Król, and M.S. Buchowski. 2014. Mulberry leaf extract intake reduces hyperglycemia in streptozotocin (STZ)-induced diabetic rats fed high-fat diet. *Journal of Functional Foods* 8: 9–17.

Jeszka-Skowron, M., E. Flaczyk, and T. Podgórski. 2017. *In vitro* and *in vivo* analyses of *Morus alba* Polish var. wielkolistna zolwinska leaf ethanol–water extract – antioxidant and hypocholesterolemic activities in hyperlipideamic rats. *European Journal of Lipid Science and Technology* 119: 1600514.

Kadar Ali, S.H., and W.A. Raja. 2019. The effect of antioxidants in acute amitriptyline poisoning. *Toxicology Reports* 6: 380–388.

Kamal-Eldin, A., and L.A. Appelqvist. 1996. The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* 31: 671–701.

Kanter, M.M., L.A. Nolte, and J.O. Holloszy. 1993. Effects of an antioxidant vitamin mixture on lipid peroxidation at rest and postexercise. *Journal of Applied Physiology* 74: 965–969.

Kar, S., H.R. Shahshahan, T.N. Kambis, S.K. Yadav, Z. Li, D.J. Lefer, and P.K. Mishra. 2019. Hydrogen sulfide ameliorates homocysteine-induced cardiac remodeling and dysfunction. *Frontiers in Physiology* 10: 598.

Karolkiewicz, J., Ł. Szczęśniak, Z. Kasprzak, and A. Nowak. 2002. An overall influence of systematic physical activity and calorie restricted diet on some parameter prooxidant – antioxidant equilibrium in obese girls. *Medicina dello Sport* 6: 217–228.

Khan, A., M.I. Khan, Z. Iqbal, Y. Shah, L. Ahmad, and D.G. Watson. 2010. An optimized and validated RP-HPLC/UV detection method for simultaneous determination of all-trans-Retinol (Vitamin A) and α-Tocopherol (Vitamin E) in human serum: Comparison of different particulate reversed-phase HPLC columns. *Journal of Chromatography B* 878: 2339–2347.

Khazim, K., D. Giustarini, R. Rossi, D. Verkaik, J.E. Cornell, S.E.D. Cunningham, M. Mohammad, K. Trochta, C. Lorenzo, F. Folli, S. Bansal, P. Fanti. 2013. Glutathione redox potential is low and glutathionylated and cysteinylated hemoglobin levels are elevated in maintenance hemodialysis patients. *Translational Research* 162(1): 16–25.

Klimant, E., H. Wright, D. Rubin, D. Seely, and M. Markman. 2018. Intravenous vitamin C in the supportive care of cancer patients: A review and rational approach. *Current Oncology* 25: 139–148.

Kokcam, I., and M. Naziroglu. 2002. Effects of vitamin E supplementation on blood antioxidants levels in patients with Behçet's disease. *Clinical Biochemistry* 35: 633–639.

Kono, Y., K. Nakamura, H. Kimura, N. Nishii, A. Watanabe, K. Banba, A. Miura, S. Nagase, S. Sakuragi, K.F. Kusano, H. Matsubara, and T. Ohe. 2006. Elevated levels of oxidative DNA

damage in serum and myocardium of patients with heart failure. *Circulation Journal* 70: 1001–1005.

Korotkova, E.I., B. Misini, E.V. Dorozhko, M.V. Bukkel, E.V. Plotnikov, and W. Linert. 2011. Study of $OH^{\bullet}$ radicals in human serum blood of healthy individuals and those with pathological schizophrenia. *International Journal of Molecular Sciences* 12: 401–410.

Lafleur, M.V., J.J. Hoorweg, H. Joenje, E.J. Westmijze, and J. Retèl. 1994. The ambivalent role of glutathione in the protection of DNA against singlet oxygen. *Free Radical Research* 21: 9–17.

Lazzarino, G., S. Longo, A.M. Amorini, V. Di Pietro, S. D'Urso, G. Lazzarino, A. Belli, and B. Tavazzi. 2017. Single-step preparation of selected biological fluids for the high performance liquid chromatographic analysis of fat-soluble vitamins and antioxidants. *Journal of Chromatography A* 1527: 43–52.

Lee, S.G., T. Wang, T.M. Vance, P. Hubert, D.O. Kim, S.I. Koo, and O.K. Chun. 2017. Validation of analytical methods for plasma total antioxidant capacity by comparing with urinary 8-isoprostane level. *Journal of Microbiology and Biotechnology* 27: 388–394.

Leeuwenburgh, C., J.E. Rasmussen, F.F. Hsu, D.M. Mueller, S. Pennathur, and J.W. Heinecke. 1997. Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. *The Journal of Biological Chemistry* 272: 3520–3526.

Lekha, G., K. Mohan, and I.A. Samy. 2010. Effect of *Celastrus paniculatus* seed oil (Jyothismati oil) on acute and chronic immobilization stress induced in swiss albino mice. *Pharmacognosy Research* 2: 169–174.

Lettieri-Barbato, D., F. Tomei, A. Sancini, G. Morabito, and M. Serafini. 2013. Effect of plant foods and beverages on plasma non-enzymatic antioxidant capacity in human subjects: A meta-analysis. *The British Journal of Nutrition* 109: 1544–1556.

Levine, R.L., D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shaltiel, and E.R. Stadtman. 1990. Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* 186: 464–478.

Liu, M.L., R. Bergholm, S. Mäkimattila, S. Lahdenperä, M. Valkonen, H. Hilden, H. Yki-Järvinen, and M.R. Taskinen. 1999. A marathon run increases the susceptibility of LDL to oxidation in vitro and modifies plasma antioxidants. *The American Journal of Physiology* 276: E1083–E1091.

Loots, J.M., G.P. Loots, and W.S. Joubert. 1979. The effect of allantoin on cellular multiplication in degenerating and regenerating nerves. *South African Medical Journal* 55: 53–56.

Lopez-Huertas, E., and J. Fonolla. 2017. Hydroxytyrosol supplementation increases vitamin C levels in vivo. A human volunteer trial. *Redox Biology* 11: 384–389.

Lykkesfeldt, J., M. Viscovich, and H.E. Poulsen. 2004. Plasma malondialdehyde is induced by smoking: A study with balanced antioxidant profiles. *The British Journal of Nutrition* 92: 203–206.

Mabry, T.J., K.R. Markham, and M.B. Thomas, eds. 1970. *The systematic identification of flavonoids*. New York: Springer-Verlag.

Mandal, M., A. Varghese, V.K. Gaviraju, S.N. Talwar, and S.S. Malini. 2019. Impact of hyperglycaemia on molecular markers of oxidative stress and antioxidants in type 2 diabetes mellitus. *Clinical Diabetology* 8: 215–222.

Margolis, S.A., and D.L. Duewer. 1996. Measurement of ascorbic acid in human plasma and serum: Stability, intralaboratory repeatability, and interlaboratory reproducibility. *Clinical Chemistry* 42: 1257–1262.

Maron, B.A., and J. Loscalzo. 2009. The treatment of hyperhomocysteinemia. *Annual Review of Medicine* 60: 39–54.

Marrocco, I., F. Altieri, and I. Peluso. 2017. Measurement and clinical significance of biomarkers of oxidative stress in humans. *Hindawi Oxidative Medicine and Cellular Longevity* 2017: 1–32, ID 6501046.

Mata-Granados, J.M., M.D. Luque de Castro, and J.M. Quesada Gomez. 2008. Inappropriate serum levels of retinol, α-tocopherol, 25 hydroxyvitamin D_3 and 24,25 dihydroxyvitamin D_3

levels in healthy Spanish adults: Simultaneous assessment by HPLC. *Clinical Biochemistry* 41: 676–680.

Mikami, T., K. Kita, S. Tomita, G.J. Qu, Y. Tasaki, and A. Ito. 2000. Is allantoin in serum and urine a useful indicator of exercise-induced oxidative stress in humans? *Free Radical Research* 32: 235–244.

Miller, N.J., C. Rice-Evans, M.J. Davis, V. Gopinathan, and A. Milner. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science* 84: 407–412.

Miller, E.R., R. Pastor-Barriuso, D. Dalal, R.A. Riemersma, L.J. Appel, and E. Guallar. 2005. Meta-analysis: High-dosage vitamin E supplementation may increase all-cause mortality. *Annals of Internal Medicine* 142: 37–46.

Milne, G.L., H. Yin, J.D. Brooks, S. Sanchez, L. Jackson Roberts, and J.D. Morrow. 2007. Quantification of F2-isoprostanes in biological fluids and tissues as a measure of oxidant stress. *Methods in Enzymology* 433: 113–126.

Miniati, E. 2007. Assessment of phenolic compounds in biological samples. *Annali dell' Istituto Superiore di Sanita* 43: 362–368.

Moosavian, S.P., A. Arab, Z. Paknahad, and S. Moradi. 2020. The effects of garlic supplementation on oxidative stress markers: A systematic review and meta-analysis of randomized controlled trials. *Complementary Therapies in Medicine* 50: 102385.

Moskaug, J.Ø., H. Carlsen, M.C. Myhrstad, and R. Blomhoff. 2005. Polyphenols and glutathione synthesis regulation. *American Journal of Clinical Nutrition* 81 (1 Suppl): 277S–283S.

Nagano, T., and T. Yoshimura. 2002. Bioimaging of nitric oxide. *Chemical Reviews* 102: 1235–1270.

Naziroglu, M., S. Akkus, and H. Celik. 2011. Levels of lipid peroxidation and antioxidant vitamins in plasma and erythrocytes of patients with ankylosing spondylitis. *Clinical Biochemistry* 44: 1412–1415.

Nicolosi, R.J., C.W. Lawton, and T.A. Wilson. 1999. Vitamin E reduces plasma low density lipoprotein cholesterol, LDL oxidation, and early aortic atherosclerosis compared with black tea in hypercholesterolemic hamsters. *Nutrition Research* 19: 1201–1214.

Niki, E. 1991. Vitamin C as an antioxidant. *World Review of Nutrition and Dietetics* 64: 1–30.

Noh, H., H. Freisling, N. Assi, R. Zamora-Ros, D. Achaintre, A. Affret, F. Mancini, M.C. Boutron-Ruault, A. Flögel, H. Boeing, T. Kühn, R. Schübel, A. Trichopoulou, A. Naska, M. Kritikou, D. Palli, V. Pala, R. Tumino, F. Ricceri, M. Santucci de Magistris, A. Cross, N. Slimani, A. Scalbert, and P. Ferrari. 2017. Identification of urinary polyphenol metabolite patterns associated with polyphenol-rich food intake in adults from four European countries. *Nutrients* 9: 796.

Ognjanović, B.I., S.D. Marković, N.Z. Ethordević, I.S. Trbojević, A.S. Stajn, and Z.S. Saicić. 2010. Cadmium-induced lipid peroxidation and changes in antioxidant defense system in the rat testes: Protective role of coenzyme Q(10) and vitamin E. *Reproductive Toxicology* 29: 191–197.

Ohkawa, H., N. Ohishi, and K. Yagi. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* 95: 351–358.

Oosterink, J.E., E.F.G. Naninck, A. Korosi, P.J. Lucassen, J.B. van Goudoever, and H. Schierbeek. 2015. Accurate measurement of the essential micronutrients methionine, homocysteine, vitamins B6, B12, B9 and their metabolites in plasma, brain and maternal milk of mice using LC/MS ion trap analysis. *Journal of Chromatography B* 998-999: 106–113.

Orhan, H., S. Coolen, and J.H. Meerman. 2005. Quantification of urinary o,o'-dityrosine, a biomarker for oxidative damage to proteins, by high performance liquid chromatography with triple quadrupole tandem mass spectrometry. A comparison with ion-trap tandem mass spectrometry. *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences* 827: 104–108.

Pan, H.Z., D. Chang, L.G. Feng, F.J. Xu, H.Y. Kuang, and M.J. Lu. 2007. Oxidative damage to DNA and its relationship with diabetic complications. *Biomedical and Environmental Sciences* 20: 160–163.

Paraskevas, G.P., E. Kapaki, O. Petropoulou, M. Anagnostouli, V. Vagenas, and C. Papageorgiou. 2003. Plasma levels of antioxidant vitamins C and E are decreased in vascular parkinsonism. *Journal of the Neurological Sciences* 215: 51–55.

Pennathur, S., J.D. Wagner, C. Leeuwenburgh, K.N. Litwak, and J.W. Heinecke. 2001. A hydroxyl radical-like species oxidizes Cynomolgus monkey artery wall proteins in early diabetic vascular disease. *The Journal of Clinical Investigation* 107: 853–860.

Peron, G., D. Santarossa, D. Voinovich, S. Dall'Acqua, and S. Sut. 2018. Urine metabolomics shows an induction of fatty acids metabolism in healthy adult volunteers after supplementation with green coffee (*Coffea robusta L.*) bean extract. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology* 38: 74–83.

Pick, E., and Y. Keisari. 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *Journal of Immunological Methods* 38: 161–170.

Pico, C., F. Serra, A.M. Rodríguez, J. Keijer, and A. Palou. 2019. Biomarkers of nutrition and health: New tools for new approaches. *Nutrients* 11: 1092.

Playdon, M.C., S.C. Moore, A. Derkach, J. Reedy, A.F. Subar, J.N. Sampson, D. Albanes, F. Gu, J. Kontto, C. Lassale, L.M. Liao, S. Männistö, A.M. Mondul, S.J. Weinstein, M.L. Irwin, S.T. Mayne, and R. Stolzenberg-Solomon. 2017a. Identifying biomarkers of dietary patterns by using metabolomics. *The American Journal of Clinical Nutrition* 105: 450–465.

Playdon, M.C., R.G. Ziegler, J.N. Sampson, R. Stolzenberg-Solomon, H.J. Thompson, M.L. Irwin, S.T. Mayne, R.N. Hoover, and S.C. Moore. 2017b. Nutritional metabolomics and breast cancer risk in a prospective study. *The American Journal of Clinical Nutrition* 106: 637–649.

Purdel, N.C., D. Margina, and M. Ilie. 2014. Current methods used in the protein carbonyl assay. *Annual Research and Review in Biology* 4: 2015–2026.

Quesada, J.M., J.M. Mata-Granados, and M.D. Luque de Castro. 2004. Automated method for the determination of fat-soluble vitamins in serum. *The Journal of Steroid Biochemistry and Molecular Biology* 89–90: 473–477.

Raha, S., and B.H. Robinson. 2000. Mitochondria, oxygen free radicals, disease and ageing. *Trends in Biochemical Sciences* 25: 502–508.

Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, and C. Rice-Evans. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine* 26: 1231–1237.

Rice-Evans, C., and N.J. Miller. 1994. Total antioxidant status in plasma and body fluids. *Methods in Enzymology* 234: 279–293.

Roberts, L.J., and J.D. Morrow. 2000. Measurement of F(2)-isoprostanes as an index of oxidative stress in vivo. *Free Radical Biology & Medicine* 28: 505–513.

Robinson, W. 1935. Stimulation of healing in non-healing wounds by allantoin in maggot secretions and of wide biological distribution. *Journal of Bone and Joint Surgery* 17: 267–271.

Rodríguez-Delgado, M., J. Díaz-Flores Estévez, F. Díaz-Flores Estévez, C. Hernández Calzadilla, and C. Díaz Romero. 2002. Fast determination of retinol and α-tocopherol in plasma by LC. *Journal of Pharmaceutical and Biomedical Analysis* 28: 991–997.

Ross, M.A. 1994. Determination of ascorbic acid and uric acid in plasma by high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications* 657: 197–200.

Rossi, R., A. Milzani, I. Dalle-Donne, D. Giustarini, L. Lusini, R. Colombo, and P. Di Simplicio. 2002. Blood glutathione disulfide: *in vivo* factor or *in vitro* artifact? *Clinical Chemistry* 48: 742–753.

Roura, E., C. Andrés-Lacueva, R. Estruch, and R.M. Lamuela-Raventós. 2006. Total polyphenol intake estimated by a modified Folin-Ciocalteu assay of urine. *Clinical Chemistry* 52: 749–752.

Salminen, I., and G. Alfthan. 2008. Plasma ascorbic acid preparation and storage for epidemiological studies using TCA precipitation. *Clinical Biochemistry* 41: 723–727.

Sánchez-Rangel, J.C., J. Benavides, J.B. Heredia, L. Cisneros-Zevallos, and D.A. Jacobo-Velázquez. 2013. The Folin-Ciocalteu assay revisited: Improvement of its specificity for total phenolic content determination. *Analytical Methods* 21: 5990–5999.

Serafini, M., D. Villano, G. Spera, and N. Pellegrini. 2006. Redox molecules and cancer prevention: The importance of understanding the role of the antioxidant network. *Nutrition and Cancer* 56: 232–240.

Shacter, E., J.A. Williams, M. Lim, and R.L. Levine. 1994. Differential susceptibility of plasma proteins to oxidative modification: Examination by western blot immunoassay. *Free Radical Biology and Medicine* 17: 429–437.

Sies, H., and W. Stahl. 1995. Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. *The American Journal of Clinical Nutrition* 62: 1315S–1321S.

Singleton, V.L., and J.A. Rossi. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* 16: 144–158.

Sjödin, B., Y. Hellsten Westing, and F.S. Apple. 1990. Biochemical mechanisms for oxygen free radical formation during exercise. *Sports Medicine (Auckland, N.Z.)* 10: 236–254.

Stalmach, A., W. Mullen, D. Barron, K. Uchida, T. Yokota, C. Cavin, H. Steiling, G. Williamson, and A. Crozier. 2009. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: Identification of biomarkers of coffee consumption. *Drug Metabolism and Disposition* 37: 1749–1758.

Stalmach, A., G. Williamson, and A. Crozier. 2014. Impact of dose on the bioavailability of coffee chlorogenic acids in humans. *Food and Function* 5: 1727–1737.

Steinberg, F.M., and A. Chait. 1998. Antioxidant vitamin supplementation and lipid peroxidation in smokers. *The American Journal of Clinical Nutrition* 68: 319–327.

Stocker, R., Y. Yamamoto, A.F. McDonagh, A.N. Glazer, and B.N. Ames. 1987. Bilirubin is an antioxidant of possible physiological importance. *Science* 235: 1043–1046.

Suarez-Pinzon, W.L., K. Strynadka, and A. Rabinovitch. 1996. Destruction of rat pancreatic islet beta-cells by cytokines involves the production of cytotoxic aldehydes. *Endocrinology* 137: 5290–5296.

Suzuki, Y. 1997. Determination of total bilirubin in serum by a color reaction using *p*-dimethylaminobenzaldehyde. *Analytical Sciences* 13: 291–294.

Szczuko, M., V. Hawryłkowicz, J. Kikut, and A. Drozd. 2020. The implications of vitamin content in the plasma in reference to the parameters of carbohydrate metabolism and hormone and lipid profiles in PCOS. *The Journal of Steroid Biochemistry and Molecular Biology* 198: 105570.

Szeto, Y.T., T.C.Y. Kwok, and I.F.F. Benzie. 2004. Effects of a long-term vegetarian diet on biomarkers of antioxidant status and cardiovascular disease risk. *Nutrition* 20: 863–866.

Tamarit, J., A. de Hoogh, E. Obis, D. Alsina, E. Cabiscol, and J. Ros. 2012. Analysis of oxidative stress-induced protein carbonylation using fluorescent hydrazides. *Journal of Proteomics* 75: 3778–3788.

Uchendu, C., S.F. Amtali, and J.O. Ayo. 2012. The organophosphate, chlorpyrifos, oxidative stress and the role of some antioxidant: A review. *African Journal of Agricultural Research* 7: 2720–2728.

Valavanidis, A., T. Vlachogianni, K. Fiotakis, and S. Loridas. 2013. Pulmonary oxidative stress, inflammation and cancer: Respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. *International Journal of Environmental Research and Public Health* 10: 3886–3907.

Vanin, A.F., A. Huisman, and E.E. van Faassen. 2002. Iron dithiocarbamate as spin trap for nitric oxide detection: Pitfalls and successes. *Methods in Enzymology* 359: 27–42.

Vipin, A.V., K. Raksha Rao, N.K. Kurrey, K.A. Anu Appaiah, and G. Venkateswaran. 2017. Protective effects of phenolics rich extract of ginger against Aflatoxin B1-induced oxidative stress and hepatotoxicity. *Biomedicine & Pharmacotherphy* 91: 415–424.

Vivekanadan-Giri, A., J.H. Wang, J. Byun, and S. Pennathur. 2008. Mass spectrometric quantification of amino acid oxidation products identifies oxidative mechanisms of diabetic end-organ damage. *Reviews in Endocrine and Metabolic Disorders* 9: 275–287.

Vivekanandan-Giri, A., J. Byun, and S. Pennathur. 2011. Quantitative analysis of amino acid oxidation markers by tandem mass spectrometry. *Methods in Enzymology* 491: 73–89.

Vuilleumier, J.P., and E. Keck. 1989. Fluorometric assay of vitamin C in biological materials using a centrifugal analyser with fluorescence attachment. *Journal of Micronutrient Analysis* 5: 25–34.

Wang, F., Z. Shang, L. Xu, Z. Wang, W. Zhao, X. Mei, J. Lu, and J.Y. Zhang. 2018. Profiling and identification of chlorogenic acid metabolites in rats by ultra-high-performance liquid chromatography coupled with linear ion trap-Orbitrap mass spectrometer. *Xenobiotica* 48: 605–617.

Washko, P.W., R.W. Welch, K.R. Dhariwal, Y. Wang, and M. Levine. 1992. Ascorbic acid and dehydroascorbic acid analyses in biological samples. *Analytical Biochemistry* 204: 1–14.

Wasowicz, W., J. Nève, and A. Peretz. 1993. Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum: Importance of extraction pH and influence of sample preservation and storage. *Clinical Chemistry* 39: 2522–2526.

Weber, D., M.J. Davies, and T. Grune. 2015. Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: Focus on sample preparation and derivatization conditions. *Redox Biology* 5: 367–380.

Weydert, C.J., and J.J. Cullen. 2010. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nature Protocols* 5: 51–66.

Whalley, L.J., R.T. Staff, A.D. Murray, S.J. Duthie, A.R. Collins, H.A. Lemmon, J.M. Starr, and I.J. Deary. 2003. Plasma vitamin C, cholesterol and homocysteine are associated with grey matter volume determined by MRI in non-demented old people. *Neuroscience Letters* 341: 173–176.

Wills, M., G. Hammond, and L.J. Calton. 2019. Simultaneous analysis of vitamin A and E in serum using LC-MS/MS for clinical research. Abstracts. *Clinica Chimica Acta* 493: S619–S639.

Wiseman, H. 1999. The bioavailability of non-nutrient plant factors: Dietary flavonoids and phytoestrogens. *The Proceedings of the Nutrition Society* 58: 139–146.

Witmer, J.R., B.J. Wetherell, B.A. Wagner, J. Du, J.J. Cullen, and G.R. Buettner. 2016. Direct spectrophotometric measurement of supra-physiological levels of ascorbate in plasma. *Redox Biology* 8: 298–304.

World Health Organization (WHO). 2001. *Biomarkers in risk assessment: validity and validation*. Geneva: WHO.

Yamaji, Y., Y. Nakazato, N. Oshima, M. Hayashi, and T. Saruta. 2004. Oxidative stress induced by iron released from transferrin in low pH peritoneal dialysis solution. *Nephrology, Dialysis, Transplantation* 19: 2592–2597.

Yang, J., F. Lv, X. Chen, W. Cui, L. Chen, X. Wen, and Q. Wang. 2013. Pharmacokinetic study of major bioactive components in rats after oral administration of extract of *Ilex hainanensis* by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 77: 21–28.

Yang, R., H. Xiu, Q. Zhou, L. Sun, H. Mu, H. Li, S. Wang, X. Li, W. Chen, and J. Dong. 2019. Application of urinary polyphenol biomarkers measured by liquid chromatography tandem mass spectrometry to assess polyphenol intake and their association with overweight and obesity in free-living healthy subjects. *Oxidative Medicine and Cellular Longevity* 2019: 1–11.

Yasui, M., Y. Kanemaru, N. Kamoshita, T. Suzuki, T. Arakawa, and M. Honma. 2014. Tracing the fates of site-specifically introduced DNA adducts in the human genome. *DNA Repair* 15: 11–20.

Young, E.G., H.P. Wentworth, and W.W. Hawkins. 1944. The absorption and excretion of allantoin in mammals. *Journal of Pharmacology and Experimental Therapeutics* 81: 1–9.

Yui, K., N. Tanuma, H. Yamada, and Y. Kawasaki. 2017. Reduced endogenous urinary total antioxidant power and its relation of plasma antioxidant activity of superoxide dismutase in individuals with autism spectrum disorder. *International Journal of Developmental Neuroscience* 60: 70–77.

Zabel, M., A. Nackenoff, W.M. Kirsch, F.E. Harrison, G. Perry, and M. Schrag. 2018. Markers of oxidative damage to lipids, nucleic acids and proteins and antioxidant enzymes activities in Alzheimer's disease brain: A meta-analysis in human pathological specimens. *Free Radical Biology and Medicine* 115: 351–360.

Zelen, I., P. Djurdjevic, S. Popovic, M. Stojanovic, V. Jakovljevic, S. Radivojevic, D. Baskic, and N. Arsenijevic. 2010. Antioxidant enzymes activities and plasma levels of oxidative stress markers in B-chronic lymphocytic leukemia patients. *Journal of B.U.ON.* 15: 330–336.

Zhang, J., M. Chen, W. Ju, S. Liu, M. Xu, J. Chu, and T. Wu. 2010. Liquid chromatograph/tandem mass spectrometry assay for the simultaneous determination of chlorogenic acid and cinnamic acid in plasma and its application to a pharmacokinetic study. *Journal of Pharmaceutical and Biomedical Analysis* 51: 685–690.

Zhou, M., Z. Diwu, N. Panchuk-Voloshina, and R.P. Haugland. 1997. A stable non-fluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: Applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Analytical Biochemistry* 253: 162–168.

Zhou, J., B. Cai, Y.P. Jang, S. Pachydaki, A.M. Schmidt, and J.R. Sparrow. 2005. Mechanisms for the induction of HNE- MDA- and AGE-adducts, RAGE and VEGF in retinal pigment epithelial cells. *Experimental Eye Research* 80: 567–580.