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Verfasser:	Barbara Stadlmayr
Matrikel-Nummer:	0204284
Studienrichtung (lt. Studienblatt):	Ernährungswissenschaften A 474
Betreuer:	Ao. Univ.-Prof. Mag. Dr. Karl-Heinz Wagner

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List of Abbreviations

(8-OHdG) ..... 8-hydroxy-2´-deoxyguanosine
$ABTS^{\bullet+}$ ..... 2,2-Azinobis-(3-ethylbenzothiazoline-6-sulphonate)
H_2O_2 ..... Hydrogen peroxide
$HO_2^{\bullet}$ ..... Hydroperoxyl radical
$O_2^{-\bullet}$ ..... Superoxide
$OH^{\bullet}$ ..... Hydroxyl radical
$ROO^{\bullet}$ ..... Peroxyl radical
$ROOH^{\bullet}$ ..... Lipid peroxide
AAPH ..... 2,2‘-Azobis(2–amidinopropane)dihydrochloride
AO ..... Antioxidant
AOPP ..... Advanced oxidation protein products
AUC ..... Area under the curve
CAT ..... Catalase
CD ..... Conjugated dienes
CV% ..... Coefficient of variation
DPPH ..... Diphenylpicrylhydrazyl radical scavening capacity
EDTA ..... Ethylenediaminetetraacetic acid
FL ..... Fluorescence
FRAP ..... Ferric reducing ability of plasma
GPX ..... Glutathione peroxidase
GSH ..... Glutathione
GSSG ..... Glutathione disulfide
HAT ..... Hydrogen atom transfer
HSP ..... Heat shock proteins
LOD ..... Limit of detection
LOQ ..... Limit of quantitation
MDA ..... Malondialdehyde
NADH ..... Nicotinamide adenine dinucleotide
NADPH ..... Nicotinamide adenine dinucleotide phosphate
Net AUC ..... Net area under the curve

oxLDL ..... Oxidised low-density lipoprotein
PUFA ..... Polyunsaturated fatty acids
RDA ..... Recommened daily allowance
RMCD ..... Randolmy methylated β-cyclodextrin
RNS ..... Reactive nitrogen species
ROS ..... Reactive oxygen species
SD ..... Standard deviation
SET ..... Single electron transfer
SOD ..... Superoxide dismutase
TBARS ..... Thiobarbituric acid reactive substances
TE ..... Trolox equivalent
TEAC ..... Trolox equivalent antioxidant capacity
TOSC ..... Total oxidant scavenging capacity
TPTZ ..... 2,4,6-tripyridyl-s-triazine complex
TRAP ..... Total radical trapping antioxidant parameter

1 Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in metabolic and physical processes [Dröge, 2002]. The formation of ROS per se is not detrimental. It is essential for the optimal function of metabolic processes, muscular contraction and immune defense [König et al., 2001]. Next to the normal appearance of radicals, exogenic factors such as ambient pollutants, ozone, UV-radition and smoke promote their formation [Berg and König, 2000]. Moreover it is claimed that exhaustive exercise is linked with an augmented production of ROS. Due to the enhancement of endogenous antioxidant system and the reduction of free radical production regular training, on the contrary leads to a better tolerance against oxidative stress [Bloch and Schmidt, 2004]. Antioxidants decrease the extent of oxidative damage by generating a less active radical or by minimizing the destroying free radical chain reaction [Knez et al., 2006].

Antioxidants have thus gained increasing importance in recent years. It is of great interest to the general public, medical and nutritional experts, health and food science researchers to be informed of the antioxidant capacity in biological samples and foods [Magalhaes et al., 2008; Somogyi et al., 2007]. Due to the complexity of the composition of foods and biological fluids, separating and analysing each antioxidant individually is expensive and time-consuming. Moreover the possible synergistic interactions among the compounds can not be determined. Therefore it is desirable for researchers to have an appropriate method for a rapid and reliable quantitation of antioxidant effectiveness [Ghiselli et al., 2000; Huang et al., 2005; Prior et al., 2005].

The present thesis will focus on the following issues:

The first objective of the thesis was to apply the oxygen radical absorbance capacity (ORAC) assay to a microplate fluorescence reader and manual performance. The ORAC assay, based on the early works of Glazer [1990] and Ghiselli et al. [1995], further developed by Cao et al. [1993] and then modified several times

by Ou et al. [2001]; Huang et al. [2005]; Prior et al. [2005], presents an innovative test to determine the antioxidant capacity of biological fluids and food samples.
A single assay would not be sufficient for validating the total antioxidant capacity of a sample, because a single test cannot affect all the different antioxidants [Magalhaes et al., 2008]. Therefore, the ORAC assay was compared with the FRAP assay and TEAC assay, two tests that were conducted for assessing the antioxidant capacity. It is important to note in this context that the ORAC, TEAC and FRAP assays are based on different biochemical principles.

The second purpose of the thesis was part of the the Austrian Science Fund-project "Risk assessment of Ironman triathlon participants". The project´s aim was to obtain more clarification of the biochemical, physiological and molecular biological effects of a single bout of ultra-endurance exercise in highly trained athletes, whether they are at health risk or not. The determination of the antioxidant capacity via ORAC assay, in the plasma of the 42 Ironman-triathletes was performed in order to give a more complete picture on the complexity of radicals and antioxidants. Furthermore correlations between the ORAC values and several parameters (training parameters, stress parameters, antioxidants) were examined.

2 Literature Review

2.1 Radicals, antioxidants, oxidative stress

Radicals

Free radicals are molecules or fragments of molecules with at least one unpaired electron in the outer orbital [Powers et al., 1999]. Due to their molecule instability, they are highly reactive and tend to catch an electron from other molecules to gain chemical stability. The attacked molecule turns into a free radical itself, resulting in a chain reaction and consequently disrupting the integrity of cells [Clarkson and Thompson, 2000].

Major types of free radicals in living organism belong to the reactive oxygen species (ROS) and to the reactive nitrogen species (RNS) [Dröge, 2002].

ROS include free radicals, such as superoxide ($O_2^{-\cdot}$), or hydroxyl radical (OH^-), as well as reactive forms of oxygens like hydrogen peroxide (H_2O_2) [Ji, 1999]. The formation of ROS per se is not detrimental. Indeed, it is basic for proper functioning of metabolic processes, muscular contraction and immune defense [König et al., 2001]. Next to the normal appearance of radicals during metabolism (mitochondria, xanthinoxidase, neutrophils) exogenic factors (ambient pollutant, ozone, UV-radition, smoke) promote their formation [Berg and König, 2000]. In case of lacking or insufficient inactivation of radicals, oxidative stress with damage or destruction to cellular macromolecules such as lipids, proteins, nucleic acids and components of the extracellular matrix may appear [König et al., 2001].

Antioxidants

An antioxidant is any substance that decreases the extent of oxidative damage by generating a less active radical or by minimizing the destroying free radical chain reaction [Knez et al., 2006]. The organism possesses a complex protection, the antioxidant system, to limit the detrimental effects of free radicals and ROS. This system is divided into an enzymatic (catalase, glutathione peroxidase, superoxide dismutase) and non-enzymatic defense [Finaud et al., 2006]. Non enzymatic antioxidants include vitamins and provitamins, flavonoids and polyphenols, proteins

or peptides containing thiol groups and various other low molecular weight components such as ubiquinone and uric acid. Some of the non-enzymatic antioxidants are formed naturally in the body (glutathione, ubichinon, uric acid), whereas others (ascorbic acid, vitamin E, β-carotin) are required from the diet. Table 2.1 gives an overview of the main functions of some antioxidants [Powers et al., 2004].

Table 2.1: Antioxidant defense system

non-enzymatic defense	**function**
Vitamin E (α,-β,-γ,-δ-tocopherol)	principle antioxidant in lipophilic compartment, major chain-breaking antioxidant
Vitamin C (ascorbic acid)	water-soluble antioxidant, in cytosol and extracellular fluid, acting as radical scavenger, recycles vitamin E
β-carotin	lipid soluble antioxidant, primarily located in biological membranes scavenges several ROS due to its structural arrangement
low-molecular-weight antioxidants	
Glutathione	non-protein thiol in cells scavenges radicals directly via hydrogen atom donating recycling of antioxidants (vitamin E, ascorbic acid)
Ubiquinones	lipid–soluble quinone derivatives antioxidant effect due to phenol ring structure reacts with ROS and recycles vitamin E reduced forms act as efficient antioxidants
enzymatic defense	**function**
Superoxide-dismutase	dismutates superoxide radicals
Catalase	transforms hydrogen peroxide into water
Glutathione-peroxidase	removes hydrogen peroxide and organic hydroperoxides

Oxidative Stress

Oxidative stress is an undesirable condition, where pro-oxidant factors overwhelm antioxidant factors. Both determinants, oxidative exposure and antioxidant defense, are highly variable. Factors including dietary habit, lifestyle, age and genetic disposition act as key contributors to the oxidant-antioxidant balance [Sen, 2001]. Normally, the body can cope with free radicals, but if antioxidants are unavailable, or if the free-radical production becomes excessive, damage can occur. It is well known that long-term oxidative stress leads to chronic disease, fastens the altering process or supports the development of cancer, arteriosclerosis and other diseases [Berg and König, 2000].

2.2 Oxidative stress and physical activity

Physical exercise has generally a positive life prolonging effect and is beneficial to health [Sen, 2001]. Nevertheless, exhaustive exercise is linked with an augmented production of free radicals. Regular training on the contrary leads to a better tolerance against oxidative stress due to the enhancement of endogenous antioxidant system and the reduction of free radical production [Bloch and Schmidt, 2004]. Exercise training is furthermore accompanied by beneficial immunological changes. Training is suggested to strengthen muscle fibres and leads to protection against muscle damage. Moreover, neutrophils of trained persons possess a minimized ability to generate ROS [Knez et al., 2006].
Davies et al., 1982 were the first to demonstrate that exercise enhances free radical production. Since then multiple studies have analysed the effects of exercise and oxidative stress [Finaud et al., 2006]. Mastaloudis et al. [2001] observed increased generation of lipid peroxidation after extreme endurance exercise, whereas Margaritis et al. [1997] could not observe oxidative stress after a triathlon race in athletes. Inconsistencies between studies can be explained by the involvement of multiple factors for the formation and effect of free radicals, the complexity to detect free radicals in vivo, different methods used to assess oxidative stress parameters, the limited transferability of data from animal model to the human and the diversity of the study designs [Berg and König, 2000; Knez et al., 2006].

2.2.1 Sources of ROS formation during sports

Potential sources of exercise induced ROS formation depend on the differences in duration, intensity and type of physical activity. Furthermore various factors like the relative proportion of aerobic and anaerobic energy production, the diverse muscle groups involved, and the extent of damaged muscles have to be taken into account when judging the role of ROS production during sports. A variety of mechanisms responsible for the augmented ROS production during aerobic or anaerobic exercise exists. The most common ones are described in addition [König et al., 2001].

Mitochondrial electron transport chain

It is basically believed that the oxygen metabolism, occuring in the mitochondria, is associated with the formation of ROS. Generally, 95-99% of the consumed oxygen is reduced into water in the respiratory chain. 1-5 % of O_2 are transformed into superoxide radicals ($O_2 + e^- \longrightarrow O_2^{\cdot -}$) [Finaud et al., 2006]. Additionally, the protonation of superoxide generates the much stronger radical hy-

droperoxyl ($O_2^{\cdot -} + H_2O \longrightarrow HO_2^{\cdot} + OH$). Moreover, superoxide can act as a Bronsted base and modify the acid-base-equilibrium to formate hydrogen peroxide ($HO_2^{\cdot} + e^- + H \longrightarrow H_2O_2$). Hydrogen peroxide possesses the capacity to generate the highly potent hydroxyl radical ($H_2O_2 + e^- \longrightarrow OH^{\cdot} + OH^-$) [König et al., 2001]. These facts suggest that physical exercise, which is associated with an increase of oxygen consumption, results in augmentation of superoxide generation [Bloch and Schmidt, 2004]. Whole body oxygen consumption rises 15 to 20 fold during maximal aerobic exercise. Furthermore it is estimated that oxygen consumption within the working muscles may reach 100 times the resting level. Although it is generally believed that a link between the augmented oxygen consumption during exercise and the amplified ROS formation exists, a direct evidence in humans has not been provided so far [König et al., 2001].

Xanthine oxidase

Xanthine oxidase is an enzyme that occurs ubiquitary in the body. Under normal conditions the major part of xanthine oxidase exists in its reduced form. Xanthine dehydrogenase plays a basic role in the purine degradation to uric acid and applies NAD (nicotinamine adenine dinucleotide) as electron acceptor [Knez et al., 2006]. Exhaustive or anaerobic exercise can lead to hypoxic conditions [Finaud et al., 2006]. Under hypoxic conditions, xanthine dehydrogenase is transformed to xanthine oxidase via intracellular proteases requiring calcium for activation [König et al., 2001]. Xanthine oxidase uses O_2 alternatively to NAD as electron acceptor resulting in the generation of the superoxide radical during the converting process of hypoxanthine to xanthin and uric acid [Knez et al., 2006]. It is hypothesised that physical training resulting in muscular ischemia and disarray of calcium homeostasis results in an environment favoring ROS production via xanthine oxidase catalyzed reactions [Finaud et al., 2006].

Inflammatory processes

Strenuous physical exercise results in activation of various cell lines within the immune system. These include neutrophils, monocytes and macrophages, all capable of generating ROS. The strongest sources of ROS production are neutrophils, capable of generating superoxide radical $O_2^{\cdot -}$ and the potent oxidant hypochlorus acid [König et al., 2001].

The activity of ROS formating cells depends on various hormones and cytokines, acting in response to the intensity and duration of an exercise bout. When signals occur from diverse chemoattractants, neutrophils migrate from the blood to the local inflammatory sites [Knez et al., 2006]. Neutrophils which infiltrate into the

damaged muscle start to remove destroyed proteins and cell debris via specific lysozymes. Even though this is a desirable response, it may contribute to an excess of inflammation due to disposal of proinflammatory mediators including interleukins and prostaglandins. This results in the activation of further inflammatory cells and rising ROS production [König et al., 2001]. Due to the limited capacity of neutrophils to distinguish between foreign and host antigens, they are likely to release their toxic agents on normal host tissues, leading to additional inflammation and oxidative stress [Knez et al., 2006].

2.2.2 Exercise induced damages

Lipid Oxidation

Polyunsaturated fatty acids (PUFA) play an important role in the constitution of cell membrans [Finaud et al., 2006]. Du to their multiple unsaturated points, they are highly susceptible to ROS attack and oxidative damage. Oxidative damage of PUFA starts with the abstraction of a H-atom of a methylene ($-CH2$) group through ROS [Sen, 2001]. The attack of polyunsaturated fatty acids results in lipid peroxidation, a chain reaction which leads to the formation of further free radicals such as $ROO^{\cdot}$ and $ROOH^{\cdot}$. Peroxidation of membrane lipids can result in numerous modifications of cell functions including an augmented membrane permeability, a decreased Ca^{++} transport in the sarcoplasmic reticulum (SR), increased mitochondrial function and generation of toxic metabolites. In addition to direct local effects, lipid peroxidation produces toxic products, capable of starting more damage at distance [Dekkers et al., 1996]. Lipid peroxidation results in the breakdown of lipids and the generation of various primary (conjugated dienes (CD)) and secondary oxidation products (MDA, pentane) [Finaud et al., 2006]. Malondialdehyde (MDA), plasma conjugated dienes (CD) and plasma thiobarbituric acid-reactive substances (TBARS) are indices of lipid peroxidation, wheras MDA and TBARS as end products of lipid peroxidation are mostly used as biomarkers [Dekkers et al., 1996; Finaud et al., 2006].

Protein Oxidation

Protein oxidation is accompanied by a loss or fragmentation of amino acids as well as by an augmentation of protein carbonly groups. In order to rebuild amino acids, oxidised proteins are catabolised, but carbonly by-products are not able to get into this proceder. Consequently, proteolysis is inhibited and accumulation of oxidised proteins is the result [Finaud et al., 2006]. Oxidative alterations of proteins may influence essential cell-regulatory processes involving,receptor modification, interfer-

ence in intracellular ionic homeostasis and modified signal transduction [Sen, 2001]. Oxidised amino acids as well as protein carbonly groups are used as common indexes for the incidence of oxidative damage [Finaud et al., 2006].

DNA Oxidation

ROS are responsible for several types of DNA damage including strand breaks, DNA-protein cross-links and base alterations [Finaud et al., 2006]. Information referring to exercise induced oxidative DNA damage, however, are limited and results are controversial. Therefore, further investigations for clarification are needed [Sen, 2001]. Regardless of the constant repair system of the DNA, each part of the DNA is predisposed to oxidative damage, particularly if the capacity of the repair system is overstretched or if modifications of the repair process occured. Consequently, DNA oxidation may result in mutagenesis and may be able to contribute to human cancer and cell aging. At present plenty of methods are used for the quantification of DNA modification. A biomarker primarily used is the oxidised nucleotide 8-hydroxy-2-deoxyguanosine (8-OHdG), which is discharged via blood and urine [Finaud et al., 2006].

2.2.3 Antioxidant defense system and exercise

The defense ability of antioxidants is of great importance concerning the protection against oxidative damage [Ji, 1999]. Enzymatic as well as non-enzymatic antioxidants are important for the protection of tissues from excessive oxidative stress [Finaud et al., 2006]. Muscle cells contain an endogenous cellular defense system (enzymatic and non-enzymatic) for the protection against exercise-induced oxidative damage. Moreover exogenous dietary antioxidants interact with endogenous antioxidants to form a cooperative network of cellular antioxidants [Powers et al., 2004]. Beside the nutritional intake, the effectiveness of the antioxidant system depends also on the endogenous antioxidant enzyme formation, which can be changed through exercise, training, nutrition and aging [Finaud et al., 2006].

Vitamin E Vitamin E, the main lipophilic antioxidant in cell membranes, is referred to at least eight structural isomers of tocopherols or tocotrienols, whereas α-tocopherol has the most potent antioxidant activity [Powers et al., 2004; Clarkson and Thompson, 2000]. Due to its abundance in cells and mitochondrial membranes and its capacity to react directly with a variety of oxygen radicals including singlet oxygen, lipid peroxide products and superoxide radical, it is known as the most important chain breaking antioxidant [Finaud et al., 2006; Clarkson and Thompson,

2000]. The interaction of vitamin E with a radical, however, leads to a loss of functional vitamin E and a vitamin E radical is generated. The vitamin E radical can be recycled to its original form via other antioxidants including vitamin C, glutathione and α-lipoic acid [Powers et al., 2004].

Vitamin C Vitamin C or ascorbic acid exists under physiological pH predominant as ascorbate anion. It is located in the aqueous phase of cells and it is said to be the most important antioxidant in extracellular fluids [Finaud et al., 2006]. Next to its capacity to directly scavenge superoxide, hydroxyl and lipid hydroperoxide radicals, vitamin C is important in the recycling of vitamin E radical. In this process, reduced vitamin C is transformed to a vitamin C (semiascorbyl) radical, which can be recycled via NADH semiascorbyl reductase or cellular thiols such as glutathione. Vitamin C can, however, also act as pro-oxidant, in higher concentrations ($1\,\mathrm{mmol.1}^{-1}$ in the existence of transition metals such as Fe^{3+}+ Cu^{2+}). Thereby, Fe^{3+} gets reduced to Fe^{2+}, which is known to catalyse the generation of free radicals [Powers et al., 2004].

β-carotin β-carotin is a lipophilic pro-vitamin of vitamin A and is mainly located in the cell membranes. Its antioxidant character is based on its ability to deactivate ROS (in particular singlet oxygen and lipid radicals) and to decrease lipid peroxidation [Finaud et al., 2006].

Glutathione Glutathione GSH is the major non-protein thiol source in muscle cells, predominantly formed in the liver. Its content among the organs can vary extremely, depending on the radical generation [Powers et al., 2004]. Operating as a substrate for the GPX (glutathione peroxidase) as well as direct inhibition of ROS are important actions of GSH [Finaud et al., 2006]. Furthermore, glutathione is involved in the regeneration process of diverse antioxidants within the cells. This includes the reduction of vitamin E radicals, or recycling of vitamin C [Powers et al., 2004].

Ubiquinone Ubiquinones are lipophilic quinone derivates, whereby the predominant form in humans is ubiquinone-10, also known as coenzyme Q [Powers et al., 2004]. Coenyzme Q10, an endogenous molecule is essential for the ATP generation and particularly attendant in mitochondrial membrane. Its antioxidant potential is attributed to its direct detoxification of peroxyl radicals and indirect action by recycling vitamin E and C. Nevertheless, coenzyme Q10 also possesses pro-oxidant

potential as it acts as a mediator for gene expression and protein formation in the muscle and, thereby, generates $O_2^{-\cdot}$ [Finaud et al., 2006].

Heat shock proteins Heat shock proteins (HSP) are proteins possessing protective effects against diverse physiological stressors. Their antioxidant capacity is based on their protection of cells and intracellular proteins against free radical induced damage [Finaud et al., 2006].

Uric acid Uric acid as an end product of the purine metabolism is known to increase in plasma during intensive physical exercise. Due to its ability to diffuse into muscle, uric acid also plays an important role as antioxidant in muscle tissues. Uric acid in muscle and plasma belongs to one of the most important antioxidants that directly act against radicals and therefore it contributes to the protection of erythrocytes, cell membranes and DNA against free radical oxidation [Finaud et al., 2006].

Albumin, bilirubin Albumin (a thiol protein) belongs to the non specific chain breaking antioxidants by giving electrons to free radicals. Bilirubin, a biliary protein derived from haemoglobin, rises with oxidative stress and possesses antioxidant ability in body fluids [Finaud et al., 2006].

Enzymatic antioxidants

There exists growing evidence that endurance exercise training contributes to the elevation of antioxidant enzymes, including total SOD and GPX activity in skeletal muscles [Leeuwenburgh et al., 1997]. Whether high-intensity exercise training is superior to low-intensity exercise concerning the up-regulation of muscle SOD and GPX activities remains unclear. Furthermore it is suggested that the up-regulation of antioxidant enzymes is limited to highly oxidative skeletal muscles [Powers et al., 1999].

SOD (superoxide dismutase) acts as first defense line against oxidative stress by catalysing the dismutation of superoxide and the production of hydrogen peroxide.

$$2\,O_2^{\cdot -} + 2\,H^+ \longrightarrow H_2O_2 + O_2 \tag{2.1}$$

Magnesium as well as copper and zinc act as cofactor for the respective SOD enzyme form [Finaud et al., 2006]. Mn-SOD is primarily situated in the mitochondria, whereas Cu-Zn SOD principally appears in the cytosol [Powers et al., 1999].

GPX (glutathione peroxidase) is mainly located in the cytosol and mitochondria. It converts hydrogen peroxide into water in the presence of glutathione, which gets transformed into its oxidised form.

$$H_2O_2 + 2\,GSH \longrightarrow 2\,H_2O + GSSG \tag{2.2}$$

The regeneration of the oxidised glutathione is accomplished via the enzyme glutathione reductase using NADPH as coenzyme.

$$GSSG + NADPH \longrightarrow GSH^{+}NADP \tag{2.3}$$

The enzyme GPX is selenium dependent and occurs in one isoform. Due to its low specificity for hydroperoxides, GPX is able to reduce a wide range of hydroperoxides, including various complex organic hydroperoxides [Powers et al., 1999].

CAT (catalase) is particularly present in peroxysomes, cytosol and mitochondria. It transforms hydrogen peroxide into water and oxygen.

$$2\,H_2O_2 \longrightarrow O_2 + 2\,H_2O \tag{2.4}$$

CAT requires Fe^{3+} as cofactor. Concerning the common function with GPX to remove H_2O_2, the two enzymes differ in their affinity to hydrogen peroxide. At low H_2O_2 concentrations, GPX is more active in eliminating H_2O_2 from the cells [Powers et al., 1999].

Adaption of the antioxidant defense system

The antioxidant ability of organ systems is well coordinated to the rates of oxygen utilisation and radical formation. Tissues implying highest oxygen uptake like liver, kidney, brain and skeletal muscles also possess the most potent antioxidant enzyme activity [Powers et al., 1999]. Moreover it is known that the antioxidant defense systems possess the ability of adaption in response to chronic exposure to oxidants [König et al., 2001]. There is good evidence that regular physical exercise results in the up-regulation of antioxidant enzyme activities as reported in several studies [Radak et al., 1999; Toskulkao and Glinsukon, 1996; Robertson et al., 1991]. The training induced adaption is not only limited to the increase in the activity of antioxidant enzymes, but can also be traced back to a higher expression of enzymes [Wilson and Johnson, 2000]. An increase in vitamin E, vitamin C and uric acid has

been shown after endurance exercise [Neubauer et al., 2008]. This could be traced back to the mobilisation of vitamin E and C from their tissue stores as well as the generation of uric acid as an endpoint of the purine metabolism. However, both processes cannot be considered as specific training adaptions against oxidative stress, although they can contribute to the exercise induced increase in the antioxidant plasma capacity [Mastaloudis et al., 2001; Liu et al., 1999; Neubauer et al., 2008].

Antioxidant Supplementation

The fact that long-term oxidative stress results in chronic diseases raises the question, whether athletes due to permanent exposure to oxidative stress are at higher risk and whether antioxidant supplementation is necessary in order to avoid deficiencies [Berg and König, 2000]. Data exists which supports the fact that antioxidant supplementation has positive effects on athletes in order to prevent them from deficiencies and detrimental consequences of free radicals [Palazzetti et al., 2004; Mastaloudis et al., 2004]. However, large amounts of antioxidants are likely to have negative effects [Finaud et al., 2006]. The increase of cellular antioxidants due to supplementation may degrade the inflammatory response to muscle damaging exercise and thus lead to limited muscle regeneration. Moreover too high amounts of supplementation may weaken cellular adaptions induced by exercise [Powers et al., 2004]. Furthermore, antioxidants received through the diet due to their balanced biochemistry and synergistic interactions, should be preferred to isolated supplement antioxidant compounds [Finaud et al., 2006]. So far, there is no scientific evidence to recommend augmented amounts of antioxidants to physically active people [König et al., 2001].

2.3 Antioxidant capacity assays

Evidence for oxidative stress and damage can be derived from direct measurement of free radicals, from measurement of damage to lipids, proteins and DNA, as described in the previous section. A further approach to validate oxidative stress is the measurement of the antioxidant status of a sample [Powers et al., 1999].

Particularly in the last years the utilisation and measurement of the antioxidant capacity in physiological fluids, foods, beverages and natural products has received increasing attention. This interest derives from the confirmed importance of antioxidants to scavenge ROS/RNS [Huang et al., 2002b; Magalhaes et al., 2008]. The antioxidant capacity tests can help to identify the conditions that have an

impact on oxidative stress in vivo, conditions like exposure to ROS and antioxidant supplementation. Moreover determining plasma antioxidant capacity is an approach to provide knowledge about absorption and bioavailability of nutrients due to variations of plasma antioxidant capacity after supplementation or after ingestion of antioxidant rich food [Ghiselli et al., 2000].
Due to the complexity of the composition of biological fluids and foods it would be expensive and time consuming to separate and analyse each antioxidant individually. Moreover the possible synergistic interactions among the compounds cannot be determined. Therefore it is desirable to researchers to have an appropriate method for a rapid quantitation of antioxidant effectiveness [Ghiselli et al., 2000; Huang et al., 2005; Prior et al., 2005]. The antioxidant capacity assays examine the cumulative action of antioxidants. Consequently, they provide an integrated parameter as they assess the synergistic interactions [Magalhaes et al., 2008]. Moreover the tests help to spare a lot of time, because generally they do not request complicated techniques and with the application of microplates, high sample throughputs are possible [Erel, 2004; Huang et al., 2002b].

Various terms exist for the measured antioxidant parameter in the literature, most frequently used include antioxidant capacity, antioxidant activity or antioxidant power [Erel, 2004]. Due to the existence of a variety of assays, it is quite difficult to compare the results of the different tests, because different probes, reaction conditions and quantitation methods are used [Huang et al., 2005]. Frankel and Meyer demonstrated the problematic of using one-dimensional methods to evaluate multifunctional food and biological antioxidants [Frankel and Meyer, 2000]. A standard assay would be needed to reliably measure the antioxidant capacity of various samples [Huang et al., 2005]. According to Prior et al. [2005] a standard assay should encompass the subsequent requirements: (a) determination of the chemistry actually present in potential applications, (b) employment of a biologically relevant radical source, (c) simplicity, (d) utilisation of a method with a defined endpoint and chemical mechanisms, (e) availability of the instrumentation, (f) good reproducibility, (g) adaption to determine hydrophilic and lipophilic antioxidants and utilisation of various radical sources, (h) adaption to high throughput analyses.

Due to the various differences in the assays, no single measurement of antioxidant status is sufficient, but a variety of assays are necessary to assess the antioxidant capacity appropriately. [Prior and Cao, 1999].

2.3.1 Chemical background

Regarding the chemical reactions involved in the assays, the majority of the antioxidant capacity tests can be classified into the two categories: Hydrogen-atom-transfer (HAT) and Single-electron-transfer (SET). Both mechanisms can appear in the same sample. The assays are intended to determine the radical scavenging capacity and not the preventive antioxidant capacity [Huang et al., 2005].

HAT based assays

Hydrogen-atom-transfer-reaction-based assays quantify the capability of an antioxidant to quench free radicals via hydrogen donation [Prior et al., 2005].

$$X^{\cdot} + AH \longrightarrow XH + A^{\cdot} \tag{2.5}$$

In general HAT based methods apply (a) a synthetic free radical generator to cause a continual flux of peroxyl radicals, (b) an oxidisable molecule probe to monitor the reaction progress and (c) an antioxidant to compete with the molecule probe for the generated radicals and consequently leading to inhibition or delay of the reaction [Huang et al., 2005; Somogyi et al., 2007]. Moreover this mechanism is pH independent and mainly quite rapid, finished within seconds or minutes [Prior et al., 2005]. The quantitation is obtained by the kinetic curves [Huang et al., 2005]. Examples for HAT based assays include the oxygen radical absorbance capacity assay (ORAC), the total radical trapping antioxidant parameter assay (TRAP) and the total oxidant scavenging capacity method (TOSC).

SET based assays

Single-electron-transfer-reaction-based assays measure the capacity of antioxidants in the reduction of an oxidant, which results in color change. The degree of color change is proportional to the concentration of antioxidants present in the sample. The endpoint of the reaction is attained when there is no more color change. The reaction mixture is based on the subsequent electron transfer reaction between oxidant and antioxidant:

$$\text{probe(oxidant)} + e^{-}(\text{fromAO}) \longrightarrow \text{reduced probe} + \text{oxidised antioxidant} \tag{2.6}$$

SET based methods can be conducted under different pH conditions, acidic, neutral and basic. The pH values have an important impact on the reducing capacity of antioxidants. A suppression of the reducing capacity may occur in an acidic milieu due to a preferred protonation of antioxidants. Basic conditions however may lead to an

augmentation of the reducing capacity. There is no competitive reaction involved, and no oxygen radical occurs, therefore the antioxidant capacity is equal to the reducing capacity which reflects one aspect of the antioxidant property [Huang et al., 2005]. Usually theses assays are slow, need long times to reach completion and are very sensitive to ascorbic acid and uric acid [Prior et al., 2005]. For obtaining results the antioxidant concentration is plotted against the change of absorbance to give a linear curve [Huang et al., 2005]. Test belonging to the SET based mechanism are the trolox equivalence antioxidant capacity assay (TEAC), the ferric ion reducing antioxidant power assay (FRAP) and the diphenyl-1-picrylhydrazyl test (DPPH).

A magnitude of assays exist, table 2.2 lists the most representative ones. The main focus of this thesis is on the ORAC assay. In addition the FRAP and TEAC assays are described in more detail, because these tests were all used for analysing the plasma samples of our study collective. We examined whether different methods provide comparable results for the same samples. Results and correlations of the applied assays are described in subsection 4.3.3.

Table 2.2: In vitro capacity assays [Magalhaes et al., 2008; Somogyi et al., 2007; Prior et al., 2005]

Assay	Mechanism	Principle Measurement	Quantification	Related articles
ORAC	HAT	Oxidation of the probe (fluorescein) leads to fluorescence decay that can be delayed by antioxidants	Lag time, AUC	[Cao et al., 1993], [Ghiselli et al., 1995], [Huang et al., 2002b]
TRAP	HAT	Oxidation of β-PE leads to fluorescence decay that can be delayed by antioxidants	Lag time	[Wayner et al., 1985]
TOSC	HAT	Ethylene, generated by the oxidation of KMBA, is measured along time using GC-FID	AUC, DT50	[Winston et al., 1998]
TEAC	HAT/SET	ABTS radical gets reduced by antioxidants, resulting in absorbance decrease	Δ Optical density	[Miller et al., 1993], [Re et al., 1999], [Van Den Berg et al., 1999]
FRAP	SET	Antioxidants reduce ferric to ferrous ion resulting in colored ferrous TPTZ complex	Δ Optical density	[Benzie and Strain, 1996], [Benzie and Strain, 1999]
DPPH	SET	DPPH radical gets reduced by antioxidants, leading to an absorbance decrease	Δ Optical density	[Brand-Williams et al., 1995]

β-PE:β-phycoerythrin, KMBA:α-keto-γ-methiolbutyric acid, GC-FID:gas chromatography with flame ionisation detector

In validating these assays, one also has to point out the critical aspects of such methods. All the tests listed above are based on chemical reactions in vitro. Therefore, there is no equivalence to biological systems [Huang et al., 2005]. Moreover the concentration of target species in the tests is usually smaller than the concentration of antioxidants. This is contradictory to in vivo conditions, where the antioxidant concentration is smaller than that of the oxidisable substrate (lipids, proteins, DNA) [Magalhaes et al., 2008]. Comparing the assays with regard to their chemical background, the HAT-based mechanism is to prefer to the SET reaction, because the peroxyl radical performed in the HAT-based assays is the superior free radical of lipid oxidation in food and biological systems [Prior et al., 2005].

Nevertheless, the tests are superior to the measurement of single antioxidants, as they assess possible synergistic interactions among the various antioxidants. Moreover we have to keep in mind that these test are yet to be developed and, therefore, it is obvious that further research and improvements are needed. An approach for improving the methods would be the application of additional radical sources with biological relevance like hydroxyl radicals, superoxide, and peroxynitrite, as well as modifications as close as possible in reaction conditions (concentration, reaction time, pH) to a physiological milieu [Prior et al., 2005; Magalhaes et al., 2008].

2.3.2 FRAP assay

The FRAP assay was initially developed by Benzie and Strain for the determination of the reducing power in plasma [Benzie and Strain, 1996]. Afterwards the method has been modified to measure antioxidants in botanicals [Ou et al., 2002; Benzie and Strain, 1999]. For more detail concerning the FRAP assay, including principle, materials, methods and discussion please see the thesis of Judit Valentini:"Entwicklung einer Mikrotiterplatten basierten Methode zur Bestimmung der ferric reducing ability of plasma (FRAP)".

Principle

FRAP belongs to the single electron based (SET-based) methods and measures the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}. The reduction of ferric to ferrous ion in acidic pH conditions leads to the colored ferrous 2,4,6-tripyridyl-s-triazine complex (TPTZ) [Benzie and Strain, 1996].

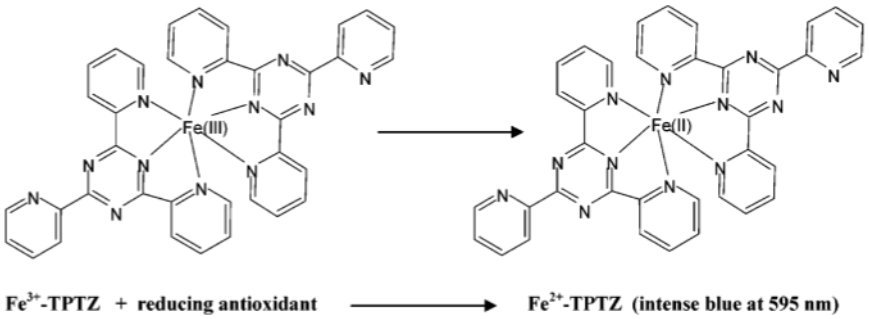

Figure 2.1: Reaction of FRAP, [Huang et al., 2005]

Characteristics
Reaction conditions include (a) a pH value of 3.6, (b) a reaction temperature of 37℃, (c) an absorption maximum of 593 nm and (d) an endpoint of the reaction after 4 minutes. The acidic condition is needed to maintain the iron stability [Prior et al., 2005].

The antioxidant capacity is equivalent to the reducing capacity

Comparing the absorbance change in the samples with those containing ferrous ions in known concentrations gives the FRAP value [Benzie and Strain, 1996].
A FRAP unit is defined as the reduction of 1 mol of Fe^{3+} to Fe^{2+} [Huang et al., 2005]. Several antioxidants, however, show differences in the relative activity, referred to as the reaction of Fe^{2+}. The activity values, shown in table 2.3, suggest that 1 mol of ascorbic acid, α-tocopherol and uric acid are able to reduce 2 mol of Fe^{3+} to Fe^{2+}, and 1 mol bilirubin could thus reduce 4 mol Fe^{3+} [Benzie and Strain, 1996]. This is controversial to the fact that bilirubin as well as the other antioxidants mentioned are two electron reductants [Huang et al., 2005]. Comparing the estimated relative contribution of various antioxidants to the FRAP value of fresh plasma, table 2.3 gives an overview. A significant correlation between FRAP values and plasma uric acid concentrations is often observed in diverse studies [Benzie and Strain, 1999; Cao and Prior, 1998]. This characteristic could also be shown in our investigations, where the correlation between FRAP values and uric acid was highly significant. Nevertheless the physiological influence of uric acid as the major antioxidant in plasma within the FRAP assay requests additional research [Benzie and Strain, 1996].

Advantages/Disadvantages The FRAP assay is fast, simple and inexpensive. Moreover this test can be carried out as an automated, semiautomated or man-

Table 2.3: Relative activity and estimated % contribution of antioxidants to FRAP

Plasma antioxidant	relative activity	estimated % contribution
Ascorbic acid	2.0	15
α-Tocopherol	2.0	5
Uric acid	2.0	60
Bilirubin	4.0	5
Protein	0.10	10
Others	-	5

ual method [Prior et al., 2005]. Single antioxidants in pure solutions as well as mixtures of antioxidants in plasma and aqueous solutions can be detected [Benzie and Strain, 1996]. A drawback of the FRAP assay concerns its quantitation, as a single–point endpoint does not represent a completed reaction [Prior et al., 2005]. It has to be taken into account that not all antioxidants are able to reduce Fe^{3+}in the timeframe of 4 minutes. Some polyphenols including caffeic acid, tannic acid and quercetin as well as thiol compounds were observed to react more slowly with Fe^{3+}TPTZ, requiring several hours. In this case the FRAP assay is inappropriate due to the long reaction time and incorrect measurement. [Ou et al., 2002; Firuzi et al., 2005; Pulido et al., 2000]. Furthermore compounds including thiols and proteins that quench radicals differently (H transfer) cannot be measured [Prior et al., 2005]. Another limitation concerns the chosen absorption maximum of 593 nm. Other substances exist such as the oxidation product of bilirubin, biliverdin, which possesses a strong absorption at 593 nm and thus leads to interferences [Ou et al., 2002]. The FRAP assay determines the reducing ability based on ferric ion. There is no oxygen involved, and the pH milieu is acidic, hence this approach does not represent in vivo conditions [Huang et al., 2005; Prior et al., 2005].
Nevertheless, one has to point out the advantages: as already mentioned, the procedure is fast and simple. In addition the results are highly reducible, the chemicals are inexpensive and the apparatus needed is available in every laboratory. Therefore, the FRAP assay can be judged as an attractive and potentially useful method. In particular the combination with methods possessing other mechanisms is advisable to give a more complete picture [Benzie and Strain, 1996].

2.3.3 TEAC assay

The TEAC assay was first developed by Miller et al. [1993]. Further modifications of the test followed by Van Den Berg et al. [1999] and Re et al. [1999].

Principle

The TEAC assay is generally grouped to the SET based methods, whereas in fact the radical $ABTS^{\cdot+}$ can be eliminated via electron transfer as well as via H atom transfer. Therefore the assay includes both mechansim, HAT and SET [Prior et al., 2005]. The assay is based on the capacity of antioxidants to scavenge the long-life radical anion $ABTS^{\cdot+}$ (2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate)). ABTS gets oxidised to its radical form $ABTS^{\cdot+}$ via peroxyl radicals or other oxidants. As the $ABTS^{\cdot+}$ radical formation starts, the absorbance increases [Somogyi et al., 2007]. The antioxidant capacity is determined by the ability of the sample to decrease the color, which is achieved by the direct interaction with the $ABTS^{\cdot+}$ radical [Prior et al., 2005].

Characteristics

Concerning the the assay conditions, various applications have been designed for the $ABTS^{\cdot+}$ formation, the appropriate wavelength and for the quantification [Magalhaes et al., 2008]. The formation of the $ABTS^{\cdot+}$ radical can be achieved (a) due to chemical reaction applying manganese dioxide, AAPH, or potassium persulfate(b) via enzymatic reaction using metmyoglobin, or (c) due to electrochemical formation [Miller et al., 1996; Van Den Berg et al., 1999; Re et al., 1999]. Enzymatic formation requests less time and in general milder reaction conditions as chemical formation [Prior et al., 2005]. The absorption maxima of the $ABTS^{\cdot+}$ radical include the wavelengths at 414, 645, 734 and 815 nm. The most frequent used wavelengths are 415 nm and 734 nm [Magalhaes et al., 2008]. Regarding the quantification, mostly the absorbance decrease of $ABTS^{\cdot+}$ in the presence of the sample or trolox at fixed time point (4-6min) is measured. The antioxidant capacity is expressed as Trolox equivalents [Prior et al., 2005].

Advantages/Disadvantages In the very first version of the TEAC assay metmyoglobin and H_2O_2 led to the generation of ferrylmyoglobin, which reacted afterwards with ABTS to create its radical form $ABTS^{\cdot+}$ [Miller et al., 1993]. The addition of the sample to the reaction milieu before the radical generation was criticised due to the fact that the antioxidants present in the sample were able to react with the oxidising part and therefore resulting in overestimation of the TEAC value [Prior et al., 2005]. Modifications of the assay also include the post addition of the sample to the reaction milieu in order to prevent troublesome interactions [Re et al., 1999; Van Den Berg et al., 1999].

From its technological point of view, the TEAC assay is rather simple, which allows the application for routine analysis [Huang et al., 2005]. The assay has been

used for the determination of various compounds and food samples [Miller et al., 1996]. Moreover TEAC has been adapted to microplates [Erel, 2004]. The $ABTS^{\cdot+}$ radical possesses solubility in water and organic milieu. This characteristic permits the determination of hydrophilic and lipophilic samples. A drawback of the assay concerns the radical $ABTS^{\cdot+}$ itself, as it does not represent a physiological radical source [Magalhaes et al., 2008]. Concerning the quantitation of the assay, endpoints with short durations of 4 or 6 minutes can result in underestimation of TEAC values due to the fact that the reaction is not finished at this time. Nevertheless, a ranking order of antioxidants can be provided by this assay [Prior et al., 2005].

3 Materials and Methods

The ORAC assay which is based on the early works of Glazer [1990] and Ghiselli et al. [1995], was further developed by Cao et al. [1993]. Afterwards it has been modified several times. Important improvement was performed by Ou et al. [2001] when applying fluorescein instead of B-phycoerythrin as the new fluorescent probe. B-phycoerythrin is a fluorescent protein which showed several shortcomings including (a) a large lot-to-lot variability in the reactivity to peroxyl radicals, (b) a photoinstability and (c) interactions with polyphenols resulting in a loss of fluorescence even without radical generator. Therefore fluorescein as a photostable fluorescent probe is now the medium of choice [Huang et al., 2005; Prior et al., 2005]. The initial versions of the ORAC assay, however, were time consuming and labor intensive, in particular for the determination of large amounts of samples. Later on the ORAC assay was semiautomated via adaption to a COBAS FARAII analyzer. The instrument is unfortunately discontinued by the manufacturer. Since then Huang et al. [2002b] have optimized the assay to a high-throughput format providing a fully automated assay from sample preparation to the final measurement. The utilised platform consists of a multichannel liquid handling system and a microplate fluorescence reader in 96- well format [Huang et al., 2002b]. Recent modifications to this assay further include the ability to determine the lipophilic, hydrophilic and total antioxidant capacity of a substance. Huang et al. [2002a] expanded the ORAC assay to lipophilic antioxidants by using 7% RMCD (randolmy methylated β-cyclodextrin) in a 50% acetone H_2O mixture-as the solubility enhancer. In the version of Prior et al. [2003] lipophilic and hydrophilic compounds are separated via different extraction conditions but the same radical generator AAPH is used. The ORAC assay possesses a wide range of applications including food samples, dietary supplements and biological samples [Cao and Prior, 1998; Otaolaurruchi et al., 2007; Ou et al., 2002]. The following chapters describe the adaption of the ORAC assay using microplates, a fluorescence reader and biological samples.

3.1 Principle of the ORAC assay

The ORAC assay is based on the inhibition of a peroxyl radical induced oxidation [Cao et al., 1993]. Thermal decomposition of the azo compound AAPH generates $ROO^{\cdot}$ that react with the fluorescent probe and consequently lead to a loss of fluorescence intensity. In presence of antioxidants $ROO^{\cdot}$ remove a hydrogen atom from the antioxidant and lead to a delay or inhibition of the reaction between $ROO^{\cdot}$ and the targeted fluorescence probe. The unique characteristic of the ORAC assay belongs to its quantitation, which is achieved by using an area under the curve (AUC) and this combines both inhibition time and inhibition degree into a single quantity [Ou et al., 2002; Huang et al., 2002b].

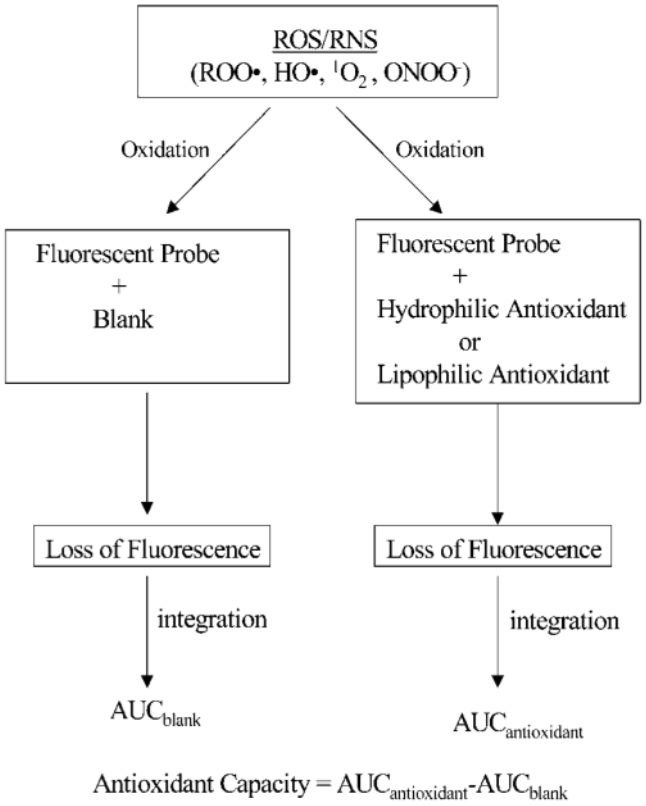

Figure 3.1: Principle of the ORAC assay, [Huang et al., 2002b]

Our investigations were applied on modified methods carried out by Huang et al. [2002b] and Prior et al. [2003]. We adapted the ORAC assay to FLUOstar Optima, a microplate fluorescence reader, coupled with a 96 well microplate and manual assessment. A further performed modification concerns the AAPH amount and the dilution of the samples, where finally an AAPH amount of 0.85284g in combination with a plasma dilution factor of 1:620 were chosen.

3.2 Chemicals, materials, apparatus

Chemicals

The chemicals and standards used are summarised in table 3.1. As a control we utilised reference plasma.

Table 3.1: Chemicals

Chemical	Manufacturer
AAPH *(2,2'–Azobis(2–amidinopropane)dihydrochloride)*	Aldrich(D)
Fluorescein disodium salt	Fluka Chemica(CH)
Phosphate buffer (pH 7.4)	Fluka Chemica(CH)
Trolox (Standard)	

Plasma (EDTA, Heparin)

Plasma was withdrawn from 42 triathletes. Analysed blood samples were previously treated with heparin or ethylenediaminetetraacetic acid (EDTA). After directly cooling the samples to 4℃, plasma or serum was separated at 1711*g for 20 min at 4℃ and subsequently aliquots were frozen at -80℃.

Apparatus

FL 600 Microplate Fluorescence Reader (FLUOstar Optima, Bio-Tek Instruments)
96-well polystyrene microplates (maximum well volume 350µl)
Water bath (37℃)
Vortex mixer
Electrical pipettes

Basic Instrument settings

FLUOstar Optima can measure fluorescence as well as absorbance. Therefore two different measuring adapters exist. It is important to check at the beginning of the measurement that the right adapter is used and that the appropriate reader configuration is adjusted. Concerning the instrument settings, table 8.1 gives an overview of the basic settings.

Table 3.2: Basic Instrument settings

Test name	ORAC
Mode	Fluorescence, Plate Mode
Microplate	Sterilin 96
No of cycle	35
No of flashes per well and cycle	10
Cycle time	60s
Filter and Integration	Exc.filter: 485 Ems.filter: 520
Temperature Control	37°C
Shaking options	orbital, 1s, before first cylce
Gain	adjusted automatically

3.2.1 Reagent, standard and sample preparation

Phosphate buffer 75mmol/l, pH 7.4: 10.96g Na_2HPO_4 + 10.21g KH_2PO_4 was dissolved in 1 liter bidest. H_2O, pH was adjusted to 7.4 with KOH. Due to the pH sensitivity of fluorescein, the pH milieu of 7.4 is important.

AAPH(97%): 0.85284g AAPH was completely dissolved in 10 ml preheated phosphate buffer to give a final concentration of 315 mmol/l. The rate of peroxyl radical generation from AAPH is temperature sensitive, so handling and timing of AAPH is a critical point. It is of great importance to start dissolving briefly before the analysis. Moreover, per each run a new AAPH solution was prepared.

Fluorescein disodium stock solution: 0.158 mg fluorescein disodium were dissolved in 100 ml phosphate buffer to give a concentration of $4.19*10^{-3}$ mmol/l. Under dark conditions at 4°C, it can last 1-2 months.

Fluorescein disodium working solution: The working solution was made daily, by dissolving 975 µl of fluorescein disodium stock solution in 50 ml phosphate buffer, to obtain a final concentration of $8.16*10^{-5}$mmol/l.

Standard (trolox): Diluting the initial trolox solution (2.5 mmol/l) with phosphate buffer to the following concentrations: 50, 25, 12.5, 6.25 µmol/l, prepared daily.

Control (reference plasma): Reference plasma was diluted carefully with phosphate buffer within the dilution factor of 1:620.

Sample (plasma): Plasma was diluted with phosphate buffer within the dilution factor of 1:620. Due to the high dilution of the samples, careful vortexing is very important.

3.2.2 Pipetting schemata

Table 3.3: Pipetting schemata (maximum volume 350µl/well)

Reagents/well	**Blank(µl)**	**Control(µl)**	**Standard(µl)**	**Sample(µl)**
Phosphate buffer	25	–	–	–
Trolox	–	–	25	–
Reference plasma	–	25	–	–
Plasma sample	–	–	–	25
Fluorescein solution	150	150	150	150
Incubation of the microplate: 10min 37°C in FLUOstar Optima				
AAPH solution*(37°C)	25	25	25	25

* Dissolving of AAPH in preheated phosphate buffer (37°C) shortly before pipetting, because AAPH is heat-sensitive. The time between preparing the AAPH solution and starting the measure should be constant each pass (<1.30min is desirable).

3.3 Implementation

The first steps were to activate the plate reader FLUOstar Optima and to adjust the temperature to 37°C. In the meantime reagents and samples were prepared as described before. Fluorescein working solution was made daily and kept under dark conditions during the day. For the AAPH solution, 10 ml phosphate buffer were kept in water bath (37°C) and the AAPH amount was provided, but not yet dissolved. The trolox standard was diluted to concentrations of 50, 25, 12.5 and 6.25 µmol/l. Subsequently the plasma samples were diluted with phosphate buffer (1:620) in test tubes (10µl plasma : 6.19ml phosphate buffer). To ensure homogeneity, careful shaking is very important. After preparing the reagents and adjusting the apparatus to the ORAC test, pipetting was started. All pipetting steps were conducted manually. Starting with 25 µl/well blank in column 1A-1D, then 25 µl/well control (reference plasama) in column 1E-H, followed by transferring 25 µl/well of the different trolox concentrations in column 2. Consequently, plasma samples were pipetted, 25 µl/well starting in column 3A to 4A (duplicate) vertiacl direction, until half of the plate was filled (column 6). Samples and trolox calibration solutions were analysed in duplicate. Via Eppendorf pipete 150 µl of fluorescein were added in each well, and immediately the plate was carried to FLUOstar Optima to incubate the plate for 10

minutes at 37℃, including 3 minutes shaking, therefore we adjusted a test program (incubation without measuring). After the incubation, AAPH was dissolved in 10 ml preheated phosphate buffer, 25 µl AAPH solution was transferred to each well, the plate was immediately carried to FLUOstar Optima, and fluorescence was measured every minute for 35 minutes in adjusted conditions (pH 7.4, 37℃). The data points were collected over the time by the evaluation software. This was then compared to the standard, trolox, and was expressed as micromoles of trolox equivalents TE/l. For calculation of data see section 3.4.

Distribution of the samples on the microplate

6 columns have to be filled per run in order to maintain the total analysing time of 35 minutes. We worked with two plates in parallel. While the first 6 columns of plate 1 were getting measured, we started to pipette the first 6 columns of plate 2. The same process was conducted with the second part (columns 7-12) of the plates. Each microplate was totally filled, but used only once.

Table 3.4: Microplates

plate1	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	B	T1	S1	S1	S9	S9	B	T1	S33	S33	S41	S41
B	B	T1	S2	S2	S10	S10	B	T1	S34	S34	S42	S42
C	B	T2	S3	S3	S11	S11	B	T2	S35	S35	S43	S43
D	B	T2	S4	S4	S12	S12	B	T2	S36	S36	S44	S44
E	C	T3	S5	S5	S13	S13	C	T3	S37	S37	S45	S45
F	C	T3	S6	S6	S14	S14	C	T3	S38	S38	S46	S46
G	C	T4	S7	S7	S15	S15	C	T4	S39	S39	S47	S47
H	C	T4	S8	S8	S16	S16	C	T4	S40	S40	S48	S48

plate2	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	B	T1	S17	S17	S25	S25	B	T1	S49	S49	S57	S57
B	B	T1	S18	S18	S26	S26	B	T1	S50	S50	S58	S58
C	B	T2	S19	S19	S27	S27	B	T2	S51	S51	S59	S59
D	B	T2	S20	S20	S28	S28	B	T2	S52	S52	S60	S60
E	C	T3	S21	S21	S29	S29	C	T3	S53	S53	S61	S61
F	C	T3	S22	S22	S30	S30	C	T3	S54	S54	S62	S62
G	C	T4	S23	S23	S31	S31	C	T4	S55	S55	S63	S63
H	C	T4	S24	S24	S32	S32	C	T4	S56	S56	S64	S64

B....Blank

C....Control (reference plasma)

T....Trolox (T1=50µmol/l, T2=25µmol/l, T3=12.5µmol/l, T4=6.25µmol/l)

S...Sample (plasma, analysed in duplicate)

3.4 Data processing

1. Calculation of the AUC (area under the curve) and the Net AUC for samples (plasma) and standard (trolox)

$$\mathbf{AUC} = 0.5 + f_1/f_0 + ...f_i/f_0 + ...f_{34}/f_0 + 0.5(f_{35}/f_0)$$

f_0= initial fluorescence reading at 0 min.
f_i= fluorescence measure at time i.

Net AUC= AUC_{sample}-AUC_{blank}

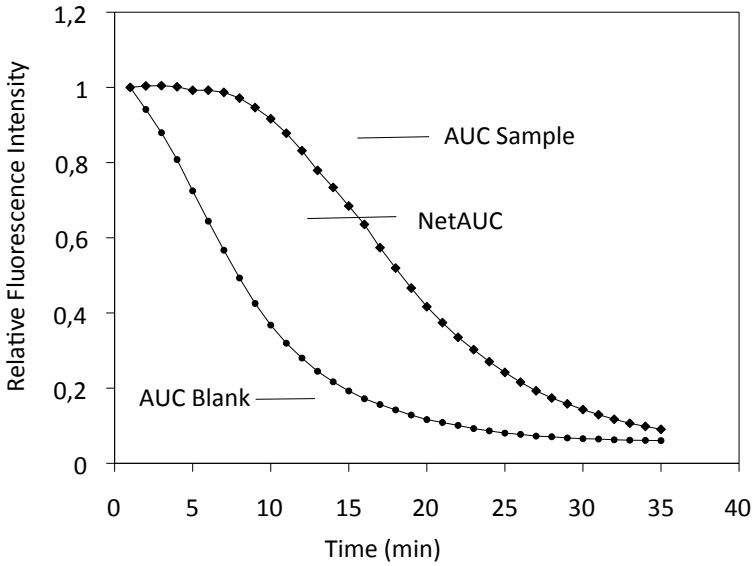

Figure 3.2: Illustration of Net AUC

2. Standard curve
   One can obtain a standard curve by plotting the concentrations of trolox 6.25, 12.5, 25, 50 [µmol/l] against the corresponding Net AUC.

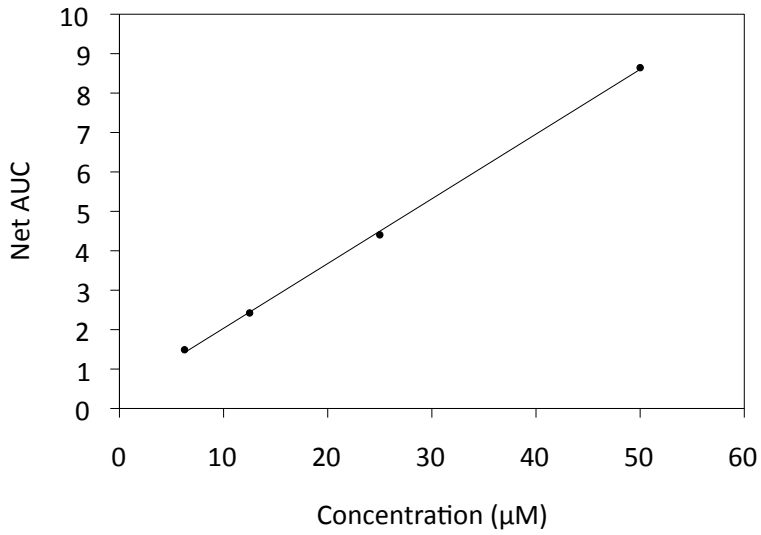

Figure 3.3: Linear plot of trolox concentrations versus the Net AUCs

3. Calculation of ORAC values via trolox regression equation
   ORAC value is expressed as µmol trolox equivalent/l
   ORAC value= $[(AUC_{sample}\text{-}AUC_{blank})/(AUC_{trolox}\text{-}AUC_{blank})]$*(molarity of trolox)

   The dilution factor of the plasma (in our case 1:620) has to be taken into account.

3.5 Subjects

48 non-professional well-trained healthy male triathletes (age 35.3 ± 7.0 years, height 180.8 ± 0.1 cm, weight 75.1 ± 6.4 kg, VO_{2peak} 56.6 ± 6.2 ml/kg/min), who attended the 2006 Ironman Austria (3.8 km swim, 180 km cycle, 42.4 km run), were included in the study. 42 participants of them finished the triathlon and all blood samplings were therefore included in the statistical analysis. The study was approved by the Ethic Commite of the Medical University of Vienna and the subjects were informed before the study about the purpose and risks.

3.6 Study design

The participating subjects were physically fit, free of acute or chronic diseases, had a normal range of body mass index and were non-smokers. Moreover they did not take any prescribed medication and avoided taking more than 100% of RDA of

antioxidants (in form of supplements additionally to their normal dietary intake) within 6 weeks before until 19 days post-race. Athletes were asked to perform a medical and health-screening, a food frequency, a supplementation questionnaire and 24 h dietary recall before each blood sampling. Furthermore they had to report their training in the six previous months to the Ironman triathlon and afterwards until the end of the study. 5 blood samples were taken within the study trail: 2 days prerace, 20 min, 1 day, 5 days and 19 days postrace. The participants fasted overnight before the blood approval days. Except on the race day and 1 day post-race, they could eat and drink ad libitum and the quantities of intake were reported. Subsequent to the triathlon the subjects performed a "recovery" training, moderate in intensity and duration until the end of the study.

3.7 Statistical analysis (SPSS, Excel)

Statistical analysis was carried out using SPSS for MAC (version16.0). Data are presented as mean ± SD. Significance was set at a p value of <0.05 and is reported as $p<0.05$, $p<0.01$ and $p<0.001$. Applying the non-parametric one sample Kolmogorov-Smirnov test, all data showed normal distribution. To account the significant differences the paired t-test was used. Potential associations between ORAC values and various parameters was calculated by Pearsons correlations.

4 Results and Discussion

4.1 Method Validation of the ORAC assay

According to the first objective of the thesis, which was to adapt the ORAC assay to a microplate fluorescent reader and manual accomplishment, the following parameters were calculated in order to validate the method.

4.1.1 Precision

Reference plasma was tested in duplicate over 5 days. Table 4.1 presents the standard deviation (SD) and the coefficient of variation (%CV) for intra–and interday. The CV% for intra-and interday was <15 and is comparable with results from Huang et al. [2002b] where the CV% was also <15. The CV% is higher than in other in vitro methods, such as the FRAP assay. This is obvious, because 35 measure points are involved in the calculation to receive one ORAC value.

Table 4.1: Precision of the ORAC assay (n=2)

	ORAC	**SD**	**%CV**
day 1 intraday	7015	878	12.52
day 2 intraday	8515	296	3.48
day 3 intraday	9981	792	7.94
day 4 intraday	8928	262	2.93
day 5 intraday	10065	691	6.86
interday av	8901	1225	14.26
ORAC values are expressed as µmol TE/l			

4.1.2 Linearity

The standard curve was linear within the tested trolox working range (6.25-50 µmol/l) and the corresponding Net AUCs. Table 4.2 summarises data (Net AUC) of trolox standard curves of different days, including the coefficient of variation(%CV)

Table 4.2: Trolox standard curves

run	**6,25µM**	**12,5µM**	**25µM**	**50µM**	**R^2**
1	3.52	6.15	9.44	15.34	0.990
2	1.45	5.24	8.10	13.88	0.967
3	2.91	6.00	9.13	14.57	0.978
4	1.54	3.73	6.73	12.25	0.994
5	3.68	5.41	9.19	15.73	0.999
6	1.91	4.12	7.81	11.93	0.969
7	1.84	5.85	8.74	14.31	0.959
8	2.40	5.34	8.84	14.39	0.981
9	2.14	4.28	9.00	14.32	0.977
10	2.28	5.28	8.13	13.62	0.981
mean	2.37	5.14	8.51	14.03	
SD	0.78	0.83	0.82	1.20	
CV %	**32.78**	**16.11**	**9.66**	**8.55**	

A smaller CV% was observed within higher trolox concentrations, which was also shown by Price et al. [2006]

and the correlation coefficient R^2. R^2 varies between 0.959-0.999, showing good results in comparison with other papers performing the ORAC assay manually such as Price et al. [2006] showing R^2 of 0.94 to 0.99.

4.1.3 Limit of quantitation LOQ, Limit of detection LOD

LOQ, limit of quantitation, is defined as the lowest concentration on the calibration curve, whereas LOD, limit of detection, is defined as the lowest amount of antioxidant that can be detected. Values cited in the literature are 6.25µM for LOQ and 5.0µM for LOD [Huang et al., 2002b].

4.2 Pre-test

The following investigations are applied on previous works performed by Huang et al. [2002b,a] and Prior et al. [2003].
The amount of AAPH and the dilution factor of plasma are important parameters to guide the reaction kinetic of the ORAC assay. They should not be changed within analysis in order to receive reproducible results. We observed that for the reproducibility of the samples, it is of great importance that the FL decay curves for blank and sample are nearly closed within the chosen timeframe of 35 minutes, so

that the reaction is driven to completion. Figure 4.1 illustrates optimal fluorescence decay curves for blank and sample.

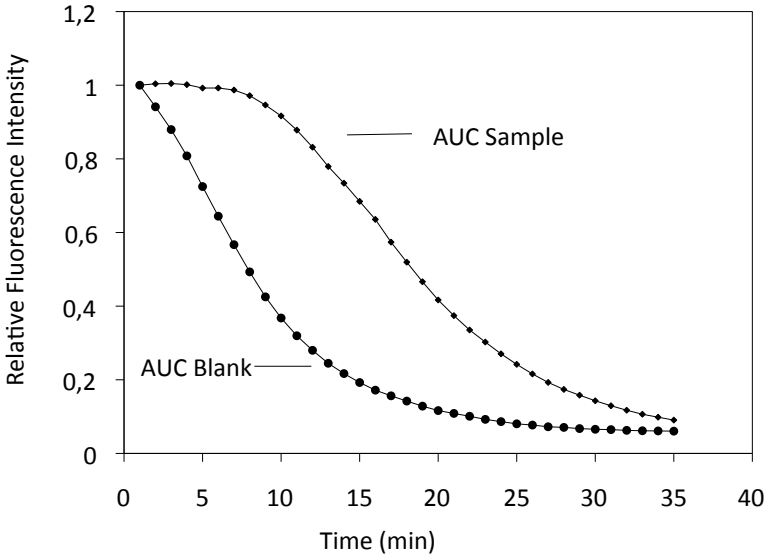

Figure 4.1: FL decay curves

4.2.1 AAPH

Figure 4.2 shows the influence of different AAPH amounts on the FL decay curves for the blank, demonstrating that AAPH determines the velocity of the reaction. Tested AAPH amounts ranged from 0.63963g to 2.34531g (+0.21321g), applied on the used AAPH amount of Huang et al. [2002b]. We have chosen an AAPH amount of 0.85284g in combination with a dilution factor of 1:620 for plasma samples. We received the best FL decay curves, within the time frame of 35 minutes and it gave good reproducible results as shown in table 4.1.

Generally it should be mentioned that too little AAPH amounts lead to an extension of the reaction. Higher amounts increase the reaction rate, decrease the reaction time and consequently decrease the Net AUC [Prior et al., 2003]. Furthermore too high amounts of AAPH are impractical due to low dissolving. In our investigation the upper level for dissolving was an AAPH amount of 2.34531g.

4.2.2 Dilution

The ORAC assay is a very sensitive method and thus samples have to be highly diluted. The conditions are the same as for the use of AAPH. Too less dilutions

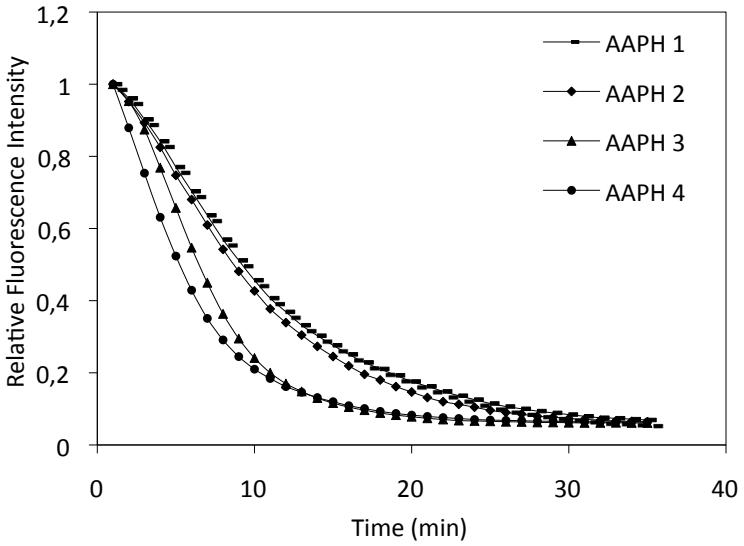

Figure 4.2: AAPH
(AAPH 1= 0.63963g, AAPH 2= 0.85284g, AAPH 3= 1.49247g, APPH 4= 2.1321g)

extent the reaction time, and too high dilutions are inappropriate due to reaching the limit of detection of the apparatus. As already mentioned at the beginning of the section, AAPH and the dilution factor are variable parameters. It is important to choose them in the right amount and ratio to each other to finish the reaction within the timeframe of 35 minutes. Comparable ORAC values for reference plasma could be reached with diverse AAPH amounts and dilution factors, as shown in table 4.3.

Table 4.3: Different ratio of AAPH and dilution factor

AAPH (g)	**Dilution factor**	**ORAC (μmol TE/l)**
0.85284	1: 620	7927
1.918896	1: 160	7087

4.2.3 Protein

Protein is a controversial issue concerning the ORAC assay. On the one hand data exists which claim that protein distorts results due to its significant antioxidative capacity, which could mask responses of small molecular anitoxidants [Prior et al., 2003]. Therefore protein was mostly eliminated with perchloric acid [Cao and Prior, 1998]. On the other hand possible negative effects of eliminating the proteins should

be taken into account. It is unknown how many other important substances, which may contribute to the total antioxidativ capacity, are lost by the extraction methods used. Especially bilirubin, which can be bound to albumin, can be affected by the extraction methods. Regarding protein from the physiological point of view, which we especially wanted to maintain in our analysis we decided that protein should not be eliminated. Different amounts of a protein solution (bovine serum albumin 2g, 4g, 8g, 12.5g, 15g) were tested and it could be demonstrated that protein does not influence the analysis. Augmented amounts of protein in the solution resulted in increased Net AUCs, which confirms the already known fact that protein posseses an antioxidativ character [Dröge, 2002; Soriani et al., 1994]. The thiol-group content of proteins reflects one contributor to the antioxidant capacity of proteins since the SH group is able to scavenge free radicals and recycle other antioxidants [Soriani et al., 1994]. Table 4.4 shows the estimated relative contribution of hydrophilic plasma antioxidants in the ORAC assay when proteins were included and precipitated [Yeum et al., 2004].

Table 4.4: Estimated % contribution of hydrophilic antioxidants

Plasma antioxidant	ORAC	ORAC protein-free
Protein/SH-group/albumin	27.8	-
Uric acid	7.1	39.2
Ascorbic acid	1.3	7.2

In the present work a highly significant correlation between ORAC and total protein was observed in the plasma of triathletes. For more details see subsection 4.3.2.

Advantages/Disadvantages The ORAC assay is a very sensitive method. It is inexpensive, no special equipment is needed, except the microplate reader, and it allows high sample throughput, so that one can save a lot of time. Working with two microplates in parallel is also recommendable in order to save time. Taking into account the running time of 35 minutes and a sample throughput of 48 samples per each run it is realistic to analyse 100 samples a day. For the calculation of the values it is advisable to configurate a good program so that the calculation per se will not delay the method.

The ORAC assay is unique concerning the AUC quantitation method, as it combines both, inhibition time and degree. An advantage of the AUC approach is

that it comprises antioxidants that show a lag phase and antioxidants that have no lag phase [Huang et al., 2005]. According to Bisby et al. [2008] the lag phase results from an equilibrium between antioxidant and fluorescein radicals. Fluorescein radicals may be repaired via antioxidants to produce a well defined lag phase. The different shapes of the curves due to different lag phases suggest that not only the quantity but also the quality of antioxidants in terms of their redox potential could be determined. Therefore, samples that produce a distinct lag phase in the ORAC assay would be more effective antioxidants [Bisby et al., 2008]. When comparing the shapes of the curves of the samples and the trolox at different concentrations we observed different forms. AUC of a trolox solution possesses a distinct lag phase in contrast to the samples.

A further advantage of the ORAC assay is its reaction conditions, which reflect a physiological milieu with a pH value of 7.4 and the induced peroxyl radicals.

A critical aspect is the temperature control, as the reaction starts with the thermal decomposition of AAPH. A good temperature control throughout the plate is important. This includes the preincubation of the phosphate buffer and the microplates before the AAPH addition in order to minimize the assay variability.

4.3 Results of the samples

The second objective of the thesis was the application of the method with biological material and considered the detection and validation of the antioxidant capacity in the plasma of 42 Ironman-triathletes. Within the project "Risk-assessment of Ironman triathlon participants" several parameters were analysed in order to entirely evaluate the impact of a single bout of ultra-endurance exercise on well trained athletes. For more details about the study see [Neubauer et al., 2008]. In the present section the results of the ORAC assay are discussed in the context with some analysed parameters of the project.

4.3.1 Time course of ORAC values

Figure 4.3.3 illustrates the time course (2d prerace, 20 min, 1d, 5d and 19d postrace) of ORAC [µmol TE/l]. Data are presented as mean ± SD. A highly significant increase of ORAC values was detected immediately postrace (***$p<0.001$) and remained higher than prerace until 1d postrace (*$p<0.05$). After five days, ORAC values returned to prerace concentrations and remained at this level to 19 days

postrace. The characteristic of the time course could also be demonstrated for the TEAC and FRAP assay. See subsection 4.3.3 for a more comprehensive overview.

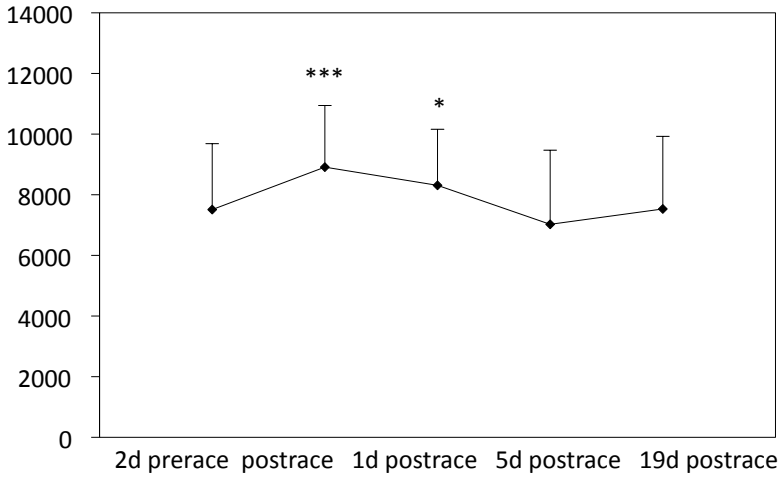

Figure 4.3: Time course of ORAC values [µmol TE/l] of 42 Ironman-triathletes

Augmentations of the antioxidant capacity could also be shown in other studies analysing the parameter in marathon runners [Liu et al., 1999; Vasankari et al., 1997]. Generally the augmentation of antioxidant capacity can be attributed to various mechanisms. On the one hand an increased antioxidant capacity could act as rapid response to an increased oxidative stress. However the exercise induced elevation in the total antioxidant capacity could also be related to an increase in the concentrations of vitamin C and α-tocopherol due to the intake of these vitamines during the race and due to tissue mobilisation [Neubauer et al., 2008]. Augmented values of total antioxidant capacity must not necessarily be a desirable condition if reflecting the response to elevated oxidative stress. A decrease in plasma of this parameter may not be an undesirable condition if reflecting the decreased formation of radicals [Prior and Cao, 1999].

When applying the time course of the measured ORAC values to the study collective, we claim that the increase in the total antioxidant capacity directly postrace is mainly due to the increase of protein and uric acid. The changes of both antioxidants (pre-to postrace) correlated with the changes (pre- to postrace) of ORAC.

4.3.2 Correlations between ORAC and other biomarkers

Protein, uric acid

No strong, but a significant correlation could be observed between ORAC and uric acid changes from pre- to postrace (r= 0,361, *p< 0.05). The raise of the uric acid immediately postrace, which was shown by Neubauer et al. [2008], is probably due to the increased purine metabolism [Finaud et al., 2006; König et al., 2001].

A highly significant correlation was found between ORAC and total protein (prerace: r= 0.443; **p<0.01, postrace: r= 0.459; **p<0.01, 5d postrace: r= 0,358; *p<0.05). These results show that proteins contribute mainly to the antioxidant capacity in the ORAC assay, which was also shown by Yeum et al. [2004]. The increase in plasma proteins within the Ironman study can mainly be traced back to the changes in the plasma volume. Due to the extreme exercise intensity, heat and dehydration lead to a loss in plasma volume. As a result, the protein contingent rises [Suzuki et al., 2006].
However, in order to achieve a physiological validation of the Ironman triathlon on the athletes, the protein was not precipitated before assessing the ORAC. Moreover, according to Soriani et al. [1994], the antioxidant potential of extracellular fluids depends by half on the protein content and by half on the small molecular weight antioxidants. If one is particularly interested in the detection of small molecular weight antioxidants, a protein precipitation is recommended to conduct.

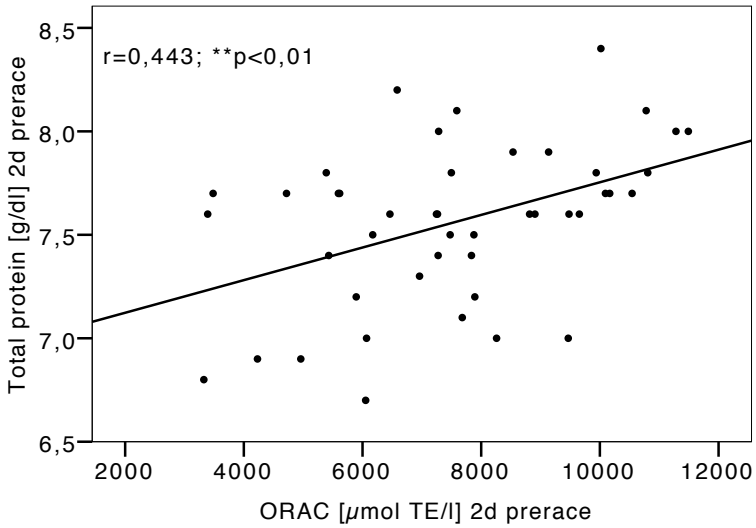

Figure 4.4: Correlations between ORAC and total protein 2d prerace

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Training status, stress parameters

No correlation of ORAC was found neither with parameters of the training status (watt peak, peak oxygen consumption, lactate threshold, relative individual anaerobic threshold), nor with lipid peroxidation and protein oxidation stress parameters (MDA, AOPP, CD, oxLDL) or the age and ORAC. The lack of correlation of ORAC and parameters related to oxidative stress could be explained as the ORAC assay is designed for the hydrophilic compartment of the plasma whereas lipid oxidation parameters describe the change in the lipophilic part.

Antioxidants

No significant correlation was found between ORAC and α-tocopherol, ascorbic acid, glutathione and bilirubin. The lack of correlations with α-tocopherol is due to the fact that it is a lipophilic antioxidant. It can thus not be determined by this test.
Concerning ascorbic acid, the lack of relation can be referred to the possible degradation of the antioxidant at the experimental conditions of the ORAC assay (37°Cfor 35 minutes). Generally, the estimated contribution of ascorbic acid to the ORAC assay, when not conducting a protein precipitation, is rather low (1.3%) [Yeum et al., 2004].
No link was found with enzymatic antioxidants such as glutathion-peroxidase, superoxid-dismutase and catalase. This seems plausible since the enzymes are located in the erythrocytes, which are not measured by the assay.

4.3.3 ORAC in comparison with FRAP and TEAC

We compared the ORAC assay with other analytical methods with similar background (FRAP and TEAC) for assessing the total antioxidant capacity of plasma. Although a similar trend of the compared methods was observed, as shown in figure 4.6, no correlation between ORAC and TEAC was found. However, correlations between ORAC and FRAP were found when testing single time points (prerace: r= 0.325; *p< 0.05, figure 4.3.3, postrace: r= 0.405; **p< 0.01, 5d postrace: r= 0.385; *p< 0.05, 19d postrace: r= 0.372; *p< 0,05), or when testing the correlations between pre-to postrace changes (r= 0.403; **p< 0.01). We suggest that the relationship between ORAC and FRAP could be due to the the uric acid effect, as uric acid correlates significantly with both tests (FRAP, uric acid: r= 0.733, *** p<0.001, ORAC, uric acid: r= 0.361, *p< 0.05).

Similar results were obtained in previous studies where a correlation between ORAC and FRAP was shown, in contrast to a lack of correlation between ORAC and TEAC [Cao and Prior, 1998].

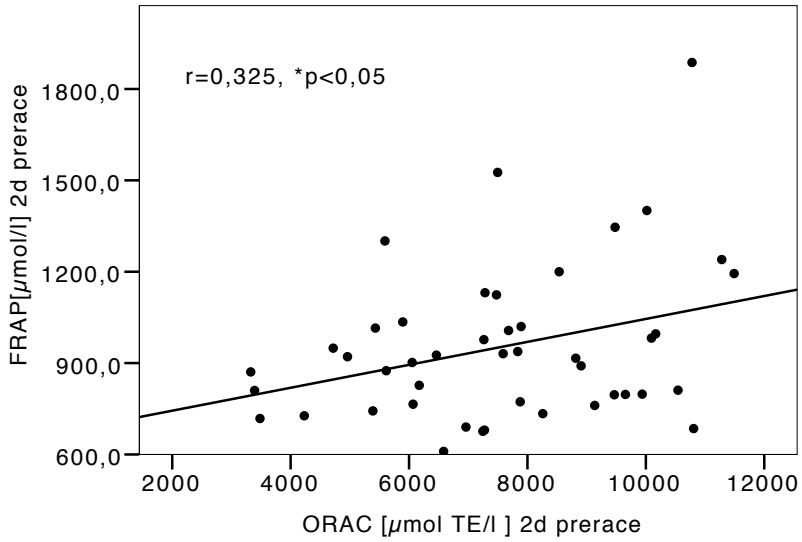

Figure 4.5: Correlation between ORAC and FRAP 2d prerace

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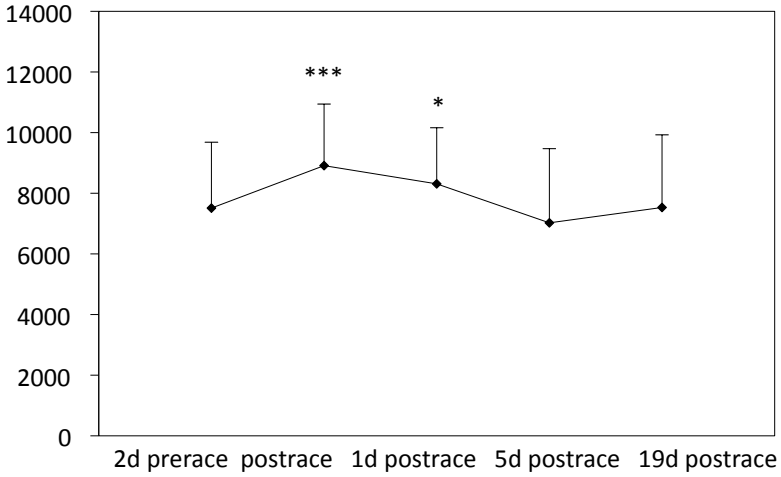
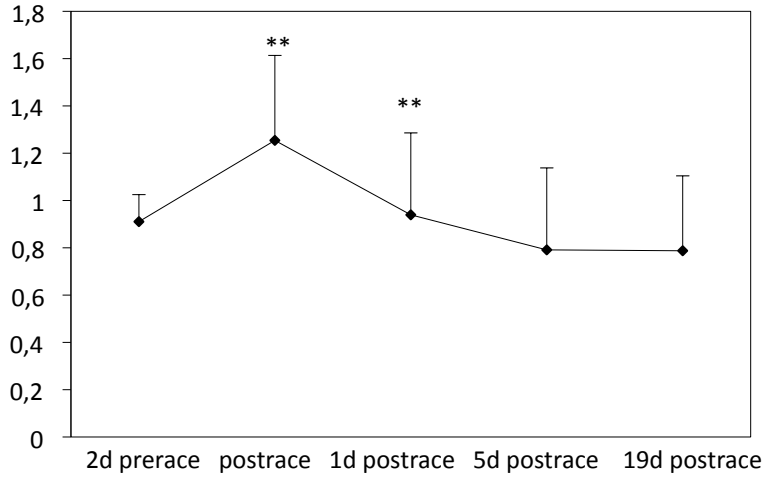
When comparing ORAC, TEAC and FRAP, it can be concluded that the ORAC assay considers most of the antioxidant capacity in plasma. This could be explained by the fact that the ORAC assay, due to its unique AUC quantitation method, is more sensitive in the detection of antioxidants than the other tests.



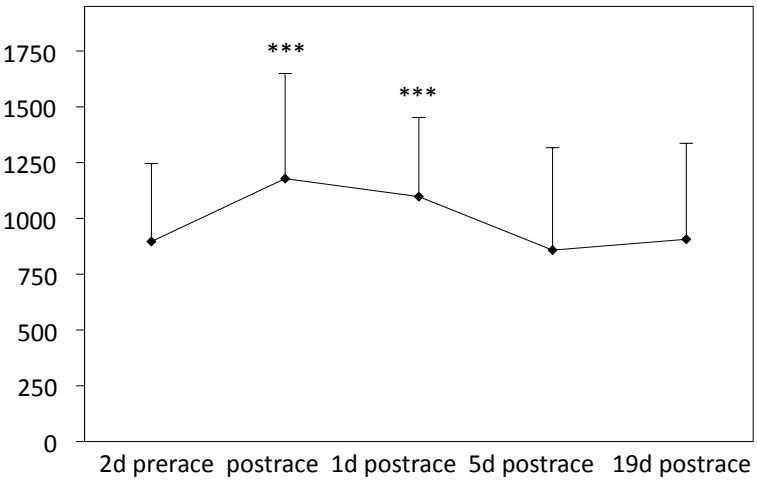
Time course of ORAC [µmol TE/l]

(a)



Time course of TEAC [µmol TE/l]

(b)



Time course of FRAP [µmol/l]

(c)

Figure 4.6: Comparison of the time course of the ORAC, TEAC and FRAP assays of 42 Ironman-triathletes

5 Conclusion

In the present thesis, the ORAC assay, which provides a simple, but very accepted method for the assessment of the antioxidant capacity, was adapted to FLUOstar Optima, a microplate flourescent reader. The data of the validation of the ORAC assay indicates that the assay shows a good reproducible ability. The coefficient of variation for inter and intraday was $< 15\%$ for plasma samples. The standard curve was linear within the tested trolox working range (6.25-50 µmol/l) and the corresponding Net AUCs. The correlation coefficient R^2 varied between 0.959-0.999 and showed good results when compared to the literature. The results also demonstrate that the ORAC assay is applicable to manual performance without robotics or machine injection systems.

We have demonstrated that the amount of AAPH and the dilution factor of the plasma are important parameters to guide the reaction kinetic. In the current work an AAPH amount of 0.85284g in combination with a dilution factor of 1:620 for plasma samples was used. The ORAC assay is a very sensitive method. We can thus confirm that it is important to dilute the samples appropriately. We further observed that the temperature control is delicate. A good temperature control throughout the plate is important in order to to minimise the assay´s variability. This could be achieved by the preincubation of the phosphate buffer and the microplates before AAPH addition.

The performed ORAC assay in this work detects preliminary hydrophilic antioxidants. The method, however, provides a wide spectrum of application and can also be expanded for the assessment of lipophilic antioxidants as conducted by Huang et al. [2002a] and Prior et al. [2003]. Moreover the test can be performed in plasma containing protein or in protein free plasma. We did not precipitate the protein in order to evaluate the physiological changes in the study collective. Nevertheless the expansion of the ORAC assay to its lipophilic format, the precipitation of the protein and the application in food samples present further approaches to be conducted.

In the second part of the thesis the applied test was used in order to detect and validate the antioxidant capacity in the plasma of Ironman-triathletes. Within the project "Risk-assessment of Ironman triathlon participants" several parameters were analysed in order to entirely evaluate the impact of a single bout of ultra-endurance exercise on well trained athletes [Neubauer et al., 2008]. Neubauer et al. [2008] demonstrates that the Ironman triathlon does not cause longer lasting alterations in systemic oxidative stress markers.

Regarding the ORAC values of the study participants we detected a highly significant increase of ORAC values immediately postrace (***$p<0.001$) and they remained higher than prerace until 1d postrace (*$p<0.05$). After five days, ORAC values returned to prerace concentrations and remained there until 19 days postrace. Correlations with different antioxidants, stress parameters and parameters of the training status were conducted. Our results indicate that the increased ORAC values can be predominantly traced back to the augmentation of protein and uric acid, as both parameters also increased immediately postrace.

For the elucidation of a complete profile of antioxidant capacity against free radicals and oxygen radical species various methods with different chemical backgrounds are needed. This was achieved by the comparison of ORAC, FRAP and TEAC, on the same study collective. We observed no strong but a significant correlation of ORAC and FRAP, which may be due to uric acid.
Generally these assays are recommendable for a dependent study design thereby measuring a time course of subjects, but they are not preferred when describing and comparing single status data. The in vitro total antioxidant capacity assays are used to provide general results, which are not based on single substances, however, they are one part of a puzzle to describe the total antioxidant network.

6 Summary

The first objective of the thesis was the application of a microplate based assay for the assessment of the oxygen radical absorbance capacity (ORAC). The second objective was referred to the detection and validation of the antioxidant capacity in plasma samples of well trained Ironman-triathletes, within the FWF fundend project "Risk-assessment of Ironman-triathlon participants".

The use of the device FLUOstar Optima in combination with 96-well microplates allowed a high sample throughput of 100 samples a day. Within the applied method we determined the antioxidant capacity in plasma of 42 Ironman-triathletes. The measured ORAC values contributed to the elucidation of the complex relationship between free radicals and antioxidants after a single bout of ultra endurance exercise. Therefore, the blood samples of the well trained triathletes of 5 different time points (prerace, 20minutes postrace, 1d, 5d, 19d postrace) were analysed.

The ORAC assay provides a good linear relationship between concentration and fluorescence. Concerning the study collective the ORAC values increased significantly immediately postrace and remained higher until 1d postrace. After five days, ORAC values returned to prerace concentrations.

The applied method offers a good index of antioxidant capacity in biological fluids within the technological equipment of every laboratory. Moreover it is inexpensive, reagents are simple to prepare and the procedure is fast. The data of the current work demonstrates that there is no persistent oxidative stress in well trained athletes after an Ironman triathlon, probably due to training and exercise induced adaptions of the antioxidant defense system.

7 Zusammenfassung

Das erste Ziel der Diplomarbeit war die Entwicklung einer auf Mikrotiterplatten basierten Methode zur Erfassung der Oxygen-radical-absorbance-capacity (ORAC). Der zweite Ansatz umfasste die Bestimmung der antioxidativen Kapazität im Plasma von Ironmantriathleten innherhalb des FWF finanzierten Projektes: Risk-assessment of Ironman-triathlon participants.

Die Verwendung des Mikroplattenlesegerätes FLUOstar Optima in Kombination mit 96-well Platten ermöglichte eine hohe Probendurchsatzrate, 100 Proben/Tag. Mit Hilfe der gewählten Methode wurde die antioxidative Aktivität im Plasma von 42 Ironmantriathleten bestimmt. Die erfaßten ORAC Werte trugen zur Aufklärung der komplexen Beziehung zwischen freien Radikalen und Antioxidantien nach intensiver physischer Belastung bei. Dafür wurden Blutproben von gut trainierten Triathleten entnommen und die Oxygen-radical-absorbance-capacity 2 Tage vor dem Ironmantriathlon, 20 Minuten, 1, 5, und 19 Tage nach dem Bewerb gemessen.

Der ORAC Test liefert reproduzierbare Ergebnisse und zeigt eine gute Linearität zwischen Konzentration und Fluoreszenz. Bezieht man die ORAC Werte auf das Studienkollektiv, zeigte sich ein Anstieg der antioxidativen Kapazität unmittelbar nach dem Bewerb. Einen Tag nach dem Bewerb lagen die Werte immer noch signifikant über den Ausgangswert. Nach fünf Tagen näherten sie sich wieder dem Ausgangsniveau.

Die eingesetzte Methode ist für die Erfassung der antioxidativen Kapazität im Plasma gut geeignet und ist mit der standardmäßigen Ausstattung eines Labors durchführbar. Außerdem erwies sich die Methode als kostengünstig, schnell und einfach in der Handhabung. Die vorliegende Arbeit zeigt, dass gut trainierte Athleten nach einem Ironmantriathlon keinen persistenten oxidativen Stress ausgesetzt sind. Dieser Effekt ist wahrscheinlich auf eine trainingsinduzierte Adaptierung des antioxidativen Systems zurückzuführen.

8 Appendix

Table 8.1: Instrument settings

SETUP	
Reader Configuration	Fluorescence
BASIC PARAMETERS	
Test name	ORAC
Mode	Fluorescence, Plate Mode
Microplate	Sterilin 96
Position delay	0.2 seconds
No. of kinetic windows	1
No. of cycles	35
Measurement start time	0.5 seconds
No. of flashes/well and cycle	10
Cycle time	60 seconds
Total measurment time	34 minutes 59 seconds
Filter settings	
Fluorescence intensity	on
Filter and Integration	Exc.filter: 485
	Ems.filter: 520
LAYOUT	
Content	S..Standard, C..Control, B..Blank, X..Sample
Groups	on

Index	constant, increase
Replicates	Number: 2 (duplicate)
	Horizontal
Reading direction	Position 11

CONCENTRATIONS/VOLUMES/SHAKING

Concentration	enter manually by standards
Shaking options	Mode: orbital
	Shaking width: 7mm
	Additional Shaking: before first cycle
	Shaking time: 1 second

TEMPERATURE CONTROL

Temperature	37°C

MEASURE

Plate out/in	
Measure	choose test in test protocols
Plate Identification	S..Standard, C..Control, B..Blank, X..Sample
Gain Adjustment	Automatic
Start test run	

Table 8.2: Collective ORAC values(μmol TE/l) of the subjects

n	2d prerace	postrace	1d postrace	5d postrace	19d postrace
1	6053	10420	11424	8467	6925
2	11491	12706	7152	5069	7450
3	8536	10851	9038	9609	11871
4	7274	7640	9684	10427	9429
5	10017	11375	9904	7958	10138
6	10780	11071	10657	10136	10817
7					
8					
9	9480	10679	11326	11987	11210
10	11282	10782	10271	8188	9014
11	7589	9720	9507	7823	9045
12	7836	8884	7253	8023	7860
13	7497	11370	8016	10173	9669
14	10094	9176	7619	8005	7193
15		10166	11658	8303	9483
16	5593	6661	8702	8397	7048
17	4230	5353	3905	4111	1444
18	9938	8437	6950		
19					
20	5431	5236	4440	4782	3160
21	4960	5221	10004	2033	4795
22	7246	8824	5322	2796	4933
23	6172	6669	6685	7174	6941
24	5389	8153	6155	7289	6275
25	10543	10214	7735	5562	4590
26	6584	9091	8077	8951	9664
27	7264	7957	7928	8211	8952
28	9136	9890	8458	5769	8578
29	5615	11969	9616	8404	10562
30	8259	11884	9231	8057	8398
31	7893	9652	12001		
32	7477	9957	10478	9240	9317
33	3391	5579	6318	3175	6156
34	5894	8573	7449	6188	6609
35	10807	11238	8635	6309	6702
36	3482	7687	9169	7838	8329
37	7877	9053	6886		
38	9467	8548	7207	5714	9711
39	9654	8950	10972	6717	9821
40	6961	8890	9176	8976	7471
41	8816	9744	9364	5706	5725
42	6462	9781	8965	9757	10471
43	8906	10864	9157	5715	5689
44	7283	8544	8097	8655	7849
45	4719	5687	5363	4365	4174
46	7681	7811	7638	1358	3647
47	3326	5069	6405	3861	5011
48	6068	6211	7296		6135

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Barbara Stadlmayr
curriculum vitae

Date of Birth	December 27, 1983
Place of birth	Wels, Austria
Nationality	Austria

Education

10/2007	Experimental diploma thesis, title: "Implementation of a microplate based assay for the assessment of the oxygen radical absorbance capacity (ORAC)", supervised by A.o.Univ.-Prof.Dr. Karl-Heinz Wagner, *Department of Nutritional Sciences, University of Vienna*
10/2002	Begin, studies of nutritional sciences, majoring in nutrition and environment, *Department of Nutritional Sciences, University of Vienna*
06/2002	School leaving examination (Matura) *Realgymnasium Lambach*, A-4650 Lambach

Internships

10/2008-dato	*Department of Nutritional Sciences, University of Vienna* Tutor
03/2007- 06/2007	*Medical University of Vienna* Internship within the Helena Project (Healthy Lifestyle in Europe by Nutrition in Adolescence)
02/2007 - 03/2007	*Department of Nutritional Sciences, University of Vienna* Trainee under supervision of A.o.Univ.-Prof.Dr. Karl-Heinz Wagner
09/2007	*La Tunella, Azienda Agricola*, I-33040 Premariacco, Italy Vintage worker
08/2005	*Gmundner Molkerei*, A-4810 Gmunden Quality control
09/2004	*S.Spitz*, A-4800 Attnang-Puchheim Quality control

Languages

German	Mother tongue
English	Written and spoken proficiency
Italian	Written and spoken proficiency

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