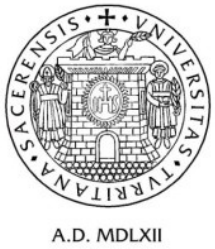



**UNIVERSITÀ DEGLI STUDI DI SASSARI**

**Scuola di Dottorato di Ricerca in**
**Scienze Biomolecolari e Biotecnologiche**

**Indirizzo: Proteomica, Metabolomica, Biochimica Clinica**
**e Biologia Molecolare Clinica**

**XXV ciclo**

# *Melanoidins from dry fruit prevent oxidative endothelial cell death by counteracting mitochondrial oxidation and membrane depolarization*

Coordinatore:
***Prof. Bruno Masala***

Tutor:
***Prof. Ciriaco Carru***

Co-Tutor:
***Prof. Gianfranco Pintus***

Tesi di dottorato di:
***Dott.ssa Gioia Gasparetti***

**Anno Accademico 2011-2012**

# *INDEX*

# *CHAPTER 1*

# *INTRODUCTION*

## *§ 1.1 Cardiovascular diseases (CVD)*

Cardiovascular disease is a class of diseases that involve the heart or blood vessels (arteries, capillaries and veins) (1). Cardiovascular disease refers to any disease that affects the cardiovascular system, principally cardiac disease, vascular diseases of the brain and kidney, and peripheral arterial disease (2). The causes of cardiovascular disease are different but atherosclerosis and/or hypertension are the most common. Besides, with aging come a number of physiological and morphological changes that alters cardiovascular function and lead to subsequently increased risk of cardiovascular disease, even in health asymptomatic individuals (3). Cardiovascular diseases remain the biggest cause of deaths worldwide, though over the last two decades, cardiovascular mortality rates have declined in many high-income countries. According to the World Health Organization, chronic diseases are responsible for 63% of all deaths in the world, with cardiovascular disease as the leading cause of death (4). At the same time cardiovascular deaths and disease have increased at a fast rate in low- and middle-income countries (5). Although cardiovascular disease usually affects older adults, the antecedants of cardiovascular disease, notably atherosclerosis begin in early life, making primary prevention efforts necessary from childhood (6). There is therefore increased emphasis on preventing atherosclerosis by modifying risk factors, such as healthy eating, exercise, and avoidance of smoking. Almost all cardiovascular disease in a population can be explained in terms of a limited number of risk factors: age, gender, high blood pressure, high serum cholesterol levels, tobacco smoking, excessive alcohol consumption, family history, obesity, lack of physical activity, psychosocial factors, diabetes mellitus, air pollution (2). While the individual contribution of each risk factor varies between different communities or ethnic groups the consistency of the overall contribution of these risk factors is remarkably strong (7). Some of these risk factors, such as age, gender or family history are immutable, however many important cardiovascular risk factors are modifiable by lifestyle change, drug treatment or social change. Age is an important risk factor in developing cardiovascular diseases. It is estimated that 87 percent of people who die of coronary heart disease are 60 and older (8). At the same time, the risk of stroke doubles every decade after age 55 (9). Multiple explanations have been proposed to explain why age increases the risk of cardiovascular diseases. One of them is related to serum cholesterol level (10). In most populations, the serum total cholesterol level increases as age increases. In men, this increase levels off around age 45 to 50 years. In women, the increase continues sharply until age 60 to 65 years (10).

Aging is also associated with changes in the mechanical and structural properties of the vascular wall, which leads to the loss of arterial elasticity and reduced arterial compliance and may subsequently lead to coronary artery disease (11). Men are at greater risk of heart disease than pre-menopausal women (12). However, once past menopause, a woman's risk is similar to a man's (12). Population based studies show that atherosclerosis the major precursor of cardiovascular disease begins in childhood. The Pathobiological Determinants of Atherosclerosis in Youth Study demonstrated that intimal lesions appear in all the aortas and more than half of the right coronary arteries of youths aged 7–9 years (13). This is extremely important considering that 1 in 3 people will die from complications attributable to atherosclerosis. In order to stem the tide education and awareness that cardiovascular disease poses the greatest threat and measures to prevent or reverse this disease must be taken. Obesity and diabetes mellitus are often linked to cardiovascular disease (14), as are a history of chronic kidney disease and hypercholesterolaemia (15). In fact, cardiovascular disease is the most life threatening of the diabetic complications and diabetics are two- to four-fold more likely to die of cardiovascular-related causes than nondiabetics (16, 17, 18). Measures to prevent cardiovascular disease may include:

- a low-fat, high-fiber diet including whole grains and plenty of fresh fruit and vegetables (at least five portions a day)
- a diet high in vegetables and fruit (19)
- tobacco cessation and avoidance of second-hand smoke;
- limit alcohol consumption to the recommended daily limits; consumption of 1-2 standard alcoholic drinks per day may reduce risk by 30% (20, 21). However excessive alcohol intake increases the risk of cardiovascular disease (22).
- lower blood pressures, if elevated, through the use of antihypertensive medications;
- decrease body fat (BMI) if overweight or obese (23);
- increase daily activity to 30 minutes of vigorous exercise per day at least five times per week;
- decrease psychosocial stress (24).

Evidence shows that the Mediterranean diet improves cardiovascular outcomes (25). In clinical trials the Dietary Approaches to Stop Hypertension (DASH) diet has also been shown to reduce blood pressure (26), lower total and low density lipoprotein cholesterol (27) and

improve metabolic syndrome (28); but the long term benefits outside the context of a clinical trial have been questioned (29). The link between saturated fat intake and cardiovascular disease is controversial and scientific studies, both observational and clinical, show conflicting results (30). Dietary substitution of polyunsaturated fats for saturated fats may reduce risk, substitution with carbohydrates does not change or may increase risk (30, 31). Increased dietary intake of Trans fatty acids significantly increases the risk of cardiovascular disease (32).

## *§ 1.2 Free radicals, oxidative stress and antioxidants in human health and disease*

Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process. These species play a dual role as both toxic and beneficial compounds. The delicate balance between their two antagonistic effects is clearly an important aspect of life. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they generate oxidative stress, a deleterious process that can damage all cell structures (33-42). Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods and/or supplements. Endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and repairing damages caused by ROS and RNS, and therefore can enhance the immune defense and lower the risk of cancer and degenerative diseases (43-47). The theory of oxygen-free radicals has been known about fifty years ago (36). However, only within the last two decades, has there been an explosive discovery of their roles in the development of diseases, and also of the health protective effects of antioxidants. ROS and RNS are the terms collectively describing free radicals and other non-radical reactive derivatives also called oxidants. Radicals are less stable than non-

radical species, although their reactivity is generally stronger. A molecule with one or more unpaired electron in its outer shell is called a free radical (33-37). Free radicals are formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron, by cleavage of a radical to give another radical and, also via redox reactions (33, 34). Free radicals include hydroxyl (OH•), superoxide ($O_2^{\bullet -}$), nitric oxide (NO•), nitrogen dioxide ($NO_2$ •), peroxyl (ROO•) and lipid peroxyl (LOO•). Also, hydrogen peroxide ($H_2O_2$), ozone ($O_3$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid ($HNO_2$), peroxynitrite ($ONOO^-$), dinitrogen trioxide ($N_2O_3$), lipid peroxide (LOOH), are not free radicals and generally called oxidants, but can easily lead to free radical reactions in living organisms (40). Biological free radicals are thus highly unstable molecules that have electrons available to react with various organic substrates such as lipids, proteins, DNA. Formation of ROS can occur in the cells by two ways: enzymatic and non-enzymatic reactions. Enzymatic reactions generating free radicals include those involved in the respiratory chain, the phagocytosis, the prostaglandin synthesis and the cytochrome P450 system (33-41). For example, the superoxide anion radical ($O_2^{\bullet -}$) is generated via several cellular oxidase systems such as NADPH oxidase, xanthine oxidase, peroxidases. Once formed, it participates in several reactions yielding various ROS and RNS such as hydrogen peroxide, hydroxyl radical (OH•), peroxynitrite ($ONOO^-$), hypochlorous acid HOCl), etc. $H_2O_2$ (a non radical) is produced by the action of several oxidase enzymes, including aminoacid oxidase and xanthine oxidase. The last one catalyses the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid.

Free radicals can be produced from non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiations. The non-enzymatic process can also occur during oxidative phosphorylation (i.e. aerobic respiration) in the mitochondria (36, 37, 40). ROS and RNS are generated from either endogenous or exogenous sources. Endogenous free radicals are generated from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer, aging. Exogenous ROS/RNS result from air and water pollution, cigarette smoke, alcohol, heavy or transition metals (Cd, Hg, Pb, Fe, As), certain drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), industrial solvents, cooking (smoked meat, used oil, fat), radiation. (36-46).

After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals. At low or moderate concentrations, ROS and RNS are necessary for the maturation process of cellular structures and can act as weapons for the host defense system. Indeed, phagocytes (neutrophils, macrophages, monocytes) release

free radicals to destroy invading pathogenic microbes as part of the body's defense mechanism against disease (37, 42). Other beneficial effects of ROS and RNS involve their physiological roles in the function of a number of cellular signaling systems (39-41). In brief, ROS/RNS at low or moderate levels are vital to human health. When produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA) (37-42). Oxidative stress can arise when cells cannot adequately destroy the excess of free radicals formed. In other words, oxidative stress results from an imbalance between formation and neutralization of ROS/RNS. For example, hydroxyl radical and peroxynitrite in excess can damage cell membranes and lipoproteins by a process called lipid peroxidation (46). Proteins may also be damaged by ROS/RNS, leading to structural changes and loss of enzyme activity (41, 46). Oxidative damage to DNA leads to the formation of different oxidative DNA lesions which can cause mutations. The body has several mechanisms to counteract these attacks by using DNA repair enzymes and/or antioxidants (38-41). If not regulated properly, oxidative stress can induce a variety of chronic and degenerative diseases as well as the aging process and some acute pathologies (trauma, stroke). Cardiovascular disease (CVD) is of multifactorial etiology associated with a variety of risk factors for its development including hypercholesterolaemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity amongst others (34, 47, 48). Recently, research data has raised a passionate debate as to whether oxidative stress is a primary or secondary cause of many cardiovascular diseases (48). Further in vivo and ex vivo studies have provided precious evidence supporting the role of oxidative stress in a number of CVDs such as atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy and congestive heart failure (34, 37, 47, 48).

## *§ 1.3 Phytochemicals antioxidants*

Phytochemicals are the chemicals extracted from plants. These chemicals are classified as primary or secondary constituents, depending on their role in plant metabolism.

Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc. Secondary constituents are the remaining plant chemicals such as alkaloids (derived from amino acids), terpenes (a group of lipids) and

phenolics (derived from carbohydrates). Antioxidants are secondary constituents or metabolites found naturally in the body and in plants such as fruits and vegetables. An antioxidant can be defined in simple terms as anything that inhibits or prevents oxidation of a susceptible substrate. Plants produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate (49). Common antioxidants include vitamin A, vitamin C, vitamin E, and certain compounds called carotenoids (like lutein and beta-carotene) (50). These plant-based dietary antioxidants are believed to have an important role in the maintenance of human health because our endogenous antioxidants provide insufficient protection against the constant and unavoidable challenge of reactive oxygen species (ROS; oxidants) (51).

Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress (52). Oxidative stress plays a role in heart diseases, malaria, neurodegenerative diseases, AIDS, cancer and in the aging process (53). This concept is supported by increasing evidence that oxidative damage plays a role in the development of chronic, age-related degenerative diseases, and that dietary antioxidants oppose this and lower risk of disease (54, 55) and thus there arises a necessity to extract these antioxidants from the plant matrices.

Antioxidants are defined as the substance that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance (56). For the *in vivo* situation the concept of antioxidants includes antioxidant enzymes, iron binding and transport proteins and other compounds affecting signal transduction and gene expression (57). In case of foods and beverages, antioxidants are related to the protection of specific oxidation substrates or the formation of specific oxidation.

Synergism, antagonism, co-antioxidants and oxidation retarders are the other useful concepts related to antioxidants. Synergism can be defined as the phenomenon in which a number of compounds, when present together in the same system, have a more pronounced effect than if they were alone. Antagonism can be defined likewise by substituting “more” with “less”, whereas coantioxidants may be defined by substituting “more” with “same”. The compounds that reduce the rate of oxidation without showing a distinct lag phase of oxidation are retarders of oxidation. Antioxidant action is measured as a decrease in over-all rate of oxidation and as the length of the lag phase. Antioxidants are divided into two classes: preventive antioxidants and chain breaking antioxidants. Preventive antioxidants inhibit

oxidation by reducing the rate of chain initiation. In most cases hydroperoxide product, ROOH of the oxidation is the cause for the initiation process. Preventive antioxidants convert the hydroperoxides to molecular products that are not potential sources of free radicals (58).
Most biological preventive antioxidants are also peroxide decomposers. Certain enzymes such as glutathione peroxidase can reduce H2O2 to H2O and also lipid hyroperoxides to the corresponding alcohol as shown in the following equation (Figure 1).


**Figure 1**

Commercial chain breaking antioxidants are generally phenols or aromatic amines.
They owe their antioxidant activity to their ability to trap peroxyl radicals are as shown in equation (Figure 2).

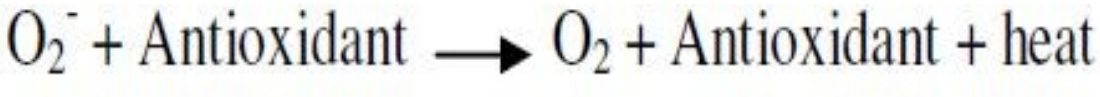


**Figure 2**

Antioxidants can also be manufactured synthetically. These belong to the class of synthetic antioxidants. The main disadvantage with these antioxidants is their side effect when taken in vivo (59). Most of the natural antioxidants are found to have higher antioxidant activity when compared with that of the synthetic ones.
Several arguments suggest that the antioxidant components of fruits and vegetables contribute in the defense effect. Epidemiological studies and intervention trials on prevention of diseases such as cancer and cardiovascular disease in people have shown the positive effects of taking antioxidant supplements (60, 61, 62).
Carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols are some of the antioxidants produced by the plant for their sustenance.

Small molecule dietary antioxidants such as vitamin C (ascorbate), vitamin E (tocopherol), and carotenoids have generated particular interest as anticarcinogens and as defenses against degenerative diseases (63). The details of these antioxidants are shown in the Table 1 (64-72).

**Table 1.** Widely used antioxidants and their applications.

| Antioxidant | Plant sources | Applications |
|---|---|---|
| Beta-Carotene<br>$C_{40}H_{56}$ | *Elaeis oleifera, Elaeis Guineensis Momordica Cochinchinnensis Spreng*<br>*Eurycoma Longifolia*<br>*Zanthoxylum Myriacanthum* | Reported to be anodyne, antidotal, aphrodisiac, diuretic, and vulnerary. Oil palm is a folk remedy for headaches, rheumatism and is used as a liniment for indolent tumors. Used as a coloring and flavoring agent in steamed glutinous rice, male aphrodisiac, stomach ache and antitumor agent. |
| Alpha-Tocopherol<br>$C_{29}H_{50}O_2$ | *Citrus Hystrix*<br>*Calamus Scipronum*<br>*Averrhoa Belimbi* | Fruit used as preservative, flavoring in both savory and sweet food. Leaves used as hair shampoo and as medicine.<br>The buds of these canes are eaten as food and have medical and antiseptic properties. They are commonly used for treatment of fever and aches.<br>The syrup of the fruit is useful in relieving thirst, febrile excitement, and also in some slight cases of hemorrhage from the bowels, stomach and internal hemorrhoids. |
| Ascorbic Acid<br>$C_6H_8O_6$ | *Apium Graveolens*<br>*Sauropus Androgynous* | Arthritis, Back Pain (lower), Nervousness, Rheumatism.<br>Insect and disease resistance. |
| Palmitic Acid<br>$CH_3(CH_2)_{14}COOH$ | *Elaeis Oleifera, Elaeis Guineensis* | Anodyne, antidotal, aphrodisiac, diuretic and vulnerary. Oil palm is source of palmitic acid and is a folk remedy for cancer, headaches, and rheumatism. |
| Beta Sitosterol<br>$C_{29}H_{50}O$ | *Morinda Citrifolia*<br>*Alpinia Officinarum*<br>*Sida Acuta* | Diabetes, high blood pressure, arthritis, skin afflictions, and conditions of aging<br>Flatulence, dyspepsia, vomiting and sickness at stomach, and recommended as a remedy for stomach cancer.<br>Entire plant for stomach ache. |
| Selenium | *Astragalus Membranaceus*<br>*Valeriana Officinalis*<br>*Achillea Millefolium* | Prevents severe side effects of chemotherapy in patients with cancer.<br>Inhibits the growth of murine renal cell carcinoma. Activation of immune system.<br>Sedative activity.<br>General tonic for the cardio-vascular system, lowers blood pressure, and slows heartbeat. |
| Anthraquinone<br>$C_{14}H_8O_2$ | *Cassia Acutifolia* | Antihelminthic, antibacterial, laxative, diuretic, for treatment of snakebites and uterine disorders. |
| Tannic acid $C_{76}H_{52}O_{46}$ | *Costus Spinosa* | Tanning of leather. |
| Quercetin$C_{15}H_{10}O_7$ | *Blumea Balsamifera* | Treatment for the swelling of pancreas. |

It has been estimated that one human cell is exposed to approximately 105 oxidative hits a day from hydroxyl radical and other such species of oxidants. ROS are normal oxidant by-

products of aerobic metabolism, and under normal metabolic conditions about 2–5% of O2 consumed by mitochondria is converted to ROS (73, 74).
Oxidative stress thus created permanently modifies the genetic material leading to numerous degenerative or chronic diseases, such as atherosclerosis and cancer (75).
Misrepair of DNA damage could result in mutations such as base substitution and deletion which could lead to carcinogenesis (76).

## *§ 1.4 Non-enzymatic browning*

Non-enzymatic browning (NEB, Maillard reaction) is one of the most important chemical phenomena that may affect food quality in processing and storage. Its effect on color and flavor as well as nutritional value, textural properties, and stability of foods has been clearly demonstrated (77, 78, 79). Much attention has been paid to control the NEB during processing and storage (80, 81, 79). Non-enzymatic browning is an amino-carbonyl reaction involving a series of condensation reactions, some of which can be considered bimolecular (82). Its mechanism and kinetics in real foods and model systems with reducing sugars and amino components as reactants have been studied intensively, and the NEB reaction is known to be affected by a number of factors, such as time, temperature, water content, water activity, pH, concentration of reactants, and the type of reactants and solvents (83, 84, 77, 85-90, 81).
Non-enzymatic browning in food materials has been suggested to be a diffusion-controlled chemical reaction (91-93) and presumably, its rate is dependent on the viscosity of the matrix material and thus affected by the physical state of the system.
Many researchers have made attempts to relate the physical state of food materials and the rate of NEB (94-101, 80, 81, 85, 88-90), especially in low- and intermediate-moisture food systems.

## *§ 1.5 The Maillard reaction*

The Maillard reaction is named after the French scientist Louis Camille Maillard (1878-1936), who studied the reactions of amino acids and carbohydrates in 1912, as part of his PhD thesis, which was published in 1913 (102).

The Maillard reaction is not a single reaction, but a complex series of reactions between amino acids and reducing sugars, usually at increased temperatures. Like caramelisation, it is a form of non-enzymatic browning. In the process, hundreds of different flavour compounds are created. These compounds in turn break down to form yet more new flavour compounds, and so on.

Each type of food has a very distinctive set of flavour compounds that are formed during the Maillard reaction.

Maillard reactions are important in baking, frying or otherwise heating of nearly all foods. Maillard reactions are (partly) responsible for the flavour of bread, cookies, cakes, meat, beer, chocolate, popcorn, cooked rice. In many cases, such as in coffee, the flavour is a combination of Maillard reactions and caramelization. However, caramelization only takes place above 120-150 °C, whereas Maillard reactions already occur at room temperature.

Although studied for nearly one century, the Maillard reactions are so complex that still many reactions and pathways are unknown. Many different factors play a role in the Maillard formation and thus in the final colour and aroma; pH (acidity), types of amino acids and sugars, temperature, time, presence of oxygen, water, water activity ($a_w$) and other food components all are important.

The first step of the Maillard reaction is the reaction of a reducing sugar, such as glucose, with an amino acid. This reaction is shown in figure 3 below and results in a reaction product called an Amadori compound.

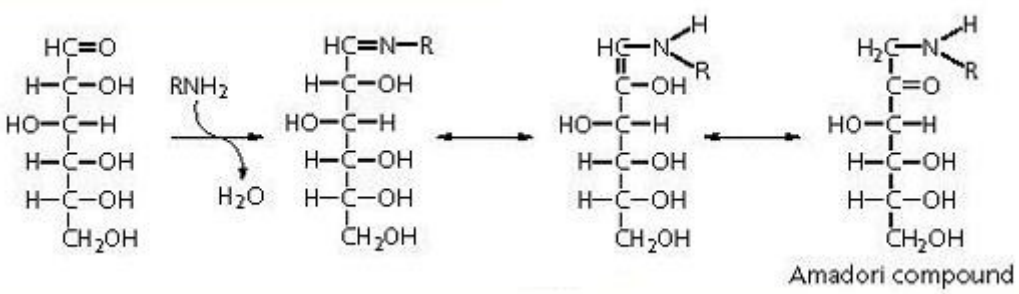


**Figure 3**: The initial step of the Maillard reaction between glucose and an amino acid ($RNH_2$), in which R is the amino acid side group (103)

As can be seen in figure 3, the Amadori compounds easily isomerise into three different structures that can react differently in the following steps. As in food generally over 5 different reactive sugars and 20 reactive amino acids are present, only the first step theoretically already results in over 100 different reaction products.

The larger the sugar, the slower it will react with amino acids. The pentose sugars (5 carbon

atoms), such as ribose, will react faster as hexose sugars (glucose, fructose) and disaccharides (sugar, lactose). From the amino acids lysine, with two amino groups, reacts the fastest and causes darker colours. Cysteine, with a sulphur group, causes specific flavours, but less colour. Sugar alcohols or polyols (sorbitol, xylitol) do not participate in the Maillard reaction. This means that bakery products sweetened with sorbitol will not or hardly change colour during baking.

The next steps differ, depending on the isomer of the Amadori compound. Either the amino acid is removed, which results in reactive compounds that are finally degraded to the important flavour components furfural and hydroxymethyl furfural (HMF). The other reaction is the so-called Amadori-rearrangement, which is the starting point of the main browning reactions, see figure 4.

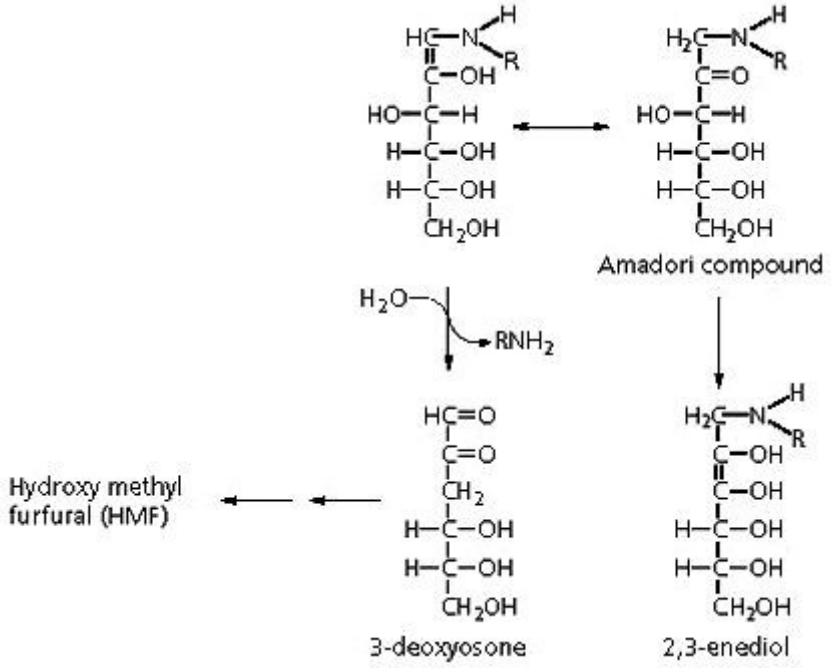


**Figure 4** : Formation of HMF and Amadori-rearrangement (103)

Furfural and hydroxymethylfurfural are characteristic flavour compounds of the Maillard reaction. Furfural is the result of a reaction with a pentose sugar (such as ribose); HMF is the result of a reaction with a hexose (glucose, saccharose).

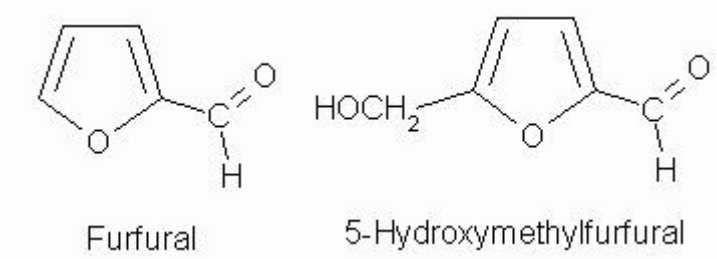


**Figure 5** : Structures of fural and HMF

After the Amadori-rearrangement three different main pathways can be distinguished:

- Dehydratation reactions,
- Fission, when the short chain hydrolytic products are produced, for example diacetyl and pyruvaldehyde,
- "Strecker degradations" with amino acids or they can be condensated to aldols.

These three main pathways finally result in very complex mixtures, including flavour compounds and brown high molecular weight pigments melanoidins.

Melanoidins are present in many foods like coffee, bread and beer. However, up to now the knowledge about structural, functional and physiological properties of this group of food components is rather limited.

The Maillard reaction products thus change the colour and flavour of food, and in most cases these changes are appreciated by people. In addition the melanoidins may have some beneficial anti-oxidant properties.

On the other hand, Maillard reactions may reduce the nutritional value of a product, as amino acids and carbohydrates may be lost. Sometimes the flavour is not appreciated, such as the 'cooking flavour' in sterilized milk.

Some of the Maillard end-products may also be toxic or carcinogenic. One of the Maillard reaction products is acrylamide, a potential toxic compound which is only formed at temperatures above 180 °C, especially in baked or fried products (French fries). When frying below 180 °C acrylamide is not formed.

In general it can be stated that Maillard products have been present in our foods for many thousands of years, and are consumed daily by nearly all people in the world.

Maillard reactions can not, or hardly, be prevented when heating foods. Only by removing the sugars or the amino acids, or making the product very acid or alkaline, the reactions can be

prevented.

## *§ 1.6 Melanoidins*

Melanoidins are coloured (brown) pigments developed during thermal treatment of foods via the Maillard reaction or by dehydration (caramelization) reactions of carbohydrates followed by polymerization (104). Basically, melanoidins are formed by interactions between carbohydrates and compounds, which possess a free amino group, such as amino acids or peptides. Recently, melanoidins – not only those from coffee – have attracted lots of interest as regards their occurrence in foods and the corresponding impact on human health. This is documented by the fact that the European Community set up a COST action (COST Action 919: 'Melanoidins in food and health') starting in 1999 and ending in 2004. This action includes research on the separation and characterization of melanoidins and related macromolecules, the flavour binding, colour, texture and antioxidant properties of melanoidins and investigation of the physiological effects and fate of melanoidins (COST, 2002). The foods of relevance specified are coffee, malt, beer, breakfast cereals and bread.

It can be anticipated that melanoidins are a very heterogeneous group of compounds as regards molecular mass as well as the chemical and biological properties. Taking this into account, one may argue that there is no justification for referring to all those compounds as melanoidins.

On the other hand, is there any alternative? This situation is comparable with other polymers in foods and beverages, e.g. the thearubigins in tea or phlobaphenes in cocoa. It is agreed that those are constituents of the beverage, and contents can be found in tables, but nobody really knows what the properties and the structures of the compounds are. What do we know about the formation of melanoidins? Most of the free mono- and disaccharides are lost during the roasting process; the same is true for free amino acids (105, 104). They are at least in part converted into melanoidins. Another part of the sugars undergoes caramelization reactions, also yielding melanoidin-like pigments (104) or is degraded yielding acidic compounds (106). Not much is yet known about the structures of melanoidins. As already mentioned above, melanoidins are formed during the Maillard reaction along with a variety of flavour compounds. The Maillard reaction in a food matrix is a complex process because of the multitude of compounds present.

Consequently, information on the Maillard reaction is best generated by model experiments with only a limited number of educts present; which, however, still give rise to a considerable number of volatile and non-volatile products (107). As with other polymers in foods, there is practically no information available on the bioavailability of the melanoidins. It seems reasonable to anticipate that the fractions with different molecular mass also will have a different bioavailability.

# *CHAPTER 2*

# *AIM OF THE WORK*

## *§ 2.1 Aim of the work*

An inverse correlation between a diet rich in plant foods and and the occurrence of cardiovascular diseases (CVD) has been reported in several epidemiological studies (108).
The vasculoprotective effect associated to fruit and vegetable consumption is thought to be due to fresh plant-contained phytochemicals, including antioxidant substances such as phenolic compounds, carotenoids and vitamins (108). However, a remarkable amount of the food intake in the human diet comes from processed foodstuffs, and whether processed plant-foods provide less benefit than unprocessed ones is uncertain remains an area of inquiry.

Food processing operations are mostly based on heating. Thermal treatments often result in non-enzymatic browning (NEB), which occurs through sugar thermal degradation (caramelisation), or, when more acidic conditions are present, by the Maillard reaction (MR) between sugar and organic acids (109). The high molecular-weight heterogeneous polymers formed in the last stage of the NEB reaction are called melanoidins. Melanoidins are widely distributed in home- and industrial-processed foodstuffs and may have various *in vitro* functional properties, including antioxidant (110, 111), antihypertensive (112) and metal-binding activities (113). Of particular interest is the antioxidant activity of melanoidins; since these products are naturally formed during food processing and storage, they can influence the oxidative and shelf life of several type of foods including cereals (114), coffee and tomatoes (115). In line with their antioxidant activity, some physiological effects, including the protection of cells from oxidative-induced damage, have been reported (116-118).
However, because of the huge complexity of both reactions and products during their chemical pathway of formation, only partial structures of melanoidins have been elucidated so far (109). Thus it is very difficult to address a specific health effect to a distinctive melanoidin chemical structure; therefore a deep and accurate exploration is needed for melanoidins derived from different foods.

Prunes are obtained by drying the fruit of certain cultivars of *Prunus domestica* L. (*Rosaceae*), and possess the highest antioxidant activity among the most commonly consumed fruits and vegetables (119). The biological effect of prunes on human health has been attributed, in part, to their high polyphenol content and antioxidant capacity (119, 120), which is due to their large amounts of caffeoylquinic acid isomers (120, 121) and flavonoids (122). *In vivo* and *in vitro* experiments indicate that prunes have high antioxidant capacity (123)

along with the ability to inhibit LDL oxidation (120) and to reduce atherosclerosis lesions (124). We have previous reported that drying two common plum varieties to produce prunes resulted in a two to three-fold increase in antioxidant activity, even though it considerably reduced the phenol content (125, 126). We hypothesized that this increase might have been due to the formation of non-enzymatic browning products (NEBPs) (e.g. Melanoidins) after drying. Thus, although the effect of polyphenolic compounds cannot be ruled out, melanoidins appear to be the prevailing contributors to the reported antioxidant activity of prunes *in vitro*. Apricot fruits are considered as a rich source of phytochemicals, which are mainly polyphenols and carotenoids (127, 128). Phenolic compounds, in particular, by acting as antioxidants, are thought to provide various *in vivo* health benefits including hepato- and cardio-protective effects (129, 130). The antioxidant properties of polyphenols in apricots have been studied in relation to ripening, cultivar and puree preparation (128, 131, 132), and contrasting results about the antioxidant activity of fresh apricot fruits have been often reported (133, 134). However, 40-45% of the total world production of apricots is processed, mainly by drying and thermal treatment (135). Similar to our previous finding on prunes (110), we found that drying apricots at high processing temperatures resulted in a significant increase of antioxidant activity, even though the phenol content was significantly reduced (136). We hypothesized that the increased in antioxidant activity observed in the dried apricots might have been due to the formation of NEB products (NEBPs), after drying (e.g. melanoidins). Thus, as reported for prunes (110), melanoidins appear to be the prevailing contributors to the maintained antioxidant activity of dried apricot *in vitro*. In this regard, although the antioxidant properties of melanoidins have been studied *in vitro* for several years their potential antioxidant effects on *in vivo* biological systems such as human cells has been little investigated and is largely unknown.

The finding that oxidative stress is a common feature in many aspects of CVD pathogenesis (137), suggests that its counteraction with antioxidants may prevent disease occurrence or ameliorate a patient's pathological condition. For this reason a great deal of attention is now focusing on naturally occurring antioxidants as potential candidates for CVD prevention and/or treatment. Endothelial cells (ECs) play a crucial role in the integration and modulation of signals within the vascular wall (138) and perturbation of such homeostasis by oxidative damage is the trigger for the development of CVD (139). Indeed, chemical characteristics, both quantitative and analytical, of compounds that participate in melanoidins formation in

prunes and apricots are known to differ and thus different melanoidins may originate from their processing (136, 140, 141).

Hence, the present work was undertaken with the intent to investigate whether the food melanoidins isolated from prunes and dried apricots might protect human ECs against $H_2O_2$-induced oxidative stress and cell damage.

# *CHAPTER 3*

# *MATERIALS AND METHODS*

## *§ 3.1. Chemicals*

Unless stated in the text all the reagents used were from Sigma (Sigma, St Louis, MO).

## *§ 3.2. Sampling and dehydration of prunes*

Prunes were prepared using fruit of the President cultivar. These were bought in a local market at an optimum stage of ripening, pre-treated and dried at 85°C as previously described (124). Before analysis, the dried fruit was packed in co-extruded plastic bags and kept in a freezer at -20 °C.

## *§ 3.3. Sampling, dehydration and blanching of apricots*

The experiments were conducted on the Cafona apricot variety, which has been chosen for its very high content in polyphenols. The fruits were purchased locally at an optimum stage of ripening and those showing defects were discarded. Fruits were size-graded, so that size difference would not affect drying times. Fruits were cut in half along the suture line with a knife and the stone carefully removed by hand. At the end of this procedure, the fruits were immediately checked to eliminate those that had been damaged and then, pre-treated and dried at 75°C as previously described (128). Before analysis, the dried fruit was packed in co-extruded plastic bags and kept in a freezer -20 °C. The blanching was executed as previously reported by boiling the selected samples in water at 90°C for 3 minutes (156).

## *§ 3.4. Determination of HMF, phenolic content and total antioxidant activity*

The polyphenol fraction, which was used to assess both phenols and hydroxymethylfurfural (HMF), was extracted and analyzed by HPLC as previously reported (124, 125) using well-established methods (119, 130).

A Hewlett Packard Series 1090 liquid chromatograph coupled with a diode array detector was used and operating conditions were as previously reported (124, 125). A Gemini 5 µm C18, 250x4.6 mm column was fitted with a Gemini pre-column C18, 4.0x3.0 mm (Phenomenex,

Torrance, CA) and used. The injection sample was 10 μL. Spectra acquisitions and quantifications for HMF and phenols were performed as detailed by us elsewhere (124). All values were expressed as milligrams per kilogram of dry matter (dm) and were calculated as the average of four measurements. The total phenol content was obtained by adding the values of the single phenols detected. Antioxidant activity was evaluated using the radical DPPH method as previously described in detail (124) and expressed as Abs-3 min-1 g-1 of dm.

## *§ 3.5. Melanoidins extraction*

The extraction of melanoidins was carried out in triplicate, following a previously published method (131, 110, 118, 157). In detail, 100 g of pitted and ground fruits were defatted with CHCl3 while stirring. After solvent evaporation, the operation was repeated twice more. Solvent traces were eliminated by rotary evaporation. 200 ml of bi-distilled water were added to this residual solid, and the resulting slurry was sonicated for 30 minutes at 40°C. The water fraction was collected and the operation repeated on the solid phase. The two water fractions were combined and centrifuged at 8400 g for 15 minutes at 15°C, and the supernatant was then evaporated under vacuum at the maximum temperature of 50°C (fraction I). The residual solid was added to that of fraction I and dissolved in 200 ml of ethanol/water (60:40 V/V), and the resulting slurry was then sonicated for 30 minutes at room temperature. This operation was repeated. The two ethanol/water fractions were combined and centrifuged at 8400 g for 15 minutes at 15°C, and the supernatant was then evaporated under vacuum at the maximum T of 50°C (fraction II). The residual solid was added to that of fraction II and dissolved in 200 mL of 2-propanol/water (50:50 V/V), and the resulting slurry was then sonicated for 60 minutes at room temperature. This operation was repeated. The two propanol/water fractions were combined and centrifuged at 8400 g C for 15 minutes at 15°, and the supernatant was then evaporated under vacuum at the maximum T of 50°C (fraction III). The remaining solid fraction, which consisted of pieces of fruit, was fraction IV. The yield of each fraction (as g per 100 g of dried fruit) was recorded.

Fraction IV had no *in vitro* antioxidant activity, and thus it was not taken into consideration.

## *§ 3.6. Determination of non-enzymatic browning (NEB)*

NEB was assessed by both browing index and color variation. The formation of brown pigment due to the NEB reaction can be estimated as a brown index from spectrophotometric readings at 420 nm (142). For this reason the polyphenolic fractions from fresh and dried samples, as well as the melanoidin fractions extracted from dried fruits were subjected to a spectrophotometric reading in absorbance mode at 420 nm in a 1 cm glass cuvette (Beckman DU 640 spectrophotometer). The samples were appropriately diluted in water to give absorbance values of <1. These values were used to give an absorbance value per g dm of each diluted fraction. Five measurements were made for each sample.

The colorimetric analysis has been carried out as proposed by Mastrocola and Lerici (143). The peel colour measurement were assessed with a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan), fitted with a CR-300 measuring head. The colour tonality was expressed as L, a*, b* Hunter scale parameters, and "a" and "b" were used to compute hue angle (tan-1 b*/a*) (144, 158). The measurements have been always done on the same set of 10 fruits selected at the start of the experiment, in order to minimise fruit colour variability.

## *§ 3.7. Cells culture, treatments, and viability assay*

ECV304 is an EC line established from the vein of an apparently normal human umbilical cord. This cell line has been proposed as a suitable model for providing novel insights into the mechanisms governing EC biology under both physiological and pathological conditions (159-163). ECV304 were provided by the European Collection of Animal Cell Cultures (ECACC Salisbury, UK). Cells were grown in medium M199 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 μg/m1penicillin, and 100 μg/m1 streptomycin (Invitrogen,). Cells were maintained in a standard culture incubator with humidified air containing 5% CO2 at 37° C. The day before each experiment, cells were plated in 24-well plates (Corning, Lowell, MA) at a concentration of 100,000 cells per well and pretreated with melanoidins for 6 hrs before oxidative stress was induced in the last 2 hrs, by treatment with the indicated concentration of hydrogen peroxide (H2O2). In accord with a previously study using coffee melanoidins on human hepatoma cells (164), the doses of 2, 6 and 12 μg/ml were

tested in our human vascular model. Cell viability for treated and untreated cell was assessed after 24 hrs by automatic cell counting (Countess® Invitrogen) and expressed as number of cells per ml.

## *§ 3.8. Cell viability and metabolic assay*

Cell viability, for treated and untreated cells, was assessed after 24 hrs by checking the leakage of the cytoplasmatic lactate dehydrogenase (LDH) from cells with a damaged membrane. The amount of LDH released in the medium by death cells was assessed using the kit CytoTox-ONE™ (Promega, Madison, WI). A standard curve with definite amounts of cells (200µ/well) was made, and the release of LDH in the medium was measured after the application of lysis solution (4µl/well). Plates containing samples were removed from the incubator and equilibrate to 22°C, and then the release of LDH from death cells was measured by supplying lactate, NAD+, and resazurin as substrates in the presence of the enzyme diaphorase. Generation of the fluorescent resorufin product, which is proportional to the amount of LDH, was measured using a GENios plus micro-plate reader (Tecan) with excitation and emission of 560 nm and 590 nm, respectively. By using the standard curve, the amount of LDH release in treated and untreated cell was conversed in number of cells per well. The Mitochondrial Metabolic Activity was assessed as previously reported in 96-well plates (BD Falcon) by using the colorimetric assay MTT (Promega, Madison, WI) (165). This colorimetric assay measures the reduction of the yellow 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide by mitochondrial succinate dehydrogenase. The yellow tetrazole compound enters the cells and passes into the mitochondria where it is reduced to an insoluble, purple colored, formazan product. The reduction of MTT in isolated cells and tissues is regarded as an indicator of "cell redox activity" (166). Although evidence exists that the MTT reduction in mammalian cells is also catalyzed by a number of non-mitochondrial enzymes, this reaction is attributed mainly to mitochondrial enzymes and electron carriers (166-168). After treatments cells were added with 20µl MTT solution (5mg/ml) in medium M199 and incubated at 37°C in a cell incubator for 60 minutes. At the end of the incubation period, the medium was removed and the cell monolayer was washed twice with HBSS. The converted dye was solubilized with acidic isopropanol (0.04 N HCl in absolute isopropanol), and plates were analyzed at 570 nm using a GENios plus micro-plate reader (Tecan) with

background subtraction at 650 nm. Results were expressed as percent of untreated control cells.

## *§ 3.9. Determination of cellular redox status*

Cellular redox status was investigated by using the redox-sensing green fluorescent protein (roGFP), which reports the redox status of the GSH/GSSG pool *in vivo* in both plant and mammalian cells (149, 150). Plasmids coding for roGFP2 expression were obtained starting from p*CVU55762-roGFP2* (kindly provided by Dr. Andreas J. Meyer, University of Heidelberg, Germany). Cyt-roGFP2 was obtained by restriction cloning using *Bam*HI and *Not*I restriction enzymes into pcCDNA3 vector (Invitrogen); mito-roGFP2 was obtained by cloning a PCR amplification product into pCMV/myc/mito (Invitrogen) using *Pst*I and *Xho*I sites. Plasmids containing cytoplasmic roGFP2 (cyto-RoGFP) and a mitochondrial targeted roGFP2 (mito-RoGFP2) were transfected in HCV304 by using the lipofectamine 2000 reagent following the provider protocol (Invitrogen).

Transfected cells were selected using 0.8 mg/mL of G418 in the media for 3 to 4 weeks. Positive stably transfectants were selected by serial dilution of G418-resistant clones which constitutively expressed both cyto- and mito-RoGFP2 under a fluorescence microscope (Olympus XI70). RoGFP has two fluorescence excitation maxima at 400 (oxidized form) and 485 nm (reduced form) and display rapid and reversible ratiometric changes in fluorescence in response to changes in ambient redox potential. The ratios of fluorescence from excitation at 400 and 485 nm indicate the extent of oxidation and thus the redox potential while canceling out the amount of indicator and the absolute optical sensitivity (150). In place of confocal imaging analysis we used a recently developed fluorometer-based method for monitoring roGFP oxidation (169). Fluorescence measurements were performed in clear 24-well plates (Corning, Lowell, MA) on a fluorescence plate reader GENios plus (Tecan, Männedorf, CH) from the upper side using multiple reads per well (the read pattern was square, and the number of reads was 2 x 2). Cells were excited by using 400 and 485 nm filters and fluorescence values were measured using 535 nm emission filter. For background correction emission intensities were determined for non-transformed cells (4 discs each experiment) exposed to same excitation wavelengths under the same conditions. These values were averaged and subtracted from the fluorescence values of roGFP2. The degree of oxidation of

the roGFP2 was estimated from the ratios of light intensities obtained during 1-min intervals under 400- and 485-nm excitation. Treatment-induced variations of roGFP2 oxidation were estimated by comparison with roGFP oxidation in control untreated cells.

## *§ 3.10. Measurement of mitochondrial membrane potential*

Measurement of mitochondrial membrane potential (MMP) was performed with the JC-1 stain (Invitrogen), a lipophilic cation fluorescent dye that accumulates in mitochondria in a MMP-dependent manner, showing red fluorescent JC-1 aggregates (590 nm emissions) at higher MMP. When MMP decreases, JC-1 aggregates depart from mitochondria and change to green fluorescent JC-1 monomers (535 nm emissions). Therefore, the ratio of the red signal to the green can been used to detect the occurrence of MMP depolarization in the early stages of cell death due to mitochondrial damage (170, 171). After treatments cells were incubated at room temperature in the dark with 5μg/ml JC-1 in HBSS for 30 minutes. The cells were then washed twice with HBSS and fluorescence levels were immediately acquired with excitation and emission wavelengths set at 535 and 590 nm, respectively, for red fluorescence, and 485 and 535 nm, respectively, for green fluorescence. Measurements were performed in clear 24-well plates (Corning, Lowell, MA) on a fluorescence plate reader GENios plus (Tecan, Männedorf, CH) from the upper side using multiple reads per well (the read pattern was square, and the number of reads was 2 x 2). For each sample, the results were calculated as the ratio (red/green) of fluorescence of sample, averaged after the fluorescence values had been corrected for the background and protein content.

## *§ 3.11. Staining and fluorescence visualization*

MitoTracker Red CMXRos (Invitrogen, catalog # M7512) is the oxidized form of a dye that can be taken up into the mitochondria of live cells utilizing their uniquely high membrane potential. This dye is retained in the mitochondria after fixation and therefore can be used to label/stain mitochondria followed by additional immunocytochemistry. For the staining, cells have been grown on glass coverslips inserted inside the multi-wells. At the end of experiments, the culture media has been removed and the cells monolayer washed with pre-warmed PBS. Then the pre-warmed solution containing the MitoTracker® probe has been

added to each well (final probe concentration of 300 nM) and the cells have been incubated at 37°C for 30 minutes. After the staining was complete, cells were fixed with a solution of 4% formaldehyde in complete growth medium at 37°C for 15 minutes, and the permeabilized for 10 minutes in PBS containing 0.2% Triton® X-100. Hoechst 33342 (SIGMA, catalog# B2261), is part of a family of blue fluorescent dyes commonly used to stain DNA. After fixation/ permeabilization, the Hoechst dye has been added to the cells at a final concentration of 0.12 µg/ml. The dye has been left to incubate with the cells for 15 minutes and then the cells monolayer has been washed for five times with PBS before visualization. Fluorescence visualization of fixed cells has been performed on a Olympus BX 51 microscope, using a 20, 40, and 100x objectives with Numerical Aperture (NA) of 0,70, 1,00 and 1,35 respectively.

## *§ 3.12. Statistical analysis*

Data were expressed as means ± S.D. of three or four different experiments. One-way analysis of variance (ANOVA) followed by a post-hoc Newman-Keuls Multiple Comparison Test were used to detect differences of means among treatments with significance defined as $P < 0.05$. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

# *CHAPTER 4*

# *RESULTS*

## *§ 4.1 Changes in chemical parameters elicited by prunes processing*

Using a previously published procedure (125), fresh fruit was processed by standardized drying and heating conditions, then both fresh fruit (plums) and processed (prunes) were chemically characterized on the basis of commonly recognized parameters (125, 126), namely the presence of intermediate (hydroxymethylfurfural) and final (NEBPs) products of the MR. The compound hydroxymethylfurfural (HMF) is practically absent in fresh food, but it is naturally created in sugar-containing food during heat-treatments like drying or cooking. HMF is formed in the MR and is one of the intermediate products of the NEB (120, 128). Despite the decreased total phenol content (Figure 1A) an increase in antioxidant capacity was elicited by the food transformation process, which strongly correlated with the paralleled increase of HMF content (Figure 1B). The processing-induced increase of HMF was also accompanied by a significant rising in the browning index (Figure 1C), suggesting NEBPs may be responsible for the increase in chain-breaking activity observed after fruit transformation. Indeed, the absorbance at 420 nm, which represents the browning index, is an important parameter for the presence of NEBPs and is related to the brown pigment formation caused by the NEB reaction during plum processing (Figure 1C) (142).

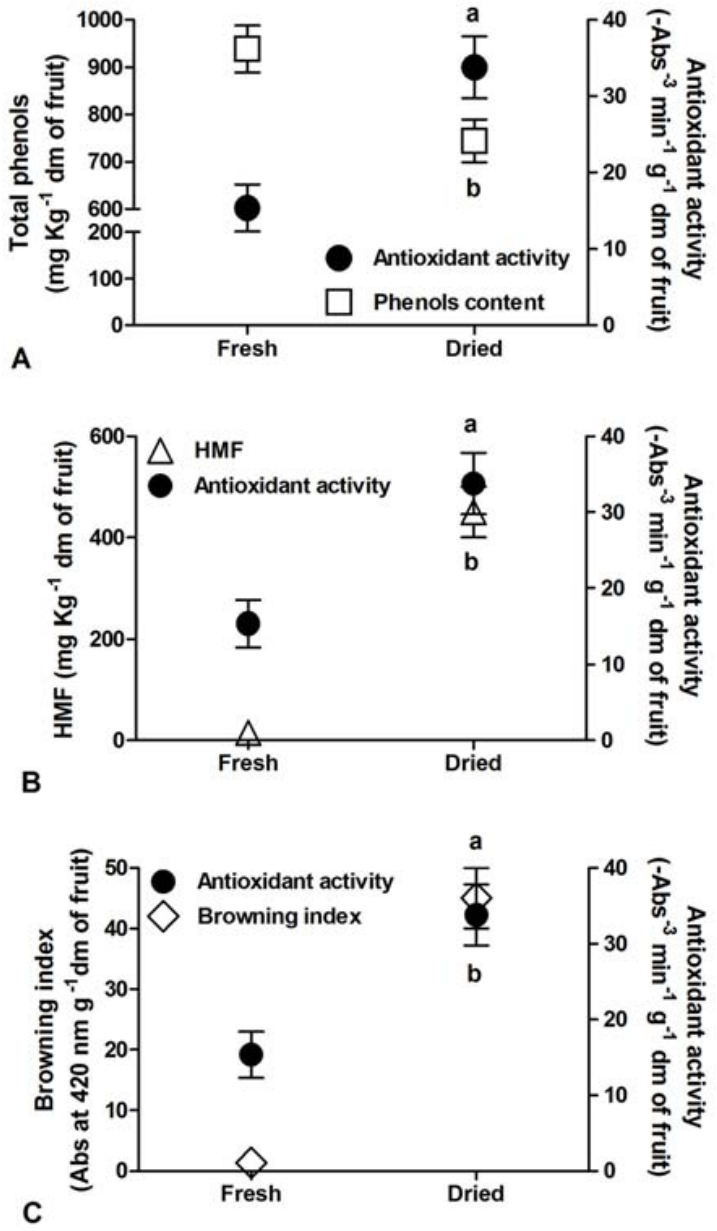


**Figure 1.** Changes in chemical parameters elicited by fruit processing. Changes in antioxidant activity (A-C), Phenols content (A), hydroxymethylfurfural (HMF) (B) and Browning index (C) during transformation of fresh fruit (plums) to dried (prunes). Data are the mean ± standard deviation (SD) from four measurements. (A-B) a; b, significantly different from the fresh sample.

The measurements of the browning index indicate that NEBPs were presents in all melanoidin fractions extracted from dried samples, while (as with whole fresh fruit samples) they were completely absent on those melanoidin fractions extracted from fresh fruit (Table 1). Noteworthily, the amount of NEBPs in the whole dried fruit samples was basically the sum of the NEBPs detected in the three melanoidins fractions, indicating they were present in the same amount in the prunes and in the melanoidin fractions extracted from prunes (Table 1).

Fraction I showed the highest amount of NEBPs among all the melanoidin fractions, and was therefore chosen to be tested for its
antioxidant activity on cells exposed to oxidative stress.

**Table 1.** browning index of whole fruit sample and of melanoidins extracted from fresh plums and dried prunes

| Samples | Fraction I[1] | Fraction II[1] | Fraction III[1] | Total[2] |
|---|---|---|---|---|
| Fresh plums | 0 | 0 | 0 | |
| Dried prunes | 23.02 ±2.00[3] | 11.49±1.45[3] | 12.43±2.20[3] | 46.95±5.24[3] |

[1] melanoidins fractions, [2] whole fruit, [3] non enzymatic browning products (NEBPs), Abs at 420nm $g^{-1}$ dm of fruit. Data are the mean ± standard deviation (SD) from four measurements

## *§ 4.2 Thermal treatment increases NEB in processed apricots*

A significant increase in antioxidant capacity was elicited by fruit drying, which strongly correlated with the paralleled increase of color found in the same polyphenolic extract (Fig. 2A). Colour was indeed dramatically changed by the drying process with a significant reduction of tonality (Fig 2B). In particular, the blanching of sample resulted in a significantly lower reduction of the drying-induced variation of color, which is expressed as a hue angle in the Hunter scale (143, 144). In fact, a more pronounced shift to a redder and deeper zone in the Hunter scale is evident in the dried, control fruits, with respect to the blanched ones, which might depend on the enzymatic browing contribution (142). However, in blanched fruits where no enzymatic browning is present (145), the observed color variation can be explained only by the formation of NEB during the drying process (Fig 2B). Browing index analysis of the melanoidin fractions isolated from fresh and processed apricots indicated that NEBPs were present only in dried fruits, thus confirming melanoidins presence in the extract obtained from the processed samples (Fig 2C). Among all the melanoidins fractions, Fraction I showed the highest amount of NEBPs as measured at 420 nm (data not shown), and was therefore chosen to be tested for its antioxidant activity on cells exposed to oxidative stress.

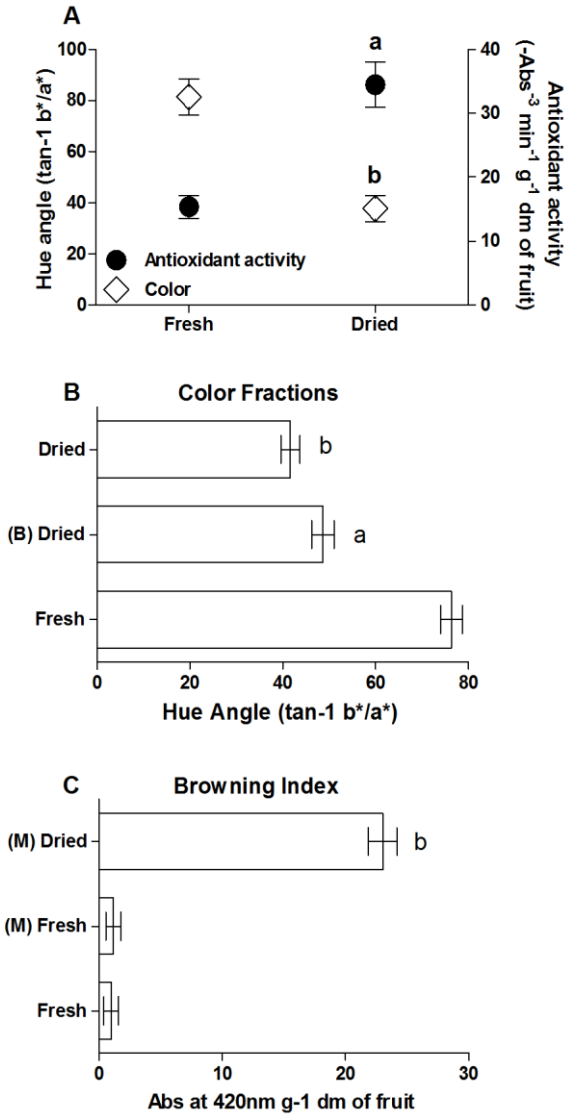


**Figure 2:** Changes in chemical parameters elicited by fruit processing. (A) Changes in antioxidant activity and color expressed as hue variation (tan - 1 b*/a*). (B) Changes in color expressed as hue variation (tan - 1 b*/a*). (C) Changes in color expressed as browning index (Abs at 420nm $g^{-1}$ dm of fruit). Fresh, fresh fruits; M, melanoidins; B, blanching. Data are the mean ± standard deviation (SD) from four or five measurements. (A-C) a; b, significantly different from the fresh sample.

## *§ 4.3 Prune Melanoidins protect human endothelial cells from hydrogen peroxide-induced cell death*

A variety of pathogenic stimuli can increase ROS production within the EC, and oxidative-induced EC dysfunction is emerging as the probable initial step in the progression toward pathological conditions such as atherosclerosis and hypertension (139). Given the pivotal role

played by the endothelium in cardiovascular homeostasis and the involvement of EC dysfunction in CVD pathogenesis (139), it was reasonable for us to use a human EC line to investigate the effect of melanoidins on H2O2-induced oxidative damage. In order to mimic oxidative damage and set standard conditions for the following experiments, we first investigated the effect of different doses of H2O2 on ECV304 cell death and mitochondrial damage. As expected, 2hr-treatment of ECV304 cells with H2O2 resulted in a dose-dependent decrease of cell survival as evidenced by the significant decrease in the number of viable cells in comparison with the untreated control group (Figure 3A). Some pathological stimuli, including ROS, can trigger an increase in mitochondrial membrane permeability promoting the release of toxic factors and the dissipation of MMP. The consequence of such mitochondrial dysfunction is a bioenergetic catastrophe culminating in the disruption of plasma membrane integrity with ensuing cell death (146). Thus regulation of cell death has emerged as a second major function of mitochondria, in addition to their established role in energy metabolism (146).

Our experimental results indicated that H2O2-induced cell death was indeed associated with a superimposable loss of MMP, which strongly implicated mitochondria in the cell death elicited by H2O2 (Figure 3A). Based on these experiments, around 50% of H2O2-induced mitochondrial impairment and cell death was observed at 0.1 mM of H2O2. We therefore used this concentration as the standard condition to mimic oxidative-induced cell damage in the following experiments. Previous studies indicate that pretreatment of human hepatoma cells with coffee (117) and biscuit (118) melanoidins exerts remarkable protection against oxidative induced cell death; therefore we investigated whether prune melanoidins would exert any protective effect on the observed H2O2-induced cell damage. To this end, cells were treated with melanoidins for 6 hrs and H2O2 was added during the last 2 hrs of incubation to induce oxidative stress. As reported in Figure 3B melanoidin pretreatment was able to dose-dependently counteract both cell death and MMP impairment as induced by 0.1 mM H2O2, strongly indicating a protective effect of these polymeric compounds against oxidative stress and mitochondrial-mediated cell death.

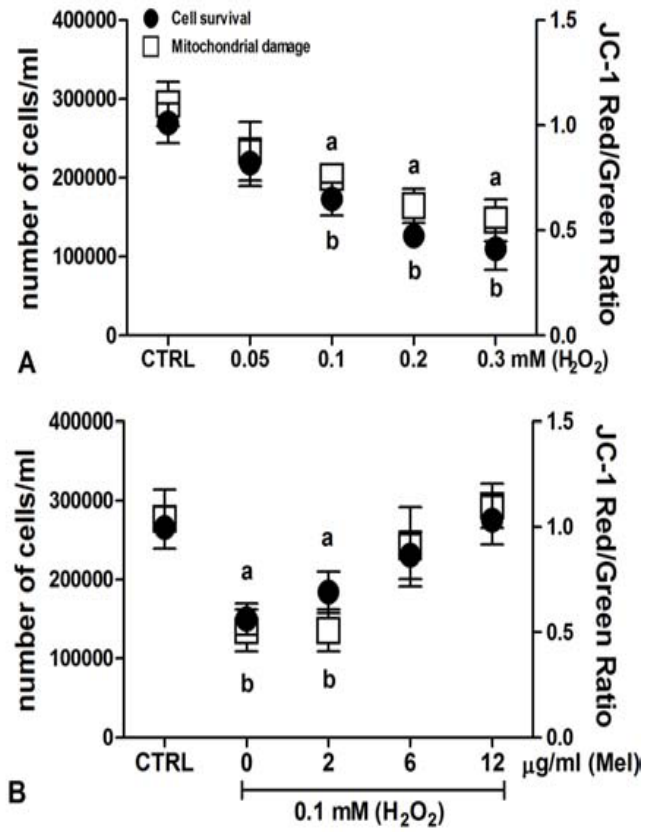


**Figure 3.** Prune Melanoidins protect human endothelial cells from hydrogen peroxide-induced cell death. Dosedependent effect of hydrogen peroxide (H2O2) on cell survival and mitochondrial damage (A). Dose-dependent effect of melanoidins on H2O2-induced mitochondrial damage and cell death (B). Data are the mean ± standard deviation (SD) of four experiments. (A-B) a; b, significantly different from the control.

## *§ 4.4 Apricot melanoidins protect endothelial cells from hydrogen peroxide-induced mitochondrial damage and cell death*

We first tested apricot melanoidins for potential toxicity in our human endothelial cells model. Based on previous observations concerning melanoidins from other sources of food such as coffee (117) and biscuit (118), we tested apricot-melanoidin at the concentrations of 2, 6 and 12 µg/ml at 48 hrs of stimulation, then melanoidin-treated cells were compared to untreated ones for both cell viability and MMA. Results shown in figure 4, which depict respectively lactate dehydrogenase (LDH) release and 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction, clearly indicate that apricot melanoidins did not produce toxic effects for the cells under the employed experimental conditions. We have just demonstrated that pretreatment of human ECs with prune

melanoidins exerts remarkable protection against oxidative-induced cell death. However, melanoidins from different foods may have different structures and activities (109). Indeed, the exact sequence of reactions from which melanoidins originated, as well as their chemical structures, in different food remain largely unknown (109). We therefore asked whether, as with those isolated from prunes, melanoidins isolated from apricots could exert a protective effect on the observed $H_2O_2$-induced cell impairment. To this end, cells were treated with apricot melanoidins for 6 hrs and then $H_2O_2$ was added during the last 2 hrs of incubation to induce oxidative stress. As shown in figure 5A melanoidins pretreatment was able to dose-dependently counteract the decrease in cell viability induced by 100 μM $H_2O_2$. Failure of oxidant in eliciting MMP and MMA impairment in melanoidin-pretreated cells, strongly indicates a protective effect of these compounds against oxidative stress and mitochondrial-mediated ECs death (Fig. 5B-C).

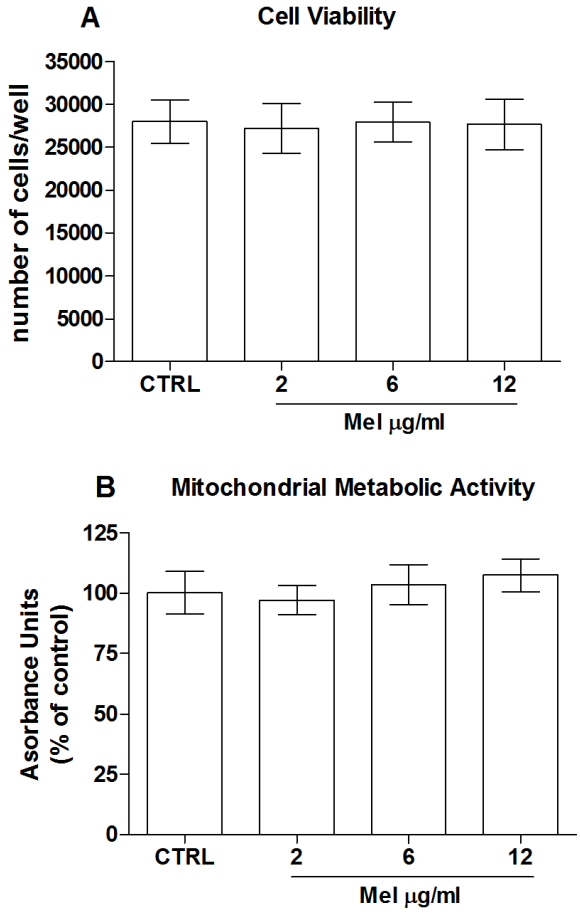


**Figure 4:** Apricots melanoidins are not toxic for endothelial cells. Effect of different concentrations of apricot melanoidins on (A) cell viability and (B) mitochondrial metabolic activity. Data are the mean ± standard deviation (SD) of four experiments.

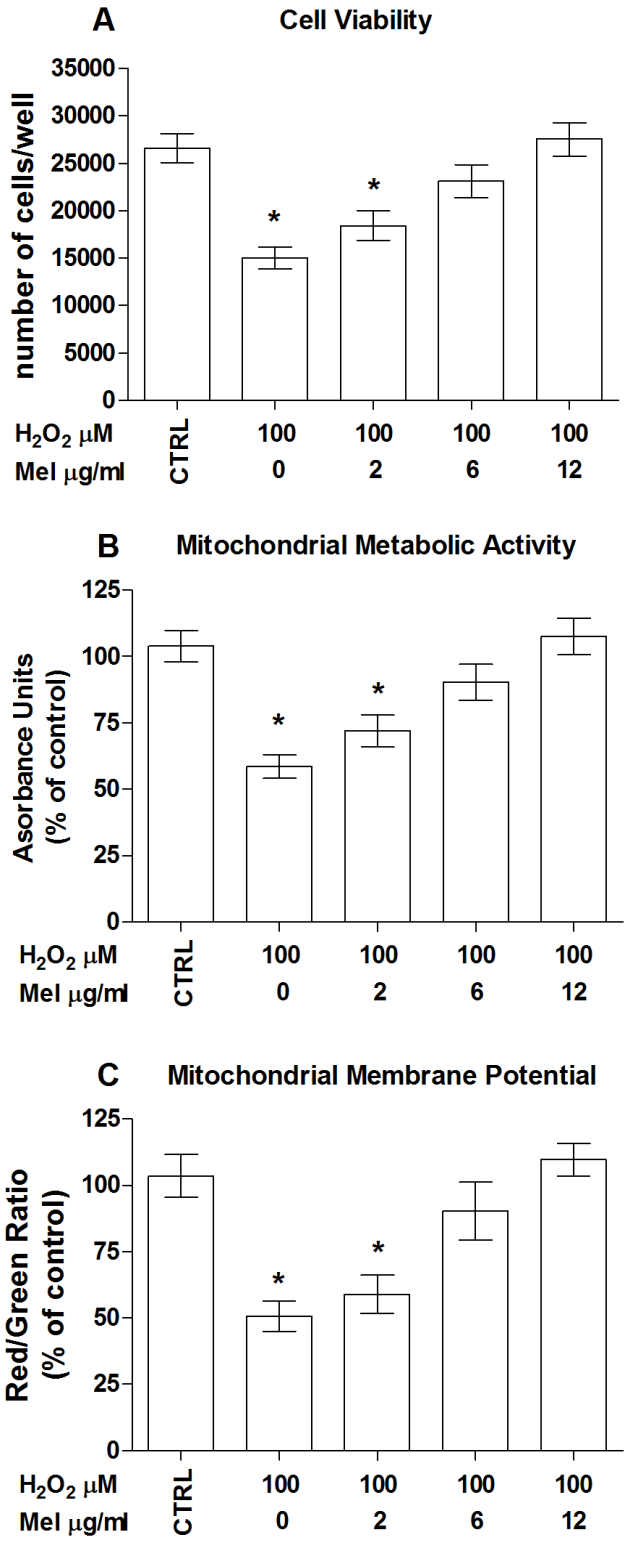


**Figure 5:** Melanoidins protect endothelial cells from hydrogen peroxide-induced mitochondrial damage and cell death. Dose-dependent effect of melanoidins on hydrogen peroxide ($H_2O_2$)-induced (A) cell death, (B)

mitochondrial metabolic activity and (C) mitochondrial membrane potential. Data are the mean ± standard deviation (SD) of four experiments. (A-C) *, significantly different from the control.

## *§ 4.5 Hydrogen peroxide induces oxidation of both cytosolic and mitochondrial compartments*

With the intention of elucidating the molecular mechanisms underpinning prune and apricot melanoidin protection, we used a redox-sensitive green fluorescent protein (roGFP) to investigate the effect of these polymers on H2O2-induced intracellular oxidative change. Fluorescence imaging of ROS in live cells has been widely used to assess intracellular oxidation in different cellular compartments and under various experimental conditions (147). However, many of the methods so far employed to determine the levels of intracellular ROS suffer from various pitfalls (147).

Because electrons for ROS detoxification derive at least in part from the GSH pool, variations of ROS levels are manifested in concomitant changes in the thiol redox potential, which is reflected in the reduced to oxidized glutathione ratio (GSH/GSSG) (148). RoGFP2 can be targeted to various cellular compartments and due to two engineered cysteine thiols is sensitive to environment redox change resulting in a thiol-thiol bond (149, 150). Dithiol formation causes reciprocal changes in roGFP emission intensity when excited at two different wavelengths. Thus the analysis of roGFP fluorescence measures the redox status of the intracellular GSH/GSSG pool (149, 150).

To follow intracellular redox changes during our experimentation we have used two human EC ECV304 lines constitutionally expressing the redox-sensing green fluorescent protein (roGFP) in both the cytosolic (cyto-roGFP) and mitochondrial (mito-roGFP) compartment (Figure 6). In particular, the fluorescence photo shown in figure 6B corresponds to ECV304 cells expressing the cyto-roGFP, while the fluorescence image shown in figure 6D represents ECV304 cells expressing mito-roGFP. Expression of the mito-roGFP in the mitochondrial compartment is confirmed by the image shown in figure 6F, which depicts the merged photo of ECV304 cells expressing the mito-roGFP (D) and ECV304 cells stained with the mitochondrial marker MitoTracker Red (E). While the two images displayed in panels A and C of figure 6, depict the merged photos of ECV304 cells expressing the cytoplasmic (A) and the mitochondrial (C) form of the roGFP (green), along with the bright-field (40X magnification). From A-F, the nuclei are

stained with Hoechst (blue). These two cell lines allowed us to specifically follow potential changes of the cytosolic and mitochondrial redox state during our experimentation.

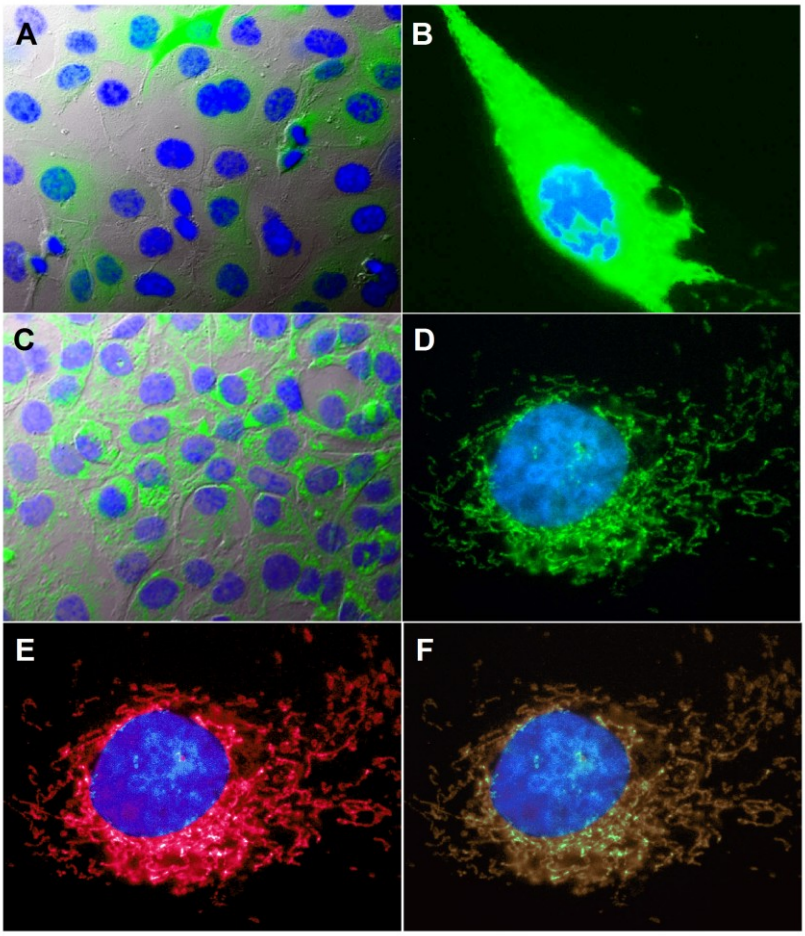


**Figure 6.** ECV304 cells lines constitutively expressing the cytoplasmic (cyto-roGFP) and mitochondrial (mito-roGFP) form of roGFP. Cells were grown in glass chamber slides at concentrations to allow 50-70% confluence in 24 hrs. On the day of experiments, cells were washed with PBS three times, counterstained with the mitochondrial marker MitoTracker Red and the nuclear marker Hoechst**,** fixed with 4% paraformaldehyde and mounted for fluorescence microscopy visualization. Images A and C depict respectively merged photos of ECV304 cells expressing the cyto-and mito-*roGFP* (green) protein, Hoechst staining (blue) and bright-field (40X, NA=1.00). Images B and D depict respectively merged photos of ECV304 cells expressing the cyto-and mito-*roGFP* (green) protein, counterstained with Hoechst (blue) (100X, NA=1.35). The figure F depicts the merged photo of ECV304 cells expressing the mito-roGFP protein (D) and ECV304 cells stained with the mitochondrial marker MitoTracker Red (E). (100X, NA=1,35).

## *§ 4.6 Prune Melanoidins protect cytosolic and mitochondrial compartments from hydrogen peroxide-induced oxidative redox changes*

As reported in Figure 7A, the treatment of roGFP expressing cells with different H2O2 concentrations, dose-dependently shifted the ECV304 intracellular redox status toward a more

oxidative condition in both mitochondrial and cytosolic compartments, indicating that under our experimental conditions roGFP2 has a significant dynamic range and responds linearly to increasing doses of a well-known oxidant. Note that the observed increase of intracellular oxidative conditions elicited by the applied doses of oxidant was paralleled by a corresponding dose-dependent increase in mitochondrial damage and cell death (Figure 3A), confirming the relationship between these H2O2-elicited phenomena.

We next wanted to determine whether the cellular protection elicited by prune melanoidins was due to counteraction of H2O2-induced intracellular oxidation. To this end, roGFP expressing cells were treated with prune melanoidins for 6 hrs and 1 mM of H2O2 was added during the last 2 hrs of incubation. At the end of the experiment both mito- and cyto-roGFP fluorescence were recorded. Data reported in Figure 7B indicate that melanoidins were able to dose-dependently inhibit intracellular oxidation elicited by 0.1 mM H2O2 and reestablish an intracellular redox state similar to that of control cells. Consistent with this antioxidant effect is the observed dose-associated protection exerted by prune melanoidins against H2O2-induced MMP depolarization and cell death (Figure 3B), indicating a tight link between their antioxidant activity and cellular protection.

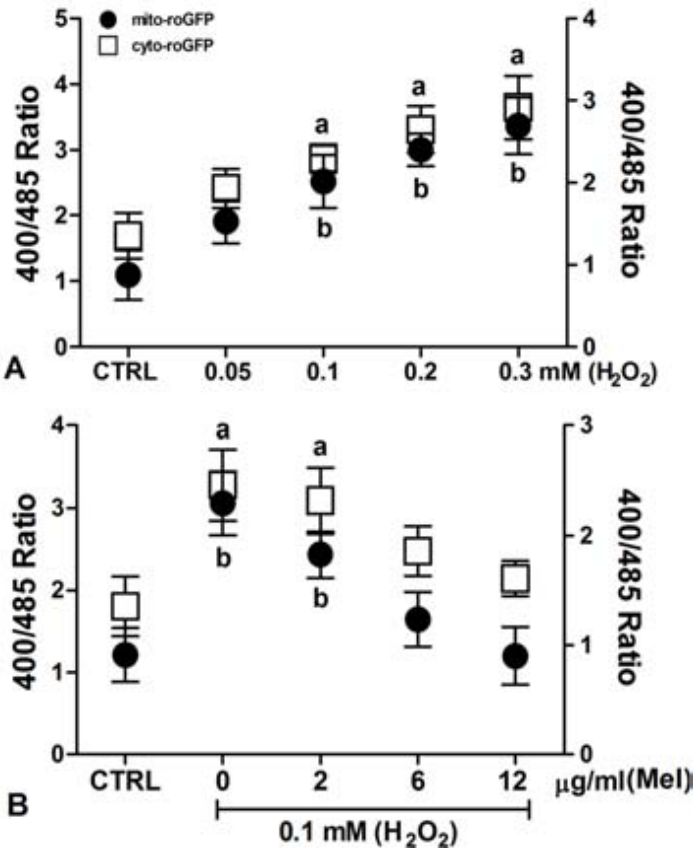

**Figure 7.** Prune Melanoidins protect human endothelial cells from hydrogen peroxide-induced oxidative stress. Dose-dependent effect of hydrogen peroxide (H2O2) on cytoplasmic (cyto-roGFP) and mitochondrial (mito-roGFP) ro GFP2 oxidation (A). Dose-dependent effect of melanoidins on H2O2-induced cytoplasmic (cyto-roGFP) and mitochondrial (mito-roGFP) ro GFP2 oxidation. Data are the mean ± standard deviation (SD) of four experiments. (A-B) a; b, significantly different from the control.

## *§ 4.7 Apricot melanoidins protect cytosolic and mitochondrial compartments from hydrogen peroxide-induced oxidative redox changes*

Just as we had done previously for the prune melanoidins, we next wanted to determine whether the cellular protection elicited by apricot melanoidins was due to the counteraction of $H_2O_2$-induced intracellular oxidation. To this end, roGFP expressing cells were treated with apricot melanoidins for 6 hrs and 100 μM of $H_2O_2$ was added during the last 2 hrs of incubation. At the end of the experiment both mito- and cyto-roGFP fluorescence were recorded. Data shown in figure 8 A-B indicate that melanoidins were able to dose-dependently inhibit intracellular oxidation induced by 100 μM $H_2O_2$ maintaining an intracellular redox state similar to that of control cells.

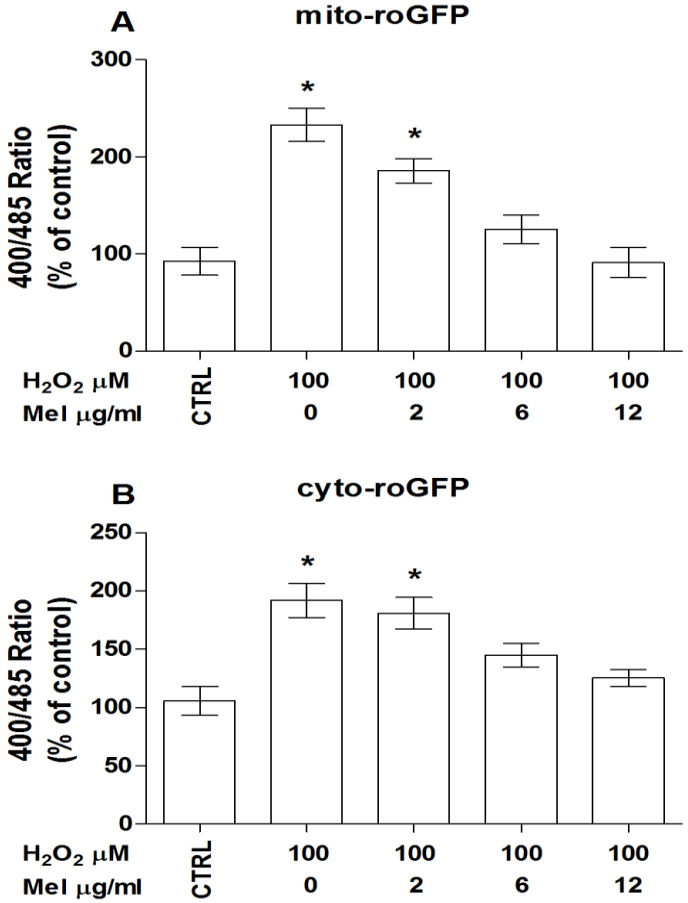


**Figure 8.** Melanoidins protect human endothelial cells from hydrogen peroxide-induced intracellular oxidative stress. Dose-dependent effect of melanoidins on $H_2O_2$-induced cytoplasmic (cyto-roGFP) and mitochondrial (mito-roGFP) roGFP oxidation. Data are the mean ± SD of four experiments. (A-B) *, significantly different from the control.

Fluorescent microscopy results confirming the protective effect of apricot-melanoidins on $H_2O_2$-induced intracellular oxidation are reported in figure 9. A yellow fluorescence pattern, which is the overlapping of the mito-roGFP (green) and MitoTracker Red, is clearly visible in both control and $H_2O_2$-treated cells pretreated with apricot-melanoidins (Fig 9 A and D). On the contrary, $H_2O_2$-induced mitochondrial damage is clearly evident in oxidatively stressed cells, which lack the above-mentioned yellow pattern. H2O2-treated cells, have indeed clear mitochondrial damage, and therefore unable to take the Red MitoTracker dye inside (Fig. 9B). As indicated in figure 9C melanoidin alone did not produce intracellular oxidative stress in cultured ECs. Quite similar results are shown concerning ECs expressing the cyto-roGFP (Figure 9E-H), although a clear yellow pattern is not visible due to the expression of roGFP in the cytoplasm. However, a bigger and clearer, punctate red patter is evident in both control and $H_2O_2$-treated cells pretreated with apricot-melanoidins (Fig. 9E and H), as compared with the H2O2-treated ones (Fig. 9F). This is due to the compromised mitochondrial function in $H_2O_2$-treated. No oxidative damage has been produced by melanoidins alone (Fig. 9G).

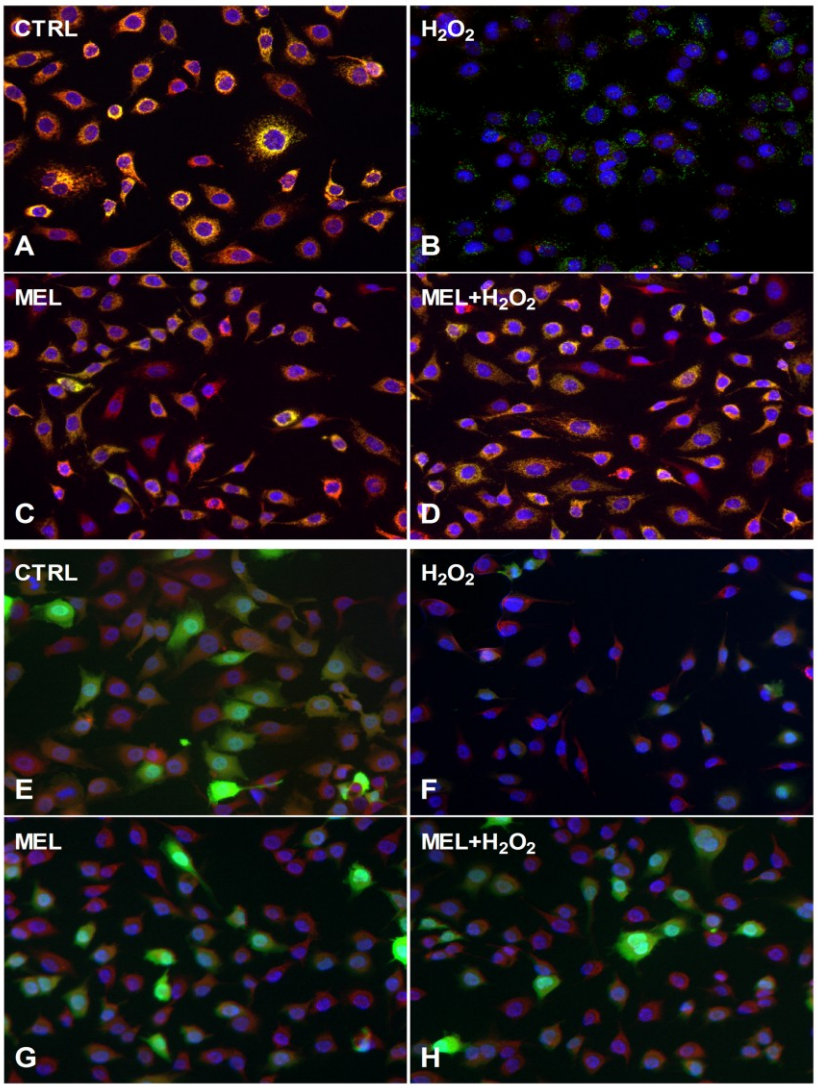


**Figure 9.** Apricot Melanoidins protect human endothelial cells from hydrogen peroxide-induced intracellular oxidative stress. Cells were grown in glass chamber slides at concentrations to allow 50-70% confluence in 24 hrs. On the day of experiments, cells were treated with apricot melanoidins for 6 hrs and then $H_2O_2$ was added during the last 2 hrs of incubation to induce oxidative stress. Then cells were washed with PBS three times, counterstained with the mitochondrial marker MitoTracker Red and the nuclear marker Hoechst**,** fixed with 4% paraformaldehyde and mounted for fluorescence microscopy visualization. Images (A-D) depict merged photos of ECV304 cells expressing the mito-*roGFP* (green) protein, counterstained with both MitoTraker Red and Hoechst. Images (E-H) depict merged photos of ECV304 cells expressing the cyto-*roGFP* (green) protein, counterstained with both MitoTraker Red and Hoechst**.** From A-H, magnification was 20X and NA 0,70).

# *CHAPTER 5*

# *DISCUSSION*

## *§5.1 Discussion*

During their lifetime cells are subjected to oxidative damage, which is reported to be associated with several pathological conditions including cancer and CVD (137, 151). Although endogenous antioxidants play an important role in protecting cells against oxidative insults, additional antioxidants (e.g. dietary antioxidants) appear to be required to prevent or to protect living cells from oxidation (108). In this context, health benefits exerted by plant-derived compounds and extracts have been mainly ascribed to their antioxidant potential and the resulting capability to counteract oxidative-induced damage (108).

However, during food processing and storage, chemical reactions among food components lead to both destruction and formation of phytonutrients (115), therefore whether processed plant foods provide the same benefits as those ascribed to unprocessed ones is uncertain. For instance, melanoidins are heterogeneous polymeric structures formed during food processing in the last stage of the MR whose effects on human health are largely unknown. Indeed, although the antioxidant properties of melanoidins have been studied *in vitro* for several years their potential antioxidant effects on *in vivo* biological systems such as human cells has been little investigated and is barely known. In this light, we explored the effect of melanoidins formed during the transformation of fruits against H2O2-induced oxidative stress and cell damage.

As we wrote previously, it has been a reasonable choice for us to use a human ECs line to investigate the effect of apricot melanoidins on H2O2-induced oxidative damage, considering the role of endothelium in cardiovascular homeostasis and in the CVD pathogenesis (139). A pathology-associated rise of ROS can trigger mitochondrial membrane permeability promoting the dissipation of MMP and ultimately cell death (146). In fact, even in our experimental model of oxidative cell death we found a superimposable loss of both MMP and MMA, which clearly indicated the implication of mitochondria in the cell death induced by $H_2O_2$. The direct link between oxidative stress, MMP depolarization and cell death is now clearly demonstrated and widely accepted (152).

In our vascular cell model H2O2 was able to dose-dependently elicit all three of the above-mentioned phenomena, clearly indicating that cell death occurred because of the intracellular oxidative conditions and the mitochondrial damage. Pretreatment of cultured cells with prune

and apricots melanoidins, significantly counteracted and ultimately abolished H2O2-induced intracellular oxidation, MMP depolarization and cell death.

In this context, we believe our results may be of importance, since they indicate for the first time that melanoidins isolated from prunes and apricots, exert a significant cellular protection against H2O2-induced oxidative stress and mitochondrial-mediated EC death, even considering that compounds that participate in melanoidin formation in prunes and apricots differ both analytically and quantitatively, and that alternate forms of melanoidins may originate from the processing of these two fruits (136, 140, 141).

Fluorescence imaging of ROS in live cells has been widely used to assess oxidative stress in different cellular compartments and under various experimental conditions (147). However, many of the methods so far employed to determine the levels of intracellular ROS suffer from various pitfalls (147). A new approach was therefore used in this work to follow $H_2O_2$-induced intracellular oxidation and its possible counteraction by melanoidins. By employing two ECs lines, which constitutionally express the redox-sensitive proteins mito- and cyto-roGFP (Fig. 6), we were able to selectively follow changes of the redox state in the cytosolic and mitochondrial compartment. Due to the ability to measure the ratio between the oxidized (GSSG) and reduce form of glutathione (GSH), these two cells lines provided a useful tool for assessing the variation of intracellular redox state, showing significant dynamic range and linear response to increasing doses of the well-known oxidant, $H_2O_2$. Of note, the increased oxidative conditions induced by the applied doses of oxidant were paralleled by a corresponding dose-dependent rise in mitochondrial damage and cellular death, suggesting a relationship between these $H_2O_2$-induced phenomena. Under these experimental conditions, apricot and prunes melanoidins were able to counteract $H_2O_2$-induced oxidation, maintaining the intracellular redox conditions similar to that of control cells. Consistent with this antioxidant effect is the observed dose-associated protection exerted by apricot melanoidins against the $H_2O_2$-induced mitochondrial impairment and cell death, indicating a tight link between their antioxidant activity and cellular protection.

To our knowledge, this is the first work on the protective effect of apricot and prunes melanoidins against oxidative-induced cell death. Moreover, using a novel genetically engineered fluorescence protein to ratiometrically assess the intracellular redox state in living cells, our data confirm and reinforce previously published observations using coffee (117), biscuit (118) and synthesized melanoidins (116). Melanoidins indeed, appear to work as antioxidants by positively modulating the GSSG/GSH ratio in favor of the reduced form, and

thus favorably preparing the cell to face oxidative insult. In addition, we also detailed the mechanism of cellular protection afforded by melanoidins, which clearly involves protection against intra-mitochondrial oxidation and oxidative-induced mitochondrial impairment assessed as MMA and MMP depolarization. We believe this work adds new insight concerning the effect of processed plant foods on cellular physiology. Indeed, melanoidins from different sources could have different effects, and because of the lack of knowledge in this field, it is imperative that various melanoidins be evaluated under different experimental conditions to determine their effects. Although further studies are required to better characterize the molecular mechanism of melanoidin protection, our findings support the general observation that natural antioxidants from fruits and vegetable can have a cardiovascular protective effect against oxidative stress. To our knowledge, plasma, organs and tissue levels of melanoidins in people are so far unknown, therefore whether the protective effect exerted by melanoidins in cultured cells may be translated in vivo remains to be elucidated. However, there is circumstantial evidence of melanoidin absorption in vivo (153), and consistent with this observation, their antioxidant activity in human volunteers has indeed been reported (154, 155, 152). In addition, data obtained with gravimetric techniques allow estimation of a daily intake of about 10 g of melanoidins in a Western diet (153). These observations indicate that melanoidins may reach in vivo concentrations comparable to the ones we used in vitro, suggesting that our results could be representative of a physiologically relevant in vivo mechanism.

# *CHAPTER 6 BIBLIOGRAFY*


1. Maton, Anthea (1993). *Human Biology and Health*. Englewood Cliffs, New Jersey: Prentice Hall.

2. Bridget B. Kelly; Institute of Medicine; Fuster, Valentin (2010). *Promoting Cardiovascular Health in the Developing World: A Critical Challenge to Achieve Global Health*. Washington, D.C: National Academies Press

3. Dantas AP, Jimenez-Altayo F, Vila E (August 2012). "Vascular aging: facts and factors". *Frontiers in Vascular Physiology* 3 (325): 1-2.

4. World Health Organization, "United Nations High-level Meeting on Noncommunicable Disease Prevention and Control". Downloaded from http://www.who.int/nmh/events/un_ncd_summit2011/en/index.html

5. Mendis, S.; Puska, P.; Norrving, B.(editors) (2011), *Global Atlas on cardiovascular disease prevention and control*

6. McGill HC, McMahan CA, Gidding SS (March 2008). "Preventing heart disease in the 21st century: implications of the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study". *Circulation* 117 (9): 1216–27.

7. Yusuf S, Hawken S, Ounpuu S, *et al.* (2004). "Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study". *Lancet* 364 (9438): 937–52

8. "Understand Your Risk of Heart Attack". American Heart Association. http://www.heart.org/HEARTORG/Conditions/HeartAttack/UnderstandYourRiskofHeartAttack/Understand-Your-Risk-of-Heart-Attack_UCM_002040_Article.jsp#

9. Mackay, Mensah, Mendis, et al. The Atlas of Heart Disease and Stroke. World Health Organization. January 2004.

10. Jousilahti Vartiainen, Tuomilehto Puska (1999). "Sex, Age,Cardiovascular Risk Factors, and coronary heart diease". *Circulation* 99: 1165–1172.

11. Jani B, Rajkumar C (2006). "Ageing and vascular ageing". *Postgrad Med J* 82: 357–362.

12. http://www.world-heart-federation.org/cardiovascular-health/cardiovascular-disease-risk-factors

13. Vanhecke TE, Miller WM, Franklin BA, Weber JE, McCullough PA (Oct 2006). "Awareness, knowledge, and perception of heart disease among adolescents". *Eur J Cardiovasc Prev Rehabil.* 13 (5): 718–23.

14. Highlander P, Shaw GP (2010). "Current pharmacotherapeutic concepts for the treatment of cardiovascular disease in diabetics". *Ther Adv Cardiovasc Dis.* 4: 43–54.

15. NPS Medicinewise (1 March 2011). "NPS Prescribing Practice Review 53: Managing lipids". Retrieved 1 August 2011.

16. Kvan E., Pettersen K.I., Sandvik L., Reikvam A. (2007). "High mortality in diabetic patient with acute myocardial infarction: cardiovascular co-morbidities contribute most to the high risk". *Int J Cardiol* 121: 184–188.

17. Norhammar A., Malmberg K., Diderhol E., Lagerqvist B., Lindahl B., Ryde *et al.* (2004). "Diabetes mellitus: the major risk factor in unstable coronary artery disease even after consideration of the extent of coronary artery disease and benefits of revascularization. J". *Am Coll Cardiol* 43: 585–591.

18. DECODE , European Diabetes Epidemiology Group (1999). "Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria". *Lancet* 354: 617–621.

19. Ignarro, LJ; Balestrieri, ML, Napoli, C (2007 Jan 15). "Nutrition, physical activity, and cardiovascular disease: an update.". *Cardiovascular research* 73 (2): 326–40

20. World Heart Federation (5 October 2011). "World Heart Federation: Cardiovascular disease risk factors". Retrieved 5 October 2011.

21. The National Heart, Lung, and Blood Institute (NHLBI) (5 October 2011). "How To Prevent and Control Coronary Heart Disease Risk Factors - NHLBI, NIH". Retrieved 5 October 2011.

22. Klatsky AL (May 2009). "Alcohol and cardiovascular diseases". *Expert Rev Cardiovasc Ther* 7 (5): 499–506.

23. McTigue KM, Hess R, Ziouras J (September 2006). "Obesity in older adults: a systematic review of the evidence for diagnosis and treatment". *Obesity (Silver Spring)* 14 (9): 1485–97.

24. Linden W, Stossel C, Maurice J (April 1996). "Psychosocial interventions for patients with coronary artery disease: a meta-analysis". *Arch. Intern. Med.* 156 (7): 745–52.

25. Walker C, Reamy BV (April 2009). "Diets for cardiovascular disease prevention: what is the evidence?". *Am Fam Physician* 79 (7): 571–8

26. Sacks FM, Svetkey LP, Vollmer WM, *et al.* (January 2001). "Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group". *N. Engl. J. Med.* 344 (1): 3–10.

27. Obarzanek E, Sacks FM, Vollmer WM, *et al.* (July 2001). "Effects on blood lipids of a blood pressure-lowering diet: the Dietary Approaches to Stop Hypertension (DASH) Trial". *Am. J. Clin. Nutr.* 74 (1): 80–9.

28. Azadbakht L, Mirmiran P, Esmaillzadeh A, Azizi T, Azizi F (December 2005). "Beneficial effects of a Dietary Approaches to Stop Hypertension eating plan on features of the metabolic syndrome". *Diabetes Care* 28 (12): 2823–31.

29. Logan AG (March 2007). "DASH Diet: time for a critical appraisal?". *Am. J. Hypertens.* 20 (3): 223–4.

30. Siri-Tarino Patty W, Sun Qi, Hu Frank B, Krauss Ronald M (2010). "Saturated fat, carbohydrate, and cardiovascular disease". *American Journal of Clinical Nutrition* 91 (3): 502–509

31. Micha, R; Mozaffarian, D (2010 Oct). "Saturated fat and cardiometabolic risk factors, coronary heart disease, stroke, and diabetes: a fresh look at the evidence.". *Lipids* 45 (10): 893–905.

32. Mozaffarian, D; Aro, A; Willett, WC (2009 May). "Health effects of trans-fatty acids: experimental and observational evidence.". *European journal of clinical nutrition* 63 Suppl 2: S5-21.

33. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 4th ed. Oxford, UK: Clarendon Press; 2007.

34. Bahorun T, Soobrattee MA, Luximon-Ramma V, Aruoma OI. Free radicals and antioxidants in cardiovascular health and disease. Internet J Med Update 2006;1:1-17

35. Valko M, Izakovic M , Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 2004, 266: 37-56.

36. Valko M, Leibfritz D, Moncola J, Cronin MD, et al. Free radicals and antioxidants in normal physiological functions and human disease. Review. Int J Biochem Cell Biol 2007;39:44-84.

37. Droge W. Free radicals in the physiological control of cell function. Review. Physiol Rev. 2002;82:47-95.

38. Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Review. Crit Rev Food Sci Nutr 2004;44:275-95.

39. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315-424.

40. Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Review. Cell Signal. 2007;19:1807-19

41. Halliwell B. Biochemistry of oxidative stress. Biochem Soc Trans. 2007;35:1147-50.

42. Young I, Woodside J. Antioxidants in health and disease. J Clin Pathol. 2001;54:176–86.

43. Valko M, Rhodes CJ, Moncol J, Izakovic M, et al. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Mini-review. Chem Biol Interact. 2006;160: 1–40.

44. Valko M, Morris H, Cronin MTD. Metals, toxicity and oxidative stress. Curr Med Chem. 2005;12:1161-208.

45. Parthasarathy S, Santanam N, Ramachandran S, Meilhac O. Oxidants and antioxidants in atherogenesis: an appraisal. J Lipid Res. 1999;40:2143-57.

46. Frei B. Reactive oxygen species and antioxidant vitamins. Linus Pauling Institute. Oregon State University. 1997, http://lpi.oregonstate.edu/ f-w97/reactive.html

47. Chatterjee M, Saluja R, Kanneganti S, et al. Biochemical and molecular evaluation of neutrophil NOS in spontaneously hypertensive rats. Cell Mol Biol. 2007;53:84-93.

48. Ceriello A. Possible role of oxidative stress in the pathogenesis of hypertension. Review. Diabetes Care. 2008 ;31(Suppl 2):S181-4.

49. Hollman PCH (2001). Evidence for health effects of plant phenols: local or systemic effects?. J. Sci. Food Agric. 81: 842–852.

50. Hayek MG (2000).Dietary vitamin E improves immune function in cats. In Reinhart G. A and Carey D. P. Eds. Recent Advances in Canine and Feline Nutrition, Iams Nutrition Symposium Proceedings.

51. Fridovich I (1998). Oxygen toxicity, a radical explanation.The J. Experimental Biol. 201: 1203–1209.

52. Mikulikova L, Popov P (2001). Oxidative stress, metabolism of ethanol and alcohol-related diseases. J. Biomed. Sci. 8: 59–70.

53. Sian BA (2003). Dietary antioxidants—past, present and future? Trends in Food Sci. Technol. 14: 93-98. Somchit BN, Reezal I, Nur V, Mutalib AR (2003). In vitro antimicrobial activity of ethanol and water extracts of Cassia alata. J. Ethnopharmacol. 84: 1-4

54. Atoui AK, Mansouri A, Boskou G, Panagiotis K (2005). Tea and herbal infusions Their antioxidant activity and phenolic profile. Food Chem. 89: 27-36.

55. Alasalvar CM, Al-Farsi PC, Quantick F, Shahidi R, Wiktorowic Z (2005). Effect of chill storage and modified atmosphere packaging (MAP) on antioxidant activity, anthocyanins, carotenoids, phenolics and sensory quality of ready-to-eat shredded orange and purple carrots. Food Chem. 89: 69–76.

56. Halliwell B, Gutteridge JMC (1989). Free Radicals in Biology and Medicine, 2 Ed., Clarendon, UK, Oxford science publications. pp. 22–85.

57. Gutteridge JM (1989). Iron and oxygen. a biologically damaging mixture. Acta Paediatrica Scandinavia. 36: 78-85.

58. Burton GW, Foster DO, Perly B, Slater TF, Smith ICP, Ingold, KU (1985). Biological antioxidants. philosophical society of royal transactions of London. Series B, Biol. Sci. 311: 565-576.

59. Chen CH, Pearson AM, Gray JI (1992). Effects of synthetic antioxidants (BHA, BHT and PG) on the mutagenicity of IQ-like compounds. Food Chem. 3: 177-183.

60. Ames BN, Shigenaga MK, Hagen TM (1993). Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences of the United States of America. 90: 7915-7922.

61. Enstrom JE, Kanim LE, Klein MA (1992). Vitamin C intake and mortality among a sample of the United States population. Epidemiology. 3: 194-202.

62. Rimm EB, Stampfer MJ, Ascherio A, Glovannucci E, Colditz GA, Rosner B, Willett W C (1993). Vitamin E consumption and the risk of coronary heart disease in men. New England J. Med. 328: 1450-1456.

63. Leo MA, Lieber CS (1999). Alcohol, vitamin A, and ß-carotene: adverse interactions, including hepatotoxicity and carcinogenicity. Am. J. Clin. Nut.. 69:1071-1085

64. Heinerman J (1996). Heinerman's Encyclopadia of healing herbs and spices. Englewood cliffs, New Jersey, Parker publishing company. p. 425.

65. Hashimoto H, Yoda T, Kobayashi T, Young AJ. (2002). Molecular structure of carotenoids as predicted by MNDO-AMI molecular orbital calculations. J. mole. struct. 604: 125-146.

66. Cao Jh QY (1993). Studies on the chemical constituents of the herb huanghuaren (Sida acuta Burm. f.) leaves. China Journal of Chinese Material Medicia. 18: 681-2.

67. Nessa F, Ismail Z, Mohamed N, Hakim MR, Haris M (2004). Free radical-scavenging activity of organic extracts and of pure .flavonoids of Blumea balsamifera. Food Chem. 88: 243–252.

68. Somchit BN, Reezal I, Nur V, Mutalib AR (2003). In vitro antimicrobial activity of ethanol and water extracts of Cassia alata. J. Ethnopharmacol. 84: 1-4.

69. Burton GW, Foster DO, Perly B, Slater TF, Smith ICP, Ingold, KU (1985). Biological antioxidants. philosophical society of royal transactions of London. Series B, Biol. Sci. 311: 565-576.

70. Halkes BA, Vrasidas I, Rooijer GR, Van Den B, Albert JJ, Liskamp RMJ, Pieters RJ (2002). Synthesis and biological activity of polygalaloyl-dendrimers as stable tannic acid mimics. Bioorganic and Medicinal Chemistry Letters. 12: 1567–1570.

71. Burkill IH (1993). A Dictionary of the economic products of the Malay Peninsula. 3rd printing. Malaysia, Publication Unit, Ministry of Agriculture.

72. Oomen HAPC, Grubben GJH (1998). Tropical leaf vegetables in human nutrition. Amsterdam, Royal Tropical Institute & Orphan publishing Co.

73. Lopaczynski W, Zeisel SH (2001). Antioxidants, programmed cell death, and cancer. Nutrition Research. 21:295-307.

74. Dreher D, Junod AF (1996). Role of oxygen free radicals in cancer development. European J. of Cancer. 32A: 30–8.

75. Ames BN, Shigenaga MK, Hagen TM (1993). Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences of the United States of America. 90: 7915-7922.

76. Barcellos-Hoff MH (2005). Integrative radiation carcinogenesis interactions between cell and tissue responses to DNA damage. Seminars in Cancer Biology.15: 138-148.

77. Labuza TP, Baisier WM. 1992. The kinetics of nonenzymatic browning. In: Schwartzberg HG, Hartel RW, editors. Physical chemistry of foods. New York: Marcel Dekker Inc. p 595–649.

78. Friedman M. 1996. Food browning and its prevention: an overview. J. Agric Food Chem 44:631–53.

79. van Boekel MAJS. 2001. Kinetic aspects of the Maillard reaction: a critical review Nahrung 45:150–9.

80. Roos YH, Himberg MJ. 1994. Non-enzymatic browning behavior, as related to glass transition of a food model at chilling temperatures. J Agric Food Chem 42(4):893–8.

81. Lievonen SM, Laaksonen TJ, Roos YH. 1998. Glass transition and reaction rates: nonenzymatic browning in glass and liquid systems. J Agric Food Chem 46:2778–84.

82. Karel M, Buera MP, Roos Y. 1993. Effects of glass transitions on processing and storage. In: Blanshard JMV, Lillford PJ, editors. The glassy state in foods. Loughborough, U.K.: Nottingham Univ. Press. p 13–34.

83. Labuza TP, Tannenbaum SR, Karel M. 1970. Water content and stability of lowmoisture and intermediate-moisture foods. Food Technol 24:543–50.

84. Labuza TP, Saltmarch M. 1981. The nonenzymatic browning reaction as affected by water in foods. In: Rockland LB, Stewart GF, editors. Water activity: influences on food quality. New York: Academic Press. p 605–50.

85. Buera MP, Karel M. 1995. Effect of physical changes on the rates of non-enzymatic browning and related reaction. Food Chem 52:167–73.

86. Bell LN, White KL, Chen YH. 1998. Glycine loss and Maillard browning as related to the glass transition in a model food system. J Food Sci 63:625–8

87. White KL, Bell LN. 1999. Glucose loss and Maillard browning in solids as affected by porosity and collapse. J Food Sci 64:1010–4.

88. Burin L, Jouppila K, Roos Y, Kansikas J, Buera MP. 2000. Color formation in dehydrated modified whey powder systems as affected by compression and Tg. J Agric Food Chem 48:5263–8.

89. Burin L, Buera MP, Hough G, Chirife J. 2002. Thermal resistance of _-galactosidase in dehydrated dairy model systems as affected by physical and chemical changes. Food Chem 76:423–30.

90. Lievonen SM, Laaksonen TJ, Roos YH. 2002. Non-enzymatic browning in food models in the vicinity of glass transition: effects of fructose, glucose, and xylose as a reducing sugar. J Agric Food Chem 50(24):7034–41.

91. Karel M, Saguy I. 1991. Effects of water on diffusion in food system. I: Levine H, Slade L., editors. Water relationships in foods. New York: Plenum Press. p 157–74.

92. Slade L, Levine H. 1991. Beyond water activity: recent advances based on an alternative approach to the assessment of food quality and safety. Crit Rev Food Sci Nutr 30:115–360.

93. Karel M, Buera MP. 1994. Glass transition and its potential effects on kinetics of condensation reactions and in particular on nonenzymatic browning. In: Labuza TP, Reineccius GA, Monnier VM, O'Brien J, Baynes JW, editors. Maillard reactions in chemistry, food and health. Cambridge U.K.: Royal Society of Chemistry. p 164–9.

94. Levine H, Slade L. 1989. Interpreting the behavior of low-moisture foods. In: Hartman TM, editor. Water and food quality. London: Elsevier Applied Science. p 71–134.

95. Slade L, Levine H. 1991. Beyond water activity: recent advances based on an alternative approach to the assessment of food quality and safety. Crit Rev Food Sci Nutr 30:115–360.

96. Karmas R, Buera MP, Karel M. 1992. Effect of glass transition on rates of non-enzymatic browning in food systems. J Agric Food Chem 40(5):873–9.

97. Roos Y, Karel M. 1993. Effects of glass transitions on dynatic phenomena in sugar containing systems. In: Blanshard JMV, Lillford PJ, editors. The glassy state in foods. Loughborough, U.K.: Nottingham Univ. Press. p 207–22.

98. Karmas R, Karel M. 1994. The effect of glass transition on Maillard browning in food models. In: Labuza TP, Reineccius GA, Monnier VM, O'Brien J, Baynes JW, editors. Maillard reactions in chemistry, food and health. Cambridge, U.K.: Royal Society of Chemistry. p 182–7.

99. Roos YH. 1995. Phase transitions in foods. New York: Academic Press. 360 p.

100. Schebor C, Buera MP, Karel M, Chirife J. 1999. Color formation due to non-enzymatic browning in amorphous, glassy, anhydrous model systems. Food Chem 65(4):427–32

101. Craig ID, Parker R, Rigby NM, Crairns P, Ring SG. 2001. Maillard reaction kinetics in model preservation system in the vicinity of glass transition: experiment and theory. J Agric Food Chem 49:4706–12.

102. Three references by L. C. Maillard from http://www.lc-maillard.org/:

- Réaction générale des acides aminés sur les sucres. Journal de Physiologie, 1912 tome 14 page 813;
- Action des acides aminés sur les sucres: formation des mélanoïdines par voie méthodique. Compte-rendu de l'Académie des sciences, 1912 tome 154, page 66-68.
- Réaction générale des acides aminés sur les sucres: ses conséquences biologiques. Compte-rendu Société de Biologie, 1912, tome 72 (LXXII), page 599 à 601 (559-561)
- Thèse présentée à la Fac des Sciences de Paris. Action de la glycérine et des sucres sur les acides x-aminés : cyclo-glycil-glycines & polypeptides; mélanoïdines et matières humiques. Laval . Imp. De L. Barnéoud 1913. In 8° XV 425 pages . Bibliothèque Nationale 4° R 489. 1508.série A n° 738 & n° 1508

103. Owen R. Fennema, Food Chemistry, 3rd Edition, 1996

104. Bradbury A.G.W., Carbohydrates, R. J. Clarke and O. G. Vitzthum (eds), Coffee – Recent Developments. Oxford: Blackwell Science, 2001

105. Trautwein E., Untersuchungen über den Gehalt an freien und gebundenen Aminosäuren in verschiedenen Kaffee-Sorten sowie über das Verhalten während des Röstens, Thesis University of Kiel, Germany, 1987

106. Ginz M., Balzer H.H., Bradbury A.G.W. and Maier H.G., Formation of aliphatic acids by carbohydrate degradation during the roasting of coffee, Eur. Food Res. Technol., 2000

107. Flament I., The volatile compounds identified in green coffee beans, Coffee Flavor Chemistry. Chichester: J. Wiley & Sons, 2001

108. Kris-Etherton, P. M., K. D. Hecker, A. Bonanome, S. M. Coval, A. E. Binkoski, K. F. Hilpert, A. E. Griel & T. D. Etherton: Bioactive compounds in foods: Their role in the prevention of cardiovascular disease and cancer. *Am J Med*, 113, 71-88 (2002)

109. Martins, S., W. Jongen & M. van Boekel: A review of Maillard reaction in food and implications to kinetic modelling. *Trends Food Sci. Technol*, 11, 364-373 (2000)

110. Madrau MA, Sanguinetti AM, Del Caro A, Fadda C, Piga A (2010) Contribution of Melanoidins to the Antioxidant Activity of Prunes. Journal of Food Quality 33: 155-170.

111. Tagliazucchi D, Verzelloni E, Conte A (2010) Contribution of Melanoidins to the Antioxidant Activity of Traditional Balsamic Vinegar during Aging. Journal of Food Biochemistry 34: 1061-1078.

112. Rufian-Henares JA, Morales FJ (2007) Angiotensin-I converting enzyme inhibitory activity of coffee melanoidins. Journal of Agricultural and Food Chemistry 55: 1480-1485.

113. Rufian-Henares JA, de la Cueva SP (2009) Antimicrobial Activity of Coffee Melanoidins-A Study of Their Metal-Chelating Properties. Journal of Agricultural and Food Chemistry 57: 432-438.

114. Lerici, C. & M. Nicoli: Chemical and physico-chemical properties affecting the quality and stability of bakery products. *Adv food sci*, 18, 229-233 (1996)

115. Nicoli, M., M. Anese, M. Parpinel, S. Franceschi & C. Lerici: Loss and/or formation of antioxidants during food processing and storage. *Cancer lett*, 114, 71-74 (1997)

116. Valls-Belles, V., M. C. Torres, P. Muniz, L. Boix, M. L. Gonzalez-Sanjose & P. Codoner-Franch: The protective effects in adriamycin-induced of melanoidins oxidative stress in isolated rat hepatocytes. *J Sci Food Agric,* 84, 1701-1707 (2004)

117. Goya, L., C. Delgado-Andrade, J. Rufian-Henares, L. Bravo & F. Morales: Effect of coffee melanoidin on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. *Mol Nutr Food Res*, 51, 536-545 (2007)

118. Martin, M. A., S. Ramos, R. Mateos, J. A. Rufian-Henares, F. J. Morales, L. Bravo & L. Goya: Biscuit melanoidins of different molecular masses protect human HepG2 cells against oxidative stress. *J Agric Food Chem*, 57, 7250-7258 (2009)

119. Stacewicz-Sapuntzakis, M., P. E. Bowen, E. A. Hussain, B. Damayanti-Wood & N. R. Farnsworth: Chemical composition and potential health effects of prunes: A functional food? *Crit Rev Food Sci Nutr*, 41, 251-286 (2001)

120. Donovan, J. L., A. S. Meyer & A. L. Waterhouse: Phenolic composition and antioxidant activity of prunes and prune juice (Prunus domestica). *J Agric Food Chem*, 46, 1247-1252 (1998)

121. Kayano, S., N. F. Yamada, T. Suzuki, T. Ikami, K. Shioaki, H. Kikuzaki, T. Mitani & N. Nakatani: Quantitative evaluation of antioxidant components in prunes (Prunus domestica L.). *J Agric Food Chem*, 51, 1480-1485 (2003)

122. Vinson, J., Y. Dabbagh, M. Serry & J. Jang: Plant flavonoids, especially tea flavonols, are powerful antioxidants using an *in vitro* oxidation model for heart disease. *J Agric Food Chem*, 43, 2800-2802 (1995)

123. Prior, R. L., G. Cao, H. Hoang, X. Wu & L. Gu: *In vivo* antioxidant capacity in human subjects following consumption of prune juice, prunes or blueberries. *FASEB J*, 16, A1010-A1010 (2002)

124. Gallaher, C. M. & D. D. Gallaher: Dried plums (prunes) reduce atherosclerosis lesion area in apolipoprotein Edeficient mice. *Br J Nutr*, 101, 233-9 (2009)

125. Piga, A., A. Del Caro & G. Corda: From plums to prunes: Influence of drying parameters on polyphenols and antioxidant activity. *J Agric Food Chem*, 51, 3675-3681 (2003)

126. Del Caro, A., A. Piga, I. Pinna, P. M. Fenu & M. Agabbio: Effect of drying conditions and storage period on polyphenolic content, antioxidant capacity, and ascorbic acid of prunes. *J Agric Food Chem*, 52, 4780-4784 (2004)

127. Sass-Kiss A, Kiss J, Milotay P, Kerek MM, Toth-Markus M (2005) Differences in anthocyanin and carotenoid content of fruits and vegetables. Food Research International 38: 1023-1029.

128. Dragovic-Uzelac V, Levaj B, Mrkic V, Bursac D, Boras M (2007) The content of polyphenols and carotenoids in three apricot cultivars depending on stage of maturity and geographical region. Food Chemistry 102: 966-975.

129. Parlakpinar H, Olmez E, Acet A, Ozturk F, Tasdemir S, et al. (2009) Beneficial effects of apricot-feeding on myocardial ischemia-reperfusion injury in rats. Food and Chemical Toxicology 47: 802-808.

130. Yurt B, Celik I (2011) Hepatoprotective effect and antioxidant role of sun, sulphited-dried apricot (Prunus armeniaca L.) and its kernel against ethanol-induced oxidative stress in rats. Food and Chemical Toxicology 49: 508-513.

131. Ruiz D, Egea J, Tomas-Barberan FA, Gil MI (2005) Carotenoids from new apricot (Prunus armeniaca L.) varieties and their relationship with flesh and skin color. Journal of Agricultural and Food Chemistry 53: 6368-6374.

132. Scalzo J, Politi A, Pellegrini N, Mezzetti B, Battino M (2005) Plant genotype affects total antioxidant capacity and phenolic contents in fruit. Nutrition 21: 207-213.

133. Guo CJ, Yang JJ, Wei JY, Li YF, Xu J, et al. (2003) Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. Nutrition Research 23: 1719-1726.

134. Ishiwata K, Yamaguchi T, Takamura H, Matoba I (2004) DPPH radical-scavenging activity and polyphenol content in dried fruits. Food Science and Technology Research 10: 152-156.

135. Crivelli G (1997) Italus Hortus 4: 48-53.

136. Madrau MA, Piscopo A, Sanguinetti AM, Del Caro A, Poiana M, et al. (2009) Effect of drying temperature on polyphenolic content and antioxidant activity of apricots. European Food Research and Technology 228: 441-448.

137. Inagi R (2006) Oxidative stress in cardiovascular disease: a new avenue toward future therapeutic approaches. Recent Pat Cardiovasc Drug Discov 1: 151-159.

138. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, et al. (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 91: 3527-3561.

139. Cai H, Harrison DG (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 87: 840-844.

140. Piga A, Del Caro A, Corda G (2003) From plums to prunes: Influence of drying parameters on polyphenols and antioxidant activity. Journal of Agricultural and Food Chemistry 51: 3675-3681.

141. Del Caro A, Piga A, Pinna I, Fenu PM, Agabbio M (2004) Effect of drying conditions and storage period on polyphenolic content, antioxidant capacity, and ascorbic acid of prunes. Journal of Agricultural and Food Chemistry 52: 4780-4784.

142. Manzocco, L., S. Calligaris, D. Mastrocola, M. C. Nicoli & C. R. Lerici: Review of non-enzymatic browning and antioxidant capacity in processed foods. *Trends Food Sci. Technol*, 11, 340-346 (2000)

143. Mastrocola D. LCR (1991) Colorimetric measurements of enzymatic and non-enzymatic browning in apple purees. Italian Journal of Food Science 3: 219–229.

144. Little A (1975) Off on a tangent. Journal of Food Science 40: 410–411.

145. Severini C, Baiano A, De Pilli T, Romaniello R, Derossi A (2003) Prevention of enzymatic browning in sliced potatoes by blanching in boiling saline solutions. Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology 36: 657-665.

146. Orrenius, S., V. Gogvadze & B. Zhivotovsky: Mitochondrial oxidative stress: implications for cell death. *Annu Rev Pharmacol Toxicol*, 47, 143-83 (2007)

147. Halliwell, B. & M. Whiteman: Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol*, 142, 231-55 (2004)

148. Marty, L., W. Siala, M. Schwarzlander, M. D. Fricker, M. Wirtz, L. J. Sweetlove, Y. Meyer, A. J. Meyer, J. P. Reichheld & R. Hell: The NADPH-dependent thioredoxin system constitutes a functional backup for cytosolic glutathione reductase in Arabidopsis. *Proc Natl Acad Sci USA*, 106, 9109-14 (2009)

149. Meyer, A. J., T. Brach, L. Marty, S. Kreye, N. Rouhier, J. P. Jacquot & R. Hell: Redox-sensitive GFP in Arabidopsis thaliana is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer. *Plant J*, 52, 973-986 (2007)

150. Dooley, C. T., T. M. Dore, G. T. Hanson, W. C. Jackson, S. J. Remington & R. Y. Tsien: Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. *J Biol Chem*, 279, 22284-93 (2004)

151. Nishikawa, M.: Reactive oxygen species in tumor metastasis. *Cancer Lett*, 266, 53-59 (2008)

152. Esposito F, Morisco F, Verde V, Ritieni A, Alezio A, et al. (2003) Moderate coffee consumption increases plasma glutathione but not homocysteine in healthy subjects. Alimentary pharmacology & therapeutics 17: 595-601.

153. Morales FJ, Somoza V, Fogliano V (2012) Physiological relevance of dietary melanoidins. Amino Acids 42: 1097-1109.

154. Dittrich R, Dragonas C, Kannenkeril D, Hoffmann I, Mueller A, et al. (2009) A diet rich in Maillard reaction products protects LDL against copper induced oxidation ex vivo, a human intervention trial. Food Research International 42: 1315-1322.

155. Bichler J, Cavin C, Simic T, Chakraborty A, Ferk F, et al. (2007) Coffee consumption protects human lymphocytes against oxidative and 3-amino-1-methyl-5H-pyrido [4,3-b]indole acetate (Trp-P-2) induced DNA-damage: Results of an experimental study with human volunteers. Food and Chemical Toxicology 45: 1428-1436.

156. Piga A, Pinna I, Ozer KB, Agabbio M, Aksoy U (2004) Hot air dehydration of figs (Ficus carica L.): drying kinetics and quality loss. International Journal of Food Science and Technology 39: 793-799.

157. Lindenmeier M, Faist V, Hofmann T (2002) Structural and functional characterization of pronyl-lysine, a novel protein modification in bread crust melanoidins showing in vitro antioxidative and phase I/II enzyme modulating activity. Journal of Agricultural and Food Chemistry 50: 6997-7006.

158. Clydesdale FM (1993) Color as a Factor in Food Choice. Critical Reviews in Food Science and Nutrition 33: 83-101.

159. Lin, R., J. Liu, W. Gan & C. Ding: Protective effect of quercetin on the homocysteine-injured human umbilical vein vascular endothelial cell line (ECV304). *Basic Clin Pharmacol Toxicol*, 101, 197-202 (2007)

160. Zhang, J., P. Xiao & X. Zhang: Phosphatidylserine externalization in caveolae inhibits Ca2+ efflux through plasma membrane Ca2+-ATPase in ECV304. *Cell Calcium*, 45, 177-84 (2009)

161. Liu, H. T., W. M. Li, G. Xu, X. Y. Li, X. F. Bai, P. Wei, C. Yu & Y. G. Du: Chitosan oligosaccharides attenuate hydrogen peroxide-induced stress injury in human umbilical vein endothelial cells. *Pharmacol Res*, 59, 167-75 (2009)

162. Debidda, M., B. Sanna, A. Cossu, A. M. Posadino, B. Tadolini, C. Ventura & G. Pintus: NAMI-A inhibits the PMA-induced ODC gene expression in ECV304 cells: involvement of PKC/Raf/Mek/ERK signalling pathway. *Int J Oncol*, 23, 477-82 (2003)

163. Pintus, G., B. Tadolini, A. M. Posadino, B. Sanna, M. Debidda, F. Bennardini, G. Sava & C. Ventura: Inhibition of the MEK/ERK signaling pathway by the novel antimetastatic agent NAMI-A down regulates c-myc gene expression and endothelial cell proliferation. *Eur JBiochem*, 269, 5861-70 (2002)

164. Goya, L., C. Delgado-Andrade, J. Rufian-Henares, L. Bravo & F. Morales: Effect of coffee melanoidin on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. *Mol Nutr Food Res*, 51, 536-545 (2007)

165. Pasciu V, Posadino AM, Cossu A, Sanna B, Tadolini B, et al. (2010) Akt Downregulation by Flavin Oxidase-Induced ROS Generation Mediates Dose-Dependent Endothelial Cell Damage Elicited by Natural Antioxidants. Toxicological Sciences 114: 101-112.

166. Marshall NJ, Goodwin CJ, Holt SJ (1995) A Critical-Assessment of the Use of Microculture Tetrazolium Assays to Measure Cell-Growth and Function. Growth Regulation 5: 69-84.

167. Kaneko I, Yamada N, Sakuraba Y, Kamenosono M, Tutumi S (1995) Suppression of Mitochondrial Succinate-Dehydrogenase, a Primary Target of Beta-Amyloid, and Its Derivative Racemized at Ser Residue. Journal of Neurochemistry 65: 2585-2593.

168. Pereira C, Santos MS, Oliveira C (1998) Metabolic inhibition increases glutamate susceptibility on a PC12 cell line. Journal of Neuroscience Research 51: 360-370.

169. Rosenwasser, S., I. Rot, A. J. Meyer, L. Feldman, K. Jiang & H. Friedman: A fluorometer-based method for monitoring oxidation of redox-sensitive GFP (roGFP) during development and extended dark stress. *Physiol Plant*, 138, 493-502 (2010)

170. Salvioli, S., A. Ardizzoni, C. Franceschi & A. Cossarizza: JC-1, but not DiOC(6)(3) or rhodamine 123, is a reliable fluorescent probe to assess Delta Psi changes in intact cells: Implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett*, 411, 77-82 (1997)

171. She, M. R., J. G. Li, K. Y. Guo, W. Lin, X. Du & X. Q. Niu: Requirement of reactive oxygen species generation in apoptosis of leukemia cells induced by 2-methoxyestradiol. *Acta Pharmacol Sin*, 28, 1037-1044 (2007)