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Evaluation of the putative healthy effects of food phytochemicals: effect on blood antioxidant status, human cells and enzyme activities

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ABSTRACT

During the last few decades, dietary antioxidants have received increasing attention, especially within biological, medical and nutritional fields, owing to their putative protective roles against the deleterious oxidative-induced reactions implicated in the pathogenesis of several human diseases. A considerable number of analytical assays has been developed claiming to ensure a fast, simple, convenient, and reliable *in vitro* determination of total antioxidant capacity (AC) of pure compounds or complex matrices, such as foods and biological samples. Nevertheless, methods for *in vitro* AC measurement show many technical and conceptual limitations. Moreover, *in vitro* AC measurements of foods alone have not been demonstrated to be relevant for the biological effects of specific bioactive compounds since they do not provide information about bioavailability of food antioxidants, as well as their *in vivo* stability, retention by tissues and *in situ* reactivity.

Therefore, the target of this research was of developing innovative methodologies/approaches able to provide information as much as possible reflecting the *in vivo* response. The model of study adopted to carry out the research consists of three sequential levels of study.

The first level regards AC measurement of plant foodstuffs. At this level, a first methodological advancement of "QUENCHER$_{ABTS}$" (QUick, Easy, New, CHEap and Reproducible) method has been attempted (see **Article 1**). It is based on the direct reaction of ABTS$^{\bullet+}$ reagent with fine solid food particles without extraction of antioxidants. In particular, by adopting a new slope calculation procedure, the applicability of QUENCHER$_{ABTS}$ method was extended to the study of larger particles (up to 0.5 mm). This may be of interest in the case of some cereal milling products.

In addition to the QUENCHER$_{ABTS}$ calculation upgrade, research relative to the first level of investigation has regarded the development of the novel Lipoxygenase (LOX)-Fluorescein (FL) method (see **Article 2**). The new LOX-FL method is based on the LOX-1 isoenzyme reaction to generate physiological reactive species and provides an AC assessment more reliable from a physiological point of view with respect to other widely used assays. This method was applied on both extracts from the antioxidant-rich dietary cereal supplement Lisosan G and serum (seven subjects) during 240 min after intake of 20 g of supplement. Interestingly, LOX-FL method discriminated *in vitro* AC of four different Lisosan G extracts similarly to ORAC and TEAC methods, used for comparison. Contrarily, only LOX-FL method was able to highlight a general increase of serum AC (up to 40% after 30 min) after Lisosan G intake, thus confirming its physiological effectiveness by *ex vivo* serum assay.

For the property to be applied to both *in vitro* and *ex vivo* AC measurements, LOX-FL assay was used also in the second level of investigation, concerning the evaluation of human blood

antioxidant status after food intake. Unfortunately, contrasting results have been obtained from blood AC assessment after consumption of antioxidant-enriched foods, suggesting the unsuitability of blood AC measurements alone and the need to consider also changes of serum oxidative status. In the light of this, we propose the use of a novel parameter, the "Antioxidant/Oxidant Balance (AOB)", representing the ratio between serum AC and serum oxidant status, evaluated as "Peroxide Level" (PxL) (see **Article 3**). AOB approach was applied for the first time to evaluate effects on serum antioxidant status during the first four hours after intake of two antioxidant-supplemented pastas, the Bran Oleoresin (BO) and Bran Water (BW) pastas, enriched respectively with either lipophilic or hydrophilic/phenolic antioxidants extracted from durum wheat bran. Interestingly, similarly to the highly active Lisosan G, intake of BO pasta allowed a significant improvement of serum AOB, until 70% as evaluated as LOX-FL/PxL. Contrarily, BW pasta induced a serum oxidative effect so as a Reference pasta and glucose, used for comparison. Overall, these findings indicate that AOB approach appears an excellent tool in highlighting effects of antioxidant-enriched food consumption, which cannot be predicted by *ex vivo* analysis of AC alone, as well as by *in vitro* measurements of cooked foods.

The third level of investigation concerns the evaluation of effects exerted by bioactive compounds at the cellular and sub-cellular levels. In particular, biological effects of some phytochemicals were evaluated on activity of: *i)* Glyoxalase I, implicated in enzymatic cell defence against dicarbonyl stress *ii)* sirtuins, a family of NAD^+- dependent deacetylases involved in the regulation of cellular transcription, apoptosis, cell division and metabolism.

Concerning Glo I, two studies were carried out. In the first study described in the **Article 4,** the effect of sulforaphane (SR), an isothiocyanate abundant in *Brassica* vegetables, on the expression and activity of GloI and on the levels of reduced glutathione (GSH) in peripheral blood mononuclear cells (PBMCs) was investigated. Incubation for 24 h and 48 h of PMBCs with 2.5 μM SR (simulating a daily consumption of a broccoli portion) did not induce a substantial increase in GloI activity and expression while caused a reduction until 73% in GSH levels compared to the control cells. This suggests the formation of a GSH-SR adduct able to significantly reduce the actual SR concentration within the cells during incubation, so explaining the lack of a substantial effect on GloI expression observed in this study.

In the second study described in the **Article 5,** the effect of some phytochemicals was evaluated on GloI activity in highly purified mitochondrial fraction obtained from durum wheat seedlings (WM). Mitochondrial GloI was chosen since mitochondria represent one of the major targets of MG-mediated carbonylation/oxidative stress. Interestingly, a hight GloI activity was measured in WM, showing a hyperbolic dependence on substrate concentration, with Km and Vmax values

equal to 0.27 mM and 0.133 EU/mg of protein, respectively. Concerning the study of modulation by phytochemicals, curcumin and quercetin were found to strongly inhibit WM-GloI activity in a competitive manner.

In the study described in the **Article 6**, a novel experimental approach for reliably measuring sirtuin activity in cell extracts and/or subcellular organelles was proposed. It involves the combined use of very different enzymatic assays, the bioluminescent SIRT-Glo™ and HTRF® technology-based based SIRT1 assays, and a comparative determination of activity of a recombinant human sirtuin 1 isoform (hSIRT1). By using the newly proposed approach, a high and nicotinamide-sensitive specific sirtuin activity was determined in WM, equal 268±10 $mU \cdot mg^{-1}$ protein, as maeasured by HTRF® assay, and 166±12 ng hSIRT1 $eq. \cdot mg^{-1}$ protein, as evaluated by the bioluminescent assay. Concerning sirtuin modulation, no significant effect of resveratrol and quercetin was found on WM-sirtuin and hSIRT1 activities by using HTRF® assay.

Taken together, results reported in this PhD thesis strongly demonstrate the crucial importance of the methodological approach in assessment of putative healthful properties of dietary phytochemicals; it is necessary to avoid incorrect and misleading data interpretation about antioxidant properties of foods.

INTRODUCTION

This research has concerned the use of innovative methodological approaches for the evaluation of the potential health value and the biological effects of phytochemicals obtained from foods and/or foods themselves characterized by a high antioxidant capacity (AC).

The study focused on different aspects.

The first one concerned the determination of AC of plant foodstuffs. With respect to this point, two novel advanced methods, based on more physiological approach with respect to the assays widely used in literature, were developed to measure *in vitro* AC; in particular, they uses different biologically relevant radical species and allow to simultaneously detect many antioxidant functions as well as synergistic interactions among antioxidants, so providing a more comprehensive and integrated determination of AC.

Another important aspect regarded the evaluation of a new approach consisting in associating the *in vitro* analysis of AC to the *ex vivo* study of blood (serum/plasma) after food intake. This measure may take into account bioavailability and metabolism, so giving an integrated information of a true effect on blood antioxidant status, which is beyond the original AC of the ingested food. With respect to this point, a novel parameter was developed, the Antioxidant/Oxidant Balance (AOB), based on evaluation of both antioxidant and oxidant status of blood after food intake.

Finally, the actions of bioactive compounds at cellular and sub-cellular levels were studied. In particular, the effects of some phytochemicals were assessed on two different classes of enzymes: *i*) Sirtuins, a family of NAD^+dependent deacetylases able to regulate a wide variety of cellular processes such as cellular senescence/aging, cellular apoptosis/proliferation, differentiation, metabolism and cell cycle regulation; *ii)* Glyoxalase I, an enzyme involved in the defence against carbonyl stress.

This introduction has been divided into three sections.

In the **Section I**, the characteristics and mechanisms of action of the most important classes of antioxidants are described. Moreover, the features of the main methods to evaluate *in vitro* AC of food are reported. In the **Section II**, the properties of Sirtuins and Glyoxalase I are reported; as mentioned above, these two enzymes were studied in order to obtain information about the biological roles of interesting phytochemicals. The **Section III** contains the aims of the research.

Section I- Food antioxidants and methods for measuring antioxidant capacity

1. Reactive Oxygen Species (ROS) and oxidative stress

Reactive Oxygen Species (ROS) are the main pro-oxidant compounds present in the cellular environment; these species include free radicals such as superoxide anion ($O_2^{\cdot -}$), hydroxyl ($^{\cdot}OH$) and peroxyl radicals ($ROO^{\cdot}$), and non-radical species, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). In the presence of Fe^{2+} and Cu^{2+}, H_2O_2 can generate, through the Fenton reaction, the hydroxyl radical ($^{\cdot}OH$). These chemical species are characterized by high reactivity, limited half-life, which varies from few nanoseconds to hours and rarely days, and finally the ability to damage cellular macromolecules as nucleic acids, proteins and cell membranes (Bjørklund et al., 2017).

In the cell, mitochondria are the main sites of ROS production. In these organelles, about 1-2% of the molecular oxygen is physiologically converted into $O_2^{\cdot -}$ and other ROS, in the course of oxidative phosphorylation that yields ATP. ROS are also produced by enzymes with oxidase activity (xanthine oxidase, NADPH oxidase, lipoxygenase, cyclooxygenase), by endoplasmic reticulum through the activity of Cytochrome P450 (Ames et al., 1989), by peroxisomal beta-oxidation and by activated phagocytic cells (Gutteridge et al., 1993, Poljsak et al., 2013, Gilca et al., 2007). In addition to endogenous ROS production, ionizing radiations, ultrasounds, cigarette smoke, pesticides and pollutants also contribute to increasing the intracellular pool of oxidant substances (Bjørklund et al., 2017).

Although ROS are known mainly for their harmful effects, they can play an important role in many physiological processes both at the cellular and systemic level. In particular, ROS, in low amounts, represent signalling molecules that are involved in the activation of growth factors in vascular smooth muscle cells (Baas and Berk, 1995), the elimination of non-functional proteins (Moldovan and Moldovan, 2004), the processes of aging, programmed cell death and regulation of gene expression (Rusterucci et al., 1999; Maccarrone et al., 2000; Jacobson, 1996). Their generation by phagocytes is essential in the defence mechanism against various strains of bacteria or fungi (Poljsak et al., 2013).

When an imbalance between ROS generation and antioxidative protection systems is established in the cell, a state of oxidative stress occurs (Pisoschi and Pop, 2015). In presence of oxidative stress macromolecules such as lipids, nucleic acids and proteins can be damaged. In particular, oxidation of proteins can occur with side-chain oxidation, backbone fragmentation, unfolding and misfolding, resulting in activity loss. ROS can damage nucleic acids, causing DNA-protein crosslinking, strand breaking, and alteration in purine and pyridine bases structure, having as

outcome DNA mutations. Lipids are the most sensitive to oxidation: membrane lipids peroxidation lead to the loss of the membrane structure and functionality that result in deleterious effects on intracellular environment (Pisoschi and Pop, 2015). Numerous studies have demonstrated that the oxidative stress is involved in the onset of numerous chronic degenerative diseases as diabetes, cancer, cardiovascular and neurodegenerative diseases (Galili et al., 2007; Furukawa et al., 2004).

2. Cellular antioxidant systems

The harmful action of ROS is countered in the cell by the presence of compounds with antioxidant activity. Antioxidants are substances able to react directly with the oxidizing species in order to generate more stable products that cannot damage biomolecules (Svilaas et al., 2004 and references within). Therefore, antioxidants, already at low concentrations, are able to delay, control or prevent oxidative processes leading to initiation and propagation of degenerative diseases in the body (Altemimi et al., 2017).

Antioxidants can be endogenous or can be introduced with the diet.

The endogenous antioxidant defence system includes compounds like glutathione, proteins (ferritin, transferrin, ceruloplasmin, and albumin) low molecular weight scavengers like uric acid, coenzyme Q and lipoic acid as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, etc. (Meng et al., 2017).

Dietary antioxidants such as vitamins, carotenoids, and phenolic compounds protect cell from oxidative damage by means of chain-breaking action, *i.e.* removing ROS and interrupting radical propagation reactions (Lee et al., 2004). These bioactive compounds present in plant foods, better known as phytochemicals, are an heterogeneous class of molecules able to slow or block oxidative reactions through different mechanisms of action.

A first mechanism involves the transfer of a hydrogen atom (Hydrogen Atom Transfer, HAT) from the antioxidant (AOH) to the peroxyl radical species ($ROO^{\cdot}$):

$$ROO^{\cdot} + AOH \rightarrow ROOH + AO^{\cdot}$$

In another mechanism of action, the antioxidant deactivates free radicals by transferring a single electron (Single Electron Transfer, SET); with the subsequent transfer of a proton, the same final product of the HAT mechanism is obtained:

$$ROO^{\cdot} + AOH \rightarrow ROO^{\cdot} + AOH^{\cdot +}$$
$$AOH^{\cdot +} + ROO^{\cdot} \rightarrow ROOH + AO^{\cdot}$$

The antioxidants can also act as oxidative enzyme inhibitors, chelator of pro-oxidant metals (see Figure 1), such as Fe^{2+} and Cu^{+}, and antioxidant enzyme cofactors.

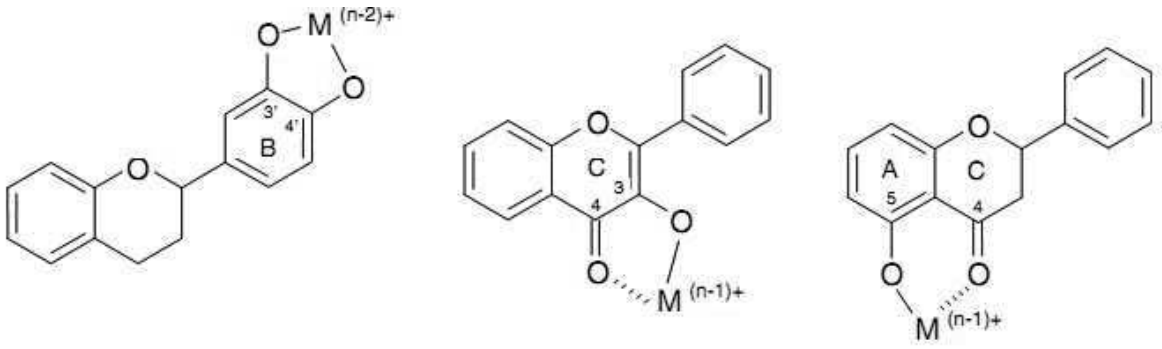


Figure 1. Metal ions complexed by flavonoids (adapted from Laguerre et al., 2007).

3. Food antioxidants

An increasing interest towards food antioxidants has arisen over the last two decades due to growing evidence of their healthy effects. Epidemiological studies indicated an inverse association between the intake of foods rich in antioxidants and incidence of chronic diseases, such as cardiovascular disease, diabetes mellitus, neurodegeneration and cancer (Redondo-Blanco et al., 2017; Gothai et al., 2016; Bomba et al., 2012; Shankar et al., 2016).

Many plant bioactive compounds have been discovered. These compounds vary widely in chemical structure and functions. The major classes of compounds with antioxidant activity are vitamins (vitamin C and vitamin E), carotenoids (carotenes and xanthophylls) and polyphenols (flavonoids, phenolic acids, lignans and stilbenes).

3.1 Vitamins

3.1.1 Vitamin C

Vitamin C (L-ascorbic acid, Figure 2) is a water-soluble vitamin. Unlike most mammals and other animals, humans have lost the ability to synthesize vitamin C as a result of a mutation of gene coding for L-gulonolactone oxidase, an enzyme required for the biosynthesis of vitamin C via the glucuronic acid pathway; thus, vitamin C must be obtained from the diet. This vitamin is especially plentiful in fresh fruit, in particular citrus fruit, and vegetables. A lack of vitamin C in the diet causes the deficiency disease scurvy (Combs and Gerald, 2012).

Vitamin C is a potent reducing agent; in our body, vitamin C plays two main functions that are essential cofactor in numerous enzymatic reactions and potent antioxidant (Carr and Frei, 1999). Vitamin C is the primary water-soluble non-enzymatic antioxidant in plasma and tissues.

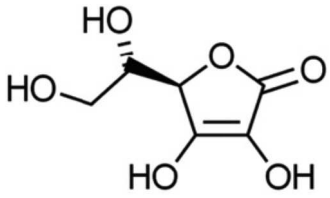


Figure 2. Vitamin C

It readily acts in scavenging $O_2^{\cdot-}$, $^{\cdot}OH$, H_2O_2, $^1O_2$ and reactive nitrogen species, thereby effectively protecting other substrates from oxidative damage (Carr and Frei, 1999). Vitamin C can also act as a coantioxidant by regenerating α-tocopherol (vitamin E) from its oxidized form. This is a potentially important function because *in vitro* experiments have shown that α-tocopherol can act as a prooxidant in the absence of coantioxidants such as vitamin C (Bruno et al., 2006).The role of vitamin C as a cofactor is also related to its redox potential. By maintaining enzyme-bound metals in their reduced forms, vitamin C assists mixed-function oxidases in the biosynthesis of collagen, carnitine, and catecholamines. Procollagen-proline dioxygenase (proline hydroxylase) and procollagen-lysine 5-dioxygenase (lysine hydroxylase), two enzymes involved in procollagen biosynthesis, require vitamin C for maximal activity. A deficiency of vitamin C results in a weakening of collagenous structures, causing tooth loss, joint pains, bone and connective tissue disorders and poor wound healing, all of which are characteristics of scurvy. Two dioxygenases involved in the biosynthesis of carnitine also require vitamin C as a cofactor for maximal activity. Carnitine is essential for the transport of activated long-chain fatty acids into the mitochondria; as a result, vitamin C deficiency results in fatigue and lethargy, early symptoms of scurvy. In addition, vitamin C is used as a cofactor for catecholamine biosynthesis, in particular in the conversion of dopamine to norepinephrine catalysed by dopamine β-monooxygenase. Depression, hypochondria, and mood changes frequently occur during scurvy and could be related to deficient dopamine hydroxylation (Carr and Frei, 1999).

Some researches also suggest that vitamin C is involved in the metabolism of cholesterol to bile acids, which may have implications for blood cholesterol levels and the incidence of gallstones (Simon and Hudes, 2000). Finally, vitamin C increases the bioavailability of iron from foods by enhancing intestinal absorption of non-heme iron (Combs and Gerald, 2012).

Numerous studies indicate that the intakes of vitamin C from diet or supplements is associated with reduction of lipid peroxidation (Shahkar et al., 2015), lowering serum uric acid levels resulting in a lower incidence of gout (Roddy and Choi, 2014), significant decreases in the risk of cataract formation (Mathew et al. 2012) as well as cardiovascular disease (CVD), including coronary heart disease (Ye and Song, 2008) and stroke (Yokoyama et al., 2000).

3.1.2 Vitamin E

The term vitamin E describes a family of eight fat-soluble molecules with antioxidant activities: four tocopherol isoforms (α-, β-, γ-, and δ-tocopherol) and four tocotrienol isoforms (α-, β-, γ-, and δ-tocotrienol).

In particular, α-tocopherol (Figure 3) appears to be the form of vitamin E with the greatest nutritional significance. The main function of α-tocopherol in humans is that of a fat-soluble antioxidant; it functions as a chain-breaking antioxidant, preventing the propagation of free radicals in membrane lipids and plasma lipoproteins. When a molecule of α-tocopherol neutralizes a free radical, it is oxidized and its antioxidant capacity is lost. Other antioxidants, such as vitamin C, are capable of regenerating the antioxidant capacity of α-tocopherol (Traber, 2012).

In the human liver, α-tocopherol is the form of vitamin E that is preferentially bound to α-tocopherol transfer protein (α-TTP) and incorporated into lipoproteins that transport α-tocopherol in the blood for delivery to extrahepatic tissues. Therefore, it is the predominant form of vitamin E found in the blood and tissues (Traber, 2012).

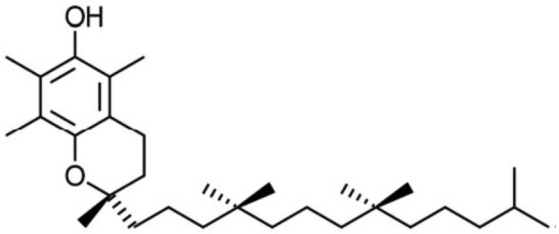


Figure 3. α-tocopherol

Vitamin E deficiency can be caused by fat malabsorption disorders or by genetic abnormalities that affect vitamin E transport. Severe deficiency symptoms include vitamin E deficiency-induced ataxia, peripheral neuropathy, muscle weakness, and damage to the retina of the eye (Traber, 2014).

It has been assessed that through the intake of high-dose of vitamin E supplements an inhibition of proatherogenic processes can be achieved. In addition, clinical evidences suggest that vitamin E supplementation may be beneficial for managing age-related macular degeneration (Evans and Lawrenson, 2012) and fatty liver diseases secondary to type 2 diabetes mellitus (Xu et al., 2014; Montero et al. 2014).

3.2 Carotenoids

Carotenoids are a class of more than 750 naturally occurring pigments synthesized by plants, algae, and photosynthetic bacteria. Fruit and vegetables provide most of the 40 to 50 carotenoids found

in the human diet. The main carotenoids present in the daily diet are carotenes, such as α-, β-carotene and lycopene, and the hydroxyl carotenoids or xanthophylls, among which zeaxanthin and lutein (Figure 4). Carotenoids are important not only for their provitamin A activity, but also for a spectrum of other actions in biological systems. Carotenes and xanthophylls are effective $^1O_2$ quenchers and free radical scavengers (Ramel et al., 2012). Some studies indicate that lycopene is one of the most effective $^1O_2$ quenchers among carotenoids. Furthermore some evidences suggest that carotenoids, particularly lycopene, and/or their metabolites may upregulate the expression of antioxidant and detoxifying enzymes via the activation of the nuclear factor E2-related factor 2 (Nrf2)-dependent pathway.

Carotenoids have been shown to play important roles in the prevention of health disorders, including cardiovascular disease and atherosclerosis, cancer, diabetes and inflammatory bowel diseases (Jomova and Valko, 2013).

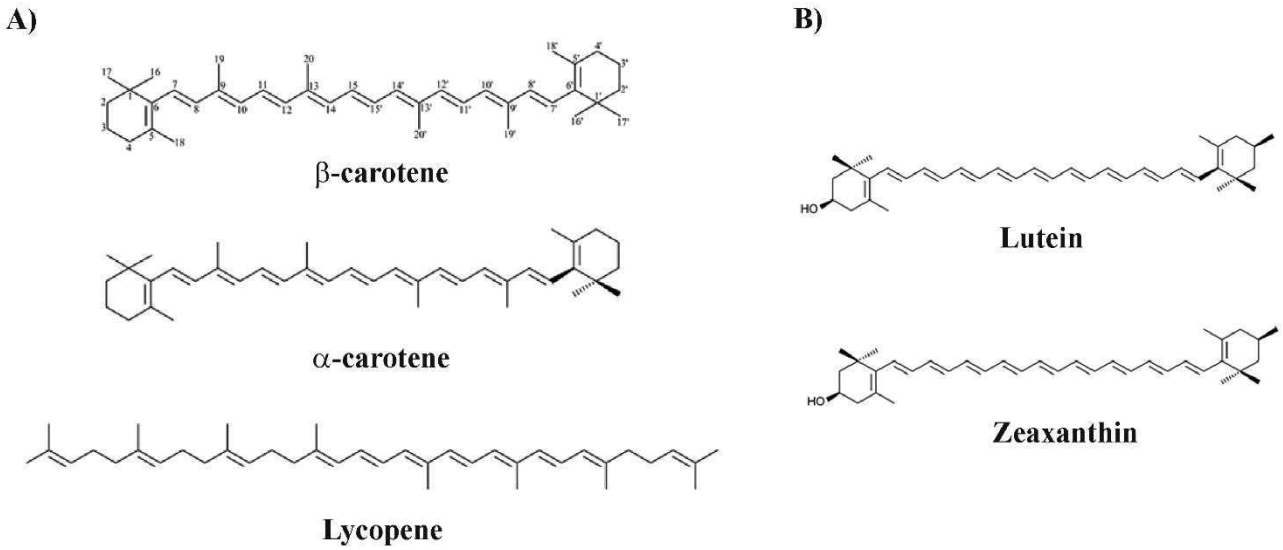


Figure 4. A) Carotenes; B) Xanthophylls

3.3 Polyphenols

Polyphenols are present in many plant foods such as fruits, vegetables, oil, wine, tea, chocolate and other cocoa-based products (Manach et al., 2004). It is a very large and heterogeneous group of secondary metabolites synthesized by plants during normal development and in response to biotic and abiotic stress conditions: in plants, polyphenols exert a protective action against ultraviolet rays and oxidative stress (Manach et al., 2004).

In addition to their role in plants, numerous studies show that phenols introduced by diet have beneficial effects on human health. The consumption of foods rich in phenolic compounds seems

to be associated with reduced incidence of several diseases, such as atherosclerosis, cancer, osteoporosis, allergies, diabetes and neurodegenerative diseases (Nijveldt et al., 2001; Manach et al., 2004).

Polyphenols act as antioxidants through numerous mechanisms. In particular, they function as scavenger of $HO^{\cdot}$ and $ROO^{\cdot}$ radicals, thus inhibiting lipid peroxidation, as chelators of metals with a pro-oxidant action (flavonoids), as inhibitors of pro-oxidant enzymes or activators of antioxidant enzyme systems (Naczk and Shahidi, 2004).

The main groups of polyphenols are flavonoids, phenolic acids, stilbenes and lignans.

Flavonoids are the most important class of polyphenols in plants (Figure 5). They can be further divided into several subclasses, of which the most representative are: flavones, flavanones, flavonols, flavanols (also called flavan-3-ols or catechins), anthocyanidins and isoflavones (Spencer et al. 2008; Ziberna et al. 2014). Flavonoids, can act as antioxidants indirectly by inhibiting enzymes that produce ROS, such as xanthine oxidase and NADPH oxidase (Orallo et al., 2002); through the inhibition of enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX), they can perform also an anti-inflammatory activity (Kim et al., 1998). Moreover, flavonoids cooperate with antioxidative vitamins (A, E, β-carotene), increasing their effectiveness and decreasing their degradation (Mojžiš and Mojžišová 2001).

Phenolic acids (Figure 6) can be divided into two categories depending on their structure: derivates of benzoic acid and derivates of cinnamic acid. Hydroxybenzoic acids include gallic, *p*-hydroxy-benzoic, protocatechuic, vanilic and syringic acids. The most important hydroxycinnamic acids are caffeic, ferulic, *p*-coumaric and sinapic acids (Abramovič, 2015). Phenolic acids are powerful antioxidants and have been reported to exert antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory actions (Lima et al., 2014; Mudnic et al., 2010).

Stilbenes are important because that their health effects have been reported to occur in lower concentrations compared to other phenolic compounds. Resveratrol (Figure 7), an important stilbene, has been found in peanuts, grapes, red wine, and some berries. In preclinical studies, resveratrol has been shown to possess numerous biological activities. As antioxidant, it neutralizes free radicals and other oxidants (Leonard et al., 2003), inhibits LDL oxidation (Brito et al., 2002) and induces antioxidant enzymes, including SOD, thioredoxin, glutathione peroxidase-1, heme oxygenase-1, and catalase. Resveratrol has been found also to inhibit the activity of several inflammatory enzymes *in vitro*, including COXs and LOXs (Donelly et al., 2004) as well as pro-inflammatory transcription factors, such as NFκB or AP-1 (de la Lastra and Villegas., 2005). Moreover, resveratrol might also function as an estrogen agonist, *i.e*. it might bind to estrogen receptors and elicit similar responses to endogenous estrogens (Yurdagul et al., 2014).

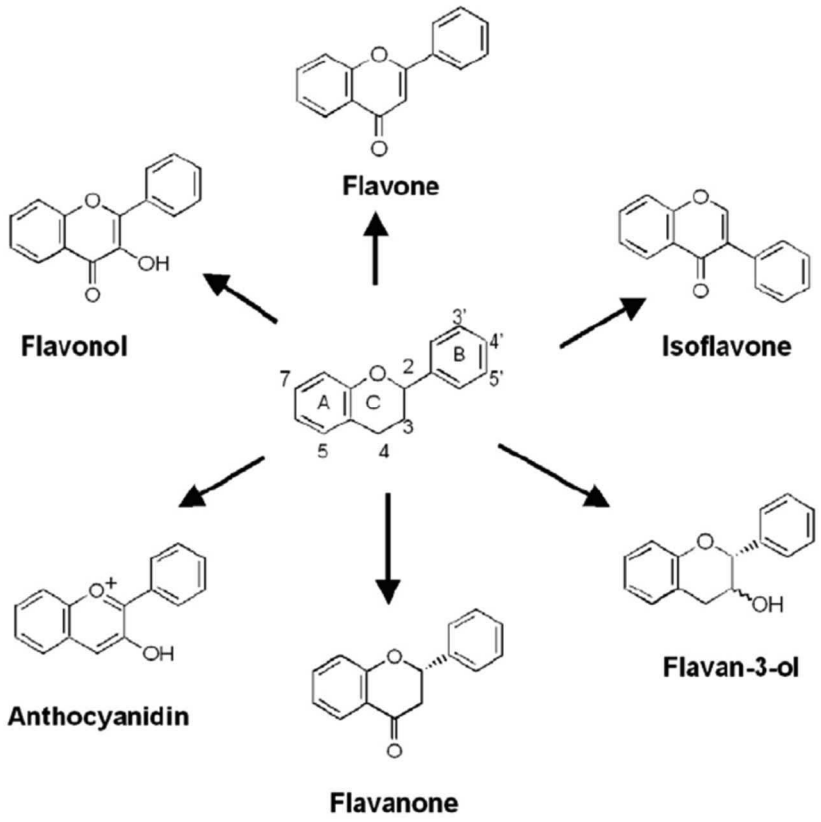


Figure 5. Flavonoids

Resveratrol has been found to induce cell cycle arrest and/or apoptosis in cancer cell lines (Stewart et al., 2003) and to inhibit angiogenesis and tumor invasion in both *in vitro* (Igura et al., 2001) and *in vivo* (Kanavi et al., 2014) studies.

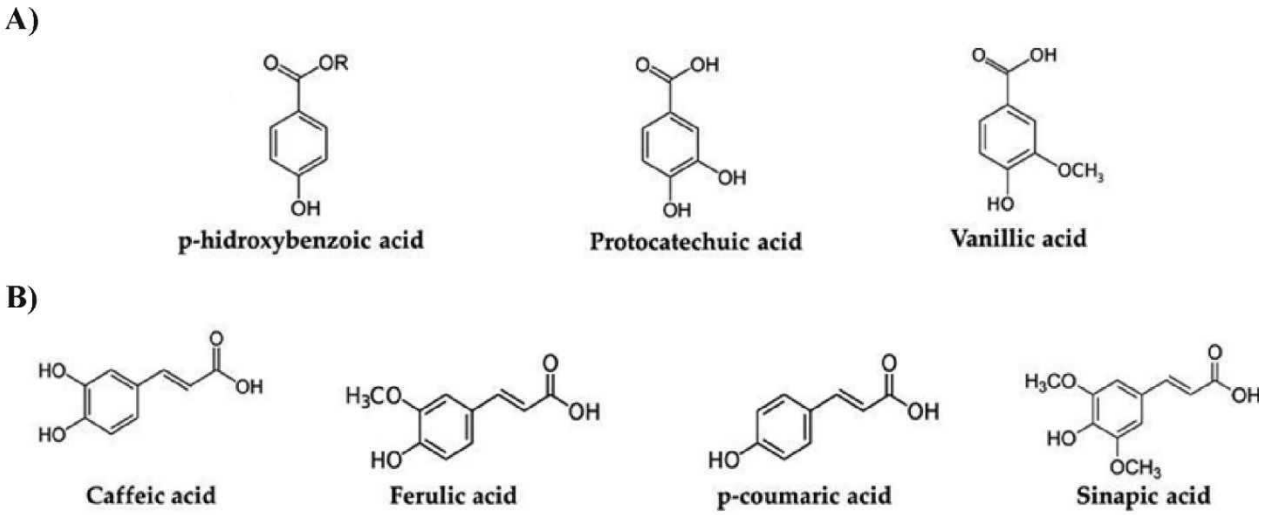


Figure 6. A) Hydroxybenzoic acids; B) Hydroxycinnamic acids

Finally, resveratrol was reported as potent activators of Sirtuins, a family of NAD^+ dependent deacetylases that have been shown to regulate a wide variety of cellular processes such as cellular senescence/aging, cellular apoptosis/proliferation, differentiation, metabolism, cell cycle regulation (Chung et al., 2010). In particular, yeast grown in media supplemented with resveratrol showed lifespan extension and this effect was directly dependent on resveratrol's ability to activate yeast Sir2 (Howitz et al., 2003). Resveratrol has also been shown to mimic anti-aging effects in lower organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* in a Sirtuin dependent manner (Wood et al., 2004). However, the activation mechanism of Sirtuins by resveratrol is still the subject of an intense debate (Hubbard and Sinclair, 2014), as better explained in the **Article 6**.

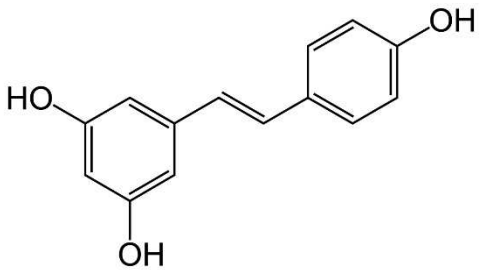


Figure 7. Stilbenes: trans-resveratrol

Lignans are a group of phenolic compounds, which occur in high concentrations in flaxseed and other seeds (Landete, 2012), roots, leaves, fruits, woody parts of vascular plants (Ekiert et al., 2013) and grains (Gerstenmeyer et al., 2013). The enterolignans, enterodiol and enterolactone (Figure 8), are formed by the action of intestinal bacteria on lignan precursors found in plants. Because enterodiol and enterolactone can mimic estrogen effects, their plant-derived precursors are classified as phytoestrogens. Lignans act as antioxidants and play an important role in normal colon functioning; recent findings have shown that lignans inhibit/delay the growth of mammary cancer (Landete, 2012).

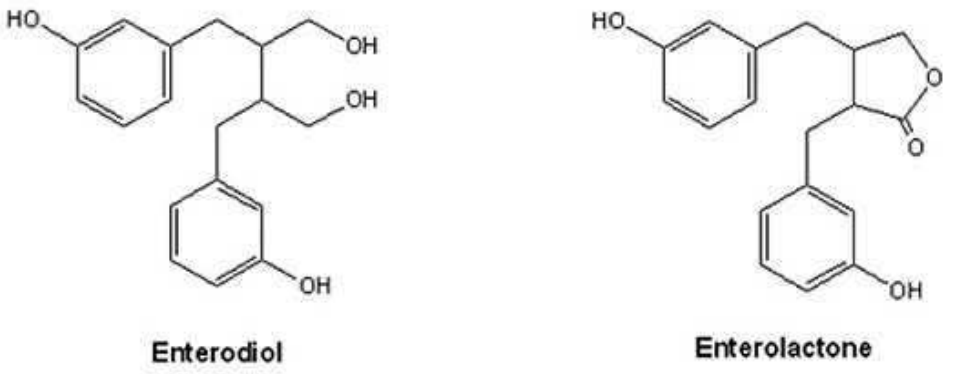


Figure 8. Enterolignans

4. Antioxidant capacity assays

Numerous methods to evaluate *in vitro* AC of food are available (Prior et al., 2005; Huang et al., 2005). Since these methods are simple, rapid and inexpensive, it was possible to spread them on a large scale. Based on the chemical reactions involved, major AC assays can be roughly divided into two categories: (1) SET reaction based assays and (2) HAT reaction based assays.

SET reaction based assays use a redox reaction in which an oxidant molecule is reduced by antioxidant compounds with consequent variation of its spectral properties; therefore, these methods allow to evaluate the antioxidant ability to function as scavengers of oxidant species or reducing agents.

Some methods belonging to this class are reported below:

- TEAC (Trolox Equivalent Antioxidant Capacity);
- DMPD (N,N-dimethyl-*p*-phenylenediamine);
- FRAP (Ferric Reducing Antioxidant Power);
- Test PCL (Photochemiluminescence);
- DPPH (2,2-diphenyl-1-picrylhydrazyl);
- CUPRAC (Total Antioxidant Potential Assay using Cu^{2+} complex as an oxidant).

Conversely, in the HAT reaction based assays, a competitive reaction occurs; in particular, an antioxidant and a probe molecule compete for binding the peroxyl radicals generated by the thermal decomposition of a diazo-compound and its reaction with oxygen. Also in this case the reaction between probe and the peroxyl radical causes a change in the spectral properties of the probe; however, in the presence of antioxidant, this reaction is delayed. Therefore these methods allow evaluating the ability of antioxidant molecules to act as chain breaking (Huang et al., 2005).

Some methods belonging to this class are reported below:

- ORAC (Oxygen Radical Absorbance Capacity);
- TRAP (Total Peroxyl Radical-Trapping Antioxidant Parameter);
- TOSC (Total Oxyradical Scavenging Capacity);
- Crocin bleaching.

According to some authors, TEAC may be considerate a HAT based methods (Prior et al., 2005).

4.1 SET reaction based methods

The SET reaction based methods imply the presence of antioxidants and an oxidizing molecule in the same reaction environment; the use of an oxidizing molecule with known spectral characteristics allows monitoring the electronic transfer reaction:

probe (oxidized) + antioxidant → probe reduced + oxidized antioxidant.

The reaction between the probe and antioxidants results in probe absorbance loss that is proportional to the amount of antioxidants present in the reaction medium.
The methods based on the SET reaction, including TEAC, DMPD, FRAP, DPPH, because of their simplicity, are in greater numbers than those based on the HAT reaction.

4.1.1 TEAC assay

TEAC assay was first developed by Miller et al. (1993) as a simple and convenient method for total antioxidant capacity (TAC) determination. The assay measures the ability of antioxidants to scavenge the radical cation $ABTS^{\cdot+}$, characterized by an intense blue-green colour, with consequent loss of absorbance.
The radical cation $ABTS^{\cdot+}$ can be generated from ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) in the presence of strong oxidizing agents. Antioxidants can neutralize the radical cation $ABTS^{\cdot+}$ by either direct reduction via electron donation or by radical quenching via hydrogen atom donation, and the balance of these two mechanisms is generally determined by antioxidant structure and pH of the medium (Prior et al., 2005).
In the original TEAC assay, metmyoglobin and H_2O_2 were used to generate an intermediate ferrylmyoglobin radical, which then reacted with ABTS to form $ABTS^{\cdot+}$ (Miller et al., 1993). However, the application of this method has several complications: the metmyoglobin has to be purified chromatographically; furthermore, this test does not allow studying AC in a wide pH range and shows a low reproducibility (Miller et al., 1993).
Several modifications in terms of the methods used to generate $ABTS^{\bullet+}$, wavelengths used to monitor the reaction and quantification methods led to many variants of this assay.
According to Lissi et al. (1995), the ABTS can be oxidized to $ABTS^{\cdot+}$ by 2,2-azobis- (2-amidinopropane) hydrochloride (ABAP) as a result of its high temperature decomposition (45 °C). The addition of antioxidants causes scavenging of the $ABTS^{\cdot+}$ resulting in absorbance decrease, which is recorded at the wavelength of 414 nm, at a fixed time and used as parameter for AC determination. Miller and Rice-Evans (1997) proposed the chemical oxidation of the ABTS with manganese oxide (MnO_2) and, as parameter for AC quantification, the absorbance decrease at 730 nm recorded at a fixed time.
In 2001, Arnao et al. employed an enzymatic system, consisting of the horseradish peroxidase in the presence of H_2O_2, to generate $ABTS^{\cdot+}$; in this case the parameter used to quantify AC of sample is the absorbance decrease at 414 nm evaluated at a fixed time after the addition of the antioxidant to the reaction medium.

In the currently most used version (Re et al., 1999) the cationic radical is generated through the reaction between ABTS and potassium persulphate and the parameter used to evaluate the AC of the sample is the absorbance decrease at 734 nm recorded at a fixed time.

The TEAC assay is simple and can be performed as a routine analysis without to need sophisticated equipment. $ABTS^{\cdot+}$ can be solubilized in both hydrophilic and lipophilic media and is not affected by ionic strength of the medium (Arnao et al., 2001). However, limitations of the TEAC assay have been noted, including the poor selectivity of the cationic radical towards electron donor species: it is shown that $ABTS^{\cdot+}$ reacts with any compound that presents an aromatic hydroxylated ring, regardless of its real antioxidant capacity (Roginsky and Lissi, 2005). Moreover, TEAC assay has also been challenged for its lack of biological relevance due to use of the artificial ABTS radical cation that is not found in food or biological systems. Nevertheless, the usefulness of these assays for ranking of antioxidant activity of similar substrates under comparable test conditions cannot be ignored (Shahidi and Zhong, 2015).

4.1.2 QUENCHER assay

The $QUENCHER_{ABTS}$ (QUick, Easy, New, CHEap and Reproducible) is a simple and direct procedure for AC assessment of food matrices avoiding any extraction and hydrolysis step: it is based on the direct reaction of food solid particles with the ABTS radical cation reagent (diluted in a mixture of ethanol and water), followed by centrifugation to obtain an optically clear supernatant for absorbance measurement (Serpen et al., 2008). So, the $QUENCHER_{ABTS}$ assay takes advantage from liquid-liquid interactions occurring in the solvent between soluble antioxidants and free radical molecules, but, interestingly, it is able to evaluate AC of insoluble moiety by surface reactions occurring at the solid–liquid interface between $ABTS^{\cdot+}$ molecules and antioxidant groups bound to insoluble matter (Serpen et al., 2007; 2008; Gökmen et al., 2009).

A considerable advantage of the direct procedure is that AC information obtained avoiding any pretreatment of food samples may be directly related to the antioxidant action in foods or in human gastrointestinal trait (Serpen et al., 2007; 2008; Gökmen et al., 2009), thus increasing the physiological relevance of results with respect to determination with the classical extraction-dependent TEAC assay.

Interestingly, the QUENCHER procedure has been successfully performed also by measuring the quenching of the DPPH radical reagent (Gökmen et al., 2009; Serpen et al., 2012a,b,c) instead of the $ABTS^{\cdot+}$ bleaching, as well as the colour generation of FRAP (Serpen et al., 2012 a,b) and CUPRAC reagents (Tufan et al., 2013) or fluorescence as in ORAC protocol (Amigo-Benavent et al., 2010; Kraujalis et al., 2013).

The QUENCHER procedure has been applied to measure AC of different cereal whole grains and some milling fractions (Serpen et al., 2007; 2008; Gökmen et al., 2009; Žilić et al., 2012), showing AC values generally higher than that obtained by different extraction and hydrolysis procedures used for comparison. AC of some fruits and vegetables has been also evaluated using the QUENCHER procedure (Serpen et al., 2012a). Moreover, this direct assay has been proved to be particularly suitable to assess AC of thermally processed foods: fried products including potato crisps (Serpen et al., 2009), bakery products such wheat bread and multigrain containing breads (Ciesarova et al., 2009; Serpen et al., 2012b), roasted pulses, nuts and seeds (Acar et al., 2009) , corn-based breakfast cereals (Rufian-Henares et al., 2009) raw and cooked meat samples (Serpen et al., 2012c). The QUENCHER protocol has been also applied to soybean seed coats and de-hulled beans (Žilić et al., 2013) and soy proteins (Amigo-Benavent et al., 2010).

Recently, the QUENCHER approach was used to study AC of durum wheat (*Triticum durum* Desf.) grains (Laus et al., 2015a). In this study was firstly assessed which kind of antioxidants determines QUENCHER response. This has been performed by comparing AC measured by $QUENCHER_{ABTS}$ and that measured by classical $TEAC_{ABTS}$ in four different extracts from whole flour of 10 durum wheat varieties containing lipophilic, hydrophilic, insoluble-bound phenolic (IBP) and free-soluble phenolic (FSP) compounds. After this preliminary analysis, the $QUENCHER_{ABTS}$ assay was applied for the first time to a screening study. In particular, the AC of grains from 12 modern Italian durum wheat varieties was compared with that of 24 old genotypes/landraces to evaluate whether genetic variability in AC, possibly related to health potential, exists between old and modern genotypes cultivated in the twentieth century.

Obviously, in this approach, total surface area and solid food particle sizes may play a crucial role in determining the reaction rate and, in turn, the measured AC (Gökmen et al., 2009). To date, the QUENCHER procedure has been generally applied to finely ground food solid particles having generally a diameter not exceeding 0.2-0.3 mm. Nevertheless, some food matrices of interest may be produced and used as larger size particles. So, in the **Article 1**, the applicability of the $QUENCHER_{ABTS}$ protocol to solid particles having large size was investigated.

4.1.3 FRAP assay

The FRAP assay (Benzie and Strain, 1996) is a typical SET-based method that measures the reduction of ferric ion (Fe^{3+})-ligand complex to intensely blue coloured ferrous (Fe^{2+})-ligand complex by antioxidants in acidic media. AC is determined as absorbance increase at 593 nm, at a fixed time (4 min), and is expressed as reduced Fe^{2+} equivalents. Unlike other SET-based

methods, FRAP assay is carried out under acidic pH conditions (pH 3.6) in order to maintain iron solubility and more importantly to drive electron transfer (Hegerman et al., 1998).
The original FRAP assay uses 2,4,6, -tripiridil-s-triazine (TPTZ) as the iron-binding ligand, while alternative ligands have also been employed for ferric binding, such as ferrozine for ascorbic acid reducing power evaluation (Molina-Diaz et al., 1998). More recently, potassium ferricyanide has been the most popular ferric reagent used in FRAP assays.
The chemical reaction underlying the FRAP method consists in the transfer of a single electron from the antioxidant molecule (AOH), which acts as an electron donor, to the ferric ion complexed with TPTZ.

$$Fe\ (TPTZ)_2\ (III) + AOH \rightarrow Fe\ (TPTZ)_2\ (II) + AOH^{\cdot +}$$

For a correct measure of the total reducing capacity some conditions must be met: only antioxidants should reduce ferric ion, the reaction must take place in short times (within 4 min) and finally, both the antioxidant and the secondary products of the reaction must not absorb at 593 nm. However, these conditions are hardly verifiable (Ou et al., 2002).

4.1.4 DPPH assay

The DPPH assay (Hogg et al., 1961) is the oldest method for AC determination. This method uses the $DPPH^{\bullet}$ radical, one of the few stable organic nitrogen radicals, which bears a deep purple colour. It is commercially available and does not need to be generated prior to the assay like $ABTS^{\cdot +}$. This assay is based on the measurement of the reducing ability of antioxidants toward $DPPH^{\cdot}$. The reaction is accompanied with the loss of DPPH colour at 515 nm, and the discolouration acts as an indicator of the antioxidant efficacy.
The percentage of the DPPH remaining is calculated as:

$$\%\ DPPH^{\cdot}{}_{REM} = 100 \times [DPPH^{\cdot}]_{REM} / [\ DPPH^{\cdot}]_{T=0}$$

The percentage of remaining $DPPH^{\cdot}$ ($DPPH^{\cdot}{}_{REM}$) is proportional to the antioxidant concentration. Sánchez-Moreno et al. (1998) further introduced another parameter to express antioxidant capacity, called “antiradical efficiency (AE)”. It was defined as:

$$AE = (1/EC_{50})\ T_{EC50}$$

in which EC_{50} is defined as the concentration that causes a decrease in the initial $DPPH^{\cdot}$ concentration by 50% and T_{EC50} is the time needed to reach the steady state with EC_{50}.

The radical DPPH· is currently one of the most used method for the evaluation of AC.
The advantages of this method are the stability and practicality of use of this radical, while the main limits are the insolubility of DPPH· in aqueous environment and its sensitivity to pH. Moreover, result interpretation is complicated when the test compounds have spectra that overlap DPPH· at 515 nm (Prior et al., 2005): carotenoids, in particular, interfere (Noruma et al., 1997).

4.2 HAT reaction based methods

HAT-based methods are composed of a synthetic free radical generator, generally a diazo-compound such as AAPH [2,2'-azobis (2- amidinopropane) dihydrochloride], a probe molecule, which allows to monitor the progress of the reaction and a parameter linked to the reaction kinetic for the AC estimation.
In particular, these methods use the thermolysis of the diazo-compound to generate, in O_2 saturated solution, the peroxyl radicals:

$$R\text{-}N{=}N\text{-}R \rightarrow 2\,R^{\cdot} + N_2$$
$$2R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$$

The peroxyl radicals generated in this way are able to oxidize the probe. However, in the presence of an antioxidant, a competition between antioxidant and probe for the peroxyl radical binding is established, and this results in the probe oxidation, inhibition or delay.

4.2.1 ORAC assay

The ORAC method, developed by Cao et al. (1993), measures the scavenger or chain breaking ability of antioxidants against not physiological peroxyl radicals, generated by the thermal dissociation of AAPH at 37 °C, which causes the oxidation of a fluorescent probe with consequent loss of its fluorescent properties. The addition to the reaction medium of antioxidant molecules able to compete with the fluorescent probe for the binding to peroxyl radicals results in inhibition or delay of probe oxidation.
The first version of the ORAC assay (Cao et al., 1993) employed β-phycoerythrin, a protein isolated from *Porphyridium cruentum*, as the probe. However, the use of β-phycoerythrin present several disadvantages: it has a large lot-to-lot variability; it is photo bleached under plate-reader conditions; it interacts with polyphenols due to the nonspecific protein binding and loses fluorescence even without added radical generator. To solve these problems, Ou et al. (2001)

replaced β-phycoerythrin with fluorescein (FL). FL is a synthetic non-protein probe and overcomes the limitations of β-phycoerythrin.

The parameter used for the quantification of AC is the integrated area under the kinetic curve (Area Under Curve, AUC) that describes the loss of the fluorescence over time. In the presence of antioxidants the fluorescence decrease is delayed and AUC increases; the net area (AUCnet) is obtained from the difference between AUC values in the presence and in the absence (blank) of antioxidants. A standard antioxidant, usually Trolox, is used to obtain a calibration curve and ORAC values of the tested antioxidants are expressed as Trolox equivalents. The ORAC method is a very versatile method, now automated, which is able to evaluate AC of both hydrophilic and lipophilic antioxidants of food as well as of biological samples; these features make it one of the most widespread and used methods today (Prior et al., 2003).

4.2.2 TRAP assay

Similar to ORAC, TRAP consists of an azo-compound as peroxyl radical generator, *e.g.* AAPH or [2,2'-Azobis(2-methylpropionamidine) dihydrochloride] (ABAP), a fluorescent molecule, *e.g.* R-phycoerythrin or dichlorofluorescin diacetate, and an antioxidant. The basic reactions of the assay are similar to those of ORAC. AC is determined as extension of the lag time for appearance of the oxidized probe when antioxidants are present. TRAP values are usually expressed as Trolox equivalents (Prior et al., 2005).

4.2.3 TOSC assay

The TOSC method (Winston et al., 1998) uses a diazo-compound, such as ABAP, that, in the presence of O_2, generates peroxyl radicals; the substrate oxidized in this assay is α-keto-γ-methiolbutyric acid (KMBA), which forms ethylene. The time course of ethylene formation is followed by gas chromatography, and the AC is quantified by the ability of the antioxidant to inhibit ethylene formation. In particular, the amount of sample needed to cause a 50% inhibition (EC_{50}) is used as an index to calculate the total AC.

5. Methods based on the use of the lipoxygenase enzyme: LOX/RNO and LOX-FL assays

The LOX/RNO assay (Pastore et al., 2009) is a method for AC assessment in food extracts, based on the bleaching of 4-nitroso-*N,N*-dimethylaniline [abbreviated as RNO (Pastore et al., 2000), because it belongs to the family of C-nitroso compounds (RN=O, R = alkyl or aryl group)] catalyzed by soybean lipoxygenase. LOXes (linoleate:oxygen oxidoreductase, EC 1.13.11.12) constitute a large gene family of non-heme-iron-containing fatty acid dioxygenases, which occur

in both the plant and animal kingdoms. LOXes catalyze the regio- and stereospecific insertion of molecular oxygen into polyunsaturated fatty acids containing 1,4-cis,cis-pentadiene structures to produce the corresponding hydroperoxyl derivatives (Baysal and Demirdöven, 2007).

In particular, LOX/RNO assay uses the soybean LOX-1 isoenzyme that catalyzes the linoleate (LH) dioxygenation to 13-hydroperoxylinoleate (LOOH). Under conditions of limited oxygen, reached with the progress of the reaction, LOX-1 is also involved in several secondary anaerobic reactions that generate, in the presence of LH and LOOH preformed in the course of the aerobic cycle, some radical species, which also occur *in vivo* (and so referred to as "physiological"): fatty acid radicals, including the linoleate alkylic ($L^{\cdot}$), alkoxyl ($LO^{\cdot}$), and peroxyl ($LOO^{\cdot}$) radicals, and oxygen radical species, such as the $^{\cdot}OH$ and $^1O_2$ (Pastore et al., 2009 and references cited therein). It is known that these radicals may cause the oxidation of plant pigments and the oxodiene generation (Siedow et al., 1991 and references cited therein). Moreover, some of these radicals ($LO^{\cdot}$, $LOO^{\cdot}$, and $^{\cdot}OH$, as well as $^1O_2$), are able to induce the bleaching of RNO in a biochemical pathway coupled with oxodiene formation (Pastore et al., 2000). The soybean LOX-1-dependent RNO bleaching may be delayed, inhibited, or even prevented by antioxidant compounds by different mechanisms: scavengers of one or more free radical species; chelating or reducing agents of the iron ion essential for the catalysis; inhibitors of the apoenzyme (antiperoxidative action). Therefore, the peculiarity of the soybean LOX-1-mediated RNO bleaching (LOX/RNO reaction) is to simultaneously detect, under conditions of low oxygen supply, the scavenging capacity toward "physiological" and biologically relevant radical species together with other important antioxidant functions. Thus, a method based on the LOX/RNO reaction (LOX/RNO method) is expected to provide more integrated and comprehensive information about the food antioxidant capacity (Pastore et al., 2009).

The LOX/RNO method was used to evaluate AC of *i)* pure compounds (Pastore et al., 2009) *ii)* hydrophilic, lipophilic and phenolic extracts from durum wheat whole grains (Pastore et al., 2009; Laus et al., 2012b), quinoa (Laus et al. 2012a) and the antioxidant-rich food supplement Lisosan G (Laus et al., 2013a) *iii)* carotenoid-enriched extracts from peach fruits (Laus et al., 2015b).

In all cases, the LOX/RNO method was able to better highlight variability among the different matrices tested and provided CA values higher, depending on the type of extract tested, than DMPD, TEAC as well as ORAC methods, used as a comparison.

Moreover, the LOX/RNO method showed a greater capacity, compared to TEAC and ORAC, to highlight synergistic interactions among *i)* food-grade antioxidants: exstracts enriched in catechins, quercetin, resveratrol, tyrosol/hydroxytyrosol and lycopene (Laus et al., 2013b) *ii)* different extracts: hydrophilic, phenolic and lipophilic extracts from durum wheat whole flour

(Pastore et al., 2009; Laus et al. 2013b) and *iii)* antioxidants contained in the same extract: phenolic compounds from durum wheat whole flour (Laus et al. 2012b).

This result can be explained by the ability of the LOX/RNO method to evaluate, as already mentioned, more antioxidant actions simultaneously, while others methods measure, mainly reducing power (TEAC and DMPD) or peroxyl radical scavenging capacity (ORAC).

A second method based on the use of soybean LOX-1 as system to produce physiological radical species is LOX-FL method. The development of this method has been one of the experimental activities carried out during the course of this research, so in the **Article 2** the features of this assay as well as its application in measuring AC of both the extracts from the antioxidant-rich food supplement Lisosan G and serum after the intake of the supplement, have been reported.

6. Limits of AC assessment on food

The methods for AC measurement owe their success to the fact that they are generally simple, rapid and inexpensive. Moreover, recent population studies found significant associations between AC of the food consumed (obtained from food-frequency questionnaires) and a lower risk of heart failure (Rautiainen et al., 2013), strokes (Del Rio et al., 2011; Rautiainen et al., 2012) and glucose intolerance (Okubo et al., 2014). On the other hand, *in vitro* AC measurements of foods alone have not been demonstrated to be relevant for the biological effects of specific bioactive compounds (Fraga et al., 2014; Huang et al., 2005), since they do not provide information about bioavailability of food antioxidants, as well as their *in vivo* stability, retention by tissues and *in situ* reactivity. Moreover, methods for AC measurement show a lot of technical and conceptual limitations.

In the light of these considerations, the U.S. Department of Agriculture (USDA) removed its ORAC database for selected foods in 2012 from its nutrient data laboratory website (Pompella et al., 2014). However, this choice is not fully shared and remains to a certain extent questionable (Rautiainen et al., 2013). All these aspects are better explained in the **Article 2**.

7. Evaluation of blood AC

An approach that move the focus from the analysis of the AC of foods to the analysis of AC of serum/plasma after food intake may be more reliable. This measure may take into account bioavailability and metabolism, so giving an integrated information of a true effect on blood antioxidant status, which is beyond the original AC of the ingested food.

Nevertheless, also this approach shows some weakness (see **Article 2**), so results about effects of food antioxidants on blood AC are rather controversial. In many cases, assays of AC in

serum/plasma appear to be not suitable tools to derive information on the quality of foods, to quantify the AC in humans or to infer effects on human health.

This inconsistency may be, at least in part, dependent on some unsuitability of methodological approach. So, to measure the antioxidant status in serum, we propose the use of a novel parameter, the Antioxidant/Oxidant Balance (AOB). The AOB represents the ratio between the serum AC and an indicator of the level of oxidation of serum, that we named Peroxide Level (PxL). The reliability of the novel AOB approach in evaluating serum antioxidant status after food intake was assessed in a study reported in the **Article 3**.

8. Methods to evaluate the blood level of peroxidation

To evaluate the level of oxidative stress in humans, numerous methods have been proposed in order to measure the oxidation of different macromolecules including lipids, proteins and DNA (Hageman et al., 1992): TBARS (Thiobarbituric Acid Reactive Substances) in blood or urine (Gutteridge and Tickner, 1978; Yagi et al., 1987; Boyd and McGuire, 1990), the measurement of oxidized LDL in the blood (Harats et al., 1989) and the assay of volatile compounds such as ethane and pentane in the exhaled (Refat et al., 1991). The measurement of the glutathione/glutathione disulfide ratio in the blood (Hughes et al. 1990), of eicosanoids in the urine (Judd et al., 1989), and of eicosanoids not derived from cyclo-oxygenase activity in the plasma (Morrow and Roberts, 1991) are also used. Moreover, peroxide level in the blood are measured by using FOX (Ferric-xylenolorange, Erel et al., 2005) and d-ROMs methods (Diacron Reactive Oxygen Metabolites, Alberti et al., 2000).

Among these methods, d-ROMs is particularly simple. It is a spectrophotometric method that allows to determine, in a biological sample, the concentration of hydroperoxides (ROOH) produced in cells by ROS oxidative attack towards various biochemical substrates (glucides, lipids, amino acids, proteins, nucleotides, etc.). This method is based on the capability of hydroperoxide groups to react with Fe^{2+} and generate the corresponding alkoxyl radical, according to the Fenton reaction. This newly formed radical, whose quantity is related to the peroxide content, is chemically trapped with *N,N*-diethyl-phenylenediamine (DPPD), leading to formation of the corresponding radical cation, which was determined at 512 nm. The reaction can be monitored as both absorbance increase (kinetic mode) and absorbance at the end-point. The concentration of hydroperoxides is expressed as Carratelli Unit (corresponding to 0.0235 μmoli H_2O_2/ml).

However, the d-ROMs method presents some critical points (Erel et al., 2005). In fact, as proposed by Diacron, the Fe^{2+} required to catalyze the Fenton reaction, should be released from the serum proteins under acidic conditions. However, blood iron is mostly transported by transferrin;

moreover, in the blood the small amount of free Fe^{2+} ions is rapidly converted to Fe^{3+} ions by enzymes having ferroxidase activity (Erel et al., 1998). For these reasons, the d-ROMs method has been improved by adding an adequate Fe^{2+} amount in order to promote the reaction. Both the technical features and an example of application of this new method, named Peroxide Level (PxL) assay, have been reported in the **Article 3**.

Section II-Sirtuins and Glyoxalase system: enzymatic reaction, physiological role and modulation

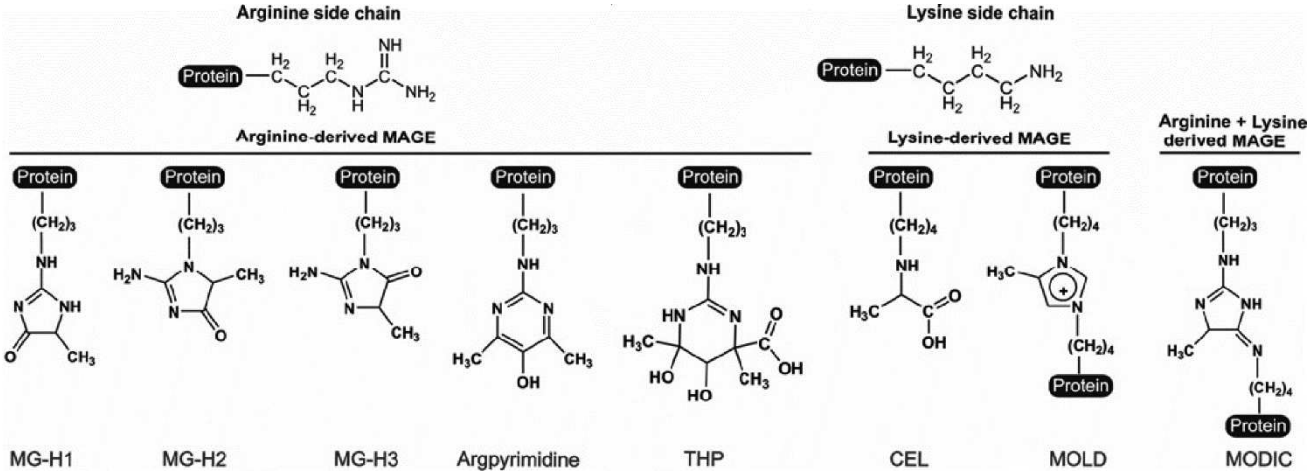
1. Glyoxalase system

The glyoxalase system is a cytoplasm enzymatic pathway, which provides the primary defence against dicarbonyl glycation by catalysing the metabolism of methylglyoxal (MG), a α,β-dicarbonyl ketoaldehyde. MG is physiologically produced in living organisms through both enzymatic and non-enzymatic pathways. Spontaneous production of MG in eukaryotic cells occurs as a consequence of glycolysis, from the non-enzymatic decomposition of the triose phosphates glyceraldehyde-3-phosphate (GA3P) and dihydroxyacetonephosphate (DHAP); other non-enzymatic sources of MG are the Maillard and lipoperoxidation reactions. The enzymatic formation of MG occurs through the oxidation of aminoacetone in the catabolism of L-threonine, mediated by the enzyme semicarbazide-sensitive amine oxidase (SSAO) and the oxidation of acetone by cytochrome P450 in the catabolism of ketone bodies (Rabbani and Thornalley, 2012; Rabbani et al., 2016). The only known enzyme that specifically catalyses MG formation is methylglyoxal synthase. This enzyme was found, until recently, in bacteria and catalyses the formation of MG from the triose phosphate DHAP (Silva et al., 2013). The rate of MG formation depends on the organism, tissue, cell metabolism and physiological conditions.

MG is the most relevant and reactive glycation agent *in vivo*. Glycation is a process of post-translational modification of proteins in which free reducing sugars and toxic aldehydes, such as MG, react non-enzymatically with amino groups leading to the formation of advanced glycation end products (AGEs).

As shown in Scheme 1, MG reacts preferentially with arginine side chains to form Hydroimidazolones (MG-H1, MG-H2 and MG-H3), Argpyrimidine and Tetrahydropyrimidine (THP). The reaction between lysine residues and MG leads to the formation of Nε-(carboxyethyl) lysine (CEL) and MG–lysine dimers (MOLDs). A cross-link between arginine and lysine residues produces MG-derived imidazolium cross-linking (MODIC). The MG-H1 is one of the most quantitatively and functionally important AGEs in physiological systems.

Proteins modified by dycarbonyl compounds are recognised as misfolded and directed to the proteasome for proteolysis (Silva et al., 2013). Other AGE-modified proteins exert cellular effects via interaction with specific AGE receptors that trigger an inflammatory response at the cellular level, accounting for AGE toxicity. Proteins known to be modified by MG include albumin, haemoglobin, 20S proteasome subunits, mitochondrial proteins, lipoproteins, extracellular matrix proteins.

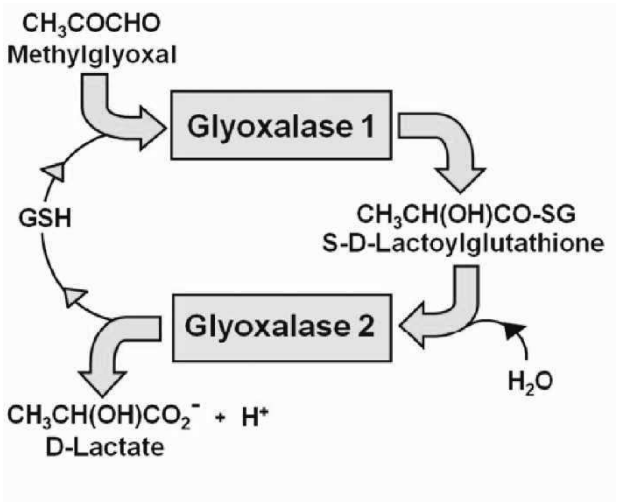


Scheme 1. MG-derived AGEs (from Silva et al., 2013).

Besides proteins, the amino groups of nucleic acids and basic phospholipids can also be irreversibly modified by glycation reactions: DNA can undergo cross-links, strand breaks, and permanent mutations; lipids are easily oxidizable, and membranes, being rich in lipids, are prone to these modifications (Silva et al., 2013). Increased formation and accumulation of AGEs in humans has been detected and implicated in development and progression of several diseases, including diabetes and its associated vascular complications, neurodegenerative disorders, renal failure, cirrhosis and aging (Silva et al., 2013).

The glyoxalase system prevents the accumulation of MG by catalyzing the conversion of MG to D-lactate via the intermediate S-D-lactoylglutathione (SLG, Scheme 2). It comprised two enzymes, Glyoxalase I (GloI) and II (GloII) and a catalytic amount of reduced glutathione (GSH). GloI catalyses the isomerisation of the hemithioacetal, formed spontaneously from MG and GSH, to SLG. GloII is a thiolesterase and catalyses the hydrolysis of SLG to D-lactate and reforms GSH consumed in the GloI-catalysed reaction step (Thornalley, 1990).

GloI enzyme has been characterized in many different organisms, from mammals to plants, yeast, bacteria and protozoan parasites. Structurally, GloI enzyme is a homodimer containing a zinc or nickel metal centre, essential for catalysis, and a thiol-binding pocket located at the interface of the two subunits (Silva et al., 2013). It is commonly accepted that eukaryotic GloI enzymes contain zinc, including human, yeast and *L. infantum*, whereas the prokaryotic enzymes have nickel, such as the bacteria *E. coli*, *Pseudomonas aeruginosa, Yersinia pestis* and *Neisseria meningitides* (Silva et al., 2013). The expression of GloI gene, is under stress-responsive control of nuclear factor erythroid 2–related factor 2 (Nrf2) through the antioxidant response element (ARE).



Scheme 2. Glyoxalase system (from Xue et al., 2016)

Nrf2, a redox sensitive transcription factor, regulates gene expression of many antioxidant enzymes and phase II detoxification enzymes, including GloI. Under physiological conditions, Nrf2 interacts with Kelch-like ECH-associated protein 1 (Keap-1), a cytosolic repressor protein that limits Nrf2-mediated gene expression. Upon activation, Nrf2 is free from Keap-1 and translocates into the nucleus where it binds ARE within promoter regions of the encoding target genes to exert cellular defense effects (Rabbani et al. 2014).

Due to its relevant role in protecting cells from the accumulation of AGEs, glyoxalase system has been extensively investigated. In particular, mammalian GloI has been received increasing attention; during the "Glyoxalase Centennial conference" it was highlighted the importance of searching dietary compounds that can enhance the Glo activity (Rabbani and Thornalley, 2014).

The effect of different dietary bioactive compounds on both GloI gene expression and activity has been evaluated by *in vitro* studies. Several studies have reported that some polyphenols, among which resveratrol, mangiferin and fisetin, were able to enhance GloI function by promoting activation of Nrf2/ARE signalling pathway (Cheng et al., 2012; Liu et al. 2016; Maher et al., 2011). Regarding resveratrol, it has been observed that it was able to activate the extracellular signal-regulated kinase (ERK) pathway leading to Nrf2 nuclear translocation with consequent elevation of GloI expression levels in Hep G2 cells (Cheng et al., 2012). GloI activation by resveratrol has been confirmed also by a recent *in vivo* study. In particular, the regular consumption of resveratrol and hesperetin for 8 weeks was found to increase GloI activity by 27% in highly overweight subjects (Xue et al., 2016).

Also sulforaphane (SR), an isothiocyanate formed upon the hydrolysis of glucoraphanin, which is a glucosinolate present in *Brassica* vegetables, was found able to enhance GloI activity by

upregulation of its genetic expression. After incubation of both human hepatoma Hep G2 cells and BJ fibroblasts with 2 μM SR, GloI activity was increased (by 2-3-fold) and a related dose-dependent increase in GloI mRNA was reported (Xue et al., 2012). Another study in which cells isolated from primary neonatal rat cardiomyocytes were incubated with 5 μM SR for 24 h and subsequently exposed for other 24 h to1 mM MG, showed that SR treatment significantly counteracted MG-induced damage (Angeloni et al. 2013). Additionally, SR protected the cardiomyocytes against apoptosis induced by the MG (Angeloni et al. 2013). Finally, GloI activity (by Western blot analysis and GloI activity assay) was found significantly increased in SH-SY5Y neuroblastoma cells after their incubation with 2.5 μM of SR for 24 and 48 h (Angeloni et al. 2015). In the **Article 4** a study aimed to assess the effect of SR on the GloI gene expression and activity in peripheral blood mononuclear cells (PBMCs) was carried out. PBMCs were chosen because they are human primary cell models, thus a good model to estimate what could be the *in vivo* effect of SR. In particular, PBMCs isolated from human volunteers were incubated with 2.5 μM SR for 24 h and 48 h to simulate a daily consumption of a broccoli portion and the GloI activity/expression were analysed.

In addition to its well-defined protection role against carbonylation damage, numerous literature studies have demonstrated an abnormal expression or high activity of GloI in many human tumors including colon, prostate, and lung (Davidson et al., 1999; Sakamoto et al., 2001). GloI has been shown to be highly expressed also in anti-tumor agent-resistant human leukemia cells (Sakamoto et al., 2000). These observations indicate that the increase of GloI expression is closely associated with carcinogenesis and anti-tumor drug resistence. So, inhibitors of GloI are expected to inhibit carcinogenesis and overcoming drug resistance; GloI inhibition, in fact, may result in MG accumulation and subsequent apoptosis of tumor cells (Takasawa et al., 2008). Recently, natural inhibitors of GloI enzymatic activity have been selected from flavonoids. In particular, Santel et al. (2008) found that curcumin was able to inhibit GloI activity and that this inhibitory action was stronger than other flavonoids as quercetin, myricetin, kaempferol, luteolin and rutin.

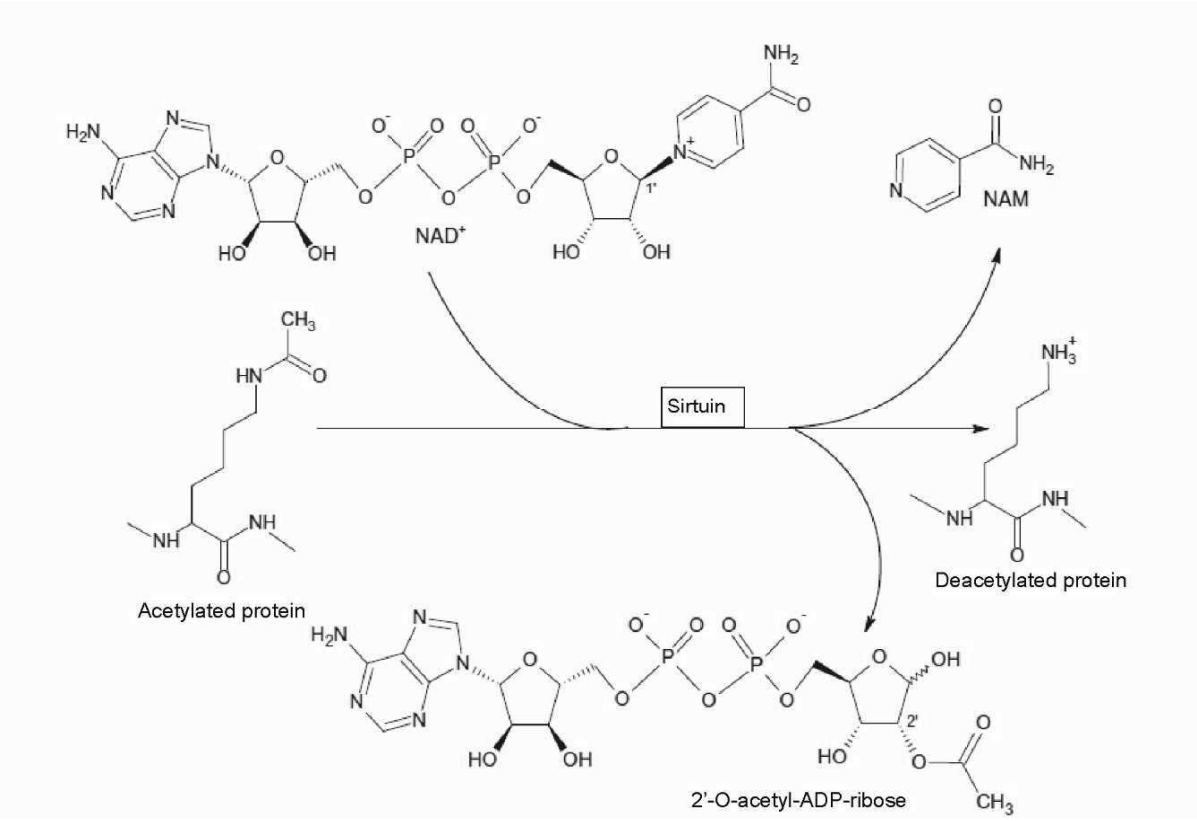
Concerning plants, in the last three decades, numerous studies have reported various functional roles for plant glyoxalases. In addition to its well-established roles in conferring abiotic (salinity, drought, mannitol, extreme temperatures and heavy metal stress) and biotic (living organisms such as bacteria, fungi, viruses, parasites, and insects) stress tolerance, it has been shown that glyoxalase enzymes are markers for cell division and play an important role both in plant reproduction and in the regulation of protein turn-over (Sankaranarayanan et al., 2017).

However, to date, the modulation of plant glyoxalase activity has been little investigated. In the **Article 5** is reported a study in which the effects of some phytochemicals on the activity of a plant

GloI were evaluated; this in order to found possible physiological modulators of plant glyoxalase activity. The study was carried out on a mitochondrial GloI because this organelle is one of the main targets of the harmful effects exerted by the MG (Li, 2016 and references therein). In particular, durum wheat mitochondria (WM) were used, since for these mitochondria an isolation procedure with high yield of pure and intact organelles is available (Pastore et al., 1999).

2. Sirtuins

Sirtuins (silent information regulator 2, SIR2 proteins), firstly discovered in yeasts and then demonstrated as ubiquitous and highly conserved throughout all kingdom life, belong to class III of histone deacetylases ($HDAC_S$). Sirtuins catalyze a specific two-stage NAD^+-dependent deacetylation of histon/non-histone proteins: the first step consists of hydrolysis of glycosidic bond between nicotinamide and ADP-ribose residue of the NAD^+ molecule; in the second step, acetyl moiety is transferred from the ε-*N*-acetylated protein sequences to the ADP-ribose residue. So, the overall reaction products are nicotinamide (NAM), the deacetylated protein and 2'-*O*-acetyl-ADP-ribose (Szućko, 2016 and references therein; Chung et al., 2010, Scheme 3).



Scheme 3. Deacetylation reaction by Sirtuins (from Kupis et al., 2016).

In addition to deacetylation, some sirtuins may also exhibit a NAD^+-dependent desuccinylation, demalonylation and defatty-acylation of lysine residues (Li et al., 2015 and references therein), as well as a mono-ADP-ribosyltransferase activity (Szućko, 2016 and references therein).

The numbers of sirtuins varies in different organisms; it increases with the complexity of the organism, ranging from one in bacteria to five in fungi and seven in vertebrates. Phylogenetic analyses of the conservative amino acids sequence motif forming the core of the catalytic NAD^+-domain show that gene encoding sirtuin proteins can be divided into five major classes. Classes from I to IV comprise sirtuins for both eukaryotic and prokaryotic organisms, while the fifth class (U group) is specific only for prokaryotes (Szućko, 2016; Cucurachi et al., 2012 and references therein). The yeast (*Saccharomyces cerevisiae*) SIR2 belongs to class I. Seven members of the sirtuin protein family (SIRT1-SIRT7) have been identified in mammals: SIRT1/SIRT2/SIRT3 belonging to class I, SIRT4 to class II, SIRT5 to class III and SIRT6/SIRT7 to class IV (Houtkooper et al., 2012). These mammalian proteins differ in tissue and cell localization, enzymatic activity, substrates and biological implications (Szućko, 2016 and references therein). Animal sirtuins have recently emerged as key regulators of life-span, cellular senescence/aging, cellular apoptosis/proliferation, differentiation, metabolism, cell cycle regulation (Chung et al., 2010 and references therein). Among sirtuins, the best-characterized is human SIRT1.

Sirtuins have been extensively investigated in last decades. Over the years, several modulators of sirtuin activity have been discovered; some act specifically, others across the entire family. Howitz et. al. (2003) screened a library of plant polyphenols to identify compounds that could modulate the activity of SIRT1 and its homologs. Several plant derived polyphenols seemed to activate SIRT1 (Howitz et al., 2003); among these, the most potent activator identified was resveratrol, a flavonoid synthesized by several plant species including grapes. However, the mechanism of SIRT1 activation by resveratrol has been debated and recent findings have shown that the direct activation of SIRT1 by resveratrol is a false positive hit (Borra et al., 2005; Pacholec et al., 2010). Therefore, the resveratrol-mediated *in vivo* effects may occur through a different molecular mechanism independent of direct SIRT1 activation.

In addition to plant bioactive compounds, synthetic SIRT1 activators with high potency, solubility, and bioavailability have been identified. These small molecules (e.g., SRT1460, SRT1720, and SRT2183) do not share any structural similarity to polyphenols and were shown to be up to 1000 fold more potent in activating SIRT1 compared to resveratrol (Milne et al., 2007). Inhibitors of SIRT1 have been also identified and characterized over the past several years (Blum et al., 2011), The majority of them are based on peptide mimics and NAD^+/NAM analogs. Some of these inhibitors such as Ex-527 and Suramin inhibit SIRT1 in the nanomolar range, whereas others such

as Sirtinol, Tenovin and Cambinol and their derivatives inhibit in the micromolar range. These last compounds were used as analogues to develop more potent and bioavailable inhibitors that were shown to possess antitumor activity.

As for measurement of sirtuin enzymatic activity, up to now different assays have been developed (Li et al., 2015). In particular, some approaches are based on direct detection of either substrate (acetylated peptides and NAD^+) consumption or product (deacylated peptides, NAM and acylated-ADP-riboses) formation by using MS or HPLC technologies or MS coupled with HPLC to achieve better performance. These assays show high accuracy and reliability; however, most of these are still time consuming and not qualified to undergo high-throughput format (Li et al., 2015). Some radio-isotope-labeled assays are available, based on use of radioactive acetylated peptides (^{3}H-acetylated histone peptides or ^{14}C-acetylated P53 peptides) or ^{14}C- or ^{32}P-labeled NAD^+. Despite high sensitivity, these methods have the drawback of high costs, of health hazard and of management of radioactive wastes; most of these assays are not suitable for high-throughput screening. Indirect luminescence assay has been also developed (Promega, SIRT-Glo™ Assay). This is based on a coupled enzymatic system in which an acetylated luminogenic peptide can be firstly deacetylated by sirtuin activity, and then cleaved by a specific protease, resulting in release of amino luciferin; free amino luciferin can be quantified using a firefly luciferase reaction to produce a stable, persistent emission of light. Other indirect assays, involving coupled two-step enzymatic assays for detection of reaction products, are fluorescence-based approaches (Li et al., 2015). For detection of NAM, nicotinamidase and glutamate dehydrogenase have been used as coupling enzymes. For detection of deacylated peptides there are two different strategies. The fluorogenic assays couple the sirtuin-dependent deacylation to the trypsin-catalyzed digestion: in particular, an acetylated tetrapeptide conjugated at the C-terminus with the fluorophore 7-amino-4-methylcoumarin (AMC) undergoes first deacetylation by sirtuin and then cleavage by trypsin at the carboxyl side of the deacetylated lysine, releasing AMC that emits fluorescence at 460 nm. A different strategy characterizes the fluorescence resonance energy transfer (FRET)-based assays. These methods exploit a FRET effect that occurs between a fluorescent donor dye and a non-fluorescent quenching group connected respectively to the carboxyl and amino terminus of a peptide sequence containing a single acetyl-lysine residue; this causes quenching of fluorescence emission from the donor dye. The deacetylation followed by trypsin digestion disrupts the FRET signal, resulting in increase of fluorescence from the donor dye (Li et al., 2015). A large variety of FRET assays has been developed over the years for different sirtuin assays using different specific substrate peptides (besides AMC-acetyl peptides, also AMC-succinyl and AMC-myristoyl peptides for SIRT5-6 assays) and fluorophores [for example 4-(dimethylaminoazo)benzene-4-

carboxylic acid (DABCYL), 5-(2-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS)] or combination of FRET pairs (Li et al., 2015). In terms of a high-throughput format, the fluorescence-based assays have the advantage to be easily miniaturized and automated. However, since these assays are indirect methods coupling two enzymatic reactions, they can generate false positives. For examples, the use of a fluorogenic assay exploiting AMC-peptide substrates has previously became the cause of false positive hits, thus brining the controversial on the effects of sirtuin activators (resveratrol and its analogues) (Borra et al., 2005; Pacholec et al., 2010).

In the light of this, a careful selection and a combinatory application of appropriate assays is necessary to achieve reliable results. In the **Article 6** a study aimed at developing a new approach for assessment of enzymatic activity in biological samples, based on combined use of different enzymatic assays and the comparative measurement of purified commercial enzyme (human SIRT1 isoform) is described. Since the only well-characterized plant sirtuin is a mitochondrial isoenzyme showing a key role in regulating mitochondrial metabolism (König et al., 2014), the new approach was applied to study sirtuin activity within the isolated durum wheat mitochondria (WM) fraction; moreover, the effect of two polyphenols, such as resveratrol and quercetin, was evaluated on WM-sirtuin activity.

Section III-Aims of the research

The objective of this research was to evaluate potential health value and biological effects of food antioxidants by using innovative methodological approaches. This was achieved by three different levels of investigation.

1.1 First level of study: determination of AC of foods by using advanced methodologies

The first level concerns the determination of the AC of plant foodstuffs. To date, a large number of analytical methods for *in vitro* AC assessment of foods has been developed; some of these assays are simple, rapid and inexpensive, so they have been extensively applied. On the other hand, it should be outlined that *in vitro* AC measurement show many technical and conceptual limitations. A technical limitation may derive from the preliminary extraction of antioxidants from food matrices that is required before AC measurement by the majority of AC assays. In fact, the use of different extraction procedures, showing different efficiency and recovery of antioxidants, can represent an important source of variation in the reported AC values. This limits a proper inter-laboratory comparison of data and it can explain many discrepancies among independent studies.

In the light of this, the **Article 1** reports a study aimed to extend the potentiality of a new procedure for AC assessment of food matrices avoiding any extraction and hydrolysis step. This new approach, abbreviated as QUENCHER$_{ABTS}$ (QUick, Easy, New, CHEap and Reproducible), involves a direct reaction of food solid particles with ABTS radical cation (Serpen et al., 2008). However, the QUENCHER procedure has been generally applied to finely ground food solid particles having generally a diameter not exceeding 0.2-0.3 mm. So, the goal of this study was to improve the range of applicability of the QUENCHER$_{ABTS}$ protocol, since some food matrices of interest may be produced and used as larger size particles.

Another important limit of the most commonly used assay is that they do not measure an actual total AC, because they highlight only one or a few possible mechanisms of antioxidant protection against oxidative damage. In fact, they are able to measure only the capacity of antioxidant compounds to break the radical propagation chain by hydrogen atom or electron donation or to reduce oxidant species, failing to detect also the capacity of chelating or reducing metal ions involved in radical generation and of inhibiting pro-oxidant enzymes. Furthermore, according to the majority of the published assays, measurements are carried out under experimental conditions not resembling the physiological ones, by using in most cases non-physiological radical species.

To overcome these problems, an innovative assay for AC assessment, indicated as LOX/RNO (Pastore et al., 2009), was developed by our research group; it is based on the use of the isoform 1

of soybean lipoxygenase (LOX-1) as a system to generate different physiological radicals and of RNO as a probe to detect these radicals. Compared to other AC assays, LOX/RNO method may provide a more comprehensive and integrated AC determination of food antioxidants, since it is able to simultaneously detect many antioxidant functions as well as synergistic interactions among antioxidants (Pastore et al., 2009; Laus et al., 2012b; 2013b). An aspect of the research of this thesis has regarded the development of another advanced LOX-1-based assay, which derives from LOX/RNO method but uses as a probe the highly sensitive fluorescein, similarly to ORAC assay. It is named LOX-FL method and described in detail in the **Article 2**. In particular, performance of LOX-FL assay was evaluated with respect to AC measurement of extracts obtained from the antioxidant-rich dietary wheat grain supplement Lisosan G and of human serum after Lisosan G intake and compared to that of the widely used ORAC and TEAC methods.

1.2 Second level of study: determination of human blood antioxidant status after food intake

As already reported (see **Section I**), *in vitro* AC measurements of foods alone have not been demonstrated to be relevant for the biological effects of specific bioactive compounds (Fraga et al., 2014; Huang et al., 2005), since they do not provide information about bioavailability of food antioxidants, as well as their *in vivo* stability, retention by tissues and *in situ* reactivity. With respect to AC analysis of foods, a more physiologically relevant information about potential health effects of foods may be provided by *ex vivo* study of blood antioxidant status after food intake. Assessment of serum/plasma AC after antioxidant consumption may provide a more integrated information, taking into account antioxidant bioavailability and metabolism. Unfortunately, also this approach shows some weakness, leading to some controversial results (Fernández-Pachón et al., 2008; Lettieri-Barbato et al., 2013).

In an attempt to overcome these literature inconsistencies, we proposed a new approach considering simultaneously the effect of food intake on both AC and oxidation level of serum. To this aim, a novel parameter for measuring serum antioxidant status was developed, indicated as Antioxidant/Oxidant Balance (AOB). AOB represents the ratio between the serum AC and an indicator of the serum oxidation level, that we named Peroxide Level (PxL). In the **Article 3**, effectiveness and reliability of the novel AOB approach in highlighting changes in serum antioxidant status after food intake was assessed by evaluating the effects of two antioxidant-supplemented pastas during four hours after their consumption. Results were compared with that obtained with the dietary supplement Lisosan G and glucose, able to show high AC or to induce pro-oxidant effects, respectively.

1.3 Third level of study: effects of food antioxidants at cellular/sub-cellular level

A third advanced level of investigation of CA of dietary antioxidants was also proposed, concerning the evaluation of effects exerted by bioactive compounds at cellular and sub-cellular levels. In particular, biological effects of some phytochemicals were evaluated on activity of *i*) Glyoxalase I, an enzyme involved in the defence against carbonyl stress and *ii*) Sirtuins, NAD^+-dependent deacetylases having a key role in a wide variety of cellular processes.

Concerning GloI, two studies were carried out. In the **Article 4** the effect of sulforaphane (SR), a compound abundant in *Brassica* vegetables, on both GloI gene expression and activity in peripheral blood mononuclear cells (PBMCs) was investigated. PBMCs were chosen because they are human primary cell models, so they may represent a good model to estimate possible *in vivo* effects of SR. For this purpose, PBMCs isolated from human volunteers were incubated with 2.5 μM SR for 24 h and 48 h to simulate a daily consumption of a broccoli portion and GloI activity/expression were analysed.

In the **Article 5**, the possible modulation of a plant GloI activity by phytochemicals was studied. For this purpose, the effects of some polyphenols, including curcumin, quercetin and resveratrol, as well as the most relevant antioxidants of durum wheat grains such as ferulic acid, sinapic acid and α- tocopherol (Laus et al., 2012b), were evaluated on GloI activity in the highly purified durum wheat mitochondrial (WM) fraction. The study was carried out on a mitochondrial GloI, because this subcellular organelle is one of the main targets of carbonylation stress (Li, 2016 and references therein).

As for sirtuins, a study was carried out (**Article 6**) aimed at developing a new approach for assessment of enzymatic activity in biological samples, based on combined use of different enzymatic assays and the comparative measurement of purified commercial enzyme (human SIRT1 isoform). Since the only well-characterized plant sirtuin is a mitochondrial isoenzyme showing a key role in regulating mitochondrial metabolism (König et al., 2014), the new approach was applied to study sirtuin activity within WM fraction; moreover, the effect of two polyphenols, such as resveratrol and quercetin, was evaluated on WM sirtuin activity.

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Article 1:

Antioxidant capacity of durum wheat large flour particles may be evaluated by QUENCHER$_{ABTS}$ assay by adopting a proper calculation mode

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Abstract

Assessment of Antioxidant Capacity (AC) of foods is useful to consider cumulative/synergistic action of all dietary antioxidants, thus providing a more integrated information than the simple sum of measurable antioxidants. Among the different AC assays, the QUENCHER$_{ABTS}$ (QUick, Easy, New, CHEap and Reproducible) procedure is based on the direct reaction of ABTS$^{\bullet+}$ reagent with fine solid food particles without extraction of antioxidants. This assay is able to measure both soluble and insoluble antioxidants, that simultaneously come into contact with ABTS$^{\bullet+}$ molecules by either liquid-liquid or solid-liquid interactions, respectively. These interactions may change depending on the particle diameter. Usually, particles having 0.1-0.3 mm size are used. Here, AC was evaluated on whole flour (WF), derived from a mix of grains of ten durum wheat varieties, characterized by three different particle sizes: a smaller one, ≤0.2 mm (control, WF$_{0.2}$), and two larger ones, ≤0.5 mm and ≤1 mm (WF$_{0.5}$ and WF$_1$, respectively). Moreover, a novel AC calculation procedure based on the slope value of the regression line of ABTS$^{\bullet+}$ response *vs* flour amount is presented in detail.

The classical QUENCHER$_{ABTS}$ procedure provided for WF$_{0.2}$ an AC value of 42.0±2.7 μmol eq. Trolox/g d.w. A similar result was obtained for WF$_{0.5}$ (38.3±0.9 μmol eq. Trolox/g d.w.), thus indicating that these large particles may be analyzed by the QUENCHER$_{ABTS}$ assay provided that the "slope" calculation procedure is used. On the contrary, WF$_1$ showed about half AC (20.3±0.2μmol eq. Trolox/g d.w.), thus showing that very large particles cannot be used even adopting the "slope" calculation.

Running title: Particle size and QUENCHER$_{ABTS}$ in wheat flour.

Keywords: Antioxidant capacity; QUENCHER$_{ABTS}$; durum wheat grains; particle size.

Abbreviations: ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); AC, Antioxidant Capacity; d.w., dry weight; f.w., fresh weight; QUENCHER, QUick, Easy, New, CHEap and Reproducible; Trolox, ±6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; WF, Whole Flour.

1. Introduction

To date, a large diversity of analytical methods for *in vitro* measurement of Antioxidant Capacity (AC) of foods is available. These assays differ from each other mainly in terms of chemical mechanism/s involved, and oxidant species/probe(s) and techniques used to monitor the reaction (Magalhaes et al., 2008; Carocho et al., 2013). Moreover, some assays provide results of questionable physiological relevance and often not related to individual dietary antioxidants or to different phytochemicals synergically acting. These aspects are discussed in Pastore et al. (2009) and Laus et al. (2012; 2013). From a methodological point of view, most of the AC assays used up to now requires the preliminary extraction of antioxidants from food matrices before AC measurement. The use of different extraction procedures, showing different efficiency and recovery of antioxidants, is an important source of variation in the reported AC values. This limits a proper inter-laboratory comparison of data and it can explain many discrepancies among independent studies (Serpen et al., 2007; 2008; Gökmen et al., 2009).

During recent years, a new simple and direct procedure for AC assessment of food matrices avoiding any extraction and hydrolysis step has been proposed (Serpen et al., 2008). It involves a direct reaction of food solid particles with the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation, followed by centrifugation to obtain an optically clear supernatant for absorbance measurement at 734 nm. The new approach, abbreviated as "QUENCHER$_{ABTS}$" (QUick, Easy, New, CHEap and Reproducible), takes advantage from liquid-liquid interactions occurring in the solvent between soluble antioxidants and $ABTS^{\bullet+}$ molecules, and it may also assess AC of antioxidants bound to insoluble matter by surface solid-liquid interactions (Serpen et al., 2007; 2008; Gökmen et al., 2009). This direct procedure, avoiding any pretreatment of food samples, may give AC values generally higher than that obtained by different extraction and hydrolysis procedures used for comparison (Serpen et al., 2008). Moreover, these values are potentially related to a true antioxidant action in food or in human gastrointestinal trait (Serpen et al., 2008; Gökmen et al., 2009), thus showing possible physiological relevance of results. At this regard, only complex *in vitro* enzymatic digestion designed to mimic digestion in the gastrointestinal tract may be more related to physiological effects (Gong et al., 2013). The QUENCHER procedure has been successfully applied to measure AC of very different foods (Serpen et al., 2008; 2009; 2012a,b,c; Acar et al., 2009; Ciesarova et al., 2009; Rufian-Henares et al., 2009; Amigo-Benavent et al., 2010; Delgado-Andrade et al., 2010; Žilić et al., 2012; 2013). Moreover, the QUENCHER assay has been successfully performed also replacing $ABTS^{\bullet+}$ as radical probe with DPPH (Gökmen et al., 2009; Serpen et al., 2012a,b,c), as well as using the colour generation of FRAP (Serpen et al., 2012a,b) and CUPRAC reagents (Tufan et al., 2013) or

fluorescence as in ORAC protocol (Amigo-Benavent et al., 2010; Kraujalis et al., 2013).
Obviously, in this approach, total surface area and solid food particle sizes may play a crucial role in determining the reaction rate and, in turn, the measured AC (Gökmen et al., 2009). To date, the QUENCHER procedure has been generally applied to finely ground food solid particles having generally a diameter not exceeding 0.2-0.3 mm. Nevertheless, some food matrices of interest may be produced and used as larger size particles. At this purpose, industrial milling process of wheat grains generates by-products showing different particle size distribution mainly dependent on milling process and grain hardness (Devaux et al., 1998). Large particle by-products may display special properties. As for wheat bran, the addition of bran having a larger particle size (≥0.5 mm) has been reported to positively influence technological performance, as well as quality and sensory characteristics of some integrated fiber-rich foods (Noort et al., 2010; Chen et al., 2011). Moreover, large particle-bran has been also shown to induce a greater acetate production in an *in vitro* fermentation system (Stewart et al., 2009) and to influence the extent of starch retrogradation and in turn starch digestibility in bran-enriched bread during storage (Cai et al., 2014), so suggesting that large particles may exert physiological effects. In the light of this, AC determination of large particles may have a physiological interest.
So, the goal of this study was to test the applicability of the $QUENCHER_{ABTS}$ protocol to solid particles having large size. This was performed by analyzing whole flour (WF) derived from a mix of grains of ten durum wheat (*Triticum turgidum* L. subsp. *durum*) varieties, characterized by three different particle sizes: a smaller one (≤0.2 mm, control, $WF_{0.2}$) and two higher ones (≤0.5 mm and ≤1 mm, $WF_{0.5}$ and WF_1, respectively), larger than that so far reported in literature. To do this, a novel AC calculation procedure based on the slope value of the regression line of $ABTS^{\bullet+}$ response *vs* flour amount was used for $WF_{0.5}$ and WF_1 and results were compared with that obtained for $WF_{0.2}$ using classical procedure.

2. Material and Methods

2.1 Chemicals

ABTS, ethanol, potassium persulfate, ±-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Corp. (St. Louis, Mo., U.S.A.).

2.2 Plant material

Grain samples from ten durum wheat varieties (Quadrato, Torrebianca, Pietrafitta, Vendetta, Alemanno, Principe, Cannavaro, Gattuso, Simeto and Duilio) were stored under vacuum at 4°C for no longer than 2 months. Before use, a balanced mix of whole grains were daily milled by

means of a Cyclotec 1093 Sample Mill (using 1 mm or 0.5 mm sieves). Ground samples were passed through 1 mm, 0.5 mm and 0.2 mm certified test sieves (Giuliani Tecnologie, Turin, Italy) to obtain WF_1 (as not passing through 0.5 mm sieve), $WF_{0.5}$ (as not passing through 0.2 mm sieve) and $WF_{0.2}$ (as passing through 0.2 mm sieve), respectively.

2.3 Determination of AC by the direct QUENCHER$_{ABTS}$ procedure

The $ABTS^{\bullet+}$ radical cation was generated by chemical oxidation with potassium persulfate as described by Re *et al.* (1999) and then diluted in a mixture of ethanol:water (50:50, v/v) to obtain an absorbance value at 734 nm (A_{734}) of 0.70±0.02. Measurements were carried out in triplicate by adding the $ABTS^{\bullet+}$ diluted solution with flour sample and vigorously stirring the suspension to facilitate a surface reaction between the solid particles and the $ABTS^{\bullet+}$ reagent. Then, optically clear supernatant obtained after centrifugation at 9200x*g* for 2 min was used to measure A_{734}. The (%) decrease of A_{734} measured after sample incubation (A_f) with respect to A_{734} of $ABTS^{\bullet+}$ solution (A_0) was calculated by the following equation: (%) decrease of $A_{734}=[1-(A_f/A_0)]x100$. To develop a properly adapted protocol for $WF_{0.5}$ and WF_1, different reaction times (ranging from 5 to 300 min for WF_1 and from 5 to 270 min for $WF_{0.5}$) and whole flour amount (ranging from 0.40 to 1.66 mg fresh weight, f.w./mL of $ABTS^{\bullet+}$ solution for WF_1 and from 0.10 to 1.60 mg f.w./mL for $WF_{0.5}$) were analyzed. Note that, depending on the particle size, the interactions between $ABTS^{\bullet+}$ molecules and insoluble-bound antioxidants are different. As a consequence, different flour amounts were necessary to assure AC values giving linearity of response. This linear dependence of the (%) decrease of A_{734} on sample amount was verified for each incubation time by linear regression analysis of data. AC was obtained by comparing the slope derived by linear regression analysis with that of the Trolox-derived calibration curve. As for $WF_{0.2}$, measurements were carried out for 60 min and using flour amount ranging from 0.10 to 0.35 mg f.w./mL of $ABTS^{\bullet+}$. In this case, AC was classically calculated by linear interpolation of data.

3. Results

Firstly, the QUENCHER$_{ABTS}$ procedure was applied to $WF_{0.2}$ particles, having a diameter ≤0.2 mm, that is included in the 0.1-0.3 mm range generally used in literature. In **Fig. 1** the linear dependence of (%) decrease of A_{734} on the ratio between $WF_{0.2}$ amount and volume of $ABTS^{\bullet+}$ solution is shown. The straight line obtained by linear regression analysis of $WF_{0.2}$ data, as well as the calibration curve obtained with Trolox, showed *y*-axis intercepts very close to zero. Linear interpolation of $WF_{0.2}$ data into Trolox calibration curve provided an AC value for $WF_{0.2}$ of 42.0±2.7 μmol eq. Trolox/g of dry weight, d.w.

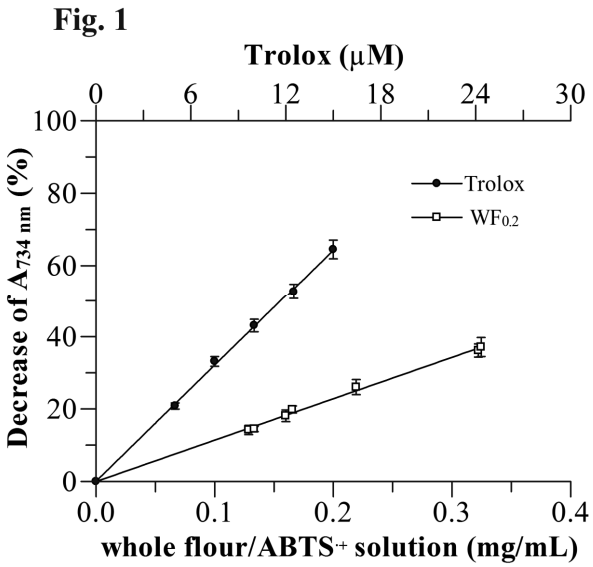

Fig. 1 *Dependence of the absorbance decrease (%), measured at 734 nm by using the QUENCHER$_{ABTS}$ assay, on the amount of durum wheat whole flour (≤0.2 mm particle size, WF$_{0.2}$).* Measurements were carried out as described in Methods, after 60 min incubation of ABTS$^{•+}$ diluted solution with different amounts (f.w.) of WF$_{0.2}$ mix. The straight line obtained by linear regression analysis of data relative to WF$_{0.2}$ mix is reported. Calibration curve obtained with Trolox is also shown. Data are reported as mean value ± SD (n=3 different experiments).

In order to test the applicability of the QUENCHER$_{ABTS}$ procedure to solid particles having diameter higher than 0.2 mm, possible methodological adaptation of classical QUENCHER$_{ABTS}$ assay was evaluated, with particular attention to both reaction time and flour amount. So, both WF$_{0.5}$ and WF$_1$ particles were analyzed by carrying out the reactions for increasing incubation times and using increasing amounts of flour sample (see also Methods). Results relative to both WF$_{0.5}$ and WF$_1$ are shown in **Fig. 2**. In particular, in **Figs. 2a** and **d** the profile of (%) decrease of A$_{734}$ *vs* the incubation time is reported for each tested amount of WF$_{0.5}$ and WF$_1$, respectively.

The profile relative to the ABTS$^{•+}$ diluted solution in the absence of sample is also shown. An undesired complete bleaching of the ABTS$^{•+}$ reagent (100% decrease of A$_{734nm}$) was observed in the presence of both 1.60 mg WF$_{0.5}$/mL and 1.66 mg WF$_1$/mL, thus indicating that ABTS$^{•+}$ molecules are limiting with respect to the sample.

This saturation response is a condition that prevents AC determination. However, using lower flour amounts, ABTS$^{•+}$ quenching curves were obtained tending to a different plateau below saturation, so allowing AC quantification. To define the most appropriate measurement condition, data of **Figs. 2a** and **d** were plotted as (%) decrease of A$_{734}$ *vs* the flour amount/ABTS$^{•+}$ volume ratio (**Figs. 2b** and **e**). In both cases, two distinct flour amount ranges were found, in which a different relationship between WF amount and (%) decrease of A$_{734}$ was observed (ranges I and II).

Fig. 2

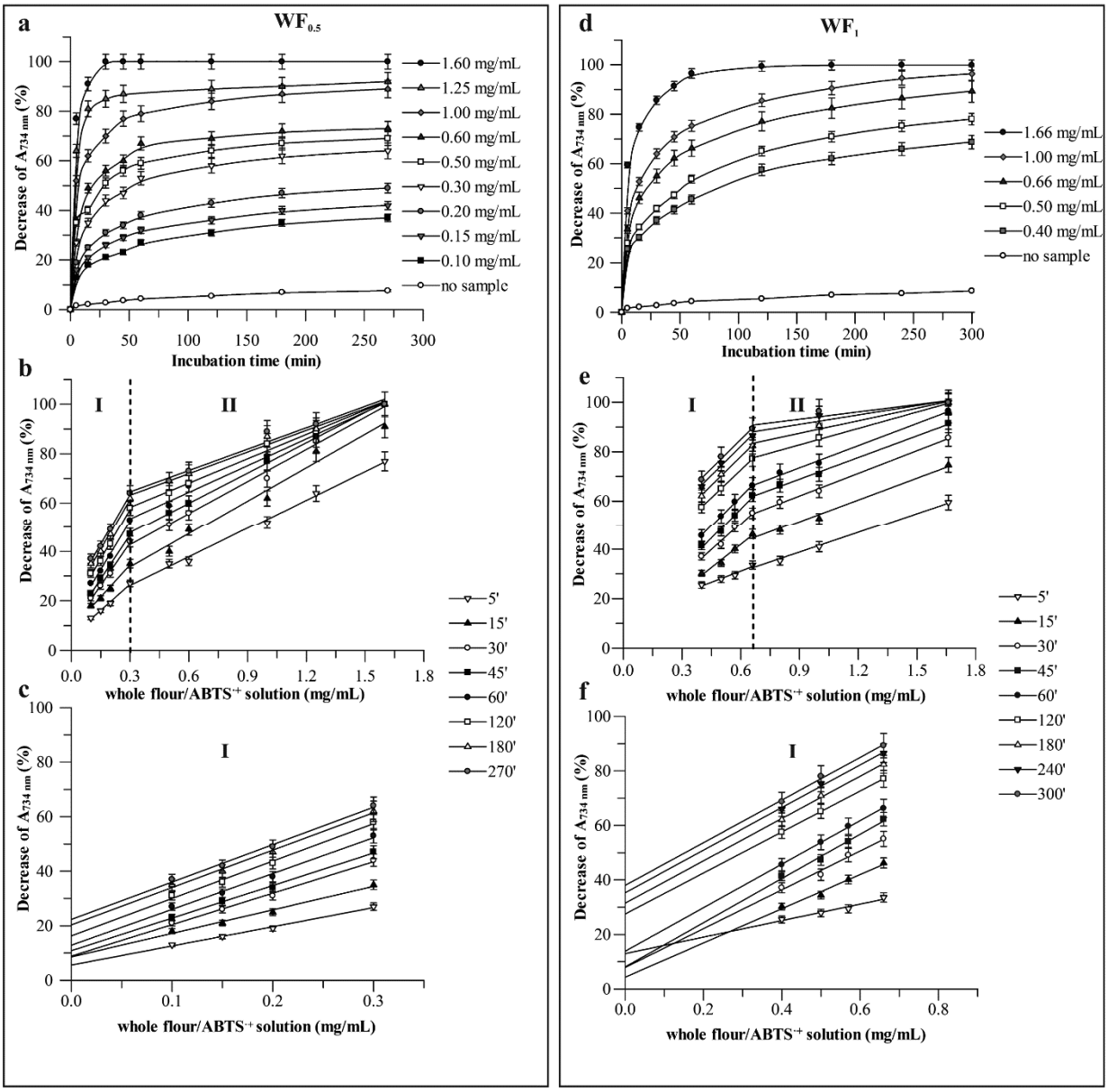

Fig. 2 *Dependence of the absorbance decrease (%), measured at 734 nm by using the QUENCHER$_{ABTS}$ assay, on the reaction time of the ABTS$^{\bullet+}$ radical cation with durum wheat whole flour (a, d) and on the amount of whole flour (b, c, e, f).* ABTS$^{\bullet+}$ diluted solution was incubated for different times with different amounts (f.w.) of either $WF_{0.5}$ or WF_1. In (**a**) and (**d**) the profiles *vs* reaction time are reported for $WF_{0.5}$ and WF_1, respectively. In (**b**) and (**e**) the straight lines obtained by two separate linear regression analyses of data in the ranges I and II (0.1-0.30 and 0.30-1.60 mg (f.w.) $WF_{0.5}$/mL and 0.40-0.66 and 0.66-1.66 mg (f.w.) WF_1/mL, respectively) are shown. In (**c**) and (**f**) the extrapolation to *y*-axis of the curves obtained by linear regression analyses of data in the range I of Fig. 2b and Fig. 2e is shown in detail. Data are reported as mean value ± SD (n=3 different experiments).

A possible explanation of this phenomenon is that the increasing of the "insoluble antioxidants/ABTS$^{\bullet+}$" ratio over a given value might result in a lowering of the reaction rate constant.

Table 1. Antioxidant Capacity (AC) evaluated by the $QUENCHER_{ABTS}$ assay of durum wheat whole flour having ≤0.5 mm ($WF_{0.5}$) and ≤1 mm (WF_1) particle sizes.

$WF_{0.5}$		
Time of incubation (min)	**Equation of straight line**	**AC* $QUENCHER_{ABTS}$**
5	y = 72.86x + 4.04	20.2 ± 0.4
15	y = 90.82x + 6.23	25.2 ± 0.6
30	y = 116.7x + 6.26	32.4 ± 0.8
45	y = 123.30x + 8.10	34.3 ± 0.7
60	y = 137.95x + 9.07	38.3 ± 0.9
120	y = 138.57x + 13.45	38.5 ± 1.2
180	y = 140.30x + 17.41	39.0 ± 1.5
270	y = 138.28x + 19.16	38.4 ± 1.3
WF_1		
Time of incubation (min)	**Equation of straight line**	**AC* $QUENCHER_{ABTS}$**
5	y = 29.60x + 13.25	7.7 ± 0.3
15	y = 61.31x + 4.96	16.0 ± 0.5
30	y = 69.51x + 8.63	18.1 ± 0.5
45	y = 79.24x + 8.90	20.7 ± 0.5
60	y = 77.86x + 14.72	20.3 ± 0.2
120	y = 73.20x + 28.36	19.1 ± 0.1
180	y = 75.54x + 32.36	19.7 ± 0.4
240	y = 76.04x + 36.21	19.8 ± 0.5
300	y = 76.09x + 38.94	19.8 ± 0.5

Equations of straight lines obtained by the linear regression analysis of data relative to $WF_{0.5}$ (Fig. 2c) and WF_1 (Fig. 2f). In the equations, *x* represents the (%) decrease of A_{734} and *y* represents whole flour amount expressed as mg f.w./mL of $ABTS^{\bullet+}$ solution. AC values were calculated by comparing the slope of each straight line with that of the Trolox calibration curve of Fig. 1, having a slope value of 4.28.

*Data are expressed as μmol eq. Trolox/g d.w. and reported as mean value ± SD (n=3 different experiments). Moisture content was 15% and 11% in the $WF_{0.5}$ and WF_1 experiments, respectively.

This effect is more pronounced as the reaction time increases, since the unreacted available $ABTS^{\bullet+}$ radical decreases. As expected, in the light of a lower diameter and higher total surface

area of $WF_{0.5}$ with respect to WF_1, the ranges I and II are different between the two particle sizes. For both $WF_{0.5}$ and WF_1, linear regression analyses of data in the range I (0.10-0.30 mg $WF_{0.5}$/mL and 0.40-0.66 mg WF_1/mL) generated straight lines generally almost parallel whatever the reaction time (**Figs. 2c** and **f**). However, none of these straight lines passed through the origin of axes, but the lines of range I showed intercepts to the *y*-axis much closer to zero with respect to the regression curves obtained in the range II. Since the theoretical intercept is zero (see $WF_{0.2}$ and Trolox in **Fig. 1**), the range I has to be used to calculate AC values. Moreover, the values of *y*-axis intercepts have to be adequately subtracted in AC calculation. To do this, the equation of the linear regression curve is calculated; then, the slope value is divided by that of the Trolox calibration curve (slope value=4.28) of **Fig. 1**. The final AC value is expressed as μmol eq. Trolox and referred to grams of d.w. All calculations are reported in **Table 1**. In particular, equations of the linear regression curves are reported, obtained in the linearity range I for each incubation time using either $WF_{0.5}$ (**Fig. 2c**) or WF_1 (**Fig. 2f**). The corresponding AC values obtained by the "slope" calculation mode are also reported. The linear regression analysis of data relative to $WF_{0.5}$ generated curves with higher slopes, up to about a doubling, with respect to WF_1. Consistently, AC values obtained for $WF_{0.5}$ by the slope calculation mode resulted up to about 2-fold higher than that calculated for WF_1. For both $WF_{0.5}$ and WF_1, AC values were found to increase with increasing incubation time up to 45 or 60 min, respectively. On the contrary, AC values remained statistically equal over from 45-60 min, with the ones obtained at 60 min (38.3±0.9 for $WF_{0.5}$ and 20.3±0.2 μmol eq. Trolox/g d.w. for WF_1) affected by the lowest experimental error. On the basis of these results, the reaction time of 60 min appears to be the most suitable.

In the whole, AC value obtained for $WF_{0.5}$ using 60 min reaction time and by exploring the range 0.10-0.30 mg of flour/mL, resulted similar to the one measured for $WF_{0.2}$ (42.0±2.7 μmol eq. Trolox/g d.w.) by the classical interpolation mode, so indicating as appropriate this experimental condition for $WF_{0.5}$ measurements. On the contrary, no experimental condition useful for WF_1 analysis was identified.

4. Discussion

The $QUENCHER_{ABTS}$ method allows for AC evaluation of compounds without preliminary extraction when they are still bound to the insoluble food matrix. Recently, we have shown that in whole flour of durum wheat the $QUENCHER_{ABTS}$ assay highlights AC mainly due to bound phenols (Laus et al., 2015). So $QUENCHER_{ABTS}$ assay appears to be a useful approach to prevent some misjudgements; for example, acid hydrolysis of bound phenolic compounds may produce 5-hydroxymethyl-2furfural and derivatives, able to display AC, that may induce an incorrect AC

determination when extracts are analyzed (Chen et al., 2014).

As for AC measurements of cereal grains by using this direct procedure, it has been reported that an accurate grinding is required to obtain particles having a diameter ranging between 0.1 and 0.3 mm (Gökmen et al., 2009). Nevertheless, since the use of larger particles of some cereal milling products has been reported to exert positive effect on technological performance and quality of some derived foods (Noort et al., 2010; Chen et al., 2011), as well as to induce some physiological effects (Stewart et al., 2009; Cai et al., 2014), the study of AC of larger particles may be of interest. Here, we show that adopting little changes with respect to the original QUENCHER$_{ABTS}$ method, it is possible to analyze large particles up to 0.5 mm, without mistakes in measured AC values. This result extends the findings of Serpen et al. (2008), who found no relevant changes (within 20%) in AC value in the 0.105-0.177 mm particle size range. In particular, the observed feature of large particles to generate straight lines that do not pass through origin appears to be a characteristic of the QUENCHER$_{ABTS}$ procedure. Under these conditions, the calculation procedure based on comparison between slopes is strongly advisable to avoid AC overestimation, while classical AC measurement through interpolation would lead to incorrect values. So, our QUENCHER$_{ABTS}$ approach extends the potentiality of the QUENCHER procedure and improves its range of applicability. On the contrary, as expected in the light of the strong total surface area/volume ratio decrease due to the doubling of particle size, the AC measured was much lower when particles up to 1 mm were analyzed. So, to avoid AC underestimation, analysis of 1 mm-particles by the QUENCHER procedure should be avoided. On the whole, when comparing literature AC data obtained by means of the QUENCHER procedure, it is very important to know the adopted experimental conditions with particular attention to particle size.

5. Conclusions

In conclusion, by adopting the novel mode of calculation based on the slope value of the regression line of $ABTS^{\bullet+}$ response *vs* flour amount, it is possible to use the QUENCHER$_{ABTS}$ method to measure, without alteration of results, AC of particles having larger size (up to 0.5 mm) than that believed so far. This finding extends the applicability of the method. However, whatever the calculation mode, very large particles having 1 mm size cannot be analyzed by the QUENCHER$_{ABTS}$ method.

6. Acknowledgements

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Article 2:

The soybean Lipoxygenase-Fluorescein reaction may be used to assess antioxidant capacity of phytochemicals and serum

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Abstract

Evaluation of putative benefic effects of food antioxidants by *in vitro* assays of Antioxidant Capacity (AC) of food extracts sometimes appears questionable. A more realistic evaluation of antioxidant effectiveness may derive from integration of *in vitro* assays with *ex vivo* assays of blood/serum/plasma AC after food intake. The aim of this work was to develop the novel Lipoxygenase-Fluorescein (LOX-FL) method advisable to assay AC of both food extracts and serum/plasma. This method was applied on both extracts from the antioxidant-rich dietary cereal supplement Lisosan G and serum (seven subjects) during 240 min after intake of 20 g of supplement. The widely used ORAC and TEAC methods were used for comparison. The new LOX-FL method is based on the reaction between soybean LOX-1 isoenzyme and FL in the presence of linoleic acid, that undergoes hydroperoxidation thus generating physiological reactive species including LOO·, LO·, HO· and $^1O_2$, able to quench FL. The quenching rate is slowed by antioxidants; this inhibition may be calibrated in terms of Trolox equivalents, so assessing AC. Interestingly, LOX-FL method discriminated *in vitro* AC of four different Lisosan G extracts similarly to ORAC and TEAC methods. Contrarily, only LOX-FL method was able to highlight a general increase of serum AC (up to 40% after 30 min) after Lisosan G intake, thus confirming its physiological effectiveness by *ex vivo* serum assay. This performance of LOX-FL method probably comes from the ability to highlight simultaneously different antioxidant mechanisms and to show very well synergy among food phenols and serum endogenous antioxidants.

1. Introduction

A direct measurement of Antioxidant Capacity (AC) of antioxidant-rich foodstuffs, in particular, fruits, vegetables, whole cereal grains, wine, tea and chocolate, has been the subject of a very large number of articles in the last years. AC has been evaluated by a lot of different *in vitro* systems (ORAC, TEAC, TRAP, FRAP, DPPH etc.).[1–3] The interest for these measurements derives from the idea that food AC is related to prevention of diseases. Consistently, recent population studies found significant associations between AC of the food consumed (obtained from food-frequency questionnaires) and healthy effects including positive effects on chronic diseases, several tumours as well as lower risk of heart failure and strokes.[4–7] On the other hand, methods for AC measurement show many technical and conceptual limitations. The technical trouble is mostly related to the chemistry behind the different AC assays making difficult to compare AC values obtained using different methods and different experimental conditions.[2] By a conceptual point of view, an important limitation is that the values indicating *in vitro* AC have not been demonstrated to be relevant for the biological effects of specific bioactive compounds.[8] Moreover, these assays do not measure bioavailability, *in vivo* stability, retention of antioxidants by tissues, and reactivity *in situ*. Finally, there is growing evidence that the metabolic pathways associated to the prevention or amelioration of chronic diseases by bioactive compounds is often dependent on enzyme/protein and/or gene expression regulation rather than by a true antioxidant effect.[9,10] In the light of these considerations suggesting that AC values of foods have no relevance to the effects of specific bioactive compounds on human health, the U.S. Department of Agriculture (USDA) removed its ORAC database for selected foods in 2012 from its nutrient data laboratory website.[11] However, this choice is not fully shared and remains to a certain extent questionable.[12]

A possible different, more physiological approach may be to move the focus from the analysis of AC of foods to the analysis of AC of serum/plasma after food intake. This measure may take into account bioavailability and metabolism, so giving an integrated information of a true effect on blood antioxidant status, which is beyond the original AC of the ingested food. In addition, the assessment of the antioxidant status of blood may be *per se* an important target. In fact, blood plays a central role in the homeostasis of cellular redox status by carrying and releasing antioxidants in the body; moreover, the maintenance of blood physiological antioxidant status may preserve endothelial function, which is thought to be an essential determinant of healthy aging.[13] Given the simplicity of AC assays and the easy access to human blood, AC measurements of serum/plasma flourished in the last years generating a large amount of data.[14] Unfortunately, in short-term studies (minutes or hours after ingestion), foods very rich in antioxidants often induced

limited AC increase.[15] These results may be, at least in part, dependent on some weakness of analytical methods. For example, TEAC is less performing than other methods.[14] Therefore, in this paper, we describe the use of the novel well performing Lipoxygenase (LOX)-Fluorescein (FL) method. It essentially derives from the LOX/4-nitroso-*N,N*-dimethylaniline (LOX/RNO) method, that is advisable on food extracts,[16–18] but shows low sensitivity towards serum (unpublished data). Similarly to the LOX/RNO method, the LOX-FL assay utilizes the soybean LOX-1 reaction with linoleate to generate linoleate hydroperoxide (LOOH) and several physiological reactive species, mainly the linoleate alkoxyl (LO·) and peroxyl (LOO·) derivatives, HO· and $^1O_2$,[19] able to cause the quenching of FL.

Here, we compared the LOX-FL method with the widely used ORAC and TEAC methods to measure AC of food extracts from the antioxidant-rich dietary wheat grain supplement Lisosan G and of serum after Lisosan G intake. Lisosan G was chosen in the light of its well-documented bioactivity, including the protective role on carbon tetrachloride- and cisplatin-induced toxicity in rat tissues,[20] the induction of antioxidant and detoxifying systems in rat hepatocytes,[21] the antimutagenic and antioxidant activity in *Saccharomyces cerevisiae*[22] and the improvement of human Endothelial Progenitor Cell (EPC) function.[23]

Interestingly, LOX-FL method, similarly to ORAC and TEAC methods, was found to be very suitable to assess AC in hydrophilic and phenolic extracts of Lisosan G and able to discriminate among the different extracts. On the other hand, only the new method was able to evaluate strong synergistic effects among phenols and serum and to highlight very well an increase of serum AC after Lisosan G intake. So, the LOX-FL method may be successfully applied to both *in vitro* analysis of food extracts and *ex vivo* analysis of serum.

2. Material and Methods

2.1 Chemicals

All reagents at the highest commercially available purity were purchased from SIGMA Chemical Co. (St. Louis, MO, USA). 3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one (fluorescein, FL), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were dissolved in different media, depending on the assay used to determine AC. 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbate, bilirubin, urate, albumin were dissolved in deionized water. An ammonium sulfate suspension of soybean LOX type V (LOX-1 isoenzyme, E.C. 1.13.11.12) was

used, properly diluted with 100 mM Na-borate buffer, pH 9.0. The linoleate solution was prepared and assayed as reported in Pastore et al.[24]

2.2 Antioxidant-enriched food

Lisosan G, an antioxidant-enriched wheat grain preparation produced by Agrisan Company (Larciano, PT, Italy), was kindly provided by Dr Vincenzo Longo from "Istituto di Biologia e Biotecnologia Agraria" (CNR, Pisa, Italy). It is registered by the Italian Ministry of Health as a dietary supplement. In the production process, the bran and germ are separated, collected, mixed with water and inoculated with selected microbic starting cultures, typically consisting of a mix of lactobacillus and natural yeast strains. Finally, once the product is fermented, it is dried and packaged.[21]

2.3 Oxygen uptake catalyzed by soybean LOX-1

Oxygen uptake was monitored at 37 °C by means of a 5300A YSI (Yellow Spring, OH, USA) oxygraph equipped with a 5331 YSI Clark-type electrode. The reaction medium (2 mL) consisted of 100 mM Na-borate buffer, pH 9.0, 400 μM Na-linoleate, 1 μL Tween 20/μmol linoleate; the reaction was started by adding 0.5 Enzymatic Units (EU) of soybean LOX-1.

2.4 FL quenching catalyzed by soybean LOX-1

FL quenching in the course of linoleate hydroperoxidation by soybean LOX-1 was fluorimetrically monitored by measuring the FL fluorescence decrease (λex 485 nm; λem 515 nm) at 37 °C using a Perkin Elmer LS 55 fluorescence spectrometer. The reaction mixture (2 mL) contained 100 mM Na-borate buffer, pH 9.0, 400 μM Na-linoleate, 1 μL Tween 20/μmol linoleate and 6.3 nM FL; the reaction was started by adding 0.5 EU of soybean LOX-1. The rate of the reaction, expressed as Δ Arbitrary Units of Fluorescence (AUF)/s, was calculated as the highest slope to the experimental curve.

In **Fig. 1C**, FL quenching was monitored spectrophotometrically at 485 nm and 37 °C using the reaction medium above reported, with the exception of FL concentration that, in this case, ranged from 1 to 12.5 μM. A Perkin Elmer LAMBDA 45 spectrophotometer was used. Rates of reaction were expressed as ΔA_{485}/min.

2.5 Extraction and assay of antioxidant compounds

2.5.1 Extraction of hydrophilic, lipophilic and phenolic compounds from Lisosan G.

Hydrophilic extracts were prepared as described in Laus et al.[17] by extracting Lisosan G with deionized water at a (w/v) ratio equal to 1 g/5 mL in an ice-water bath for 1 h. Lipophilic compounds were extracted as in Laus et al.[17] and reconstituted in ethanol. Insoluble Bound (IB) phenolic and Free Soluble (FS) phenolic compounds from Lisosan G were extracted as reported in Laus et al.[17] and reconstituted in water. All extracts were daily used.

2.5.2 Determination of total phenols and flavonoids. Total phenols were determined using the colorimetric Folin-Ciocalteu method and quantified by means of a proper calibration on a gallic acid basis, as reported in Laus et al.[17] Flavonoids were determined as reported in Laus et al.[17] and quantified by means of a proper calibration curve on a catechin basis. Protein content in the hydrophilic extract from Lisosan G was evaluated as reported in Laus et al.[17] using bovine serum albumin as a standard.

2.6 Sera collection

Sera were obtained from seven volunteers (4 men and 3 women with age ranging from 24 to 33 years) at a laboratory of clinical analysis in agreement with ethical policy of the SAFE Department of the University of Foggia. Each volunteer provided informed written consent to participate in this study. For 2 days before analysis, the volunteers followed a low antioxidant diet, avoiding phenolic-rich foods, namely all fresh fruits and vegetables and derived products (tea, coffee, fruit juices, wine, chocolate). The working window was free from interference by different variables (physical exercise, energy expenditure, etc.).

After an overnight fast, the volunteers ingested 20 g of Lisosan G suspended in 500 mL of water. This is an amount able to induce a serum AC increase, as verified in preliminary experiments. Venous samples at baseline (T_0) and at 30-60-90-120-240 min after Lisosan G consumption were collected in vacutainers for serum collection. Sera were obtained by centrifugation (3000 x *g*, 5 min), distributed in portions and frozen at -80 °C until analysis.

2.7 AC determination by means of the LOX-FL, ORAC and TEAC methods

2.7.1 LOX-FL method. The LOX-FL reaction was carried out at 37 °C as already described for the LOX-1 dependent FL quenching, in both the absence (control) and the presence of sample (extract or serum or pure/standard antioxidant). The inhibition of the LOX-FL reaction was determined by calculating the (%) decrease of the rate of the FL quenching measured in the presence of sample (or standard antioxidant) (v_a) with respect to the control (v_c), according to the equation: Inhibition (% of the control) = $[1 - (v_a/v_c)] \cdot 100$. AC was calculated by means of a dose-response curve

obtained with Trolox by plotting the (%) decrease of the rate of FL quenching as a function of the standard antioxidant concentration. Since the rate of the LOX-FL reaction is affected by ethanol, when the lipophilic extract of Lisosan G, reconstituted in ethanol, was evaluated, a constant volume of ethanol was maintained in the assay mixture.

2.7.2 ORAC method. The ORAC protocol described in Ou et al.[25] was applied with some modifications by using a CLARIOstar - BMG Labtech microplate reader. Every working well of a 96-well plate contained the assay mixture (final volume 0.2 mL) consisting of 75 mM Na-phosphate buffer, pH 7.4, 10 nM FL and an appropriate volume of sample (extract or serum or pure/standard antioxidant). The reaction was started by adding 40 mM AAPH. FL fluorescence intensity decay was monitored at 37 °C at the excitation and emission wavelengths of 483 nm (bandwidth 14 nm) and 530 nm (bandwidth 30 nm), respectively. Since lipophilic antioxidants extracted from Lisosan G were reconstituted in ethanol, in this case a constant volume of ethanol was maintained in the assay mixture.

2.7.3 TEAC method. TEAC assay described in Re et al.[26] was applied with slight modifications as reported in Laus et al.[27] The $ABTS^{\cdot+}$ solution was diluted with 5 mM Na-phosphate buffer, pH 7.4 (or ethanol for lipophilic extract of Lisosan G).
For all three AC methods, measurements were carried out in triplicate for three different amounts of sample and AC was quantified by means of a dose-response curve obtained with Trolox.[18]

3. Results and discussion

3.1 Characterization and setting of the LOX-FL reaction as new tool to evaluate AC

A first characterization of the LOX-1-dependent FL quenching is reported in **Fig. 1**. The addition of the enzyme to the reaction mixture, containing excess of linoleate (400 μM) and limiting oxygen amount (200 μM at 37 °C, temperature of measurement), caused the disappearance of both excitation and emission FL fluorescence spectra in the Vis region (**Fig. 1A**). The time course of this reaction can be easily monitored by continuously measuring the FL fluorescence decrease (**Fig. 1B**). In this experiment, the LOX-1-mediated FL quenching (curve a) and oxygen uptake (dotted curve, b) were simultaneously monitored. It should be noted that LOX-1 addition to the reaction mixture caused a rapid oxygen consumption due to linoleate dioxygenation, whereas no simultaneous FL fluorescence change was observed. The FL quenching started about 20-25 s after enzyme addition, that is, only when a very low oxygen concentration approaching anaerobiosis was reached in the test sample. Therefore, the observed lag phase represents the time necessary to

consume oxygen during the primary aerobic LOX-1 reaction of linoleate peroxidation.[18,19] On the contrary, FL quenching is essentially a secondary anaerobic reaction. A study about kinetics of the LOX-1 dependent FL quenching was also carried out; to do this, since the high FL concentration necessary in this experiment is incompatible with fluorescence measurements, the reaction was monitored spectrophotometrically at 485 nm. By plotting the rate of FL quenching vs FL concentration, a hyperbolic relationship was obtained (**Fig. 1C**). Data were also plotted as Lineweaver-Burk (inset), Hanes, Eadie-Hofstee and Eadie-Scatchard plots (not shown). All plots were linear, thus confirming hyperbolic kinetics having Km and Vmax equal to 1.53 ± 0.08 (SD) μM and 0.28 ± 0.01 (SD) $\Delta A_{485\ nm}$/min, respectively, calculated by means of GRAFIT 5.0 software.

FL sensitivity to some relevant physiological reactive species, some of which generated in the course of LOX-1 mediated anaerobic reactions,[18,19] was investigated (not shown). In particular, FL resulted insensitive to H_2O_2 as well as to $O_2^{\cdot-}$ and LOOH, generated *via* xanthine/xanthine oxidase[28] and LOX/linoleate systems, respectively. On the contrary, FL was sensitive to LO· and HO· generated *via* Fenton reaction from LOOH or H_2O_2, respectively, in the presence of $CoCl_2$.[29] Similarly, FL quenching was also observed in the presence of $^1O_2$ generated *via* Na_2MoO_4/H_2O_2 system.[30]

In another set of experiment, the sensitivity of the FL quenching reaction to antioxidants was evaluated. Firstly, the sensitivity of the FL quenching to the standard antioxidant, Trolox, an α-tocopherol analogue with enhanced water solubility, was tested.

In **Fig. 2A**, the experimental curves of the FL quenching reactions carried out in both the absence (control, trace a) and presence of Trolox at different concentrations (10, 15 and 20 μM; traces b, c and d, respectively) are reported. Trolox was found to inhibit the FL quenching by causing a decrease of the reaction rate consistent with its antiradical activity.

In the inset of **Fig. 2A**, Trolox-dependent inhibition, expressed as (%) decrease with respect to the control trace, is reported as a function of the standard antioxidant concentration: a linear dependence of the inhibition from about 15% to 45% on Trolox concentration ranging between 5 and 20 μM was obtained (see the equation in figure caption). Interestingly, kinetic analysis highlights competitive inhibition mechanism of Trolox on the LOX-1 mediated FL quenching with a Ki value equal to about 5 μM (not shown).

This result implies that the antioxidant/oxidant interaction occurs at the level of the enzyme macromolecule rather than in the bulk phase of solution.

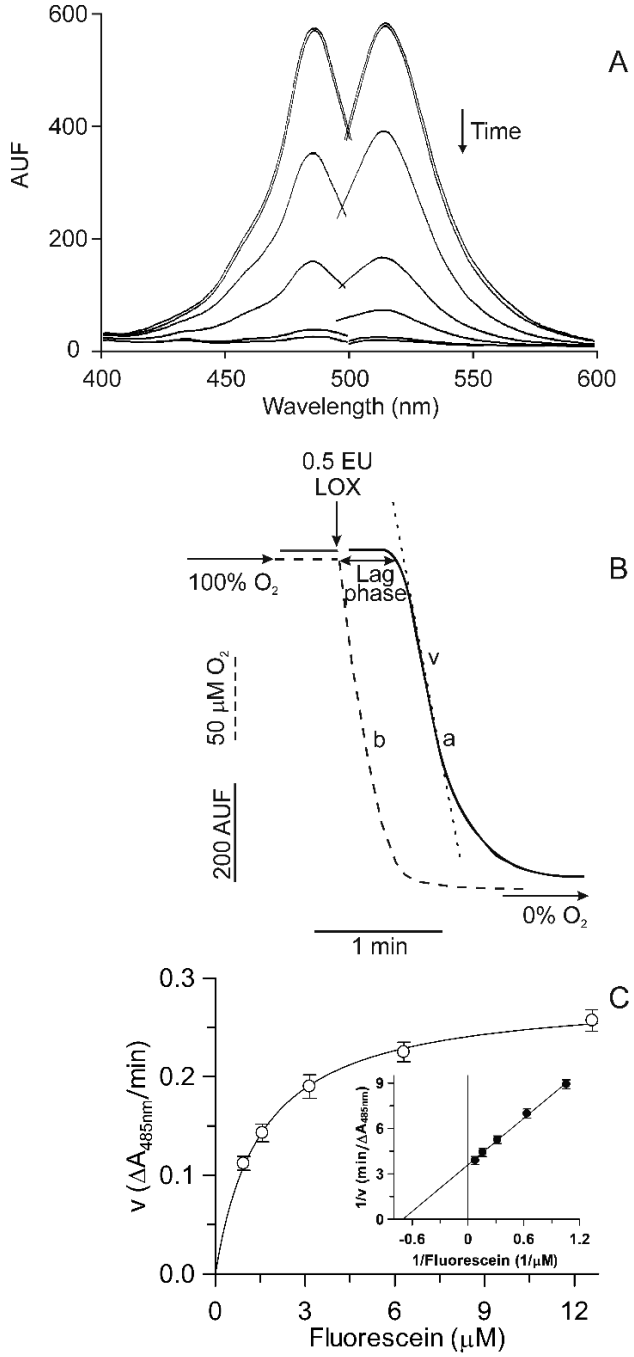

Figure 1. *Kinetics of the FL quenching catalyzed by soybean LOX-1*. The LOX-1-dependent FL quenching (A and B, trace a) was fluorimetrically monitored (λex 485 nm; λem 515 nm) at 37 °C in 2 mL of 100 mM Na-borate buffer, pH 9.0, containing 6.3 nM FL, 400 µM linoleate and 1 µL Tween 20/µmol linoleate (see Experimental Section). In (A) the reaction was started by adding 0.05 Enzymatic Units (EU) soybean LOX-1 and monitored by recording spectra every 90 s. In (B) the reaction was monitored as time course and simultaneously oxygen uptake (dotted line, curve b) was oxygraphycally measured (see Experimental Section). At time indicated by the arrow, 0.5 EU of LOX were added. The rate of the FL quenching (v) and the lag phase are also indicated. In (C), measurements were carried out spectrophotometrically at 485 nm by using the same reaction medium described in (B), but in presence of the reported FL concentrations. The rates of FL quenching, expressed as $\Delta A_{485\ nm}$/min, are reported as Michaelis-Menten and Lineweaver-Burk (inset) plots.

Since the LOX-1 mediated FL quenching reaction is sensitive to reactive species and also shows inhibition by Trolox, that may be easily quantified, we adopted this reaction as a new tool to measure AC. Interestingly, this new LOX-FL method shows several advantages.

Hyperbolic dependence of the reaction rate on FL concentration indicates that the reaction cannot occur in the bulk phase of the reaction mixture, but at the LOX surface, in particular, at the level of a definite number of FL binding sites, generating a ternary enzyme-radical-FL complex. Therefore, the effect of an antioxidant should be considered more physiological, as it refers to a reaction taking place in the body of a biological macromolecule. In addition, the LOX-FL assay evaluates the oxidant-antioxidant competition at low oxygen concentration as often occurs in human cells. Moreover, the LOX-FL method may evaluate antioxidants acting in different manner: as scavengers of different physiological radicals, including LO· and LOO· radical derivatives of linoleate, HO· as well as $^1O_2$, but also as reducing agents or chelators of the non-heme Fe^{3+} necessary for LOX catalysis or as direct inhibitors of peroxidation reaction catalysed by LOX, thus providing a comprehensive AC evaluation.[18]

A first application of the new LOX-FL method was carried out on the dietary supplement Lisosan G. The capability of the LOX-FL assay to evaluate AC in FS and IB phenols, hydrophylic and lipophilic extracts of Lisosan G was tested. In particular, in **Fig. 2B** typical experimental traces of FL quenching in the presence of FS phenols of Lisosan G are reported. Small amounts of extract (obtained from 2.5 to 10 mg dry weight, d.w., of Lisosan G) were found to inhibit the LOX-FL reaction. In this experiment, the FS phenols inhibited the rate of the FL quenching from about 20% to 50% with a linear relationship between inhibition and amount of sample (inset of **Fig. 2B**).

Comparison with linear regression of Trolox, carried out as reported in Pastore et al.,[18] gave a calculation of AC value of 4.3 ± 0.4 (SD) μmol Trolox eq./g (d.w.) (see also **Table 2**).

LOX-FL assay was tested also to evaluate AC in human serum sample. Analogously, in **Fig. 2C** typical experimental traces in the presence of serum are reported. Also in this case, small volumes of serum (ranging from 10 μL to 40 μL) were found to linearly inhibit (from 20% to 50%) the LOX-FL reaction (inset of **Fig. 2C**). In this experiment, AC value, in terms of Trolox, was 1.21 ± 0.03 (SD) μmol Trolox eq./mL serum. Interestingly, LOX-FL method may be used on both serum and plasma (not shown). However, caution has to be paid when plasma is obtained using EDTA or citrate as anticoagulants since they may chelate iron necessary to LOX activity. On the contrary, the LOX/RNO assay was found to require too high volume of both biological fluids (unpublished data).

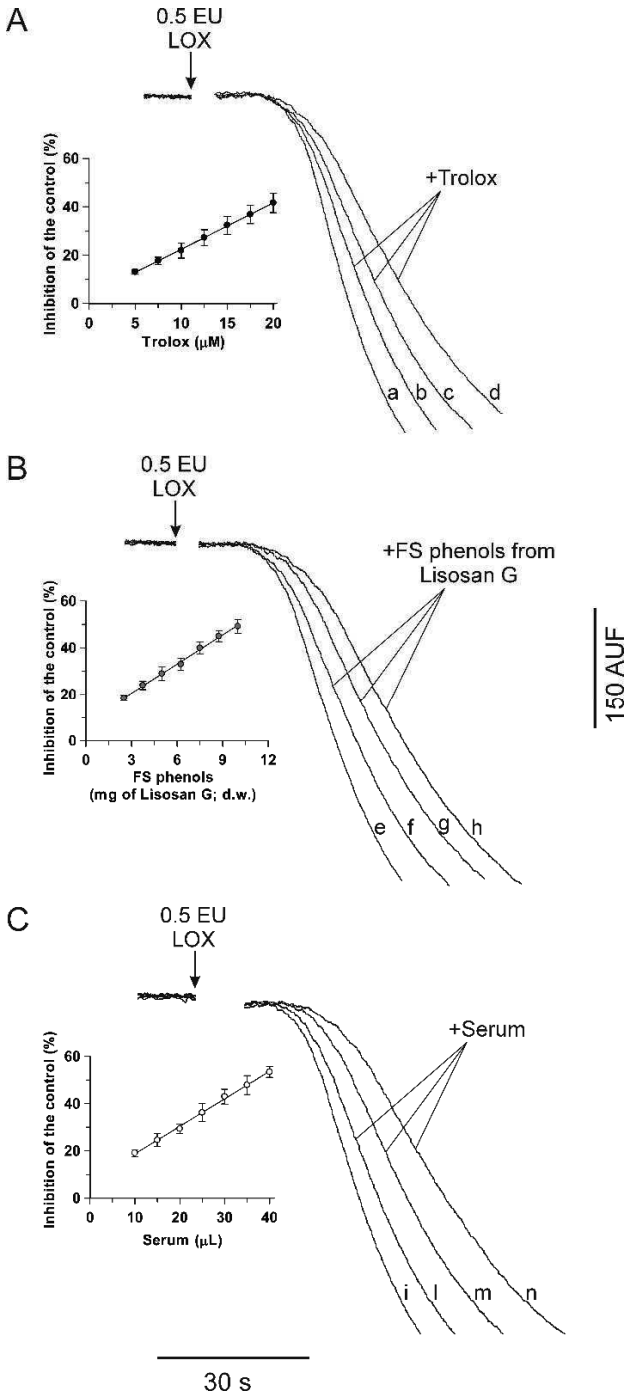

Figure 2. *Inhibition of the LOX-1-dependent FL quenching by Trolox (A), FS phenols from Lisosan G (B) and human serum (C).* The LOX-FL reaction was monitored as FL fluorescence decrease at 37 °C as reported in Fig. 1B and in Experimental section. Measurements were carried out in both the absence (control, traces a, e and i) and in presence of (A) Trolox (10, 15 and 20 μM; traces b, c and d, respectively), (B) FS phenols from Lisosan G (obtained from 5, 7.5 and 10 mg of Lisosan G, d.w.; traces f, g and h, respectively) and (C) human serum (10, 20 and 35 μL; traces l, m and n, respectively). The rates of the FL quenching, expressed as percentage decrease with respect to the control, are reported as a function of Trolox concentration (inset of Fig. 2A), FS phenols from Lisosan G (inset of Fig. 2B) and volume of serum (inset of Fig. 2C). The resulting equations are: inhibition (%) = 1.920[Trolox] + 3.23 ($r = 0.9996$, $p<0.001$) (inset of Fig. 2A); inhibition (%) = 4.154(Lisosan G) + 8.12 ($r = 0.9985$, $p<0.001$) (inset of Fig. 2B); and inhibition (%) = 1.165(serum) + 7.10 ($r = 0.9989$, $p<0.001$) (inset of Fig. 2C), where Trolox, Lisosan G and serum are expressed in μM, mg d.w. and μL, respectively.

In order to check the capability of the LOX-FL assay to evaluate AC of the main antioxidant compounds contained in serum samples, *i.e.* albumin, bilirubin, urate and ascorbate,[2,11] they were individually investigated (**Table 1**).

Table 1. Effect of different compounds on the LOX-FL and ORAC reactions.

LOX-FL		
Antioxidant	Range (μM)	IC_{50} (μM)[a]
Trolox	5 - 20	26 ± 2
Albumin	7.5 - 30	25 ± 2
Bilirubin	1.5 - 6.0	7.6 ± 0.6
Urate	10 - 25	40 ± 2
Ascorbate	150 - 1000	1360 ± 10
ORAC		
Antioxidant	Range (μM)	IC_{50} (μM)[b]
Trolox	2.5 - 10	2.5 ± 0.2
Albumin	0.075 - 0.225	0.16 ± 0.02
Bilirubin	0.15 - 0.90	2.0 ± 0.2
Urate	1 - 4	1.6 ± 0.1
Ascorbate	1.5 - 18	11.1 ± 0.1

[a]For LOX-FL, the IC_{50} values represent the antioxidant concentration able to make half the rates of the LOX-FL reaction.
[b]As for ORAC, the IC_{50} values represent the antioxidant concentration able to make double the area of the blank ORAC reaction.

Trolox was used as analogue of α-tocopherol. A comparison with the ORAC assay is also reported. All tested compounds were found to show AC, as measured by both the LOX-FL and ORAC methods. Interestingly, IC_{50} values are 4-160 fold higher for the LOX-FL than for the ORAC assay, thus indicating a general lower sensitivity of LOX-FL.

Consistently, AC values of sera measured by means the LOX-FL method show a reference interval from about 1 to 1.6 μmol Trolox eq./mL (see also caption of **Fig. 4**), about tenfold lower than that of ORAC as well as about twofold lower than TEAC. Interestingly, this ability to measure lower basal AC in serum may help to better highlight AC changes after food antioxidant ingestion. In another set of experiments, the effect due to the addition of a FS phenolic extract to serum (**Figs. 3A** and **C**) and the possible synergism among the antioxidant compounds reported in **Table 1**

(**Figs. 3B** and **D**) were also evaluated. Once again, LOX-FL and ORAC were compared. In each experiment, AC of each sample was measured and the sum of the AC values of all tested compounds was calculated (AC_{Sum}). Then, the same samples were mixed and AC was determined again (AC_{Mix}).

In the experiment of **Fig. 3A**, two equal amounts (0.5 mL) of serum and FS phenols were mixed. For the resultant mixture (1 mL) an AC_{Mix} value of 4.41 ± 0.01 μmol Trolox eq., 124% higher than the AC_{Sum} (1.97 ± 0.08 μmol Trolox eq.), was measured. In **Fig. 3B**, to mimic serum composition,[31] the mix was a solution (1 mL) containing 250 μM urate, 50 μM ascorbate, 10 μM bilirubin, 600 μM albumin and 30 μM Trolox.

This last was used instead of α-tocopherol due to its solubility in aqueous solution. Interestingly, an AC_{Mix} value equal to 1.48 ± 0.05 (SD) μmol Trolox eq. was obtained, 74% higher than AC_{Sum} (0.85 ± 0.05 μmol Trolox eq.).

By using the same approach, low synergism (16% increase, **Fig. 3C**) or no significant synergistic effect (**Fig. 3D**) was observed using the ORAC method. Therefore, the LOX-FL assay may highlight the synergistic effect among food and serum antioxidants as well as among serum antioxidants better than ORAC.

This is consistent with the ability of the LOX-FL reaction to generate, as reported above, more than one oxidant species having relevant physiological significance and to evaluate simultaneously different antioxidant mechanisms. These findings are in accordance with the ability of the LOX/RNO method to highlight very well antioxidant synergisms.[17,18]

An interesting result coming from **Table 1** and **Fig. 3** is the major role exerted by albumin as serum antioxidant, whatever the AC method used for the assay; this is in good agreement with previous literature findings.[31]

3.2 Ability of the new LOX-FL method to evaluate both AC of food extracts (in vitro measurements) and the effect of food intake on the AC of serum (ex vivo measurements).

To better investigate the ability of the new LOX-FL method to measure *in vitro* AC of food extracts, three different antioxidant extracts from Lisosan G were investigated in some detail. AC was also evaluated by means of the ORAC and TEAC methods (**Table 2**).

As expected, depending on the different chemistry of the assays, the absolute AC values measured by the different methods are very different each other, but all three methods measured the highest AC values in the hydrophilic extract of Lisosan G, probably mainly attributable to proteins, as already demonstrated for wheat grain.[17]

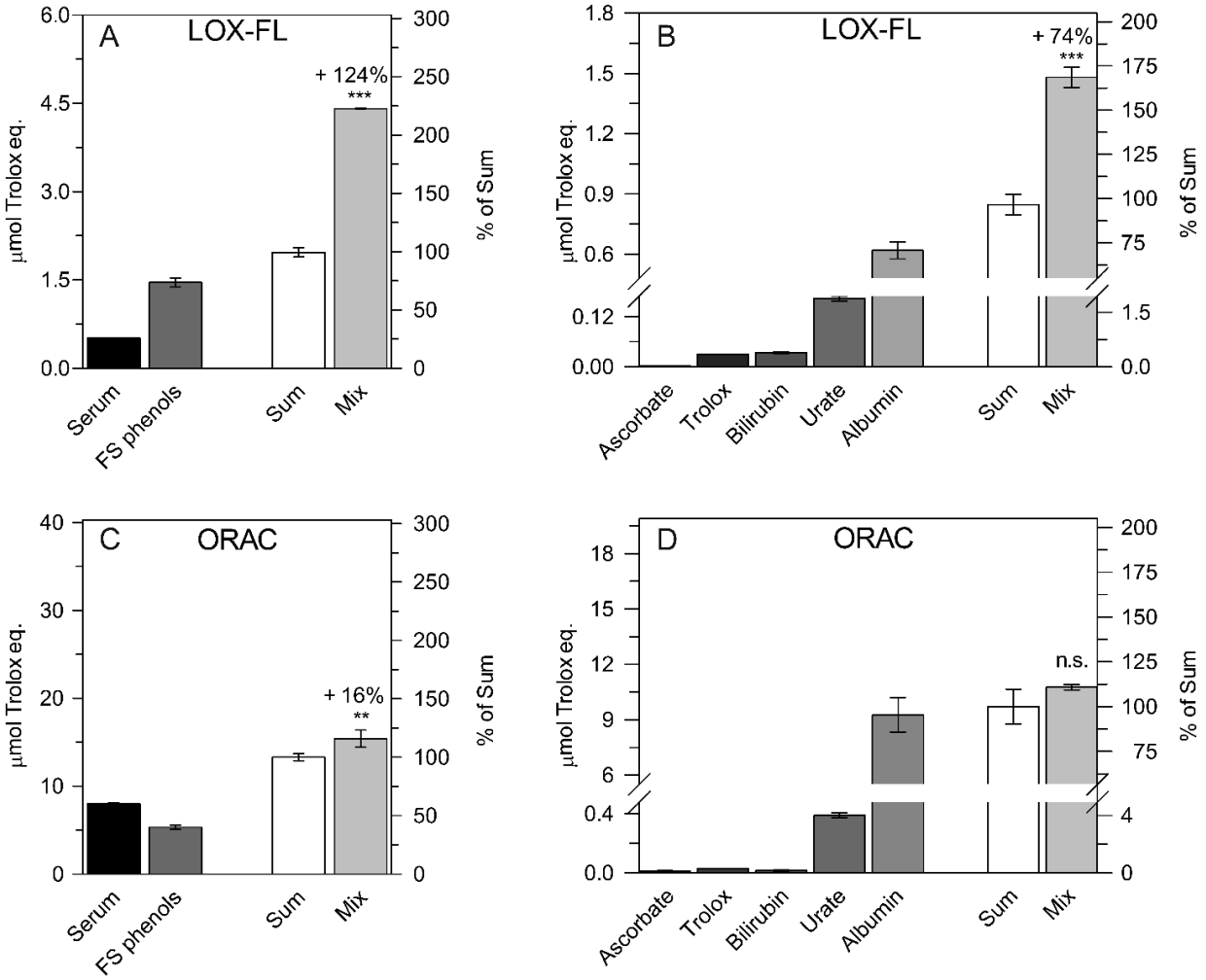

Figure 3. *Synergism among human serum and FS phenols (A and C) and among ascorbate, Trolox, bilirubin, urate and albumin (B and D), evaluated by means of the LOX-FL (A and B) and ORAC (C and D) methods*. In each plot, AC values of each sample, as well as the Sum (AC_{Sum}) and the AC of the Mix (AC_{Mix}) are shown as µmol Trolox eq. and as percentage of the AC_{Sum}. Synergism is reported as percentage increase of AC_{Mix} value with respect to AC_{Sum} value according to the equation: synergistic increase (%) = $[(AC_{Mix}/AC_{Sum}) - 1] \cdot 100$. Data are reported as mean value ± SD. The probability level (**, $p \leq 0.01$; ***, $p \leq 0.001$; n.s. = not significant) according to the Student's *t*-test is also reported.

With regards to phenolic extracts of Lisosan G, all three methods measured much higher AC values in the IB phenolic extract with respect to the FS phenolic one; these data are in good agreement with many literature data reporting IB phenolic compounds as the major phenolic component in cereal whole grain[32]. Consistently, total phenolic and flavonoid contents were higher in the IB phenolic extract than in the FS phenolic one. Lipophilic extract was also evaluated, but only negligible activity was measured (not shown), with AC values equal to about 0.6%, 0.6% and 4% of the total activity as evaluated by the LOX-FL, ORAC and TEAC methods, respectively. On the whole, these results indicate a very high total AC of Lisosan G resulting from about 2- to 10-fold higher than that of whole grain from different wheat species.[16,17] This result is in agreement with

the well-documented bioactivity of Lisosan G. In fact, it has been already shown that Lisosan G protects rats against toxicity induced in liver by carbon tetrachloride and in liver, kidney and testis by cisplatin, possibly by attenuating oxidative stress and by preserving antioxidant enzymes.[20] Moreover, it induces a decrease of intracellular ROS concentration and H_2O_2-dependent mutagenesis in *Saccharomyces cerevisiae*,[22] activates the NAD(P)H:quinone oxidoreductase and heme oxygenase-1 in rat hepatocytes,[21] glutathione peroxidase-1 and superoxide dismutase-2 expression in EPCs exposed to oxidative stress[23] and decreases H_2O_2-induced toxicity in rat hepatocytes.[21]

Furthermore, activation of the nuclear Nrf-2/ARE in rat hepatocytes[21] and EPCs exposed to oxidative stress[23] has been reported. So, bioactivity of phytochemicals from Lisosan G may be exerted in several different modes, possibly including AC properties, but also *via* direct modulation of metabolic pathways and/or gene expression. In order to evaluate the ability of LOX-FL method to assess changes of AC of blood, in comparison with ORAC and TEAC, *ex vivo* measurements of serum were carried out before and 30, 60, 90, 120 and 240 min after the intake of 20 g of Lisosan G (18 g in terms of d.w.).

On the basis of data reported in **Table 2**, this implies the intake of about 1700, 3700 and 1600 μmol Trolox eq. as evaluated by means of the LOX-FL, ORAC and TEAC methods, respectively. The effect on serum AC is reported as percentage change of initial (T_0) AC (**Fig. 4**). As shown, the ingestion of Lisosan G caused an increase of about 40% of the AC, evaluated by means of the LOX-FL assay, after 30 min. Then, AC decreased after 90 min, until to reach an AC value similar to T_0, and increased again at 120 min. This behaviour might be compatible with a prompt release and absorption of FS phenols (see **Table 2**) within 30-60 min in the small intestine followed by either a delayed release of IB phenols at 120 min probably due to the activity of gut microbiota or to metabolism increasing antioxidant activity. Finally, the AC value gradually decreased and reached about the same value of T_0 after 240 min. On the contrary, the ORAC method did not evaluate a significant increase of AC after Lisosan G intake even showing an incoherent decrease at 120 min (about 20%). TEAC method also did not show relevant changes in serum AC. Therefore, under the same experimental conditions different methods may give very different results, thus suggesting that the use of different assays represents a main source of incoherence in a lot of literature data.

Table 2. AC of Lisosan G extracts evaluated by means of the LOX-FL, ORAC and TEAC methods, and content in some antioxidant compounds.

Extract	AC (μmol Trolox eq./g d.w)			Antioxidant compounds		
	LOX-FL	ORAC	TEAC	Protein content[a]	Phenolic content[b]	Flavonoid content[c]
Hydrophilic	81.7 ± 4.7[d]	123 ± 6	48 ± 3	131.7 ± 2.6	n.d.	n.d.
FS phenolic	4.3 ± 0.4	25.6 ± 0.7	7.1 ± 1.2		34.5 ± 1.1	2.76 ± 0.15
IB phenolic	7.1 ± 0.1	56.6 ± 4.8	29.5 ± 2.1		81.1 ± 0.8	26.6 ± 1.1

[a]data are expressed as mg/g d.w.; [b]data are expressed as mg of gallic acid eq./100 g d.w.; [c]data are expressed as mg of catechin eq./100 g d.w.; [d]data are reported as mean value ± SD. n.d., not determined.

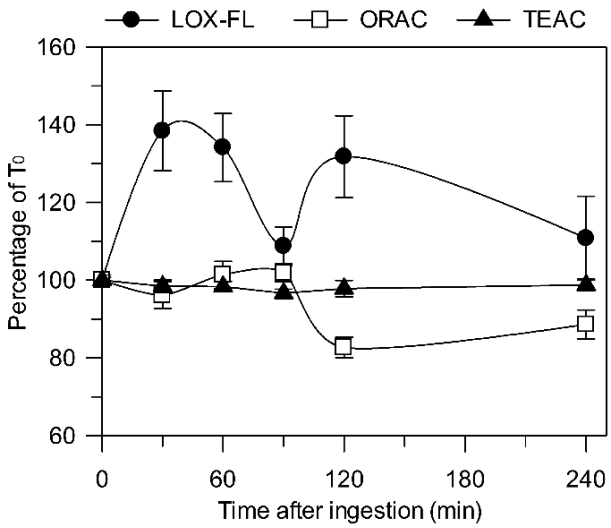

Figure 4. *Percent changes of serum AC, evaluated by means of the LOX-FL, ORAC and TEAC methods, during 240 min after consumption of 20 g of Lisosan G in seven subjects*. A different curve was obtained for each subject, having a T_0 value ranging from 1.04 to 1.65, from 9.32 to 26.18 and from 1.78 to 3.71 µmol Trolox eq./mL of serum, as calculated by LOX-FL, ORAC and TEAC methods, respectively. Then, for each AC method, the seven curves were mediated. Data are reported as mean value ± SE.

Overall, it seems that ORAC and TEAC methods appear to be less performing with respect to LOX-FL; once again, this is probably due to the ability of this method to evaluate simultaneously different antioxidant mechanisms. On the contrary, ORAC assay only measures the chain-breaking AC against a peroxyl radical according to a hydrogen atom transfer (HAT) reaction, whereas TEAC method evaluates the capability of antioxidants to induce the quenching of the non-physiological $ABTS^{\cdot+}$ radical cation, mainly according to a single electron transfer (SET) redox reaction, this last having a minor role *in vivo*.[1] The different rationales for the different methods easily explain why results of LOX-FL, ORAC and TEAC are unrelated each other.

4. Conclusions

The LOX-FL method is able to give a physiological and comprehensive evaluation of AC, so better highlighting the synergistic interaction among the different classes of antioxidants. This novel tool may be used to obtain both *in vitro* measurements of food extracts and *ex vivo* measurements of serum after food ingestion. This ability allows an integrate evaluation of food, using the same assay method, by testing whether foods showing strong AC may give a realistic physiological effects after ingestion. Interestingly, using this approach on the antioxidant-rich dietary wheat grain supplement Lisosan G, it was shown that its consumption may deeply improve the antioxidant status of serum.

This cannot be obtained when ORAC and TEAC methods were used. In fact, although all methods are able to give coherent information about AC of *in vitro* extracts of Lisosan G, LOX-FL was found to be able to highlight a remarkable AC increase of serum after Lisosan G ingestion, while under the same experimental condition ORAC and TEAC failed to do this.

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Article 3:

Different effectiveness of two pastas supplemented with either lipophilic or hydrophilic/phenolic antioxidants in affecting serum as evaluated by the novel Antioxidant/Oxidant Balance approach

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Abstract

Effectiveness in improving serum antioxidant status of two functional pastas was evaluated by the novel Antioxidant/Oxidant Balance (AOB) parameter, calculated as Antioxidant Capacity (AC)/Peroxide Level ratio, assessed here for the first time. In particular, Bran Oleoresin (BO) and Bran Water (BW) pastas, enriched respectively with either lipophilic (tocochromanols, carotenoids) or hydrophilic/phenolic antioxidants extracted from durum wheat bran, were studied. Notably, BO pasta was able to improve significantly (+65%) serum AOB during four hours after intake similarly to Lisosan G, a wheat antioxidant-rich dietary supplement. Contrarily, BW pasta had oxidative effect on serum so as conventional pasta and glucose, thus suggesting greater effectiveness of lipophilic than hydrophilic/phenolic antioxidants under our experimental conditions. Interestingly, no clear differences between the two pastas were observed, when AC measurements of either serum after pasta intake or pasta extracts by *in vitro* assays were considered, thus strengthening effectiveness and reliability of AOB approach.

Keywords: durum wheat antioxidants; functional pasta; human serum; LOX-FL; ORAC; TEAC; Lisosan G.

Abbreviations

AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; ABTS, diammonium-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate); AC, Antioxidant Capacity; AOB, Antioxidant/Oxidant Balance; AUC, area under curve; BO, bran oleoresin; BW, bran water; DPPD, *N,N*-diethyl-*p*-phenylenediamine; d.w., dry weight; FL, fluorescein, 3',6'-dihydroxy-3H-spiro[2-benzofuran-1,9'-xanthen]-3-one; FSP, Free Soluble Phenolic; f.w., fresh weight; HAT, Hydrogen Atom Transfer; LDLox, oxidized low-density lipoproteins; ln, natural logarithmic; LOX, Lipoxygenase; ORAC, Oxygen Radical Absorbance Capacity; PxL, Peroxide Level; R, reference; RNO, 4-nitroso-*N,N*-dimethylaniline; SC-CO_2, supercritical carbon dioxide; SET, Single Electron Transfer; sr, square root; TEAC, Trolox Equivalent Antioxidant Capacity; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

Chemical compounds studied in this chapter

AAPH, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (PubChem CID: 76344); ABTS, diammonium-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (PubChem CID: 9570474); fluorescein, 3',6'-dihydroxy-3H-spiro[2-benzofuran-1,9'-xanthen]-3-one (PubChem CID: 16850); linoleic acid sodium salt (PubChem CID: 23702140); DPPD, *N,N*-diethyl-*p*-phenylenediamine (PubChem CID: 7120); Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (PubChem CID: 40634).

1. Introduction

Food industry makes continuous effort to place on the market newer functional products enriched of nutrients and bioactives. Being pasta a widely consumed food product with long shelf life, it has been the object of several supplementation strategies, acting as carrier of vitamins, minerals, polyunsaturated fatty acids, composite plant extracts, and dietary fibers. The most recent functionalizations of pasta include a variety of non-traditional ingredients, such as orange by-product fiber (Crizel, Rios, Cruz, Thys, & Flôres, 2015), mushroom beta-glucans (Kim, Lee, Heo, & Moon, 2016), carob flour (Sęczyk, Świeca, & Gawlik-Dziki, 2016), oregano and carrot leaf (Boroski et al., 2011), not to mention the employment of alternative cereals or pseudo-cereals, such as buckwheat flour and bran (Biney & Beta, 2014), amaranth, teff, quinoa (Kahlon & Chiu, 2015), or the use of legumes, such as soy (Clerici et al., 2011), white bean, yellow pea, and lentil (Wójtowicz & Mościcki, 2014).

Besides reducing the glycemic index, raising protein content and improving the profile of amino acids and fatty acids, one of the main objects of pasta functionalization is improving Antioxidant Capacity (AC) of the end-product. In previous researches, the authors produced durum wheat (*Triticum durum* Desf.) Bran Oleoresin (BO) (Durante, Lenucci, Rescio, Mita, & Caretto, 2012) and durum wheat Bran Water (BW) (Pasqualone et al., 2015) antioxidant extracts, by applying respectively supercritical carbon dioxide (SC-CO_2) or ultrasound-assisted extraction. BO and BW extracts were then used to supplement pasta with lipophilic (tocochromanols, carotenoids) or hydrophilic/phenolic antioxidants, respectively (Pasqualone et al., 2016).

In the present paper, a study was carried out to check both *in vitro* AC of cooked BO and BW pastas and, mostly, their effectiveness in improving the antioxidant status of blood (in particular of serum) by means of *ex vivo* analysis. The latter may take into account bioavailability and metabolism, giving therefore an integrated information of a real effect on blood antioxidant status, which is beyond the original AC of the ingested food. In particular, the analyses were carried out during digestion, just few hours after food intake, *i.e.* in the window time when glucose released from starch digestion is expected to induce an oxidative effect on blood (Ceriello et al., 2014; Khor et al., 2014). Although the latter may appear as a major aspect to assess functionality of antioxidant-enriched pastas, at our best knowledge, so far only one study reported some information about changes of plasma AC two hours after the intake of pasta containing wholegrain sorghum flour (Khan, Yousif, Johnson, & Gamlath, 2015). On the contrary, many investigations studied *in vitro* AC (Biney & Beta, 2014; Boroski et al., 2011; Sęczyk et al., 2016) or effects of long-term consumption of antioxidant-enriched pastas on serum/plasma AC (Clerici et al., 2011; Durazzo et al., 2014; Whittaker et al., 2016). Unfortunately, in short-term studies (minutes or hours

after ingestion), foods often induced limited AC increases, even though very rich in antioxidants (Fernández-Panchón, Villano, Troncoso, & Garcia-Parrilla, 2008; Soccio, Laus, Alfarano, & Pastore, 2016). This finding may depend, at least in part, on some weakness of the analytical approach, which takes into account only changes of AC without considering changes of the oxidative status of serum. This is a central point; in fact, several reports showed that the intake of food antioxidants may induce an increase of AC or a decrease of the oxidation level of serum/plasma or both (Alvarez-Suarez et al., 2014; Khan et al., 2015; Torabian, Haddad, Rajaram, Banta, & Sabaté, 2009). In practice, a fraction of antioxidants is consumed to counteract oxidation and thus cannot be revealed by AC assays. Therefore, a more effective evaluation of antioxidant intake effect should consider simultaneously the effect on AC and that on the oxidation level of serum. With this aim, in this paper, the serum antioxidant status was evaluated by means of a novel approach, presented for the first time in this research. It is based on the determination of "Antioxidant/Oxidant Balance" (AOB), representing the ratio between serum AC and serum oxidant status, evaluated as "Peroxide Level" (PxL). In particular, serum AC was evaluated by three different methods. The first one was the new Lipoxygenase-Fluorescein (LOX-FL) method (Soccio et al., 2016), derived from the LOX/4-nitroso-*N,N*-dimethylaniline (LOX/RNO) one (Pastore, Laus, Tozzi, Fogliano, Soccio, & Flagella, 2009; Pastore, Trono, Padalino, Di Fonzo, & Passarella, 2000), which is able to highlight simultaneously several antioxidant functions and synergy among serum antioxidants (Soccio et al., 2016). The other two methods were the widely used Oxygen Radical Absorbance Capacity (ORAC) and Trolox Equivalent Antioxidant Capacity (TEAC) assays, which mainly give information about antioxidant mechanisms based respectively on Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET) reactions (Huang, Boxin, & Prior, 2005). The combined use of these assays, which have a very different basic rationale, may allow a more complete picture of AC and related AOB.

On the whole, the aims of this paper are to assess the reliability of the novel AOB approach in evaluating serum antioxidant status after food intake and to evaluate effects on serum during four hours after BO and BW pasta intake using the new AOB approach. To these purposes, the two antioxidant-supplemented pastas were compared with other foods able to show high AC or to induce pro-oxidant effects (see section 2.2).

2. Materials and methods

2.1 Chemicals

Chemicals and solvents at analytical and HPLC-grade purity were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). In the LOX-FL assay, a dilution in 100 mM Na-borate buffer pH 9.0

of an ammonium sulfate suspension of soybean LOX type V (LOX-1 isoenzyme, E.C. 1.13.11.12) was used, as well as a sodium linoleate solution prepared as described in Pastore et al. (2000). A competitive ELISA kit obtained from Mercodia (Uppsala, Sweden) was used to assay the oxidized low-density lipoprotein (LDLox). Glucose was purchased from Baxter (Rome, Italy). Other chemicals are reported in the sections where their use is described.

2.2 Tested foods

Foods under study included: *i*) pasta enriched with durum wheat BO extract; *ii*) pasta enriched with durum wheat BW extract; *iii)* non-supplemented reference (R) pasta; *iv)* the wheat grain dietary supplement Lisosan G; *v)* glucose; *vi)* R pasta consumed together with Lisosan G.

In particular, Lisosan G is a nutritional supplement certified by Italian Ministry of Health, produced by Agrisan Company (Larciano, PT, Italy) from lysed fine bran and germ of organic wheat (*Triticum aestivum* L.) grains (Pozzo et al., 2015). It was kindly provided by Dr Vincenzo Longo (Institute of Agricultural Biology and Biotechnology-CNR, Pisa, Italy).

As regards extracts, BO was produced by SC-CO_2 extraction accordingly to Durante et al. (2012). Briefly, durum wheat bran, provided by Tomasello milling industry (Casteldaccia, Palermo, Italy), was firstly oven dehydrated at 60 °C to a residual moisture content of 3%. Then, aliquots (3 kg) of dehydrated wheat bran matrix were extracted by SC-CO_2 for 3 h using the following operative parameters: gaseous CO_2 flow rate = 18-20 kg/h; pressure = 35 MPa; temperature = 60 °C. BW extract was obtained by ultrasound-assisted extraction of durum wheat bran (3.5 kg) mixed with tap water (35 L) by means of a pilot plant assembled by Weal (Milano, Italy). Extraction was performed at 20 °C for 25 min; thirty-second recirculation steps of the suspension into the extraction chamber were carried out every 5 min of ultrasonic treatment. Finally, the suspension was filtered through a metal grid with 1 mm holes to recover the liquid phase (Pasqualone et al., 2015).

As for pastas, the BO-enriched pasta (BO pasta) was obtained by adding 525 g of BO to 9.475 kg of durum wheat (*Triticum durum* Desf., cv. Vertola) semolina; then, the BO-added semolina was mixed with 3 L tap water. As for the BW-supplemented pasta (BW pasta), this was obtained by adding semolina with 3 L of BW extract. The ingredients were processed by using a MAC 60 VR vacuum extruder (Italpast, Fidenza, Italy) at the following conditions: 15 min kneading; 1 bar chamber vacuum; 40 °C die temperature; 25 rpm extruder auger speed. The dough was extruded through a Teflon-coated spaghetti die. Then, pastas were dried in a static dryer (LAB, Namad Impianti, Rome, Italy) according to a high temperature drying program (T max = 78 °C) with linear decrease of relative humidity into the drier from 95% to 40% during the entire drying process (8

h and 50 min). The temperature linearly increased from 40 to 60 °C in 120 min, then from 60 to 68 °C in 120 min, and from 68 to 78 °C until the end of the drying cycle (Pasqualone et al., 2015, 2016). The BO and BW pastas showed color characteristics, cooking performances, viscoelastograph properties and textural parameters resulting not significantly different compared to a conventional pasta in an overall sensory judgment (Pasqualone et al., 2016).

The non-supplemented R pasta was spaghetti produced by TAMMA food industry (Foggia, Italy). This pasta was chosen because in preliminary experiments it showed AC very close to average AC of 20 pastas purchased at local market differing for brand, shape and size (Pastore et al., unpublished results).

Energy/nutrient composition profile of pastas under study is reported in **Table 1**. Analyses were performed by BonassisaLab research and analysis centre (Foggia, Italy) according to international guidelines.

Table 1 - Energy/nutrient composition of pastas under study.

	BO pasta[1]	**BW pasta[2]**	**R pasta[3]**
Energy (kJ/100 g)	1482	1500	1487
Humidity (%)	10.2	9.5	9.7
Ash (%)	0.83	0.84	0.78
Protein (%)	12.7	12.4	12.5
Total carbohydrate (%)	68.9	68.9	72.7
-soluble sugars (%)	1.03	2.78	1.0
sucrose (%)	*0.18*	*0.28*	*0.22*
maltose (%)	*0.85*	*2.50*	*1.23*
Total fat (%)	1.83	1.73	1.50
-saturated (%)	0.33	0.42	0.30
-saturated (% of total fat)	18.1	24.2	20.0
-monounsaturated (% of total fat)	26.1	18.8	23.9
-polyunsaturated (% of total fat)	55.8	57.0	56.1
Total dietary fibre (%)	5.5	6.6	3.0
Sodium (mg/kg)	12.3	17.4	n.d.

[1] BO pasta: pasta supplemented with bran oleoresin; [2] BW pasta: pasta supplemented with bran water extract; [3] R pasta: reference pasta; n.d.: not determined.

2.3 Extraction of antioxidant compounds and sera collection

2.3.1 *Preparation of cooked pasta samples for in vitro AC determination*

Pasta cooking was carried out by adding about 100 g of pasta to 1 L of boiling distilled water. All pasta samples were cooked at their optimal time, determined as reported in Pasqualone et al. (2015, 2016), *i.e.* 9, 6 and 5 min for the BO, BW and R pastas, respectively. Cooked pasta samples were then drained with a Buchner funnel. Cooked samples were frozen at -20 °C and then freeze-dried by using the "LIO-5PDGT" lyophilizer (Tecnochimica Moderna, Roma, Italy). Freeze-dried samples were milled using the cryogenic grinder "Cryo Mill" (Retsch, Bergamo, Italy) and stored at -20 °C until needed.

2.3.2 *Preparation of hydrophilic, lipophilic and phenolic extracts from cooked pasta samples for in vitro AC determination*

Hydrophilic extracts were prepared as described in Laus, Tozzi, Soccio, Fratianni, Panfili, & Pastore (2012b) by extracting samples with deionized water in an ice-water bath for 1 h at a (w/v) ratio equal to 1 g/6 mL.

Lipophilic compounds were extracted according to the procedure described in Laus et al. (2012b), by saponification at 70 °C of samples (2 g) with 2 mL of 60% (w/v) KOH, 5 mL of 6% (w/v) ethanolic pyrogallol, 2 mL of 1% (w/v) NaCl, 2 mL of 96% (v/v) ethanol. After saponification, the suspension was extracted four times with 15 mL of *n*-hexane/ethyl acetate (9:1, v/v). Lipophilic compounds were reconstituted in ethanol.

Free Soluble Phenolic (FSP) compounds were extracted as reported in Laus et al. (2012b), with some modifications. In particular, samples (1.5 g) were extracted twice with 30 mL of 80% (v/v) ethanol for 10 min at room temperature and centrifuged at 14000x*g* for 10 min at 20 °C. The combined supernatants were evaporated under vacuum at 40 °C using a Buchi evaporator and concentrated to approximately 6 mL; then, they were acidified to pH 2-3 using 1 M HCl and centrifuged at 14000x*g* for 10 min at 20 °C. The resultant supernatant was extracted twice with *n*-hexane (at an *n*-hexane/water phase ratio equal to 1:1 by vol.); then, the combined water phases were subjected to three extractions in ethyl acetate (at an ethyl acetate/water phase ratio equal to 1:1 by vol.). The ethyl acetate fractions were combined and evaporated to dryness under vacuum at 40 °C; the dry residue was reconstituted in 1.5 mL of water. All extracts were immediately assayed.

2.3.3 Preparation of hydrophilic, lipophilic and phenolic extracts from Lisosan G for in vitro AC determination

Extraction of hydrophilic compounds from Lisosan G was performed as reported in Laus et al.

(2012b), by adopting a (w/v) ratio equal to 1 g Lisosan G/5 mL of deionized water. Extraction of lipophilic and FSP compounds was carried out as described in Laus et al. (2012b), with final resuspension of dry residues in ethanol and water, respectively. The extracts were straightaway assayed.

2.3.4 Collection of sera from volunteers

Seven healthy subjects (3 women and 4 men aged between 24 and 33 years) participated in this study, after providing informed written consent. The study was conducted in according to the guidelines laid down in the Declaration of Helsinki and in accordance with relevant Italian laws and institutional ethical policies. Experiments were approved by both the Board of the SAFE Department of University of Foggia (Italy) and the Scientific Committee of Regional Technological District DARe-Puglia (Italy). The seven volunteers were free of diabetes, cardiovascular, liver, gastrointestinal and kidney diseases. Two days before analysis, the subjects were asked to abstain from alcohol and vigorous physical activity and to follow a diet poor in phenolic antioxidant compounds, avoiding all fresh fruits and vegetables and derived products including fruit juices, tea, chocolate, coffee and wine. Each volunteer attended six sampling sessions at 15 days intervals. At each session each subject consumed 20 g fresh weight, f.w. (18 g dry weight, d.w.) of Lisosan G, or 50 g of glucose, or 70 g f.w. of BW or BO or R pasta, or 70 g of R pasta consumed together with 20 g (18 d.w.) of Lisosan G. Dose of Lisosan G was determined in preliminary experiments as able to induce an increase of serum AC (Soccio et al., 2016). As for serving of pasta, 70 g, representing a typical meal, was chosen according to Khan et al. (2015) as an amount able to decrease significantly plasma antioxidant status. Dose of glucose represents the amount released from pasta serving size. After 12 h fast, the subjects consumed one of the test foods within 10 min. The subjects assumed glucose as 500 mL of 10% solution or Lisosan G as resuspended in 500 mL of water; pasta was consumed with drinking 500 mL of mineral water. Venous blood samples were collected at baseline (T_0) and exactly 30, 60, 90, 120 and 240 min after food consumption. Blood samples were centrifuged at 3000x*g* for 5 min and the resulting serum samples were stored at -80 °C until analysis.

2.4 AC determination by means of the LOX-FL, ORAC and TEAC methods

2.4.1 LOX-FL method

AC determination by LOX-FL assay was performed as recently described in Soccio et al. (2016). The quenching of FL (3',6'-dihydroxy-3H-spiro[2-benzofuran-1,9'-xanthen]-3-one) was monitored at 37 °C at the excitation and emission wavelengths of 485 and 515 nm, respectively,

by means of a LS 55 fluorescence spectrometer (Perkin Elmer, Waltham, MA, USA). The assay mixture (2 mL), consisting of 100 mM Na-borate buffer pH 9.0, 6.3 nM FL, 400 µM Na-linoleate and 1 µL Tween 20/µmol linoleate, was added with 0.5 enzymatic units of soybean LOX-1 to start the reaction. The LOX-FL measurements were performed in both the absence (control) and the presence of sample (extract or serum or the standard antioxidant (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Trolox). The (%) decrease of the rate of LOX-FL reaction measured in presence of sample was calculated compared to the control. At least three different amounts of sample were analysed in triplicate and the slope of the resulting regression line was obtained. AC was calculated by comparing the slope derived by linear regression analysis of the extract or serum with that of the calibration curve obtained by using Trolox (Di Benedetto et al., 2015; Soccio et al., 2016).

Concerning lipophilic antioxidants reconstituted in ethanol (see sections 2.3.2 and 2.3.3), measurements were carried out in presence of a constant ethanol concentration in the reaction mixture to exclude ethanol effect on LOX activity.

2.4.2 ORAC method

ORAC measurements were performed as reported by Ou, Hampsch-Woodill, & Prior, (2001), properly modified as in Soccio et al. (2016). A CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) and 96-well plates were used for measurements. Assays were conducted in a mixture (final volume of each well: 0.2 mL) consisting of 75 mM Na-phosphate buffer pH 7.4 and 10 nM FL (solubilized in 75 mM Na-phosphate buffer pH 7.4), in tabsence (blank) and presence of sample (extract, serum or Trolox). FL fluorescence decrease was started by adding 40 mM 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, solubilized in 75 mM Na-phosphate buffer pH 7.4) and monitored by recording fluorescence (λ_{ex}=483 nm, bandwidth 14 nm; λ_{em}=530 nm, bandwidth 30 nm) at 37 °C every 30 s. Four different amounts of sample were analysed in triplicate. In order to quantify AC, the difference was calculated between the area under the fluorescence decay kinetic curve (area under curve, AUC) of sample and the AUC of the blank. AC was determined using a dose-response curve obtained by using Trolox. As for measurements of ethanolic extracts, the assay mixture also contained a fixed concentration of ethanol.

2.4.3 TEAC method

TEAC assay described in Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, (1999) was applied with slight modifications as reported in Laus et al. (2015). The aqueous solution of the

diammonium-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\cdot+}$) was diluted with 5 mM Na-phosphate buffer pH 7.4 (or ethanol in AC measurements of lipophilic extracts). Measurements were carried out in triplicate for three different amounts of sample (extract or serum or Trolox) and AC was quantified using a proper calibration curve obtained with Trolox by plotting the (%) decrease of absorbance at 734 nm as a function of standard antioxidant concentration.

For all three methods, as for *in vitro* measurements of food extracts, AC values were calculated as μmol Trolox eq./g d.w.; then, this value was multiplied for grams of serving size. So, in **Table 2** AC values of pasta and Lisosan G extracts were referred to AC per serving. As for *ex vivo* measurements, AC values were calculated as μmol Trolox eq./mL of serum and reported in Table 1 of Laus et al. (2016). On the other hand, in Figs 1 and 2, in order to facilitate comparison, data were reported as (%) variation with respect to T_0 value.

2.5 Serum PxL and LDLox determination

Serum PxL was spectrophotometrically measured at 37 °C by using a SpectraMax® M5 Multimode Plate Reader (Molecular Devices, Wokingham, UK). The method is based on the capability of hydroperoxide groups to react with Fe^{2+} and generate the corresponding alkoxyl radical, according to the Fenton reaction. This newly formed radical, whose quantity is related to the peroxide content, is chemically trapped with *N,N*-diethyl-*p*-phenylenediamine (DPPD), leading to formation of the corresponding radical cation, which was determined at 512 nm. The method has been preliminarily assessed on the basis of the literature remarks (Erel, 2005) by adding an adequate Fe^{2+} amount in order to promote reaction. Moreover, we adopted as measured parameter the absorbance at the end-point rather than the initial reaction rate, thus significantly improving reproducibility of results. According to Inchingolo et al. (2014) and Simiakakis, Kapsimalis, Chaligiannis, Loukides, Sitaras, & Alchanatis, (2012), we found that alkylamine reaction strongly enhances response to serum peroxides; although this effect amplifies sensitivity, it also implies calculation of a very high equivalent content of H_2O_2 in comparison with the physiological one (Erel, 2005). For this reason, in this paper we refer the oxidation status of serum to a conventional “Peroxide Level” rather than to a misleading “Peroxide Content”.

Briefly, every working well of a 96-well plate contained the assay mixture consisting of 0.2 mL of 100 mM Na-acetate buffer pH 4.8, 6 mM DPPD (solubilized in 100 mM Na-acetate buffer pH 4.8), 30 μM $FeSO_4$ in absence (blank) or in presence of serum. Calculations were based on absorbance at 512 nm evaluated at the end-point (at least after 6 h). Results are expressed as

equivalents of H_2O_2 using a proper calibration curve. Three different amounts of sera were evaluated in triplicate for each subject. Serum PxL values after consumption of each food under study are reported in Table 1 of Laus et al. (2016), while in Fig. 1, to facilitate comparison, data were reported as (%) variation of T_0 value.

LDLox were measured by a commercially available ELISA kit (Mercodia oxidized LDL Competitive ELISA, Uppsala, Sweden).

2.6 Determination of AOB of serum

AOB values of serum after consumption of a tested food were obtained as AC/PxL ratio, *i.e.* by calculating the LOX-FL/PxL, ORAC/PxL and TEAC/PxL ratios. Data were expressed as (%) of T_0 value. It should be outlined that this calculation was carried out individually for each subject, rather than as ratio between the AC and PxL averages of the subjects. So, AOB profiles *vs* time reported in Figs 1 and 2 were obtained by averaging the seven individual AOB (%) profiles obtained by the subjects enrolled in the study.

2.7 Calculation of AC-Area, PxL-Area, AOB-Area, AOB-Index and statistical analysis

Areas under the AC, PxL and AOB profiles *vs* time (expressed as % of T_0 *vs* time) were calculated by means of the trapezoidal rule using a Microsoft Excel spreadsheet; then, these areas were expressed as (%) of the basal area, *i.e.* the area below the value at baseline (T_0), and indicated as AC-Area, PxL-Area or AOB-Area. The AOB-Index was calculated, in analogy with the glycemic index, as the ratio between the area under the AOB profile of the tested food and the area under AOB profile of 50 g of glucose, and expressed as (%). Also in this case, the calculation was carried out individually for each subject, rather than as a ratio between the averages of the subjects. In this manner, each subject represented the control of himself.

As for statistical analysis, preliminarily data distribution was evaluated using the Shapiro-Wilk and Jarque-Bera tests. Homogeneity of variances was verified by the Bartlett's test. Where necessary, either a natural logarithmic (ln) or square root (sr) transformation was performed (see **Table 2**). Data were submitted to analysis of variance (ANOVA) and the mean separation was tested by Duncan's test at 0.05 *P* level of significance. ANOVA was performed using Statistica (data analysis software system), version 7.1 (StatSoft, Tulsa, Oklahoma, USA).

3. Results and discussion

In this paper, BO and BW pastas were compared each other by means of *ex vivo* assessment of antioxidant status of serum in terms of the novel AOB approach after pasta intake. Preliminarily,

an *in vitro* analysis of AC on pasta extracts was carried out.

3.1 In vitro AC measurements of cooked pastas and Lisosan G

After cooking, BO and BW pastas were compared each other as well as with conventional R pasta and antioxidant-rich dietary supplement Lisosan G in terms of AC determined by *in vitro* assays (**Table 2**). This was made by using LOX-FL, ORAC and TEAC methods on extracts highly enriched of hydrophilic, lipophilic and FSP compounds. Although the insoluble bound phenolic fraction represents the major phenolic component in cereal whole grains (Laus et al., 2012b and refs therein), it was not considered in this study. This fraction, in fact, evades small intestine absorption and might be freed by the gut microbiota with a possible absorption through the colon (Visioli et al., 2011). Therefore, its absorption is delayed and exceeds the four hours chosen to evaluate effectiveness of antioxidants to counteract serum oxidation by glucose.

AC values of extracts were determined per serving of pasta or Lisosan G (see section 2.3.4). Values of Lisosan G were recalculated from Soccio et al. (2016). All data were also reported as (%) of AC of reference (R) pasta (**Table 2**).

As expected, because of chemical differences among the assays, LOX-FL, ORAC and TEAC showed different AC values. As regards comparison among different types of extract from the same food, the highest AC values were measured in H component; interestingly, this is observed for every food under study and for each AC assay adopted in this study. As for comparison among different tested foods against the same antioxidant fraction, Lisosan G showed the highest AC value, as evaluated by LOX-FL assay, in both hydrophilic and FSP fractions. Consistently, Lisosan G showed very high protein and phenolic/flavonoid content in hydrophilic and FSP extracts, respectively: 2.37±0.05 g bovine serum albumin eq., 6.21±0.20 mg gallic acid eq. and 0.50±0.03 mg catechin eq. per serving, respectively (Soccio et al., 2016). The most active FSP component following Lisosan G according to LOX-FL method was obtained in BW pasta, resulting 80% compared to Lisosan G and 2.7- and 3.2-fold higher than R and BO pastas, respectively. As for lipophilic component, the highest AC value among all tested foods was obtained by LOX-FL assay in BO pasta, resulting even about 4- and 11-fold higher than R (and BO) pasta and Lisosan G, respectively. This is in general agreement with the different supplementation of BW and BO pastas. In fact, BW extract showed a total phenolic content equal to 1.3 g ferulic ac. eq./L and retained a significant level of ferulic and *p*-coumaric acids (0.31±0.01 and 0.25±0.01 mg/g d.w., respectively), as reported in Pasqualone et al. (2015, 2016). BO extract showed a very high content of lutein, β-carotene and zeaxanthin (4.1±1.5, 1.9±0.4 and 1.6±0.2 µg/g BO, respectively), as well as a high level of tocotrienols and tocopherols (5.2±1.5 and 4.3±0.7 mg/g BO, respectively), as

reported in Durante et al. (2012). Similar to LOX-FL, the ORAC method pointed out a remarkable AC in the hydrophilic and FSP extracts of Lisosan G, as well as a low AC of lipophilic extract. On the contrary, ORAC failed to highlight statistical differences among pastas for FSP fraction.

Table 2 - *Antioxidant Capacity (AC), evaluated by LOX-FL, ORAC and TEAC methods, of hydrophilic (H), Free Soluble Phenolic (FSP) and lipophilic (L) extracts obtained from BO, BW, R pastas and Lisosan G.* AC values were reported per serving: 70 g (f.w.) of pasta or 20 g (18 g d.w.) of Lisosan G. Percentages with respect to R pasta values are reported between brackets. In the case of all TEAC measurements and of the LOX-FL measurements of H extracts, statistical analysis was performed using ln-transformed data; as for the LOX-FL measurements of FSP, sr-transformed data were used. Within the same column, different letters indicate significant differences at 0.05 *P* level, according to the Duncan's test. Data are reported as mean value ± SD (n=3 independent experiments).

	AC_{LOX-FL} (μmol Trolox eq. per serving)		
	H	**FSP**	**L**
R pasta[1]	179 ± 6 ^{c} *(100)*	24 ± 1.4 ^{c} *(100)*	29 ± 2.8 ^{b} *(100)*
BW pasta[2]	167 ± 6 ^{c} *(94)*	64 ± 2 ^{b} *(271)*	25 ± 1.4 ^{b} *(85)*
BO pasta[3]	205 ± 8 ^{b} *(115)*	20 ± 0.7 ^{d} *(85)*	111 ± 7 ^{a} *(385)*
Lisosan G	1471 ± 85 ^{a} *(824)*	77 ± 7 ^{a} *(325)*	10 ± 1.1 ^{c} *(34)*
	AC_{ORAC} (μmol Trolox eq. per serving)		
	H	**FSP**	**L**
R pasta[1]	361 ± 39 ^{bc} *(100)*	102 ± 11 ^{b} *(100)*	78 ± 8 ^{a} *(100)*
BW pasta[2]	322 ± 14 ^{c} *(89)*	105 ± 2 ^{b} *(103)*	77 ± 7 ^{a} *(99)*
BO pasta[3]	422 ± 34 ^{b} *(117)*	118± 13 ^{b} *(116)*	60 ± 8 ^{b} *(77)*
Lisosan G	2214 ± 108 ^{a} *(614)*	461 ± 13 ^{a} *(454)*	23 ± 0.4 ^{c} *(30)*
	AC_{TEAC} (μmol Trolox eq. per serving)		
	H	**FSP**	**L**
R pasta[1]	160 ± 14 ^{b} *(100)*	14 ± 2.1 ^{c} *(100)*	10 ± 1.4 ^{c} *(100)*
BW pasta[2]	164 ± 8 ^{b} *(102)*	20 ± 0.7 ^{b} *(145)*	11 ± 0.7 ^{bc} *(107)*
BO pasta[3]	149 ± 7 ^{b} *(93)*	13 ± 0.7 ^{c} *(95)*	13 ± 0.2 ^{b} *(130)*
Lisosan G	864 ± 54 ^{a} *(539)*	128 ± 22 ^{a} *(913)*	67 ± 14 ^{a} *(680)*

[1] R pasta: reference pasta; [2] BW pasta: pasta supplemented with bran water extract; [3] BO pasta: pasta supplemented with bran oleoresin.

Concerning lipophilic extract, ORAC even showed some superiority of BW and R pastas compared to BO. Interestingly, ORAC pointed out a higher AC value of hydrophilic extract of BO pasta than BW; this is in accordance with results obtained by LOX-FL, also showing a more active hydrophilic fraction of BO in respect of both BW and R pastas. Really, this result is rather unexpected and merits further investigation.

As for TEAC measurements, Lisosan G showed the highest AC in all extracts; unlike LOX-FL and ORAC, TEAC showed also a considerable AC value of lipophilic extract of Lisosan G. Among pastas, TEAC assay highlighted a higher AC value of lipophilic extract of BO pasta compared to R pasta and of FSP extract of BW pasta in respect of both BO and R pastas. Regarding the latter point, it should be reported that using the DPPH assay an AC 20% higher than the control was found in BW pasta (Pasqualone et al. 2015), which is the one obtained by adopting the HT1 drying diagram in that paper.

As for the antioxidant compounds present in the type of extracts reported in **Table 2**, a characterization was already carried out in durum wheat grains. In particular, AC of hydrophilic extract was mainly dependent on flavonoids, as well as on proteins, which are able to show significant *in vitro* antioxidant properties (Laus et al., 2012b); ferulic acid was the most abundant among phenols, while sinapic, vanillic and protocatechuic acids were together about 10% (Pastore et al., 2009); lipophilic antioxidants were essentially β-tocotrienol, α-tocopherol, α-tocotrienol, β-tocopherol and lutein (Laus et al., 2012b).

On the whole, all methods showed a very strong AC of hydrophilic and FSP fractions of Lisosan G, resulting from about 2- to 10-fold higher than other wheat species, depending on the cereal species and AC assay (Laus, Gagliardi, Soccio, Flagella, & Pastore, 2012a; Laus et al., 2012b). As for AC of lipophilic extract, only TEAC indicated the highest AC of Lisosan G, while LOX-FL and ORAC gave an opposite result. Some contrasting data were observed also for pastas. Among all tested foods, BO pasta had the highest AC value in lipophilic component only according to LOX-FL, while TEAC pointed out a superiority of lipophilic fraction of BO only with respect to R pasta. As for BW pasta, LOX-FL and TEAC assays, but not ORAC, highlighted a higher AC of FSP extract of BW pasta with respect to both BO and R pastas. These partly incoherent findings may be attributed to the different antioxidant mechanisms highlighted by the three assays. In particular, TEAC mainly assesses a reducing power. Our classical ORAC measurements mainly highlight scavenging activity against peroxyl radicals (Huang et al., 2005), although a recent new version of ORAC may highlight five different free radical species ($ORAC_{MR5}$, Prior et al., 2016). The highly performing LOX-FL method is able to simultaneously detect scavenging capacity against different physiological radicals as well as other antioxidant functions, thus providing a more comprehensive AC evaluation (Soccio et al., 2016).

Although this kind of analysis is useful to characterize a food, in general, the values indicating *in vitro* AC have not been demonstrated to be relevant for the biological effects of specific bioactive compounds (Fraga, Oteiza, & Galleano, 2014). Therefore, the study was enlarged to *ex vivo* analysis of the antioxidant status of serum.

3.2 Effect of intake of Lisosan G, glucose and pastas on the antioxidant status of serum. Assessment of the novel AOB parameter

3.2.1 Lisosan G, glucose and AOB approach

In order to evaluate the effect of functional pasta consumption on serum antioxidant status, the reliability of the novel AOB experimental approach was preliminarily verified by comparing AC and AOB evaluation (**Fig. 1**). In particular, the capability to highlight changes in serum antioxidant status after food antioxidant intake was checked by studying the effect of both an antioxidant-rich food and a pro-oxidant food. In the light of its well-documented high antioxidant properties and bioactivities (Pozzo et al., 2015 and refs therein), the dietary supplement Lisosan G was chosen as a strong source of wheat antioxidants lacking any significant glycemic effect (not shown). On the other hand, glucose was used as pro-oxidant food since its consumption is known to induce serum oxidation (Ceriello et al., 2014; Khor et al., 2014). So, the use of Lisosan G and glucose may impose opposite conditions giving to the serum high antioxidant supply and high oxidation, respectively.

Fig. 1 shows changes of serum AC, evaluated by the three assays, and PxL in comparison with the derived AOBs during four hours after ingestion of either Lisosan G (Fig. 1 A, B and C) or glucose (Fig. 1 A', B' and C'). With the aim of better highlighting changes of the different parameters, data were reported as (%) variation of T_0 value. Lisosan G induced a general increase of AC and decrease of oxidation of serum as measured respectively by LOX-FL and PxL (Fig. 1 A). The data clearly agree with those reported by Torabian et al. (2009) who found an increase of plasma AC and a decrease of plasma lipid peroxidation after consumption of polyphenol-rich walnuts or almonds. These findings indicate that AC and PxL are related each other in such a manner that an antioxidant may both increase AC and/or decrease PxL. Therefore, the novel AOB parameter, considering simultaneously both AC and PxL, may really evaluate in a more comprehensive manner the antioxidant status of serum. Consistently, $AOB_{LOX\text{-}FL/PxL}$ is able to highlight a very strong improvement of antioxidant status of serum up to about 80% at 30-60 min (Fig. 1 A).

The trend appears biphasic: a first increase at 30-60 min, compatible with a rapid release and absorption of FSP compounds in the small intestine, was followed by a minimum at 90 min and a subsequent new increase. Although further investigation is required to explain this finding, a possible explanation might be either a secondary release of covalently bound antioxidants or an AC increase following antioxidant metabolism (Visioli et al., 2011). As expected, glucose increased oxidation and decreased AC (Fig. 1 A') in accordance with Khor et al. (2014) who reported an increase of lipid peroxidation and a decrease of AC at 2 and 4 h after sugar eating, respectively.

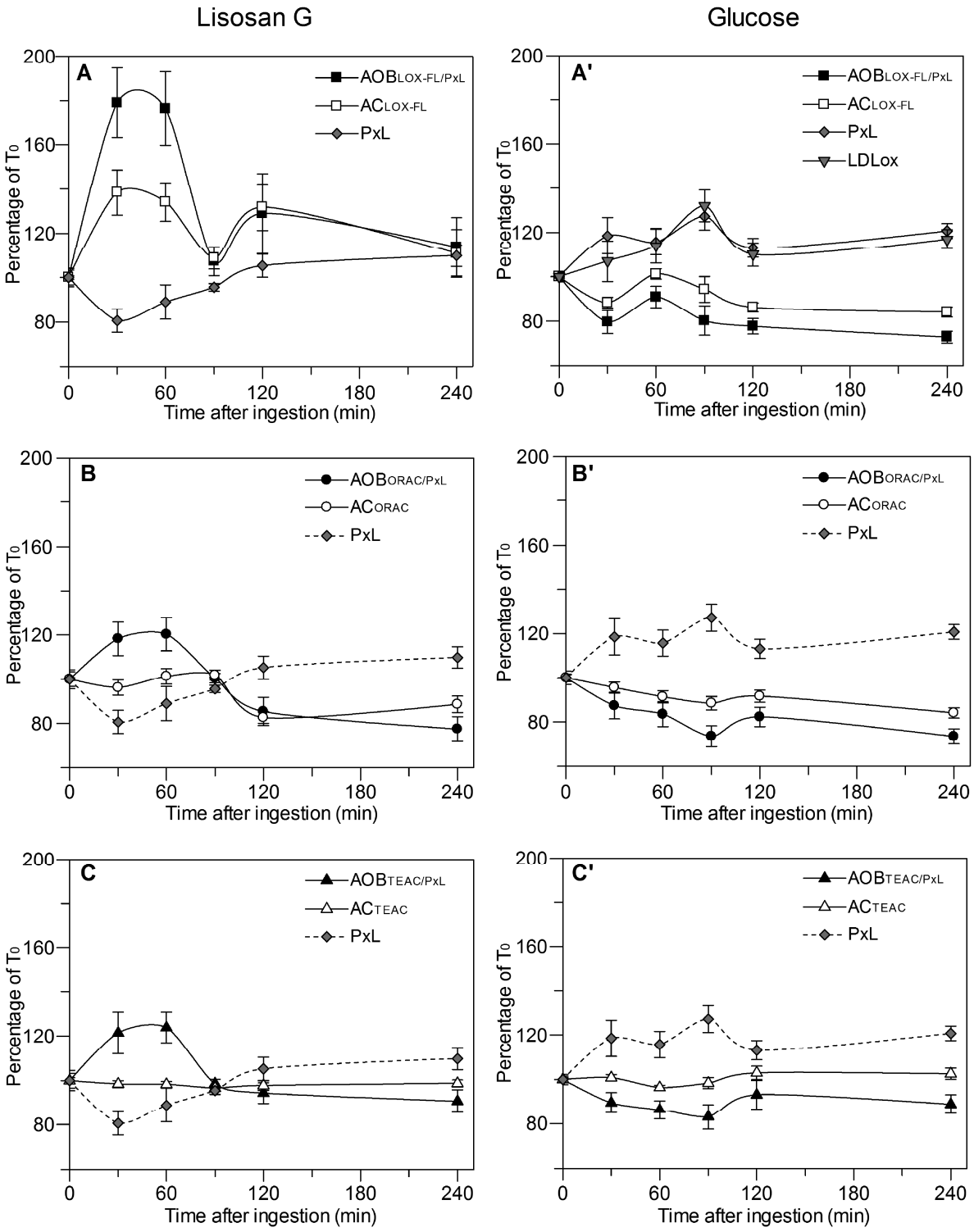

Fig. 1. *Serum Antioxidant Capacity (AC), Peroxide Level (PxL) and Antioxidant/Oxidant Balance (AOB) after consumption of Lisosan G or glucose.* Seven subjects ingested either 20 g (18 g d.w.) of Lisosan G or 50 g of glucose. AC was evaluated by means of LOX-FL, ORAC and TEAC methods. AOB was evaluated as LOX-FL/PxL, ORAC/PxL and TEAC/PxL ratios. PxL values shown in A and A' are reported as dotted lines also in B and C and in B' and C', respectively. The oxidized low-density lipoproteins (LDLox) were also assayed after glucose consumption (A'). AC profiles of Lisosan G shown in A, B, C are from Soccio et al. (2016), here reported to compare them with PxL and AOB profiles. Data are expressed as (%) of T_0 value and are reported as mean value ± SE (n = 7 subjects).

In this experiment, the reliability of PxL response was also validated by comparison with the direct determination of serum LDLox. Interestingly, the PxL profile resulted very similar to that of LDLox, with a generally high positive correlation (r=0.94, $P<0.01$) of areas under the PxL and LDLox profiles in different experiments. Since PxL determination is easier and inexpensive, it was routinely preferred. As for $AOB_{LOX\text{-}FL/PxL}$, also in the case of glucose it highlighted better than $AC_{LOX\text{-}FL}$ changes of antioxidant status by showing a general decrease up to -30% at 240 min (Fig. 1 A'). ORAC and TEAC methods were less performing than the LOX-FL one. ORAC even showed some AC decrease after 90 min following Lisosan G intake (Fig. 1 B), but highlighted a clear AC decrease due to glucose (Fig. 1 B'). TEAC showed no or little changes after Lisosan G or glucose intake, respectively (Fig. 1 C and C'). These data are in accordance with previous results (Lettieri-Barbato, Tomei, Sancini, Morabito, & Serafini, 2013; Soccio et al., 2016), stressing different performances of different AC methods and, in particular, a poor ability of TEAC to assess serum AC changes. These failures may be easily overcome by using AOB; in fact, although results were less performing than those obtained as $AOB_{LOX\text{-}FL/PxL}$, both $AOB_{ORAC/PxL}$ and $AOB_{TEAC/PxL}$ profiles were able to unmask antioxidant (Lisosan G, Fig. 1 B and C) and oxidant (glucose, Fig. 1 B' and C') effects that were hidden when AC alone was measured.

As a whole, AOB parameter, taking simultaneously into account both AC and oxidant status of serum, can be considered a much more appropriate and powerful tool than AC measurements for detection of changes in serum antioxidant status due to food intake.

3.2.2 Quantification of the effects of Lisosan G, glucose and pastas

In order to quantify changes of serum antioxidant status, areas under profiles of AC, evaluated using the three different methods, and PxL were considered (**Table 3**). In particular, areas were normalized in respect of the area measured in the absence of food intake, *i.e.* the area below the AC or PxL values at baseline (T_0). In comparison with Lisosan G and glucose, the effects of intake of four pastas, containing both antioxidants and starch, were analyzed: BO, BW and R pastas, as well as R pasta consumed with Lisosan G.

Unfortunately, the (%) changes were limited and AC-Area of ORAC and TEAC were mostly unable to assess differences between Lisosan G and glucose. Only AC-Area of LOX-FL was able to point out statistical differences between Lisosan G and glucose, showing respectively some increase and decrease of AC. Symmetrically, PxL-Area also showed some decrease and increase of PxL for Lisosan G and glucose, respectively. As a whole, in agreement with data from Fig. 1, data from **Table 3** make the ranks obtained by using separately AC and PxL inconclusive.

A completely different outline emerges from the use of AOB-derived parameters (**Table 4**).

Table 3 - *"Antioxidant Capacity"-Area (AC-Area), evaluated by LOX-FL, ORAC and TEAC methods, and "Peroxide Level"-Area (PxL-Area) of serum after consumption of different foods in seven subjects.* For each tested food, AC-Area and PxL-Area values are reported, representing the area under profiles of AC or PxL *vs* time (from 0 to 240 min) of the tested food respectively, expressed as (%) of basal area. Data are reported as mean value (n = 7 subjects). Within the same column, different letters indicate significant differences at 0.05 *P* level, according to the Duncan's test.

Food	Serving size (g f.w.)	AC-Area (% of basal area)			PxL-Area (% of basal area)
		LOX-FL	ORAC	TEAC	
BO Pasta[1]	70	107 [b]	96 [b]	101 [ab]	87 [d]
Lisosan G	20	123 [a]	92 [bc]	98 [abc]	100 [c]
R Pasta[2]+Lisosan G	70+20	98 [bc]	85[c]	102 [a]	113 [b]
BW Pasta[3]	70	103 [b]	96 [b]	94 [c]	128 [a]
R Pasta[2]	70	86 [d]	105 [a]	96 [bc]	96 [a]
Glucose	50	90 [cd]	91 [bc]	101 [ab]	116 [b]

[1] BO pasta: pasta supplemented with bran oleoresin; [2] R pasta: reference pasta; [3] BW pasta: pasta supplemented with bran water extract.

AOB-Area has the same meaning of AC-Area, indicating the (%) change of the area under AOB profile in respect of the basal area, thus showing how serum AOB changes as a result of food intake. On the other hand, AOB-Index is calculated like glycemic index, *i.e.* as ratio between area under AOB profile of the tested food and area under AOB profile of 50 g of glucose. This last parameter may take into account the effect on AOB of the starch component of pasta (in terms of glucose released from starch digestion). In all cases, a clear highly significant superiority of the very active antioxidant supplement Lisosan G is highlighted in respect of the pro-oxidant glucose (**Table 4**), thus making reliable the analysis. A remarkable serum antioxidant effect, equal or even higher to that of Lisosan G, is attributed to BO pasta by AOB-Area,
evaluated as LOX-FL/PxL or as ORAC/PxL and TEAC/PxL ratios, respectively. As expected, the non-supplemented R pasta showed an effect statistically equal or slightly lower than that of glucose, in terms of AOB-Area determined as LOX-FL/PxL and ORAC/PxL or TEAC/PxL, respectively. Unfortunately, both the BW pasta and the R pasta added with Lisosan G, unlike BO pasta, resulted incapable of exerting a beneficial antioxidant effect on serum, as their AOB-Area values resulted equal or even lower compared to that of glucose (**Table 4**). AOB-Index essentially confirmed data of AOB-Area, but had the added dimension of further enhancing the changes, with more than 65% increase of BO pasta in respect of the R one in the case of LOX-FL/PxL assay.
AOB-Index also pointed out a significant advantage of R pasta consumed with Lisosan G compared to R pasta alone when analyzed by LOX-FL/PxL and TEAC/PxL.

Table 4 - *"Antioxidant/Oxidant Balance"-Area (AOB-Area) and "Antioxidant/Oxidant Balance"-Index (AOB-Index) of serum, evaluated as LOX-FL/PxL, ORAC/PxL and TEAC/PxL ratios, after consumption of different foods in seven subjects.* For each tested food, AOB-Area and AOB-Index values are reported. The AOB-Area values represent the area under the profile of AOB *vs* time (from 0 to 240 min) of the tested food, expressed as (%) of basal area. As for the AOB-Index, values represent area of each tested food expressed as (%) of area relative to consumption of glucose. Data are reported as mean value (n=7 subjects). Within the same column, different letters indicate significant differences at 0.05 *P* level, according to the Duncan's test.

Food	Serving size (g f.w.)	AOB-Area (% of basal area)		
		LOX-FL/PxL	ORAC/PxL	TEAC/PxL
BO Pasta[1]	70	127 [a]	113 [a]	119 [a]
Lisosan G	20	133 [a]	95 [b]	101 [b]
R Pasta[2]+Lisosan G	70+20	89 [b]	76 [c]	89 [c]
BW Pasta[3]	70	82 [b]	78 [c]	76 [d]
R Pasta[2]	70	66 [c]	80 [c]	74 [d]
Glucose	50	79 [bc]	80 [c]	89 [c]
Food	**Serving size (g f.w.)**	**AOB-Index (% of glucose area)**		
		LOX-FL/PxL	**ORAC/PxL**	**TEAC/PxL**
BO Pasta[1]	70	165 [a]	145 [a]	137 [a]
Lisosan G	20	173 [a]	123 [b]	119 [a]
R Pasta[2]+Lisosan G	70+20	132 [b]	106 [bc]	120 [a]
BW Pasta[3]	70	110 [bc]	99 [c]	87 [b]
R Pasta[2]	70	98 [c]	111 [bc]	98 [b]

[1] BO pasta: pasta supplemented with bran oleoresin; [2] R pasta: reference pasta; [3] BW pasta: pasta supplemented with bran water extract.

On the contrary, no beneficial effect was observed after BW and R pasta intake, showing an AOB-Index close to 100 or lower; therefore, BW and R pastas are not different from each other and from glucose as for *ex vivo* AC tests. Interestingly, highly statistically significant positive correlations were obtained between AOB-Area and AOB-Index calculated as LOX-FL/PxL (r=0.988, $P \leq 0.01$), as well as ORAC/PxL (r=0.976, $P \leq 0.01$) and TEAC/PxL (r=0.925, $P \leq 0.05$). Moreover, significant positive correlations were found also among AOB-Area data obtained as different AC/PxL ratios (LOX-FL/PxL *vs* ORAC/PxL r=0.814, $P \leq 0.05$; LOX-FL/PxL *vs* TEAC/PxL r=0.896, $P \leq 0.05$; ORAC/PxL *vs* TEAC/PxL r=0.920, $P \leq 0.05$). This last point strengthens the effectiveness and reliability of AOB approach, able to provide the same results whatever the assay adopted to measure AC. Differences among BO, BW and R pastas, observed in **Table 4** in terms of AOB-Area and AOB-Index, are better highlighted in **Fig. 2**, reporting serum AOB profiles of pastas obtained using all three AC methods. The intake of BO pasta caused a very high increase of AOB

measured as LOX-FL/PxL up to about 70% after 60 min (Fig. 2 A). Then, AOB gradually decreased, to reach a value of about +20% after 240 min. A significant increase (of about 40%) of serum antioxidant status after BO pasta intake was highlighted also by AOB measured as ORAC/PxL and TEAC/PxL. On the contrary, the ingestion of both BW (Fig. 2 B) and R (Fig. 2 C) pastas caused a general decrease of AOB values measured as LOX-FL/PxL, up to about -30% and -50% after 90 min, respectively. In both cases, AOB measured by both ORAC/PxL and TEAC/PxL also pointed out a significant decrease. Data from Fig. 2 indicate that, whatever the assay used, BO pasta is able to improve AOB of serum, while BW induces a worsening similar to a conventional pasta. In practice, BO pasta is not only able to counteract the detrimental effect of starch/glucose, but even to enhance serum antioxidant status. These results are in accordance with Khor et al. (2014), showing that the ingestion of a phytonutrient-poor food and its individual fat/protein or sugar components rapidly increases plasma oxidative activity, whereas this is not observed after ingestion of a kilojoule-equivalent phytonutrient-rich food. It should be noticed that serum AOB response to the pastas studied in this paper cannot depend on different energy content or different content of macronutrients (**Table 1**). In particular, only dietary fibre content significantly differed in BO and BW pastas compared to R pasta; nevertheless, AOB responses (**Table 4**, Fig. 2) appear unrelated to fibre content.

These observations strongly suggest that different AOB response after pasta consumption cannot depend on different energy intake in terms of kJ, as well as on different nutrient composition reported in **Table 1**, but it may be due to the different composition in terms of antioxidant compounds. In particular, BO pasta showed very high total tocochromanol and carotenoid contents (1511±180 and 23±3 μg per serving, respectively), with an enrichment of 3.1 and 3.4 times compared to the non-enriched pasta, respectively (Pasqualone et al., 2016). Specifically, among tocochromanols, α-tocopherol, β-tocopherol and α-tocotrienol even increased about 25, 17 and 10 times, respectively, while, among carotenoids, the highest increase of about 6 times was obtained in both zeaxanthin and β-criptoxanthin contents (Pasqualone et al., 2016).

On the contrary, BW pasta enriched in hydrophilic/phenolic compounds failed to induce a positive effect. This may also depend on the low final enrichment in antioxidants in BW pasta, showing a total phenolic content equal to 89±0.7 mg ferulic acid eq., on a serving size basis, with an increase of only 1.3 times compared to the non-enriched pasta (Pasqualone et al., 2016).

So, lower amount of antioxidants taken with this pasta in respect of BO pasta may induce lower AOB response.

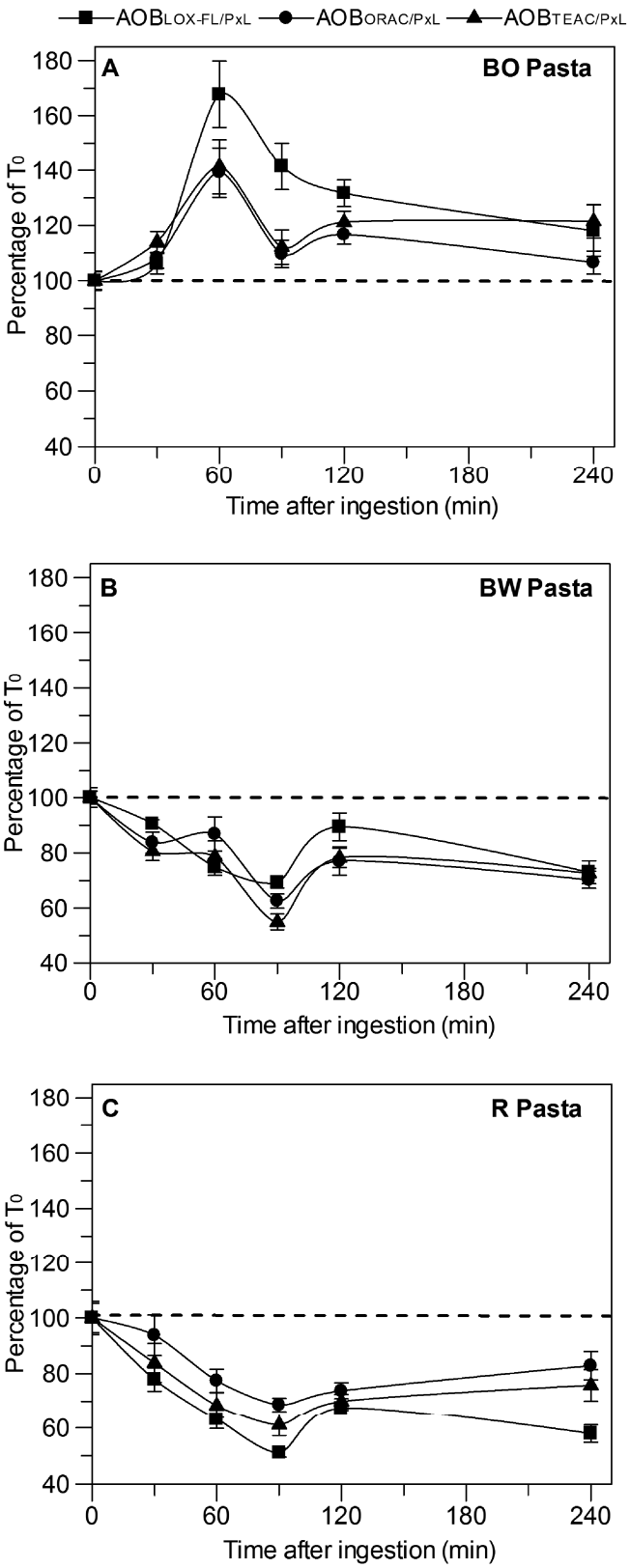

Fig. 2. *Serum Antioxidant/Oxidant Balance (AOB) after consumption of bran oleoresin (BO)-supplemented pasta (A) or bran water extract (BW)-supplemented pasta (B) or reference (R) pasta (C).* Seven subjects ingested 70 g (f.w.) of BO or BW or R pasta. AOB was evaluated as LOX-FL/PxL, ORAC/PxL and TEAC/PxL ratios. Data are expressed as (%) of T_0 values and reported as mean value ± SE (n = 7 subjects).

Nevertheless, it should be noticed that also the addition to R pasta of Lisosan G, providing a high content of hydrophilic/phenolic antioxidant compounds (see 3.1 section), cannot elicit increase when evaluated as AOB-Area, while only limited improvement in relation to R pasta was observed when evaluated as AOB-Index. Consequently, addition of pasta with lipophilic rather than hydrophilic/phenolic antioxidants appears more effective in preserving and improving antioxidant status of serum.

No statistically significant correlations were found between data of **Tables 2** and **4**, under our experimental conditions, thus confirming that AC measurements of food extracts by *in vitro* assays may not be predictive of food effects on serum antioxidant status assayed by *ex vivo* approach. However, in the case of Lisosan G, the very high activity measured *in vitro* may explain the high antioxidant effect observed on serum after the dietary supplement intake. As for pastas, only the LOX-FL method was able to highlight a higher AC of lipophilic antioxidants of BO pasta. This result is in accordance with generally good performances of methods based on soybean LOX-1 secondary reactions (Pastore et al., 2000, 2009; Soccio et al., 2016), but the resounding effect on serum AOB remained quantitatively unpredictable on the basis of *in vitro* analysis. This observation is in agreement with several reports stating that assessment of putative beneficial effects of food antioxidants on consumers only by means of widely used *in vitro* AC assays seems to be questionable and rather unrealistic (Fraga et al., 2014; Huang et al., 2005; Pompella et al., 2014).

However, accurate *in vitro* dissection of AC of food extracts containing different classes of antioxidants may contribute to characterization of antioxidant-rich foods and may help to point out the more promising ones deserving an in depth investigation by physiological approaches. On the other hand, *ex vivo* analysis of serum AOB after food intake may have a series of advantages. First of all, AOB may implicitly take into account bioavailability, *i.e.* the fraction of an ingested nutrient or compound that reaches the systemic circulation and the specific sites where it can exert its biological action (Visioli et al., 2011). This is an important point, since it has been shown that bioavailability of phytochemicals varies depending on food source and dose. Moreover, AOB will consider antioxidant metabolism, *i.e.* possible transformations by gut microbiota as well as intestinal and hepatic metabolism (*e.g.* glucuronidation, sulfation, and methylation). Gut microbiota, in particular, may deeply transform phenolic compounds (Visioli et al., 2011). Obviously, many transformations can take place beyond the four hours taken into consideration here.

By a methodological point of view, the new AOB approach has very high reliability so that, whatever the assay used, the results are the same, thus showing certainty of outcome, while

information from serum AC measurements is somehow unclear and also dependent on the assay method adopted.

4. Conclusions

The lipophilic antioxidant-enriched BO pasta, containing high level of tocotrienols, tocopherols and carotenoids, extracted by SC-CO_2 technology from durum wheat bran, allows an improvement of antioxidant status of serum after intake. BO pasta has the capability to compensate serum oxidation due to glucose released from starch and, notably, even increases antioxidant status similarly to the highly active wheat antioxidant-enriched food supplement Lisosan G. This is a prominent effect since blood plays a central role in the homeostasis of cellular redox status by conveying and releasing antioxidants in the body; moreover, the maintenance of blood physiological antioxidant status may preserve endothelial function, which is thought to be an essential determinant of healthy aging (El Assar, Angulo, & Rodríguez-Mañas, 2013). On the contrary, the hydrophilic/phenolic antioxidant-enriched BW pasta induces no different effect on serum antioxidant status than a conventional pasta. Also the conventional reference pasta added with a Lisosan G, which is strongly enriched with hydrophilic/phenolic antioxidants, cannot improve AOB so as BO pasta. These results suggest less effectiveness of hydrophilic/phenolic compounds in respect of the lipophilic ones during the time window when the glucose released from starch digestion induces blood oxidation.

These findings were obtained by using the novel AOB approach. The good performance of this approach depends on the simultaneous evaluation of AC and PxL, the latter being lowered by antioxidants in a manner that cannot be accounted when the AC alone is measured. Therefore, a more comprehensive and effective assessment of antioxidant status of serum after food intake may be carried out. Further studies about serum AOB approach regarding other foods different from pasta are worthwhile.

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Article 4:

The effect of sulforaphane on Glyoxalase I expression and activity in peripheral blood mononuclear cells

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Abstract

Recent studies suggested that sulforaphane (SR), a compounds formed in *Brassica* vegetables, can increase expression/activity of Glyoxalase I (GloI), the enzyme involved in the degradation of methylglyoxal (MG). The activation can be achieved at doses corresponding to the consumption of a broccoli portion. Such effect has been shown, using pure SR, in rat cardiomyocytes, human hepatoma HepG2 cells, BJ fibroblasts and neuroblastoma cells. In this study, we investigated the effect of SR on the expression/activity of GloI in peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from 8 human volunteers and incubated with SR (2.5 μM) for 24 and 48 h. GloI activity/expression, reduced glutathione (GSH) and the expression of genes that encode the glutathione-S-transferase (GSTP1, GSTT2 and GSTM2) were measured. SR was not able to induce a substantial change of GloI expression/activity. GSTP1 expression slightly increased after 24 h incubation (1.08-fold), but not after 48 h incubation while the isoform GSTT2 and GSTM2 were under the detection limit. Reduced glutathione (GSH) sharply decreased upon incubation with SR, suggesting the formation of a GSH-SR adduct that could significantly influence the actual concentration of SR available within the cells.

Keywords: Glyoxalase I; sulforaphane; peripheral blood mononuclear cells; glutathione; glutathione-S-transferase

1. Introduction

1.1 Glyoxalase and Methylglyoxal physiological relevance

Glyoxalase I (GloI) is a key enzyme of the enzymatic defense against the dicarbonyl compound methylglyoxal (MG), which is mainly formed as a by-product of glycolysis [1]. MG is the major precursor in the formation of advanced glycation end products (AGEs) and the GloI protects human body from the accumulation of the AGEs. GloI catalyzes the formation of S-D-lactoylglutathione from hemithioacetal formed non-enzymatically from reduced glutathione (GSH) and α-oxoaldehyde [1, 2]. Glyoxalase II, another enzyme of the glyoxalase system, catalyzes the hydrolysis of S-D-lactoylglutathione to the corresponding aldonic acid, reforming the GSH that is consumed in the GloI-catalysed reaction. In patients affected by diabetes and other age-related diseases, AGEs accumulate in most sites of diabetes complications, including kidney, retina, and atherosclerotic plaques [1, 2]. Moreover, new findings suggest that AGEs may play a role in promoting neurodegenerative diseases [1, 3]. Neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's diseases, are characterized by an abnormal accumulation and aggregation of proteins, which is triggered by AGEs [1, 3]. An increase of Glo enzymes expression is a way to protect our body against the accumulation of MG and the importance of the consumption of dietary compounds that can enhance the Glo activity was recently highlighted [4].

1.2 Sulforaphane can modulate Glyoxalase and glutathione S-transferases activity

The regular consumption of resveratrol and hesperetin in humans, which are dietary bioactive compounds, for 8 weeks has been recently studied and the results showed an increase of GloI activity by 27% [5]. However, the concentration of those compounds cannot be achieved by diet, since those compounds are present in low concentration in grapes and citrus fruit. Sulforaphane (SR) is an isothiocyanate that is also a dietary bioactive compound. It is present in *Brassica* vegetables; broccoli (*Brassica oleracea* var. italica) is particularly rich in this compound [6]. Epidemiological studies show correlation between dietary intake of *Brassica* vegetable and lower occurrence of cancer and various chronic diseases [7-9]. The bioactivity of SR is the results of a multitude of molecular mechanisms that act simultaneously [7-9]. Among those, SR is reported to be involved in modulation of xenobiotic metabolism by enhancing the expression of phase II enzymes, such as glutathione S-transferases (GST) [9]. GST catalyzes the conjugation of GSH with xenobiotic compounds for detoxification. Moreover, studies have shown that SR can enhance GloI activity by upregulation its genetic expression. After incubation with 2 μM of SR both human hepatoma HepG2 cells and BJ fibroblasts, the GloI activity increased (by 2-3-fold) and a related

dose-dependent increase in GloI mRNA was reported [10]. Another study showed that cells isolated from primary neonatal rat cardiomyocytes and treated with 5 μM of SR for 24 h and subsequently exposed for other 24 h to MG (1 mM), showed a significant lower MG-induced damage [11]. In another study, SH-SY5Y neuroblastoma cells were incubated in 2.5 μM of SR and after 24 and 48 h, the GloI activity increased significantly [12]. Most importantly, the concentrations of SR that were tested on SH-SY5Y neuroblastoma cells, human hepatoma HepG2 cells and BJ fibroblasts can be achieved by consuming broccoli [13, 14].

1.3 Aim of the study

The aim of this study was to investigate the effect of SR on the GloI expression and activity in peripheral blood mononuclear cells (PBMCs). PBMCs were chosen because they are human primary cell models, thus they are a good model to estimate what could be the *in vivo* effect of SR. For this purpose, PBMCs were isolated from 8 human volunteers and were incubated in 2.5 μM SR for 24 h and 48 h to simulate a daily consumption of a broccoli portion and the GloI activity/expression were analyzed after 24 and 48 h of incubation. Moreover, the total glutathione concentration and the GSTP1, GSTT2 and GSTM2 expressions, which encode the GST, were analyzed.

2 Material and Methods

2.1 Chemicals

Chemicals and solvents at analytical and HPLC-grade purity were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). WST-1 Cell Proliferation Assay was purchased from Roche Diagnostics (Mannheim, Germany). D,L-Sulforaphane (SR) was obtained from Merck, Germany (574215). HT Glutathione Assay Kit was purchased from Trevigen, Inc. (Gaithersburg, USA). Glyoxalase I human recombinant, expressed in E. coli (SRP6125, Sigma) Glutathione S-Transferase from equine liver (G6511, Sigma) Other chemicals are reported in the sections where their use is described.

2.2 PBMC isolation

Peripheral blood samples were collected from 8 healthy adult donors (4 women and 4 men aged between 30 and 55 years). The samples were collected after signing written informed consent by donors. About 50 ml of blood from each donor was collected into Cell Preparation Tube (CPT, 362782, Sanabio) and PBMCs were isolated as following. After blood collection, the CPT tubes were gently inverted about 10 times and centrifuged at 1800 g for 20 min at room temperature.

After centrifugation, plasma was removed and the white cloudy layer, containing PBMCs, was carefully transferred into a 15 mL tube and washed twice with Phosphate Buffered Saline (PBS) by centrifugation at 300 g for 10 min. Cell pellet was dissolved in 1 mL of RPMI-1640 medium supplemented with 10% heat–inactivated FCS and 1% penicillin-streptomycin and cells were counted by using Trypan blue staining.

2.3 Cell culture and treatments

PBMCs were grown in RPMI-1640 in the absence (control) and in the presence of 2.5 μM SR, at 37 °C, in a humidified incubator containing 5% CO_2 for 24 and 48 h.

2.4 Assessment of PBMC viability

Cell viability of PBMCs incubated in the absence (positive control) and the presence of 2.5 μM SR for 24 and 48 h was evaluated by using the WST-1 Cell Proliferation Assay (Roche). It is a colorimetric assay based on the cleavage of a tetrazolium salt, 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) by mitochondrial dehydrogenases in viable cells to form formazan, an orange compound which absorb at the wavelength of 450 nm. Briefly, at the end of each experiment 10% Triton X-100 was added to the culture medium of PBMCs used as negative control. After 15 min of incubation at 37 °C, cells were centrifuged at 219 g for 5 min and about 170 μL of supernatant was removed. At this point 70 μL of fresh medium and 10 μL of WST-1 reagent were added and cells were incubated at 37 °C and 5% CO2 in a humidified atmosphere. The absorbance was measured at 450 nm every 30 min using a Multiskan Ascent reader (Thermo Labsystem) until OD of the positive control was around 1-1.5 value. The viability of both SR-treated cells and negative control was calculated as (%) variation in absorbance at 450 nm with respect to the positive control.

2.5 GloI activity assay

GloI activity in PBMC lysate was assessed according to the method initially described by Clelland and Thornalley (1990) [24] and recently reviewed by Arai et al (2014) [25], with minor modifications. Briefly, PBMCs were collected, washed once with PBS and lysed in cold 10 mM sodium phosphate buffer pH 7.0 supplemented with 0.1% Triton X-100 and inhibitor protease cocktail. The GloI activity assay was performed by the addition of increasing amounts (from 20 to 200 μL) of cell lysate to a reaction mixture (final volume = 1 mL) containing 50 mM sodium phospate buffer at pH 6.6 and 3 mM MG / GSH mixture, which had been equilibrated at 37 °C for 10 min before sample addition. The reaction was monitored spectrophotometrically by following

the absorbance increase at the wavelength of 240 nm due to the formation of *S*-D-lactoylglutathione (Cary 60 UV-Vis, Agilent Technologies). The reaction rate, expressed as ΔA240 / min, was calculated as the tangent of the experimental curve at which the highest variation of absorbance per min was reached. Enzymatic activity, expressed in EU/mg of protein, was calculated using a molar absorption coefficient (Δμ240) equal to 2.86 $mM^{-1}cm^{-1}$. The protein content in PBMC lysate was determined by Bradford's method using BSA as a standard.

2.6 Determination of the reduced glutathione (GSH) content in the PBMC lysate

The GSH content in PBMC lysate was determined spectrophotometrically by using HT Glutathione Assay Kit (Trevigen). In this assay sulfhydryl groups of GSH reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce both a yellow colored 5-thio-2-nitrobenzoic acid (TNB), that absorbs at 405 or 414 nm, and the mixed disulfide, GSTNB, that is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to the concentration of glutathione in the sample. This method allows to measure the total glutathione because the oxidized glutathione (GSSG) present in the sample is reduced by glutathione reductase, present in the reaction mixture, to two molecules of GSH. To measure GSSG content, free thiols present in the reaction must be masked with 2 M of 4-Vinylpyridine. The concentration of GSH is thus estimated by subtracting the measured GSSG levels from the measured total (GSH plus GSSG) glutathione in each sample.

After incubation PBMCs were collected, washed once with PBS, deproteinized with 5% (w/v) metaphosphoric acid and lysed by the addition of 0.1% Triton X-100 and inhibitor protease cocktail to the cell suspension. A Multiskan Ascent reader (Thermo Labsystem) and 96-well plates were used for measurements. The reaction medium (final volume of each well: 0.2 mL) consisted of 1X assay buffer and samples (untreated PBMC lysates or 4-Vinylpyridine-treated PBMC lysates for the determination of total glutathione and GSSG, respectively). The reaction was started by adding 150 μL of freshly-prepared reaction mix, containing DTNB and the glutathione reductase, and monitored by recording the absorbance increase at 414 nm, due to TNB production, at 1 minute intervals over a 10 minute period. At least three different amounts of sample were analysed in triplicate and the slope from the maximum linearity portion of the each curve was determined. Glutathione (total and GSSG) concentrations for each sample, was determined by comparing the slope of the samples with that of the standard curve obtained by using a GSSG standard. GSH content was thus calculated and expressed in nmol / mg of protein. The protein content in PBMC lysate was determined by Bradford's method using BSA as a standard.

2.7 Determination of change in GSH concentration after 24 h of incubation with SR

The change in the GSH content of a standard solution prepared with the commercial GSH (Sigma Aldrich) after 24 h of incubation with SR at 37 °C, was evaluated according to Browne and Armstrong (1998) with slight modifications. A CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) and 96-well plates were used for measurements. The assay was conducted in a mixture containing 50 mM sodium phosphate buffer (pH 8.0) plus 5 mM ethylenediaminetetraacetic acid (EDTA) and GSH in the absence (control) and in the presence of SR (2.5 and 50 µM). The same solutions were prepared with the addition of GST enzyme (1 U / mL). After the addition of 0.38 mM of o-phthaldialdehyde (1 mg/mL in ethanol), the reaction mixture was incubated at 37 °C for 15 min. Fluorescence was measured using wavelengths of excitation and emission of 350 and 420 nm, respectively. The change in GSH concentration after SR treatment was calculated as (%) fluorescence decrease with respect to control.

2.8 Real Time PCR

After treatment, PBMCs were washed once with PBS before addition of Trizol reagent (750 µL /1*106 cells) to lyse the cells and cells were subsequently frozen. After thawing, samples were kept at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Next, chloroform (150 µl; Merck) was added to the PBMCs before shaking the samples vigorously for 15 seconds. After 15 minutes incubation (room temperature), the resulting mixture was centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube containing 5 µl Glycogen (5mg/ml;Fisher Scientific Emergo). Next, 375 µl 2-propanol (Sigma) was added and the samples were mixed by vortexing. After 10 minutes incubation (room temperature), the samples were centrifuged at 12,000 × g for 10 minutes at 4°C. Supernatant was removed and the RNA pellet was washed by adding 1 ml of 75% ethanol. Samples were vortexed and centrifuged at 12,000 × g for 5 minutes at 4°C. The washing step was repeated once. The RNA pellets were air dried for 20 minutes after complete removal of ethanol. The RNA pellet was dissolved in 20 µl RNAse free water. RNA (250 ng) was used to make cDNA using the iScript cDNA synthesis kit (Biorad 1708891) according to the manufacturer's instructions and for the rtPCR IQ SensiMix SYBR master mix (Bioline, London, UK), was used. Next, cDNA (10 ng) was used for each Real Time PCR reaction on a Biorad CFX. Housekeeping genes were RPS18 and RPLPO.

2.9 Statistical Analysis

A paired T-test two-tailed distribution has been used to determine significant difference for cell viability between the control cells and the cells incubated with SR (two tests, one to compare control and incubation at 24h, and one test to compare control and 48 h incubation, both the tests). A paired T-test one-tailed distribution to determine significant difference for GloI activity, GloI and GSTP1 expression and GSH concentration between the control cells and the cells incubated with SR (two tests, one to compare control and incubation at 24h, and one test to compare control and 48 h incubation).

3. Results

PBMCs viability was not significantly affected by treatment with SR (2.5 μM) indicating no cytotoxic effect of SR on PBMC (**Table 1**). Moreover, since SR was dissolved in dimethyl sulfoxide in a preliminary experiment the potentially cytotoxicity of this solvent on PBMCs was tested, and no cytotoxic effect was showed (data not shown).

Table 1. *Cell viability, GSH content and GSTP1 gene expressions of PBMCs grown for 24 h and 48 h in absence (control) and in presence of SR (2.5 μM).* Data are reported as absolute value (mean values ± S.E., n=8 subjects). The *p* values were calculate by comparing the control values with 24 h SR incubation samples, and with the 48 h SR incubation samples (paired T-test two-tailed distribution for the cell viability and paired T-test one-tailed distribution for GSH and GSTP1 expression).

Assays	24 h			48 h		
	Control	2.5 □M SR	*p*	Control	2.5 □M SR	*p*
Cell viability	1.71 ± 0.22[a]	1.65 ± 0.18[a]	0.470	1.38 ± 0.16[a]	1.60 ± 0.16[a]	0.06
GSH	34.75 ± 3.63[b]	9.49 ± 1.51[b]	< 0.001	30.8 ± 3.26[b]	12.15 ± 1.35[b]	< 0.001
GSTP1 gene expression	7.56 ± 0.27[c]	8.21 ± 0.17[c]	0.002	7.67 ± 0.17[c]	7.56 ± 0.32[c]	0.322

[a] $A_{450\ nm}$; [b] nmol / mg of protein; [c] Δ CT.

In **Figure 1**, the effects of SR on both GloI gene expression and activity in PBMCs are shown. The bars at 24 h represent the expression and activity relative to the control at 24 h without SR. The bars at 48 h represent the expression and activity relative to the control at 48 h without SR. SR did not affect the GloI activity. Regarding the gene expression, after 48 h of SR treatment a slight significant increase (1.03-fold) was measured. GSH plays an important role in the glyoxalase system, thus the effect of SR treatment on intracellular GSH levels was evaluated (**Table 1**).

No oxidized glutathione (GSSG) was detected, only the reduced form. SR treatment caused a strong decrease in GSH levels compared to GSH levels of the control cells (**Table 1**), with a reduction by 73% and 61% of intracellular GSH concentration after 24 and 48 h of SR treatment, respectively.

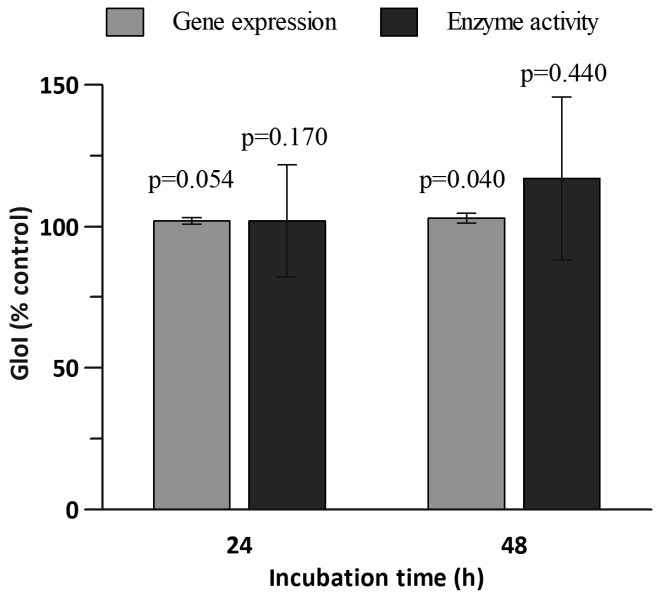

Figure 1. *Effect of SR treatment on GloI gene expression and activity*. PBMCs were treated with 2.5 μM SR for 24 and 48 h followed by RT-PCR on GloI gene and GloI enzymatic activity assay. Data are shown as percentage of control: the bars at 24 h represent the expression and activity relative to the control at 24 h without SR, and the bars at 48 h represent the expression and activity relative to the control at 48 h without SR. Each bar represents means ± SE; n = 8 subjects. The p values were calculate by comparing the control values with 24 h SR incubation samples, and with the 48 h SR incubation samples (paired T-test one-tailed distribution).

To verify if the GSH concentration was lower due to adducts formed with SR, the direct chemical interaction between GSH and SR under our experimental conditions was performed (**Figure 2**). Different concentrations of GSH standard solution were incubated at 37 °C for 24 h in absence of SR (control) and in presence of SR (50 μM). GSH and SR concentrations were chosen to mimic the ratio GSH/SR achieved in the cell culture experiments. GSH levels were measured using two different methods. Data obtained from the two methods are plotted as A_{414} nm (**Figure 2A**) and Arbitrary Units of Fluorescence (AUF, **Figure 2B**) *vs* the ratio nmol GSH / nmol SR as well as *vs* GSH concentration. In both cases, SR decreased the slope of the linear regression in comparison to that of the control (no SR). This suggests an interaction between GSH and SR and that the formation of a GSH-SR adduct may reduce the levels of GSH and SR in PBMCs. In order to understand if GST enzyme could increase the formation rate of those adducts, GSH was also incubated at 37 °C for 24 h with 50 μM SR in presence of 1U/ml of GST (**Figure 2**).

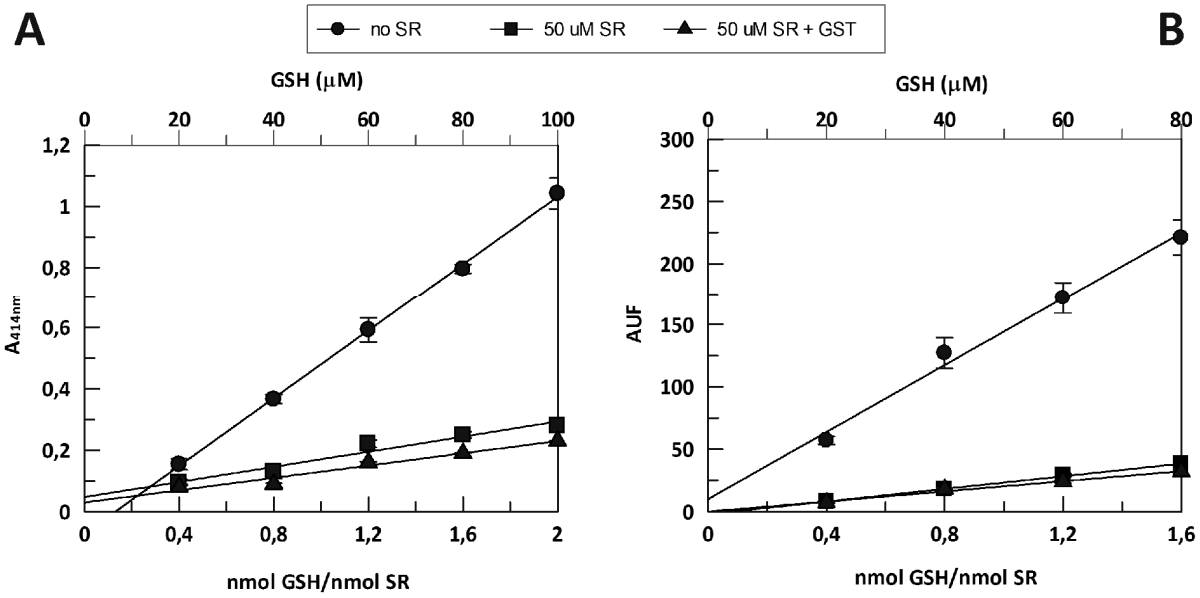

Figure 2. *Effect of presence of SR on GSH assayed in vitro*. A GSH standard solution (20-100 μM in A, 20-80 μM in B) was incubated for 24 h at 37 °C in absence of SR (control) and in presence of 50 μM SR alone or 50 μM SR + 1U/ml GST. GSH levels were measured using both HT Glutathione Assay Kit (A) and the fluorescence probe o-phthaldialdehyde (B) as reported in Methods. Vertical bars represent ± S.D. (n = 3).

The slopes of the lines in presence (50 μM SR + GST) and in the absence of GST (50 μM SR) were similar, suggesting that the chemical conjugation between the two compounds was dominant. GSTT2 and GSTM2 expression was too low to be detected while the values of GSTP1 are reported in **Table 1**. After 24 h, a slight but significant increase of GSTP1expression was measured (1.08-fold);, however after 48 h such increase was not confirmed, suggesting that the incubation with SR did not lead to an increase in GSTP1 expression.

4. Discussion

In this research, we found that at physiological conditions, SR treatment in PBMCs from healthy volunteers did not have an effect on GloI expression and activity (**Figure 1**). In contrast to our study, the incubation with SR led to a significant increase of GloI activity (by 2-3-fold) and to a related dose-dependent increase in GloI mRNA levels in human hepatoma HepG2 cells and BJ fibroblasts [10]. In SH-SY5Y neuroblastoma cells treated with SR, the GloI activity and expression increased significantly (by around 2-fold).

Since GloI catalyses the formation of S-D-lactoylglutathione from the hemithioacetal formed non-enzymatically from MG and GSH, we decided to measure the intracellular concentration of GSH. Results showed that SR treatment caused 73% and 61% reduction in GSH levels compared to the control cells after 24 and 48 h of SR treatment, respectively (**Table 1**). This finding was opposite

to the previous one on neuroblastoma cells: the concentration of GSH found in SH-SY5Y cells after 24 h incubation with SR, were significantly higher than the control [12, 15]. Previous studies showed that isothiocyanates molecules like SR, undergo reversible Michael addition reactions with nucleophiles including protein thiols and GSH. The reaction with GSH occurs both enzymatically, catalyzed by GST, [16] and non-enzymatically at physiological pH. The data showed in **Figure 2** clearly indicates that the interaction between GSH and SR led to the formation of a GSH-SR adduct. This is relevant because it implies that a reduced concentration of SR is actually available at the cellular level (**Figure 2**). The conjugation of SR with GSH was observed in another study in which SR and GSH were incubated in sodium phosphate buffer (pH 6.5), and the addition of cloned human GST, significantly increased the conjugation between GSH and SR [17]. Whereas, in this study, the addition of GST, did not increase the conjugation between GSH and SR (**Figure 2**), suggesting that at the applied conditions, the chemical conjugation between the two compounds was prevalent.

Moreover, we found no substantial increase of the expression of GSTP1. Evidences support the hypothesis that the modulating effect of SR on phase II enzymes is caused by the interaction of SR with Kelch-like ECH-associated protein 1 (Keap-1). Keap-1 is involved in the fast degradation of Nuclear Factor–like 2 (Nrf2), a transcription factor which binds to the antioxidant response element (ARE) in the upstream promoter region of many antioxidative genes, up-regulating their transcription among those, the genes encoding GST [18, 19]. In a study in which single aortic smooth muscle cells from spontaneously hypertensive rats or normotensive Wistar-Kyoto rats were isolated and incubated with SR (0.05 – 1.00 μM), it was shown that during 24 h incubation, SR increased GSH level in both the cells significantly and such increase was concentration-dependent [20]. Moreover, SR (0.1 μM) increased the activity of GST by 1.47-fold in cells from hypertensive rats and a slight increase of the GST activity in the normotensive cells from Wistar-Kyoto rats was detected at 0.5 μM SR [20]. In another study, rat aortic smooth muscle A10 cells were incubated with SR (0.25–5 μM) for 48 h, and the results showed an increase of cellular GSH and GST activity [21]. In a study in which human prostate cells (LNCaP) were incubated with 0.25–5 μM for 72 h, the results showed a dose dependent (5-10 μM) increase in GSH levels after 48 h incubation, while at lower doses, as the dose used in this study, no effect was detected [22]. Moreover, no changes in GSTA1 band (transcriptional response) appeared even after 72 h incubation [22].

Studies that have shown the potential enhancing effect on GloI expression and activity of compounds that are naturally present in foods such as SR, have inspired the design of this study. PBMCs were chosen as because, although it is still an *in vitro* study, those cells represent human

primary cell models [23] and the results may be a good base to design an human intervention to test the effect of SR *in vivo*.

In this study, at the conditions applied, SR did not increase the GloI expression/activity and the GSTP1 expression substantially. The potential formation of the GSH-SR adducts suggests that the actual concentration of SR available to the cells during incubation may have been lower and this may explain the lack of a substantial effect on GloI, and GSTP1 expression seen in this study. A longer exposure of PBMCs to SR could not be tested and longer exposure might have shown different results. Although, the use of PBMCs has the advantage of representing human primary cell models, the disadvantage is that the cells cannot be incubated for more than 48 h thus limiting the significance of the results.

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6. Author Contributions

Michela Alfarano contributed designing and performing the experiments and writing the article; Donato Pastore contributed designing the experiments and writing the article; Vincenzo Fogliano contributed designing the experiments and writing the article; Casper G. Schalkwijk contributed performing the experiments (Real Time PCR) and writing the article; Teresa Oliviero contributed designing the experiments and writing the article

7. Conflicts of Interest

All the authors declare that they don’t have conflict of interest.

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Article 5:

First evidence of the existence of Glyoxalase I in durum wheat mitochondria and study of modulation by phytochemicals

Article in progress

Abstract

The glyoxalase system, which includes GloI and GloII, is an enzymatic pathway that provides the primary defence against dicarbonyl glycation by catalysing the metabolism of methylglyoxal (MG), a toxic α,β-dicarbonyl ketoaldehyde. In plants, MG is mainly produced during glycolysis reactions and in the Calvin cycle of photosynthesis. In addition to its well-established roles in conferring abiotic and biotic stress tolerance, glyoxalase system has been found to play an important role both in plant reproduction and in the regulation of protein turn-over. Despite the relevant functional roles attributed to plant glyoxalases, modulation of these enzymes have been little investigated. Little is known also about glyoxalase activity in subcellular organelles.

In this study, activity and possible modulation by phytochemicals of GloI were evaluated in the highly purified mitochondrial fraction obtained from durum wheat (WM). A mitochondrial GloI was studied because this organelle is one of the main targets of the harmful effects of carbonylation/oxidative stress induced by MG. In particular, WM were chosen, since they were demonstrated to be an early target of ROS effects, as well as to possess some efficient dissipative systems able to counteract oxidative stress. Comparative measurements of highly purified recombinant human GloI enzyme were also carried out.

Interestingly, for the first time in plant mitochondria, a high GloI specific activity was measured in WM, showing hyperbolic kinetics, with Km and Vmax values equal to 0.27±0.02 mM and 0.133±0.001 EU/mg of protein, respectively. Moreover, the effect on WM-GloI activity of curcumin and quercetin, known as GloI inhibitors in mammals, and of resveratrol, able to activate both GloI activity and gene expression, as well as of the most relevant antioxidants of durum wheat grains, such as ferulic acid, sinapic acid and α- tocopherol, was assessed. Among the tested compounds, curcumin and quercetin were found to inhibit WM-GloI activity and, as expected, hGloI. A competitive nature of WM-GloI inhibition by curcumin and quercetin was demonstrated, with Ki values equal to 20 μM and 55 μM, respectively. Competitive inhibition by curcumin and quercetin was also confirmed for hGloI (Ki values equal to 90 μM and 2 μM, respectively). In conclusion, results of this study provide first insights on the existence of GloI activity in WM, as well as the identification of the first highly efficient modulators of this enzyme. With respect to this last point, possible physiological role of the inhibition of WM-GloI activity by curcumin and quercetin merits further investigations.

1. Introduction

In plants, methylglyoxal (MG), which is a highly reactive, cytotoxic α-oxoaldehyde, is mainly produced during glycolysis reactions and in the Calvin cycle of photosynthesis (Sankaranarayanan et al., 2017). Under normal plant growth conditions, MG is maintained at low basal levels; however, MG concentrations in plant cells rapidly increase, from 2- to 6-fold compared with normal conditions, under abiotic stress conditions such as salinity, drought, and cold stress (Yadav et al., 2005). A consistent increase in MG levels is also observed in aging plants; in broccoli (*B. oleracea* L var. italica), higher MG levels were observed (3.9 μM) in leaves of 65-day-old plants compared to 9-day-old plants (2.8 μM) (Rabbani and Thornalley, 2014). Light and dark cycles (diurnal cycles) also contribute to changes in the levels of MG-derived glycation adducts in plants. In *Arabidopsis*, MG-derived AGEs have been shown to display an oscillatory, diurnal behavior where maximal residual contents were observed in the middle of light and dark cycles (Bechtold et al., 2009).

Like animals, the MG detoxification process is primarily mediated by the glyoxalase system comprising GloI and GloII. GloI catalyses the isomerization of the hemithioacetal (HA) formed non-enzymatically from methylglyoxal (MG) and reduced glutathione (GSH) to S-D-lactoylglutathione (SLG). GloII catalyses the hydrolysis of SLG to D-lactate and reforms GSH consumed in the GloI-catalysed reaction step. A single detoxification step of MG to D-lactate has been described due to a unique glyoxalase pathway consisting of Glyoxalase III (GloIII); this class of enzyme was first identified in *E. coli* and does not require any cofactor. Contrary to the conventional GloI/II enzymes, GloIII required higher substrate concentrations and had very low specific activity, suggesting that it would come into action under extreme stress conditions when plants accumulate massive amounts of MG (Ghosh et al., 2016; Kwon et al., 2013).

Unlike most microbial and animal systems, in plants, glyoxalases exist as a multigene family. It has been reported that rice has a total of 19 GloI proteins encoded by 11 genes and 4 GloII proteins encoded by 3 genes (Mustafiz et al., 2011, 2014); while the model plant *Arabidopsis* has 22 GloI proteins encoded by 11 genes and 9 GloII proteins encoded by 5 genes (Mustafiz et al., 2011). The molecular mechanism, subcellular localization, and functional roles of these diverse isoforms are yet to be uncovered. However, the existence of multiple forms of these genes in plants probably indicates possible diverse, tissue-specific roles that the glyoxalase system may have, besides detoxification of MG (Sankaranarayanan et al., 2017). In the last three decades, numerous studies have reported various functional roles for plant glyoxalases, in addition to its well-established roles in conferring abiotic (salinity, drought, mannitol, ABA, extreme temperatures and heavy metal

stress) and biotic (living organisms such as bacteria, fungi, viruses, parasites, and insects) stress tolerance. In particular, it has been shown that glyoxalase enzymes are markers for cell division and play an important role both in plant reproduction and in the regulation of protein turn-over (Sankaranarayanan et al., 2017).

Despite the important functional roles attributed to the plant glyoxalases, the modulation of GloI enzyme has been little investigated. Moreover, to the best of our knowledge, no information is available about GloI activity assessment in subcellular organelles in plant systems and, in particular, in mitochondria. Nevertheless, mitochondria have been demonstrated as one of the main targets of MG-mediated carbonylation stress; in particular, MG can inhibit mitochondrial electron transfer chain and induce an increase in the level of H_2O_2 and $O_2^{\cdot -}$ (Li, 2016 and references therein). In the light of this, in this study GloI activity and its possible modulation by phytochemicals were studied in durum wheat mitochondria (WM). WM were used, since for these mitochondria an isolation procedure providing high yield of pure and intact organelles is available (Pastore et al., 1999). Moreover, WM were demonstrated as an early target of oxidative stress (Pastore et al., 2002), as well as to possess some transport pathways, having a relevant role in controlling ROS production under stress conditions (Pastore et al., 2007).

2. Materials and methods

2.1 Chemicals and plant material

All chemicals at the highest commercially available purity were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Curcumin, quercertin, sulforaphane and α-tocopherol were dissolved in dimethylsulfoxide (DMSO). Certified seeds of durum wheat (*Triticum durum* Desf. cv Ofanto) were kindly provided by the CREA-Cereal Research Centre (Foggia, Italy).

2.2 Durum wheat mitochondria isolation

WM purification was performed according to Pastore et al. (1999) with minor modifications, as reported in the Article 6. Protein content was determined by the method of Lowry modified according to Harris (1987) using BSA as a standard.

Purified mitochondria showed high intactness of inner and outer membranes and a good functionality, as evaluated by means of fluorimetric measurements of electrical membrane potential according to Pastore et al. (1999), Laus et al. (2008; 2011) and Soccio et al. (2013).

2.3 GloI activity assay

GloI activity was assessed according to the method initially described by Clelland and Thornalley (1991) and recently reviewed by Arai et al (2014). According to this method GloI activity was spectrophotometrically measured by following the absorbance increase at 240 nm due to the conversion of HA into the SLG. HA was preformed by incubation of 3 mM MG and 3 mM GSH in 50 mM sodium phospate buffer pH 6.6 at 37 °C for 10 min. HA solution was freshly prepared as it slowly isomerizes to SLG non-enzymatically.

GloI assay was performed by using a SpectraMax® M5 microplate reader and 96-well plates; the reaction medium (final volume of each well: 0.2 mL) consisted of 50 mM sodium phospate buffer pH 6.6 and the source of GloI enzyme (20 µg WM, previously lysed with 0.1% Triton X-100 or 0.1 µg hGloI); the reaction was started by adding the MG/GSH mixture.

The reaction rate, expressed as $\Delta A_{240} \cdot min^{-1}$, was calculated as the highest slope to the experimental curve. Enzymatic activity (EU) was calculated using the molar absorption coefficient ($\Delta\varepsilon_{240}$) obtained by subtracting ε_{240} of HA from ε_{240} of SLG (both able to absorb at 240 nm):

$$\mathbf{\Delta\varepsilon_{240} = \varepsilon_{240}\,[SLG] - \varepsilon_{240}\,[HA] = 2.86\ mM^{-1} \cdot cm^{-1}}$$

GloI activity assay was carried out in both the absence (control) and presence of phytochemicals. In this case measurements were carried out at different substrate concentrations using different (at least three) amount of phytochemicals. The GloI activity was determined as percentage (%) variation of the reaction rate measured in the presence of phytochemicals with respect to the control. Concerning antioxidants reconstituted in DMSO, measurements were carried out in presence of a constant DMSO concentration in the reaction mixture to exclude DMSO effect on GloI activity.

3. Results and discussion

To the best of our knowledge, no information is available about GloI activity assessment in subcellular organelles of plant systems. In the light of this, firstly, experiments were carried out in order to evaluate GloI activity in WM. Interestingly, SLG generation was observed by adding WM to the reaction mixture, occurring at a rate progressively increasing in the presence of increasing amount of WM proteins. Parallel measurements were always carried out using the easily available human recombinant GloI (hGloI, Sigma).

In figure 1, the dependence of reaction rate, expressed as EU (µmol/min), *vs* protein amount is reported for both WM-GloI (A) and hGloI (B). WM-GloI activity showed a linearly dependent behavior in the range from 10 to 30 µg of proteins. Similarly, the activity of hGloI was linearly dependent in the range from 0.025 to 0.25 µg of protein.

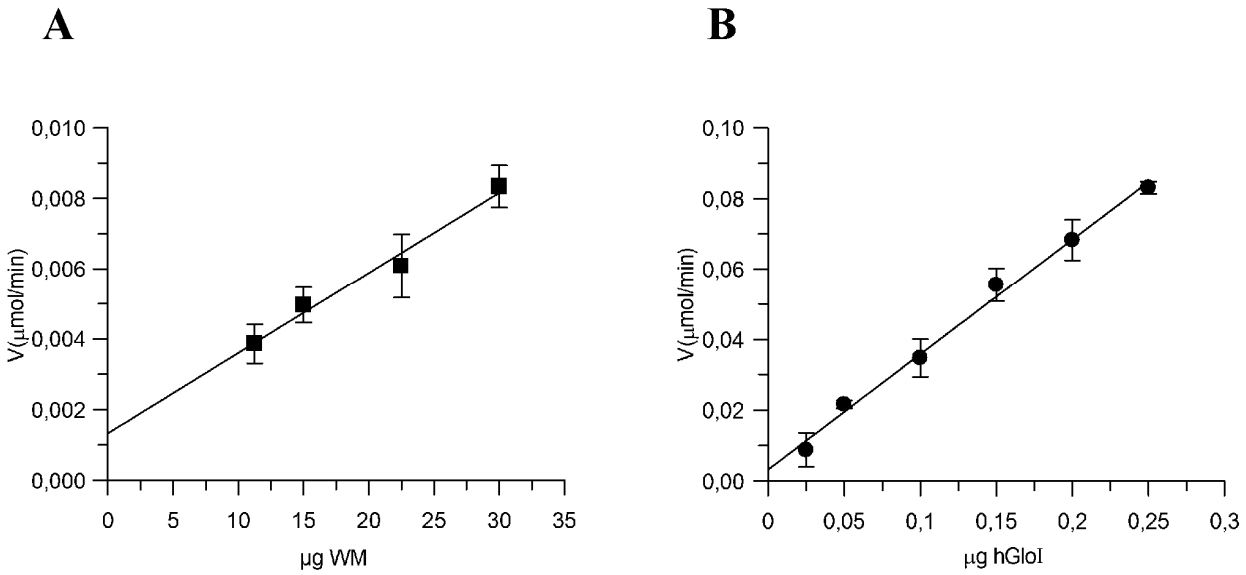
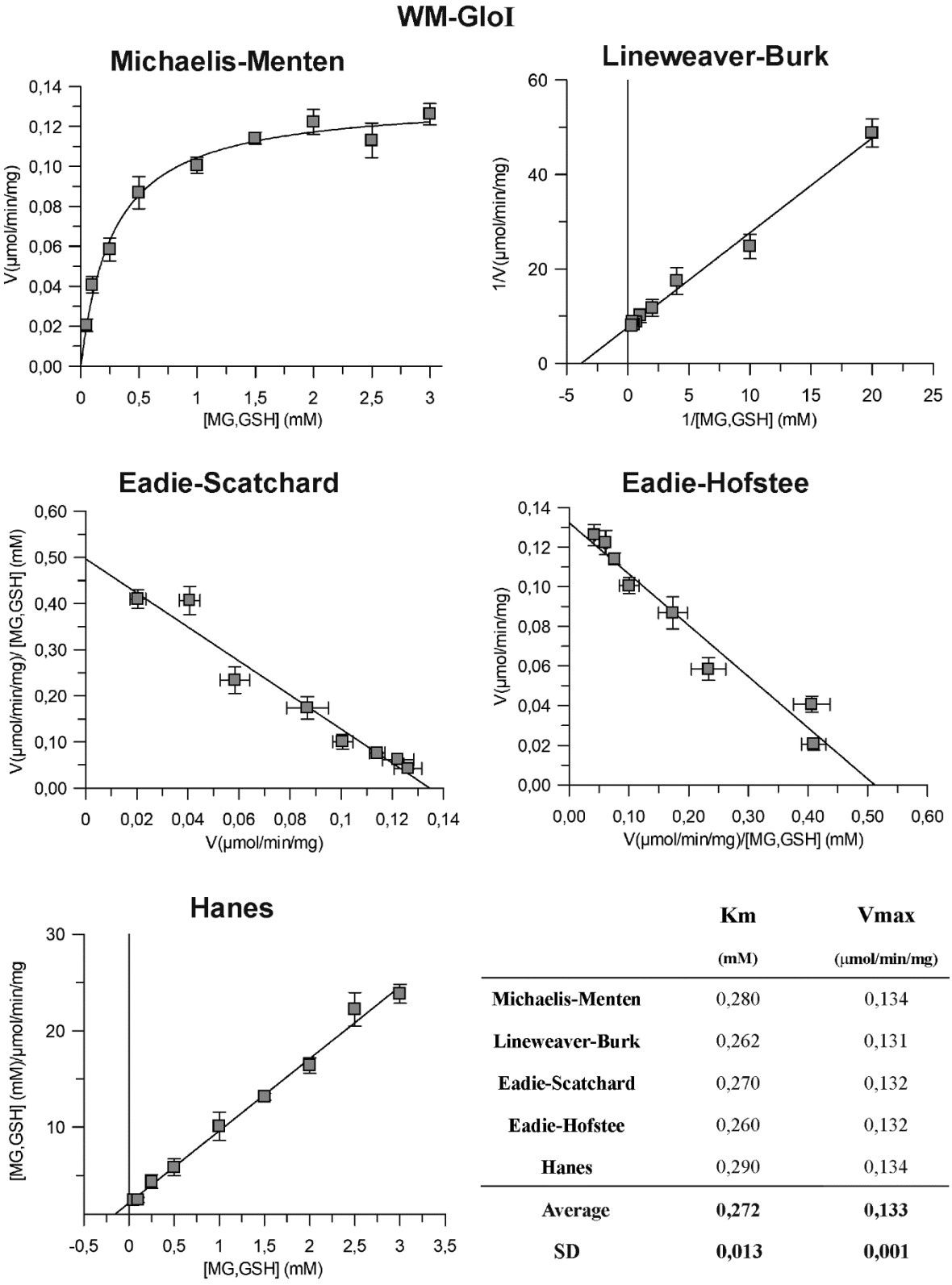


Figure 1. *Dependence of WM-GloI (A) and hGloI (B) activities on protein amount.* The reaction rates are expressed as EU (µmol/min).Vertical bars represent ± S.D. (n = 3).

A study of kinetics of WM-GloI was also carried out (Figure 2); by plotting WM-GloI reaction rate *vs* the MG/GSH concentration, a hyperbolic relationship was obtained according to the Michaelis-Menten equation. Data were also reported as Lineweaver–Burk, Eadie–Hofstee, Eadie–Scatchard and Hanes plots. In this experiment, the Km and Vmax mean values from all plots were equal to 0.27± 0.02 mM and 0.133 ± 0.001 EU/mg of protein, respectively.

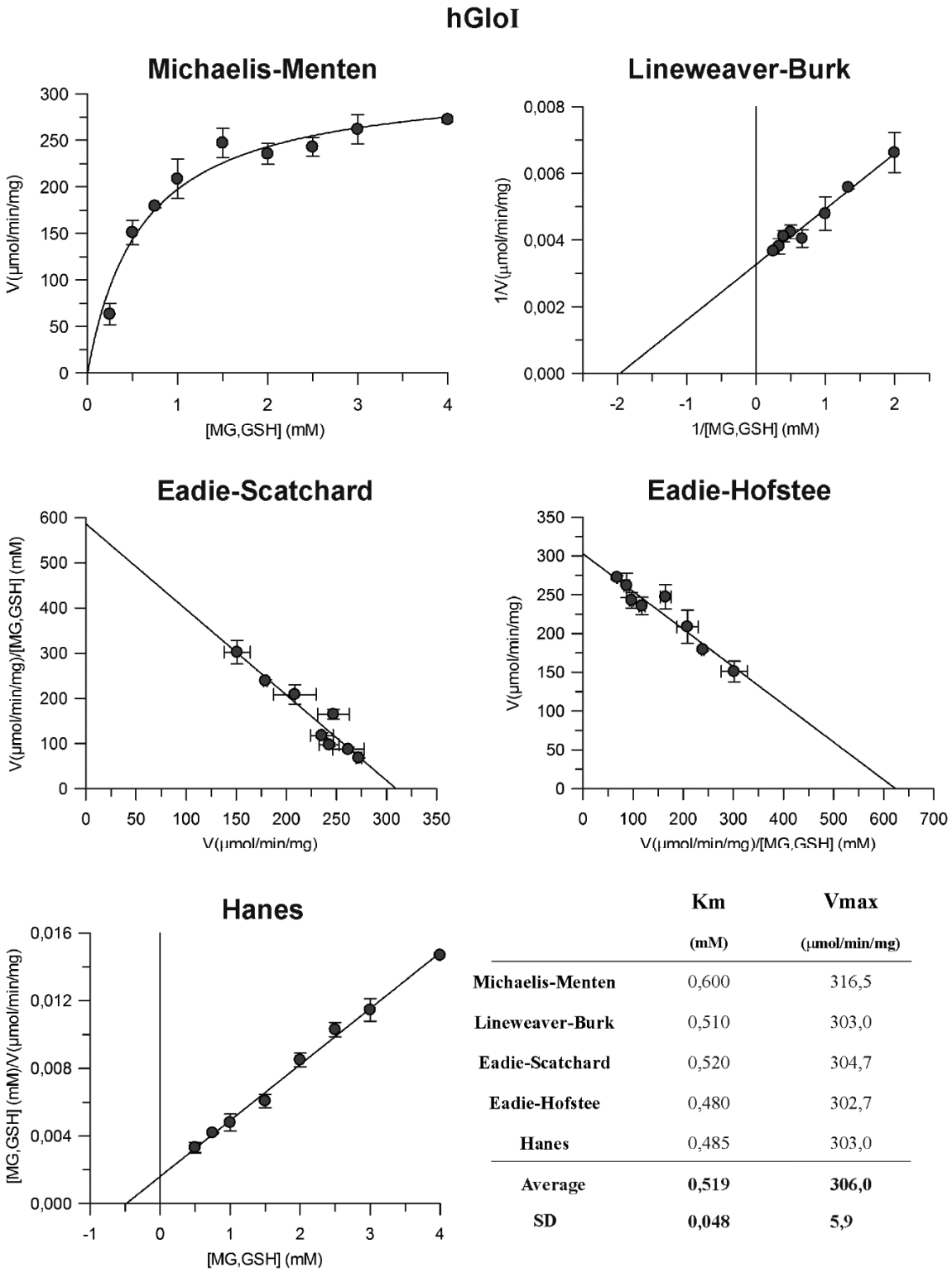
Also for hGloI, a study of kinetics was carried out (Figure 3). As expected, the rate of the hGloI reaction was found to exhibit hyperbolic dependence on MG/GSH concentration as predicted by the Michaelis-Menten equation. Saturation kinetics were confirmed by plotting the data according to Lineweaver-Burk, Eadie-Hofstee, Eadie-Scatchard, and Hanes plots. All plots were linear, thus confirming the hyperbolic kinetics, with Km and Vmax equal to 0.52 mM and 306 EU/mg of protein, respectively.

The hyperbolic dependence of reaction rate on substrate concentration (Figure 2) and the linear dependence on protein amount (Figure 1) confirm the enzymatic nature of SLG generation reaction measured in WM. The behaviour similar to hGloI allows attributing this reaction to GloI enzyme activity. Based on results of experiment reported in Figure 1A, a GloI specific activity in WM is quantified, equal to 0.20 ± 0.03 EU /mg of protein.



	Km (mM)	Vmax (µmol/min/mg)
Michaelis-Menten	0,280	0,134
Lineweaver-Burk	0,262	0,131
Eadie-Scatchard	0,270	0,132
Eadie-Hofstee	0,260	0,132
Hanes	0,290	0,134
Average	**0,272**	**0,133**
SD	**0,013**	**0,001**

Figure 2. *Dependence of WM-GloI reaction rate on substrate concentration.* The reactions were carried out by using 20 µg WM previously lysed with 0.1% Triton X-100 and in the presence of increasing concentrations of the mixture [MG, GSH]. Data are plotted according to Michaelis-Menten, Lineweaver-Burk Eadie-Scatchard, Eadie-Hoffstee and Hanes equations. The values are reported as mean ± S.D. (n = 3). The table shows Vmax and Km values calculated from each graph and the averages of the kinetic constants.



	Km (mM)	Vmax (µmol/min/mg)
Michaelis-Menten	0,600	316,5
Lineweaver-Burk	0,510	303,0
Eadie-Scatchard	0,520	304,7
Eadie-Hofstee	0,480	302,7
Hanes	0,485	303,0
Average	**0,519**	**306,0**
SD	**0,048**	**5,9**

Figure 3. *Dependence of hGloI reaction rate on substrate concentration.* The reactions were carried out by using 0.1 µg hGloI and in the presence of increasing concentrations of the mixture [MG, GSH]. Data are plotted according to Michaelis-Menten, Lineweaver-Burk Eadie-Scatchard, Eadie-Hoffstee and Hanes equations. The values are reported as mean ± S.D. (n = 3). The table shows Vmax and Km values calculated from each graph and the averages of the kinetic constants.

To date, the modulation of plant glyoxalase activity has been little investigated. Therefore, the effect on WM-GloI activity of some plant bioactive compounds was evaluated. In particular, the effect of curcumin and quercetin, known as GloI inhibitors in mammals (Santel et al., 2008), and resveratrol, able to activate both GloI activity and gene expression (Cheng et al., 2012), were assessed on WM-GloI activity. In addition, the most relevant antioxidants of durum wheat grains such as ferulic acid, sinapic acid and α- tocopherol (Laus et al., 2012), were tested. This in order to evaluate a possible role of these phytochemicals as physiological modulators of plant glyoxalase activity.

The possible modulation of the WM-GloI reaction was studied in the presence of increasing concentrations of the compound to be tested and at substrate concentrations close to Km (0.3 mM MG/GSH), to avoid that a saturating MG/GSH concentration could hide inhibitors acting by a competitive mechanism. Since the phytochemicals were dissolved in DMSO, it was added in all wells in the same amount (5 µL) to exclude non-specific effects of this solvent.

In Table 1, the effect of the phytochemicals on WM-GloI activity is shown. In particular, curcumin and quercetin was found to inhibit WM-GloI activity, while resveratrol, ferulic acid, sinapic acid and α- tocopherol were not able to exert any effect in the range of the investigated concentrations.

Table 1. Effect of some phytochemicals on WM-GloI activity. For each compound, the range of concentrations tested and the possible effect on the activity of WM-GloI are reported.

Compounds	Concentration range	Effect on WM-GloI activity
Curcumin	25 - 100 µM	Inhibition
Quercetin	25 - 75 µM	Inhibition
Resveratrol	75 µM	No effect
Ferulic acid	100 µM	No effect
Sinapic acid	75 µM	No effect
α-tocopherol	75 µM	No effect

In Figure 4, the phytochemical-dependent inhibition, expressed as (%) decrease with respect to the control of the rate of WM-GloI activity, is reported as a function of curcumin (A) and quercetin (B) concentrations.

A linear dependence of the inhibition from 35 to 70% on curcumin concentration ranging between 20 and 100 µM and from 15 to 45% on quercetin concentration ranging between 22.5 and 75 µM was obtained.

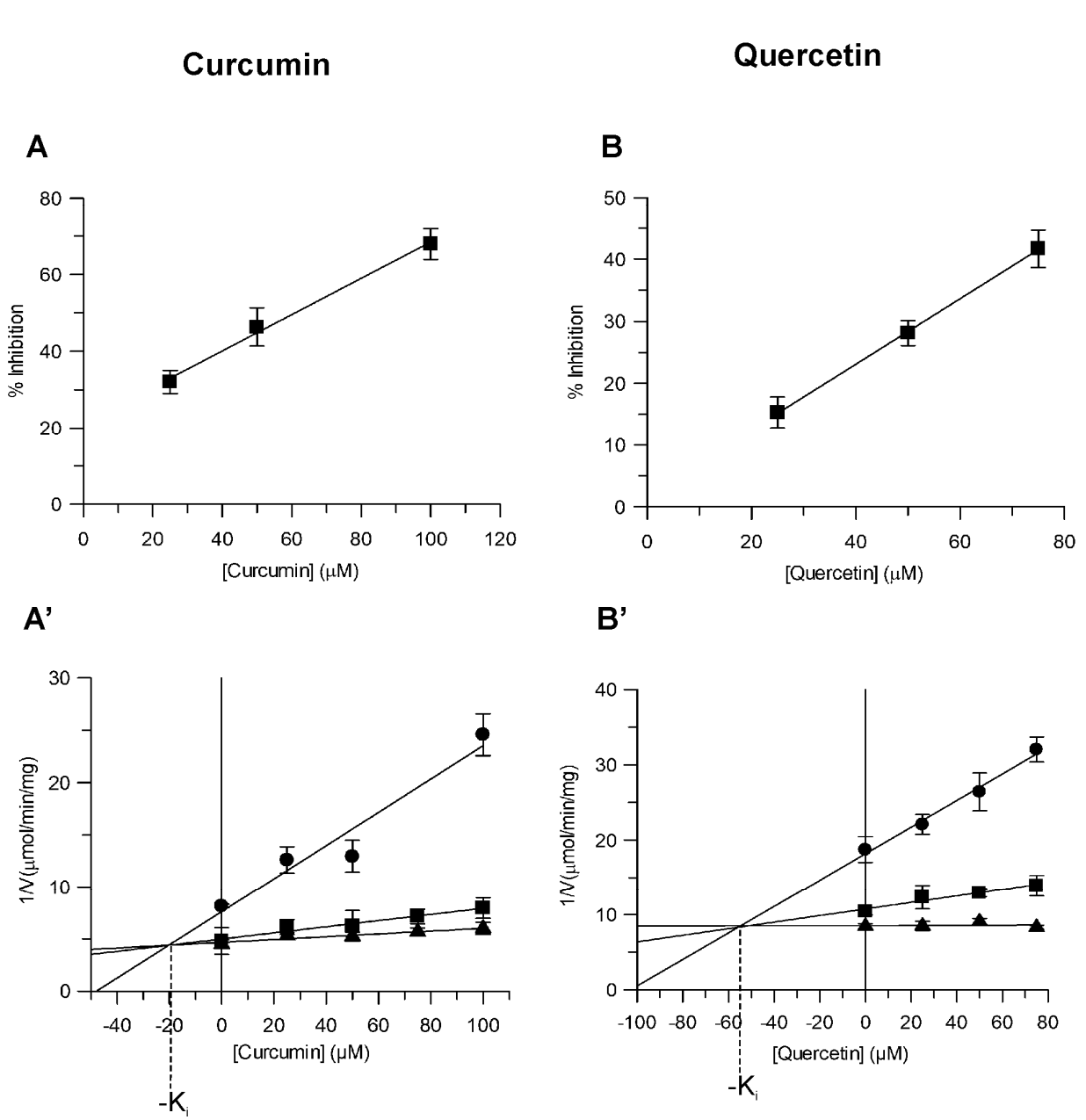


Figure 4. *Inhibition of WM-GloI by curcumin and quercetin.* In A and B the inhibition of WM-GloI activity, expressed as (%) inhibition with respect to the control, is reported as a function of curcumin and quercetin concentrations, respectively; data are expressed as mean value ± SD (n = 3). The reactions were carried out by using 30 µg WM previously lysed with 0.1% Triton X-100, 0.3mM [MG, GSH] and curcumin or quercetin at different concentrations. In A' and B' Dixon graphs, obtained by plotting 1/v *vs* phytochemical concentration at different substrate concentrations, are shown. (A'): 25, 50, 75, 100 µM curcumin, 0.300, 0.625, 1 mM [MG, GSH] and 30 µg WM-GloI; (B'): 25, 50, 75, 25 µM quercetin, 0.375, 0.625, 1 mM [MG, GSH] and 30 µg WM-GloI. *Ki* values were calculated from the Dixon plots.

A detailed inhibition study was also performed; this in order to identify the nature of the inhibition and to determine the inhibition constant (Ki). To this purpose, the reaction rate was measured in the presence of different substrate and phytochemical concentrations. Data were plotted according to Dixon (Figures 4A' and 4B'). Dixon plots clearly show that both curcumin and quercetin are able to inhibit the rate of the WM-GloI reaction in a competitive manner, with a Ki value equal to 20 μM and 55 μM for curcumin and quercetin, respectively. These results suggest that these phytochemicals are both able to compete with the substrate for binding to the active site, but curcumin shows a higher inhibition efficiency (2.75 times greater than quercetin).

Similarly to the WM-GloI, a possible modulation of hGloI activity by phytochemicals such as curcumin, quercetin, apigenin and sulforaphane was studied. Also in this case, hGloI reaction was studied at substrate concentrations close to Km (0.6 mM MG/GSH).

The results are shown in Table 2. Among the tested compounds, only curcumin and quercetin were able to inhibit the hGloI activity, in agreement with previous literature data (Santel et al., 2008).

Table 2. Effect of some phytochemicals on hGloI activity. For each compound, the range of concentrations tested and the possible effect on the activity of hGloI are reported.

Compounds	Concentration range	Effect on hGloI activity
Curcumin	15-100 μM	Inhibition
Quercetin	10-25 μM	Inhibition
Sulforaphane	100-300 μM	No effect
Apigenin	45-200 μM	No effect

Therefore, a detailed study of their inhibition was carried out (Figure 5); the inhibition of hGloI activity linearly increases with the phytochemical concentration in the investigated ranges that were 15-100 μM and 10-25 μM for curcumin (A) and quercetin (B), respectively.

According to Dixon plots (Figure 5A' and 5B') both curcumin and quercetin inhibit the rate of the hGloI reaction in a competitive manner, with Ki value equal to 90 μM and 2 μM for curcumin and quercetin, respectively. It is interesting to highlight that, despite the high phylogenetic distance between human and durum wheat species, the two phytochemicals show the same inhibitory effect on both WM-GloI and hGloI, but with a very different efficiency.

In fact, quercetin show an efficiency 28 times higher in WM with respect to hGloI, while curcumin is an inhibitor about 4.5 times more efficient of WM-GloI than hGloI. Further investigations are necessary in order to understand the physiological role of the inhibition of WM-GloI activity by curcumin and quercetin.

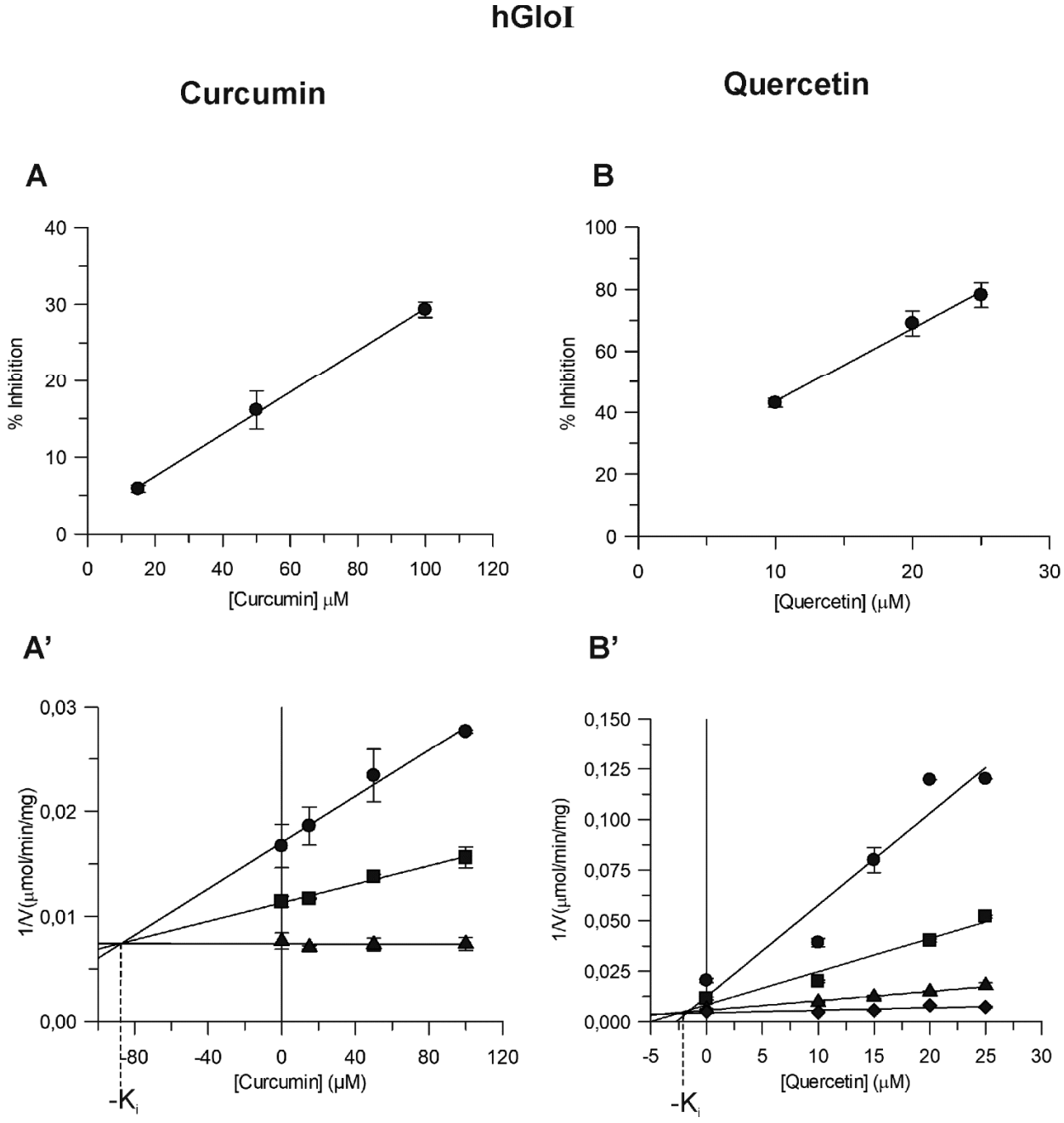


Figure 5. *Inhibition of hGloI by curcumin and quercetin.* In A and B the inhibition of hGloI activity, expressed as (%) inhibition with respect to the control, is reported as a function of curcumin and quercetin concentrations, respectively; data are expressed as mean value ± SD (n = 3). The reactions were carried out by using 0.1 μg hGloI, 0.625 mM [MG, GSH] and curcumin or quercetin at different concentrations. In A' and B' Dixon graphs, obtained by plotting 1/v *vs* phytochemical concentration at different substrate concentrations, are shown. (A'): 15, 50, 100 μM curcumin, 0.375, 0.625, 1 mM [MG, GSH] and 0.1 μg hGloI; (B'): 10, 15, 20, 25 μM quercetin, 0.375, 0.625, 1, 3 mM [MG, GSH] and 0.1 μg hGloI. *Ki* values were calculated from the Dixon plots.

4. Conclusions

Results of this study shed light on the existence of GloI activity in WM. The generation of SLG from MG in the presence of GSH was easily measured in these mitochondria. Similarly to hGloI reaction, the rate of SLG generation resulted linearly dependent on WM proteins and showed a hyperbolic relationship on substrate concentration. The kinetic study of WM-GloI allowed calculating Km and Vmax values equal to 0.27± 0.02 mM and 0.133 ± 0.001 EU/mg of protein, respectively. Moreover, for the first time in plants, modulation of WM-GloI activity was studied, in comparison with hGloI. The first inhibitors of WM-GloI were identified: curcumin and quercetin, confirmed in this study as hGloI inhibitors, resulted both able to inhibit in a competitive manner and with high efficiency WM-GloI activity. The role of curcumin and quercetin inhibition of plant mitochondrial GloI activity remains to be established.

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Article 6:

Measuring activity of native plant sirtuins

The wheat mitochondrial model

Submitted for publication as: M. Soccio, M.N. Laus, M. Alfarano and D. Pastore

Abstract

Sirtuins are NAD^+-dependent deacetylase enzymes that have gained considerable interest in mammals for their recognized importance in gene silencing and expression and in cell metabolism. Conversely, knowledge about plant sirtuins remains limited, although a sirtuin-mediated regulation of mitochondrial energy metabolism has been recently reported in *Arabidopsis*. However, so far, no information is available about measurement of intracellular plant sirtuin activity, *i.e.* in cell extracts and/or subcellular organelles. In this study, a novel approach was proposed for reliable evaluation of native sirtuin activity in plant samples, based on *i*) an adequate combinatory application of enzymatic assays very different for chemical basis and rational and *ii*) a comparative measurement of activity of a recombinant human sirtuin 1 isoform (hSIRT1). In particular, two sirtuin assays were used, based on bioluminesce emission and Homogeneous Time-Resolved Fluorescence (HTRF®) technology. For the first time in plants, this new approach allowed measuring a high and nicotinamide-sensitive sirtuin activity in highly purified mitochondrial fraction obtained from wheat (WM). WM-sirtuin activity resulted 268±10 $mU \cdot mg^{-1}$ protein, as evaluated by HTRF® assay, and 166±12 ng hSIRT1 $eq. \cdot mg^{-1}$ protein, as evaluated by the bioluminescent assay and calculated by normalizing data based on hSIRT1 activity. Moreover, effects of resveratrol and quercetin, reported as potent hSIRT1 activators, but whose activation mechanism is still debated, were also studied. No effect of resveratrol was found on both WM-sirtuin and hSIRT1 activities, while only a slight increase, up to about 20%, of hSIRT1 activity by quercetin was observed. In the whole, results of this study indicate that WM may represent a good model system for studying native plant sirtuins. In fact, the high yield of purified WM and their high sirtuin activity, together with use of microplate readers, allow performing a large number of measurements from the same preparation, so qualifying the approach for application to large-scale high-throughput screening. Moreover, WM may also represent an excellent tool to investigate physiological role and modulation of plant sirtuins under experimental conditions more physiologically relevant with respect to recombinant purified enzymes.

Keywords: plant sirtuin, wheat mitochondria, bioluminescent sirtuin activity assay, HTRF® sirtuin activity assay, resveratrol, quercetin.

Running title: sirtuin assay and wheat mitochondria

1. Introduction

Sirtuins are ubiquitous enzymes belonging to class III of histone deacetylases that catalyze the specific NAD^+-dependent deacetylation of ε–*N*-acetyl lysine residues of both histones and non-histone proteins to produce nicotinamide (NAM), 2'-*O*-acetyl-ADP-ribose and the deacetylated polypeptide (Chung et al., 2010; Haigis and Sinclair, 2010; Michan and Sinclair, 2007; Szućko, 2016). Sirtuin-mediated deacetylation of proteins represents a highly regulated post-translational modification having a strong impact on protein functions, enzyme activities, as well as on protein-protein and protein-DNA interactions (König et al., 2014). Multiple substrates of sirtuin activity have been identified including numerous regulatory proteins involved in many metabolic processes and defense mechanisms in response to stress (Chung et al., 2010)In mammals, seven sirtuin isoforms (SIRT1-7) have been identified showing nuclear (SIRT1/6/7) (Haigis and Sinclair, 2010; Michan and Sinclair, 2007), mitochondrial (SIRT3/4/5) (Bell and Guarente, 2011; Gertz and Steegborn, 2010) and cytosolic (SIRT2) (Black et al., 2008; North et al., 2003) localization. As for biological implications, animal sirtuins have been shown to regulate a wide variety of processes. In particular, for the best-characterized human SIRT1 an important role in regulating pathogenesis of diabetes, obesity, cancer, as well as neurodegenerative, cardiovascular, and chronic renal and pulmonary diseases is reported (Chung et al., 2010).

Concerning plant sirtuins, only few studies can be retrieved from literature and regard only few species. Compared to other eukaryotes, plant have relatively fewer sirtuin-related genes coding for nuclear (SRT1) (Cucurachi et al., 2012; Huang et al., 2007; Zhao et al., 2015) and mitochondrial (SRT2) (Chung et al., 2009; Cucurachi et al., 2012; König et al., 2014) isoforms, although a nuclear/cytosolic localization has been obtained for SRT2 in tomato (Zhao et al., 2015). The knowledge about sirtuin functions in plants is still limited. Plant sirtuins were suggested to have a protection role against genome instability and cell oxidative damage required for plant cell growth (Huang et al., 2007), as well as to be implicated in gametogenesis, development and ripening of fruits (Zhao et al., 2015), leaf senescence and regulation of photosynthetic activity (Cucurachi et al., 2012) and auxin signaling (Grozinger et al., 2001; Hollender and Liu, 2008). Interestingly, a relevant role in fine-tuning of mitochondrial energy metabolism has been recently demonstrated for *Arabidopsis* SRT2 (König et al., 2014).

Given their relevant role in a wide variety of cellular processes, sirtuins have been extensively investigated in last decades. In particular, mammalian sirtuins have been received increasing attention as important attractive drug targets and some modulators of sirtuin activity have been shown to have very promising therapeutic value for treating many human chronic and degenerative diseases (Chung et al., 2010; Hubbard and Sinclair, 2014; Sánchez-Fidalgo et al., 2012).

Much of current knowledge about functional characterization and modulation of sirtuin-dependent deacetylase activity in biological systems is inferred from gene-expression studies that are often not accompanied by enzymatic activity measurements (Chung at al., 2010). It should be also considered that information available about sirtuin activity and modulation is generally referred to purified recombinant enzymes. Although catalytic activity and effects of modulators highlighted on purified enzyme should be confirmed in cellular systems, to date only few data can be retrieved from literature reporting direct assessment of sirtuin activity in yeast/animal cell extracts (Chai et al., 2017; de Boer et al., 2006; Rogacka et al., 2018). In plants, so far, sirtuin-dependent deacetylase activity has never been measured in cell lysates; only one paper concerning plant sirtuin activity is available, in which activity was measured by using an *Arabidopsis* SRT2 protein overexpressed and purified from *E. coli* (König et al., 2014). Moreover, to the best of our knowledge, no information is available about sirtuin activity assessment in subcellular organelles, both in animal and plant systems. The difficulty of carrying out direct sirtuin activity measurements in cell/cell lysates can depend on the complexity of the commonly employed enzymatic tools. Most of these assays imply indirect measurements that can be influenced and distorted by potentially interfering physiological compounds present in the biological extract. The use of these indirect assays can further affect results leading to a compromised data interpretation in the study of direct modulation of sirtuin activity by chemical compounds. For example, the use of a fluorimetric assay exploiting peptide substrates conjugated to the 7-amino-4-methylcoumarin (AMC) fluorophore has led to controversies on the mechanism of SIRT1 activation by resveratrol and its analogues (Borra et al., 2005; Pacholec et al., 2010).

In the light of above reported observations, appropriate assays for measuring sirtuin enzymatic activity should be carefully selected. Firstly, these assays should be able to achieve reliable and reproducible results and to be easily applicable directly on the "native" enzyme, *i.e.* on the enzyme in its biological environment (within the cell/subcellular organelle). Moreover, assays should be based on methodological approaches that are *i*) able of providing an appropriate quantification of sirtuin activity to allow comparison among different experimental conditions and systems, and *ii*) as free as possible from false positives or negatives due to interfering molecules present in the reaction mixture/biological sample. With respect to the last point, an appropriate combinatory application of different methods can be an effective tool for highlighting undesirable interfering effects.

In the light of this, in the present study, sirtuin activity was measured by using two enzymatic assays: a bioluminescent assay (SIRT-Glo™, Promega) and a Homogeneous Time Resolved Fluorescence (HTRF®)-based assay (HTRF® SIRT1, Cisbio). They were chosen as very different

in terms of reaction mechanisms, experimental conditions, methodologies used for monitoring the reaction progress and for quantifying results, so that their comparison could clearly unmask possible interfering effects. For both methods, parallel assessment of activity of the commercially available human recombinant SIRT1 isoform (hSIRT1) was carried out. This experimental approach was applied to measure for the first time a "native" plant sirtuin activity. As a plant model system, mitochondrial sirtuin was studied, since this is the best-characterized plant sirtuin showing significant relevance in regulating mitochondrial energy metabolism (König et al., 2014). To this aim, wheat mitochondria (WM) were used, since an isolation procedure providing high yield of pure and intact organelles is available (Pastore et al., 1999) and bioenergetics aspects of these mitochondria have been investigated in detail (Laus et al., 2008, 2011; Pastore et al., 2007; Soccio et al., 2010, 2013, Trono et al., 2011, 2013).

Moreover, by using the developed approach, the effect on both hSIRT1 and WM-sirtuin activity of two polyphenols, such as resveratrol and quercetin, was evaluated. This in order *i*) to assess in our experimental conditions the effect of these compounds, reported as potent natural SIRT1 activators (Howitz et al., 2003), but whose activation mechanism has been the subject of an intense debate (Hubbard and Sinclair, 2014), and *ii*) to evaluate a possible role of these phytochemicals also as physiological modulators of plant sirtuin activity.

2. Materials and Methods

2.1 Chemicals and plant materials

All chemicals at the highest commercially available purity were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The SIRT-Glo™ and the HTRF® SIRT1 assays were purchased from Promega Co. (Madison, WI, USA) and Cisbio US (Bedford, MA, USA), respectively.

Oligomycin was dissolved in ethanol; resveratrol and quercetin were dissolved in ethanol or dimethylsulfoxide (DMSO) for sirtuin activity measurements using the SIRT-Glo™ or the HTRF® SIRT1 assays, respectively.

Certified seeds of durum wheat (*Triticum durum* Desf., cv Ofanto) were kindly supplied from the CREA-Cereal Research Centre (Foggia, Italy).

2.2 Wheat mitochondria (WM) isolation

WM were purified from 72-h-old etiolated seedlings, as reported in (Pastore et al., 1999) with minor modifications. The grinding and washing buffers were: (*i*) 0.5 M sucrose, 4 mM cysteine, 1 mM EDTA, 30 mM Tris-HCl (pH 7.50), 0.1% (w/v) defatted bovine serum albumin (BSA), 0.6% (w/v) polyvinylpyrrolidone (PVP)-360; and (*ii*) 0.5 M sucrose, 1 mM EDTA, 10 mM Tris-HCl

(pH 7.40), 0.1% (w/v) defatted BSA, respectively. Washed mitochondria were subjected to an isopycnic centrifugation in a self-generating density gradient containing 0.5 M sucrose, 10 mM Tris-HCl (pH 7.20) and 28% (v/v) Percoll (colloidal PVP coated silica, Sigma-Aldrich) in combination with a linear gradient of 0% (top) to 10% (bottom) PVP-40 (Moore and Proudlove, 1987) to obtain the purified mitochondrial fraction. For determination of sirtuin and marker enzyme activities in the different fractions obtained in the course of WM purification (Table 3), purified mitochondria were recovered, as well as the initial homogenate and the combined pellets obtained by the first and third centrifugations according to protocol described in (Trono et al., 2013). Protein content was determined by the method of Lowry modified according to Harris (1987) using BSA as a standard. WM showed high intactness of inner and outer membranes and a good functionality, as evaluated by means of fluorimetric measurements of electrical membrane potential according to (Laus et al., 2008, 2011; Pastore et al., 1999; Soccio et al., 2013).

a. Enzymatic assays

2.3.1 Sirtuin assays

SIRT-Glo™ Assay (Promega)

As shown in **Fig. 1**, the assay uses a pro-luminescent substrate containing an acetylated lysine attached to aminoluciferin. In particular, it is a sirtuin-optimized amino acid sequence based on a consensus sequence derived from p53; it also contains an amino-terminal blocking group (Z) that prevents non-specific cleavage. The NAD^+-mediated deacetylation by sirtuin produces NAM, 2'-*O*-acetyl-ADP ribose and the deacetylated peptide. This last is then cleaved by a specific protease resulting in aminoluciferin release. The free aminoluciferin is then quantified using an ATP-dependent firefly luciferase reaction to produce a stable and persistent "glow-type" light emission that is proportional to sirtuin deacetylase activity. Measurements were performed by using a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) and 96-well plates and the manufacturer's protocol was applied with slight modifications. The reaction mixture (final volume of each well: 200 µL) consisted of the SIRT- Glo™ Buffer and the sample, *i.e.* the sirtuin enzyme source (hSIRT1 or WM). For sirtuin activity measurements in WM, the reaction mixture also contained: 0.1% Triton X-100, in order to lyse mitochondria so releasing mitochondrial sirtuin; 30 µM P^1,P^5-di(adenosine-5') pentaphosphate (Ap5A) and 4 µg oligomycin, able to inhibit adenylate kinase and ATPase, respectively, to avoid consumption by mitochondrial metabolism of ATP, necessary for the firefly luciferase reaction.

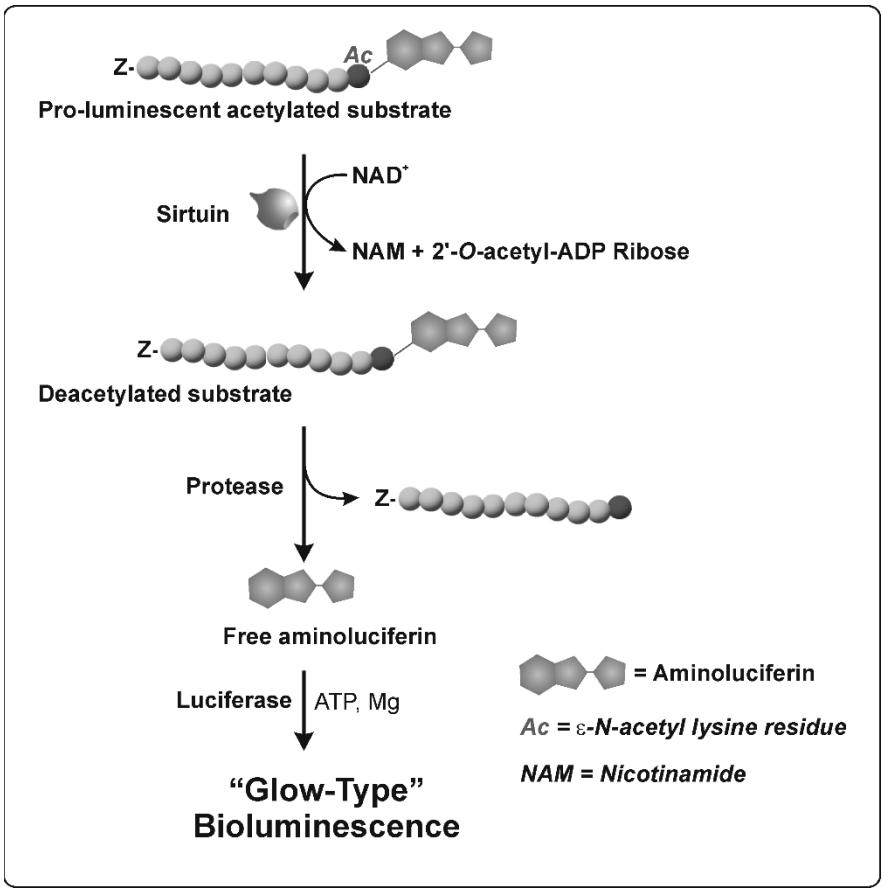

Figure 1. *Bioluminescent assay (SIRT-Glo™, Promega).* The three reactions involved in the sirtuin assay are shown: NAD^+-dependent deacetylation by sirtuin of the pro-luminescent substrate; cleavage by a specific protease of the deacetylated substrate resulting in aminoluciferin release; luciferase reaction producing a stable and persistent emission of light. "Z" represents an amino-terminal blocking group that protects the substrate from nonspecific cleavage. All three enzymatic events occur in coupled. For more details see the text. Adapted from Technical Manuals of SIRT-Glo™ Assay and Screening System (Promega) and HTRF® SIRT1 assay (Cisbio).

For sirtuin activity measurements in WM, the reaction mixture also contained: 0.1% Triton X-100, in order to lyse mitochondria so releasing mitochondrial sirtuin; 30 μM P^1,P^5-di(adenosine-5') pentaphosphate (Ap5A) and 4 μg oligomycin, able to inhibit adenylate kinase and ATPase, respectively, to avoid consumption by mitochondrial metabolism of ATP, necessary for the firefly luciferase reaction. Control was made that Triton X-100, Ap5A and oligomycin had no significant effect on sirtuin activity detection system.

The reaction was started by adding the SIRT-Glo™ Reagent (prepared according to the manufacturer's protocol by combining the SIRT-Glo™ Substrate Solution and the Developer Reagent and containing substrate, NAD^+, ATP, protease and luciferase) and monitored by recording the bioluminescence increase at 25 °C, until to steady-state signal. The maximum signal expressed as Relative Luminescence Unit (RLU) was determined and "signal to noise ratio" was calculated, *i.e.* the ratio between luminescent signal of sample and that of no-sirtuin (buffer

background) control. The reaction rate was also calculated as the highest slope of the experimental curve and expressed as RLU·min^{-1}.

Determinations were carried out in triplicate by analysing at least four different amounts of sample. A linear dependence of both signal to noise ratio and RLU·min^{-1} on the protein amount of hSIRT1 or WM was verified by linear regression analysis of data. WM-sirtuin activity was obtained by comparing the slope derived by linear regression analysis with that of the curve relative to hSIRT1 and expressed as hSIRT1 equivalent (ng hSIRT1 eq./mg of WM protein).

In experiments aimed at evaluating the effect of resveratrol and quercetin on sirtuin activity, the compound to be tested was added to the reaction mixture and incubated for 30 min before starting the reaction. In order to detect false positives due to inhibition of protease and/or luciferase, measurements were also carried out using a non-acetylated control substrate under the same experimental conditions as the acetylated SIRT-Glo™ Substrate. Since oligomycin, resveratrol and quercetin were dissolved in ethanol that can affect sirtuin activity, a constant volume of ethanol (5 µL) was maintained in the reaction mixture.

HTRF® SIRT1 assay (Cisbio)

This assay combines standard Fluorescence Resonance Energy Transfer (FRET) technology with time-resolved measurement of fluorescence. As shown in **Fig. 2**, the assay uses a peptide containing a single acetylated lysine (substrate-d2) and bound to the XL665 fluorophore (λ_{em}= 665 nm), a phycobiliprotein pigment purified from red algae which acts as acceptor. The acetylated substrate-d2 can be recognized and bound by an anti-acetyl mouse monoclonal antibody labelled with a second fluorophore, the Eu^{3+}-cryptate (λ_{ex} =337 nm, λ_{em}= 620 nm), that is the donor. In the absence of sirtuin activity (**A**), the interaction between substrate and antibody brings the two fluorophores in close proximity; the excitation of Eu^{3+}- cryptate triggers an energy transfer towards the XL665, which in turn emits specific fluorescence at 665 nm. Conversely, in the presence of sirtuins that deacetylate the substrate-d2, the interaction between substrate and antibody does not occur, resulting in loss of FRET and extinction in signal (**B**).

Measurements were performed by carrying out an enzymatic step followed by a detection step. During the enzymatic step, a mixture (volume of each well: 10 µL) consisting of the Enzymatic Buffer containing 1 mM DTT, 6 nM substrate-d2, 500 µM NAD^+, was incubated at 25 °C for 30 min in both the absence (No Enzyme Control, NoE) and the presence of sample, *i.e.* the sirtuin enzyme source (hSIRT1 or WM).

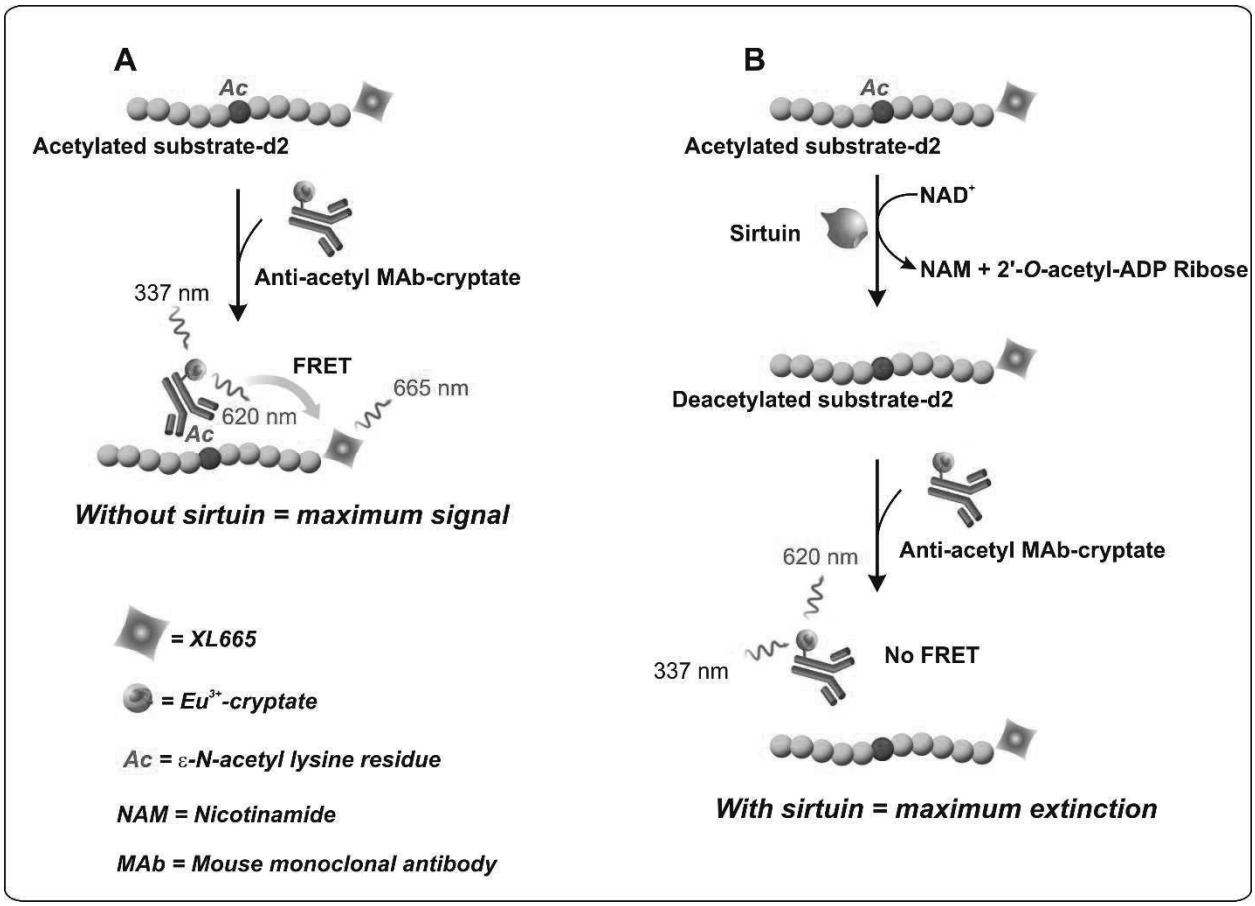

Figure 2. *HTRF® assay (HTRF® SIRT1 Cisbio).* In this assay an anti-acetyl specific Eu^{3+}-Cryptate labelled antibody and an acetylated d2 substrate labelled with the phycobiliprotein pigment XL665 are used. In absence of sirtuin activity (A) the anti-acetyl Eu^{3+}-Cryptate binds the acetylated substrate-d2. If the Eu^{3+}-Cryptate is excited at 337 nm a FRET signal is obtained. On the contrary, in the presence of sirtuin (B) d2 labeled substrate is deacetylated thus preventing the binding with the antibody. In this case, no FRET is obtained. Therefore, the maximum signal is obtained in absence of sirtuin activity (A) and decreases proportionally to the deacetylation process (B). Adapted from the Technical Manual of HTRF® SIRT1 assay (Cisbio).

For sirtuin WM measurements, the reaction mixture also contained 0.1% Triton X-100, in order to lyse mitochondria and release mitochondrial sirtuin; control was made that Triton X-100 had no significant effect on sirtuin activity detection system. Another substrate-d2-free mixture, consisting of the Enzymatic Buffer containing 1 mM DTT, 500 µM NAD^+, 0.1% Triton X-100 (for WM-sirtuin measurements) was incubated for 30 min in the presence of sample (hSIRT1 or WM) and used as Negative Control (Neg). NoE and Neg controls were used to define the upper and lower limit of FRET signal, respectively.

In the detection step, the enzymatic reaction was stopped by the addition of 10 µl of anti-acetyl antibody dissolved in the Detection Buffer containing EDTA and NAM, a sirtuin inhibitor. The final volume of each well was 20 µL. After incubation from 5 h to overnight at 25 °C, the fluorescence was measured at both 620 nm (emission of Eu^{3+}-cryptate) and 665 nm (emission of

XL665) by using a SpectraMax® M5 Multimode Plate Reader (Molecular Devices, Wokingham, UK) and HTRF® 96-well low volume plates (Cisbio). The 665 nm/620 nm fluorescence ratio (*Ratio*) was calculated and used to determine: *i*) $\Delta F/\Delta F_{max}$ according to the formula: ($Ratio_{Sample}$ - $Ratio_{Neg}$)/($Ratio_{NoE}$ - $Ratio_{Neg}$); *ii*) the substrate deacetylation (%) according to the formula: 100 - ($Ratio_{Sample}/Ratio_{NoE} \cdot 100$).

Determinations were carried out in triplicate by analysing different amounts of sample. A linear dependence of both $\Delta F/\Delta F_{max}$ and substrate deacetylation (%) on the amount of sample was verified by linear regression analysis of data. Sirtuin activity was calculated from the substrate deacetylation (%) and expressed as mU (1 U = 1 pmol $\cdot$ min^{-1}).

In experiments aimed at assessing possible modulation of sirtuin activity by resveratrol and quercetin, hSIRT1 and WM were incubated for 30 min with the compound at tested concentration before starting the enzymatic step. Resveratrol and quercetin were also added to NoE and Neg controls. Since resveratrol and quercetin were dissolved in dimethyl sulfoxide (DMSO), a constant volume of DMSO (0.25 µL) was maintained in the reaction mixture.

2.3.2 Marker enzyme assays

Phosphoenolpyruvate carboxylase (PEPC) and cytochrome c oxidase (COX) were assayed as marker enzymes of cytosol and mitochondria, respectively. PEPC activity was measured by monitoring spectrophotometrically the absorbance decrease at 340 nm due to NADH oxidation in the course of the PEPC/malate dehydrogenase coupled assay by means of a Perkin Elmer Lamba 45 UV/VIS spectrometer as reported in (Soccio et al., 2013; Trono et al., 2013). COX assay was checked oxygraphically by following oxygen consumption due to cytochrome c oxidation by means of a Gilson Oxygraph (model 5/6-servo Channel pH5) equipped with a Clark-type electrode (5331 YSI, Yellow Spring, OH) as reported in (Trono et al., 2013).

2.4 Bioinformatic analysis

BLASTP search of the putative wheat sirtuins was performed using the EnsemblPlants (http://plants.ensembl.org/index.html) website. Predictions of subcellular localization were made using iPSORT (http://ipsort.hgc.jp/), TargetP (http://www.cbs.dtu.dk/services/TargetP/), Predotar (https://urgi.versailles.inra.fr/predotar/) and MitoProt (https://ihg.gsf.de/ihg/mitoprot.html) websites.

3. Results

In the present study, experiments were carried out to develop a new experimental approach able to reliably and accurately measure sirtuin activity in plant biological samples, by means of a protocol broadly applicable, technically simple and adaptable for high-throughput analysis. To achieve this goal, a combinatory application of enzymatic assays, differing in terms of substrates, principles of measurement, detection systems and quantification, was proposed. In particular, the luminescence SIRT-Glo™ (Promega) and (HTRF®)-based SIRT1 (Cisbio) assays were applied. The SIRT-Glo™ is based on indirect detection of deacetylated peptides by three coupled sequential enzymatic events involving a substrate containing a ε-*N*-acetylated lysine residue bound to aminoluciferin. Briefly, upon acetyl group removal by sirtuin, aminoluciferin is released by specific proteolytic cleavage and then oxidized by firefly luciferase, thus inducing bioluminescence emission, whose magnitude is directly related to sirtuin deacetylase activity (for details see Methods and **Fig. 1**). A very different strategy characterizes the HTRF® SIRT1 assay (Cisbio). Briefly, in this case, substrate deacetylation by sirtuin is detected by exploiting a FRET effect occurring between a fluorescent donor dye, consisting of Eu^{3+}-cryptate bound to anti-acetyl mouse monoclonal antibody, and a fluorophore acceptor, represented by the XL665 phycobiliprotein connected to a substrate containing a single acetylated lysine (for details, see Methods and **Fig. 2**). In the absence of sirtuin (**A**), close proximity of two fluorophores due to substrate/antibody interaction ensures the maximum energy transfer from the excited Eu^{3+}-cryptate towards XL665. Conversely, acetyl group removal by sirtuin activity prevents antibody binding to substrate, resulting in maximum extinction of FRET signal (**B**). Signal decrease is directly correlated to substrate deacetylation.

This methodological approach based on combinatory use of SIRT-Glo™ and HTRF® SIRT1 assays was first applied for studying native plant sirtuin activity in WM. Mitochondria were chosen as plant model system, since plant mitochondrial SRT2 isoforms have been demonstrated as key regulators of mitochondrial energy metabolism (König et al., 2014), and WM, in particular, are well-characterized from a bioenergetics point of view (Laus et al., 2008, 2011; Pastore et al., 2007; Pastore et al., 1999; Soccio et al., 2010, 2013).

To this purpose, a preliminary investigation was carried out to identify sequences coding for putative sirtuins in *Triticum aestivum* in EnsemblPlants (http://plants.ensembl.org/index.html) database. Based on a BLASTP search, by using *Oryza sativa* SIR2b (*Os*SIR2b) protein as query sequence, two putative SRT2 and a putative SRT1 were identified in wheat database (data not shown). In particular, the A0A1D5YSJ0 sequence, which we named *Wh*SRT2, shows very high homology with respect to *Os*SIR2b (86% identical at both CDS and amino acid sequence levels)

as well as to SRT2 counterpart from *Arabidopsis thaliana* (*At*SRT2, 64% and 59% identical at CDS and amino acid sequence levels, respectively) (**Table 1**).

Table 1. Protein and coding DNA sequence (CDS) identity among SRT2 from rice, wheat and *Arabidopsis*.

	% of identity	
	CDS	**Protein**
***Wh*SRT2 *vs Os*SIR2b**	85.5	86.3
***Wh*SRT2 *vs At*SRT2**	63.8	58.6
***Os*SIR2b *vs At*SRT2**	63.4	59.6

Bioinformatics analysis of subcellular localization of *Wh*SRT2 in comparison with *At*SRT2 and *Os*SIR2b was performed by using the iPSORT, TargetP, Predotar and MitoProt prediction software (**Table 2**).

All the applied tools predicted a mitochondrial localization of *At*SRT2; this result is in agreement with data from König et al. (2014) that demonstrated the exclusive *At*SRT2 targeting to mitochondrial compartment.

A prediction of mitochondrial/chloroplast localization was obtained for both *Os*SIR2b, as already reported by Chung et al. (2009), and *Wh*SRT2. It should be outlined that for *Os*SIR2b an exclusive mitochondrial localization by using transient expression in tobacco BY2 cells has been demonstrated (Chung et al., 2009). All together, these results strongly suggest the existence of a putative mitochondrial SRT2 protein in wheat.

As for sirtuin activity measurements, a first set of experiments was carried out to define experimental conditions suitable to assay this activity in WM. To this purpose, a comparison with a highly purified recombinant hSIRT1 was always carried out.

As for measurements by using the bioluminescent SIRT-Glo assay, typical experimental traces are reported in **Fig. 3A**. The addition of the SIRT-Glo™ Reagent (acetylated substrate, NAD^+, ATP, protease and luciferase, see Methods) to the reaction mixture containing hSIRT1 caused an increase of bioluminescent signal until reaching a maximum steady-state value after about 25 min. In particular, an increasing steady-state signal was observed in the presence of increasing amounts (30, 60, 120 and 240 ng) of hSIRT1 (traces c, d, e and f, respectively).

Table 2. Predicted subcellular localization of SRT2 from *Arabidopsis*, rice and wheat.

Name	Protein ID	Organism	Program			
			iPSORT[a]	TargetP[b]	Predotar[c]	MitoProt[d]
*At*SRT2	AYO45873[e]	*A. thaliana*	M	M	M	0.985
*Os*SIR2b[*]	ABA95936[e]	*O. sativa*	M	C	P	0.917
*Wh*SRT2	A0A1D5YSJ0[f]	*T. aestivum*	M	C	P	0.997

[a]iPSORT (http://ipsort.hgc.jp/); [b]TargetP (http://www.cbs.dtu.dk/services/TargetP/); [c]Predotar (https://urgi.versailles.inra.fr/predotar/); [d]MitoProt (https://ihg.gsf.de/ihg/mitoprot.html); [e]Genebank accession numbers; [f]UniProt accession number. M = Mitochondria; C = Chloroplasts; P = Plastid.
[*]Data from Chung et al. (2009).

Bioluminescent signal was completely abolished after boiling of hSIRT1 at 100 ºC for 10 min (trace a), as well as in the presence of 5 mM NAM (trace b), the most potent physiological inhibitor of all sirtuin family enzymes (Bitterman et al., 2002; Jackson et al., 2003). These results demonstrate the correct functioning of the bioluminescent sirtuin/protease/luciferase coupled assay. As for WM, a similar behaviour was observed. In particular, increasing amounts (50, 100, 150 and 200 µg) of WM proteins (**Fig. 3B**, traces i, j, k and l, respectively) caused an increase of bioluminescent steady-state signal. It should be underlined that in this case the reaction mixture also contained 0.1% Triton-X-100, 30 µM Ap5A, 4 µg oligomicyn, in order to lyse mitochondria so releasing mitochondrial sirtuin and to avoid ATP consumption by mitochondrial metabolism. Similarly to hSIRT1, the activity was abolished in boiled WM and in presence of 5 mM NAM (traces h and g, respectively). Overall, these results demonstrate that the bioluminescence generation observed in WM is an enzyme-mediated reaction and it can be attributable to sirtuin activity. An increase of reaction rate ($RLU \cdot min^{-1}$) was also observed in the presence of increasing amounts of both hSIRT1 and WM proteins. A linear dependence of bioluminescent signal, expressed as signal to noise ratio (**Fig. 3C**), on hSIRT1 amount was obtained in a wide protein range (25-250 ng). Similarly, the sirtuin activity in WM showed linear response over protein amount ranging from 50 to 250 µg (**Fig. 3D**). The result of Fig. 3C, relative to hSIRT1, can be also used as a calibration curve to quantify the sirtuin activity in WM. Interestingly, WM displayed a significant sirtuin activity, equal to 166 ± 12 ng hSIRT1 eq. $\cdot$ mg^{-1} of WM protein. Linear responses over both hSIRT1 and WM proteins were also obtained when the bioluminescent signal was expressed as reaction rate (**insets of Figs 3C** and **D**). Interestingly, signal to noise values resulted statistically positively correlated to reaction rate both in the case of hSIRT1 ($r=0.996$, $P< 0.001$) and WM-SRT ($r=0.994$, $P< 0.001$), respectively. These results show that reaction rate may be also used to quantify sirtuin activity, thus allowing reducing the time of analysis.

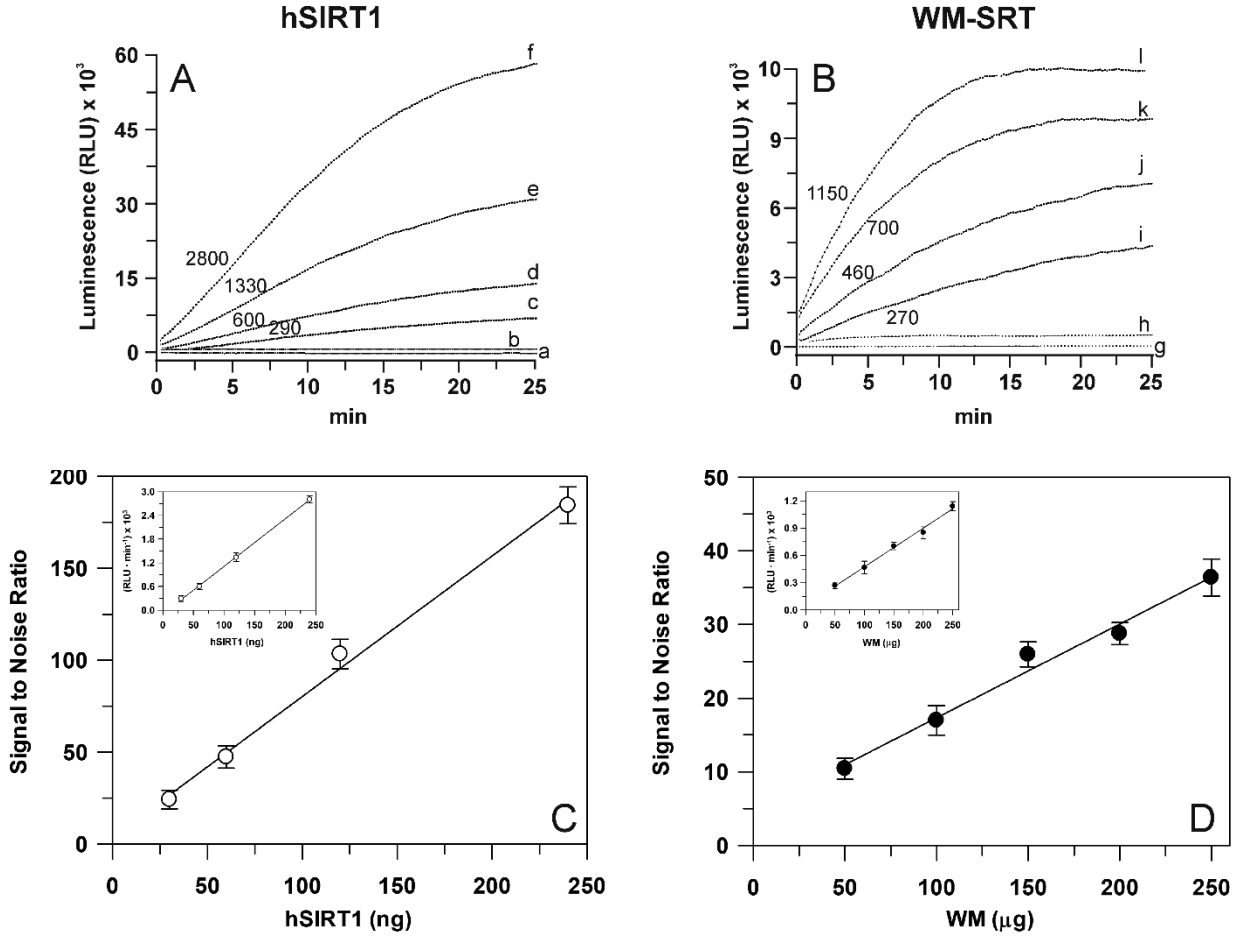

Figure 3. *Luminescent signal mediated by the recombinant hSIRT1 (A) and WM-SRT (B) and dependence of deacetylase activity by hSIRT1 (C) and WM (D) amount.* Sirtuin-mediated bioluminescent signal was monitored using the SIRT-Glo™ assay (Promega) as described in "Material and Methods" section in presence of 30, 60, 120 and 240 ng of hSIRT1 (traces c, d, e and f, respectively) and 50, 100, 150 and 200 µg of WM (traces i, j, k and l, respectively).
In traces (b) and (h) measurements were carried out in presence of 5 mM NAM using 120 ng hSIRT1 and 150 µg WM, respectively. In traces (a) and (g) measurements were carried out after boiling, at 100° C for 10 min, of 120 ng hSIRT1 and 150 µg WM, respectively. Numbers on the traces refer to the reaction rate expressed as RLU·min^{-1}.
In (C) and (D) sirtuin activity, expressed as "signal to noise ratio" and RLU·min^{-1} (insets), is reported as a function of hSIRT1 and WM amount, respectively. Data are reported as mean value ± SD (n=3)

By using the bioluminescent assay, another set of experiments was performed in order to assess whether sirtuin activity evaluated on WM is a true mitochondrial activity or a contamination due to other cellular fractions. At this purpose, the activities of sirtuin and of both PEPC and COX, marker enzymes of the cytosol and mitochondria respectively (Soccio et al., 2013; Trono et al., 2013), were evaluated in the total homogenate, in the combined pellets (containing nuclei) of the first and third centrifugations (see Methods), and in the purified mitochondrial fraction. As shown in **Table 3**, PEPC specific activity strongly decreased in mitochondrial fraction; conversely, COX activity showed a strong specific activity enrichment of about 30-fold in mitochondrial fraction.

These results confirm that the employed protocol is suitable to remove contamination due to cytosolic enzymatic activities and to obtain a highly purified mitochondrial fraction (Trono et al., 2013). As for sirtuin activity, no enrichment with respect to homogenate was observed in both pellet and mitochondrial fractions, thus suggesting that sirtuin activity is likewise present in both cellular compartments. Nevertheless, sirtuin enrichment in mitochondrial fraction was about two-fold higher than that observed in pellet fraction. This probably depends on the higher cytosolic contamination in pellet fraction with respect to the mitochondrial one, as confirmed by the higher PEPC recovery. The activity detected in all tested fractions was found to be NAM-sensitive, thus confirming that the assayed activity is really due to a sirtuin activity. These results support even more the choice of WM as a plant model system to study native plant sirtuin activity.

As for sirtuin activity measurements by using the HTRF® assay, experiments were carried out by running enzymatic reaction for 30 min in the presence of increasing enzyme amounts (0.1-160 ng). As shown in **Fig. 4A**, $\Delta F/F_{max}$ parameter was found to decrease with increasing amounts of hSIRT1 (control). As above reported, this depends on the hSIRT1-mediated deacetylation of the substrate-d2, causing a loss of FRET signal with a consequent reduction of $\Delta F/F_{max}$ parameter (see also Methods and Fig. 2). $\Delta F/F_{max}$ parameter was found to remain constant at the maximum value after boiling of hSIRT1 at 100 ºC for 10 min, as well as in the presence of 5 mM NAM.

The dependence of hSIRT1 activity on protein amount was also reported both in terms of percentage of deacetylated substrate and mU (**Fig. 4A'**). As expected, an opposite behaviour was obtained with respect to $\Delta F/F_{max}$ parameter.

In particular, highly statistically significant inverse correlation (r=-0.987, $P< 0.001$) was found between $\Delta F/F_{max}$ parameter and (%) deacetylation (or mU values), thus indicating the correct functioning of the assay. A linear dependence of $\Delta F/F_{max}$ parameter (**inset of Fig. 4A**), as well as of percentage of deacetylated substrate and mU (**inset of Fig. 4A'**), on hSIRT1 protein amount was obtained in 0.1-20 ng range.

It should be underlined that in parallel a time course study was performed in which the enzymatic step was carried out for increasing incubation times ranging from 15 to 240 min; a linear dependence from 15 to 90 min was found, thus confirming that 30 min is an appropriate incubation time for the enzymatic step (data not shown). A profile similar to hSIRT1 was obtained for $\Delta F/F_{max}$ parameter in the presence of WM proteins ranging from 0.05 to 80 μg WM (**Fig. 4B**, control).

It should be considered that, in order to allow the complete lysis of the organelles and make free the sirtuin activity, in this case the reaction mixture contained 0.1% Triton-X-100. Similarly to hSIRT1, activity was abolished in boiled WM and in the presence of 5 mM NAM.

Table 3. Protein content and SRT, phosphoenolpyruvate carboxylase (PEPC) and cytochrome c oxidase (COX) activities in different fractions obtained in the course of WM purification. Protein content and enzymatic activities were carried out as reported in Materials and Methods. For each enzyme, total activity (TA) and specific activity (SA) with respect to 1 g homogenate proteins, as well as the enrichment (*E*) of SA with respect to homogenate, are reported for all tested fractions. Data are expressed as mean value ± S.D. (n=3).

Fraction	**Protein (mg)**	**SRT**		**PEPC**		**COX**	
		TA[a]	**(SA[b], *E*)**	**TA[c]**	**(SA[d], *E*)**	**TA[e]**	**(SA[f], *E*)**
Homogenate	1000	429000±58000	(429, *1.00*)	52000±4750	(52, *1.00*)	13800±720	(13.8, *1.00*)
Pellet	35.1± 3.8	3300±360	(94, *0.22*)	1544±170	(44, *0.85*)	1035±82	(29.5, *2.14*)
Mitochondria	4.1±0.3	720±85	(176, *0.41*)	49±6	(12, *0.23*)	1566±185	(382, *28*)

[a] ng of hSIRT1 eq.; [b] ng hSIRT1 eq. · mg^{-1} protein; [c] nmol of NADH oxidised · min^{-1}; [d] nmol NADH oxidised · min^{-1} · mg^{-1} protein; [e] nmol of oxygen taken up · min^{-1}; [f] nmol of oxygen taken up · min^{-1}· mg^{-1} protein.

An opposite profile with respect to $\Delta F/F_{max}$ parameter was obtained for WM-sirtuin expressed as percentage of deacetylated substrate and mU (**Fig. 4B'**). Also in the case of WM, a significant inverse correlation (r=-0.999, $P< 0.001$) was found between $\Delta F/F_{max}$ parameter and (%) deacetylation (or mU values), thus confirming the correct functioning of the assay also in purified mitochondrial fraction. Similarly to hSIRT1, WM-SRT activity showed linear response over protein amount ranging from 0.05 to 5 µg, evaluated as $\Delta F/F_{max}$ (**inset of Fig. 4B**), as well as percentage of deacetylated substrate and mU (**inset of Fig. 4B'**). As already reported for the bioluminescent assay, these results demonstrate that FRET signal changes observed in WM can be attributable to sirtuin activity. Interestingly, WM-SRT activity equal to 268 ± 10 mU · mg^{-1} of WM protein was measured.

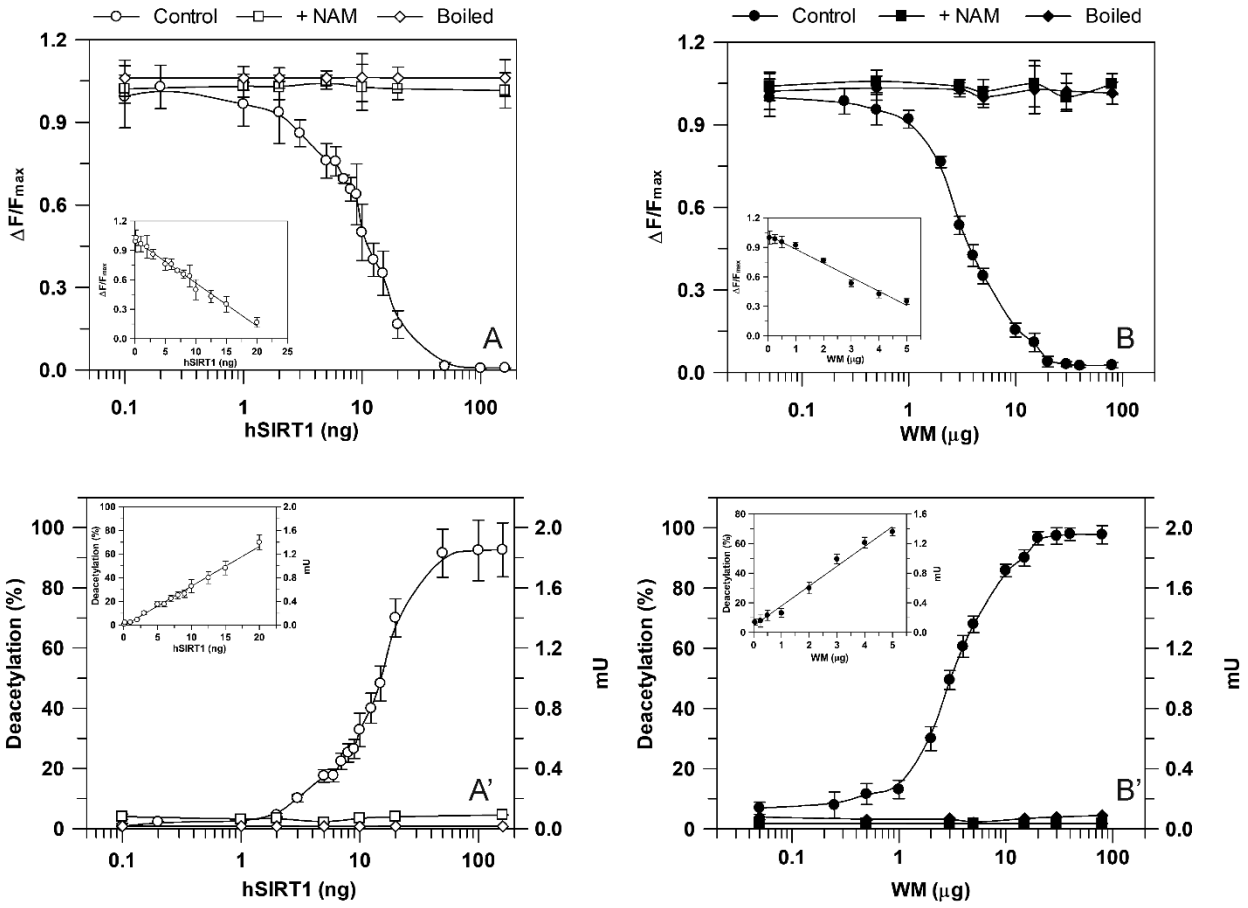

Figure 4. *Dependence of deacetylase activity, evaluated using HTRF® based method, on hSIRT1 (A and A') and WM (B and B') amount.* Sirtuin activity was monitored using the HTRF® SIRT1 assay (Cisbio) as described in "Material and Methods" using different hSIRT1 (A and A') and WM (B and B') amount, in absence (control) and in presence of 5 mM NAM as well as after boiling (100°C for 10 min) of hSIRT1 and WM, respectively. Data are expressed as $\Delta F/F_{max}$ (A and B) and as both deacetylation (%) and mU (A' and B'). In the insets, protein ranges, in which a linear dependence was obtained, are shown. All data are reported as mean values ± SD (n=3).

The newly developed approach was then applied to study modulation of sirtuin activity by resveratrol and quercetin, known as sirtuin activating compounds (Howitz et al., 2003), but whose

activation mechanism has raised controversies (Hubbard and Sinclair, 2014). Also in this case, a comparison between hSIRT1 and WM-SRT activities was made. As shown in **Fig. 5A**, in 50-200 µM range a complete inhibition on hSIRT1 by both resveratrol and quercetin was observed when the bioluminescent assay was applied. A complete inhibition was observed also by using the non-acetylated control substrate (data not shown), thus allowing to exclude a direct inhibition of resveratrol and quercetin on hSIRT1; conversely, the observed effect can depend on the capability of resveratrol and flavonoids to inhibit the firefly luciferase activity (Auld et al., 2008; Bakhtiarova et al., 2006; Zhang et al., 2017). A similar behaviour was observed also for WM-SRT (**Fig. 5B**). Interestingly, a different response was observed when the HTRF® assay was used.

In this case, the effect of both phenolic compounds was evaluated in 25-200 µM range. As shown in **Fig. 5A**, no effect was observed on hSIRT1 in the presence of resveratrol and only a slight increase, up to about 20%, was measured in the presence of 200 µM quercetin. As for WM (**Fig. 5B**), no effect on sirtuin activity was observed both in presence of resveratrol and quercetin.

4. Discussion

4.1 Novel approach for sirtuin activity measurements: use of methods based on different rationale and comparison with hSIRT1

This study arises in an attempt to overcome the difficulty of carrying out direct sirtuin activity assessment in plant cellular systems. To this purpose, a novel approach for measuring sirtuin activity in plant cell extracts was proposed that combines the use of multiple appropriately selected methods. The main requirement for choosing the assays to be combined is that methods are based on different reaction mechanisms and employ different substrates, experimental conditions, detection methodologies and quantification modes.

Moreover, the following criteria should be also met: *i)* assays should use simple protocols involving a readily available instrumentation, easily applicable to cell extracts and able of providing good performance in terms of accuracy and reproducibility; *ii)* they should be as far as possible able to avoid potential artifacts due to interfering molecules present in the reaction mixture/biological sample.

With respect to the last point, intense debate on the effects of some putative SIRT1-activating compounds (STACs) has arisen from the use of fluorimetric assays employing acetylated substrate peptides conjugated to AMC or carboxytetramethylrhodamine (TAMRA) fluorophores.

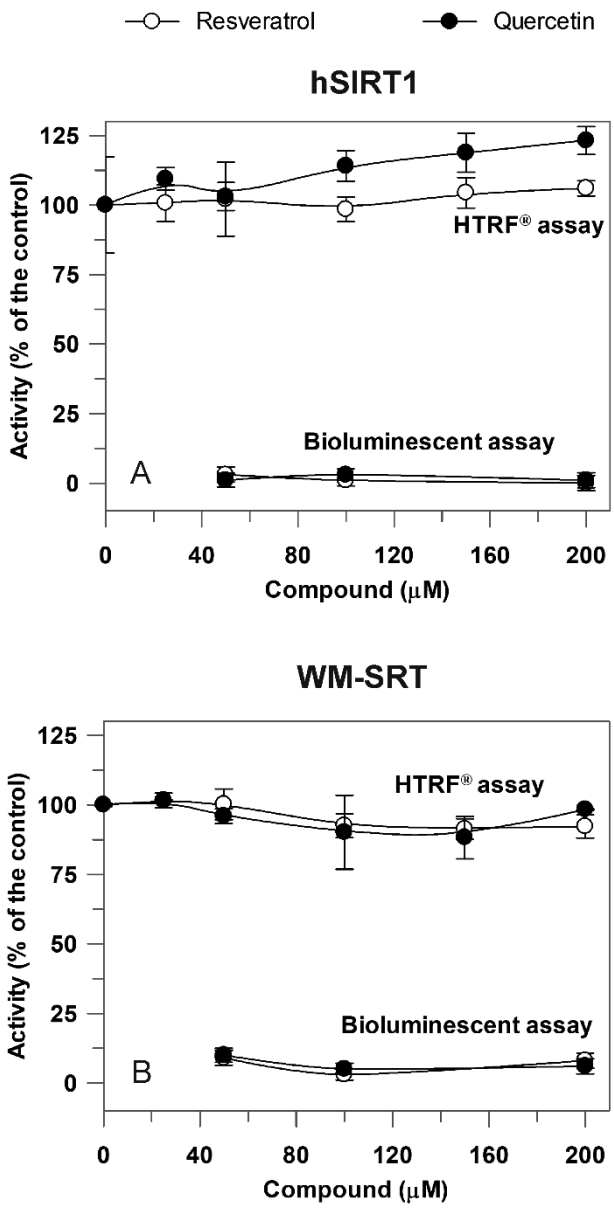

Figure 5. *Effect of resveratrol and quercetin on hSIRT1 (A) and WM-SRT (B) activities evaluated by both the luminescent and HTRF® assays.* (A) hSIRT1 activity was evaluated by HTRF® and luminescent assays, as reported in "Materials and Methods", by using 15 and 120 ng hSIRT1, respectively. (B) WM-SRT activity was evaluated by HTRF® and luminescent assays, as reported in "Materials and Methods", by using 3 and 150 μg WM, respectively. Measurements were carried out in the absence (control) and in presence of different concentrations of resveratrol and quercetin. Data are expressed as % of the control and reported as mean value ± SD (n=3).

By using the commercially available Fluor de Lys kit based on AMC-fluorescent assay, some physiological STACs were identified (Howitz et al., 2003), among which the most effective was resveratrol (Howitz et al., 2003). Subsequently, by using TAMRA-tagged substrates several synthetic STACs were also discovered (Dai et al., 2010; Hubbard et al., 2013; Milne et al. 2007). On the other hand, by using isotopic methods in comparison with Fluor de Lys assay, two independent research groups demonstrated that resveratrol is not a direct activator of SIRT1, being

the activation completely dependent on the presence of covalently attached fluorescent moieties in the peptide substrate (Borra et al., 2005; Kaeberlein et al., 2005). The fluorophore-specific activation of SIRT1 by resveratrol was also demonstrated by means of a fluorimetric assay based on quantification of remaining NAD^+ after deacetylation (Feng et al., 2009), as well as by direct HPLC detection and quantification of acetylated/deacetylated TAMRA-p53-derived peptide substrates (Pacholec et al., 2010). On the contrary, another report showed that some STACs activate the deacetylation of unlabeled peptides composed only of natural amino acids (Dai et al., 2010). A subsequent study demonstrated that *in vitro* SIRT1 activation by resveratrol and synthetic STACs requires hydrophobic residues in specific positions with respect to the acetylated lysine in some native peptide substrates (Hubbard et al., 2013). Finally, using peptide microarrays a separate research group demonstrated that effects of resveratrol are substrate sequence-selective, with activation occurring only with a few physiological acetylation sites, but not with other native sequences (Lakshminarasimhan et al., 2013).

In the light of these issues, an adequate combination of enzymatic methods deeply differing for chemical basis and rational may represent an effective strategy for revealing any interfering compounds and unmasking possible false positive/negative hits, so allowing a correct data interpretation.

In particular, in the present study sirtuin activity was evaluated by combinatory use of the luminescence SIRT-Glo™ (Promega) and (HTRF®)-based SIRT1 (Cisbio) assays. Both methods have the advantage of being a highly sensitive assay of sirtuin activity. Nevertheless, for the SIRT-Glo™ assay the activity assessment is indirectly obtained by measuring bioluminescence emission associated to aminoluciferin oxidation occurring in three coupled enzymatic events involving sirtuin/protease/firefly luciferase; moreover, sirtuin activity may be quantified in terms of arbitrary steady state signal. The advanced HTRF® SIRT1 assay directly measures the FRET signal level depending on the proximity between Eu^{3+}-cryptate donor and XL665 acceptor fluorophores (bound to anti-acetyl antibody and acetylated substrate, respectively), that in turn depends on substrate deacetylation by sirtuin activity. With respect to bioluminescence assay, the HTRF® assay shows the advantage *i*) of using the ratio between the donor and acceptor emission signals able to provide information useful for recognition of interfering substances, and *ii)* of allowing a quantification of sirtuin activity in terms of enzymatic units. It should be outlined that both used assays can be easily performed by using microplate readers and used in high-throughput analysis.

In addition to the appropriate selection of assays, the newly proposed approach for sirtuin activity determination in biological samples involves the parallel measurement of a commercial highly purified recombinant hSIRT1. Firstly, the use of hSIRT1 allows verifying the correct functioning

of detection systems. Moreover, the similar behavior observed in both hSIRT and biological sample under the same experimental conditions strongly suggests the existence of sirtuin activity also in the tested sample. In the case of bioluminescence assay and for all assays providing an arbitrary quantification of sirtuin activity, the parallel measurement of hSIRT1 allows an internal calibration, able to provide a relative quantification of sirtuin activity of the tested sample in terms of hSIRT1 equivalents. Therefore, the use of hSIRT1 has the advantage of normalizing sirtuin activity measurement in plant samples, thus allowing cross comparison among results obtained from different biological systems, experimental conditions, research groups and laboratories.

4.2 WM as a model system for evaluating plant sirtuin activity

The combinatory application of bioluminescence SIRT-Glo™ and HTRF® SIRT1 assays was used for the first time for studying sirtuin activity in WM, in comparison with hSIRT1 activity.

The interest in mitochondrial sirtuins arises from the relevant role in fine-tuning of mitochondrial energy metabolism recently reported for *At*SRT2 protein isoforms, able to catalyze the specific deacetylation of ATP/ADP carrier and ATP synthase (König et al., 2014).

The choice of WM takes into account different aspects. Firstly, the existence of a putative mitochondrial sirtuin activity is expected in wheat. This is in the light of recent literature data demonstrating the existence of plant mitochondrial sirtuin in the dicotyledonous *Arabidopsis thaliana* (König et al., 2014), as well as in a monocotyledonous cereal species phylogenetically far from *Arabidopsis*, but closely related to wheat, such as rice (Chung et al., 2009). In fact, by means of an *in silico* analysis we identified in wheat a full-length *Wh*SRT2 cDNA having *i*) highly homology to SRT2 from rice and *Arabidopsis* and *ii*) a very high probability to localize into the mitochondria, as supported by bioinformatic tools. These results strongly support the occurrence of sirtuin activity in WM.

Another aspect that should be considered is that highly pure, intact and functional mitochondria may be isolated with high yield from wheat seedlings (Pastore et al., 1999). Moreover, by measuring sirtuin activity, in comparison with cytosol and mitochondria marker enzymes, in different fractions obtained in the course of WM isolation, the highly purified mitochondrial fraction resulted *i)* characterized by sirtuin enrichment about two-fold higher than that observed in nuclear fraction (one of the major sites of sirtuin activity), and *ii)* affected by only a negligible cytosolic contamination compared to nuclei. In particular, a significant sirtuin activity equal to 268±10 mU · mg^{-1} protein and 166±12 ng hSIRT1 eq. · mg^{-1} protein was measured in WM, as evaluated by using HTRF® and the bioluminescence assays, respectively.

High recovery of mitochondrial proteins guaranteed by WM isolation protocol and significant sirtuin activity levels in purified mitochondrial fraction allow performing a large number of measurements from the same preparation (about 15-50 in RLU and 500-2000 in HTRF®). Moreover, for both the bioluminescence and HTRF® sirtuin assays the use of microplate readers allows for screening applications, thus strongly increasing the number of processed samples and reducing analysis times and assay cost per sample, also maintaining high repeatability of the results.

Taken together, these results strengthen the use of WM as a well-characterized good plant model system for studying plant mitochondrial sirtuin.

An interesting aspect should be emphasized. By using an appropriately developed experimental strategy, the present study reports the first measurement of catalytic activity of a "native" plant sirtuin, *i.e.* the first direct assessment of intracellular sirtuin activity and, in particular, within a subcellular system represented by purified mitochondrial fraction. In this regard, only one paper reports assessment of plant mitochondrial sirtuin activity, but determination was performed on overexpressed and purified *At*SRT2 protein from *E. coli* (König et al., 2014).

In light of this significant specific activity, an important physiological role can be suggested for sirtuin in WM, in analogy with key role in fine regulation of energy metabolism of SRT2 in *Arabidopsis* (König et al., 2014). The possible implication of WM-sirtuin activity in post-translational regulation of some transport pathways, extensively studied in WM and having a role in controlling ROS generation and mitochondrial bioenergetics under environmental/oxidative stress conditions (Trono et al., 2014; 2015 and refs therein), merits future investigations.

4.3 Study of modulation of WM-sirtuin activity by resveratrol and quercetin

The developed approach was applied for studying the effects on both hSIRT1 and WM-SRT activities of two phenols, such as resveratrol and quercetin. These compounds have been reported as potent hSIRT1 activators (Howitz et al., 2003), but with respect to their activation mechanism inconsistent and conflicting findings have been reported (Hubbard and Sinclair, 2014). The combinatory use of bioluminescence/HTRF® sirtuin assays proved to be crucial in identifying the false inhibition effect of both tested compounds shown by SIRT-Glo™ assay, which actually may be attributed to the capability of resveratrol and flavonoids to inhibit the firefly luciferase activity (Auld et al., 2008; Bakhtiarova et al., 2006; Zhang et al., 2017). Interestingly, by using HTRF® assay no effect of resveratrol was observed on both WM-SRT and hSIRT1 activities, while only a slight increase, up to about 20%, of hSIRT1 activity by quercetin was observed, much lower than that reported in previous studies using Fluor de Lys assay (de Boer et al., 2006; Howitz et al.,

2003). So, although a fluorophore-labeled substrate was used in HTRF® assay, we did not found any activation of hSIRT1 by resveratrol, unlike what was observed in previous studies reporting activation using AMC- or TAMRA-tagged substrates but not unlabeled peptides (Borra et al., 2005; Kaeberlein et al., 2005; Pacholec et al., 2010). The lack of a significant effect of both resveratrol and quercetin on hSIRT1 observed in our study may be explained in the light of findings of Lakshminarasimhan et al. (2013), showing either SIRT1 activation or no effect or inhibition by resveratrol depending on native peptide sequences used as substrate to measure activity.

Interestingly, we found a similar effect of resveratrol and quercetin on both hSIRT1 and WM-SRT. Considering the high phylogenetic distance between wheat and human species, this result may allow generalizing effects of these compounds to sirtuins from different plant sources under the same experimental conditions. This allows hypothesizing the use of WM to obtain preliminary information about modulation of other plant sirtuins characterized by very low and not easily detectable activity. Overall, these results strongly support the use of WM as a model system to study plant sirtuin activity.

5. Conclusions

Results of this study demonstrate that, by combining the use of two different enzymatic assays to the comparative measurement of activity of a highly purified recombinant enzyme (hSIRT1), a reliable and reproducible quantification of native sirtuin activity in plant biological samples can be achieved. For assessing sirtuin activity in plant extracts, a good model system has been identified in WM, able to be isolated with high purity and good yields and showing high sirtuin specific activity. The proposed methodology using WM and microplate readers can be qualified for application to large-scale high-throughput screening. In the light of this, the newly developed approach may represent an excellent tool to investigate the physiological role and modulation by phytochemical of plant sirtuin activity in experimental conditions (subcellular lysate) more biologically relevant, *i.e.* more resembling the physiological ones, compared to pure/recombinant enzymes. As a first application of the novel study model, the inability of resveratrol and quercetin to act as direct modulators of sirtuin activity in WM model system was shown in the applied HTRF® experimental conditions.

6. Abbreviations

AMC, 7-amino-4-methylcoumarin; Ap5A, P^1,P^5-di(adenosine-5') pentaphosphate; BSA, bovine serum albumin; COX, cytochrome c oxidase; DMSO, dimethyl sulfoxide; WM, wheat

mitochondria; FRET, Fluorescence Resonance Energy Transfer; hSIRT1, human SIRT1 isoform; HTRF®, Homogeneous Time Resolved Fluorescence; NAM, nicotinamide; PEPC, phosphoenolpyruvate carboxylase; PVP, polyvinylpyrrolidone; RLU, Relative Luminescence unit; SIRT, mammalian sirtuin isoforms; SRT, plant sirtuin isoforms; STAC, SIRT1-activating compound; TAMRA, carboxytetramethylrhodamine; XL665, phycobiliprotein pigment purified from red algae.

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9. Author Contributions

MS, MNL and MA performed the experiments and processed the experimental data. MS and MNL wrote the manuscript with support from MA. DP conceived the study, supervised the research and co-wrote the manuscript.

10. Conflict Of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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DISCUSSION AND CONCLUSIVE REMARKS

During the last few decades, dietary antioxidants have received increasing attention, especially within biological, medical and nutritional fields, owing to their putative protective roles against the deleterious oxidative-induced reactions implicated in the pathogenesis of several human diseases (Shahidi and Zhong, 2015). A considerable number of analytical assays has been developed claiming to ensure a fast, simple, convenient, and reliable *in vitro* determination of total antioxidant capacity (AC) of pure compounds or complex matrices, such as foods and biological samples. By using different reaction mechanisms, oxidant and target/probe species, reaction conditions, detection methodologies and result expression, these methods are able to measure mostly the scavenging capacity against certain free radical species and the reducing power of antioxidants (Huang et al, 2005; Magalhaes et al., 2008). Nevertheless, *in vitro* AC determination by analytical assays using one chemical reaction remains rather unrealistic and questionable for the difficulty in reflecting the *in vivo* situation (Fraga et al., 2014; Huang et al., 2005; Pompella et al., 2014; Shahidi and Zhong, 2015). An adequate combinatory application of AC assays showing different chemical basis could represent a valid strategy for a more biologically relevant AC assessment. However, this could not enough for obtaining reliable information on potential of dietary antioxidant as health promoting agents. Therefore, proposing new advanced methodologies and innovative approaches aimed at ensuring an AC determination really correlated to healthful effects of food antioxidants on consumers can be worthwile.

The research of PhD course has been developed in this context. In particular, very different aspects related to AC measurement of dietary phytochemicals have been critically tackled, with the common target of developing innovative methodologies/approaches able to provide information as much as possible reflecting the *in vivo* response.

An absolute novelty has been the model of study adopted to carry out the research: it consists on an approach with three sequential levels of study.

First level of study: in vitro AC determination of food matrices. The first level regards AC measurement of plant foodstuffs. At this level, a first methodological advancement has been attempted with respect to AC assessment of food matrices by means of "QUENCHER$_{ABTS}$" (QUick, Easy, New, CHEap and Reproducible) approach. This procedure allows AC determination of both soluble and insoluble food antioxidants, by exploiting direct either liquid-liquid or solid-liquid interactions of food solid particles with 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), avoiding any preliminary

extraction and hydrolysis step (Serpen et al., 2008). These interactions depend on particle size and, in the case of cereal grains, particles having 0.1-0.3 mm size are generally used. Research of this PhD thesis has allowed to propose a novel mode of calculation for $QUENCHER_{ABTS}$ procedure based on the slope value of the regression line of $ABTS^{\bullet+}$ response *vs* flour amount (Di Benedetto et al., 2015, see **Article 1**). By adopting this new slope calculation procedure, AC measurements of particles having larger size (up to 0.5 mm) have been achieved with results similar to that obtained using classical procedure. This extends applicability of $QUENCHER_{ABTS}$ method to the study of larger particles. This may be of interest in the case of some cereal milling products, that in the form of larger particles have been reported to positively affect technological quality of some derived foods (Noort et al., 2010; Chen et al., 2011), as well as to induce some beneficial effects on consumers (Stewart et al., 2009; Cai et al., 2014).

In addition to the $QUENCHER_{ABTS}$ calculation upgrade, research relative to the first level of investigation has pursued the relevant goal to design novel methods able to provide an AC assessment more reliable from a physiological point of view with respect to other widely used assays. This has been achieved through the development of the novel Lipoxygenase (LOX)-Fluorescein (FL) method (Soccio et al., 2016, see **Article 2**). It merges the advantages of LOX/RNO method (Pastore et al., 1999), deriving from the use of soybean LOX-1 isoform as a system to generate different physiological radicals, and the high sensitivity of ORAC assay due to use of fluorescein as a probe (Ou et al., 2001). Interestingly, the main requirements/criteria proposed by Prior et al. (2005) for standardization of AC determination may be considered sufficiently satisfied by LOX-FL method. In fact, it employs a protocol *i*) technically simple and involving a readily available instrumentation, *ii*) able of ensuring good reproducibility of results and *iii*) easily applicable to either single pure antioxidant compounds or food extracts, both fat-soluble and water-soluble. Moreover, similarly to LOX/RNO reaction, LOX-FL measurements are carried out in experimental conditions resembling those occurring *in vivo*. In fact, (*i*) more than one oxidant species having relevant biological significance are involved in LOX-FL reaction, (*ii*) the oxidant-antioxidant competition is evaluated under condition of low oxygen concentration, and (*iii*) it occurs at the surface of a biological macromolecule (the LOX enzyme) rather than in the bulk phase of the reaction mixture. Moreover, LOX-FL assay is able to simultaneously evaluate different antioxidant mechanisms (scavenging of different radicals, reducing or chelating of iron ions necessary for LOX-1 catalysis and direct inhibition of LOX-catalysed peroxidation reaction). In addition to being used for *in vitro* measurements of food extracts, the novel LOX-FL assay has the added dimension with respect to LOX/RNO method to be a suitable tool also for *ex vivo* analysis of human serum after food ingestion. With respect to *in vitro* AC assessment, LOX-FL

method resulted, in particular, capable to discriminate among different extracts obtained from the antioxidant-rich dietary wheat grain supplement Lisosan G and to assess very high values in hydrophilic and phenolic extracts of Lisosan G, similarly to the widely used ORAC and TEAC methods. As for *ex vivo* serum AC assessment, the new LOX-FL method was able to evaluate AC of the main blood antioxidants. Interestingly, unlike ORAC and TEAC assays, LOX-FL proved able to measure strong synergistic effects among blood antioxidants and between serum and food (phenols from cherry) antioxidants, as well as to reveal a significant increase of serum AC (up to 40% after 30 min) after Lisosan G intake. High performance of LOX-FL method in both *in vitro* and *ex vivo* measurements has the advantage to provide an integrated AC evaluation of food antioxidants: by using the same assay, it is possible to verify if foods showing strong AC may provide a real beneficial effect after ingestion. By means of LOX-FL method, Lisosan G, that was chosen in the light of its well-documented bioactivity (Longo et al., 2011; Frassinetti et al., 2012; La Marca et al., 2013; Lucchesi et al., 2014), was verified to show a strong *in vitro* AC and to significantly improve blood antioxidant status after ingestion. Conversely, incoherent information was provided by ORAC and TEAC assays, able to highlight a remarkable *in vitro* AC of Lisosan G extracts, but not in *ex vivo* serum analysis after Lisosan G consumption.
Interestingly, for the property to be applied to both *in vitro* and *ex vivo* AC measurements, LOX-FL assay can play an important role also in the second level of investigation, involving the analysis of AC of serum/plasma after food intake.

Second level of study: evaluation of human blood antioxidant status after food intake. It is a necessary level of study. In fact, *ex vivo* AC values of serum/plasma after food intake reflect bioavailability of dietary phytochemicals, *i.e.* the fraction of an ingested antioxidant compound reaching the systemic circulation; AC values measured in serum/plasma also depend on metabolism of food antioxidants, *i.e.* on the possible transformations of phytochemicals by gut microbiota and by intestinal and hepatic metabolism (Del Rio et al., 2010). In the light of this, *ex vivo* AC analysis may provide an integrated information about the real effect food antioxidant assumption on blood antioxidant status, which cannot be deduced by only *in vitro* AC measurements of the ingested food.
Unfortunately, contrasting results have been obtained from blood AC assessment after consumption of antioxidant-enriched foods, showing only limited AC increase (Khan et al., 2015; Torabian et al., 2009, as well as no effect (Fernandez-Pachon et al., 2008; Lettieri-Barbato et al., 2013) and, in some cases, even an AC decrease (Lettieri-Barbato et al., 2013). It should be also considered that some reports showed no AC change accompanied by a decrease of serum/plasma

oxidation level (Matthaiou et al., 2014). These findings suggest the unsuitability of blood AC measurements alone to derive information on food antioxidant effectiveness and the need to consider also changes of serum oxidative status. In the light of this, aimed at overcoming this weakness of the methodological approach, we attempted an advancement with respect to measurement of serum antioxidant status by proposing a novel parameter, named as "Antioxidant/Oxidant Balance (AOB)", representing the ratio between serum AC and serum oxidant status, evaluated as "Peroxide Level" (PxL) (Laus et al., 2017, see **Article 3**). AOB approach was applied for the first time to evaluate effects on serum antioxidant status during the first four hours after intake of two antioxidant-supplemented pastas, the Bran Oleoresin (BO) and Bran Water (BW) pastas, enriched respectively with either lipophilic (tocochromanols, carotenoids) or hydrophilic/phenolic antioxidants extracted from durum wheat bran. For comparison, a non-supplemented reference (R) pasta, as well as the dietary supplement Lisosan G and glucose, showing high AC or inducing a pro-oxidant effect, were also evaluated. Interestingly, similarly to the highly active Lisosan G, intake of BO pasta allowed a significant improvement of serum AOB, of about 70% as evaluated as LOX-FL/PxL and 40% as measured as ORAC/PxL or TEAC/PxL. Contrarily, BW pasta induced a serum oxidative effect so as R pasta and glucose; a decreasing trend of serum antioxidant status was also observed after consumption of R pasta added with Lisosan G. These results suggest the inefficacy, under our experimental conditions, of hydrophilic/phenolic antioxidants with respect to the lipophilic ones in compensating serum oxidation due to glucose release from starch digestion, as well as the capability of lipophilic compounds not only of counteracting detrimental effect of starch/glucose but also of improving serum antioxidant status. A quantification of changes of serum antioxidant status after food intake was also proposed by evaluating area under AOB profiles *vs* time, thus obtaining two AOB-derived parameters, indicated as AOB-Area and AOB-Index. In both cases, the remarkable superiority of Lisosan G was obtained, as well as the relevant beneficial effect on serum of BO pasta (+65% increase in the case of AOB-Index) and the worsening induced by BW pasta intake similar to that induced glucose and R pasta. On the contrary, area under profile relative to *ex vivo* serum AC analysis (AC-Area) was able to discriminate between Lisosan G and glucose only in the case of LOX-FL measurements, while ORAC and TEAC failed to do this. As for *in vitro* AC measurements of cooked pastas by means of LOX-FL, ORAC and TEAC methods, all three methods resulted in agreement only in highlighting a remarkable AC of hydrophilic and free-soluble phenolic fractions of Lisosan G, but contrasting results were provided on pastas. Despite the different final antioxidant enrichment of BO and BW pastas (equal to 1.3 times of total phenolic content in the case of BW pasta compared to the non-enriched pasta, and 3.1 and 3.4 times in total

tocochromanol and carotenoid content for BO past, respectively), only LOX-FL method was able to highlight a higher AC in BO pasta.

Overall, these findings indicate suitability and reliability of AOB approach in assessing short-term changes of blood antioxidant status after antioxidant intake. AOB approach appears an excellent tool in highlighting effects of antioxidant-enriched food consumption, which cannot be predicted by *ex vivo* analysis of AC alone, as well as by *in vitro* measurements of cooked foods, thus indicating that AOB is more performing than both *in vitro* and *ex vivo* AC determinations alone. Interestingly, AOB parameter provides the same results whatever the assay used to measure AC, although higher performance was obtained by using the novel LOX-FL method probably due to its capability to provide a more biologically relevant AC information. The use of AOB may solve some misleading results obtained by *ex vivo* serum AC determination alone. This good performance of AOB approach depends on the simultaneous evaluation of AC and PxL, which allows accounting for both AC and the AC fraction not directly measureable as consumed to compensate serum oxidation. Whether a positive effect on blood antioxidant status can be related to a health beneficial effect remains to be established. However, AOB may provide a biologically relevant information, since the maintenance of blood physiological antioxidant status is required to prevent aging-related endothelial dysfunction and consequent cardiovascular disease (El Assar et al., 2013). The reliability of the AOB approach was also assessed in long-term studies carried out both on humans (Soccio et al., 2018) and sheeps (unpublished data).

Third level of study: effects of food antioxidants at cellular/sub-cellular level. A part of PhD research activity was carried out in an attempt to define and develop a third level of investigation of antioxidant properties of dietary phytochemicals. After the study of food matrix (first level, *in vitro* measurements) and blood antioxidant status after food consumption (second level, *ex vivo* measurements), the third level of investigation concerns evaluation of effects exerted by bioactive compounds at the cellular and sub-cellular levels. To achieve this goal, biological effects of some phytochemicals were evaluated on activity of: *i)* Glyoxalase I, implicated in enzymatic cell defence against dicarbonyl compounds such as Methylglyoxal (MG), representing the major precursor of advanced glycation end products (AGEs) and *ii)* sirtuins, a family of NAD^+- dependent deacetylases of both histone and non-histone proteins, involved in several important cellular processes, including genomic stability, genetic material repair, transcriptional silencing, apoptosis, cell division and metabolism.

Glyoxalase I

Concerning Glo I, two studies were carried out. In the first study described in the **Article 4,** peripheral blood mononuclear cells (PBMCs) were used to investigate the effect of sulforaphane (SR), an isothiocyanate abundant in *Brassica* vegetables, on *i)* the expression and activity of GloI, *ii)* the levels of reduced glutathione (GSH) and *iii)* the expression of genes encoding for glutathione-S-transferase (GSTP1, GSTT2 and GSTM2). PBMCs were chosen as model system to estimate possible *in vivo* effects of SR, because they are human primary cell models. Incubation for 24 h and 48 h of PMBCs with 2.5 μM SR (simulating a daily consumption of a broccoli portion) did not induce a substantial increase in GloI activity and expression. Moreover, GSTP1 expression slightly increased after 24 h incubation, but not after 48 h incubation while the isoform GSTT2 and GSTM2 were not detected. Conversely, SR treatment caused 73% and 61% reduction in GSH levels compared to the control cells after 24 and 48 h of SR treatment, respectively; this suggests the formation of a GSH-SR adduct able to significantly reduce the actual SR concentration within the cells during incubation, so explaining the lack of a substantial effect on GloI and GSTP1 expression observed in this study. Our results are in contrast with previous studies reporting a significant increase of GloI activity and expression upon SR treatment of SH-SY5Y neuroblastoma cells (Angeloni et al., 2015), human hepatoma HepG2 cells and BJ fibroblasts (Xue et al., 2012). Also for GSH level, literature data showed a significant increase after 24/48 h incubation with SR of SH-SY5Y cells (Angeloni et al., 2015; Tarozzi et al., 2009), as well as of single aortic smooth muscle cells from spontaneously hypertensive rats or normotensive Wistar-Kyoto rats (Brooks et al., 2001) and of rat aortic smooth muscle A10 cells (Fuentes et al., 2015). Nevertheless, our data are in agreement with that reported by Navarro et al. (2011), showing an increase in GSH levels after 48 h incubation of human prostate cells with high (5-10 μM) SR concentrations, but no effect at lower doses, as that used in this study. In the light of these observations, further investigations are necessary to establish if high rate of GSH-SR adduct generation can be dependent on the applied experimental conditions or limited to the particular cell type used in our study. Moreover, higher SR doses could be tested, but not a longer cell exposure to SR, since PBMCs representing human primary cells are a well performing cell model in short-term studies.

This research was carried out at the "Food Quality and Design" Department of Wageningen University and Research (Wageningen, Netherlands).

In the second study described in the **Article 5,** the effect of some phytochemicals was evaluated on GloI activity in highly purified mitochondrial fraction obtained from durum wheat seedlings (WM). We chose to study mitochondrial GloI since mitochondria represent one of the major targets of MG-mediated carbonylation/oxidative stress (Li, 2016); therefore, an efficient MG

detoxification system is expected in these organelles. WM were chose in the light of twenty-year experience in the study of bioenergetics aspects of these mitochondria by the research group. In particular, WM have been demonstrated to be early damaged by ROS (Pastore et al., 2002), as well as to have some transport pathways, having a role in controlling ROS generation under environmental/oxidative stress conditions (Pastore et al., 2007; Trono et al., 2014, 2015). A parallel assessment of activity of the commercially available human recombinant GloI was carried out. Interestingly, a hight GloI activity was measured in WM, showing a hyperbolic dependence on substrate concentration, with Km and Vmax values equal to 0.27 mM and 0.133 EU/mg of protein, respectively. In order to identify possible physiological modulators of plant GloI reaction, the effect on WM-GloI activity of some polyphenols such as curcumin, quercetin and resveratrol, known as modulators of mammalian GloI (Cheng et al., 2012; Santel et al., 2008), as well as of the most relevant antioxidant compounds of durum wheat grains, including ferulic acid, sinapic acid and α- tocopherol (Laus et al., 2012), was assessed. Interestingly, curcumin and quercetin were found to strongly inhibit WM-GloI activity. A detailed inhibition study allowed identifying the competitive nature of inhibition by these compounds, with Ki values equal to 20 μM and 55 μM for curcumin and quercetin, respectively. In agreement with previous literature data (Santel et al., 2008), competitive inhibition by curcumin and quercetin was also confirmed for purified human GloI. While modulators of human GloI activity may have promising therapeutic value for cancer treatment (Sakamoto et al., 2001; Takasawa et al., 2008), further investigations are worthwhile aimed at understanding the physiological role of the inhibition of WM-GloI activity by curcumin and quercetin. It should be emphasized that this study reports the first direct assessment of GloI activity in plant mitochondria and the first investigation of its modulation by phytochemicals; therefore, this PhD thesis has allowed a significant advancement of knowledge regarding these issues.

Sirtuins

The attempt to improve some methodological aspects has played a central role in the research focused on sirtuin activity. In fact, to overcome some difficulty of carrying out direct assessment of intracellular sirtuin activity probably due to complexity of the commonly employed enzymatic tools, firstly, a novel experimental approach for reliably measuring sirtuin activity in cell extracts and/or subcellular organelles was proposed (see **Article 6**). It involves *i)* the combined use of multiple appropriately selected enzymatic assays, very different in terms of substrates, principles of measurement, detection systems and quantification, and *ii)* a comparative determination of activity of a recombinant human sirtuin 1 isoform (hSIRT1). In particular, the bioluminescent

SIRT-Glo™ and HTRF® technology-based based SIRT1 assays were used. The first indirectly measures bioluminescence emission associated to aminoluciferin oxidation occurring in three coupled enzymatic events involving sirtuin/protease/firefly luciferase. The HTRF® assay measures extinction of FRET signal between Eu^{3+}-cryptate donor and XL665 acceptor fluorophores, connected to anti-acetyl antibody and acetylated substrate, respectively, which depends on substrate deacetylation. For the first time, performance of this new approach was evaluated in measurement of sirtuin activity in WM. The interest in mitochondrial sirtuins arises from their implication in metabolic diseases, cancer, neurodegeneration, ageing-related disorders (Osborne et al., 2016); for plant mitochondrial sirtuins, in particular, a relevant role in fine-tuning of mitochondrial energy metabolism has been recently demonstrated *in Arabidopsis* (König et al., 2014). As already explained above, WM were chosen as a model system, since highly pure and functional organelles may be obtained with high yield from wheat seedlings (Pastore et al., 1999) and these mitochondria are well characterized from a bioenergetics point of view (Pastore et al., 2007; Trono et al., 2014, 2015). By using the newly proposed approach, a high and nicotinamide-sensitive specific sirtuin activity was determined in WM, equal 268±10 $mU \cdot mg^{-1}$ protein, as maeasured by HTRF® assay, and 166±12 ng hSIRT1 $eq. \cdot mg^{-1}$ protein, as evaluated by the bioluminescent assay and obtained after data normalization by using hSIRT1 activity. It should be emphasized that this is the first measurement of catalytic activity of a "native" intracellular plant sirtuin and, in particular, the first determination of activity within a subcellular system, consisting of a purified mitochondrial fraction. Effects of two naturally occurring dietary phenolic compounds, such as resveratrol and quercetin, known as potent hSIRT1 activators, but with a mechanism largely debated (Hubbard and Sinclair, 2014), were also studied. No significant effect of resveratrol and quercetin was found on WM-sirtuin and hSIRT1 activities by using HTRF® assay. This could be attributable to peptide sequences used as substrate to measure activity; according to findings of Lakshminarasimhan et al. (2013), these sequences can be responsible of contrasting effects (inhibition or activation or no effect) exerted by resveratrol and other modulators and observed in different separate studies (Hubbard and Sinclair, 2014 and refs therein). Overall, these results indicate WM as a good model system for studying native plant sirtuins; due to high WM sirtuin specific activity and good yields of pure organelles ensured by isolation protocol, a single WM preparation is sufficient to perform by using microplate readers numerous activity measurements, thus allowing application in high-throughput screening. With respect to the use of purified recombinant enzymes, WM appear also as a valid tool to obtain a more biologically relevant information about physiological role and modulation of plant sirtuins, as it is referred to enzyme within its environment (cell/subcellular organelles).

It is interesting to outline that our results obtained by using plant mitochondria can be considered from the standpoint of a *xenohormetic* approach. *Xenohormesis* explains how certain molecules such as plant polyphenols, that are secondary metabolites involved in the plant adaptive response to unfavourable both abiotic and biotic stress conditions (*hormesis*), can exert in consumers (*i.e.* mammals) a very similar adaptive response with beneficial health effects. It is evident that the understanding of the mechanisms responsible for the protective effect of phytochemicals in plants can provide useful information to understand the mechanisms of adaptive response in humans.

Taken together, results reported in this PhD thesis strongly demonstrate the crucial importance of the methodological approach in assessment of putative healthful properties of dietary phytochemicals. In fact, the selection of appropriate methodologies is necessary to avoid incorrect and misleading data interpretation about antioxidant properties of foods. With respect to this issue, an advanced methodological approach has been proposed. It combines *i) in vitro* AC measurements of food extracts by means of innovative assays based on the soybean LOX-1 reactions able to produce several physiological radical species in experimental conditions approaching the cellular ones and to reveal different antioxidant attributes and *ii) ex vivo* analysis of serum after food consumption, involving both AC and peroxidation level determination and calculation of AOB parameter as AC/PxL ratio. By using this innovative approach, high antioxidant properties of the dietary supplement Lisosan G have been confirmed, as well as the pro-oxidant effect of glucose ingestion. Interestingly, the same approach has allowed highlighting a strong efficacy in enhancing serum antioxidant status of a pasta supplemented with durum wheat bran oleoresin extract, as well as excluding a possible biological effect of another putative functional pasta, added with phenolic compounds from bran water extracts. In the light of these results, our approach appears a highly performing tool able to provide a more integrated and trustworthy information about potential health value of food antioxidants, which is unpredictable by AC analysis alone. The picture emerging from this combined *in vitro/ex vivo* study of antioxidant properties of dietary antioxidants should be appropriately completed by evaluation of biological effects of phytochemical at cellular/subcellular level. With respect to this point, new and very interesting results are reported in the PhD thesis regarding effects of some phytochemical on enzymatic activity of both sirtuin and glyoxalase I measured within a highly purified mitochondrial fraction obtained from wheat. This encourages deepening this innovative mode of studying bioactive properties of phytochemicals, which can allow evaluation of biological action exerted by these antioxidant compounds on mitochondrial function, with particular interest on the capacity of these organelles to counteract carbonylation and oxidative stress.

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