POTENTIAL ROLES OF POLYPHENOL OXIDASE IN APPLE TISSUES: EFFECT ON MICROBIAL GROWTH AND ANTIOXIDANT CAPACITY

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Experience is what you get when you didn't get what you wanted. The brick walls are there for a reason; they are not there to keep us out, but to give us a chance, to show how badly we want something, because brick walls are there to stop people who don't want it badly enough. I'm dying, and I'm having fun, and I'm going to keep having fun every day I have left, because there's no other way to play it. Never lose the childlike wonder, it's just too important: it's what drives us.

Randy Pausch

POTENTIAL ROLES OF POLYPHENOL OXIDASE IN APPLE TISSUES: EFFECT ON MICROBIAL GROWTH AND ANTIOXIDANT CAPACITY

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Polyphenol oxidase (PPO) is a typical food enzyme considered very important in the food industry mainly for its damaging effects. It is responsible for tissue darkening after fruit cuts or bruises, resulting in a nutritional value loss and a general decrease in food acceptability. However, this colour development is desirable, too, for certain products like coffee or apple cider. Despite all these considerations, it is not completely clear which roles this compound may have in cell metabolism. In this project, its possible antimicrobial and antioxidant effects were examined. Analyses were carried out in two distinct ways: properties of standard polyphenolic substances oxidized by PPO were assayed alongside tests on *Golden delicious* apple extract, whose phenolics were previously isolated.

Toxicity investigations were performed against three bacteria belonging to *Lactobacillus* spp. (*L. brevis*, *L. casei* and *L. plantarum*) and three strains of *Saccharomyces cerevisiae*, all isolated from fruits. For these organisms, Minimum Inhibitory Concentration (MIC) test was executed and results between oxidized and not oxidized solutions were compared. Simultaneously, Total Antioxidant Capacity (TAC) was researched, for the same samples. In this case, data were collected with DPPH test and crocin bleaching assay and results were compared to a Trolox C® standard solution.

From the MIC analyses of four different standard polyphenols (catechol, chlorogenic acid, epicatechin and quercetin) antimicrobial capacity seemed to be

affected by PPO only for the first, and just against *L. brevis* and *L. plantarum*. Besides that, quercetin promoted microbial growth, despite the inconsistent outcomes of PPO influence on that: the enzyme was able to reduce such promotional effect for one strain (*S. cerevisiae* SC/D/1P) while in another case it was accentuated (*S. cerevisiae* SC/B/4A). On the other hand, results obtained from apple extracts showed how fruit phenolics, either treated or not with PPO, were able to accentuate microbial and yeast growth. *S cerevisiae* SC/B/4A and SC/D/1P increased their metabolism, effect confirmed for *L. brevis*, even if in a much lower manner.

Examining TAC data, an increase in free radical scavenging activity for the action of PPO arose for catechol and chlorogenic acid, while quercetin exerted an opposite behaviour. Despite the lower TAC values of apple extracts, they appeared to confirm results collected for the first two phenolics, especially when measured with DPPH test.

A mia madre, che ha sempre creduto in me…

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LIST OF ABBREVIATIONS AND ACRONYMS

AA: Amino acid

AAPH: 2,2′-azobis(2-methylpropionamidine) dihydrochloride

BHA: Butyl hydroxyl anisole

BHT: Butyl hydroxyl toluene

CCT: Tropical culture collection

CHS: Chalcone synthase

DAD: Diode array detector

DNA: Deoxyribonucleic acid

DPPH: 2,2-diphenil-1-picrylhydrazyl

ε: molar absorptivity

$(E)_0$: Initial concentration of the enzyme

EC: Enzyme classification

EMP: Embden-Meyerhof-Parnas

F: Stabilization factor

FDP: Fructose diphosphate

IP_0: Induction period in the absence of an inhibitor

IP_{inh}: Induction period in the presence of an inhibitor

k_m: Michaelis-Menten constant

k_{cat}: Turnover value

LDL: Low density lipoprotein

MIC: Minimum inhibitory concentration

MIC_{50}: Minimum inhibitory concentration to 50 % inhibit microbial growth

MIC_{90}: Minimum inhibitory concentration to 90 % inhibit microbial growth

MRS: Man-Rogosa-Sharpe

NAD^+/NADH: Nicotinamide dinucleotide

OD: Optical density

ORR: Oxidation rate ratio

PAL: Phenylalanine ammonia-lyase

PHPPA: 3-(4-hydroxyphenyl)-propionic acid

PODs: Peroxidases

PPO: Polyphenol oxidase

PV: Peroxide value

ROS: reactive oxygen species

$(S)_0$: Initial concentration of the substrate

SC/A/7A: *Saccharomyces cerevisiae* strain extracted from apple

SC/B/4A: *Saccharomyces cerevisiae* strain extracted from apple

SC/D/1P: *Saccharomyces cerevisiae* strain extracted from plum

SD: Standard deviation

Sen: Sensitizer

TAC: Total antioxidant capacity

tBHQ: *tert*-butylhydroquinone

Trolox (m)eq.: Trolox C® (milli) equivalents

v: initial slope of the curve trend were calculated for cuvettes containing an amount of antioxidant

v_0: initial slope of the curve trend for cuvettes without samples

W_0: Oxidation rate in the absence of an inhibitor

W_{inh}: Oxidation rate in the presence of an inhibitor

YPD: Yeast-peptone-dextrose

INTRODUCTION

1. OXIDATION PROCESSES

An oxidation is a general reaction involving a loss of electrons by a molecule, atom or ion. However, it mainly involves unsatured compounds (as lipids) and oxygen.

In nature, molecular oxygen behaves as a biradical, having two unpaired electrons in its ground state: this form is called triplet ($^3O_2$) state because of the number of the possible electron configurations. In this form, a molecule cannot react directly with unsatured substances, due to its spin barrier (Frankel, 2007), so they have to be activated by a number of initiative mechanisms, such as singlet oxygen, partially reduced activated oxygen species (H_2O_2, $O_2^{\bullet -}$, $HO^{\bullet}$) and metals, giving radicals. This process is named autoxidation and this phase is commonly known as the "initiation step" (Figure 1).

After the formation of the radical, almost instantly this reacts with triplet oxygen, giving a peroxyl radical: the rate of this step is 10^6–fold greater than the others (at atmospheric oxygen pressure), so the concentration of alkyl radicals $L^{\bullet}$ is basically zero. All the reactions involving a radical transfer contribute to form the "propagation step".

Finally, the "termination step" includes the formation of non-radical products.

In the first two steps, the hydrogen atom close to an isolated double bond is removed and the alkyl radical is stabilized by resonance (α-methylene mechanism) (Farmer, *et al.*, 1943). However, in polyunsatured fatty acids, bis-allylic carbons are present. The lower bond energies of their hydrogens and the allylic ones versus methylene hydrogens (75 and 88 versus 100 kcal/mol), combined with a better resonance and stabilization, lead to a much easier

oxidation of polyunsatured compounds (Robaugh & Stein, 1986; Vajda, *et al.*, 1986; Gardner, 1989).

INITIATION $RH \rightarrow R^{\bullet} + H^{\bullet}$

PROPAGATION $R + {}^{3}O_2 \rightarrow ROO^{\bullet}$

$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$

$RO^{\bullet} + ROOH \rightarrow ROO^{\bullet} + ROH$

$RO^{\bullet} + RH \rightarrow ROH + R^{\bullet}$

$RH + {}^{\bullet}OH \rightarrow ROH + R^{\bullet}$

TERMINATION $2R^{\bullet} \rightarrow RR$

$2OR^{\bullet} \rightarrow ROOR$

$ROO^{\bullet} + R^{\bullet} \rightarrow ROOR$

$RO^{\bullet} + R^{\bullet} \rightarrow ROR$

$2ROO^{\bullet} \rightarrow ROOR + {}^{1}O_2$

$2ROO^{\bullet} \rightarrow ROOH + RO + {}^{1}O_2$

Figure 1: Steps of autoxidation processes.

The primary products of these reactions will be hydroperoxides if the oxidised substances were lipids, whereas epoxides, ciclic peroxides and polyperoxides will be formed in case of other types of olefins (Yanishlieva, 1971). Hydroperoxides are tasteless and without smell because they are too big and heavy to reach olfactory receptors of human beings and to stimulate papillae. An entire series of secondary reactions breaks these compounds, producing molecules having the typical taste and smell of oxidized food (Shahidi, 1997).

The generation of singlet oxygen ($^{1}O_2$), leads to a different type of oxidation:

the photoxidation. This phenomenon occurs when a sensitizer (typically a natural pigment such as chlorophyll, pheophytin, porphyrin or riboflavin) absorbs energy from light to transfer it to triplet oxygen, exiting it (Figure 2). Even in this case, the presence of coenzymes, metal salts and transitional metal complexes may increase the process (Min & Boff, 2001). In the excited state, oxygen can react directly with organic compounds as spin barriers no longer exist (Conte, 2004).

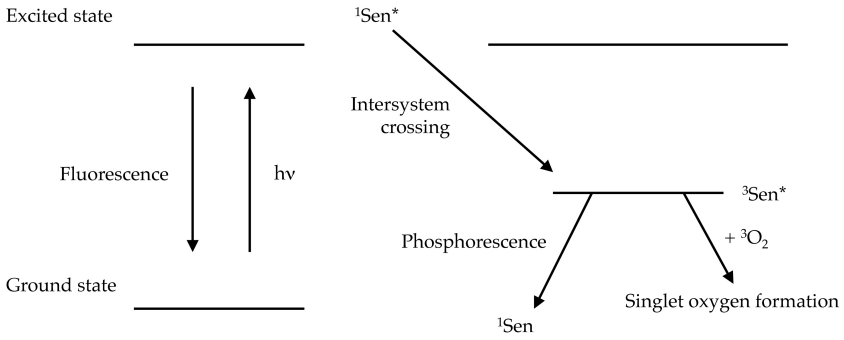

Figure 2: Chemical mechanism for the formation of singlet oxygen in the presence of a sensitizer.

Once the sensitizer is oxidized (^{3}Sen*), the reaction can continue following two different pathways (Figure 3). It may release its energy surplus to a compound, generating a radical (this is a common autoxidation initiation step), or it can activate a triplet oxygen molecule, bringing it to its singlet state ($^1O_2$). The sensitizer returns to its ground state (^{1}Sen) and may begin the cycle again while the oxygen reacts directly with double bonds to make hydroperoxides. In this case the configuration of double bonds changes from *cis* to *trans*.

The rate of Type II pathway is strongly correlated to oxygen solubility and concentration in the food system. Thus, the change from one type to another can be achieved just depleting the gas. Considering that oxygen is more soluble in lipids than in water (Golovanov & Zhenodarova, 2005), aqueous food systems favour Type I pathway due to the lower availability of this compound.

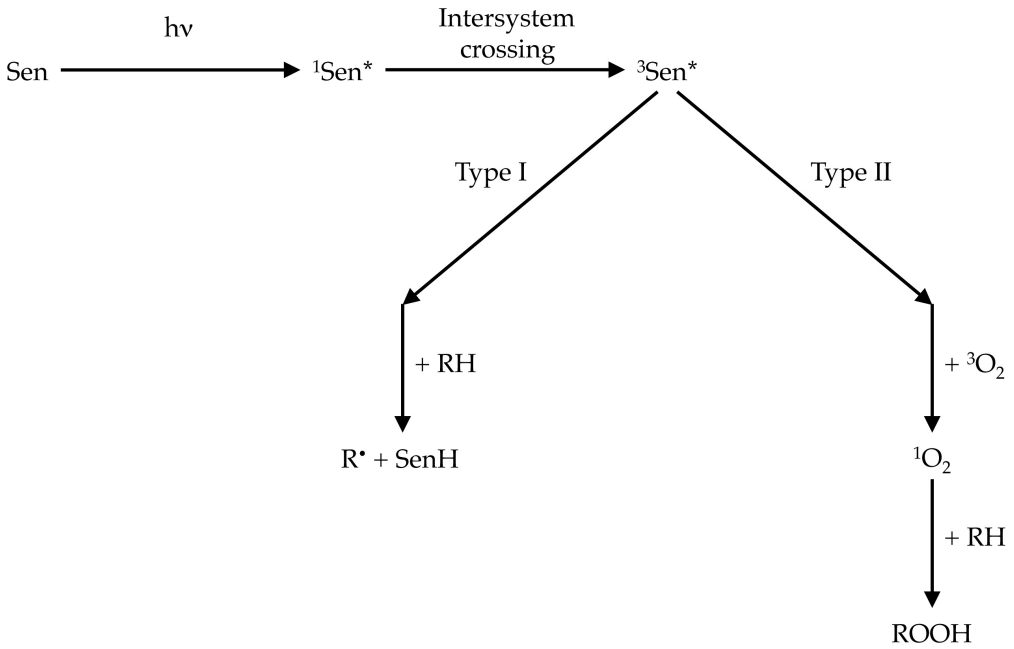

Figure 3: Formation of excited triplet sensitizer (^{3}Sen*) and its reaction with substrate via Type I and Type II reactions.

These two types of reactions will enhance oxidation by either the formation of reactive radical compound species or the production of singlet oxygen. The competition between compounds and triplet oxygen for the excited sensitizer is another reason that determines whether the reaction pathway is Type I or Type II. Of course photoxidation may change its pathway during the reaction as the concentration of reagents could evolve. The case of aqueous-lipid biphasic systems is interesting, where the longer half-life of excited oxygen in the lipid phase favours Type II pathway.

1.1. Classification

Being the antioxidant capacity a functional property, a univocal classification of this broad group of molecules does not exist. Antioxidants belong to a very heterogeneous group of substances sharing the characteristic to slow down oxidative processes as long as possible. It is a functional classification (not

chemical) and total inhibition of such phenomena is impossible to achieve, only its delay is possible.

However, their action in inhibiting either the initiation or the propagation steps of peroxidation could be useful to start to divide them. Besides that, other classes of antioxidants exist, such as oxygen scavengers and photoxidation inhibitors (Frankel, 2007).

Table 1: Effect of EDTA and gallic acid on lipid oxidation in mayonnaise containing 16 % fish oil.
(Jacobsen, *et al.*, 2001)

Sample	Hydroperoxyl triacylglycerol	Hydroperoxyl cholesterols esters	Fishy flavor (Scale of 0 to 9)	Fishy aroma (Scale of 0 to 9)
Control	7.86	0.34	2.8	2.0
+ Emulsifier (Em)	6.87	0.26	3.0	1.7
+ Gallic acid	3.13	0.19	3.4	2.5
+ Gallic acid + Em	2.49	0.13	3.0	2.1
+ EDTA	1.86	0.18	0.3	0.3
+ EDTA + Em	1.60	0.13	0.1	0.2

If the target of the action of antioxidants is to reduce radical generation, different strategies may be followed. They can remove or chelate metals, changing their oxidoreductive potential, and they have to be selected carefully to avoid the activation of metals by altering their redox potential. Ascorbic acid may act as a metal chelator but it can be converted into a powerful pro-oxidant if transitional metals are present (Retsky, *et al.*, 1993; Kondakçi, *et al.*, 2013). Chelators in which oxygen atoms are bind to the metal tend to prefer the oxidized form of iron or copper and decrease their redox potential. These types of antioxidant substances, together with metal binding proteins (such as lactoferrin), are more effective than the compounds targeting the propagation step because they are particularly susceptible to oxidations. Lactoferrin is an iron transport non-heme glycoprotein

present in a wide range of biological secretions that may inhibit oxidations of different lipid systems. However, as described for ascorbic acid, this peptide could exert a pro-oxidant activity, if its concentration is high enough (Nielsen, *et al.*, 2004). Other important chelating proteins found in human plasma include transferrin, which removes iron, and albumin, which binds copper while Table 1 shows how EDTA proved to be more effective than gallic acid in lowering oxidative and flavor deterioration of mayonnaises enriched with fish oil.

In Table 2, the strength of different antioxidant substances is presented. They were analysed in three different lipid systems, varying the temperature of the test. In this case, antioxidant capacity was found calculating the ratio between the stabilization factor (F) and the oxidation rate ratio (OOR) parameters (Equation 1). The first was estimate comparing the induction period of the oxidation in presence (IP_{inh}) and absence (IP_0) of an inhibitor molecule, while for the other one the two oxidation rates were used, with (W_{inh}) and without (W_0) inhibitor (Yanishlieva, 2001).

(I) $$A = \frac{F}{ORR} = \frac{IP_{inh}/IP_0}{W_{inh}/W_0}$$

Another strategy to inhibit the initiation step is to stabilize the excited sensitizers formed during photoxidation. Certain substances simply transfer light energy without free radical formation. Carotenoids are the most important inhibitors of Type II photosensitizer oxidation. They act interfering with the activation of triplet oxygen to singlet oxygen by an energy transfer mechanism from singlet oxygen to carotene (Ramel, *et al.*, 2012).

Table 2: Activity of some natural antioxidants during autoxidation at different temperatures. (Yanishlieva, 2001)

Antioxidant	Lipid system	Antioxidant concentration (M * 10^4)	Temperature (°C)	A	Reference
Ferulic acid	TGL	10.3	100	5.2	1
Caffeic acid	TGL	11.1	100	10350	1
Ferulic acid	TGOO	10.3	100	20	2
Caffeic acid	TGOO	11.1	100	4867	2
Ferulic acid	TGSO	10.3	100	4.3	1
Caffeic acid	TGSO	11.1	100	448	1
α-tocopherol	TGL	2.33	22	24.6	2
α-tocopherol	TGL	2.33	90	221.4	2
α-tocopherol	TGSO	2.33	22	6.9	3
α-tocopherol	TGSO	2.33	90	167.5	3
Esculetin	TGL	5.6	100	324	4
Fraxetin	TGL	4.8	100	764	4
Esculetin	TGSO	5.6	100	97	4
Fraxetin	TGSO	4.8	100	88	4
Quercetin	TGL	4.44	22	86.7	5
Morin	TGL	4.44	22	84.2	5
Quercetin	TGL	4.44	90	22.1	5
Morin	TGL	4.44	90	195.7	5
Quercetin	TGSO	4.44	22	21.9	3
Morin	TGSO	4.44	22	14.4	3
Quercetin	TGSO	4.44	90	310	3
Morin	TGSO	4.44	90	48.6	3
Thymol	TGL	13.3	22	7.1	6
Carvacrol	TGL	13.3	22	5.5	6
Thymol	TGSO	13.3	22	75.0	6
Carvacrol	TGSO	13.3	22	2.8	6

TGL: triacylglycerols of lard
TGOO: triacylglycerols of olive oil
TGSO: triacylglycerols of sunflower oil
A: Antioxidant power, function of the induction period and the oxidation rate with and without inhibitor (see text for details)

[1] Yanishlieva & Marinova (1995)
[2] Marinova & Yanishlieva (1996)
[3] Marinova & Yanishlieva (1998)
[4] Marinova, *et al.* (1994)
[5] Yanishlieva & Marinova (1996)
[6] Yanishlieva, *et al.* (1999)

Unfortunately, carotenoids are quickly destroyed by light or hydroperoxides;

thus to be effective, they have to be protected by chain-breaking antioxidants. The effectiveness of β-carotene as an inhibitor of photosensitized oxidation in unsatured lipids depends to a large extent on its protection against oxidation by natural tocopherols, which also can reinforce its activity (α-tocopherol may react with singlet oxygen, quenching it and forming stable addition products). If not protected, carotenoids behave as pro-oxidant substances, forming free radicals (Zeb & Murkovic, 2013). β-carotene and other carotenoids such as lycopene have been classified as antioxidants as they behave as relatively weak free radical scavengers (if low oxygen pressures are present). However, this dual effect of β-carotene as a strong singlet oxygen quencher and weak free radical scavenger has led to much confusion and controversy in biochemical and nutrition literature.

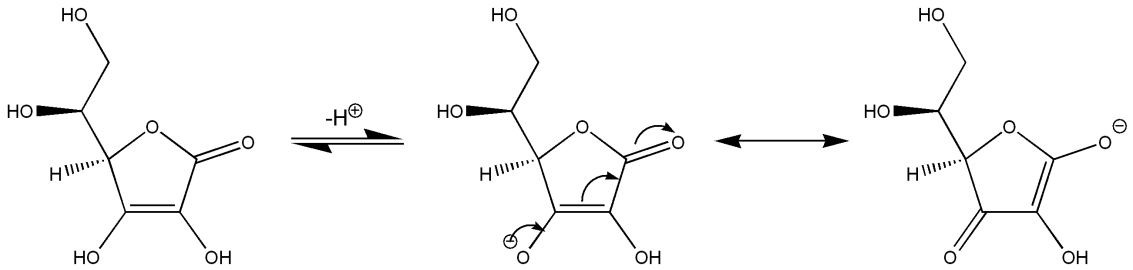

Figure 4: Ascorbic acid resonance structures that allow its radical form to be more stable.

Finally, there are even compounds that convert hydroperoxides into stable hydroxyl products. Ascorbic acid and tocopherols at stoichiometric concentrations can reduce hydroperoxides to produce stable hydroxyl compounds. Sodium borohydride has been used to reduce hydroperoxides accumulated during vegetable oil processing, but it has never been adopted commercially. Several phosphorus and sulphur reducing compounds have been used in industrial olefin applications, but they are not suitable for foods (Frankel, 2007).

On the other hand, compounds that convert peroxyl radicals into stable

products can be classified as inhibitors of the propagation step. Generally, these molecules lose hydrogens, becoming radicals, and stabilize themselves with multiple resonance structures (Figure 4).

1.2. Relations between antioxidant capacity and health

Several situations such as infections, inflammations, ultraviolet radiation, and tobacco smoke can increase free radical production. Oxidation compounds are connected with a series of diseases and illness and they are responsible for a large number of cell damages, interacting with biological tissues (Fitó, *et al.*, 2007). Targets for free radicals may be lipids, of course, but deoxyribonucleic acid (DNA) and proteins can suffer interactions with these compounds, too. Oxidations lead to a plethora of mutagenic DNA lesions in purines, pyrimidines, deoxyribose, and DNA single- and double-strand breaks (Poulsen, *et al.*, 1998; Whiteman, *et al.*, 2002). These mutation accumulations from oxidative DNA damages are hypothesized to be a crucial step in human carcinogenesis (Evans, *et al.*, 2004).

In these last few decades, several studies correlated a reduced risk of coronary heart disease with a dietary intake of foods rich in natural antioxidants (Hertog, *et al.*, 1993; Stampfer, *et al.*, 1993). A possible explanation of this effect may be the interruption of lipid peroxidation operated by polyphenols. Even the oxidative degradation of the low-density fraction of lipoproteins (LDL) is supposed to be implicated in the first stages of arteriosclerosis and heart diseases (Itabe, 2009). However, the simple shielding of LDL from reactive oxygen species (ROS) controlled by antioxidants may not be enough to totally explain the activity of such compounds. Several polyphenols might affect cellular response to different stimuli, including cytokines and growth factors.

Some polyphenols may also influence bacterial enzymes, contributing to

decrease the overall cancer risk.

Sengottuvelan & Nalini (2006) supplemented rats with resveratrol (8 mg/kg body weight/day, intragastrically), finding a reduction in colon cancer incidence, if compared to control rats. However, it is still not clear if this effect was due to modifications of enzymatic activity within a subpopulation of gut microorganisms or just a change in the proportions of specific bacteria (Cardona, *et al.*, 2013). The anti-inflammatory activity of this polyphenol includes inhibition of proinflammatory mediators, modification of eicosanoid synthesis and inhibition of certain enzymes (Namasivayam, 2011).

Miene, *et al.* (2011) analysed the influence of polyphenol metabolites on modulating the activity of some enzymes implicated in detoxification and inflammation of human adenoma cells. These authors hypothesized a correlation between that regulation and the chemopreventive potential of polyphenols, after degradation in the gut. Besides that, coffee and caffeic acid proved to specifically inhibit colon cancer metastasis in mice (Kang, *et al.*, 2011) and tea-derived catechins exert anticancer activity, mediating different cellular events (Butt & Sultan, 2009; Singh, *et al.*, 2011). Another strong antioxidant molecule is quercetin, a flavonoid found in apples and vegetables, which proved to be anticancer by inhibiting cell proliferation and the induction of apoptosis (Gibellini, *et al.*, 2011).

Whether the concentration of these compounds can be sufficiently achieved in human diets to exert these beneficial effects is not known but, in this scenario, it is obvious that factors influencing system antioxidant strength in food are to be considered very carefully.

1.3. Factors influencing antioxidant capacity

Total Antioxidant Capacity (TAC) depends not only from the activity of

antioxidants present in the environment. A wide range of factors, indeed, contributes to affect such property. Antioxidant concentration, temperature, light, type of substrate, physical state of the system and micrcomponents acting as pro-oxidants or synergists may influence oxidant stability.

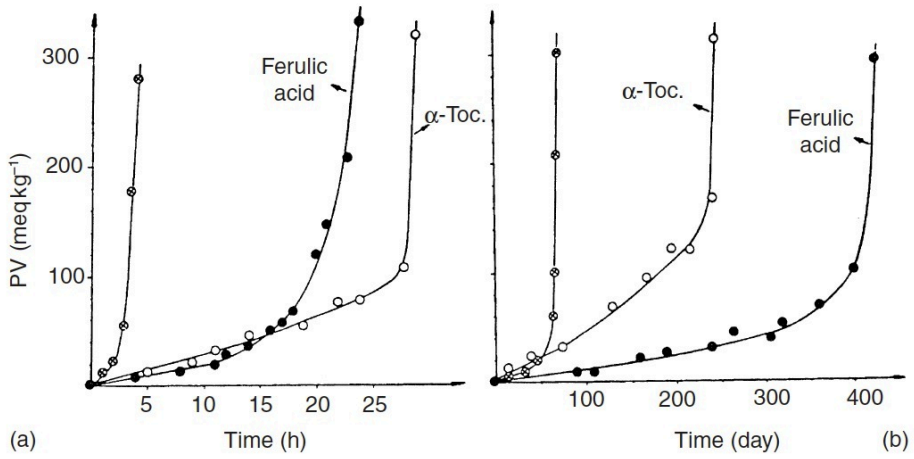

Figure 5: Kinetic curves of peroxide accumulation during inhibited oxidation of triacylglycerols heated (a) or kept at room temperature (b).

Among the physical factors, oxygen pressure, heating and irradiation play the major role, causing an acceleration of the chain initiation and propagation of the oxidation process. Naz, *et al.* (2005) investigated the effect of air, light, heat and deep-frying on three different edible oils, finding a clear increase in peroxide and *p*-anisidine values. Different temperatures may change even the mechanism of action of some antioxidants (Marinova & Yanashlieva, 1992). In Figure 5 the oxidation kinetics (expressed as peroxide values, PV, versus time) of triacylglycerols heated (a) or kept at room temperature (b) is illustrated. An extension of the induction time is clearly visible if a 2.4 mM solution of ferulic acid or α-tocopherol was added but more interesting is the different behaviour of the two substances: the acid proved to be more effective at 100 °C, if compared to vitamin E while an opposite performance was detected at room temperatures.

Considering also physical factors, antioxidant behaviour is system-dependent, too. Lampi & Kamal-Eldin (1998) stated that fatty acid composition influences oxidative stability more than minor antioxidants, if the system is at frying temperatures. Otherwise, if not heated, oil oxidation is more affected by the latter. The effect of antioxidants in stabilizing the environment depends even if the fatty acid mixtures are either bound to triglycerides or to monohydric alcohols (Yanishlieva & Marinova, 1995; Marinova & Yanishlieva, 1996). Free fatty acids also slow down oxidative stability and the same effect may be observed if other lipid microcomponents are present in the moiety, such as mono- and diglycerides or fatty alcohols (Popov & Yanishlieva, 1969; Kortenska & Yanishlieva, 1995).

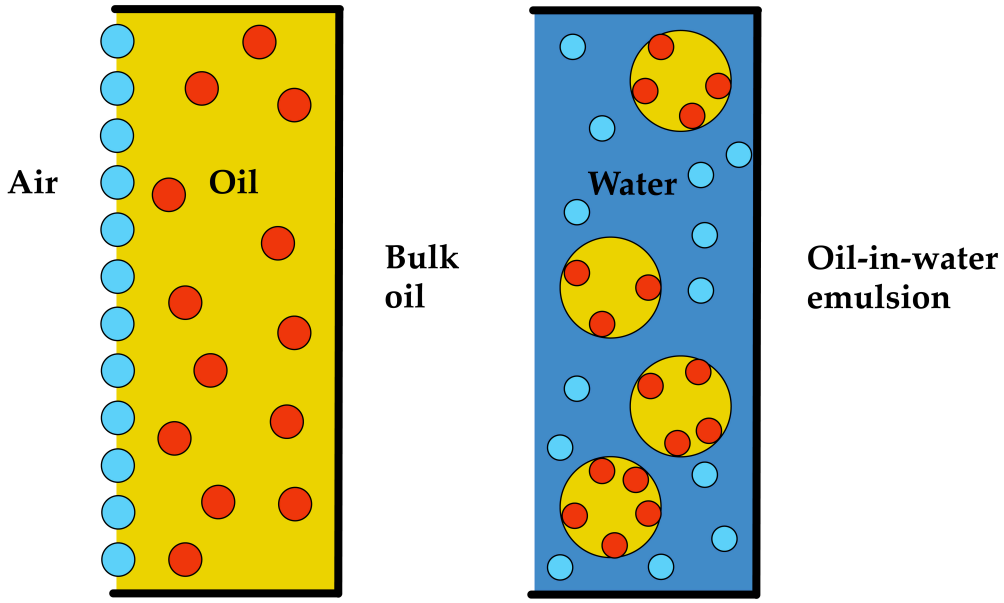

Figure 6: Arrangement of hydrophilic (light blue circles) and lipophilic (red circles) antioxidant molecules in bulk oil and in oil-in-water emulsion.

Another factor strictly influencing oxidation is the physiochemical state of the system. In the past years, a lot of evidence pointed out the contrast in relative effectiveness of phenolic antioxidants, depending on the hydrophilic/lipophilic balance of the mixture. Frankel, *et al.* (1994) proved that α-tocopherol is more

effective in oil-in-water emulsions than bulk oil, while Trolox C® (its hydrophilic analogue) showed the opposite trend. Besides that, they found that carnosol proved to have a stronger antioxidant capacity in oil-in-water emulsions than the more polar rosmarinic and carnosic acids; again, a reverse behaviour can be observed in bulk oils (Frankel, *et al.*, 1996). Finally, Yanishlieva, *et al.* (1994) demonstrated that the order in antioxidant power for α-tocopherol and caffeic acid was reversed passing from a bulk phase to a liposome oxidation, the acid having a stronger activity in the former case but a weaker one in the latter. This apparent contradiction between the polarity of the substances with stronger activity and the polarity of the system has been described by the term of "polar paradox" (Porter, *et al.*, 1989). A possible explanation of this phenomenon could be given observing where the antioxidants are placed in the two phases (Figure 6). In bulk oil, hydrophilic antioxidants arrange themselves in the oil-air interface, protecting the fat matrix better than the hydrophobic compounds dissolved in the homogenous lipid phase. On the contrary, if an oil-in-water emulsion was taken in exam, non-polar antioxidants were found to migrate towards the surface of the lipid droplets, exerting their action in a more efficient way than the hydrophilic ones. Moreover, this already complex situation is complicated by the interfacial phenomena taking place in heterogeneous systems: hydrophilic polyphenolic compounds may vary in their partition behaviour between water and oil phases and their interface (Schwarz, *et al.*, 1996; Huang, *et al.*, 1997; Heinonen, *et al.*, 1998).

2. ENZYMES AND ANTIOXIDATION SYSTEMS

Enzymes play a crucial role among microcomponents present in food that may influence oxidative stability. A lot of these proteins are implicated in

oxidoreductive processes, regulating the oxidation balance in tissues or just minimizing damage caused by the wide range of secondary substances produced during cell metabolism.

Enzyme classification divides all these compounds into six main groups. The first of them (EC 1) is strictly dedicated to oxidoreductases. These may act transferring hydrogen or electrons; otherwise they can add oxygen to the substrates.

Superoxide dismutase (EC 1.15.1.1) is one of the most characteristic enzymes that protect cells from ROS. This protein can use different elements as coenzymes, including manganese and iron, and operate a dismutation between two superoxide anions, producing a less reactive hydrogen peroxide and a molecule of oxygen (Halliwell & Gutteridge, 1999).

Catalase (EC 1.11.1.7) lowers hydrogen peroxide amount, transforming it into water and oxygen. Plants have multiple catalases, each one encoded by a different gene, and they act operating a dismutation: one molecule of hydrogen peroxide is reduced to water while another one is oxidized to oxygen (Halliwell & Gutteridge, 1999). Besides that action, this enzyme contributes to convert hydroperoxides to less dangerous alcohols (Chance & Herbert, 1950; Maehly, 1954).

Even glutathione peroxidase (EC 1.11.1.9) is able to reduce hydrogen peroxide levels inside tissues but this selenoenzyme operate in a different way: it oxides two glutathione molecules, combining them together (Mills, 1957; Christophersen, 1968; Little & O'Brien, 1968).

Alcohol dehydrogenase (EC 1.1.1.1) transfers two hydrogens from ethanol to nicotinamide adenine dinucleotide (NAD^+), both acceptor substrate and cofactor, producing NADH, a proton and a molecule of acetaldehyde (Ribas de Pouplana,

et al., 1991).

Examining apple tissues, enzymes proved to affect antioxidant capacity very strongly, even if post-harvest treatments, climatic conditions and genes seemed to significantly modify composition and concentration of such phytochemicals. However, despite all the genetic and environmental factors, Łata, *et al.* (2005) proved that apples maintain their nutritional value during long-term storage. The same authors stated that apple antioxidants contribute in keeping fruit quality and play a crucial role in apple responses to different storage conditions.

2.1. Antioxidant interaction

Enzymes fighting oxidation stress cooperate in an efficient network, built to regenerate reducing cofactors and to reinforce their mutual protection (Frankel, 2007).

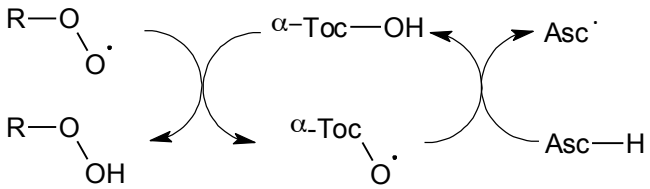

Figure 7: Tocopherol-ascorbate redox cycle.

α-tocopherol exerts its action donating a hydrogen (Lass & Sohal, 1998) to stabilize peroxyl radicals but it may be restored to its reduced form by the activity of ascorbic acid (free radical translocation), due to the higher reduction potential of the latter (Figure 7). The different polar nature of the two compounds is bypassed because of the polar paradox: in phospholipid liposomes, the oxidized tocopherols arrange themselves exposing radicals towards the aqueous phase of the bilayer interface; in this way, they can easily be reduced by the polar ascorbic acid molecules. However, this radical translocation is not finished as the ascorbate

may be reduced by glutathione, with the resulting disulphide in turn restored by NADH (and glutathione peroxidase).

Another example of this network might be dephosphorylation. Phosphatases action can be modulated by antioxidant enzymes (preventing their oxidative inactivation) and by 5-lipoxygenase activity in leucocytes.

2.2. Synergism

The large number of interactions between antioxidants is also referred to as synergism. A possible classification of all these processes divides them into three main classes, based on their mechanism of action.

Homosynergism happens when two antioxidant molecules act with the same principle, as tocopherol and ascorbic acid. This type of interaction may be also observed between two chain-breaking compounds such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) or BHA and propyl gallate. Even mixtures of phenolic compounds, lecithin polyols and amino acids may be synergists, scavenging metals. Moreover, lecithins could help the system exerting their emulsifying properties to improve contact between polar antioxidants and non-polar substrates.

On the other hand, if the two compounds act through different mechanisms, heterosynergism occurs. Citric acid may be used in combination with BHA, BHT or *tert*-butylhydroquinone (tBHQ) to better protect food: the first is a well-known chelating agent while the others are hydrogen donators. Even vegetable oils containing tocopherol mixtures could be stabilized by the addition of this organic acid: the compound protects the system from metal catalysed oxidation. Phenolics were found to have synergistic effects with lactoferrin, too. The highest effects were found more in liposomes than in in emulsions (Medina, *et al.*, 2002).

Finally, autosynergism happens when the same molecule can exert two different antioxidant actions. Flavonoids are able to scavenge free radicals, deactivate metals (complexing them), inhibit enzymatic generation of free radicals and inhibit membranes and LDL oxidation. In this scenario, molecules help each other to fight oxidation stress and the resulting antioxidant capacity is exponential.

2.3. Phenolic compounds

Among the most powerful antioxidant substances, polyphenols certainly play a crucial role. This family of compounds is widely distributed in vegetable and fruit products, performing numerous functions. Some polyphenols are responsible for the colour of flowers and for a contribution to certain flavours; besides that, they are supposed to play crucial roles against microorganism proliferation and insect attack (Lattanzio, *et al.*, 2012) and protection from UV damage (Landry, *et al.*, 1995; Booij-James, *et al.*, 2000).

These substances may be modified after a methylation catalysed by *o*-methyltransferases, or by acylation and glycosylation of secondary metabolites. Such compounds have different volatility, polarity and chemical stability: for this reason, polyphenols biological activity and their interaction with other metabolites may vary significantly. Phenolics production is strictly regulated by a matrix of overlapping signals, including developmental signals (such as anthocyanins production during flower and fruit development) and environmental signals (protection against biotic and abiotic stresses) (Cheynier, *et al.*, 2013).

An evolutionary adaption led plants to accumulate these secondary metabolites in response to adverse environmental conditions (Matern & Grimmig, 1993; Harborne, 1995). Finally, phenolics may be a way to store carbon molecules

(accumulated from photosynthesis), when nitrogen access is limited (Lattanzio, *et al.*, 2012).

In the last decades, polyphenolic compounds were largely examined. Particularly, the attention focused on all the effects that phenolics may exert on human health, after digestion. The most studied sources of such substances are red wine (Ghiselli, *et al.*, 1998) and olive oil (Cioffi, *et al.*, 2010) but, nonetheless, apples are rich in polyphenols, especially present in skins. The research conducted by Huber & Rupasinghe (2009) found that chlorogenic acid, epicatechin, phloretin, proanthocyanidin and quercetin are the most representative phenolics present in apples. Results partialy conferming a study conducted by Veberic, *et al.* (2005), where the most typical polyphenols seem to be chlorogenic acid, epicatechin, protocatechuic acid, *p*-coumaric acid and phloridzin.

3. POLYPHENOL OXIDASE

Polyphenol oxidase (PPO) was discovered in mushrooms in 1856 by (Schoenbein, 1856).

This enzyme has several names, it is also known as tyrosinase, phenolase, catecholase, catechol oxidase, *o*-diphenol oxidase, monophenol oxidase, and cresolase. PPO nomenclature is so complex due to the two general types of substrates it may act on: monophenols (Figure 8, a) are hydroxylated in the *o*-position (Monophenol : oxygen oxido reductases, EC 1.14.18.1) while diphenols (Figure 8, b) are oxidized removing a hydrogen from the hydroxyl groups (1,2-benzendiol : oxygen oxido reductases, EC 1.10.3.1), forming benzoquinones (NC-IUBMB & Webb, 1992). If the second hydroxyl group is bond to the *p*-carbon, however, such substances may give dark melanoidinic pigments (Figure 8, c).

Finally, even laccases (benzendiol : oxygen oxido reductases, EC 1.10.3.2) contain copper, but oxidation mechanism differs from that of the *o*-diphenol oxidases (Figure 8, d).

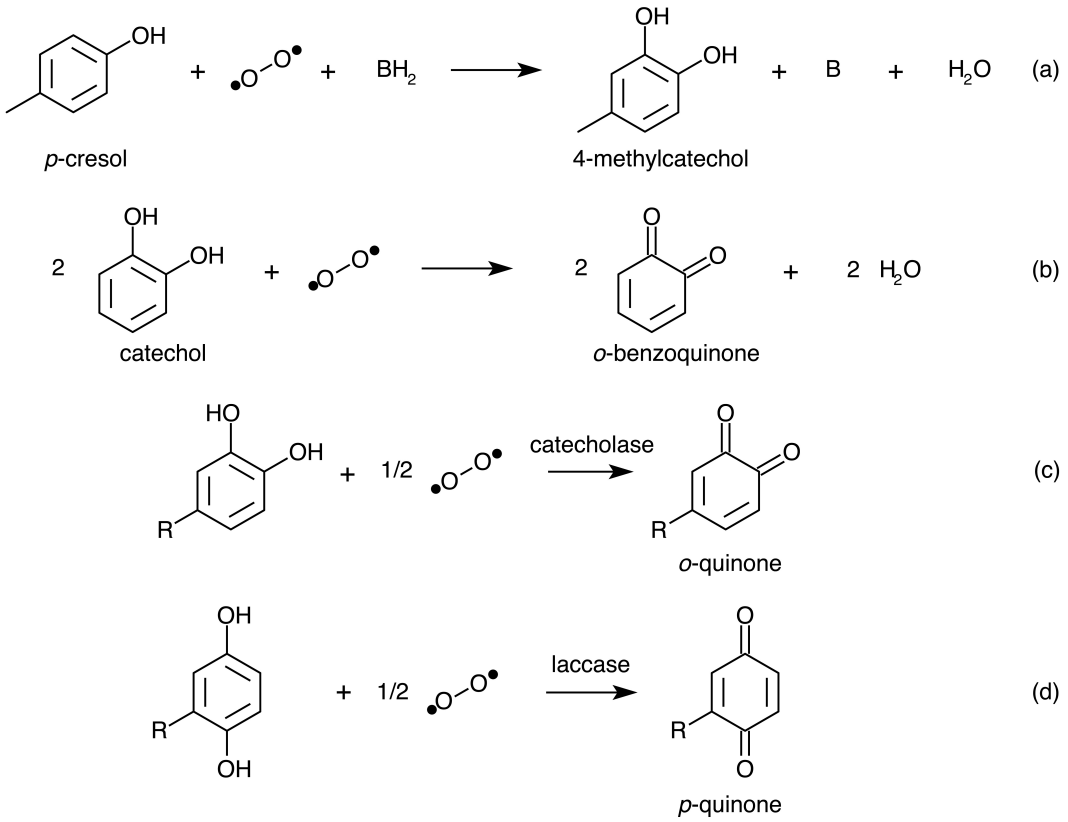

Figure 8: Phenolic oxidation catalysed by different polyphenol oxidases.

Different types of PPO are present in many plant tissues (Rocha, *et al.*, 1998; Mishra, *et al.*, 2012), and in many animals, including insects (Wang, *et al.*, 2013), shrimps (Zamorano, *et al.*, 2009), mice (Silvers, 1980) and humans (Witkop, 1985). This large array among so phylogenetically different organisms makes PPO functions even more interesting and worth of investigations.

3.1. Importance in apple quality

PPOs were studied a lot in the past years, mainly for the technological

damage they can cause in apples during harvesting and/or storage (Vámos - Vigyázó & Haard, 1981; Nicolas, *et al.*, 1994; Chisari, *et al.*, 2007). If these fruits are accidentally cut or bruised, oxygen manages to penetrate inside cells, reacting with PPOs and polyphenols to give undesirable products. These substances comprehend dark melanoidins and off-taste compounds, producing simultaneously a general loss of nutritional value. Brown spots on the surface of damaged apples are a clear example of such reactions.

A lot of methods exist to inhibit PPO, including thermal processes (Benlloch-Tinoco, *et al.*, 2013), pH variations (Liu, *et al.*, 2013), addition of antioxidants (Holzwarth, *et al.*, 2013), and the use of physical barriers to prevent contact between enzyme and oxygen (Jiang, *et al.*, 2005; Thongsook & Tiyaboonchai, 2011). The latter approach, accomplished with modified atmospheres, coatings or edible films, is what normally plants do in nature, with waxed skins (Ramírez, *et al.*, 2003).

Nonetheless, colour development due to PPO activity is required for certain products. Tea, coffee, cocoa, apple cider, prunes, black raisins, Black Mission figs and zapote need this enzyme to acquire their complete sensorial profile (Lea, 1984; Mazzafera & Robinson, 2000).

3.2. Structure

Like every enzyme, PPO structure is organized in four levels; each of them is fundamental to the overall activity of the enzyme.

Nowadays, primary amino acid sequences of several PPOs are identified, thanks to the modern techniques of gene analysis. Comparing together such structures of different PPOs belonging to higher plants, fungi and higher animals, Whitaker (1995) found interesting results. The author calculated a similarity of

96.6 % between the two potato DNAs extracted, 92.2 % between potato and tomato but just 38.1 % between potato and broad bean. If fungi are analysed, *Streptomyces glaucescens* is sharing 87.5 % of similarities with *S. antibioticus* and 17.0 % with *Neurospora crassa*. The comparison between humans and mice gave a similarity of 41.0 % while potatoes have a similarity from 10 to 20 % with the other species examined. Of course, such percentages increase if only the active-site regions A and B were taken in exam. The site A includes 26 amino acids (AA), and it has similarities between PPOs ranging from 31 to 100 %, while in the greater site B (56 residues) the similarities vary from 34 to 96 % (Table 3).

Table 3: Amino acids sequence similarities between nine polyphenol oxidases in active-site regions A and B.
(Adapted from Whitaker (1995))

Source	Active-site region A		Active-site region B	
	AA sequence region	% Similarity	AA sequence region	% Similarity
Potato (a)	110-135	100	241-296	100
Potato (b)	110-135	100	241-296	96
Tomato	110-135	100	241-296	96
Broad bean	110-135	96	241-295	62
Streptomyces glaucescens	54-76	50	189-229	36
Streptomyces antibioticus	54-77	42	189-229	34
Neurospora crassa	96-121	42	277-320	36
Human	190-215	31	350-390	34
Mouse	191-216	31	352-393	38

Being PPO similarities so various, it is not surprising that even their molecular weights are widely spread (Table 4). To be precise, it must be taken in consideration that part of this variability may be due to a partial proteolysis of the enzyme during isolation. Furthermore, a whole family of genes codes for these peptides, so genetic variability could play an important role in the definition of their primary structure, and consequently their molecular weights (van Gelder, *et*

al., 1997; Mayer, 2006).

Table 4: Comparison between polyphenol oxidase molecular weights isolated from different sources.

Source	Molecular weight (kDa)
Potato (a)	56.5 [a]
Potato (b)	56.6 [a]
Tomato	56.5 [a]
Broad bean	58.1 [a]
Streptomyces glaucescens	30.9 [a]
Streptomyces antibioticus	30.7 [a]
Neurospora crassa	46.0 [a]
Mushroom	128.0 [a]
Eggplant	56.0 [b]
Human	62.6 [a]
Mouse	57.9 [a]

[a] (Whitaker, 1995)
[b] (Mishra, *et al.*, 2012)

For mushroom tyrosinase, a lot of β-sheets were discovered, especially in the C-terminal domain (van Gelder, *et al.*, 1997). On the contrary, in human PPO Oetting & King (1992) found α-helices for the two copper-binding regions. In sweet potatoes PPO clearly showed seventeen α-helices with just four β-sheets (Eicken, *et al.*, 1998; Klabunde, *et al.*, 1998). These secondary structures have β- and γ- turns and random coils. Therefore, PPO appears as a α+β globular protein with two disulphide bridges between the residues Cys11-Cys28 and Cys27-Cys89. Each of the two active-site coppers appear to be coordinated by three histidine residues on α-helices: copper of site A is bond to His88 of helix α2 and His109 and His118 of helix α3 while copper of site B to His240 and His244 of helix α6 and His274 of helix α7.

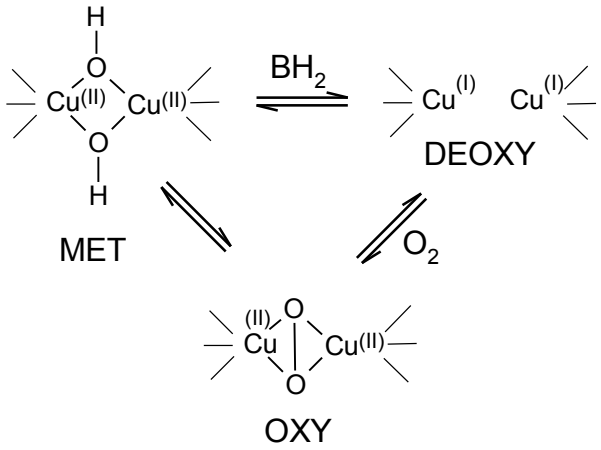

Figure 9: Three different oxidative states of polyphenol oxidase. BH_2 refers to a generic reducing molecule.
(Wong, *et al.*, 1971)

Spectroscopy studies revealed transformations between three distinct forms of the enzyme. Particularly, Himmelwright, *et al.* (1980) found that the so-called *oxy* form predicts a copper ion in a hypothetic tetragon, whose vertexes are three histidine residues (two strong equatorial and one weaker axial N-ligand) and an exogenous oxygen molecule, bound as peroxide (Figure 9).

One of the first PPO 3D-structure modelled so far refers to sweet potato and it was performed by Eicken, *et al.* (1998) using x-ray crystallography. Figure 10 depicts how this PPO is formed by two isozymes (whose molecular weight is 39 and 40 kDa respectively). Furthermore, the authors found that the smaller subunit has 345 amino acids residues and it is a monomeric ellipsoid molecule (55 x 45 x 45 Å).

EPR, UV/Vis and RAMAN spectroscopy data revealed a surprising similarity between PPO and both arhropodan and molluscan hemocyanins, especially regarding copper ligation and oxygen binding (van Gelder, *et al.*, 1997). Even these oxygen-carrying proteins consist of two domains, one of them containing copper. Sequence alignments and secondary structure predictions

carried out by Marusek, *et al.* (2006) seemed to point out that the C-terminals domains of the two families of proteins share a lot of similarities in their tertiary structure.

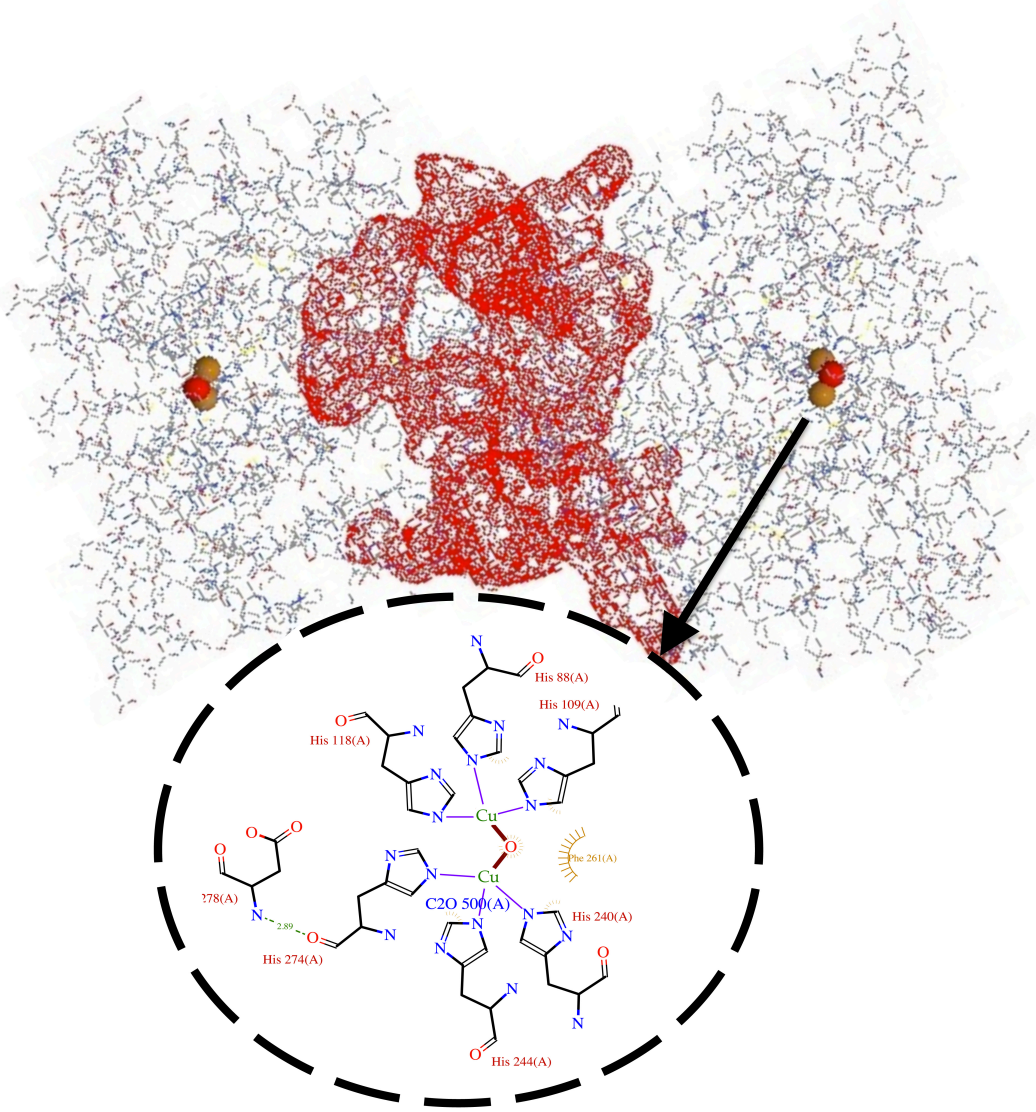

Figure 10: 3D structure of the sweet potato (*Ipomoea batatas*) polyphenol oxidase. One of the two active sites, with copper ions inside, is enlightened in the circle.

In addition to that, mushroom tyrosinase appears to own four subunits (Ismaya, *et al.*, 2011), contributing to its quaternary configuration. Their similarity is not clear, though, as sequencing the genes, even in this case two isozymes seem

to exist (Strothkamp, *et al.*, 1976; Seo, *et al.*, 2003).

3.3. Action and characterization

PPOs group can exert its action on different substrates, both diphenols and monophenols (in a weaker manner). Catechol is the more characteristic diphenol processed but catechins, chlorogenic acid, quercetin, caffeic acid and gallic acid may be oxidized too. On the contrary, among monophenolic compounds, tyrosine, *p*-cresol and *p*-coumaric acid are the best reactive substances (Gandía-Herrero, *et al.*, 2005) but even betaxanthins are found to be a suitable substrate for PPO (Yoruk & Marshall, 2003).

Table 5: Polyphenol oxidase activities of four plants for several substrates, classified by number of hydroxyl substituents. All data are expressed compared to catechol activity (100 %).

	Activity relative to catechol (%)			
Substrate	**Potato [a]**	**Peach [b]**	**Broad bean leaf [c]**	**Pear [d]**
Di- or triphenolic compounds				
Catechol	100	100	100	100
4-Methylcatechol	nd	51.5	200-225	72.3
d-Catechin	nd	31.8	nd	7.79
Chlorogenic acid	140	22.2	8	71.8
Caffeic acid	76.5	0	12.5	4.41
Protocatechuic acid	nd	16.3	0.11	nd
3,4-Dihydroxy-L-phenylalanine	54.3	40.5	50	nd
Dopamine	nd	45.6	nd	15.6
Gallic acid	nd	25.7	0.22	nd
Pyrogallol	nd	nd	85.95	nd
Monophenolic compounds				
p-Cresol	5.5	0	4	nd
p-Coumaric acid	nd	0	0.05	0

[a] pH 7.0 (Macrae & Duggleby, 1968)
[b] for isozyme A of Clingstone peach at pH 6.8 and 30 °C (Wong, *et al.*, 1971)
[c] (Robb, *et al.*, 1966)
[d] for isozyme B at pH 6.2 and 35 °C (de Jesus Rivas & Whitaker, 1973)

In Table 5 different plant PPOs are compared. No activity of peach PPO

could be observed for caffeic acid and the two monophenols tested. In the same table, the lower activity of such PPO on monophenolic molecules can be detected (from 0.05 to 5.5 %, compared to catechol). Another interesting behaviour arousing from those data is the different performance of PPOs depending on the substrate: for example, chlorogenic acid seems to be a more efficient substrate than catechol for potato PPO, while its activity is reduced for peach and pear PPO and being just 8 % in the case of broad bean leaves PPO.

Ramírez, *et al.* (2003) proposed interactions between different substrates (4-methylcatechol and p-cresol), resulting in the entire elimination of the initial lag-phase of the reaction.

The proposed mechanism of action is depicted in Figure 11. In the upper part oxidation pathway for *o*-diphenol is presented (a) whereas the lower refers to monophenolic compounds (b).

The resting form of the enzyme is hypothesised to be the *met* form (1), where no substrate is present. When catechol (or a generic reducing agent BH_2) is added to the medium, a bound between them occurs, resulting in the production of *o*-benzoquinone and the *deoxy* [Cu(I)] form of the enzyme (2). The protein is then able to bind oxygen giving the oxy form (3), and to process another molecule of catechol (4) to produce a ternary complex removing two protons [Cu(II) · O_2 · catechol]. Eventually, the substrate is oxidized and a molecule of benzoquinone is released (5), leaving the *met* form of the enzyme to start another cycle again. However, if monophenols are added to the mix, a different mechanism of action may happen. *Oxy* form could bind the substrate (1'), oxidizing and rearranging it to form an *o*-diphenol (2' and 3'). Now the enzyme is ready to pass in the *deoxy* form (releasing a quinone, 4') and finally in the *oxy* form (capturing on oxygen molecule, 5').

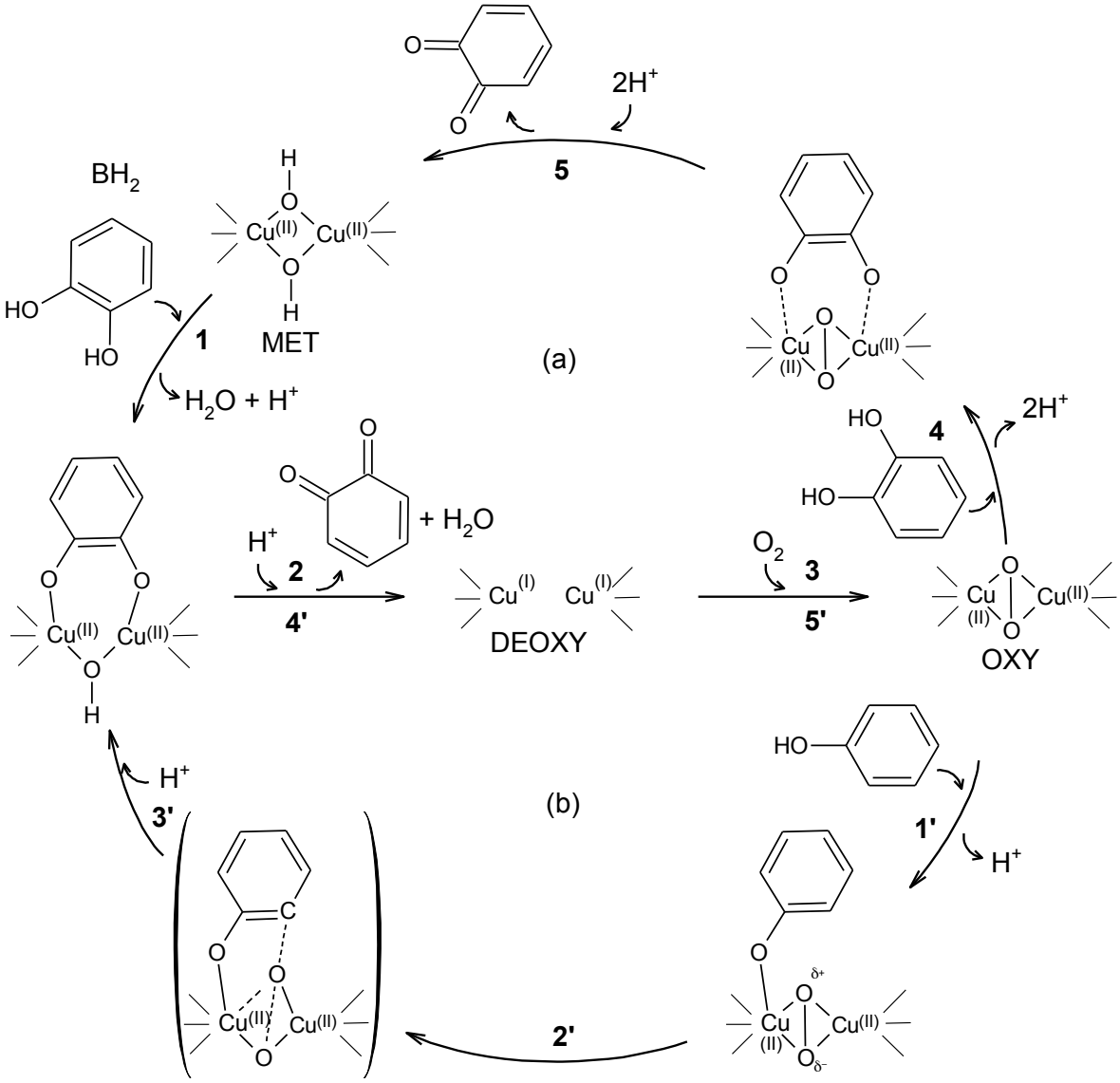

Figure 11: Overall proposed mechanism for the action of *N. crassa* polyphenol oxidase. *o*-diphenol (a) and monophenol (b) oxidation pathways are depicted. See text for details. (Lerch, 1983; Solomon, *et al.*, 1992)

Other ways exist for the *met* form to be oxidized to the *deoxy* one. Gutteridge & Robb (1975) investigated the action of reducing compounds such as ascorbic acid and hydrogen peroxide in the presence of oxygen: the results clearly showed the formation of oxygenated complexes between PPO and the other molecules.

The action of PPO on its substrates leads to a decrease in the activity. Golan-

Goldhirsh & Whitaker (1985) calculated that mushroom tyrosinase appears to be inactivated after 5000 turnovers with almost a 0.02 % efficiency. Such inactivation seemed to be due to a free radical-catalyzed fragmentation of the histidine residues present in the active sites. The loss leads consequently to a copper release (Nelson & Dawson, 1944; Dietler & Lerch, 1982; Golan-Goldhirsh & Whitaker, 1985). Later, Golan-Goldhirsh, *et al.* (1992) discovered that ascorbic acid, copper and oxygen seemed to mime this reaction also with other proteins, such as ovalbumin, bovine serum albumin and small histidine-containing peptides.

Table 6: Polyphenol oxidase activity of four edible mushrooms, measured at different pHs

Source	Activity at different pHs (μM/min/mg protein)				
	4.0	**5.0**	**6.0**	**7.0**	**8.0**
Macrolepiota mastoidea	164 ± 2.2	145 ± 2.0	78.9 ± 0.8	17.3 ± 0.5	10.2 ± 0.5
Lepista nuda	99.5 ± 1.5	99.4 ± 1.6	82.1 ± 0.8	77.4 ± 1.2	75.2 ± 1.4
Handkea excipuliformis	17.2 ± 0.5	15.0 ± 0.2	4.3 ± 0.2	3.7 ± 0.2	No activity
Amanita rubescens	97.2 ± 1.7	83.7 ± 1.3	No activity	No activity	No activity

PPO is strictly influenced by the pH of the medium. However, this correlation depends from numerous factors: plant source, nature of the phenolic substrate, temperature and extraction buffer may affect it (Yoruk & Marshall, 2003). Kolcuoğlu, *et al.* (2007) compared the activities on different compounds of *Macrolepiota mastoidea* PPO; these authors found out that with 3-(4-hydroxyphenyl)-propionic acid (PHPPA), a monophenolic substance, the protein showed one peak of activity in correspondence of pH 6.0, whereas two distinct peaks were depicted at pH 4.0 and 7.0 using 4-methylcatechol (a diphenolic compound). The second peak might be explained considering the presence of different diphenolase isoforms (van Gelder, *et al.*, 1997). Table 6 summarize the activity at several pHs for PPO extracted from different edible mushrooms but

even among plants a lot of variability exists: subtropical fruits are more active at neutral pHs (Das, *et al.*, 1997; Jiang, 1999), while apple and peach are more active at 3.5-4.5 (Marqués, *et al.*, 1994; Fraignier, *et al.*, 1995).

In *M. mastoidea*, optimum temperatures for monophenolase and diphenolase activity are 30 °C and 20 °C respectively and it seems that the second one is more heat-sensitive. On the contrary, other sources proved to own similar behaviours comparing the two families of substrate: 18 °C for *Amasya* apple (Oktay, *et al.*, 1995), 25 °C for artichoke (Aydemir, 2004), and 30 °C for potato (Duangmal & Apenten, 1999).

3.4. Activity determinations

Ramírez, *et al.* (2003) classified different methods to assay PPO activity, based on the type of substrate.

If monophenolase strength has to be determined, different approaches may be applied:

- Tritium labelled substrate: this method foresees the use of a 2-tritiated *p*-cresol solution. When PPO is added, the enzyme will add an oxygen atom (along with an hydrogen) to form a 4-methyl catechol molecule. During this process, tritium will be released in the aqueous phase, and it will be easily revealed, giving a good measure of the initial velocity of the hydroxylation step.
- ^{18}O-labelled O_2: exploiting the same principle as described before, the oxygen could be labelled, instead of marking the substrate. Eventually, the unincorporated $^{18}O_2$ can be flushed from the solution by N_2, or the marked 4-methyl catechol can be obtained. In both cases,

the differences in labelled oxygen concentration between before and after the reaction will give a good measure of the activity.

- Spectrophotometric method: an easier way to determine the 4-methyl catechol produced is to read the increase of the solution absorbance at 280 nm.
- Diphenol conversion rate to benzoquinone: this approach is a little more complicated than the others. When the Michaelis-Menten constant (k_m) and the turnover (k_{cat}) values of the mono- and the o-diphenol substrates are similar, the observed rates for benzoquinone formation will be influenced by both rate constants (Equation II).

(II)
$$v_0 = \frac{k_2 * (E)_0 * (S)_0}{\frac{k_2 + k_3}{k_2 * k_3} + (S)_0}$$

where v_0 is the conversion rate to benzoquinone in the first minutes of the reaction, $(E)_0$ and $(S)_0$ are the initial concentrations of enzyme and substrate respectively, k_2 is the constant rate between substrate-enzyme complex and product-enzyme complex and k_3 the constant rate between product-enzyme complex and the final products.

On the other hand, if diphenolase activity has to be assayed, the main methods are spectrophotometry and polarography:

- Spectrophotometric method: this procedure is fast and easy, and it takes advantage of a catechol solution oxidation, simply following recording absorbance at 395 nm. Both pH and temperature should be controlled; besides that, a recording spectrophotometer should be used, in order to precisely calculate v_0, being the reaction very quick.

As an alternative, other substrates than catechol may be used: chlorogenic acid, epicatechin and quercetin are all substances suitable for this assay, each of them with an optimal reading wavelength.

- Polarographic method: oxygen-uptake rate could be measured using an O_2-electrode. Another approach is to determine oxygen pressure but it does not give linear responses between oxygen uptake and enzyme concentration (Mayer, *et al.*, 1966).

4. MINIMUM INHIBITORY CONCENTRATION AND MICROORGANISMS

Several approaches can be used to assay whether or not a certain substance exerts germicide activity. One of the most developed techniques implies working with a microplate reader.

This useful tool was standardized in the 1990s, enabling more robotic and machine reading of their contents than ever before. For this reason, in the years that followed, multiwell plate readers obtained more and more value. Nowadays, samples may be incubating at fix temperatures with shaking, allowing to cultivate living cells for long periods (Marešková & Sychrová, 2007). The aim is to monitor changes in absorbance during specific time periods, thus tracing kinetic measurements: substrate degradation or product formation may be revealed; otherwise cellular growth can be followed. For the last approach, a mere optical density of the well will be assayed, rather than a real absorbance.

In bibliography, a lot of works implied the use of microplate readers. They might be employed to identify clinical yeast-like isolates (St.-Germain & Beauchesne, 1991), to diagnose infections by immunocapture techniques (Aubert,

et al., 1995), to detect the effect of various chemicals (Hasenbrink, *et al.*, 2006) or test antimicrobial susceptibility (Riesselman, *et al.*, 2000).

4.1. Minimum inhibitory concentration (MIC) assay

The lowest compound concentration able to inhibit bacterial (or yeast) growth represents the minimum inhibitory concentration (MIC). Sometimes this value may refer to the concentration entailing an inhibition of 90 % of cell growth (MIC_{90}) but, usually, the inhibition threshold taken is the 50 % (MIC_{50}) (Prescott & Baggott, 1993).

However, these concentrations are required just to compare the results between different laboratories or different compounds. Even if a MIC_{50} or a MIC_{90} could not be determined, nonetheless this assay remains a useful tool in assaying the damage effects of the compound of interest towards biological targets. As previously stated, the application of a microplate reader to this test can implement its reproducibility and automatize all the process.

Each well will be loaded with the appropriate medium, the microorganism and the compound under exam. Afterwards, optical densities of the solutions will be evaluated (usually at 600 nm) and the plates will remain 24 h (or sometimes 48 h) in an incubator, at the optimal temperature for the chosen cells to grow. At the end of the selected time, optical densities will be determined again, to observe if a microbial growth has occurred. Microplate readers allow the operator to investigate different concentrations simultaneously, increasing replicates number at the same time.

Among biological targets, *Lactobacillus* spp. and *Saccharomyces cerevisiae* are certainly very suitable. The authors de Oliva-Neto & Yokoya (2001) compared several antimicrobial compounds acting on *S. cerevisiae* and two species of

lactobacilli (*L. fermentum* and *L. mesenteroides*). Their aim was to find out which substances may be used against bacterial contaminants from distilleries, without affecting the yeasts.

4.2. Lactic acid bacteria

Lactobacillus spp. are a Gram + non-sporing microorganism genre. They can grow both in presence or absence of oxygen but generally they are microaerophilic. Inside the human body, these bacteria can easily grow in mouth, gastrointestinal tract and female genitourinary tract (Slover & Danziger, 2008), and they have been used largely as fermentation starters in dietary manufacturing products, infant's food formulae and pharmaceutical products.

Based on their metabolic pathways, *Lactobacillus* spp. might be divided into three main groups: homofermentatives, facultative heterofermentatives and obligately heterofermentatives (Jafarei & Ebrahimi, 2011). All of them are able to produce lactic acid from substrates, thus they are usually referred as Lactic Acid Bacteria (LAB). Homofermentative LAB ferments hexoses almost exclusively to produce lactic acid by the Embden-Meyerhof-Parnas (EMP) pathway while the facultative bacteria are also able to metabolize pentose carbohydrates and gluconate, having both frutose diphosphate (FDP) aldolase and phosphoketolase. Finally, the last group degrade hexoses and pentoses by the phosphogluconate pathway, producing lactate, ethanol or acetic acid and carbon dioxide (Felis & Dellaglio, 2007). Table 7 presents the differences among these groups, indicating a few typical species for each.

Even fruit skin can be an ideal growth environment. Savino, *et al.* (2012) managed to isolate up to 68 colonies from apples (35 from Red Delicious apples and 33 from Golden Delicious apples) that exhibit distinct morphological traits

concerning colour, shape and size. Among them, 48 colonies proved to belong to the LAB group. At the same time, *Lactobacillus* might grow over the surface of flowers, leaves and stalks (Magnusson, *et al.*, 2003).

Table 7: Characteristic and some representative species of the three distinct groups of *Lactobacillus* spp.
(Adapted from Salminen, *et al.* (2005) and Endo & Okada (2007))

Characteristic	Homofermentatives	Facultative heterofermentatives	Obligately heterofermentatives
Pentose fermentation	-	+	+
CO_2 from glucose	-	-	+
CO_2 from gluconate	-	- [a]	+
FDP aldolase present	+	+	-
Phosphoketolase present	-	+ [b]	+
Species	*L. acidophilus* *L. delbrueckii* *L. helveticus*	*L. casei* *L. curvatus* *L. plantarum* *L. rhamnosus*	*L. brevis* *L. buchneri* *L. fermentum* *L. hilgardii* *L. kefir* *L. reuteri* *L. sanfrancisco*

[a] **when fermented**
[b] **inducible by pentoses**

4.3. *Saccharomyces cerevisiae*

Another microorganism largely distributed on the surface of skin is *Saccharomyces cerevisiae*. This yeast is responsible for the main fermentations used by humankind: baked products and alcoholic beverages would be almost impossible to obtain without *S. cerevisiae*. For these reasons, in literature it is one of the most studied organisms: it reproduces by a budding division process and its cells are round to ovoid, 5 to 10 μm in diameter.

Such microorganisms are responsible for the most common type of fermentation and might be isolated from all fruits (Valles, *et al.*, 2007; Satora, *et al.*, 2010). Martos, *et al.* (2013) investigated pectinolytic activity of yeast. Their purpose was to isolate the strains from citrus peel to find out if they are able to produce a polygalacturonase by submerged fermentation with maturation activity of raw

cassava roots. Eventually, they managed to isolate 160 different strains.

For its diffusion and its importance, *S. cerevisiae* could be used as a representative target when an antimicrobial feature of a substance has to be verified.

5. PROJECT

5.1. Roles of phenolic compounds inside cells

Plants may follow different approaches to defend themselves against environmental dangers, generally ascribed in two main strategies. If the risk is unlikely, or just rare, the organism may synthetize defence compounds after the initial damage (induced defence). On the other hand, if harm is frequent, or particularly serious, plants may invest energy creating constitutive defences. At the same time, these strategies could have either the aim to inhibit the attack from pathogens (resistance mechanism) or to reduce the consequences of such injures (tolerance strategy) (Lattanzio, *et al.*, 2006).

Both resistance and tolerance traits need a reallocation of the plant resources: this is the reason why a lot of organisms prefer the induced defence, synthetizing protective chemicals just after the pathogen attack. Such responses are supposed to be kept under strict genetic control, and are activated only when plants detect potential invaders.

In this scenario, phenolic compounds may play a crucial role. They are assumed to have antifungal properties but, if they are not enough to stop the infection process, plant cells usually increase the level of pre-existing phenols in the infection site, after an increased activity of particular enzymes, phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) (Mauch-Mani & Slusarenko,

1996; Pallas, *et al.*, 1996; Campos, *et al.*, 2003). The increased levels of such compounds provide the substrate to oxidative reactions catalysed by PPO and peroxidases (PODs), whose products are claimed to be antimicrobials (Lattanzio, *et al.*, 2006). These oxidation products are often associated with an inhibition of the cell wall degradation (Albersheim & Anderson, 1971), phenomenon operated by pathogens extracellular enzymes, and the oxidative polymerization of low molecular phenolic compounds to produce brown tannin-like molecules, called melanins (Dordick, *et al.*, 1987; Mikolasch & Schauer, 2009).

Lattanzio, *et al.* (2001) suggested that PPO, whose activity increases in infected tissues, might improve host resistance, oxidising pre-existing phenols. This mechanism could be ruled by the host-pathogen interaction, which leads to a hypersensitive response.

Finally, Beckman, *et al.* (1974) proposed the formation of a protective and impermeable barrier to parasites resulting from melanins; as quoted above, these compounds might be formed during the oxidative polymerization of phenolics.

5.2. Experimental design

The presented work had the aim to investigate which roles PPO might have in tissues. Particularly, the project focused on the microbial growth influence of PPO-treated substrates as well as their antioxidant protection.

In the following section, *Experimental Work*, three main studies will be illustrated. The first one involved chemical standards. Different PPO substrates were chosen, and then treated with the enzyme, in order to investigate their effect on *Lactobacillus* spp. and *Saccharomyces cerevisiae*. These two organisms were chosen as models of a prokaryotic and eukaryotic cell, respectively. Several analyses were accomplished, among which a MIC investigation was performed to

observe the enzyme influence on the growth of the microorganisms mentioned above.

Afterwards, the same approach was used to study a fruit extract. *Golden delicious* apples were chosen as PPO source to investigate the *in vitro* effect on microorganisms of such polypeptide; however, an incubation step with additional PPO standard was included to reinforce the action of the naturally present enzyme.

Finally, total antioxidant capacity was assayed for the same polyphenolic substances analysed before, comparing the activity of the not-treated compounds with that of the oxidized ones. The assay chosen to measure such capacity was the 2,2-diphenil-1-picrylhydrazyl (DPPH) free radical method (Bondet, *et al.*, 1997). Even for this analysis, the repetition of the assay with apple extracts followed the chemical study.

6. REFERENCES

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EXPERIMENTAL WORK

1. EFFECTS OF POLIPHENOL OXIDASE PRODUCTS ON *LACTOBACILLUS* spp. AND *SACCHAROMYCES CEREVISIAE* GROWTH.

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1.1. ABSTRACT

Polyphenol oxidase (PPO) is an apple enzyme that oxidizes polyphenols and it is studied mainly because of the damages it causes after cuts or bruises, darkening fruit skin and pulp. However, the aim of this paper is to clarify in vitro one of the possible roles PPO products may have on *Lactobacillus* spp. and *Saccharomyces cerevisiae* growth.

This is a preliminary study on standard phenolics, conducted before testing polyphenols extracted from apples (*Experimental Work, Chapter 2*).

MIC analyses were performed using three *Lactobacillus* spp. (*brevis*, *casei* and *plantarum*) and three strains of *Saccharomyces cerevisiae*, all isolated from fruit and used as a biological target. Microorganisms were incubated in a medium containing different concentrations of single polyphenols (catechol, chlorogenic acid, epicatechin and quercetin) previously oxidized by PPO. Control solutions were prepared in the same way, without the oxidation step.

The assay gave positive effects only with catechol in two Lactobacilli: in these cases when PPO oxidized the compound, the antimicrobial effect was greater. On

the contrary, quercetin (oxidized or not) proved to promote microbial growth.

1.2. INTRODUCTION

Polyphenol oxidase (PPO) is a generic name referring to a group of copper-containing enzymes whose action is to catalyse polyphenols if molecular oxygen is present (Ayaz, *et al.*, 2008). These substances can exert three different activities and so they have specific EC numbers: o-diphenol oxidoreductase (also known as catechol oxidase, EC 1.10.3.1), p-diphenol oxidoreductase (laccase, EC 1.10.3.2) and monophenol oxidoreductase (cresolase, EC 1.18.14.1) (NC-IUBMB & Webb, 1992; Sheptovitsky & Brudwig, 1996; Ramírez, *et al.*, 2003).

PPO is a typical apple enzyme, and it was mostly studied throughout the years just for its browning reactions, due to a change in the polyphenol double bonds configuration. This enzymatic browning is undesirable in the majorities of the cases, leading to off-colours, off-tastes and loss of nutritional properties, but it is required in the processes of tea, cocoa, coffee, apple cider, prunes, black raisins, Black Mission figs and zapote (Ramírez, *et al.*, 2003).

These oxidations occur mainly during fruit harvesting and storage, being caused by the penetration of oxygen inside cells after cuts or bruises, but levels of PPO and phenolics may change during fruit development and ripening which may influence the potential damage (Ayaz, *et al.*, 2008). The increased levels of phenolic compounds provide an adequate substrate to oxidative reactions catalysed by PPO (Lattanzio, *et al.*, 2006), which could suggest a possible substrate-induced expression of this enzyme.

Despite all these considerations, it is still unclear what is the real role of this protein in the cell environment. The oxidation of pre-existing phenolics may exert

antimicrobial activity, often associated with a decrease of the cell wall degradation rate operated by exogenous pathogen enzymes (Lattanzio, *et al.*, 2012). Moreover, many low molecular weight phenolics can be polymerized by oxidation yielding brown tannin-like compounds (melanins), containing quinonoid groups.

Plant resistance systems often need the reallocation of host resources, thus plants that are likely to suffer frequent and/or serious damage may invest mainly in constitutive defences, while the others may rely in the induced ones: this occurs when defence substances are synthesized only after damage by a pathogen (Purrington, 2000).

The aim of this paper is to investigate in vitro if and how the oxidation of phenolic compounds, that in apple could be lead by PPO after skin and pulp damages, may exert some pathogenic activity. Chlorogenic acid, epicatechin and quercetin were chosen as typical polyphenolic substances found in apples, while catechol, not naturally present, was analysed because it is one of the ideal substrates for PPO. In this work, both prokaryotic and eukaryotic organisms were assayed, choosing three *Lactobacillus* spp. as microorganism model of the former and *Saccharomyces cerevisiae* as model of the latter.

1.3. MATERIALS AND METHODS

- **Chemicals**

All the chemicals used in this study and the enzyme standard were purchased from Sigma-Aldrich® Co. Ltd (Poole, Dorset, UK) and were over 99 % purity. PPO standard was purchase from Sigma-Aldrich®, too, at a 3610 U/mg solid concentration. HPLC grade acetic acid and acetonitrile were purchased from Merck® (Darmstadt, Germany).

- **Instrumentation**

The HPLC used for this paper was a Dionex UltiMate® 3000 HPLC system (Dionex, Camberly, UK) equipped with a 3000 RS pump, a 3000 autosampler and a 3000 RS Diode Array Detector (DAD). The bacterial analyses were carried out using a Synergy HT Multi-Mode Microplate Reader (BioTek, UK).

- **Microorganisms**

All *Lactobacillus* spp. and strains of *Saccharomyces cerevisiae* were provided by the Department of Biology, Food and Nutritional Sciences of the University of Northumbria (UK), being isolated from apples and prunes.

- **Experimental design**

The aim of this paper is to establish whether PPO action could inhibit microorganism proliferation after fruit cuts or bruises. To verify this, some of the major apple polyphenols were treated with the enzyme and their toxicity were evaluated through the Minimal Inhibitory Concentration (MIC) assay.

After having chosen the chemicals (catechol, chlorogenic acid, epicatechin and quercetin), the first part of the work had the aim to find the amount of time required by PPO to oxidase the different substrates. An HPLC analysis was proved to be the best way to measure it.

Polyphenol solutions were then incubated with the enzyme and the toxicity of products was evaluated by MIC analysis, using 6 different microbial targets. All of them were isolated from fruit: a strain of *L. brevis*, a strain of *L. casei*, a strain of *L. plantarum* and three strains of *S. cerevisiae*.

- **Oxidation kinetics**

For each polyphenol a 0.1 M batch solution was prepared, split in 7 test tubes and finally the PPO solution (with a final concentration of 10 Units/mL) was added and let react for 24 h. To stop the reaction at fixed times (allowing to determine the amount of substrate still present) in each tube 15 μL of a 4 M hydrochloric acid solution was mixed.

After having neutralized samples, HPLC analyses were performed. A gradient of solvent A (water/acetic acid, 99:1, v/v) and solvent B (acetonitrile/acetic acid, 99:1, v/v) was applied to a reversed-phase thermal Acclaim C_{18} column (2.1 mm x 150 mm; 3μm particle size) at 25 °C. The elution program was the following:

- 0 min: 20 % solvent B, 0.2 mL/min
- 12 min: 100 % solvent B, 0.2 mL/min
- 13 min: 100 % solvent B, 1 mL/min
- 18 min: 100 % solvent B, 1 mL/min
- 20 min: 20 % solvent B, 0.2 mL/min

Detection was carried out following the analysis at 2 different wavelengths: 280 nm for catechol, chlorogenic acid and epicatechin, 426 nm for quercetin.

All measurements were performed in triplicate, in order to validate the results.

- **Media preparation**

The media used for bacterial analyses were Man-Rogosa-Sharpe (MRS) for *Lactobacilli* and Yeast-Peptone-Dextrose (YPD) for yeasts, both purchased from Sigma-Aldrich Co. Ltd (Poole, Dorset, UK). YPD was then fortified with a 15 mg/L solution of adenine.

- **MIC assay**

The first part of this phase was to trace the growth curve for each microorganism. This was required, as the assay needs them at the maximum stage of vitality.

In the previous stage of the work, measurements established that 20 h was a sufficient time to allow the action of PPO. A solution containing the analysed chemical and the enzyme was then kept for this time at 25 °C. After this period products were used in the MIC assay.

After the incubation phase, different concentration of the solution, the appropriate medium and a fixed amount of each microorganism were mixed in a 96-well plate. For all of them, the optical density at 600 nm was read before (t0) and after (t24) 24 h of incubation. The plates containing the bacteria were kept at 37 °C while the ones with the yeasts were kept at 28 °C.

All the assays were performed in triplicate and polyphenols not incubated with the enzyme were used as control solutions.

- **Statistical analyses**

For this paper, triplicate analyses were carried out to validate results. A t-test for independent samples was used to compare them in the MIC assay: differences were considered to be significant at $p < 0.05$ and the analyses were performed using IBM® SPSS® Statistics (version 20.0.0) software.

1.4. RESULTS AND DISCUSSION

- **Results**

The first part of the work was carried out to detect in which manner the

examined substrates interact with PPO, particularly observing their reaction rate. Results showed in Figure 12 illustrate the amount of each substrate remained at fixed times during the first 2 hours of reaction. Each value was divided by the mean of the initial one, in order to standardize data by these concentrations. Standard deviations of the first values belong to the mean of those data.

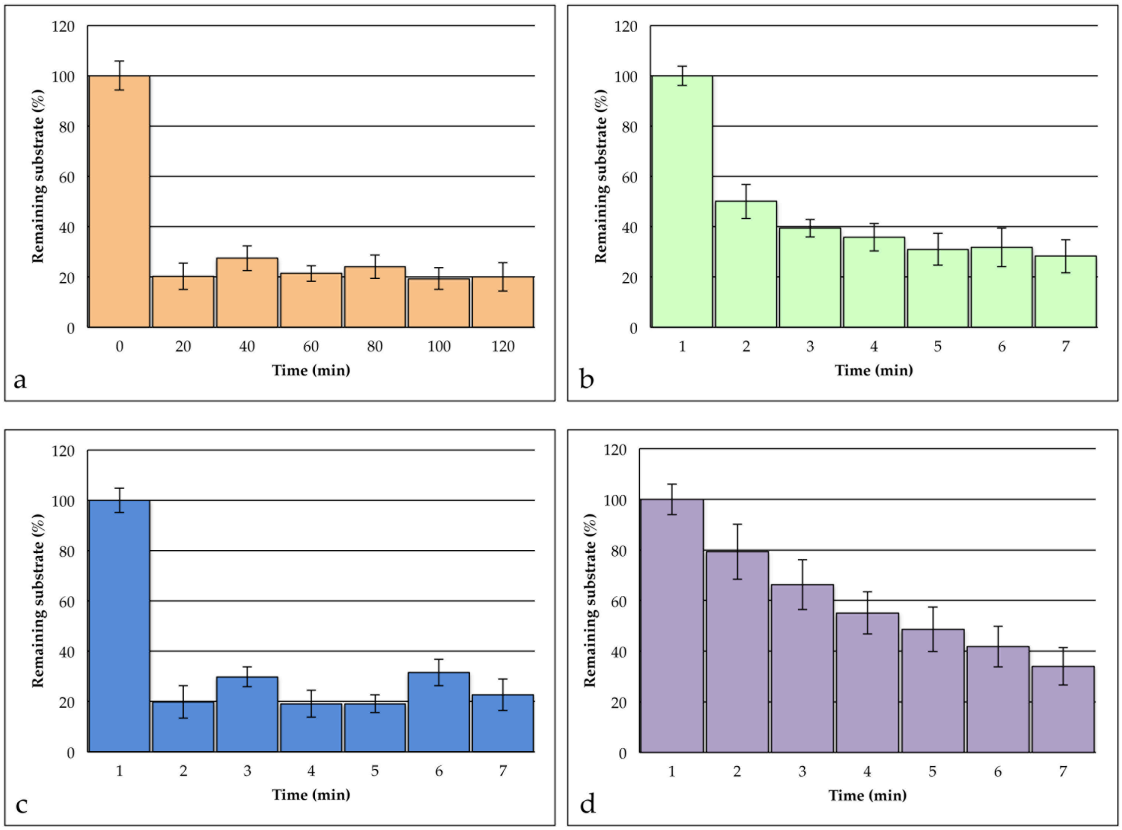

Figure 12: Amount of sample (expressed as remaining percentage of the initial concentration) for four different polyphenol oxidase substrates: catechol (a), chlorogenic acid (b), epicatechin (c) and quercetin (d).

Catechol and quercetin solutions gave the most relevant MIC outcomes. Figures 13 and 14 showed the influence of the former polyphenol on *L. brevis* and *L. plantarum* growth, whereas quercetin effect on *S. cerevisiae* growth was presented in Figures 15 and 16, considering RD/B/4A and RD/D/1P strain. Sample and control solutions were compared and results were expressed as

percentages referred to the microbial growth in absence of the polyphenol.

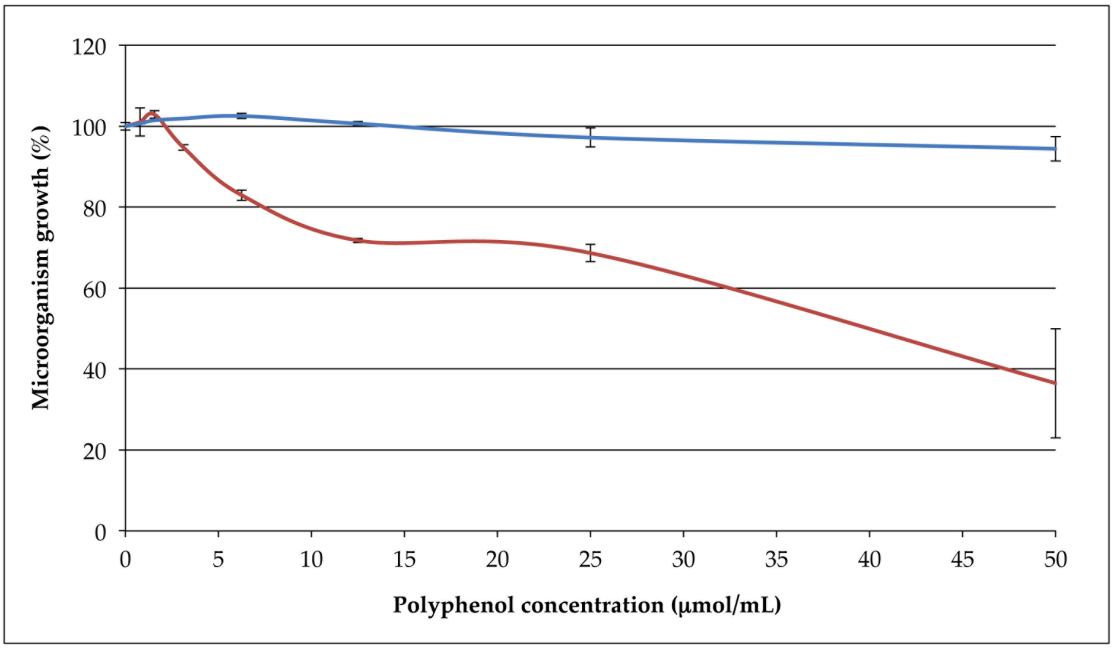

Figure 13: Influence of catechol on microbial growth for *L. brevis*. Catechol oxidized by PPO is depicted with red line, while the light blue lines represent the control solution.

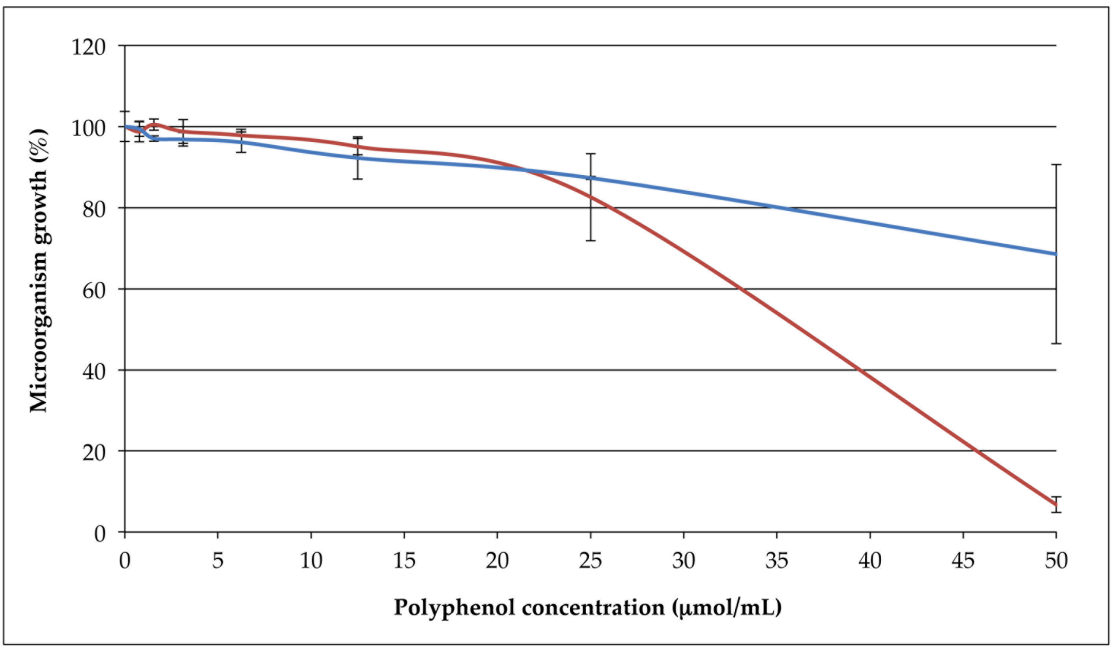

Figure 14: Influence of catechol on microbial growth for *L. plantarum*. Catechol oxidized by PPO is depicted with red line, while the light blue lines represent the control solution.

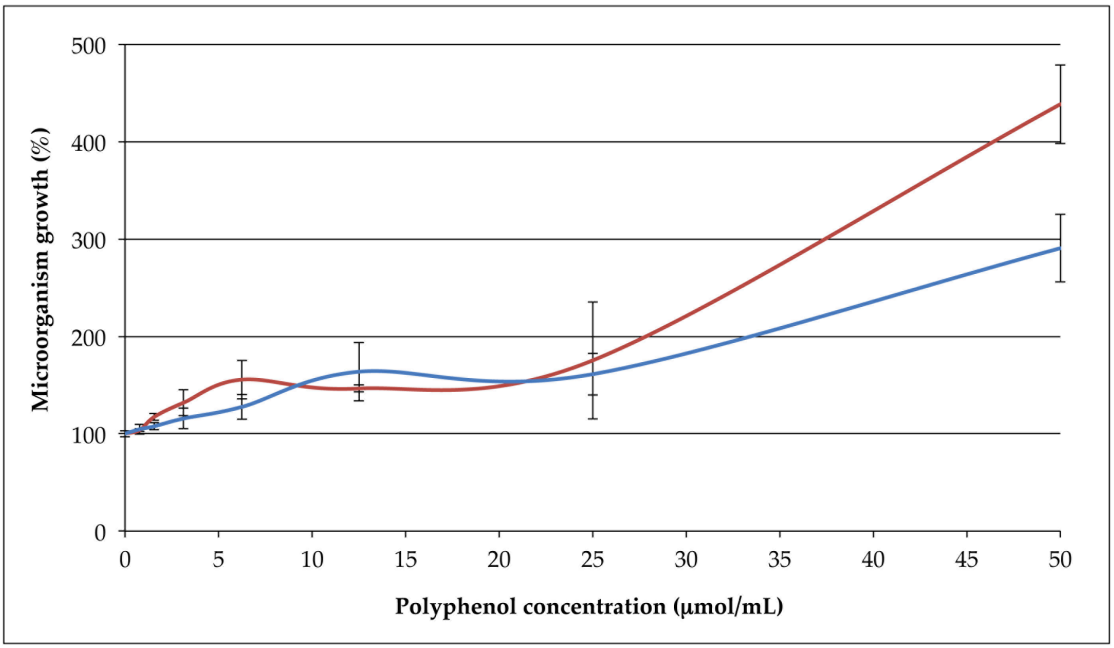

Figure 15: Influence of quercetin on microbial growth for *S cerevisiae* RD/B/4A. Quercetin oxidized by PPO is depicted with red line, while the light blue lines represent the control solution.

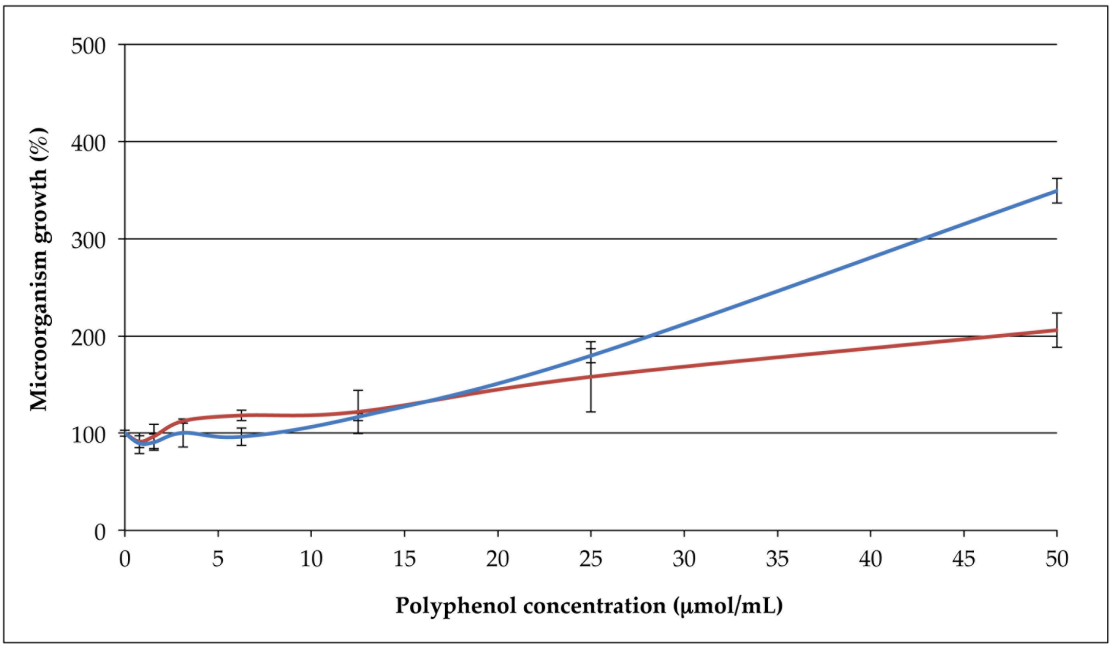

Figure 16: Influence of quercetin on microbial growth for *S cerevisiae* RD/D/1P. Quercetin oxidized by PPO is depicted with red line, while the light blue lines represent the control solution.

- **Discussion**

Observing Figure 12, a different behaviour between the various substrates can be detected. After 2 h, catechol, chlorogenic acid and epicatechin were more reactive then the other compound examined (Mishra, *et al.*, 2012; Aka, *et al.*, 2013); on the other hand, quercetin showed a slight slower affinity with the enzyme. It is worth noticing that the total period in which compounds were quantified was guaranteed by the acidification step, before injecting the samples.

Considering that after 2 h phenolics were not completely oxidized, solutions were analysed 24 h after the PPO addition, in order to allow a quantitative reaction between enzyme and substrate.

Ideally, for substances with strong antimicrobial capacity, the MIC_{50} value could be measured (representing substance concentration capable of inhibit microbial growth by 50 %). In this case, however, the inhibitory effect was so weak that in most cases a real MIC_{50} value was impossible to obtain. Nonetheless, the analysis was effective in the determination of the different effects between sample (substrates treated with PPO) and control solutions (substrates not treated with PPO). The percentage of growth was calculated comparing the absorbance at 600 nm of the medium containing microorganism and compound of interest to the absorbance of an identical well not containing the latter.

Examining all the results, the action of PPO seemed not to affect the antimicrobial activity of chlorogenic acid and epicatechin towards both *Lactobacillus* spp. and *S. cerevisiae* (data not shown). However, oxidized catechol exhibited a significant different behaviour compared to the control solution in two of the six biological targets, *L. brevis* and *L. plantarum* (Figures 13 and 14). Particularly, in *L. brevis* the effect of PPO seemed to be incisive even at low concentrations. Bacteria and yeasts are genetically very distant and for this reason

responses to antimicrobial compounds are different (de Oliva-Neto & Yokoya, 2001; Muñoz, *et al.*, 2010; Belda-Galbis, *et al.*, 2013), so it is possible for the same substance to have such different effects.

Unlike other samples, quercetin seemed to promote microbial activity, in all the lactobacilli and yeasts tested. Curiel, *et al.* (2010) suggested that this flavonoid might exert a positive effect on the fermentation capacity of *L. plantarum*, accelerating sugar consumption and lactic acid production. To reinforce this hypothesis, Figueiredo, *et al.*, (2008) found positive effects of quercetin in another bacteria, *L. hilgardii*: antioxidant capacity of the tested compound could be responsible for the increased microorganism final density, even if the observed growth rate was reduced. Despite these considerations, the effects of this flavonoid on bacteria are conflicting: Lee, *et al.* (2013) found that quercetin inhibits the formation of a biofilm among microorganisms and, moreover, this compound did not demonstrate to exert any effects on *L. acidophilus* (Hervert-Hernández, *et al.*, 2009), proving the extreme variability of bacteria. Even *Saccharomyces cerevisiae* growth was accelerated by quercetin. Vilaça, *et al.* (2012) hypothesized that this flavonoid may protect yeasts against oxidative stress, modulating cell-signalling pathways related to carbohydrate metabolism and cell integrity. However, PPO action did not affect this pro microbial property in all bacteria examined while in the case of *S. cerevisiae* results were not coherent, so further investigations will be required. Figure 15 showed how PPO seemed to reinforce quercetin effect on *S. cerevisiae* RD/B/4A growth, whereas the action of the enzyme weakened such influence in the case of *S. cerevisiae* RD/D/1P (Figure 16).

1.5. CONCLUSIONS

Different substrates demonstrated to react in different ways with PPO. Particularly, quercetin seemed to have a slower kinetic behaviour if compared to catechol and epicatechin, whereas chlorogenic acid appears to have an intermediate reaction rate.

Observing MIC results, only one compound proved to possess antimicrobial strength. Oxidized catechol seemed to be capable to inhibit bacterial growth in two of the three *Lactobacillus* spp. tested more than the non-oxidized compound, while its efficacy was not proved for the yeasts. On the contrary, the oxidation operated by PPO on chlorogenic acid and epicatechin did not affect the slight antimicrobial capacity present. Finally, quercetin showed a completely different behaviour compared to the other substances. It acted like a promoter for bacteria and yeast, increasing their growth. However, the action of PPO on pro-microbial activity of this compound gave unclear results, being ineffective in all bacteria and in one strain of *S. cerevisiae*, increasing the effect in the second strain and decreasing it in the last one.

After this *in vitro* study, an investigation using fruit extracts is required to better understand the functions of PPO. Both PPO and polyphenols in fruit may be different from the ones used in this experiment; besides that, interactions between substrates (oxidized and not) may play an important role in preventing damaged plants from infections.

1.6. ACKNOWLEDGMENTS

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2. EFFECTS OF *GOLDEN DELICIOUS* APPLE HYDROPHILIC EXTRACTS ON MICROORGANISMS: THE ACTION OF POLYPHENOL OXIDASE.

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2.1. ABSTRACT

Polyphenol oxidase (PPO) is a common fruit enzyme, which oxidizes phenolic compounds giving, generally, quinones. In apple wound tissues, the activity of this protein increases both for the rising oxygen partial pressure and for a complex plant defensive system that leads to an over-production of polyphenols in damaged areas. In this paper, *Golden delicious* hydrophilic extracts were treated with PPO and their effect on microbial growth was investigated.

MIC analyses were performed using six different microorganisms as targets: three *Lactobacillus* spp. (*L. brevis*, *L. casei* and *L. plantarum*, isolated from apples), as prokaryotic model, and three strains of *Saccharomyces cerevisiae* (isolated from apples and plums), as eukaryotic model.

Results showed that *Golden delicious* hydrophilic extracts, oxidized by PPO, were not effective against these bacteria and yeasts. On the contrary, two strains of *S. cerevisiae* appeared to be reinvigorated when grown in a medium containing the extracts. This effect may be related to the presence of compounds exerting a

promicrobial activity, like quercetin.

2.2. INTRODUCTION

Apples are one of the most common sources of dietary natural antioxidants found in markets. An investigation conducted by Cornell University (Geneva, USA) and the University of Nebraska (Nebraska, USA) established that apples are the second richest source of such beneficial compounds among fruit, next to oranges, in the North American diet (results displayed as vitamin C equivalents): this may probably be related to the elevate consumption per capita of the product, too (Chun, *et al.*, 2005). However, despite these encouraging outcomes, Konopacka, *et al.* (2010) proved that European consumption of apples appears to decline among younger generations. Considering that fruit consumption proved to bestow other beneficial effects to human health (Hyson, 2011; Larson, *et al.*, 2012; Muraki, *et al.*, 2013), beyond antioxidant capacities, it is clear how further investigations are needed to better understand the complex systems regulating such properties.

Among the healthier compounds present in apples, phenolics are certainly noteworthy. These chemicals can be classified into several sub-classes, with procyanidins being the most abundant polyphenols (between 40 and 89 %), followed by hydroxycinnamic acids, dihydrochalcones, flavonols and anthocyanins (Oleszek, *et al.*, 1988; Guyot, *et al.*, 1998; Alonso-Salces, *et al.*, 2004). Particularly, the latter are glycols of anthocyanidins; these compounds are responsible for the red colour of many fruit and they can be essentially found in skin tissue (Khanizadeh, *et al.*, 2007) (main phenolic compounds present in these cells are presented in Table 8). Besides this notable effect, some phenols are

responsible for the cider aroma (Lea & Drilleau, 2003) and others are suitable substrates for enzyme action, among which polyphenol oxidase (PPO). Hydroxycinnamates, such as chlorogenic acid, are involved in the typical colouring process occurring by the action of this substance (Holderbaum, *et al.*, 2010); besides that, Amiot, *et al.* (1992) found that the browning degree was directly correlated to hydroxycinnamic acids and procyanidins content in apple tissue.

Table 8: Mean concentration (mg/100 g DW ± standard deviation; 2 harvest seasons) and percentage distribution of phenolic compounds found in apple skin of 21 fruit genotypes. (Huber & Rupasinghe, 2009)

Phenolic compound	Retention time (min)	Parent ion (m/z)	Product ion (m/z)	Concentration		Distribution [a] (%)
				(mg/100 g DW)	(µmol/100 g DW)	
Proanthocyanidin B1	23.23	578	289	17 ± 11	29 ± 19	4
Proanthocyanidin B2	26.07	578	289	51 ± 28	88 ± 48	11
(-)-Epictechin	12.19	289	109	50 ± 27	172 ± 93	11
(+)-Catechin	9.85	289	109	7 ± 9	24 ± 31	1
Quercetin-3-O-galactoside	17.71	463	301	73 ± 34	157 ± 73	16
Quercetin-3-O-rhamnoside	23.47	447	301	59 ± 42	132 ± 94	12
Quercetin-3-O-glucoside	18.67	463	301	23 ± 18	50 ± 39	5
Quercetin-3-O-rutinoside	17.33	609	301	12 ± 10	20 ± 16	3
Cyanidin-3-O-galactoside	10.93	449	287	124 ± 107	277 ± 239	22
Phloretin-3-O-glucoside	26.41	435	273	26 ± 10	60 ± 23	6
Chlorogenic acid	10.05	353	191	42 ± 32	119 ± 90	9

[a] **Percentage distribution and significant differences were determined based on the mg phenolic compound / 100 g DW.**

All these phenolic compounds can also help in plant self-protection against infective agents. Plant defensive responses may be divided into two main approaches. The first one, called resistance, concerns traits that inhibit or limit pathogen assaults, while tolerance strategies have the aim to reduce damage

caused by harmful agents. The choice of the approach to follow depends on how likely the potential damage is and on its seriousness. Considering that both approaches need to reallocate host resources and defensive chemicals are costly, in case of less frequent damages plants prefer to synthetize protective substances only after the initial attack. If antifungal polyphenolic compounds are not sufficient in the infected area, plants may increase their amount using particular key enzymes (Campos, *et al.*, 2003; Yedidia, *et al.*, 2003). These phenolics exert antimicrobial capacity by themselves, but also provide adequate substrates for the activity of other enzymes, involved in oxidative reactions. PPO and peroxidases (PODs) transform these molecules into products supposed to exert an even higher protective action against bacteria and fungi (Lattanzio, *et al.*, 2006).

In a damaged apple, wound tissues may be subdued to microbial proliferation, because physical natural barriers no longer exist, and pathogens can attack internal cells. At the same time, PPO intensifies its action both for the increased amount of substrate, as described above, and for the higher oxygen partial pressure present in wounded regions. These double induced-response (genetic and environmental) allow plants to use energies only when needed: it is the presence of the parasite itself that activates the defensive mechanisms.

Several hypotheses were formulated about how these oxidized compounds may lower microbial damage. Simple low-molecular weight phenolics may be polymerized by oxidation (Dordick, *et al.*, 1987), giving melanins (brown tannin-like molecules containing quinonoid groups). These polymers may exert an efficient physical barrier against bacterial (or fungal) proliferation with a contemporary decrease of nutrients, crucial to fungal growth. However, antioxidant capacity and direct antimicrobial actions were suggested, too. The effects might be related to resistance against cell wall degradation operated by

microorganisms (Albersheim & Anderson, 1971). Finally, the oxidation operated by these enzymes remove oxygen from the environment, of course, transforming the medium in a less favourable place for bacteria and fungi to grow.

Table 9: Minimum inhibitory concentration of several products on different strains of *Saccharomyces cerevisiae* and lactic acid bacteria at 30 °C. (de Oliva-Neto & Yokoya, 2001)

Product	Time (h)	pH	*S. cerevisiae* (µg/mL)	*L. fermentum* (µg/mL)		*L. mesenteroides* (µg/mL)	
			CCT 0472 [a]	CCT 1400	CCT 0559	CCT 0582	CCT 0367
Copper sulphate	24	4.5	75	75	300	75	150
		6.5	140	70	70	65	140
3-sodium polyphosphate	24	4.5	5000	2500	2500	5000	5000
		6.5	1250	625	625	2500	1250
Sodium sulphite	24	4.5	5000	40	20	20	10
		6.5	5000	625	625	312	78
Sodium sorbate/Sodium phosphate (1:1)	24	6.5	1250	1250	> 2500	> 1250	1250
Sodium phosphate	24	6.5	> 12500	6250	12500	3125	3125
Sodium nitrite	24	4.5	234	117	117	<58	117
		6.5	3750	1875	469	234	234
Tannin	24	6.5	> 302	> 302	> 302	> 302	302
Lysozyme	24	4.5	> 124	> 124	> 124	> 124	> 124
Acid penicillin V	15	4.5	OF [b]	0.10	0.05	0.05	0.10
	24		OF	0.20	0.10	0.10	0.20
Clindamycin	15	4.5	OF	0.10	0.05	0.20	0.05
	24		OF	0.10	0.05	0.40	0.05
Cephamandole	15	4.5	OF	0.40	0.20	0.40	0.20
	24		OF	0.36	0.26	1.45	0.36

[a] **CCT = Topical Culture Collection, Campinas - Brazil**
[b] **OF = No effect**

One of the most versatile techniques to verify possible antimicrobial activity of a food extract (as well as standard solutions) is the Minimum Inhibitory Concentration (MIC). This assay is based on the observation of microbial growth (through optical density) in different media containing growing concentrations of the solution of interest (St.-Germain & Beauchesne, 1991; Riesselman, *et al.*, 2000). If the medium is toxic for the microorganism tested, the optical density of the solution will remain the same after 24 h; otherwise it will increase. Usually sample concentrations leading to a microbial growth of 50 % (MIC_{50}) or 10 % (MIC_{90}) are determined but even lower reductions (not necessarily up to those percentages)

may give useful information. However, Table 9 shows how the same compound might affect yeasts and Lactic Acid Bacteria (LAB) in a very different way, even for two strains belonging to the same species.

In this paper, the results of a MIC assay were presented. The aim was to verify if natural apple polyphenols, oxidized by PPO, might have antimicrobial activity. Enzyme products were isolated and their activity was compared with the same extract not treated with PPO. To attest the toxicity, three strains of *Saccharomyces cerevisiae* and three different *Lactobacillus* spp. were chosen as biological targets.

2.3. MATERIALS AND METHODS

- **Chemicals and food samples**

All chemicals were purchased from Sigma-Aldrich® Co. Ltd (Poole, Dorset, UK) and were over 99 % purity. PPO standard was purchased from Sigma-Aldrich®, too, at 3610 U/mg. Solvents were purchased from Merck® (Darmstadt, Germany).

Golden delicious apples were purchased in a local supermarket.

- **Instrumentation**

MIC analyses were performed with a Synergy HT Multi-Mode Microplate Reader (BioTek, UK), that allow the simultaneously data collection of 96 wells.

To isolate PPO products, C_{18} cartridges (125 Å pore size) were used, purchased from Sep-Pak®.

- **Microorganisms**

The three strains of *Saccharomyces cerevisiae* and the three *Lactobacillus* spp. were supplied by the Department of Biology, Food and Nutritional Sciences of the University of Northumbria (UK). All microorganisms were isolated from apples or prunes.

- **Experimental design**

This work was performed to assay weather or not apple extracts might have antimicrobial activity when PPO acts.

To verify this hypothesis, a fruit extract was prepared from *Golden delicious* apples, and then it was incubated with a PPO solution. Later, oxidized products were filtered and eluted in methanol. The dry weight of each sample was then determined letting the solvent evaporate under a slight nitrogen flow.

Once the dry products were ready, they were dissolved in methanol again and a MIC test was performed, using *S. cerevisiae* and *Lactobacillus* spp. as targets.

The same preparations, lacking of the incubation step, were set as control solutions to distinguish the effect on antimicrobial activity of the enzyme (compared to the action of the non-oxidized phenols).

- **Extraction and isolation of the oxidized products**

Apples were weighed and then homogenized with phosphate buffer (0.1 M, pH 7.0) in a 1:5 w/v ratio. The mixes were then split in two aliquots: control and sample.

In the first one, few droplets of a 4 M hydrochloric acid solution were added, in order to stop any enzymatic reaction (Rocha, *et al.*, 1998), while the other was incubated with a PPO standard solution (in a final concentration of 10 U/mL) for

24 h at 25 °C. The amount of time required for the action of the enzyme was calculated based on oxidation kinetics of different polyphenol standards (see *Experimental Work, Chapter 1*).

Both control and sample were then filtered with C_{18} cartridges and the retained compounds were eluted with methanol. Substances were finally dried under a slight flow of nitrogen and weighed.

- **Media preparation**

Both media for MIC test were purchased from Sigma-Aldrich Co. Ltd (Poole, Dorset, UK). In the case of *S. cerevisiae*, a Yeast-Peptone-Dextrose (YPD) medium fortified with a 15 mg/L adenine solution was used while, for *Lactobacillus* spp., a Man-Rogosa-Sharpe (MRS) medium was employed.

- **MIC assay**

Since MIC analysis requires microorganisms at their highest stage of vitality, preliminar growing curves were obtained for the three strains of *S. cerevisiae* and three *Lactobacillus* spp. Analyses were then performed using cells incubated for the amount of time required to reach such vitality.

Before performing the test, the dried compounds obtained from the isolation step were suspended again in methanol and several dilutions were prepared.

Later, in a 96-well plate, such compound solutions at different concentrations were added to each microorganism. Optical density (OD) was then read at 600 nm for all wells, using a plate reader, and, after a 24 h of incubation, in order to verify microbial growth the analysis was repeated. Each plate was incubated to its optimal temperature for the organism to grow, 28 °C for yeasts (Scanes, *et al.*, 1998; Yalcin & Ozbas, 2008) and 37 °C for bacteria (Hujanen & Linko, 1996).

All assays were performed in triplicate, to support data with appropriate statistical elaborations.

- **Statistical analyses**

In this work, analyses were carried out in triplicate to validate results. Differences in MIC values were considered significant with $p < 0.05$ (test t for independent samples). Statistical elaboration was accomplished using IBM® SPSS® Statistics (version 20.0.0) software.

2.4. RESULTS AND DISCUSSION

- **Results**

The aim of this project was to investigate PPO influence on toxicity against microorganisms. Particularly, the research focused on oxidized phenols effect using three species of lactic acid bacteria (*L. brevis*, *L. casei* and *L. plantarum*) and a yeast (*S. cerevisiae*) as models.

Table 10: Yield of extraction for control and sample.
Values are presented as means of several replicates.

Extract	Number of replicates	Extraction yield ($\%_{w/w}$)	Standard deviation ($\pm \%_{w/w}$)
Control	16	0.511	0.074
Sample	23	0.441	0.031

To exclude the influence of not oxidized fruit components on microbial growth, in the control solutions PPO was inhibited by acidification; on the other hand, samples were fortified with the addition of an enzyme standard solution. Table 10 depicts extraction yield for the two prepared solutions. These values were

obtained from several replicates which represent the amount of isolates retained by the C_{18} cartridges, mainly of polyphenolic nature. Data are expressed as percentages (w/w) of the initial apple wet weights.

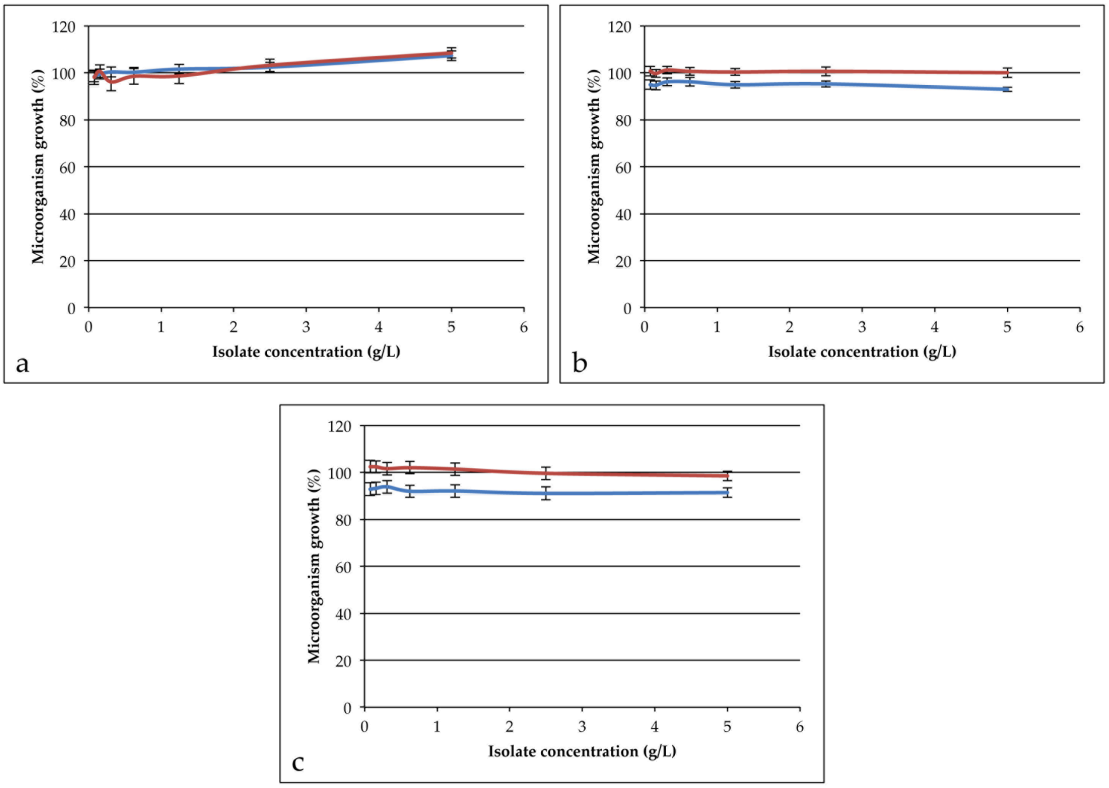

Figure 17: Influence of apple polyphenols on microbial growth for three species of lactic acid bacteria: *L. brevis* (a), *L. casei* (b) and *L. plantarum* (c).
Extracts oxidized by polyphenol oxidase are depicted with red lines, while the light blue lines represent the control solutions.

Antimicrobial activities were investigated using MIC tests and results are presented in Figures 17 and 18. In the first one, control and sample toxicity for *Lactobacillus* spp. cultures are compared, while the other figure refers to results obtained with yeasts. In each test, the abilities to inhibit microorganism growth for several concentrations of the examined solutions were assayed, and differences between sample (blue lines) and control solutions (red lines) were investigated.

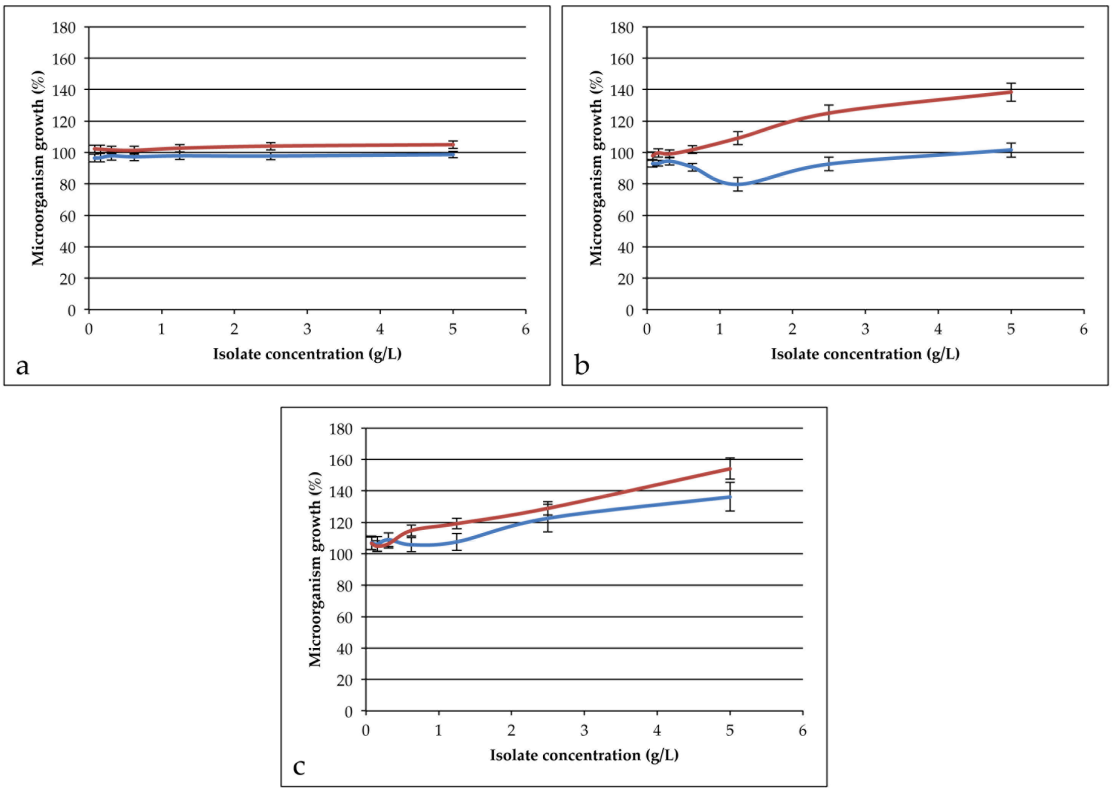

Figure 18: Influence of apple polyphenols on microbial growth for three strains of *Saccharomyces cerevisiae*: SC/A/7A (a), SC/B/4A (b) and SC/D/1P (c).
Extracts oxidized by polyphenol oxidase are depicted with the red lines, while the light blue lines represent the control solutions.

- **Discussion**

Being this project focused on apple PPO effects on microbial growth, results obtained from polyphenols oxidized and not oxidized by the action of this enzyme were compared.

Observing Figure 17, slight different behaviours might be identified for the three *Lactobacillus* spp. tested. Particularly, *L. brevis* seemed to differ from the other two bacteria in two aspects: the first was the influence of PPO, not relevant for *L. brevis* but statistically significant for *L. casei* and *plantarum*; furthermore, *L. brevis* growth seemed to be slightly promoted by apple extracts (approximately 108 % with a 5 g/L solution), effect that was absent for the other two LABs tested.

On the contrary, *L. casei* and *L. plantarum* grew to a lesser extent in a medium containing polyphenols not treated with PPO. If compared to normal activity, microbial growth reached a 92 % value while growth of the same microorganisms was not influenced if the medium contained PPO-treated polyphenols.

On the other hand, Figure 18 illustrates results for three *S. cerevisiae* strains. Even in this case, the reaction of one strain differed significantly from the other two. SC/A/7A, isolated from apples, seemed not to be affected from the extracts, oxidized or not, while for SC/B/4A (isolated from apples, too) and SC/D/1P (isolated from plums) the growth showed a very different trend. Considering that, anyway, all yeasts were affected from oxidized extracts more than from control solutions, the last two organisms revealed a growth increase that reach values about 140-150 %. This effect may be related to apple content in flavonols. These compounds are located mainly in skin but are present in the flesh, too (Lamperi, *et al.*, 2008). Influence of quercetin and quercetin-derivatives on promoting yeast growth, despite their relatively low concentration in the pulp, may explain this effect. As proved in the previous section, a quercetin standard solution increase four fold yeast growth, for certain strains: to confirm the results, SC/B/4A and SC/D/1P showed the highest effect in both studies (*Experimental Work, Chapters 1* and *2*).

The extreme variability between yeast strains (even isolated from the same matrix) is confirmed by several studies. Carreto, *et al.* (2011) highlighted how such microorganisms respond differentially to environmental changes: the authors hypothesized a possible correlation of this diversity among closely related strains to the gene expression variability. These outcomes are also confirmed by Ansel, *et al.* (2008) and they might be explained as a result of the complexes protein-protein interactions (Uetz, *et al.*, 2000). Modig, *et al.* (2008) analysed *S. cerevisiae* responses

for several strains to lignocellulose hydrolysates and even in that case they showed large different fermentation performances during the fed-batch experiments.

2.5. CONCLUSIONS

The aim of this research was to evaluate antimicrobial effects of *Golden delicious* apple hydrophilic extracts, if treated with PPO. Samples were then fortified with this enzyme and compared with a control solution of the same fruit, whose PPO action was inhibited by acidification.

Performing MIC analysis for three *Lactobacillus* spp. no activity can be detected, but a slight toxicity of the control extracts against *L. casei* and *L. plantarum* is reported. On the contrary, *L. brevis* growth seemed to be feebly promoted by both preparations.

The same effect, just more accentuated, was visible in two of the three *Saccharomyces cerevisiae* strains tested. SC/B/4A and SC/D/1P showed an increase in their growth that, for control solutions, reach values about 140-150 % of a normal activity. Such results may be correlated to the high promicrobial capacity of compounds present in both apple skin and flesh, like quercetin: this polyphenol increased microbial growth, even four fold for certain yeast strains. However, PPO activity did not prove to be effective in slow down growth for the tested microorganisms.

After these trials, it is almost clear that the focus of attention for PPO's role in apple may be conveyed elsewhere, maybe investigating the relation of such enzyme with oxidation processes happening during plant stress. These phenomena are closely related with plant defence mechanisms and may be

delayed with this peptide, despite its oxidative action.

2.6. AKNOWLEDGMENTS

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3. ANTIOXIDANT PROPERTIES OF OXIDIZED PHENOLIC COMPOUNDS AS STANDARD SUBSTANCES OR EXTRACTED FROM APPLE.

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3.1. ABSTRACT

Phenolics are a class of very common substances inside plant cells whose beneficial actions for the organisms are broadly recognized. This study foresaw the investigation about the capability of polyphenols to scavenge free radicals before and after being oxidized by a common apple enzyme, polyphenol oxidase (PPO).

Antioxidant capacity was measured with two distinct approaches: DPPH test and crocin bleaching assay. Besides this, the analyses were performed using standard phenolic compounds and fruit extracts from *Golden delicious* apple.

Among the four chemicals analysed, catechol and chlorogenic acid showed an increase in antioxidant capacity, if treated with PPO. On the contrary, quercetin appeared to behave in an opposite way. All these results were confirmed by both analyses. On the other hand, epicatechin data were not clear, depicting inconsistent results.

Apple phenolics isolates confirmed a beneficial effect of the enzyme in promoting free radical scavenging, despite total antioxidant capacity was orders

of magnitude weaker of the standard solutions.

3.2. INTRODUCTION

Polyphenolic compounds are a broad chemical class, which contains many natural and synthetic molecules. In a classification depicted by Handique & Baruah (2002), the first group is arranged by (1) proanthocyanidin derivatives, (2) galloyl and hexa hydroxydiphenyl ester derivatives, (3) hydroxycinnamic acid derivatives and (4) phloroglucinol derivatives, while in the second one (5) open chain, (6) cyclic compounds and (7) others are included. A more complex organisation was traced by Ramassamy (2006) a few years later. The author classified polyphenols in three main areas, based on their chemical structure: (1) phenolic acids, (2) non-flavonoids (such as *trans*-resveratrol) and (3) flavonoids (which in turn can be divided into various classes, such as anthocyanins and anhtoxantins). Particularly, anhtoxantin group contains flavonols, flavans, flavanols, flavones and isoflavones.

All these molecules can be found basically in every plant and, in the past years, a lot of beneficial properties for human health were discovered. Evidences were found regarding a beneficial role of tea polyphenols in lowering Parkinson's disease risk (Checkoway, *et al.*, 2002) and in protecting neurons from amyloid β-induced damage, process involved in Alzheimer's disease (Choi, *et al.*, 2001; Bastianetto, *et al.*, 2006). Other studies suggested that diets supplemented with blueberry polyphenolic extracts might protect the brain from neuronal loss and prevent the decrease of cognitive functions, having antioxidant and anti-apoptotic properties and being involved in the regulation of cell signalling: Joseph, *et al.* (1999) observed a reduction in cognitive deficits in Morris water maze

performance test in 19-month-old rats fed for 8 weeks with a blueberry extract supplementation, the relationship between flavonoids and cell-signalling is observed in several cases (Dias, *et al.*, 2005; Martínez-Flórez, *et al.*, 2005) and rats receiving blueberries extracts proved to have a caspase-3 activity lower than the control animals (Wang, *et al.*, 2005), an effect that may protect brain cells from apoptosis.

Besides these actions, several cases are recorded about a healthy role of phenolic compounds even in their oxidized form. Lattanzio, *et al.* (2006) reviewed several cases where polyphenol oxidase (PPO) products are supposed to protect plants from microorganisms and parasites. This peptide is a typical apple chemical but it may be found in several animals and fungi, too. In the past years, it was broadly studied for its damage actions during apple harvesting and ripening; however, this molecule is believed to play a positive role during infections. In addition to the toxic quinones produced (Yang & Boissy, 1999; Bittner, 2006) and the barrier-effect (against microbial proliferation) operated by the polymerised phenolics (Dordick, *et al.*, 1987), its products might increase antioxidant capacity, reinforcing plant natural defences.

Several methods exist to assay antioxidant capacity. Generally, they can be classified in two main areas: Total Antioxidant Capacity (TAC) assays or single antioxidant measurements. Even if the second approach is more precise, an overall measure may allow to obtain more useful information. Furthermore, using methods based on different chemical principles, results can be more reliable.

One of the most spread TAC analyses is the 2,2-diphenil-1-picrylhydrazyl (DPPH) assay. DPPH is a synthetic radical compound whose main characteristic is to bleach when stabilized (Equation III). If a solution having antioxidant capacity is added to a mixture containing DPPH, the radical becomes stable decolouring

the system from dark purple to light yellow.

(III) $$DPPH^{\bullet} + AH \rightarrow DPPH - H + A^{\bullet}$$

where AH is a general antioxidant molecule. A limit of this test is the absence of substrate, thus it does not provide any information on the protective activity to real foods or biological systems (Frankel, 2007); however, polyphenols are very reactive towards DPPH free radicals, producing *o*-quinones intermediates by H-abstraction (Wang, *et al.*, 1999).

Another widely used principle to observe such property is the bleaching of a β-carotene molecule, monitored during linoleic acid oxidation. Antioxidants are able to produce a delay in the absorption decay (Cao, *et al.*, 1993; Koleva, *et al.*, 2002). Deriving from this test, the crocin bleaching assay involves radicals generated by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH). A competition is set up between crocin (a carotenoid extracted from saffron) and antioxidants in reacting with radicals produced (Tubaro, *et al.*, 1996). The result is a slower decolouration of crocin in presence of compounds with high antioxidant capacity. This test exploits kinetics measurements; therefore it is not influenced by extract absorbance, even if some phytochemicals and food pigments may interfere with crocin bleaching. It is also a good choice in certain samples not suitable for the DPPH analysis, like plasma (Tubaro, *et al.*, 1998), whose proteins precipitate in the required solvents (Schilcher, *et al.*, 2012).

In this work, TAC for polyphenols was presented in comparison with that of the same compounds oxidized by the action of PPO. Two different tests were used to have more detailed information: DPPH method and crocin bleaching assay. Eventually, the antioxidant capacity of apple hydrophilic extracts was evaluated,

investigating the influence of PPO.

3.3. MATERIALS AND METHODS

- **Chemicals and food samples**

All chemicals were purchase from Sigma-Aldrich® Co. Ltd (Poole, Dorset, UK) and were over 99 % purity. PPO standards was purchased from Sigma-Aldrich®, too, at a 3610 U/mg solid concentration. Solvents were purchased from J. T. Baker® (Milano, Italy).

Golden delicious apples were purchased in a local supermarket.

- **Instrumentation**

DPPH analyses were performed with a Synergy HT Multi-Mode Microplate Reader (BioTek®, UK) that allows to collect data of 96 wells at the same time. The instrument was thermostatically controlled, shakeable and equipped with a SQ xenon flash monochromator (2.4 nm bandpass).

Crocin bleaching assay was carried out using a Varian, Inc., Cary® 50 Series UV-Vis Spectrophotometer (Agilent Technologies®, Italy). The instrument was equipped with a 18 Cell Holder Transporter and a Czerny-Turner monochromator.

PPO products isolation was conducted with C_{18} cartridges (125 Å pore size), purchased from Sep-Pak®.

- **Experimental design**

In this paper, a comparison between antioxidant properties of phenolic compounds oxidized by PPO and the same substances not oxidized is presented.

The property was measured using two separate assays that exploit different principles: DPPH test verifies how sample antioxidants might be able to transfer a hydrogen atom remaining stable while crocin bleaching assay establishes competition kinetics between the carotenoid and the sample towards the radicals generated by AAPH. Both set of data were finally compared with a Trolox C® standard solution, the hydrophilic analogue of vitamin E.

The first part of the project was carried out using four standard polyphenols: catechol, chlorogenic acid, epicatechin and quercetin. To analyse PPO influence on TAC, the enzyme was added to the mixtures and let react. Control solutions were prepared with the same substances without the addition of the PPO.

Afterwards, apple polyphenols were extracted, oxidized by the enzyme and the products were isolated. The same tests used in the previous part were accomplished for such oxidized molecules, too. A control solution was prepared, blocking PPO activity by acidification immediately after the extraction and before the isolation step.

- **Chemical preparation, apple extraction and isolation of the oxidized products**

For each phenolic compound 10 mM batch solutions were prepared. A 1 mM Trolox C© solution was prepared while DPPH was dissolved in methanol to reach a final batch concentration of 120 μM. Crocin was extracted from saffron: 0.125 g of the spice was dissolved in 5 mL of methanol and then left to rest for 12 h at 4 °C. A 0.5 M solution of the radical generator AAPH was finally prepared.

Apples were weighed and homogenized using a 0.1 M phosphate extraction buffer (pH 7.0) in a 1:5 w/v ratio.

A PPO solution was added in the sample vials, to reach a concentration of 10

U/mL. An incubation step was required: solutions are kept for 24 h at 25 °C, the time being calculated as described in *Experimental Work, Chapter 1*. In the control solution, hydrochloric acid was added to block PPO activity (just a few droplets of a 4 M solution were enough, to avoid any dilution) (Rocha, *et al.*, 1998).

Finally, all the preparations were filtered with C_{18} cartridges to isolate the substances required. The retained substances were re-eluted with methanol, dried and weighed.

- **DPPH test**

DPPH analysis was performed adapting a method used by Bondet, *et al.* (1997) for antioxidant measurements. In 96-well plates, radical preparations were diluted 1:1 with antioxidant solutions; absorbance was then read at 515 nm for 30 min, a sufficient amount of time for the system to reach the colour plateau required. For each sample, different concentrations were prepared.

Once all data were collected, absorbance (a mean value of the replicates) was graphed correlated to the polyphenolic concentration. Finally, ratio between each sample and Trolox C® slopes was calculated and the results were expressed as equivalents (eq.) of the comparative antioxidant.

- **Crocin bleaching assay**

This test was carried out starting from the procedure described by Tubaro, *et al.* (1996) for Maillard reaction products. The concentration of crocin required for the test was 10 μM, corresponding to a final absorbance value of 1.33; this value was calculated considering that in methanol, at 443 nm, molar absorptivity (ε) corresponds to $1.33 * 10^5$ $L*mol^{-1}*cm^{-1}$ (Bors, *et al.*, 1984).

Afterwards, increasing sample amounts, the volume of crocin required to

reach a 10 μM solution, and phosphate buffer were mixed in 1.5 mL plastic cuvettes. Bleaching reactions started with the addition of 50 μL of the AAPH preparation (concentration of 25 mM) and absorbance was then read at 443 nm for 30 min (the temperature required for the action of AAPH is 40 °C).

After data collection, initial slopes of the curve trends were calculated for cuvettes containing no sample (v_0) and cuvettes containing an amount of antioxidant (v). In the final graphs, ratio between sample and crocin concentrations and ratio between v_0 and v were correlated. Data were expressed as Trolox eq., referring to a standard antioxidant solution.

- **Statistical analyses**

Five independent replicates were analysed for each sample to validate results. Statistical elaboration was accomplished using IBM® SPSS® Statistics (version 20.0.0) software.

3.4. RESULTS AND DISCUSSION

- **Results**

The aim of this work was to investigate polyphenols antioxidant effect using two different methods, in order to compare and validate the outcomes. The first approach was the DPPH test, that allowed a stability evaluation of the antioxidant in its radical state; on the contrary, crocin bleaching assay measured the ability of the substances to capture radicals establishing a kinetic competition with the carotenoid. Chemicals were analysed both as standard solution (four phenolic compounds were chosen) and as fruit extracts (*Golden delicious* apples), with the aim to evaluate the influence of PPO in their antioxidant capacity.

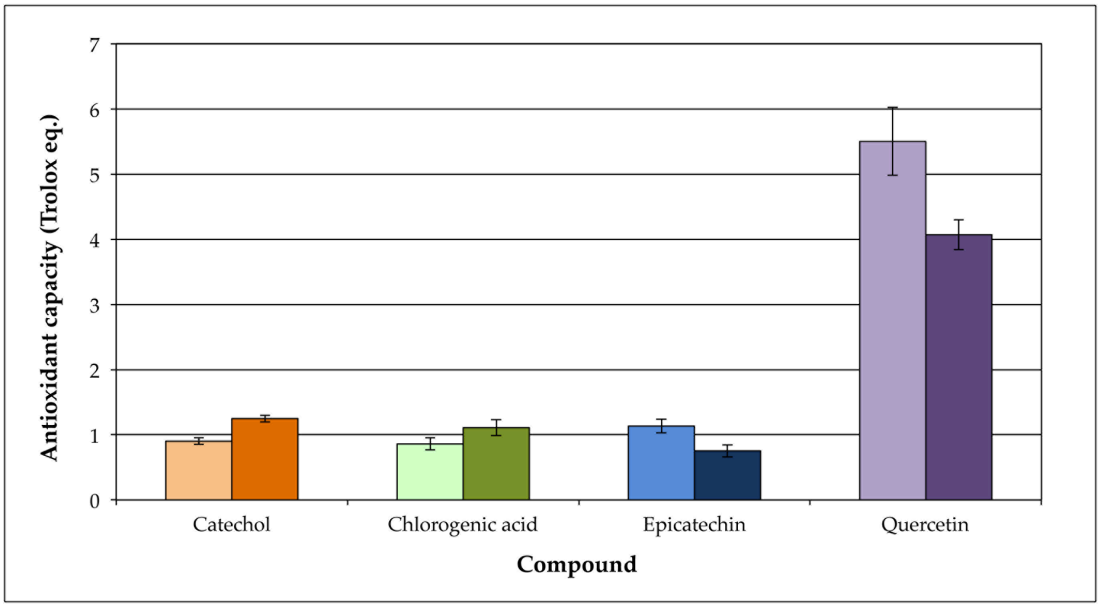

Figure 19: Antioxidant capacity for different polyphenols standards, measured with DPPH test.
Comparison between chemicals oxidized (dark bars) and not oxidized (light bars) by the action of polyphenol oxidase.

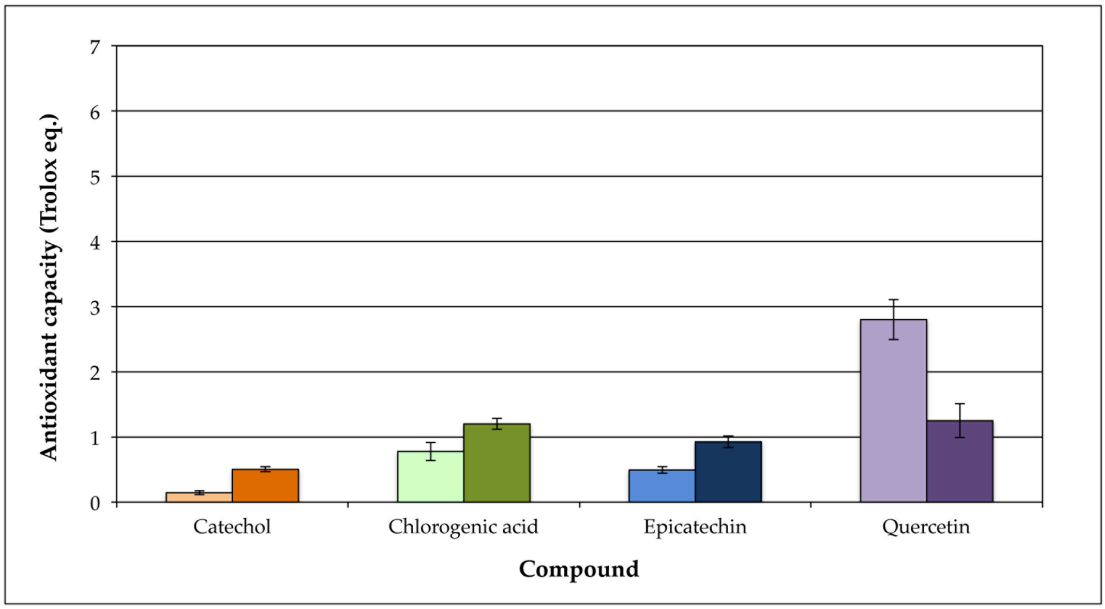

Figure 20: Antioxidant capacity for different polyphenols standards, measured with crocin bleaching assay.
Comparison between chemicals oxidized (dark bars) and not oxidized (light bars) by the action of polyphenol oxidase.

Figures 19 and 20 present TAC values of the analysed phenolic standard solutions (catechol, chlorogenic acid, epicatechin and quercetin): light bars refer to phytochemicals not oxidized by the action of the enzyme while dark ones depict results of the oxidized equivalent compounds. For both analyses, TAC was expressed as Trolox eq., comparing the effects to a Trolox C® standard solution (the hydrophilic analogue of α-tocopherol).

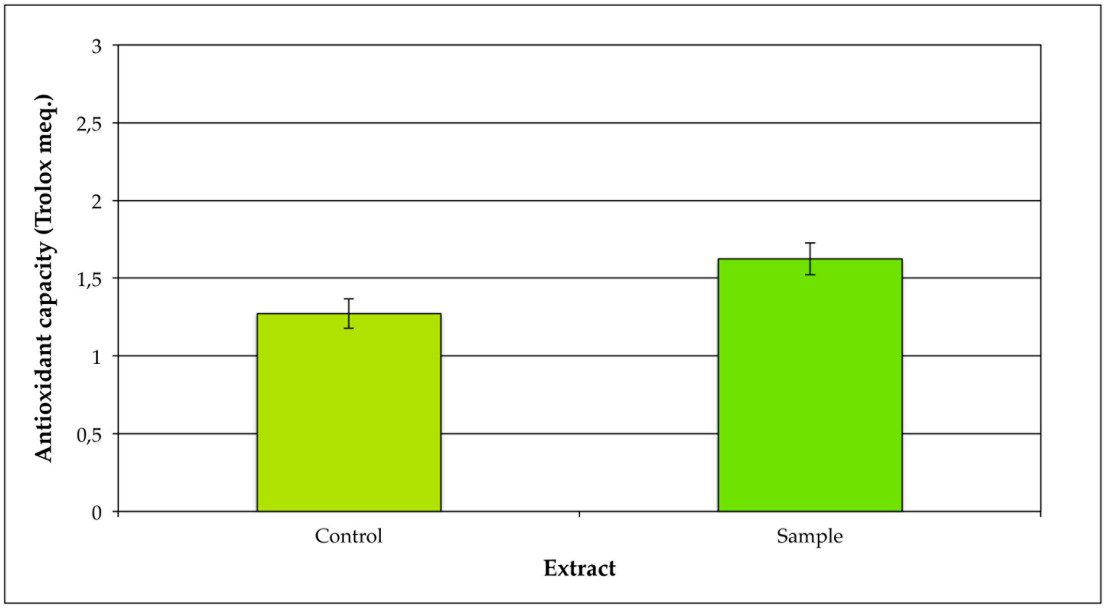

Figure 21: Antioxidant capacity for apple polyphenol extracts, measured with DPPH test.
Comparison between solutions oxidized (Sample) and not oxidized (Control) by the action of polyphenol oxidase.

Once chemical data were collected, polyphenols were extracted from apples. Results are presented in Figures 21 and 22. Sample solutions represent the extracts oxidized by PPO and then isolated with C_{18} cartridges; TAC values were depicted with the dark bars. Data for the equivalent control solutions were illustrated with light bars. In both these graphs, antioxidant capacity is expressed as Trolox meq., being the results orders of magnitude weaker than the standards.

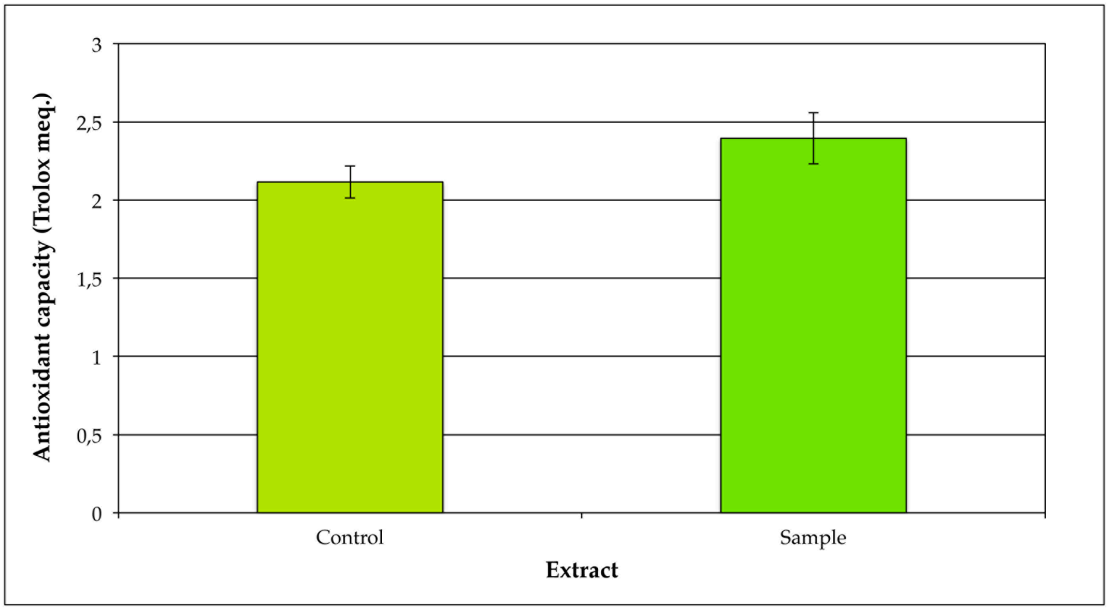

Figure 22: Antioxidant capacity for apple polyphenol extracts, measured with crocin bleaching assay.
Comparison between solutions oxidized (Sample) and not oxidized (Control) by the action of polyphenol oxidase.

- **Discussion**

Observing Figure 19, antioxidant capacity can be found for all the chemicals investigated, with Trolox eq. values ranging from 0.9 to 5.5. For catechol and chlorogenic acid, the enzyme action led to a slight increase in their activity. On the contrary, for epicatechin and, moreover, quercetin, the oxidation induced a loss in the ability to capture free radicals. However, even in its oxidized form, quercetin exerted the highest activity between the phenolic compounds tested. The behaviour of these phytochemicals in protecting the carotenoid crocin was very similar. TAC values appeared to remain similar during the second analysis, except from a slight decrease for catechol and quercetin (in both forms, oxidized and not),. Non-treated epicatechin solution was the only one that seemed to behave differently with the two methods. Figure 20 shows how TAC value of epicatechin control solution was about 50 % of the equivalent data, acquired during the DPPH

test. Having obtained pretty much the same results from both analyses, and considering the reproducibility of data, a statistically significant action of PPO may be assumed in enhancing antioxidant capacity for three of the tested polyphenols, while quercetin proved to be affected negatively. This protection made by quinone substances on the environment is in accordance with previous studies (Cotelle, *et al.*, 1991; Hino, *et al.*, 1998), and may be related to the formation of stable quinone radicals (Hendry, *et al.*, 1994).

Data collected from isolated apple polyphenols seemed to confirm such activities (Figures 21 and 22). In both the analyses, samples showed a higher TAC value than control solutions, although in this case molecules seemed to respond better with crocin bleaching assay. Differences were not pronounced, nonetheless they were statistically significant. For apple extracts, results were expressed as Trolox meq., being their TAC about 1000 times lower than the reference. Even in this case, standard deviations were satisfactory, always remaining under 8 %.

3.5. CONCLUSIONS

This work focused on possible PPO effect on antioxidant capacity in a biological system. After a phenolics standard investigation, *Golden delicious* apple extracts were prepared, to assay the strength of their polyphenolic compounds in delaying oxidation processes.

PPO increased the ability to bind free radicals for catechol and chlorogenic acid; results were confirmed by both DPPH test and crocin bleaching assay. On the contrary, a negative effect was observed for quercetin. Epicatechin did not give clear results, apparently showing a decrease in TAC with the first method and an enhancement with the latter. However, all chemicals displayed values ranging

from 10 to 550 %, compared to Trolox C® activity.

Apple extracts gave similar encouraging results, demonstrating how antioxidant capacity is favoured by the action of the tested enzyme. Control solutions seemed to exert a slower free radical scavenging activity, especially towards $DPPH^{\bullet}$, yet both extracts showed a TAC thousand times lower than the reference.

All these experiments suggested that apple PPO might play an important role against oxidative stress, too. Therefore, the greater activity of this protein in damaged tissues could help the organism to strengthen itself against pathogen attacks.

3.6. AKNOWLEDGMENTS

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CONCLUDING REMARKS AND FUTURE PERSPECTIVES

1. CONCLUSIONS

The aim of this PhD dissertation is to describe a 3 year-investigation work about polyphenol oxidase (PPO). Starting from a bibliographic research about the possible roles of this enzyme in cells, the project focused on possible antioxidant capacities of PPO substrates (treated with enzyme) and their effect on microbial growth. All the analyses were carried out using standard phenolic substances, in the first stage, and *Golden delicious* hydrophilic extracts, later. Toxicity against microorganisms was performed with Minimum Inhibitory Concentration (MIC) tests for three *Lactobacillus* spp. (*L. brevis*, *L. casei* and *L. plantarum*) and three strains of *Saccharomyces cerevisiae*, all isolated from fruit skin. Total Antioxidant Capacity (TAC) was investigated exploiting two different methods, DPPH tests and crocin bleaching assays, and results were normalized to a Trolox C® standard solution.

Among the standard phenolics tested, only catechol antimicrobial activity towards *L. brevis* and *L. plantarum* seemed to be increased by PPO; for the other microorganisms no effects were observed. Chlorogenic acid and epicatechin properties were not affected by the enzyme while quercetin clearly promoted microbial growth, even if PPO influence on that gave unclear results: the enzyme improved quercetin effect on *S. cerevisiae* RD/B/4A growth, while an opposite result was observed for *S. cerevisiae* RD/D/1P.

At the same time, outcomes obtained from the hydrophilic extracts of apples showed how they were able to promote microbial growth, effects that were accentuated for yeasts. MIC analyses proved that extracts accentuated the proliferation of *L. brevis* and, especially, the growth of two strains of *S. cerevisiae*, reaching values about 140-150 % of the cultures not supplemented with such

substances. Anyway, in all the microorganisms investigated, PPO did not influence samples activity. For this project just three *Lactobacillus* spp. and three strains of *Saccharomyces cerevisiae* were used (as prokaryotic and eukaryotic cell models, respectively), however data collected seemed to suggest that the influence on microbial growth is not the main objective of the investigated enzyme. An interesting result is the confirmation of the quercetin behaviour, which promoted microorganism growth, despite the anti-biofilm activity of this molecules were proved.

Finally, TAC investigations showed that PPO was capable to increase free radical scavenging of both catechol and chlorogenic acid, while it reduced such ability in the case of quercetin. Epicatechin gave inconsistent results, proving to be negatively affected during DPPH test and positively with crocin bleaching assay. PPO slightly activated apple extracts in the protection of the system from oxidation: this result was accentuated using DPPH radical as damaging agent, even if, in both analyses, results proved to be orders of magnitude weaker than Trolox C®. This result is just a starting point: the TAC improvement of the extracts cannot represent, alone, the biological value of PPO. As many natural compounds, this enzyme might perform different roles in apple tissues, either connected to plant defences or not. A possible function, related to a polymerization effect, is described in the next section (*Concluding Remarks and Future Perspectives, Chapter 2*).

2. FUTURE PERSPECTIVES

This research investigated just a few of the possible roles PPO might exert in cellular metabolism, during and immediately after pathogen attacks. Particularly,

the formation of a polymeric barrier against oxygen and infectious agents might represents another synergistic effect operated by this enzyme. A way to possibly determine such mechanisms could be the use of two semi-permeable barriers to support polyphenol extracts (oxidized and not by PPO). A simple oxygen detector placed after each system would complete the experiment. If the gas will not be able to flow from one side of the membrane to the other, than the barrier effect will be confirmed.