UNIVERSITÀ DEGLI STUDI DI PALERMO
Dottorato Internazionale in Frutticoltura Mediterranea
Dipartimento Scienze Agrarie e Forestali
Settore Scientifico Disciplinare AGR/03

POLYPHENOLS EXTRACTED FROM THREE APPLE CULTIVARS GROWN IN SICILY: EFFECT ON K-CASEIN AGGREGATION

PhD CANDIDATE
Dr. G. C. Rappa

PhD COORDINATOR
Prof.ssa M. A. Germanà

SUPERVISOR
Prof.ssa M. A.Germanà

CO-SUPERVISOR
Dr. P.L. San Biagio

Dr. V. Guarrasi

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Preface

Apples are considered the "health fruit" for which is valid the traditional rhyme "*an apple a day keeps the doctor away*." Nutritionists identify apple as functional food, because it has potentially positive effect on health beyond basic nutrition. The health benefits of apples are mainly attributed to their phenolic content. Apple polyphenols have elevated antioxidant capacity, enzyme modulation ability, and effects on cell signaling pathways and on gene expression. Recently, a neuroprotective and anti-amyloidogenic effect of apple polyphenols has been found in the field of neurodegeneration, but action mechanism is still unclear (Toda et al., 2011).

Object of this research project is the study of the effects of the bioactive compounds extracted from apples of three different cultivars, 'Gala', 'Granny Smith' and 'Fuji', grown in Sicily, on the aggregation process of a model amyloid fibril-forming protein, κ-casein.

The research activity has been organized in two phases, to which two different thesis chapters with specific results are associated.

The first phase has been the quality characterization of apple fruits from the three cultivars, 'Gala', 'Granny Smith' and 'Fuji', among the most cultivated apple varieties, produced in Sicily (zone of Caltavuturo) in an organic farming system. In particular, the chemical-physical parameters of the three apple varieties, the total polyphenolic content from different tissue kinds and their antioxidant capacity have been analyzed.

In the second phase, the effect of apple polyphenol extracts on the aggregation mechanism of a milk protein, κ-casein, used as amyloidogenic model fibril-forming protein, has been studied. The choice of the kind of apple extracts and the concentrations used to be tested on κ-casein has been performed on the basis of the

cell viability results obtained on a mouse fibroblast NIH-3T3 cell line, used as cellular model. Physical-chemical properties, structural features of κ-casein and the inhibition of its fibrillogenesis exerted from apple polyphenolic extracts have been analyzed by bioinformatic tools and biophysical techniques (Fluorescence Spectroscopy, Circular Dichroism, Light Scattering and Atomic Force Microscopy).

Finally, the global overall results of the work research are presented and the general conclusions are drawn.

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INTRODUCTION

1. INTRODUCTION

In this chapter, the state of the art of the main research topics is presented. First, the apples general features and three different varieties, in particular Gala, Fuji, Granny Smith, are described. Afterwards, the molecular class of polyphenols will be introduced, with particular emphasis on their beneficial effects in the field of neurodegeneration. Finally, the general problem of amyloid aggregation, and related oxidative stress, is reported and the structural features leading, κ-casein, a protein from bovine milk, used in this study as amylopidgenic model, is presented.

1.1 APPLE (*Malus domestica*)

Apple belongs to the *Rosaceae* family, subfamily of *Maloideae* and genus *Malus*.

The common cultivated apple, *Malus domestica*, is believed to be originated in central Asia and to be an interspecific hybrid complex of European apple *Malus sylvestris* and Asian species *Malus dasyphyllus* and *Malus praecox* (Ferree and Warrington, 2003).

Malus domestica is cultivated widely in temperate latitudes or at high elevations in the tropics, on all continents, except Antarctica. The fruits are consumed fresh, dried or tinned, or processed into juice, preserves or alcoholic beverages. Besides, fruits of several other species are used for medicinal purposes (Ferree and Warrington, 2003).

Apple fruit is a “false fruit” or pome, varying in size from 1–4 cm diameter in most of the wild species, to 6 cm in *Malus pumila*, 8 cm in *Malus sieversii*, and even larger in cultivated apples. The center of the fruit contains five carpels arranged star-like, each containing one to two (rarely three) seeds, and surrounded by accessory tissue. The accessory tissue is interpreted by some specialists as an extension of the

receptacle and is then referred to as "fruit cortex", and by others as a fused hypanthium or "torus". It is the most edible part of this fruit. The carpels of a pome are fused within the "core". The epicarp and mesocarp of a pome may be fleshy and difficult to distinguish one from another and from the hypanthial tissue. The endocarp forms a leathery or stony case around the seed, and corresponds to what is commonly called the core (Fig. 1). Since the fruit contains tissue not derived from the pericarp, it is called an accessory fruit. This is the typical fruit of certain members of the family *Rosaceae* (Ferree and Warrington, 2003).

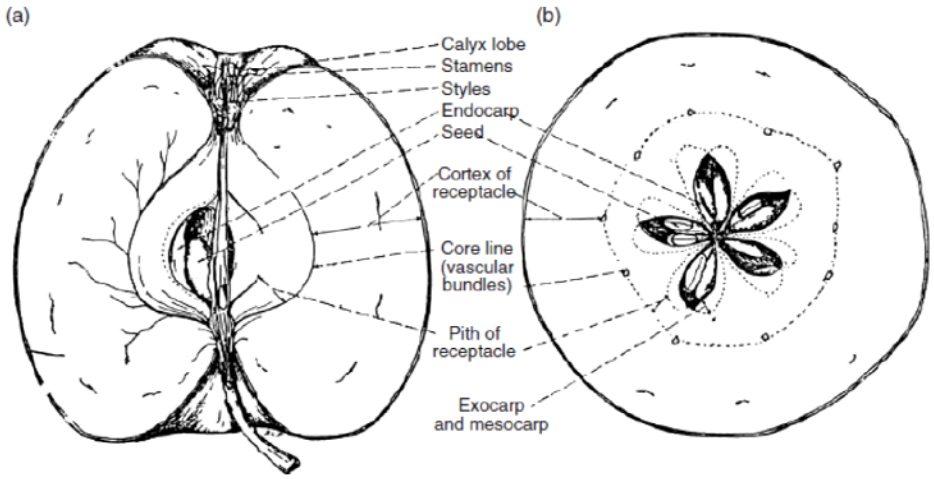


Fig. 1. Structure of a mature apple fruit. (a) Vertical section; (b) equatorial section (Robbins, 1933).

1.1.1 Apple production

Apples are fruits widely produced worldwide. From 1993 to 2013 apple production in the world is doubled achieving a production above 80 million tons. The areas mainly committed to the production of apples are Asia, which accounts for 55.6 %,

followed by Europe with 25.6% and the Americas with 14.6%. African continent and Oceania contribute to worldwide production only for 3% and 1.3% respectively (Fig. 2) (http://faostat3.fao.org).

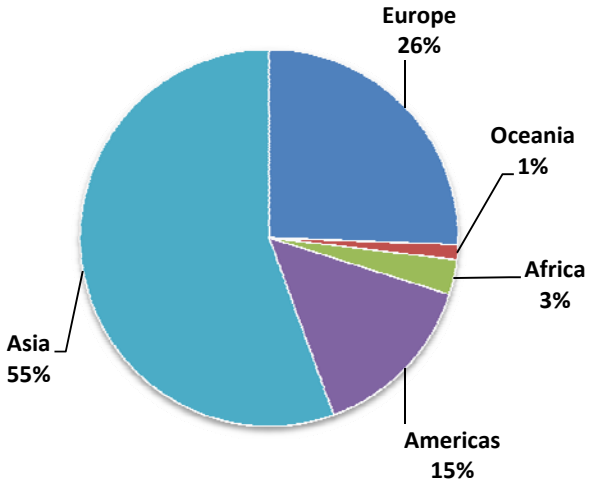


Fig. 2 Production share by region. Average 1993-2013. Source: http://faostat3.fao.org

China is the first apple producer country, with an annual production that exceeds 20 million tons, largely detached the other countries, namely the United States, in second place with a apple production of over 4 million tons, and Turkey, with an average share of 3.12 million tons of product per year. Then, among the countries of the northern hemisphere, Poland follows with 3.08 million tons and Italy with 2.21 million tons (http://faostat3.fao.org). Southern Hemisphere's largest producer is Chile, with a production of about 1.8 million tons, significantly distanced by other countries such as Brazil, Argentina and South Africa with production of 980, 825 and 706 thousand tons, respectively (www.wapa-association.org). Referring to the situation in European Union, the countries that specifically perform the role of the largest producers are Poland, Italy and France, followed by Germany and Spain (www.bmti.it; www.cn.camcom.it).

In Europe, Italy is the second Community producer of apples with over 67,000 hectares for a production that often exceeds 2 million tons per year. In Italy apple cultivation is practiced all over the Italian territory, but it is traditionally concentrated in the mountain and foothill regions such as Trentino Alto Adige with 54% of the national area invested (28201 ha), followed by Veneto, Emilia Romagna and Piemonte. In the south, the apples are grown mainly in Campania with 3.6% of the national production (www.bmti.it; www.cn.camcom.it), while Sicily records only 1.2% (619 ha). Recently, in Sicily the apple-growing shows an increase (+9.4%) thanks to the availability of new irrigated areas: recent installations have been made in the province of Palermo, on the Madonie, in environments characterized by freezing winter and not excessively hot summer (Istat 2010 – 2012).

In Europe, apples are the most popular forints, followed by bananas, citrus, and pears. In the period between 2011 and 2012 there was a slight growth in consumption of apples, thanks to higher availability and exports. In Italy, the apple is part of the eating habits of almost all Italian families. Indeed, Italians are considered great consumers, as the per capita consumption is around 20 kg per year, among the highest in the world. Due to the very wide commercial offer, and the possibility to cover with apples production the whole year, this trend can only increase. Experts estimate that in 2012 around 10% of the Italian crop was designed to the industry, while the remainder 90% was used for domestic fresh consumption and export. Italy consumes most of its domestic production, exporting less than 16% (www.fleshplaza.it; www.assomela.it; www.wapa-association.org).

1.1.2 Apple cultivars grown in Italy

Apples have been cultivated and vegetatively propagated for over two millennia. Over 10,000 named cultivars exist and breeders worldwide create many new selections annually, but, actually, only a few dozen types are widely introduced in commerce (Ferree and Warrington 2003). In most countries, growers continue to produce many cultivars that are traditional in their region. However, never cultivars have gradually been introduced for varied production and marketing reasons. Currently, there are over 5,000 cultivars. The various cultivars can be classified according to several important parameters such as:

~ Ripening of collection (they can be divided into various summer and autumn-winter cover according to collection time);

~ Families or pomological groups (Golden Delicious group, Red Delicious group, etc.); the standard cultivar, of high or medium development, and the cultivar *spur*, weak in development, are also distinguished within the following groups;

~ Use (fresh consumption, for industry, to be cooked);

~ Flowering time (early, middle or late, though, often, the difference is a few days);

~ Pollination capacity (some triploid cultivars are bad pollinating, unlike diploid ones such as Golden Delicious instead is a good pollinator).

The list of the more common and important Italian apple varieties is the following (www.unece.org):

1 ANNURCA
2 BRAEBURN
3 CAMEO
4 CRIPPS PINK
5 ELSTAR
6 FUJI
7 GALA
8 GOLDEN DELICIOUS
9 GOLDEN ORANGE
10 GOLDRUSH/COOP 38
11 GLOSTER
12 GRANNY SMITH
13 GRAVENSTEIN
14 IDARED
15 JONAGOLD
16 JONATHAN
17 FLORINA
18 MORGENDUFT
19 OZARK GOLD
20 PINOVA
21 RED DELICIOUS
22 RED CHIEF
23 RENETTA DEL CANADA
24 ROYAL GALA
25 RUBENS
26 SANSA
27 STAYMAN-WINESAP
28 SUMMERRED
29 TOPAZ

For this study the selected apple cultivars were **'Gala'**, **'Granny Smith' and 'Fuji'**, which are different for collection time, pomological groups and quality characteristics.

'Gala': *Gala* apples represent one of the most widely grown apple varieties in the world, and a mainstay of apple selection in the supermarket because it is available year round from northern and southern hemisphere suppliers. One of the unique features of Gala is that it can be grown with good quality results in both temperate and warm apple-growing regions, and it is generally regarded as a low-chill variety. *Gala* is a cross between *Kidd's Orange Red* and *Golden Delicious*. Bearing in mind that *Kidd's Orange Red* is the offspring of *Cox's Orange Pippin* and *(Red) Delicious*,

Gala is effectively a union of three of the world's most important and distinctive apple varieties. The fruits are of frusto-conical shape slightly rounded, of medium size (160g), with firm, juicy, sweet and slightly acidic pulp and with excellent organoleptic characteristics. The coloration of Gala is exactly as you would expect from a cross between a *Cox-type* variety and *Golden Delicious*. It starts out as a very light colored Cox, mainly orange streaks over yellow; mature apples are much darker, often a strong red colour. The colour is a good indicator in the supermarket of the Galas apple age: if it is very pale then it is probably the new season's crop, probably picked slightly early. If it is very dark then either it has been left deliberately on the tree to mature or it has matured over a long period in a cold store. Gala apples are of particular interest due to the high productivity and excellent agronomic characteristics. They require great care during thinning fruits and pruning, in order to reach a sufficient size (www.bmti.it; www.cn.camcom.it; www.orangepippin.com).

'Granny Smith': *Granny Smith* variety was discovered in Australia in the 1860s from Mrs Maria Smith and it is the most instantly recognized apple. It requires a warm climate to ripen properly, and it performs well in the main apple-growing regions of the southern hemisphere. In the northern hemisphere it is grown in France, Italy and the warmer zones of North America. The trademark apple-green skin requires warm days and nights. It presents a peel of a beautiful green color with numerous clear lenticels. The flesh is crisp and fresh and the acidity flavor of the fruit make it a highly regarded both at the table in the kitchen, and as ingredient irreplaceable for salads and sorbets. It is an uncompromising crisp hard apple with a very sharp taste. The flavor sweetens in storage. Nevertheless, its share of the

international market is on the decline, with supermarkets preferring to sell bi-coloured varieties with a sweeter flavor. The *Granny Smith* apple production is available on the National market from September to May, while in the summer months it is present as a product overseas (www.bmti.it; www.cn.camcom.it; www.orangepippin.com).

'Fuji': *Fuji* is surely one of the more attractive modern apple varieties. Selected in 1939 in the Japanese district of Morioka, the Fuji variety is now widely grown in many areas of the world due to its high productivity and the good taste characteristics. One of the peculiarities of this cultivar is that it has excellent quality performance even in lowland areas, normally less suitable to apple production. *Fuji* is a cross between the widely grown *Red Delicious* and *Ralls Janet*, which is much less well known but is probably the reason for Fuji's attractive pink flush.

Its main characteristic is the streaked peel that colors very late to pinkish red over a yellow-green background. The fruit is cylindrical with crisp, tasty and very sweet pulp. It is available on the market from October (northern hemisphere orchards) to June (southern hemisphere orchards) (www.bmti.it; www.cn.camcom.it; www.orangepippin.com).

1.1.3 Apple bioactive compounds and difference among tissues

Apples have a rich polyphenolic profile and the natural function of these molecules is correlated to protection of plants from diseases and ultraviolet light and prevention of damage to seeds until they germinate. Polyphenols are induced in plants under oxidative stress conditions and support the activity of other important cellular

antioxidant compounds such as glutathione, α-tocopherol, ascorbic acid, and enzymes such as peroxidase, and superoxide dismutase. Phenolic compounds are accumulated in plant organs: roots, stems, leaves, flowers, fruits, etc. These compounds vary in their composition and concentration, among cultivars and fruit tissues according to growing conditions, cultural practices, and ripeness, during harvest, post-harvest storage conditions, and processing. Several researches have reported that apple peel contain more antioxidants and antioxidant capacity than the remaining part or the whole fruit (Drogoudi et al., 2008; Vieira et al., 2009; Henríquez et al., 2010; Francini et al., 2013). Preferential localization of polyphenols in peel is set in relation with effect of light on the phenolic metabolism, as well as, with the protective role of phenolic compounds against ultraviolet radiations and other abiotic and biotic stressors (Winkel-Shirley, 2002; Lattanzio et al., 2006; Francini et al., 2013).

Apples have a variety of phenolic compounds (Scalbert et al., 2000) such as (+)-catechin and (−)-epicatechin (flavan-3- ols or flavanols), phloridzin (dihydrochalcone glycosides), quercetin (flavonols), cyaniding (anthocyanidins), cyanidin-3-O-galactoside (anthocyanins), chlorogenic acid (phenolic acids), and hydroxycinnamates (p-coumaric acid) (Cuthbertson et al., 2012; Vrhovsek et al., 2004). In general, the polyphenolic contents range between 19.6 and 55.8 flavan-3-ols, 17.7–33.1 flavonols, and 10.6–80.3 chlorogenic acid mg per apple; the lowest values were recorded for phloridzin (1.0–9.3 mg per apple) and anthocyanin (0.1–6.5 mg per apple) (McGhie et al., 2005; Francini et al., 2013).

1.1.4 Human health benefits of apple polyphenols

The general role of polyphenols in plant physiology and allelopathy has been revealed a long time ago and researchers still actively study it. These bioactive compounds, which naturally and abundantly are present in fruits, have been rediscovered by nutritionists as advantageous molecules and potentially interesting components for the production of functional food, foods that have a potentially positive effect on health beyond basic nutrition (Francini et al, 2013).

Apples have been identified as one of the main dietary sources of polyphenolic compounds. The health benefits of the polyphenols are due to their large array of biological actions such as antioxidant activity, enzyme modulation ability, effects on cell signaling pathways and on gene expression (Dell'Agli et al., 2005). Indeed, the polyphenols are linked to the ability to prevent free-radical damage that is a major cause of chronic-degenerative diseases (Henríquez et al 2010). It is reported that apple polyphenols reduce Fenton reaction-mediated lipid peroxidation and 2-deoxyribose degradation in a dose-dependent manner (Chaudhary et al., 2006).

Evidence suggests that a diet rich in apples may reduce the risk of diseases. Apples are fruits for which numerous data are available (Manach et al., 2004) and each phenolic compound might have specific health benefits. For example, the non-glycosilated form of phlorizin, phloretin, has been revealed to influence epigenetic processes that play an important role in gene expression regulation in breast cancer cells (Paluszczak et al., 2010). In animal models, apples have been revealed to prevent mammary, skin and colon carcinogenesis. Epidemiological studies indicated that regular consumption of one or more apples per day was associated with a reduction in risk of cancer compared to consumption of less than one apple per day

(Le-Marchand et al, 2000; Boyer et al., 2004; Hyson, 2011; Jedrychowski et al., 2010).

Numerous and significant mechanisms of cancer are affected by apple components, more specifically by oligomeric procyanidins. These effects *in vitro* antimutagenic activity, modulation of carcinogen metabolism, antioxidant activity, anti-inflammatory mechanisms, modulation of signal transduction pathways, anti-proliferative, and apoptosis-inducing activity, as well as novel mechanisms on epigenetic events and innate immunity (Gerhauser, 2008; Francini et al, 2013).

Researchers suggested an interesting role for apple polyphenols related to glucose control in diabetes (Marks et al 2009). Phloretin-O-glycosides, phloretin-2′-O-glucoside and phloretin-2′-O-(2″-O-xylosyl) glucoside inhibit sodium-dependent glucose transporters in the intestinal lumen, this is verified especially for phloretin-2′-O-glucoside. By decreasing the absorption of glucose, phloretin-2′-O-glucoside may reduce post-prandial blood glucose levels, and it is thought this action may be helpful to the treatment of diabetes mellitus (Johnston et al., 2002).

In addition, a neuroprotective and anti-amyloidogenic effect of apple polyphenols has been found. In particular quercetin, a potent antioxidant abundant in apples, showed protective effects against hydrogen peroxide-induced neurodegradation (Biedrzycka and Amarowicz, 2008). Recently some researchers showed that apple polyphenols may have beneficial effects on outcomes related to Alzheimer's disease: procyanidins, major components of the apple polyphenols, significantly suppressed $A\beta_{1-42}$ aggregation and dissociated $A\beta_{1-42}$ aggregates in a dose-dependent manner (Toda et al., 2011). Besides, apple juice may work in cognitive decline of normal aging suppressing over expression of presenilin-1, which is linked to the production of amyloid β-peptide, a marker of Alzheimer's (Chan et al., 2009).

An Italian research group studying the effects of ten weeks of fresh '*Annurca*' apple intake in aged rats found that regular apple consumption in aged rats restored synaptic function to the level of younger animals. In particular, researchers found that activity of superoxide dismutase (SOD), enzyme involved in producing of oxygen radical species, is increased in aged rats fed with the standard diet, whereas SOD activity in the hippocampus of the aged rats fed with *Annurca* apple was at the level of the young animals (Viggiano, 2006).

The consumption of apple fruits, as well apple juice and other derivate products, suggest their potential to affect the health of the populations (Hyson, 2011).

1.2. POLYPHENOLS: classification and molecular structure

Polyphenols are a class of molecules that has significant health properties. In general terminology, they are a large group of natural and synthetic small molecules that are composed of one or more aromatic phenolic rings with one or more hydroxyl groups (Porat. et al., 2006; Dai and Mumper, 2010; Quideau et al 2011). Phenols belong to the family of alcohols and the hydroxyl group of phenol determines its acidity whereas the benzene ring characterizes its basicity (Rappoport, 2003).

Synthetic polyphenols are commonly used as pH indicators in cell culture media and synthetic food additives (e.g., phenolsulfonphthaleine, Butylated hydroxylanisole (BHA), and Butylated hydroxytoluene (BHT)) (Porat et al 2006).

Natural polyphenols arise from plant secondary metabolism, in particular from the shikimate-derived phenylpropanoid and/or polyketide pathways (Francini and Sebastiani et al., 2013).

They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins (Dai and Mumper, 2010). Polyphenols have ability to serve multiple functions in plant–environment interactions. They act as defence against herbivores, microbes, viruses or competing plants, and signal compounds to attract pollinating or seed dispersing animals, as well as protecting the plant from ultraviolet radiation and oxidants. Moreover, plants use them in defence against reactive oxygen species produced during photosynthesis. Antioxidant activity of polyphenols is determined by their reactivity as a hydrogen- or electron-donating species, which relates to their reduction potential (Biedrzycka and Amarowicz, 2008).

Polyphenols are usually divided on the basis of the number of carbon atoms in conjunction with the structure of the basic phenolic skeleton. Plant phenols include phenolic acids, flavonoids, tannins (Figure 3) and the less common stilbenes and lignans (Figure 4) (Dai and Mumper, 2010).

***Flavonoids*:** they are the most abundant polyphenols in our diets. The basic flavonoid structure is the flavan nucleus, containing 15 carbon atoms arranged in three rings (C6-C3-C6), which are labeled as A, B and C. Flavonoid are themselves divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central C ring. Their structural distinction in each subgroup is partly due to the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation (Dai and Mumper, 2010). The most important flavonoids in food are mainly catechins, proanthocyanins,

anthocyanidins and flavons, flavonols and their glycosides (Chi-Tang Ho et al., 1991).

Phenolic acids: they can be divided into two subclasses, hydroxybenzoic acids derivatives of benzoic acid, and hydroxycinnamic acids derivatives of cinnamic acid. The hydroxybenzoic acids are present very infrequently in edible plants; this is the reason why they do not acquire a great nutritional interest. One of the polyphenols representative of this group is gallic acid. The hydroxycinnamic acids are contained in cereals, and in some types of fruit, including apples. They are found in all parts of the fruit, but the highest concentration was measured on the peel (Antolovich M et al., 2002). Among the hydroxycinnamic acids, the most common is caffeic acid, very often esterified with quinic acid as in chlorogenic acid.

***Tannins*:** they are another major group of polyphenols in our diets and are usually subdivided into two subgroups: hydrolysable tannins and condensed tannins. Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins. The great variety in the structure of these compounds is due to the many possibilities in forming oxidative linkage. Intermolecular oxidation reactions give rise to many oligomeric compounds having a molecular weight between 2,000 and 5,000 Daltons (Khanbabaee et al., 2001). Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond. They are also referred to as roanthocyanidins because they are decomposed to anthocyanidins through acid-catalyzed oxidation reaction upon heating in acidic alcohol solutions.

Stilbenes: stilbenes are not very present in the diet; the most representative of this class is resveratrol, present in more than 70 species of plants, such as grapes, berries

and peanuts. The red grape skin is particularly rich in resveratrol; this contributes to the high concentration of this compound in red wine and in grape juice (Antolovich et al., 2002).

***Lignans*:** lignans are constituted by two phenylpropane units. The main sources of this class of polyphenols are flax seeds. Interest in lignans and their synthetic derivatives is growing because of the potential applications in chemotherapy (Antolovich et al., 2002).

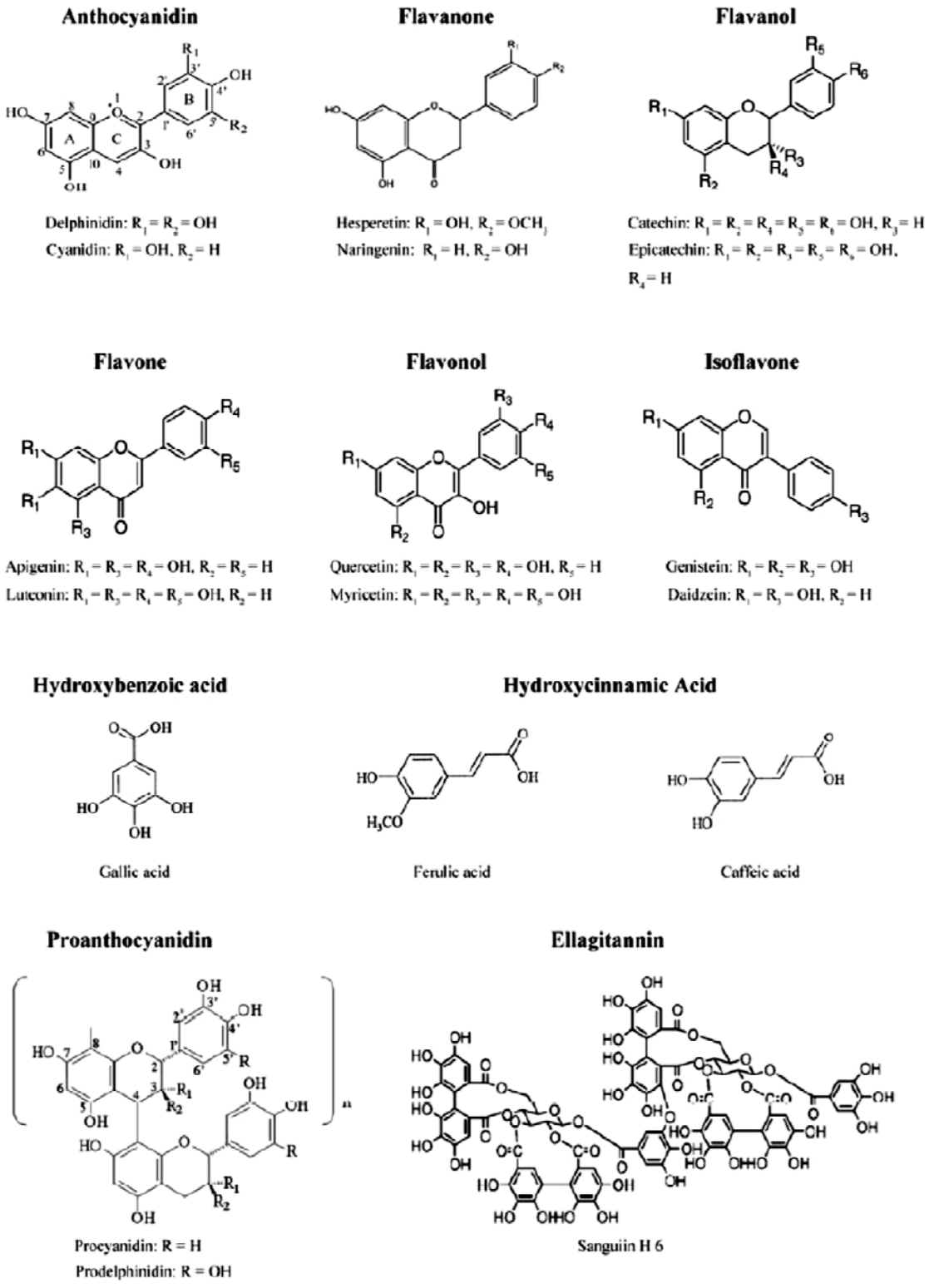


Fig. 3 Molecular structures of flavonoids, phenolic acids and tannins (Dai and Mumper., 2010)

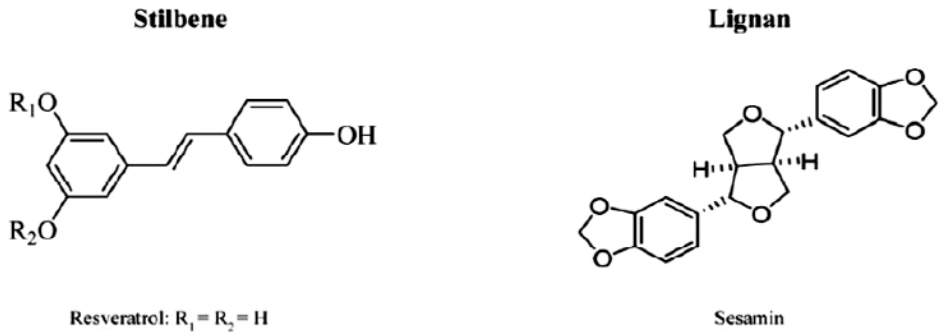


Fig. 4 Molecular structures of Stilbene and Lignan (Dai and Mumper, 2010)

The structural diversity is a result of the difference in hydroxylation pattern, stereochemistry at the three chiral centers, and the location and type of interflavan linkage, as well as the degree and pattern of methoxylation, glycosylation and galloylation (Koleckar et al., 2008; Dai and Mumper, 2010). The antioxidant action of polyphenols is of the type chain-breaking: they react with the radicals giving them a hydrogen radical and forming phenolossil radical that is less reactive thank to delocalization of the elettron on the aromatic ring.

1.2.1 Oxidative Stress and Cytoprotection

The causes of the danger of oxygen remained unknown until the publication of the free radical theory of Gershman (Gerschman et al., 1954), who attributed the toxicity of oxygen to its reduced forms. Reactive oxygen species (ROS), nitrogen (RNS) and chlorine species, are products of normal cell metabolism and play a dual role, beneficial and deleterious, depending on their concentration and localization in the organism. The positive effects of ROS are observed at low to moderate

concentrations: these species are also involved in the defense against infectious agents and in the transduction of cellular signals (Valko et al., 2007).

The damaging effects of reactive species are observed when they are involved in the "oxidative stress": a change in the delicate balance oxidants/antioxidants, which occurs when there is an overproduction of ROS or a deficiency of antioxidants. The concentration of ROS, normally generated by cellular metabolism, may be increased by external factors: ionizing radiation, pollutants, metal ions and barbiturates may directly or indirectly generate reactive oxygen species in cells (Valko et al., 2006).

The superoxide anion $O_2^{\bullet -}$ is considered the ROS "primary", which can generate ROS "secondary" by reaction with other molecules (Valko et al., 2007). The major sources of superoxide anion are the chains of electron transport which can promote the transfer of an electron to molecular oxygen.

$$O_2 + e^- \rightarrow O_2^{\bullet -}$$

The ROS secondary can then be generated for subsequent reactions when the enzyme superoxide dismutase (SOD) catalyzes the reduction of the superoxide anion to hydrogen peroxide. The SOD operates in conjugation with the enzymes catalase (CAT) and glutathione peroxidase (GSH-P_X) which proceed to the reduction of hydrogen peroxide to water and oxygen (Valko et al., 2007).

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 + 2GSH \xrightarrow{GSH\text{-}P_X} 2H_2O + GSSG$$

$$H_2O_2 \xrightarrow{CAT} H_2O + \tfrac{1}{2} O_2$$

Under conditions of stress, the presence of the superoxide anion can induce the release of ions of iron (II) by certain enzymes; such ions can participate in the Fenton reaction with hydrogen peroxide, producing the hydroxy radical $OH^{\bullet}$. The superoxide anion also acts by reducing the Fe^{3+} and Fe^{2+} making available again for the Fenton reaction. It is favored thus further production of $OH^{\bullet}$, through a chain mechanism (Valko et al., 2007).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

$$Fe^{3+} + O^{2\bullet -} \rightarrow Fe^{2+} + O_2$$

The radical $OH^{\bullet}$ is very responsive, when it is formed, it reacts instantly causing substantial damage to proteins, lipids and DNA. In proteins, for example, the oxidation of cysteine residues can lead to the formation of disulfide bonds which cause an alteration in the tertiary structure of the protein with the consequent inhibition of its biological function (Valko et al., 2007).

Cells growing aerobically are exposed to reactive oxygen species generated during metabolism, such as hydrogen peroxide, the hydroxyl radical and the superoxide anion. Although reactive oxygen species are formed during normal cellular functioning, high concentrations result in a period of oxidative stress and they can damage proteins, lipids, carbohydrates, and DNA. In respiring cells, the primary source of ROS is leakage of electrons from the mitochondrial respiratory chain: under physiological conditions, 1 to 3% of oxygen molecules present in the mitochondria are converted into superoxide, during transfer electronic respiratory chain. Another organelle in which you can have ROS production is the endoplasmic reticulum, where the cytochrome P-450 catalyzing the oxidation of xenobiotic agents

in order to transform them into less toxic molecules, using oxygen as the oxidizing agent. Finally, in the membranes the NADPH oxidase catalyzes the oxidation of NADH or NADPH with simultaneous reduction of oxygen (Valko et al., 2004). Besides, ROS have been implicated in a caspase independent mechanism activating apoptosis (Fleury et al., 2002) and they can lead to cell death.

Oxidative stress has been associated with a number of pathologies including cancer, cardiovascular disease, Down's syndrome, Friedreich's ataxia, aging, and age-related diseases (Costa et al., 2001; Busciglio et al., 1995). However, cells fight oxidative stress by using a variety of defenses including cell-cycle delay, the induction of enzymatic antioxidants such as catalases, peroxidases, and superoxide dismutases, and the synthesis of non-enzymatic antioxidants such as glutathione, vitamins C and E, and ubiquinol (Thorpe et al., 2004). Their actions limit the potential damage caused by reactive oxygen species. Antioxidants maintain homeostasis and reduce the deleterious effects providing protection to the cells (Cordier et al., 2013).

The process by which bioactive compounds provide protection to cells against harmful agents is defined "cytoprotection". Resveratrol, a polyphenolic compound found in mulberries, grapes and red wine has been demonstrated to be capable of protecting against oxidative cardiovascular pathophysiology (Yunbo Lia et al 2006). Other authors reported that a extract containing polyphenols, obtained from Annurca apple, prevents damage to human gastric epithelial cells *in vitro* and to rat gastric mucosa *in vivo* (Graziani et al. 2005). Recently Carrasco-Pozo and colleagues showed that apple polyphenols have ability of free radical scavenging and protect Caco-2 cells against indometacin-induced oxidative stress, mitochondrial damage and cytotoxicity (Carrasco-Pozo et al., 2010).

1.2.2 Mechanisms of action of the polyphenols

In general, antioxidants are defined as "any substance that is present in low concentration compared to an oxidizable substrate, able to slow down or inhibit oxidation of that substrate".

The antioxidants are classificated according their orgin in endogenous (already present in the body) and exogenous (introduct through the diet) antioxidants and according the structural characteristics such as enzymatic (eg. superoxide dismutase, glutathione peroxidase, catalysis) and non- enzymatic systems (eg. seleno-proteins, glutathione). A second type of classification can be carried out in primary and secondary antioxidants, considering their mode of action. The first ones preventatively restrict the oxidizing action of reactive oxygen species favoring the transformation of ROS in non-reactive species, chelating transition metals or regenerating (eg. enzyme systems) and bringing them back into their active forms (eg. glutathione, vitamin C). The second ones are “chain-breaking antioxidants” and have structures which allow them to block free radicals, transforming into radicals less reactive than those of departure (eg. polyphenols).

Recently, phenolic compounds have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidants than Vitamin C and E and carotenoids (Dai and Mumper, 2010). The polyphenols are defined as “exogenous antioxidant” because they are introducted in the body through the diet, mainly eating vegetables and fruit. It has been proposed that the antioxidant action of phenolic compounds can be mediated by the following mechanisms: 1) scavenging radical species such as ROS/RNS; 2) suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production; 3) upregulating or protecting antioxidant defense (Cotelle, 2001).

According the first mechanism, the polyphenols act as free radical acceptors and "chain-breakers" because they have a particular chemical structure: 1) a phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical; 2) an extended conjugated aromatic system to delocalize an unpaired electron. These characteristics allow them to trap chain-propagating, oxygen-centered free radicals, according following reaction:

$$ROO^{\cdot} + POH \rightarrow ROOH + PO^{\cdot}$$

$$PO^{\cdot} + R^{\cdot} \rightarrow POR$$

Polypnenols (POH) give a hydrogen atom from a phenolic hydroxyl group to peroxyl radicals ($ROO^{\cdot}$), converting them to hydroperoxides (ROOH). The phenoxy radical intermediates ($PO^{\cdot}$) are relatively stable due to resonance and therefore a new chain reaction is not easily initiated. Moreover, the phenoxy radical intermediates also act as terminators of propagation route by reacting with other free radicals (Rappoport, 2003; Dai and Mumper, 2010).

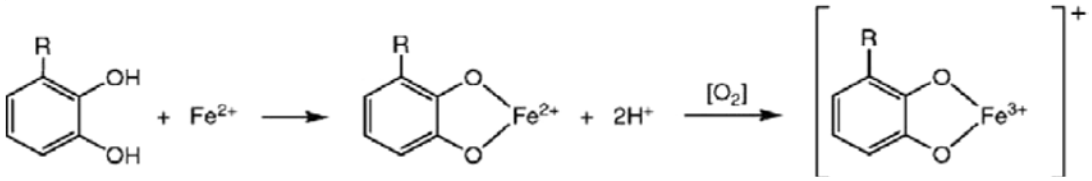
More shifted towards the second member is the reaction, higher is the ability of the polyphenol to yield a $H^{\cdot}$. This ability is linked closely to chemical structure and can be quantified through:

- the 'homolytic' dissociation energy of the O-H bond: the tendency to donate $H^{\cdot}$ increases with decreasing bond dissociation energy, which depends on the type and amount of substituents present on the molecule (e.g. the electro-attractor substituents favor the dissociation of bond);
- the position of the O-H bond: it is necessary a good mobility of the radical towards the OH group to occur the reaction between the two components;

- the stability of the phenoxy radical derived from antioxidants: unpaired electron is delocalized on the aromatic ring and this is influenced by the substituents on the ring because electron-withdrawing substituents tend to attract the unpaired electron and increase delocalization (Laguerre et al., 2007).

Another mechanism of action of the polyphenols is that to suppress ROS formation by chelating trace metals involved in free radical production. Indeed, some phenolic compounds with dihydroxy groups can conjugate transition metals, preventing metal-induced free radical formation. The redox active metal ions such as Cu^{+} or Fe^{2+} interact with hydrogen peroxide through Fenton chemistry to form hydroxyl radicals, which is the most reactive ROS known, being able to start free radical chain reactions by abstracting hydrogen from almost any molecule.

Polyphenols with catecholate and gallate groups can inhibit metal-induced oxygen radical formation either by coordination with Fe^{2+} and enhancing autoxidation of Fe^{2+} (as show in reaction below), or the formation of inactive complex with Cu^{2+}, Fe^{2+}, or Cu^{+} with relatively weaker interaction (Yoshino et al., 1998; Perron et al., 2009).



Yet, the benefit mechanisms of action of the polyphenols are mostly attributed to their antioxidative properties but many other mechanisms seem to be involves in

their molecular mode of function (Porat et al., 2006). For example, polyphenols upregulat or protect antioxidant defense (Cotelle, 2001).

Polyphenols, such as quercetin, are efficient inhibitors of sulfotransferases and may change the activity of thyroid hormones, steroids, and catecholamines (Otake et al., 2000; Marchetti et al., 2001; Coughtrie et al.,1998). Some authors demonstrate that epicatechin gallate and epigallocatechin gallate not only inhibit the free radical chain reaction of cell membrane lipids, but also inhibit mutagenicity and DNA damaging activity (Mou-Tuan Huang et al., 1992).

Polyphenols such as Curcumin, Exifone, and Myricetin exhibit modest inhibition toward fibril formation of tau peptide, which is associated with Alzheimer's disease, inducing conformational changes in the oligomer aggregate (Berhanu et al., 2015).

The different mechanisms of action that polyphenols exercise make them optimal candidates such as potential therapeutic agents in various human diseases, especially for the treatment of amyloid-associated diseases due to multi-factorial causes.

1.3. AMYLOID PROTEINS IN NEURODEGENERATIVE DISEASES

Neurodegenerative diseases affect millions of people worldwide. The most known diseases are Alzheimer, Parkinson, Huntington, Amyotrophic Lateral Sclerosis and Prion Diseases. In each pathology respectively a specific protein, β-amyloid, α-synuclein, polyglutamine superoxide dismutase, prion proteins is involved. The proteins involved in the different neurodegenerative diseases are extremely heterogeneous in terms of sequence and ⁄or 3D structure. Nevertheless, all the diseases are characterized by common and molecular mechanisms involving protein aggregation and inclusion body formation. The aggregates at the end of the

aggregation process consist of filaments, named amyloid fibrils. All amyloid fibrils have a characteristic, elongated fibrillar morphology with a diameter of 5 – 15 nm, a β-sheet-rich structure, and typical X-ray fiber diffraction with a reflection of 4.6 – 4.8 Å in the meridian (Porat, 2006; Ross and Poirier, 2004).

To aggregate are partially folded intermediate conformations that mutually associate entering the off-folding pathway. Their association is mediated by β-strand motifs, and stacking of these β-strands is the basis of fibril nucleation and elongation. Since the β-strand is a physiochemical property of the polypeptide backbone, rather than of the type of amino acids, it has been proposed that the amyloid fibril state is a generic conformation accessible to all polypeptide chains, given the appropriate conditions (Ecroyd et al., 2010). The native structure of the protein precursors is often a well-folded structure (e.g. lysozyme or transthyretin). However, many of them, like Aβ peptide in Alzheimer Disease, belong to the class of intrinsically disordered proteins (IDPs) (Ecroyd et al., 2008). The attitude of a protein to abandon its normal folding and go towards the formation of aggregates is linked to some chemical-physical parameters, such as the hydrophobicity, the net charge and the probability of forming β-strand (Uversky et al., 2009). High hydrophobicity and low net charge, together to the probability of forming β-strand, are characteristics often common to amyloid proteins.

Another aspect common to the different diseases is the specific manner by which amyloid fibrils form starting from their precursors. The process is a typical nucleation-polymerization process, with a lag phase, not entropically favored, during which initial nuclei form, and an exponential elongation phase, during which other molecules bind the initial nuclei. Each phase is characterized by specific structural conformations with peculiar morphology and toxicity potential. In fact, it has been

shown that, rather than amyloid fibrils, the more toxic species are prefibrillar oligomers that form at the beginning of the aggregation process of the proteins. They expose larger hydrophobic surfaces that strongly interact with cell membranes causing oxidative stress and variations in calcium homeostasis leading to cell necrosis or apoptosis.

The high incidence of neurodegenerative diseases provides a major push to the research of new drug therapies. To date, considerable advances in neurobiological research have occurred, but this progress is far from the design of new drugs to the central nervous system, since that the brain is an organ in which it is difficult to achieve effective therapeutic concentrations due to the almost impermeable blood-brain barrier. The criterion followed for the treatment of neurodegenerative diseases is often the polypharmacy, although the treatments approved for human use only produce a modest relief of cognitive and behavioral symptoms of patients (Rosenberg, 2005). The new trend in therapy is therefore so-called "disease modifying therapies", i.e. therapies that might slow or halt the progression of the disease by interrupting the pathophysiological processes that underlie it, such as the production and formation of neurotoxic aggregates. Therapeutic approaches to intervene in amyloid diseases follow four basic strategies that tend to:

1) block the production of amyloidogenic peptide or protein;

2) inhibit its "misfolding" or transformation from one state monomeric / oligomeric non pathogenic to one oligomeric / polymeric toxic;

3) reverse the toxic effects of amyloid structures;

4) modulate cellular pathways that promote alternative one or several of the previous approaches.

Polyphenols constitute a group of molecules that may exert antiplatelet activity against misfolded proteins by acting by all the strategies mentioned above. In this regard, the polyphenols can be taken into account for a therapeutic approach in neurodegenerative diseases field.

1.3.2 Κ-Casein: a model fibril-forming protein

Although unrelated to specific diseases, many proteins have been demonstrated to form amyloid fibrils, suggesting that any protein can take on the amyloid conformation under specific conditions (Ecroyd et al., 2008). A protein's likelihood of forming β-strands, its hydrophobicity and its overall net charge can predict its propensity to aggregate rather than fold normally. One protein not associated with specific disease and that is susceptible *in vitro* to fibril formation is κ-casein, a bovine milk protein. Amyloid fibrils of κ-casein are observed *in vitro*, but *in vivo* aggregation is prevented from the chaperone activity of the other two caseins, β- and αs1-casein, which are also naturally present in bovine milk (Leonil et al., 2008). When the chaperone-system fails, κ-casein forms corpora amylacea in the bovine mammary gland and in milk**.**

On the basis of chemical and structural characteristics, caseins are defined as reomorfic because may adapt the conformation to the conditions of the surrounding environment (Holt and Sawyer, 1993). In this sense, they are considered as belonging to the class of intrinsically disordered proteins (IDPs) (Tompa, 2002). The primary structure of the caseins is typical of IDPs due to the presence of a high number of amino acid residues proline, which prevents the protein to be folded to form more ordered structures. In particular, κ-casein is characterized from poor secondary and tertiary structures and a high percentage of disordered regions under physiological

conditions (Leonil et al., 2008). It has a structure with hydrophobic residues in the N-terminal region and a non-structured C-terminal tail (Horne, 1998).

As mentioned before, κ-casein forms *in vitro* fibrils that display several characteristics of amyloid fibrils involved in diseases. Indeed many authors reported that both the "native" and "reduce" and "carboxymetilated" κ-casein aggregate and form amyloid fibrils at 37 °C (Farrell et al., 2003; McSweeney. & Fox 2013; Chun et al., 2012). The molecular mechanism underlying the reduced κ-casein amyloid fibrillogenesis has been recently hypothesized on the basis of indirect experimental procedures. The fibrillogenesis profile of κ-casein has not the lag phase typical of nucleation-polymerization process, and, according to Ecroyd et al., this would be symptomatic that the rate limiting step involved in this protein aggregation is not the nucleation of a nucleation-polymerization process (Ecroyd et al., 2008). The rate limiting step in the case of κ-casein amyloid aggregation would be the dissociation from the oligomeric initial state of an amyloidogenic monomeric precursor provided with the proper structural features leading to the ordered fibrillar aggregation (Ecroyd et al., 2010) (Fig.5). In fact, the κ-casein collapsed monomeric conformation, arranged as a "horse a rider" structure (Kumosinski et al., 1993) (Fig. 6) presents, in correspondence of horse legs, a tyrosine rich sheet-turn-sheet hydrophobic motif that, during oligomer dissociation, would be exposed to the solvent and would lead to beta sheet stacking resulting in a ordered aggregation. A single κ-casein monomer would contribute to more than one β-strand in the final fibrillar structure. The hydrophobic region is incorporated into the core of the fibrils formed by κ-casein and this is a unique feature that would determine its high aggregation propensity. The aggregation mechanism in the case of native protein would be similar, even if reduced in extinction due to the presence of disulfide

bridges (Ecroyd et al., 2010, Thorn et al., 2005). The double β-strand structural fold formed by κ-casein would have the same biophysical features of the amyloid core formed by Aβ peptide in Alzheimer Disease (Ecroyd et al., 2010).

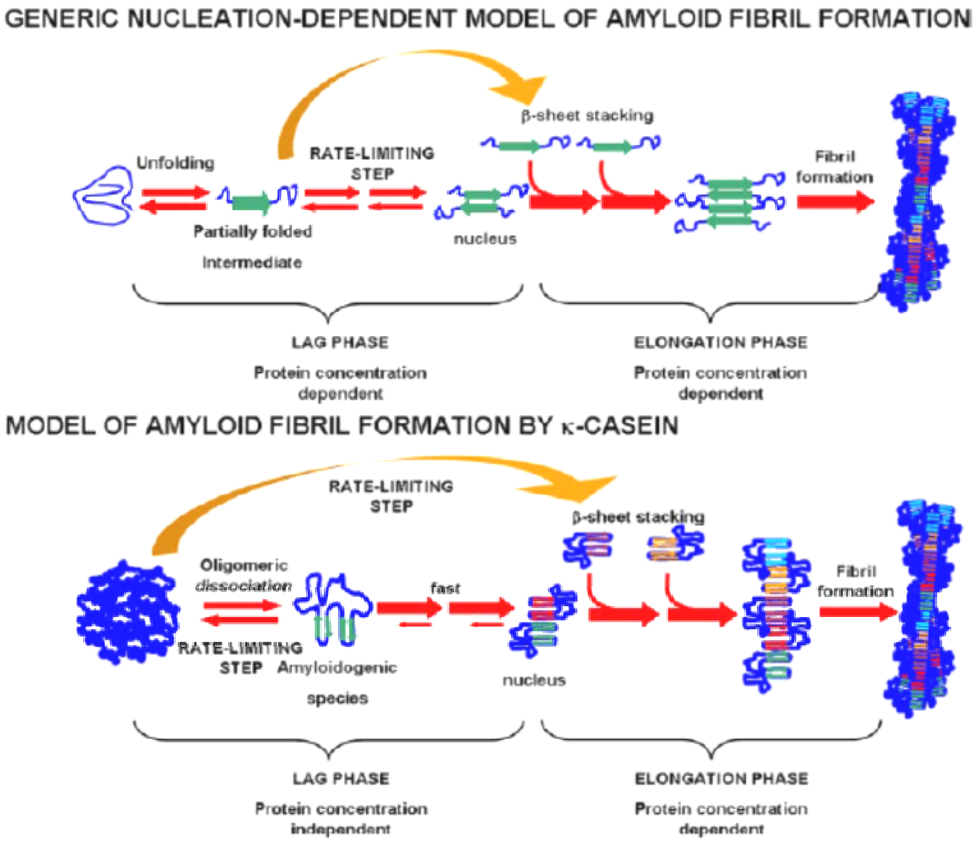


Fig. 6 Fibril formation mechanisms for β- amyloid and k-casein. Differences can be seen in the lag phases and similarities in the elongation phases (Ecroyd et al., 2008).

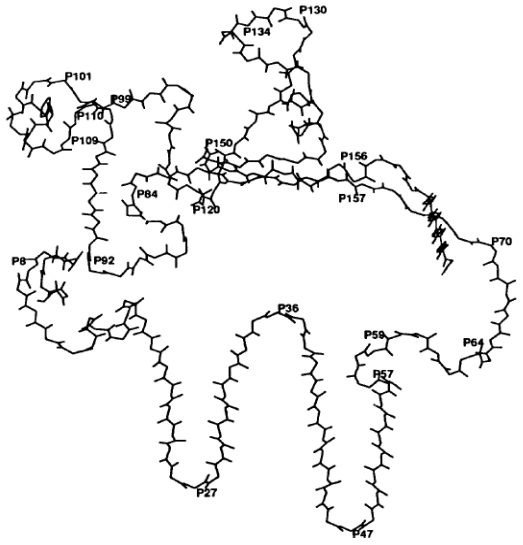


Fig. 6 Backbone of κ-casein without side chains (Kumosinski et al., 1993)

Also native κ-casein, with no reduction, is able to form amyloid fibrils. The presence of the two Cys residues, Cys11 and Cys88, creates a complex disulfide bonding pattern between κ-casein molecules bovine milk (Swaisgood et al., 1964; Farrell et al., 2003; Farrell et al.,1996). In the absence of reducing agents, κ-casein distinct polymers ranging from monomers (10%) to octamers have been detected (Farrell et al.,1996).

All of these heterogeneous polymers, however, further associate by means of electrostatic, hydrogen bonds, hydrophobic interactions, thus forming multimeric colloidal systems in native environments. During amyloid formation native κ-casein probably undergoes multimeric dissociation toward lower molecular weight disulfide linked species (Farrell et al., 1996). These species, enhancing the amount of amyloidogenic hydrophobic regions exposed to the solvent, further aggregate and cause κ-casein ordered aggregation even in the absence of reducing agents.

1.3.1 Interaction between polyphenols and amyloid proteins

Currently, there is no approved therapeutic strategy based on safe compounds directed towards the formation of oligomeric toxic assemblies, which have been recently shown to have a key role in the pathological nature of amyloidogenic proteins. One important approach in the development of therapeutic agents is the use of small molecules that specifically and efficiently inhibit the aggregation process. Very interestingly, in addition to their effectiveness in fighting oxidative stress as amyloid toxic effect, several small polyphenol molecules have been demonstrated to remarkably inhibit, by direct interactions, the formation of amyloid assemblies *in vitro* and their associated cytotoxicity. The therapeutic potentiality of polyphenols in direct interactions with amyloidogenic species is strongly based on their structural similarities. This mechanism is assuming structural constraints and specific aromatic interactions, which direct polyphenol inhibitors to the amyloidogenic core (Porat et al., 2006).

Armstrong et al. (2011) have suggested that the planar structure of phenolic compounds could contribute to their effectiveness as inhibiting aggregation by allowing them to intercalate between the monomer layers. The H-bonding analysis of Curcumin (the most flexible ligand with few hydroxyl groups) indicates that ligand flexibility and number of strong H-bond acceptors play important roles in interaction of the ligand with the aggregates (Berhanu et al., 2015). Polyphenols induce conformational changes in the oligomer aggregates and these changes disrupt the amyloid H-bonding, perturbing the aggregates structure. The H-bonding capacity of polyphenols is responsible for the observed behavior (Berhanu et al., 2015). A proper balance between molecular flexibility and number of strong H-bond donor/acceptor

groups could play a role in the designing of more potent polyphenols as aggregation inhibitors (Lemkul and Bevan, 2010).

Jan Bieschke and colleagues show that Epigallocatechina-3-gallato (EGCG) disassembles preformed amyloid fibrils. However, the specific mechanisms underlying this action remained unclear. EGCG has the ability to convert large, mature α-synuclein and β-amyloid fibrils into smaller, amorphous protein aggregates that are nontoxic to mammalian cells. Mechanistic studies revealed that the compound directly binds to β-sheet rich aggregates and mediates the conformational change without their disassembly into monomers or small diffusible oligomers. These findings suggest that EGCG is a potent remodelling agent of mature amyloid fibrils (Bieschke et al 2010).

In addition, other researchers demostrated that polyphenols such as myricetin, morin, quercetin, kaempferol, (+)-catechin and (–)-epicatechin dose-dependently inhibited the formation, extension, and destabilization of β-amyloid fibrils at pH 7.5 and 37 °C *in vitro* (Ono et al., 2003).

1.3.3 Apple polyphenols inhibit neurodegenerative diseases

Several studies reported the ability of different single polyphenols to inhibit both oxidative stress and amyloid aggregation, associated to neurodegenerative diseases. Many of the phenolic molecules, as (+)-catechin, (–)-epicatechin, quercetin, with antioxidant and antiaggregation properties have been found in polyphenolic profile of different apple cultivars (Vrhovsek et al., 2004; Karaman et al. 2012) and this suggests that polyphenols extracted from apples could be used as potential therapeutic agent in neurodegenerative diseases.

About antioxidant properties, many authors reported the ability of apple polyphenols to prevent free-radical damage that is a major cause of chronic-degenerative diseases (Henríquez et al 2010; Biedrzycka and Amarowicz, 2008; Chaudhary et al., 2006; Viggiano 2006; Shay et al., 2014).

However, few studies have been performed in order to investigate the antiaggregation action of apple polyphenols that remains still unclear. Toda and colleagues showed that apple procyanidins significantly suppressed $A\beta_{1-42}$ aggregation and dissociated $A\beta_{1-42}$ aggregates in a dose-dependent manner, indicating that they are a potent suppressor of Aβ aggregation (Toda et al., 2011).

Chan and Shea demonstrated that dietary supplementation with apple juice decreases endogenous amyloid-β levels in murine brain (Chan A. and Shea T.B. 2009). Dietary supplementation with apple juice provides also neuroprotection against ApoE deficiency, Aβ exposure and folate deficiency, moreover attenuates or prevents the overexpression of presenilin-1 implicated in increasing reactive oxygen species in Alzheimer's Disease. This finding highlights the possibility of apple juice to provide neuroprotection by mechanisms in addition to its antioxidant potential (Chan and Shea, 2006).

The combination of antioxidant and antiaggregation properties of apple polyphenols suggests them as potential therapeutic agents for neurodegenereative diseases.

GENERAL OBJECTIVES

2. GENERAL OBJECTIVES

The aim of this work is the study of the amyloid aggregation inhibitory action of an amyloidogenic model protein, κ-casein, exercised by polyphenols extracted from three apple cultivars, ‘Gala’, ‘Granny Smith’ and ‘Fuji’, grown in Sicily.

For sake of clarity, the results presentation in the thesis work has been organized in two different sections, each one referring to a specific aspect analyzed during the research study: quality characterization of the fruits in the first one and the inhibiting effects of their polyphenol extracts in the second one.

In particular, the quality characterization of three apple cultivars (‘Gala’, ‘Granny Smith’ and ‘Fuji’) is fundamental in order to maintain or affirm the product in the global market, characterized by high competitiveness, and also to promote these emergent crops for local production.

Moreover, an important aspect for human health and promotion of apple fruits is the evaluation of their polyphenol extracts ability of inhibiting amyloid aggregation process.

Results from cellular viability assays, performed as preliminary step on NHI-3T3 cell line, have revealed that, differently from polyphenol extracts of whole fruit and flesh, peel extracts, also tested at high concentration, have no toxic effect on cells. Therefore, peel polyphenol extracts have been selected for subsequent studies with κ-casein.

The evaluation of apple peel total polyphenol effects on amyloid aggregation process of a model protein system is the basis in order to considering their potential use as therapeutic agent in neurodegenerative diseases.

CHAPTER 3:

Quality characterization of three apple cultivars, ‘Gala’, ‘Granny Smith’ and ‘Fuji’, grown in Sicily

3. QUALITY CHARACHTERIZATION OF THREE APPLE CULTIVARS, ‘GALA’, ‘GRANNY SMITH’ AND ‘FUJI’, GROWN IN SICILY

3.1 Abstract

Apples are considered the “health fruit”. Indeed they contain high and low percentage of water and sugar respectively, many vitamins, minerals, polyphenols and pectins. Epidemiological studies have shown that the consumption of apples and related products is linked with a lower risk of cardiovascular diseases, lung dysfunctions and cancer. In addition, a neuroprotective and anti-amyloidogenic effect of apple polyphenols has been found *in vitro*. For all these reasons, apple intake is recommended in the daily diet.

The aim of this study was the quality characterization of apple fruits produced in Sicily, exactly at Caltavuturo, in an organic farming system. In particular, the determination of chemical - physical parameters, total polyphenolic content and antioxidant capacity in three apple cultivars: 'Gala', 'Granny Smith' and 'Fuji', was performed. Qualitative fruit measures, as weight, sizes, color, flesh firmness, starch degradation, pH, titratable acidity, soluble solids content and mineral composition were carried out for each cultivar. Total phenol content (TPC) and antioxidant capacity were measured respectively by Folin-Ciocalteu method and ORAC assay on different fruit tissues (peel, flesh and whole fruit) in each cultivar.

The results revealed variability in chemical-physical properties, phenolic content and antioxidant capacity among tissues and cultivars. As already reported, the apple peel shows a higher total phenolic content and a higher antioxidant capacity higher than flesh and whole fruit for all three cultivars studied. Moreover, a high correlation between phenolic content and antioxidant capacity was evidenced.

Experimental evidences show that Gala, Granny Smith and Fuji apples cultivated in Sicily are comparable to other productions and they can represent a considerable alternative to traditional products and a great deal for the fruit market.

3.2 INTRODUCTION

Apples are fleshy fruit characterized by high water content (about 85%) and moderated sugar content (about 10-12% of weight). Apples contain many fibers such as cellulose and pectin that stimulate intestinal transit and nourishing absorption respectively. The scarce presence of sodium and fair content of potassium give apples diuretic property (Aprikian et al 2003).

Besides, apples have been identified as one of the main dietary sources of antioxidants, mainly of polyphenolic compounds derived from secondary plant metabolism. The potential human health benefits of polyphenols are linked to ability to prevent free-radical damage that is a major cause of chronic-degenerative diseases (Henríquez et al 2010). Epidemiological studies have shown that polyphenols play an important role in the prevention of cardiovascular diseases, lung dysfunctions and cancer (Knekt et al, 1996; Eberhardt et al, 2000; Le-Marchand et al, 2000; Xing et al 2001). In addition, a neuroprotective and anti-amyloidogenic effect of apple

polyphenols has been found in vitro (Ono et al 2003; Bastianetto et al 2006; Toda et al., 2011).

Apples are fruits widely produced worldwide. In Italy, apple cultivation is practiced all over areas, but is traditionally concentrated in the mountain and foothill regions such as Trentino Alto Adige with 54% of the national area invested (28,201 ha), followed by Veneto, Emilia Romagna and Piemonte. In the south, the apples are grown mainly in Campania with 3.6% of the national production, while Sicily only records 1.2% (619 ha) (Istat 2010 – 2012). Recently, in Sicily the growing apple area shows an increase (+9.4%), thanks to the availability of new irrigated areas: recent installations have been made in the province of Palermo, on the Madonie hills, in environments characterized by freezing winter and not excessively hot summer (Grassi et al, 2000).

The present study shows the quality characterization of three apple cultivars 'Gala', 'Granny Smith' and 'Fuji' produced in Caltavuturo (Sicily, Italy), in an organic farming system.

3.3 MATERIALS AND METHODS

3.3.1 Plant materials

Gala, Granny Smith and Fuji apples, were harvested in Caltavuturo (Sicily, Italy), in the “Scannale s.r.l.” organic farm, in the period from September to November 2014. Thirty fruits for each cultivar were chosen for analytical procedure.

3.3.2 Physical -Chemical analysis

After harvest, fruits were stored at 4°C before analysis. Weight, longitudinal (LD) and transverse (TD) diameters, peel color and flesh firmness of apples from each

cultivars were detected. The fruits were individually weighted with a precision balance. Longitudinal and transverse diameters were measured with a digital caliper. The flesh firmness was measured on half of fruits with penetrometer with a cylindrical 8 mm head (EFFEGI texture analyser). Chemical parameters detected refer to starch content, titratable acidity (TA), pH, soluble solid content (SSC) and mineral composition. Starch content was determinate with iodine test (iodine solution = 40g KI + 10g I2/ L H2O) and visual rating using the "CTIFL Starch Conversion Chart for Apples" (Planton et al., 1995). Fruit pomace, obtained blending pulp fruits, was used for pH, TA, SSC and mineral composition. TA was performed by titrating an aliquot of 5 ml with a solution of NaOH 0.1 N and expressed in percentage of malic acid. The SSC were measured with digital refractometer (Atago) and reported as °Brix. Ripe Index (RI) was also calculated as the ratio between SSC, expressed in °Brix, and TA, expressed in percentage (%). All parameters were obtained by a mean of 5 measures.

The colorimetric analysis was performed with the Colorimeter CHROMA METER CR-400/410 (Konica Minolta) using standard color spaces L* a* b* (CIELAB) by measuring ten randomly selected apples on two opposite sides of the fruit, blushed side (BS) and shaded side (SS), at the level of the equatorial section.

The parameter L* indicates lightness or brightness (with 0 = black and 100 = white), a* and b* represent the chromatic coordinates (with $-60 \leqslant a^* \leqslant +60$, describing variations in the color from green (-60) to red (+60) and with $-60 \leqslant b^* \leqslant +60$ describing variations in the color from blue (-60) to yellow (+60)). The parameters L*, a* and b*, without manipulation, do not provide an indication of color shade and color appearance (*chroma-aspects*). For this reason numerical values of a* and b* were converted into hue angle ($h° = \tan^{-1}(b^*/a^*)$), indicating color shade, and

chroma ($C^*= (a^*2 + b^*2)^{1/2}$) indicating color saturation or intensity. Chroma represents the hypotenuse of a right triangle created by the union of the points (0, 0), (a*, b*) and (a*, 0), while hue is the angle between hypotenuse and 0° on the axis a*. In color wheel of 360° hue presents 0° = red-purple, 90° = yellow, 180° = bluish-green, and 270° = blue (McGuire, 1992).

3.3.3 Sample preparation for polyphenols extraction

For polyphenolic extraction three different apple tissues, "peel", "flesh" and "whole fruit" (peel+flesh), were chosen from thirty fruits of each cultivar. Peel of each fruit was separated from flesh, pooled and frozen in liquid nitrogen. For "whole sample" the fruits were sliced with an apple divider into 8 pieces and core area. The core area was discarded and two opposite cuts of each fruit were randomly chosen and immediately frozen in liquid nitrogen. Next the frozen samples were ground to fine powder with mill and stored at -80°C until extraction.

3.3.4 Chemicals

Methanol and formic acid for polyphenolic extraction were obtained from Sigma Aldrich. Folin-Ciocalteu reagent and anhydrous sodium carbonate from Sigma Aldrich (Italy) were used for total polyphenol determination. For measuring the Oxidant Radical Assay Capacity (ORAC) were used sodium phosphate buffer, fluorescein and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Sigma Aldrich. (+)-Catechin, epicatechin, gallic acid and chlorogenic acid were purchased from Sigma Aldrich.

3.3.5 Extraction of Pholyphenols

Extraction was carried out as described by Ceymann et al., 2012. Briefly, aliquots of the frozen powder (2.50 g) for each sample were mixed with 50 mL of methanol containing 1% formic acid (v/v) and homogenized for 15 minutes. The polyphenolic extract was centrifuged at 10,000 rpm for 10 minutes. For TPC and ORAC analysis the supernatants were used after filtration through 0.45 µm nylon filters. All extractions were done in triplicate and analyzed.

3.3.6 Total Phenolic Content (TPC) by Folin-Ciocalteu

The analysis of TPC was carried out as described by Ceymann et al. (2012), modifying the percentage of alcohol in final mixture, from 5% to 1%, to avoid precipitate formation and obtain reproducible results, according to Singleton and Rossi (1965), Cicco and Lattanzio (2011) and Cicco et al. (2008).

Briefly, Folin-Ciocalteu method was performed as follows: 200 µL of Folin-Ciocalteu reagent and 2,000 µL of distilled water were automatically pipetted to 200 µL of properly diluted extract. After 1 min, 800 µL sodium carbonate solution 20% and 800 µL distilled water were added, thoroughly mixed and incubated for 30 min at 37 °C. Absorption was measured automatically at 750 nm. Total polyphenol content was calculated with external standard catechin calibration curve and expressed as mg catechin equivalents (CTE) per 100 g fresh weight (FW).

3.3.7 Oxygen Radical Absorbance Capacity (ORAC)

The original method of Cao et al. (1993) was used with slight modification. Fluorescein (3I,6I-dihydroxy-spiro[isobenzofuran-1[3H],9I[9H]-xanthen]-3-one) was chosen as fluorescent probe. When the sample, with antioxidant activity, had

exhausted its capacity to trap peroxyl radicals induced by 2,2I-azobis(2-amidinopropane)dihydrochloride (AAPH) at 37° C, fluorescein became the target of the radicals and lost its fluorescence. The area under the curve (AUC) of fluorescence decay was proportional to the antioxidant capacity of the sample, and a comparative evaluation with Trolox was performed. The final reaction mixture for the assay (200 µL) was prepared as follows: 165 µL of 0.05 µM fluorescein sodium salt in 0.075 M sodium phosphate buffer, pH 7.4, 20 µL of properly diluted sample in 0.075 M sodium phosphate buffer, pH 7.4 or 100 µM Trolox (positive control). The negative control was 0.075 M sodium phosphate buffer, pH 7.4. The fluorescence was read every 1 min at 37°C using a Multiplate Fluoroskan Ascent FL Thermo at 485 nm excitation, 520 nm emission. When stability was reached, the reaction was started with 15 µL of 100 mM AAPH and fluorescence was measured until zero fluorescence was detected.

The ORAC value was calculated according to the following formula: **ORAC (µmol Trolox equivalent/g FW) = $[(A_S - A_B)/ (A_T - A_B)]$ *kah***, where A_S is the AUC of fluorescein in the presence of the sample, A_T is the AUC of the Trolox, A_B is the AUC of the control, ***k*** is the dilution factor, ***a*** is the concentration of Trolox in mmol/ L and ***h*** is the ratio between the liters of extract and the grams of sample used for the extraction.

3.4 RESULTS AND DISCUSSION

Fruit chemistry and maturity parameters

The quality of fruits, regarding the quality index for weight, flesh firmness, peel color, starch degradation rate, pH, titrable acidity, solid soluble content, etc., is important to satisfy the consumers requirements. Indeed it is necessary to regard

physical-chemical parameters before commercialization of fruits (Campeanu et al. 2009).

The three apple cultivars, studied in this work, didn't show significant difference about weight, longitudinal diameter (LD) and transversal diameter (TD), as reported in table 1.

Tab. 1 Apple fruits physical parameters

Cultivar	Weight (g)	LD (mm)	TD (mm)	Flesh Firmness (kg/cm^2)
Gala	157.78 ± 9.15	58.34 ± 3.38	67.38 ± 1.75	3.21 ± 0.32
Granny Smith	158.60 ± 12.06	62.60 ± 1.91	69.26 ± 3.10	4.26 ± 0.42
Fuji	158.76 ± 10.66	60.16 ± 2.96	66.70 ± 1.78	4.46 ± 0.45

The middle weight and diameter values are similar for all three cultivar, conferring them an oval shape (the ratio TD/LD > 1). The flesh firmness was lower in 'Gala' apples than 'Granny Smith' and 'Fuji' apples, which result crisper (Tab.1). Physical parameters, such as weight, diameters and flesh firmness, varied among cultivars but all obtained data for three cultivar were within commercially acceptable ranges as reported by Hampson et al 2003, Thompson-Witrick et al 2014.

Chemical characteristics, reported in Table 2, showed a pH significantly different among the three cultivars. The 'Granny Smith' fruits had the lowest value in comparison with the other cultivars evaluated. The titratable acidity, expressed as percentage malic acid (% of malic acid), was in agreement with pH values obtained, 'Granny Smith' had the highest value, followed by 'Gala' and 'Fuji'. The SSC was significantly different, apple flesh of 'Fuji' had the highest value of °Brix compared

to the other cultivars studied. These results confirm previous data for cv. 'Fuji' which is riper than others, with ripening index of 61.73 (Tab.2) (Kader 1999; Kvikliene et al., 2006).

The conversion of starch to sugar is one of the most important processes that indicate the ripening stage of apples (Szalay et al 2013, Jackson 2003, Watkins 2003). The three apple cultivars had starch index higher than 5, indicating that the most of the stored starch had converted to sugar.

Tab. 2 Apple fruits chemical parameters

Cultivar	pH	TA (% malic acid)	SSC (°Brix)	Starch (1-10)	RI
Gala	3.60±0.10	0.32±0.01	15.16±0.16	7.40±0.54	47.37±0.52
Granny Smith	3.40±0.10	0.56±0.04	16.04±0.16	6.20±0.83	28.64±0.28
Fuji	3.90±0.10	0.30±0.01	18.52±0.30	7.20±0.83	61.73±1.11

The mineral composition of each cultivar is shown in table 3. Their ionic content was characterized mainly regarding K^+, Na^+, NO^{3-}, Ca^{2+} and it was significantly different depending on the cultivar evaluated. Among the three apple cultivars, 'Fuji' had the highest Na^+ e NO^{3-} content, 'Granny Smith' had the highest K^+ content and 'Gala' had the highest Ca^{2+} content. The values of K^+, Na^+, NO^{3-}, Ca^{2+} defer from the dates reported by Henríquez C. et al. (2010). These differences could be explained by various growing area, climatic temperature and methodology used to determine the mineral composition.

Tab. 3 Apple fruits mineral composition: values expressed in mg /100 gr FW ± standard deviation				
Cultivar	K^+	Na^+	NO^{3-}	Ca^{2+}
Gala	80.40 ± 2.60	1.40 ± 0.10	8.52 ± 0.75	1.24 ± 0.08
Granny Smith	104.66 ± 3.05	2.13 ± 0.11	8.80 ± 0.52	0.73 ± 0.11
Fuji	98.00 ± 0.10	4.40 ± 0.10	10.60 ± 0.90	0.86 ± 0.11

Peel color

Color directly affects the appearance and the consumer acceptability of fruit. The three cultivars, 'Gala', 'Granny Smith' and 'Fuji', the most marketed apple varieties, showed significantly different coloration in peel. In particular, the cultivar 'Gala' belongs to group of "red apples", while 'Granny Smith' belong to group of "green apples" and 'Fuji' is defined "bicolor" (Drogoudi et al., 2008). The color parameters evaluated (L*, a* and b*) in the Sicilian apples showed, in general, lower values of L* and b* and higher values of a* for blushed side (BS) compared with shaded side (SS) values (Tab. 4).

'Gala' peel was redder on BS (with lower L* and b* values and higher a* value) and with yellow patches on SS (with higher L* and b* values and lower a* value), showing bicolored in agreement with Iglesias et al. (2008). 'Granny Smith' peel was green (negative a* values), lighter (higher L* values) and with b* indicating a slight yellow color mainly on SS. 'Fuji' peel color was either intermediate or similar to red or green cultivars. Indeed, peel color was red on BS (with intermediate L*, a* and b* values) and green/yellow patches on SS (with higher L* and b* values and negative a* value), typical of bicolored cultivars. These results are in agreement with Drogoudi et al., (2008) and Henríquez et al. (2010).

Tab. 4 Color parameters L*, a* and b*(CIELab color space)						
Cultivar	Blushed Side (BS)			Shaded Side (SS)		
	L*	a*	b*	L*	a*	b*
Gala	49.14 ± 6.74	35.22 ± 7.15	22.71 ± 2.91	61.71 ± 8.04	23.30 ± 6.45	27.91 ± 5.49
Granny Smith	64.43 ± 2.58	-11.77 ± 14.50	44.34 ± 0.98	68.62 ± 2.05	-16.73 ± 4.54	44.49 ± 2.15
Fuji	55.04 ± 3.87	19.69 ± 6.00	27.50 ± 7.48	70.64 ± 3.70	-4.41 ± 5.54	40.29 ± 4.89

The parameters L*, a* and b* were manipulated to obtain Chroma C* and hue angle *h*° indicating intensity and color shade respectively, that are intuitively understood by those in the marketing chain from producer to consumer. The mean values of C* and *h*° corresponding to BS and SS are shown in table 5. The cultivar 'Gala' had higher value for Chroma and lower value for hue angle on BS indicating higher coloration and brighter red, on the contrary for other side. 'Granny Smith' and 'Fuji' are characterized by lower values for Chroma and higher values for hue angle, but the first showed similar values for both sides indicating homogenous color, instead 'Fuji' had very different values for BS and SS indicating different coloration. Similar values of L*, a*, b*, C* and *h*° were reported by Henríquez et al. 2010.

Tab. 5 Color parameters C* and *h*°				
Cultivar	Blushed Side (BS)		Shaded Side (SS)	
	Chroma C*	Hue angle *h*°	Chroma C*	Hue angle *h*°
Gala	42.28 ± 4.45	32.67 ± 0.15	37.11 ± 1.65	49.84 ± 0.22
Granny Smith	47.64 ± 2.32	139.9 ± 1.12	47.69 ± 2.73	110.67 ±0.09
Fuji	34.52 ± 5.71	53.85 ± 0.22	40.79 ± 5.23	131.87 ± 1.32

The different color among the three cultivars could be explained from different content of nutrients in peel as suggested by Drogoudi et al (2008). The more nourished peel may be darker, redder and bluer in color. Besides, the difference of coloration between two sides of fruit could be attribute to different flavonols content because their biosynthesis is stimulated by light, indeed marked differences in flavonols concentration exist between sides of a single piece of fruit, depending on exposure to sunlight (Price et al., 1995; Manach et al., 2004).

Total polyphenolic content

The average content of total polyphenols was evaluated in different tissues (peel, flesh and whole fruit) of each cultivar (Gala, Granny Smith and Fuji), with significant differences depending on the apple tissue and variety (Fig. 7).

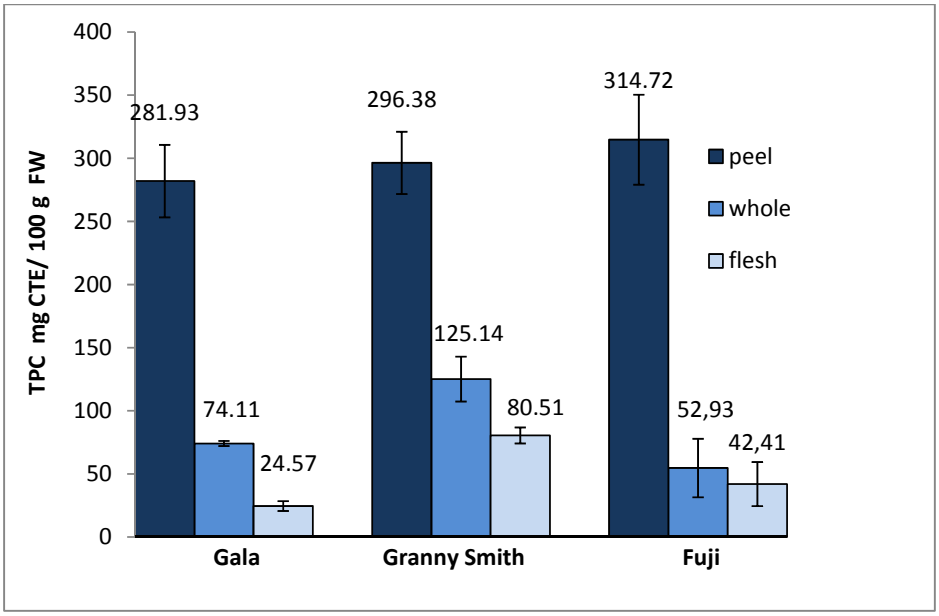


Fig.7 Total phenolic content (TPC) (data given in mg Catechin Equivalents / 100 g of FW) of peel, whole and flesh fruit of three different cultivars: Gala, Granny Smith and Fuji.

Results show higher polyphenolic content in apple peel, such as reported in literature (Carbone et al. 2011; Drogoudi et al., 2008), followed by whole fruit and flesh of all evaluated cultivar. The polyphenolic content results significantly different among analyzed tissues but it is not among cultivars. Whole fruit, in all three cultivars, was characterized by intermediate polyphenolic content between peel and flesh. In Fuji the whole apple had the lowest value of polyphenols than the other cultivars. While Gala had intermediate value and Granny Smith higher polyphenolic content, as likewise reported by Vrhovsek et al. (2004). The high phenolic content of whole fruit in the cultivar Granny Smith could be linked to high phenolic content of the flesh. Indeed flesh is the tissue with the lowest polyphenolic content and, in particular, in the cultivar Granny Smith flesh presents a considerable high content of polyphenols unlike Gala and Fuji, in agreement with data reported by Henríquez et al. 2010.

These results are in accordance with previous reports, which have shown that apple peel has a higher total phenols content compared to other edible parts of this fruit (Carbone et al. 2011;-Drogoudi et al., 2008; Vieira et al., 2009, Francini et al., 2013). In agreement with our data, different researches reported that depending on cultivar, apple peel contains about two to nine times more polyphenols than flesh (Hassimotto et al., 2005; Drogoudi et al., 2008).

Antioxidant capacity

The antioxidant capacity of the three tissues (peel, whole and flesh) for each cultivar is reported in table 6. Results show higher antioxidant capacity for peel tissue compare to whole and flesh. The highest antioxidant activity is given by Fuji for peel tissue and by Granny Smith for flesh tissue. These results are in agreement with TPC's data reported above. In particular there is a linear correlation between total

phenolic content and antioxidant capacity (Fig. 8). Besides, the ORAC values show the same range of values reported by Giomaro et al. (2013).

Tab. 6 Antioxidant capacity of peel, whole and flesh: values exspressed in µmol of Trolox equivalent / g DW ± standard deviation

Tissues	Cultivar		
	Gala	Granny Smith	Fuji
Peel	83.93 ± 16.56	93.56 ± 13.36	118.02 ± 4.99
Whole	29.06 ± 3.16	46.47 ± 3.60	21.72 ± 6.95
Flesh	9.71 ± 2.53	32.40 ± 6.98	12.77 ± 5.47

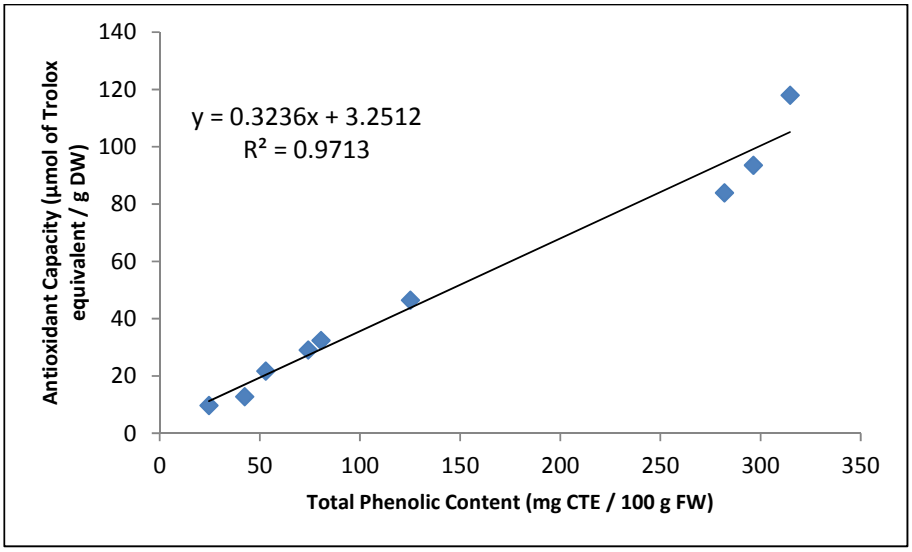


Fig. 8 Linear correlation between total phenolic content and antioxidant capacity

The difference in phenolic content and antioxidant capacity among cultivars is certainly linked at different polyphenolic pattern of the fruits (Vrhovsek et al. 2004; Karaman et al., 2012). It would certainly be interesting to know the profile polyphenolic tissue of these apples. This surely will be the next step of our studies.

Our data further confirm the fact that regular consumption of the apple with the peel in a diet can contribute an important amount of polyphenolic antioxidants. For this reason it is very interesting to produce apple fruit by an organic farming system. Moreover, the reuse of the product "peel" considered usually reject product should be promoted for foodstuff business.

3.5 CONCLUSIONS

The results obtained in this study present variability in chemical-physical properties, phenolic content and antioxidant capacity among tissues and cultivars in agreement with data reported by others authors (Hampson et al. 2003, Drogoudi et al. 2008, Henríquez et al. 2010, Thompson-Witrick et al. 2014). Apple peel in all three cultivars contains the highest total phenolic content and antioxidant capacity when compared to flesh and whole fruit, as already reported (Carbone et al. 2011, Vrhovsek et al. 2004 and Giomaro et al. 2013). Moreover, a high correlation between phenolic content and antioxidant capacity is evidenced.

These data confirm that the usual consumption of apples in a diet can provide to an important amount of antioxidants and also the importance of the growing in an organic farming system which permits to eat the fruits with peel, usually considered reject product .

In conclusion, the cultivars Gala, Granny Smith and Fuji grown in Sicily in an organic farming system, for their chemical-physical properties, total polyphenolic content and antioxidant capacity can be included within commercially acceptable ranges and comparable to other worldwide productions. Besides, the analyzed apples, grown in Sicily, present values of polyphenolic content compared to that of other

fruits growing in other Italian region (Vrhovsek et al. 2004), suggesting that Sicilian apples represent a considerable alternative to traditional Sicilian products and a great deal for the fruit market.

In a market constantly exposed to globalization, strong competition is becoming clearer and inevitable both for what concerns the productive aspect and the actors themselves of the supply chain. In order to maintain or affirm market competitiveness, it is necessary, therefore, to promote the cultivation and commercialization of emerging crops in Sicily, as the aforementioned apple cultivars.

CHAPTER 4:

Apple polyphenols inhibit amyloid aggregation of κ-casein

4. APPLE POLYPHENOLS INHIBIT AMYLOID AGGREGATION OF K-CASEIN

4.1 Abstract

The polyphenols extracted from various fruits are known for their beneficial effects on human health due to their antioxidant, anti-cancer and anti-inflammatory activity. Moreover, their neuroprotective and anti-amyloidogenic effect *in vitro* has been detected too. Indeed, recent studies have reported that some polyphenols are capable to redirect the aggregation of amyloidogenic peptides, such as the β-amyloid peptide involved in Alzheimer's disease and α-synuclein involved in Parkinson's, leading to the formation of non toxic amorphous aggregates.

The purpose of this work is to study the effect of total polyphenols extracted from three apple varieties on the process forming amyloid fibers *in vitro*, using κ-casein as model protein. K-casein is a bovine milk protein that, similarly to amyloid β-peptide, forms amyloid fibers from a monomer intrinsically disordered and contributes to the fibrillar core with a β-double strand.

In particular, polyphenols extracted from different tissues (peel, whole fruit and flesh) of three apple cultivars ('Gala', 'Granny Smith' and 'Fuji'), grown in Sicily, were used. Preliminary cellular viability assays, performed on NHI-3T3 cell line, have helped to focus the choice on testing the effects of peel extracts because they have, also at high concentration, no toxic effect on these cells unlike the whole fruit and flesh extracts .

Several biophysical techniques (Fluorescence Spectroscopy, Circular Dichroism, Light Scattering and Atomic Force Microscopy) and bioinformatics tools were used to investigate physical-chemical properties, structural features and amyloid firbrillogenesis of κ-casein, as well as the effects on the aggregation process exerted by apple peel polyphenols. Biophysical results have revealed that k-casein (50μM ≈1 mg/ml) forms fibrils and aggregates under the conditions reported for κ-casein amyloid formation (37 °C, 50 mM phosphate buffer pH 7.4). Moreover, apple peel polyphenols of all three cultivars inhibited κ-casein aggregation at 40 μg/ml.

These results give new suggestions to investigate the action of natural polyphenols as potential therapeutic agents for amyloidosis, supporting the use of small molecules inhibiting amyloid aggregation that is one of the most modern strategies searched for the treatment of neurodegenerative disease.

4.2 Introduction

Polyphenols are a class of molecules with potential human health benefits. They are natural compounds arising from plant secondary metabolism, in particular from the shikimate-derived phenylpropanoid and/or polyketide pathways. Polyphenolic compounds have more than one phenolic ring and are devoid of any nitrogen-based functional group in their most basic structural expression (Francini and Sebastiani, 2013). In plants, polyphenols have ability to serve multiple functions in plant–environment interactions. They act as defense against herbivores, microbes, viruses or competing plants, and signal compounds to attract pollinating or seed dispersing animals, as well as protecting the plant from ultraviolet radiation and oxidants. Moreover, plants use them in defense against reactive oxygen species produced

during photosynthesis. Antioxidant activity of polyphenols is determined by their reactivity as a hydrogen- or electron-donating species, which is related to their reduction potential (Biedrzycka and Amarowicz, 2008).

Natural polyphenols are been object of several studies, especially as potential therapeutic agents in human diseases. Indeed, many review articles claim beneficial effects of polyphenols on cardiovascular disease, cancer, pulmonary function and age-related cognitive decline (Leontowicz et al, 2002, Ono et al., 2003, Femia et al, 2005, Bastianetto et al., 2006; Terra et al., 2011, Vineetha et al., 2014).

The health benefits of the polyphenols are due to their large array of biological actions such as antioxidant activity, enzyme modulation ability, effects on cell signaling pathways and gene expression (Dell'Agli et al., 2005). Moreover, recent studies have provided significant information on their ability to inhibit peptide/protein aggregation in various ways and to stimulate cell defenses, leading to identify shared or specific mechanisms. In particular, some polyphenols are capable to inhibit amyloid aggregation and their associated cytotoxicity interfering directly with fibril formation through specific aromatic interaction, and to remodel performed fibrils, thus generating non toxic species (Porat et al., 2006; Hudson et al. 2009; Stefani and Rigacci, 2013). For example the quercetin, a flavonol, was shown to prevent the growth of Aβ amyloid aggregates *in vitro* and to destabilize preformed fibrils, using ThT binding and TEM analysis (Ono et al., 2004). The efficacy of quercetin as amyloid aggregation inhibitor and preformed fibril destabilizer was confirmed by experiments on insulin and α-synuclein (Wang et al., 2011; Ono et al., 2006).

The (-)-epigallocatechin-3-gallate (EGCG), one member of the family of flavan-3-ols or flavanols, prevents fibril formation of amyloid β and it is capable of rescuing

memory impairment, induced by amyloid β peptide (Lee et al., 2009). Its action does not only concern amyloid β peptide but also many others amyloidogenic peptides/proteins including α-syn, mutant htt, TTR, hIAPP, the amyloidogenic peptide PAP248–286 from prostatic acidic phosphatase, HEWL, k-casein and calcitonin (Bastianetto et al., 2006; Masuda et al., 2006; Ehrnhoefer et al., 2006; Ferreira et al., 2009; Meng et al., 2010; Hauber et al., 2009; He et al., 2009; Hudson et al. 2009; Huang et al., 2012).

Other studies reported that flavanols, such as (+)-catechin and (−)-epicatechin, reduce semantic interference on memory tasks and improved verbal learning capability (Krikorian et al., 2010; Krikorian et al., 2012).

Above-mentioned polyphenols have been found in polyphenolic profile of different apple cultivars (Vrhovsek et al., 2004; Karaman et al. 2012) and this suggests that polyphenols extracted from apples could be used as potential therapeutic agent in neurodegenerative diseases. In particular, Toda and colleagues (2011) showed that apple polyphenols, with high procyanidins content, inhibit aggregation of amyloid-β peptide *in vitro* and Chan and Shea (2009) demonstrated that dietary supplementation with apple juice decreases endogenous amyloid-β levels in murine brain.

In order to deep inside the inhibitory effect *in vitro* of the polyphenols on the process forming amyloid fibers, κ-casein, a bovine milk protein that similarly to amyloid β-peptide forms amyloid fibers, was chosen as model protein. Many authors reported that and the "native" and "reduced" and "carboxymetilated" κ-caseins aggregate and form amyloid fibrils at 37 °C (Farrell et al., 2003; McSweeney and Fox, 2013; Chun et al., 2012). The molecular mechanism underlying the κ-casein amyloid fibrillogenesis has been recently hypothesized on the basis of indirect experimental procedures. It involves the dissociation from the oligomeric initial state of an

amyloidogenic monomeric precursor provided with the proper structural features leading to the ordered fibrillar aggregation (Ecroyd et al., 2010). In fact, the κ-casein collapsed monomeric conformation, arranged as a "horse a rider" structure (Kumosinski et al., 1993) presents, in correspondence of horse legs, a tyrosine rich sheet-turn-sheet hydrophobic motif that, during oligomer dissociation, would be exposed to the solvent and would lead to beta sheet stacking resulting in a ordered aggregation. A single κ-casein monomer would contribute to more than one β strand in the final fibrillar structure. The hydrophobic region is incorporated into the core of the fibrils formed by κ-casein and this is a unique feature that would determine its high aggregation propensity. The aggregation mechanism in the case of native protein would be similar, even if reduced in extinction due to the presence of disulfide bridges (Ecroyd et al., 2010, Thorn et al., 2005).

To better understand the *in vitro* effect of apple polyphenols on amyloid aggregation, this study has been carried out using κ-casein, as model protein. Before studies with polyphenols, k-casein chemico-physical features and aggregation properties were analyzed.

4.3 Material and Methods

4.3.1 Sample preparation of apple polyphenolic extracts

For polyphenolic extraction different apple tissues (peel, flesh and whole fruit) of three cultivars, 'Gala', 'Granny Smith' and 'Fuji', were selected. Extraction was carried out as described in the previous chapter. Extracts were then concentrated by drying in a rotatory evaporator at 30 ºC to remove extraction solvent that is toxic for cells. Afterward, the concentrated samples were dissolved in water, aliquoted and kept at -20 ºC until analysis, avoiding direct contact with light and oxygen.

4.3.2 Cell culture

Mouse fibroblast NIH-3T3 cell line was obtained from Sigma Aldrich. The cells were cultured in Dulbecco's modified eagle medium (DMEM) (Sigma Aldrich) supplemented with 10% (vol/vol) heat-inactivated Calf Bovine Serum (CBS), 1% penicillin and streptomycin (10,000 U/ml and 10,000 mg/ml, respectively) in humidified 5%-CO_2 and 95%-air atmosphere at 37 °C. For routine maintenance, cells were cultured in 75 cm^2 flasks as monolayers and maintained in DMEM with 10% serum and 1% penicillin and streptomycin. Cells were harvested by trypsinization (0.1% trypsin-0.05% EDTA) once a week.

4.3.3 Viability assay

NIH-3T3 cells were seeded into 96-well plates at a density of 10^4 cells per well in 100 µl of growth medium, cultured for 24 h and then treated with filter-sterilized apple polyphenolic extract at final concentration of 80, 40, 20, 10 or 5 µg/ml for peels, and from 40 and from 20 µg/ml to 5 µg/ml for whole fruit and flesh, respectively. All treatments were performed for 24 and 48 hours of incubation and in triplicate.

Cell viability was analyzed by the CellTiter 96H AQueous One Solution Cell Proliferation Assay kit (MTS assay, Promega) following the manufacturer's instruction. In brief, after cell treatments 20 µl of the MTS solution was added to each well and incubated with cells for 3 h at 37 °C, 5% CO_2. The absorbance was read at 490 nm with the Bio-Rad iMark^{tm} Microplate Reader. Cell viability was quantified as percentage of cell viable using untreated cells as a control. All the experiments were repeated three times.

4.3.4 Bioinformatics analysis

A visual inspection of charge-hydropathy (CH) plot (Uversky et al., 2000; Vilasi. et al., 2008; Oldfield et al., 2005) gives information on chemico-physical features of proteins. Based on the rationale that protein folding is governed by a balance between hydrophobic forces (attractive) and electrostatic forces between similarly charged residues (repulsive) (Ferron et al., 2006; Farrell et al. 2006), the combination of low mean hydrophobicity and relatively high net charge turns out to be an important prerequisite for the absence of a regular structure in proteins under physiologic conditions, thus leading to "natively unfolded" proteins (Uversky et al., 2000; Vilasi et al., 2008; Oldfield et al., 2005). The two sets are separated by a straight line <charge>=2.743*<hydropathy>-1.109 representing the linear function best separating ordered and disordered proteins (Oldfield et al., 2005).

The database of disordered proteins was created using a list of natively unfolded proteins (Uversky et al., 2000) and the SWISS-PROT protein sequence data bank (Bairoch, and Apweiler 2000). The ideal database of globular proteins is available at the address http://phys.protres.ru/resources/folded_80.html (Garbuzynskiy et al., 2004; Galzitskaya et al., 2006, Vilasi et al., 2008). The sequence of κ-casein was obtained from SWISS-PROT and http://www.cbs.dtu.dk/services/SignalP/ (Nielsen et al., 1997) was used to predict the signal peptide cleavage sites for the protein.

The protein mean hydrophobicity was calculated using the Kyte–Doolittle Scale (Kyte and Doolittle, 1982) rescaled to a range of 0–1. The mean net charge was defined as the absolute value of the difference between the numbers of positively and negatively charged residues at pH 7.0, divided by the total residue number, according to Uversky et al. (2000).

Protein disorder was predicted for k-casein by the algorithms PONDR VL-XT (Iakoucheva et al, 2002).

4.3.5 Sample preparation of κ-casein

Lyophilized κ-casein was purchased from Sigma-Aldrich and used without any further purification. A fresh stock solution of the protein in 50 mM Phosphate Buffer pH 7.4 was continuously stirred for 24 h and filtered through 0.22 μm filter before of use. The protein concentration was determined by absorption at 280 nm using an extinction coefficient of 0.95 mg^{-1} ml cm^{-1} for κ-casein (McSweeney and Fox, 2013)

4.3.6 Atomic Force Microscopy (AFM)

AFM is the most commonly used form of the scanning probe microscopy (SPM) family of techniques. SPM began with the development of the scanning tunneling microscope (STM) that could only be applied to conductive or semiconductive specimens. To broaden this type of microscopy to the study of insulators, the atomic force microscope was invented (Binnig et al., 1986) and, because of its not invasive character and its resolution at nanometer scale, it represents now one of the most important techniques in the imaging of biological samples. AFM has been used to study directly the higher-order structure of amyloid assemblies made by a number of peptides and proteins as well as to analyze fibrillogenesis mechanism (Ionescu-Zanetti et al.,1999; Goldsbury et al., 1999; Kad et al., 2003; Hoyer et al., 2004).

The atomic force microscope (AFM) probes the surface of a sample with a sharp tip, a couple of microns long and often less than 100 Å. The tip is located at the free end of a flexible cantilever, 100-200 nm long. The surface is scanned by means of a piezoelectric tube scanner. The interaction force between the tip and the sample

surface causes the change of cantilever mechanical state, detection or oscillation amplitude depending, as it will be seen, on the operation mode of the microscope. During the scanning, the detection system measures the interaction parameter from its initial value and sends to the scanner control system a signal proportional to it. The control system moves the probe by means of the scanner perpendicular to the surface to bring the parameter back to its original value (feedback mechanism). Simultaneously the probe displacement value is saved in the PC memory and interpreted as the sample topography (Fig. 9). Several forces typically characterize the interaction between sample and the tip.

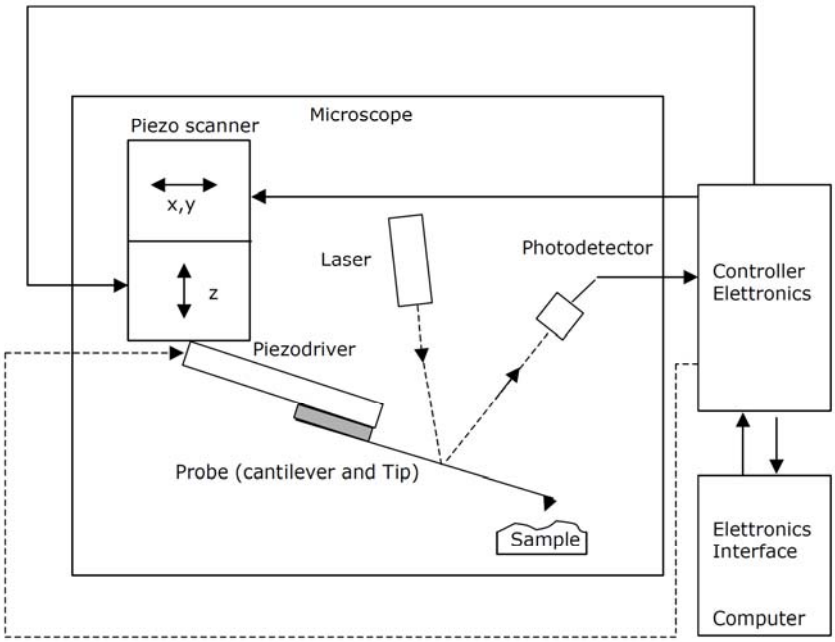


Fig. 9 A schematic rapresentation of AFM microscope

The force most commonly to be associated with atomic force microscopy is the interatomic van der Waals force. The dependence of the van der Waals force upon the distance between the tip and the sample is shown in figure 10. Two distance

regimes are labeled on figure: 1) the contact regime and 2) the non-contact regime. In the contact regime, the cantilever is held less than a few angstroms from the sample surface, and the interatomic force between the cantilever and the sample is repulsive. In the non-contact regime, the cantilever is held on the order of tens to hundreds of angstroms from the sample surface, and the interatomic force between the cantilever and sample is attractive. There is also an intermediate regime, called intermittent contact or tapping one, in which the cantilever tip vibrates near to the sample so that at the bottom of its travel it just barely hits or "taps" the sample. Tapping Mode Atomic Force Microscopy (TM-AFM) works by vibrating the tip at the end of a cantilever and bringing it into intermittent contact with the sample surface.

When the tip interacts with a surface feature, its amplitude is varied from its previous amplitude of oscillation. The AFM senses this variation, and the tip is raised away from the sample in order to re-attain, by using a feedback circuit, the previous amplitude of oscillation. In this way, the tip can be rastered across the sample to generate topographical images.

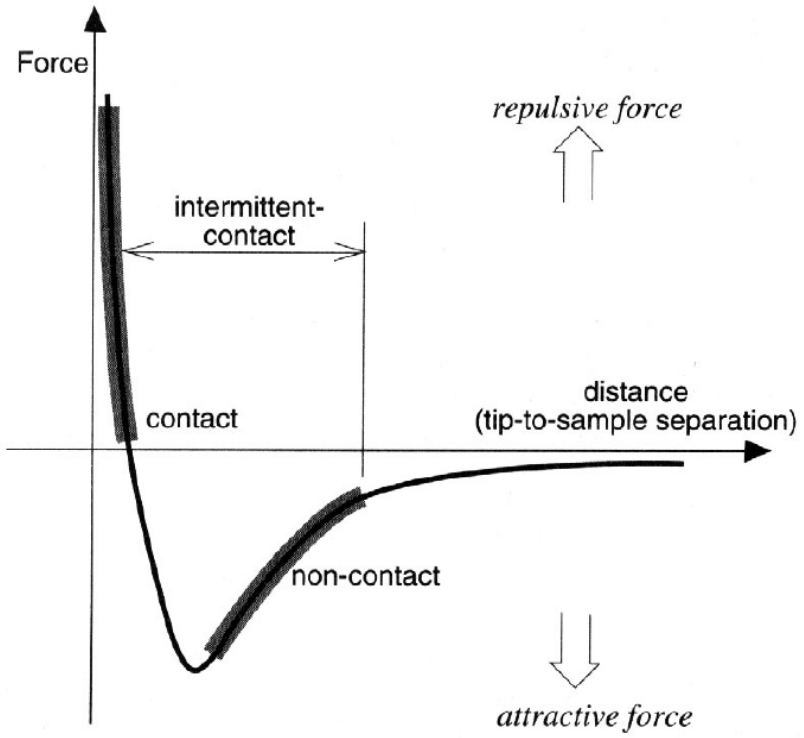


Fig. 10 Van der Waals interatomic force vs. distance curve (Berne et al., 1976)

All AFM measurements were performed by using a Nanowizard III (JPK Instruments, Germany) mounted on an Axio Observer D1 (Carl Zeiss, Germany) inverted optical microscope. V-shaped silicon nitride cantilevers (SNL, USA), with a nominal spring constant ranging from 0.12 N/m to 0.48 N/m, with a resonance frequency in air ranging from 40 kHz to 75 kHz and tip with typical curvature radius of 2–12 nm were used. The actual spring constant of each cantilever was determined in situ, using the thermal noise method (Hutter and Bechhoefer, 1993). Aliquots of fresh protein solution (50 μM) were deposited onto freshly cleaved mica surfaces (Agar Scientific, Assing, Italy) and incubated for up to 20 min before rinsing with deionized water and drying under a low pressure nitrogen flow. Imaging of the protein was carried out in intermittent contact mode in air.

4.3.7 Fluorescence experiments

The kinetic data of κ-casein aggregation at different concentrations (50, 8 or 2.4 μM) were monitored by the ThT assay. Thioflavin T is a fluorescent dye that is widely used to detect amyloid fibrils. In fact, the emission intensity of the dye increases significantly upon binding to the linear array of beta strands in amyloid fibrils (Naiki et al., 1991; LeVine, 1993; Tjernberg et al.,1999; Samuel et al., 2001; Ban et al., 2003). The experiments were performed in a plate reader setup spectrofluorimeter Fluoroskan Ascent™ FL Microplate Fluorometer (Thermoscientific). The samples were placed in the plate of 96 wells thermostated at 37 °C, continuously sheared at 180 rpm. Each sample was run in triplicate. The excitation and emission wavelengths were 450 and 485 nm, respectively. ThT concentration was 12 μM.

4.3.8 Static and dynamic Light Scattering (SLS and DLS)

The κ-casein aggregation and its inhibition by apple polyphenolic extracts were studied by Static and Dynamic Light Scattering.

With the advent of the laser and its associated detection techniques, light scattering has become an important tool for the study of many problems in chemistry, biology and physics. Light scattering techniques are particularly useful for exploring homo- or hetero-association of proteins and other biological macromolecules. It has been shown to be one of the primary experimental techniques in several studies on the kinetics of the amyloid aggregation process, especially when the kinetics is relatively slow (minutes to hours) (Nichols al., 2002).

In a light scattering experiment, light from a laser passes through a polarizer (to define the polarization of the incident beam) and then impinges on the scattering medium. Then, the scattered light passes through an analyzer, which selects a given

polarization and finally enters a detector. The position of the detector defines the scattering angle θ.

The intersection of the incident beam and the beam intercepted by the detector defines a scattering region of volume V (Fig. 11). Polarizers and analyzers are used to define the polarizations of the incident and scattered light beams.

The scattered light impinges directly on the photocathode and the output of photocathode, which is proportional to the square of the incident electric field that is the intensity of the light, passes through the autocorrelator, which calculates its time autocorrelation function.

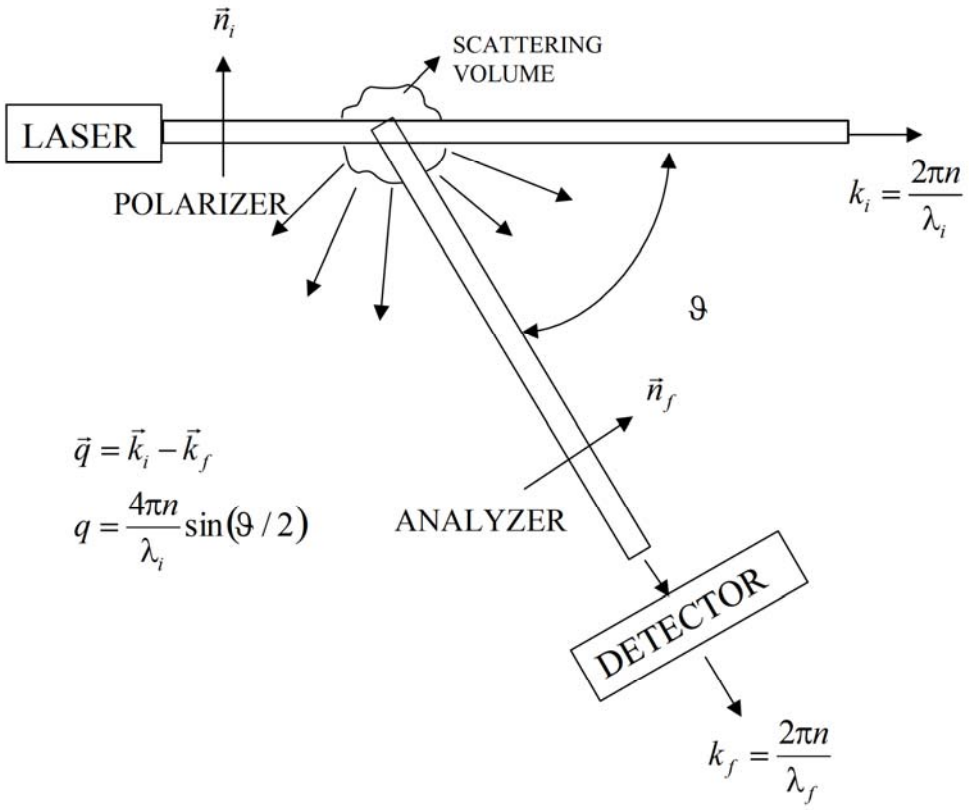


Fig. 11: A schematic representation of the light-scattering experiment

In a *static light scattering experiment*, the intensity of the light I(q), in terns of Kilo Count of Photons per Seconds (kcps) is measured. From the theory, it has been demonstrated that the scattered intensity I(q), given in terms of the Rayleigh ratio[1] is related to the weight averaged molecular mass Mw of particles by the relation: R(q) = KcMwP(q), with the instrumental factor K, c is the mass concentration, P(q) is the z-averaged form factor, ñ is the medium refractive index, λ_0 is the incident wavelength, and NA is the Avogadro's number (Pusey, 2002; Berne et al., 1976).

From this relationship, it is evident the in a static light scattering experiments that measures the static light scattered from a samples over time, we have information on the variation of molecular mass during the observation time, and therefore, it is suitable in order to monitor aggregation processes.

During a *dynamic light scattering*, what is analyzed is the autocorrelation function of the intensity g(2) (t) of scattered field. Due to their Brownian motion, particles moving in solution give rise to fluctuations in the intensity of the scattered light. In math terms, time auto-correlation of the intensity measures the degree of correlation between i at a time t and i at another time.

t + τ: It is defined by:

$$\langle i(0)i(\tau)\rangle = \lim_{T\to\infty}\frac{1}{T}\int_0^T dt\, i(t)i(t+\tau)$$

The autocorrelator measures the intensity–intensity correlation function that, for a Gaussian distribution of the intensity profile of the scattered light, is related to the electric field correlation function g(1)(t):

[1] $R(q)=I(q)/I_s r^2/V_s$, where I_s is the intensity of the laser source, V_s is the scattering volume, and r is the distance of the detector from the sample

$$g^{(2)}(q,t)^2 = \left[A + Bg^{(1)}(q,t)\right]^2$$

For polydisperse particles, *g(1)(q,t)* is given by:

$$g^{(1)}(q,t) = \int_0^\infty G(\Gamma)\exp(-\Gamma t)d\Gamma)$$

Here, $G(\Gamma)$ is the normalized number distribution function for the decay constant $\Gamma = q2DT$, where $q = (4\pi n/\lambda)\sin(\vartheta/2)$ is the scattering vector defining the spatial resolution with n, the solvent refractive index and *DT*, the translational diffusion coefficient. The hydrodynamic diameter *DH* is calculated from *DT* through the Stokes–Einstein relationship:

$$D_T = \frac{k_B T}{3\pi\mu D_H}$$

A technique for extracting the moments of the diffusion coefficient from heterodyne and homodyne functions consists in the cumulant analysis that allows to obtain the statistical characteristics of the distribution $G(\Gamma)$, among which, the average value Γ, related to the average hydrodynamic size. Therefore, by a DLS experiments over time, informations about the average hydrodynamic size over time can be obtained.

The sample was placed into a thermostatic cell compartment of a Brookhaven Instruments BI200-SM goniometer (Biophysics Institute, Palermo Unit, National Research Council). The temperature was controlled within 0.1°C using a

thermostated recirculating bath allowing temperature-programmed runs. The light scattered intensity and its autocorrelation function were measured by using a Brookhaven BI-9000 correlator and a 100mW solid-state laser (Quantum-Ventus MPC 6000) tuned at λ = 532 nm. The spatial resolution is defined by the scattering vector $q=4\pi n\lambda 0-1\sin(\theta/2)$, where n is the refraction index of the solution, λ_0 is the wavelength of the incident light, and θ is the scattering angle. Static light scattering data were corrected for the background scattering of the solvent and normalized by using toluene as a calibration liquid. All samples were filtered through 0,2 μm cellulose acetate (Millipore) syringe filters to remove contaminants.

4.3.9 Circular dichroism

Circular dichroism is a unique tool for analyzing secondary structures of dissolved proteins. Fundamentally, optical activity is a manifestation of the differential interaction of matter with left and right circularly polarized light. The interesting, particularly useful feature of this phenomenon is that it relates, in a sensitive way, to molecular geometry itself; that is, the extent to which a molecular structure interacts differently with the two circular polarizations depends directly on both its inherent conformation and its precise orientation relative to other molecules, structures or charges in its immediate environment.

Circular Dichroism (CD) refers to the differential absorption of the left and right circularly polarized components of plane polarized radiation. Let us consider the oscillating vector electric field E of a plane-polarized light viewed at varies times as it moves toward the observer in a plane normal to the plane of the paper. The vector E can be regarded as the resultant of two coherently superposed electric field vectors, one, say, EL for left circularly polarized light, and the other, say, ER, for right

circularly polarized light. The vector fields EL and ER appear to rotate counterclockwise and clockwise (respectively) as they move towards the observer. E can be split into the two circularly polarized components by passage through a modulator (usually a piezoelectric crystal such as quartz). If E passes through an optically active medium, waves of opposite circular polarizations are differentially absorbed giving rise to elliptically polarized light; the emerging electric vector field, rather then tracing a line as before, traces (with time) an ellipse when viewed head-on. The circular dichroism of the sample is found to be approximately proportional to the ellipticity of the ellipse (Fig. 12).

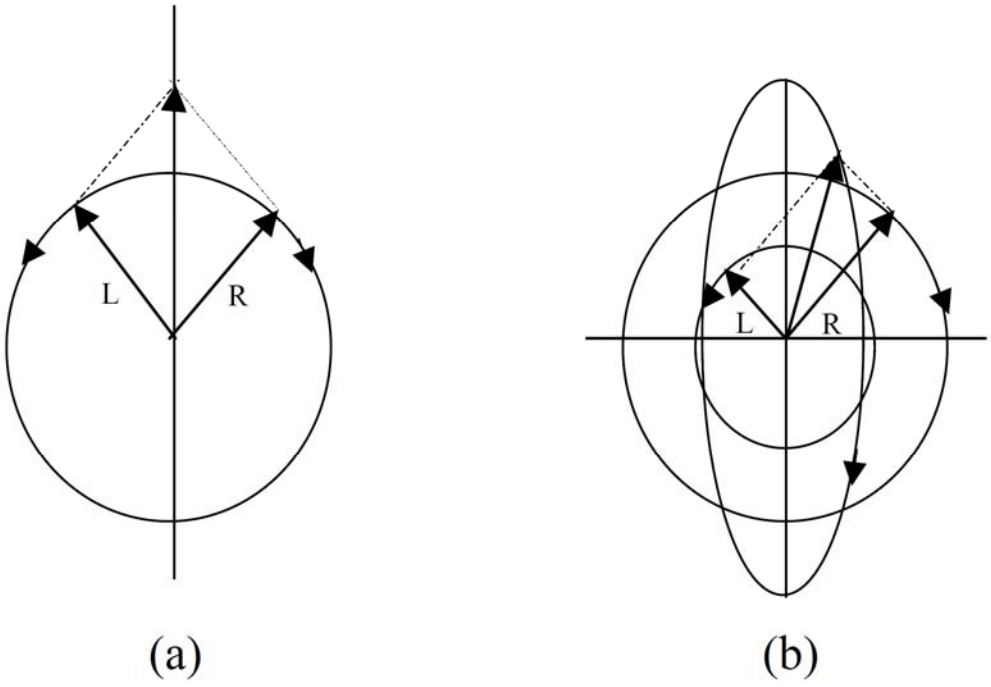


Fig. 12: Combination of the two circularly polarized electric field components, one right- handed and one left-handed (a) before the passage through an optical active medium (b) after the passage through the medium.

The CD spectra of proteins are generally divided into three wavelength ranges, based on the energy of the electronic transitions that dominate in the given range. These are: the far UV (below 250 nm), where the peptide contributions dominate, the near UV (250-300 nm), where aromatic side-chains contribute and the near UV-visible region (300-700 nm), where extrinsic chromophores contribute. Studies of the far-UV region (typically 240 nm to 190 or 180 nm) can be used to assess quantitatively the overall secondary structure content of the protein. In this region the absorbing group is principally the peptide bond.

Depending on the orientation of the peptide bonds in the arrays, the optical transitions can be split into multiple transitions, the wavelengths of the transitions can be increased or decreased, and the intensity of the transitions can be enhanced or decreased.

As a consequence, many common secondary motifs, such as the alphahelix, beta pleated sheets, turn have very characteristic CD spectra.

The alpha helical CD spectrum (Fig. 13) is characterized by two negative bands at 222 and 208 nm, and by positive band at 192 nm. The CD spectrum of the typical beta-sheet has a negative band near 216 nm and a positive band near 198 nm. There are many methods to extract protein conformation in solution from CD.

In order to analyze the variation in the total secondary structure we used an innovative algorithm β-structure selection (BeStSel), that, differently from previous tools, is able to distinguish parallel from unparallel beta sheet and has been built in order to take into account specific variation in beta structure characteristics for amyloid formation (Micsonai et al., 2015).

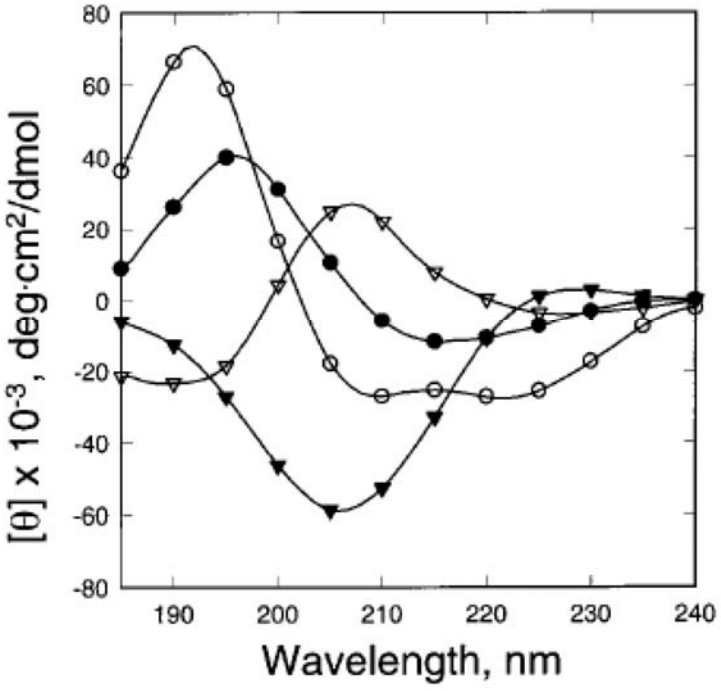


Fig. 13: Far UV CD spectra of common secondary structure motifs: alpha (empty circle); beta (full circles) and unordered (triangles)

Basically all of these methods assume that the spectrum of a protein can be represented by a linear combination of the spectra of the secondary structural elements. In these methods, spectra of either model polypeptides or of a set of reference proteins with known secondary structure are used and the CD spectrum of a given protein in the reference set is treated as a linear combination of component secondary structure spectra. The secondary structure fractions for the proteins in the reference set are determined from the corresponding crystal structures.

Performing CD analysis using different methods should help improve the reliability of predicted structural features. However, care should be taken to ensure that the protein reference set and the secondary structure assignments used are suitable for analyse the specific protein under study.

In this work we used a novel algorithm that is able to distinguish parallel from unparallel beta sheet and contains, in the data set, many amyloid beta protein structures (Micsonai et al. 2015).

An aliquot of κ-casein at the beginning and after 24 hours of incubation at 37 °C was used for circular dichroism analysis. CD spectroscopic measurements were performed at 20°C by using a JASCO J-810 spectrometer equipped with a temperature control unit. A quartz cell with a path length of 0.5 mm was used for far-UV (190–250 nm) measurements. Each CD spectrum was obtained by averaging over eight scans and subtracting the blank solvent contribution. The analysis of CD spectra was performed by using CDPro software.

4.3 Results and discussion

Structural features of k-casein investigated by bioinformatic tools.

Before studying amyloid aggregation properties of k-casein, information on its structural features was obtained by two bioinformatic tools. First, the values per residue of the mean normalized hydrophobicity and absolute net charge were compared with those reported for ideally globular or natively unfolded proteins (Fig. 14). In fact, it has recently emerged that protein disorder tends to be related to general chemical properties rather than abundance or scarcity of specific amino acids. This visual inspection of charge-hydropathy (CH) plot was reported for the sequences of κ-casein purified from bovine milk. K-casein point (yellow in the figure) falls in regions populated by ordered proteins separated from disordered proteins by the linear function. Indeed its higher mean hydrophobicity and the lower net charge per residue suggest a more folded state (Fig. 14). Secondly, when analyzed by PONDR, one of the online available protein disorder predictors (Fig.

15), κ-casein however presents high disorder content along the sequence. Actually as reported by Iakoucheva et al. (2002) a PONDR output score higher than 0.5 is symptomatic of significant disorder content in the protein along the sequence.

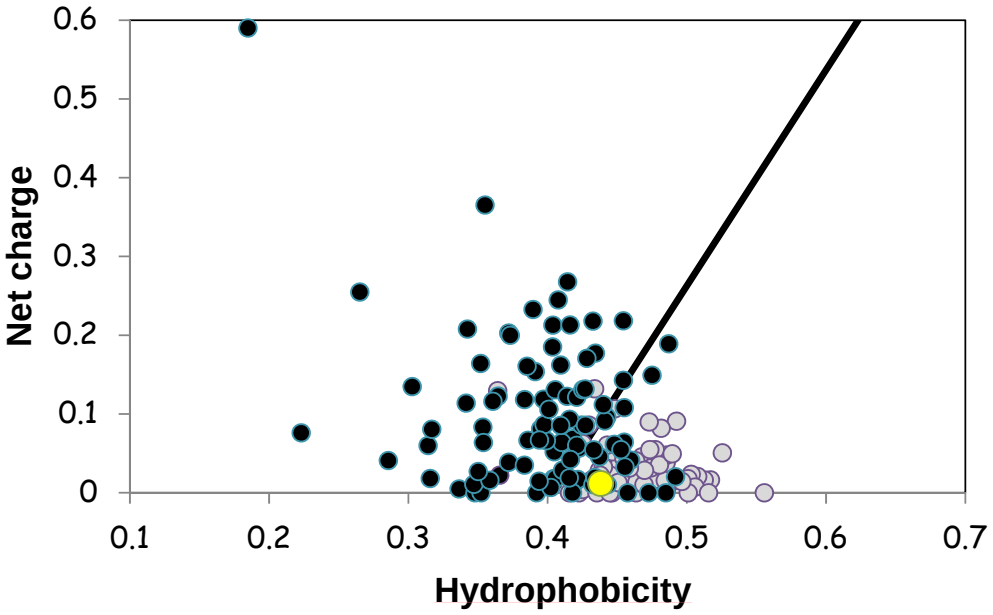


Fig.14 Analysis of κ-casein (yellow) sequences by Uversky plot. The casein net charge and hydrophobicity are compared with those of the two sets of 90 natively unfolded and 80 ideally globular proteins (black and grey symbol respectively). The two sets are separated by a straight line <charge>=2.743*<hydropathy>-1.109 representing the linear function best separating ordered and disordered proteins .

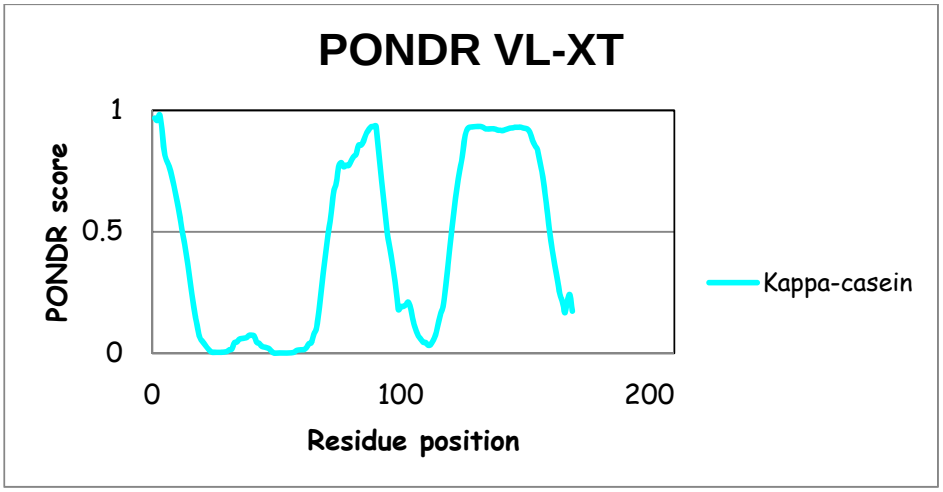


Fig. 15 Analysis of the κ-casein sequences by using the predictor PONDR

It has been reported that a protein predicted to contain significant regions of natural disordered structure, but grouped with ordered proteins on a CH plot, exists in a collapsed disordered conformation, distinct from an extended disordered one (random coil) (Jain et al., 2011, Lavery and McEwan 2008). The ensemble of collapsed conformations adopted by intrinsically disordered proteins is supposed to be modulated by the net charge per residue (Mao et al., 2010). This, according to Jain et al., (2011) would be at the basis of the chain collapse that renders κ-casein the archetypal model protein of an amyloidogenic intrinsically disordered protein.

K-casein amyloid aggregation process

Next step was the analysis of κ-casein amyloidogenic behavior, in order to better understand the effects of the polyphenols on the aggregation process. Figure 16 reports the results regarding the protein amyloid aggregation when incubated at various concentrations (8, 2.4 or 50 μM), under the conditions reported for κ-casein amyloid formation, by in situ Thioflavin T (ThT) assay. ThT in the presence of κ-casein exhibited a concentration-dependent increase in fluorescence emission, symptomatic of amyloid formation (Fig. 16), as expected by bioinformatic results.

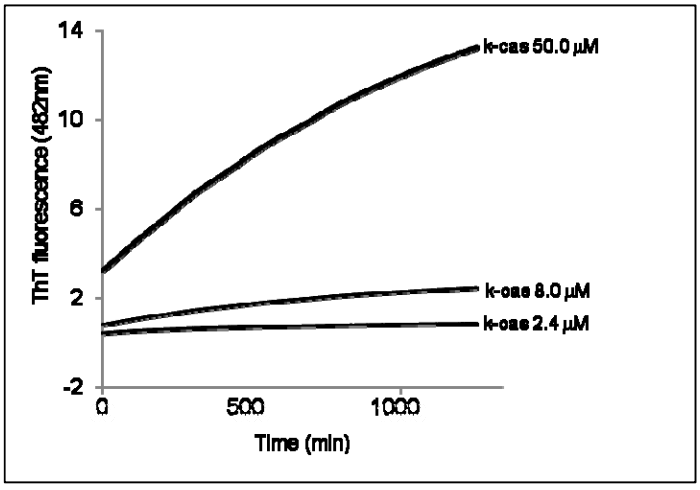


Fig. 16 K-casein amyloid aggregation monitored by ThT fluorescence at different protein concentrations

The absence of concentration-dependent variation in the lag-phase suggests for κ-casein a mechanism different from the typical nucleation-polymerization fibrillogenesis (Ecroyd et al., 2008). The initial high ThT signal, especially evident at the highest κ-casein concentration (50 μM) was due to the binding of the dye to the protein in its native state. The species formed by "native" κ-casein (50 μM), when observed by AFM, appeared as fibrillar structures stubby (Fig. 17) similar in width to those formed by the reduced and carboxymethylated κ-casein, as reported in literature (Thorn et al., 2005) but noticeably shorter.

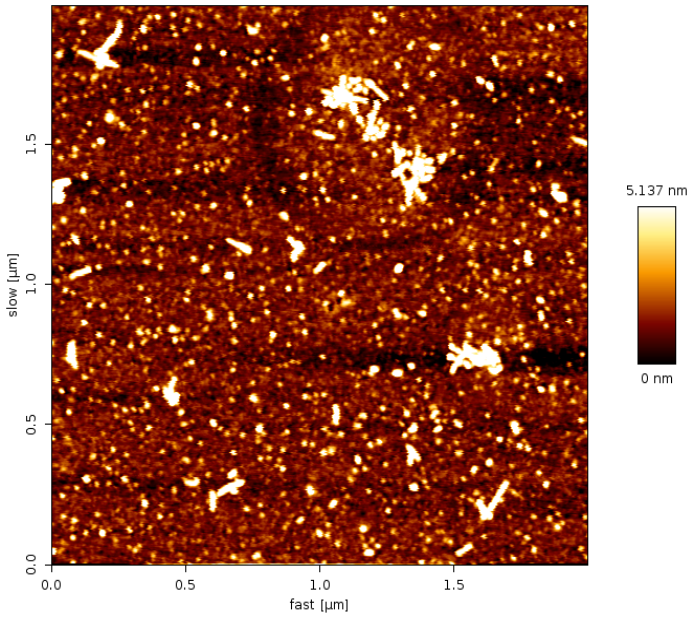
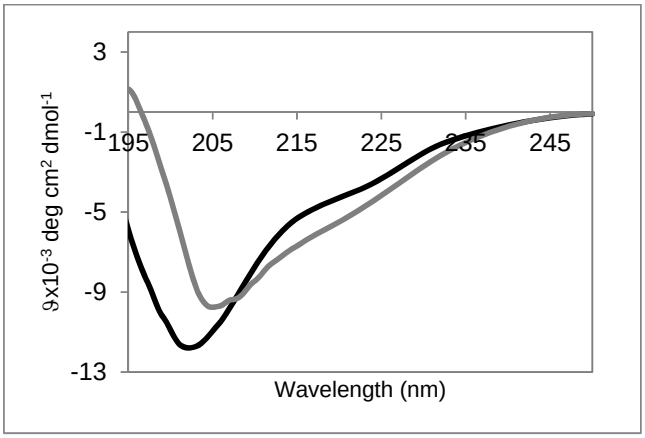


Fig. 17 κ-casein amyloid aggregates morphology at 50 μM and at the end of the kinetics profile visualized by AFM.

Structural conversion of κ-casein during amyloid formation was investigated by FAR-UV CD spectroscopy. The measures, performed at the start and at the end of kinetics process, showed that the protein undergoes a conformational transition with two distinct spectral changes (Fig. 18). The values of the secondary structure analysis, reported in the table of figure 18 reveal that at the end of kinetics process (after 24 h), k-casein presented a significant increase in the percentage of beta

parallel structures (from 0.3% at the start of aggregation process to 5.6% value at the end of aggregation process). On the whole a small variation in the total secondary structure is detected. This is coherent with the dissociation model leading to amyloid formation for κ-casein (Ecroyd et al., 2010). The dissociated species, characterized by a significant percentage of β-native structure, would be the amyloidogenic precursor of fibrillar assembly through a β-sheet stacking that would not dramatically increase the total β-structure content, but would be responsible for variations in the distribution of secondary β-parallel and antiparallel elements.

The reported results show that k-casein can be considered a good amyloidogenic model for the next studies on the polyphenols effects.



	Alpha	Beta Antiparallel	Beta Parallel	Turn	Unordered
t=0	9.2%	24%	0.3%	14.8%	51.7%
t=24h	10.9%	21.1%	5.6%	14.0%	48.5%

Fig. 18 Structural conversion accompanying 2.4 μM κ-casein amyloid formation investigated by FAR-UV CD spectroscopy. Protein spectra at the beginning of the aggregation process (black) and after 24 hours (grey) are reported. In the table, results from secondary structure analysis, performed by β-structure selection (BeStSel), are reported.

Apple polyphenols effects on cell viability

Before analyzing polyphenols influence on k-casein aggregation, cell viability assays were performed for each polyphenolic extract of peel, flesh and whole fruit from each cultivar, 'Gala', 'Granny Smith' and 'Fuji'.

Results reported in figure 19 show that NIH-3T3 cells presented nearly 30 % of cell death, for every treatment time, when higher concentration (80 µg/ml) of Fuji peel phenolic extract was used. On the contrary, using lower concentrations of peel no cell mortality was showed either after 24 or 48 hours of treatment. Different effects were found in whole fruit and flesh of Fuji, using the same concentrations and time treatments. In fact, the doses of 40 and 20 µg/ml for whole fruit and the dose of 20 µg/ml for flesh were toxic for cells. Only 10 and 5 µg/ml concentrations, both of whole fruit and flesh phenolic extracts, were not toxic (Fig. 19).

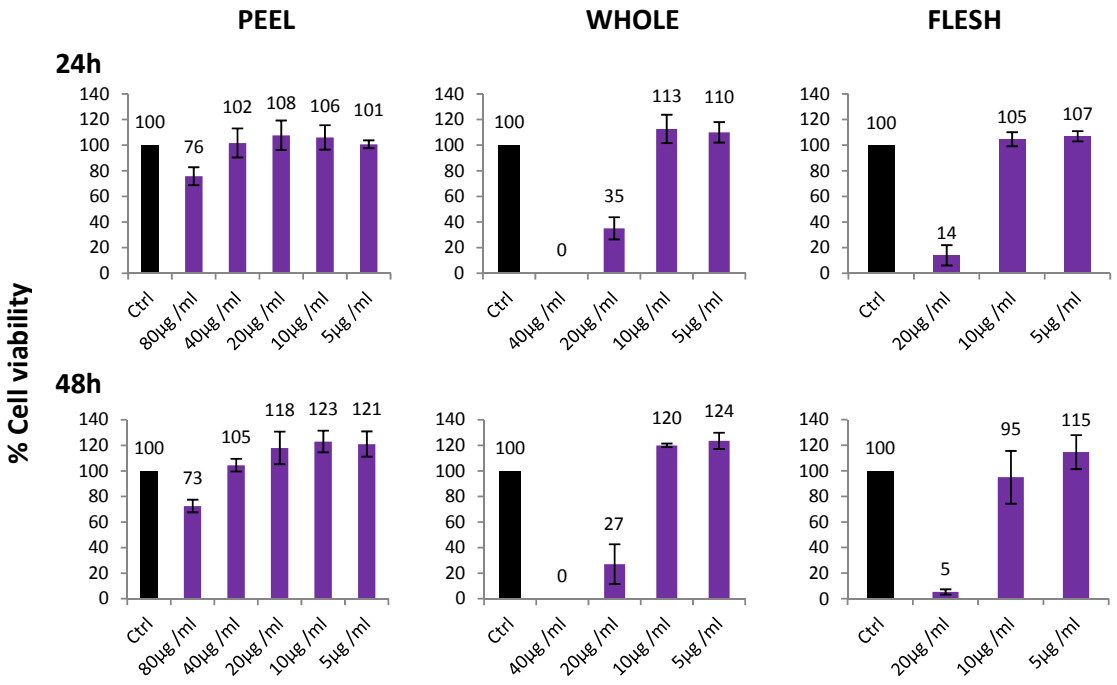


Fig. 19 Effects of a wide range of 'Fuji' apple phenolic extracts on cell viability of NIH-3T3 cells. The cells were incubated for 24, 48, 72 h with the appropriate concentrations of phenolic extracts. At the end of incubation, cell viability was measured by MTS assay as described in Materials and Methods. Values are means ± SD of cell viability calculated from at least three separate experiments.

Figures 20 and 21 show the effects on NIH-3T3 cells of total polyphenols extracted from the 'Granny Smith' and 'Gala' apples, respectively. Granny Smith peel total polyphenols showed a strong toxic effect on cells at higher concentration of 80 µg/ml (Fig. 20) differently from peel polyphenols of Gala (Fig. 21) and Fuji (Fig. 19), that at the same concentration gave a low toxicity. As regards other concentrations of Granny Smith peel polyphenols, they were not toxic excluding the concentration of 40 µg/ml, at which a slight toxicity was found, as reported in figure 20. Polyphenols extracted from whole fruit of Granny Smith showed strong toxicity only when the dose of 40 µg/ml was used. In a different way flesh polyphenols worked, in fact the only concentration of 5 µg/ml was not toxic for cells (Fig. 20).

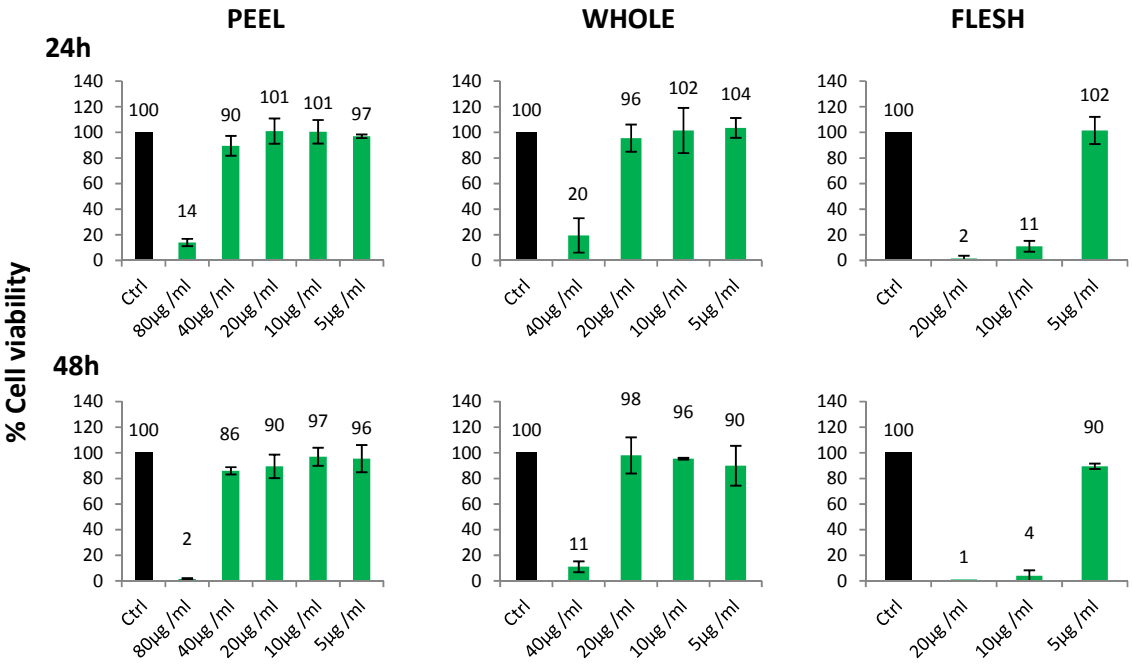


Fig. 20 Effects of a wide range of 'Granny Smith' apple phenolic extracts on cell viability of NIH-3T3 cells. The cells were incubated for 24, 48, 72 h with the appropriate concentrations of phenolic extracts. At the end of incubation, cell viability was measured by MTS assay as described in Materials and Methods. Values are means ± SD of cell viability calculated from at least three separate experiments.

Different far behavior was found for total polyphenols extracted from Gala apple tissues. The results reported in figure 21 reveal that total phenolic extracts of peel presented, more or less, a slight toxicity at all concentrations except at 5 µg/ml. Moreover, whole fruit and flesh polyphenols were more toxic than phenolic extracts of the other two cultivars, at the same concentrations analyzed (Fig. 21).

These differences among cultivars could be attribute to different polyphenol profile in each cultivar and more, inside of each cultivar, to a different distribution of polyphenols among apple tissues.

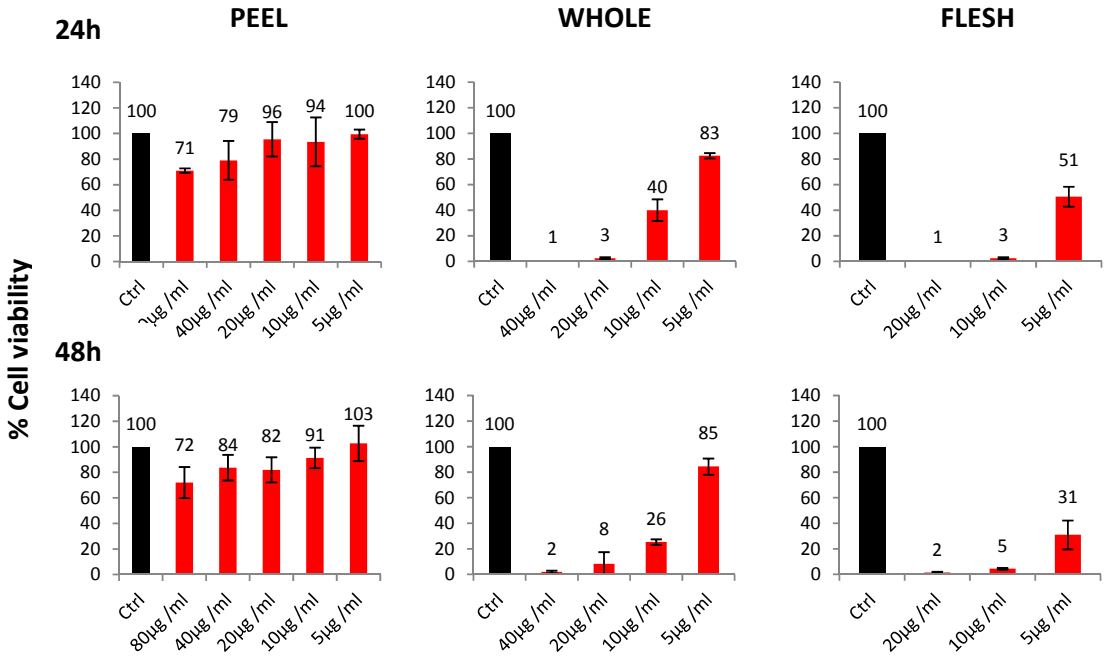


Fig. 21 Effects of a wide range of 'Gala' apple phenolic extracts on cell viability of NIH-3T3 cells. The cells were incubated for 24, 48, 72 h with the appropriate concentrations of phenolic extracts. At the end of incubation, cell viability was measured by MTS assay as described in Materials and Methods. Values are means ± SD of cell viability calculated from at least three separate experiments.

Inhibition of k-casein aggregation

The effect of peel polyphenolic extracts of each apple cultivar was investigated on k-casein aggregation by light scattering measurements at 90° angle.

The decision to perform LS experiments lies in the pitfalls sometimes presented by using other classical tests for amyloids, like ThT assay, when drugs have to be tested. ThT, for example, can be sequestered by the molecules, thus invalidating the obtained results. On the contrary, Light Scattering intensity is proportional to the molecular mass of species in solution and, therefore, is a suitable technique to monitor aggregation process.

When native k-casein is incubated in the absence of compounds (50μM =1mg/ml) under physiological conditions (37 °C, phosphate buffer pH 7.4), an increase of the scattered intensity and of particles size in solution occurred in time. The static scattered intensity, in particular, switched from 540kcps to 700kcps, from the beginning to the end of the process, with an increase of 30%. By the cumulated analysis from the intensity autocorrelation function, in the DLS modality, resulted that a correspondent increase of the average diameter of species in solution from 100 nm to 110 nm also occurred. This indicated the occurrence of an aggregation process.

When k-casein was incubated with 120μg/ml of peel polyphenolic extract of cultivar 'Fuji', the scattered intensity variation was significantly reduced. It switched from 540 kcps to 610 kcps with an increase of only 13% compared to k-casein alone condition. Moreover, no significant variation in the average diameter of species in solution was observed and it remains of about 100nm. These results show that apple peel polyphenols of cultivar 'Fuji' inhibit k-casein aggregation (Fig. 22).

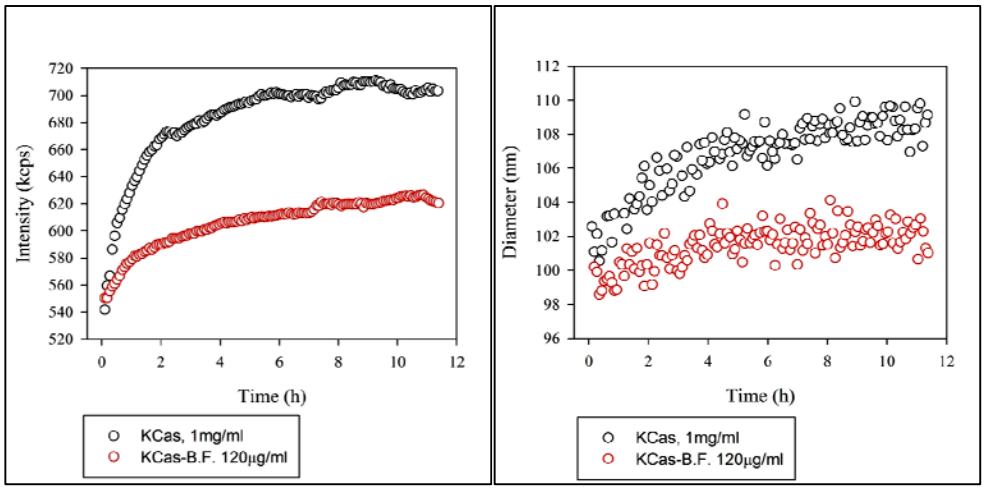


Fig. 22 Time-course of the scattered intensity of kcasein (50µM =1mg/ml) incubated in absence (black circles) and in presence of 120µg/ml of apple peel polyphenols (red circles).

In order to analyze the morphology of the κ-casein species formed at the end of kinetics in the presence or in the absence of peel polyphenolic extract of the cultivar 'Fuji', AFM experiments were performed. In the absence of polyphenols, κ-casein formed fibrillar rod-shaped structures with average height of 5.13 nm and length of 100nm (Fig. 23a). The sample of k-casein incubated in presence of peel phenolic extract (120 µg/ml) showed a significantly reduced number of fibers and amyloid aggregates compared to k-casein alone, as showed in figure 23b.

AFM results were in agreement with the data obtained by Light Scattering. These results further confirm the ability of apple polyphenols to inhibit amyloid aggregation and goes to support the use of apple polyphenols as potential therapeutic agents against amyloid diseases.

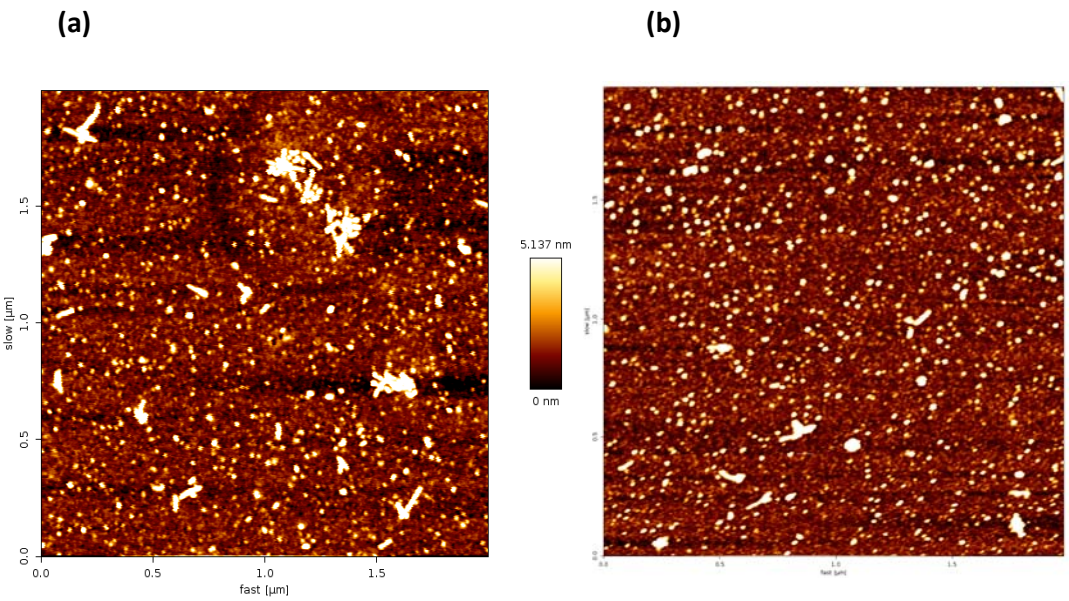


Fig. 23 κ-casein amyloid aggregates morphology (50 µM) in the absence **(a)** and in the presence of peel phenolic extract of cultivar 'Fuji'**(b)**, at the end of the kinetics profile visualized by AFM.

The concentration of 120 µg/ml of peel polyphenolic extract resulted a string inhibitor of amyloid aggregation of κ-casein. The next step was to observe the effects of polyphenols by light scattering using the lower concentration (40 µg/ml) that resulted non toxic in cell viability assay for NHI-3T3 cells. Scattering experiments were carried out incubating κ-casein with peel phenolic extract from each of the three cultivars under study at the concentration of 40µg/ml. Results revealed that with each extract a decrease in scattered intensity was observed for k-casein. In particular, in absence of polyphenols, the scattered intensity from κ-casein solution changes from 450 kcps to 640 kcps with an increase of about 40%. When k-casein was incubated with 40µg/ml of peel polyphenolic extracts, the scattered intensity switched from 450 kcps to 510 kcps with an increase of only 13%. This was observed for all the three cultivars (Fig. 24). Moreover, the kinetic profile of κ-casein incubated with peel

polyphenolic extract of cultivar 'Fuji' was characterized also from a different slope, indicative of a different aggregation rate. In fact, the less steep slope in comparison to that of other kinetic profiles suggests that the process of formation of aggregate species in the presence of peel polyphenolic extract cultivar 'Fuji' appears slowed down (Fig. 24).

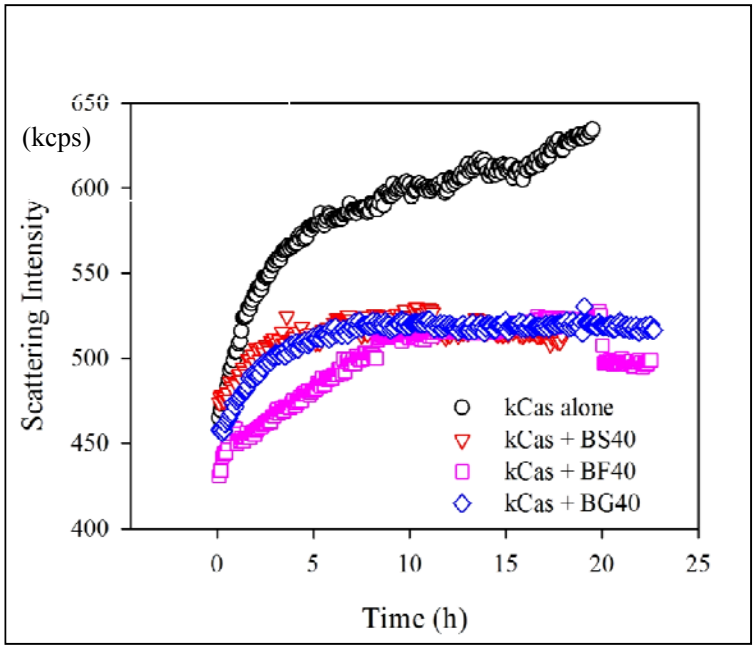


Fig. 24 Time-course of the scattered intensity of κ-casein (50μM =1mg/ml) incubated in absence (black circles) and in presence of 40μg/ml of peel polyphenols extracts of cultivar 'Granny Smith' (red triangles), 'Fuji'(pink square) and 'Gala' (blue rhombs).

4.4 CONCLUSIONS

We used native κ-casein as protein system model to test the effects of polyphenols extracted by apple fruits on the amyloid aggregation process. First, we characterized the structural feature and aggregation process of κ-casein from bovine milk, by bioinformatic tools and experimental techniques as AFM, CD and ThT assay. By analyzing amino acid composition through publicly available predictors as PONDR,

we showed that κ-casein presents high disorder content along the sequence. However the high mean hydrophobicity and low net charge per residue values analysis groups the κ-casein with ordered proteins on a Charge-Hydropathy plot. It has been reported that a protein predicted to contain significant regions of natural disordered structure, but grouped with ordered proteins on a CH plot, exists in a collapsed disordered conformation that renders κ-casein the archetypal model protein of an amyloidogenic intrinsically disordered protein.

In fact, when, incubated under physiological conditions, κ-casein goes to amyloid formation as demonstrated by increase of the ThT signal and scattered intensity. This propensity of κ-casein to form fibers is evidenced by the CD data where the k-casein, after incubation at 37 °C, is characterized by a structural conformational conversion and by the increase of the formation of beta parallel structures typical of amyloid formation. Moreover, the AFM images show fibrillar rod-shaped structures and amyloid assembly, confirming the results above reported.

Therefore, native κ-casein results a good model of amyloidogenic protein, suitable to investigate the effect of apple polyphenolic extracts on aggregation process *in vitro*. Ligth Scattering data show an effect of inhibition on the aggregation of κ-casein by peel polyphenolic extracts of the three cultivars, at the concentration of 40μg/ml, but a more strong effect is given by peel polyphenolic extract of cultivar 'Fuji', with a notable reduction of scattered intensity. In fact, the less steep slope in comparison to that of other kinetic profiles suggests that the process of formation of aggregate species in the presence of Fuji peel polyphenolic extract appears slowed down. The different effect of the three polyphenolic extracts on κ-casein aggregation can be attributed to the different polyphenolic profile in each cultivar. Indeed, 'Gala', 'Granny Smith' and 'Fuji' belong to a different apple groups and present different

phenolic compounds both qualitatively and quantitatively, as reported in literature (Vrhovsek et al., 2004; Carbone et al., 2011; Karaman et al. 2012).

The inhibitor effect of apple polyphenols is showed by AFM images too. Indeed k-casein incubated with apple polyphenols does not form a large number of amyloid assemblies but only some stocky fibrils with prevalence of granular structures.

These results agree with studies performed by Toda et colleagues (2011), which have demonstrated the ability of apple procyanidins to inhibit the aggregation of amyloid-β peptide *in vitro* promoting their potential use as anti-aggregation agents in amyloid aggregation processes. Our work provides new indications to investigate the action of natural polyphenols as potential therapeutic agents for amyloidosis, supporting the use of small molecules inhibiting amyloid aggregation, that is one of the most modern strategy searched for the treatment of neurodegenerative diseases.

GENERAL CONCLUSIONS

5. GENERAL CONCLUSIONS

Apples are one of the most produced, exported and consumed fruits in the world. Apples are also considered the "health fruit" and they are identified by human nutritionists as functional food, thank to their potentially positive effect on health beyond basic nutrition. The health benefits of apples are mainly attributed to their phenolic content.

The three apple cultivars, 'Gala', 'Granny Smith' and 'Fuji', grown in Sicily in an organic farming system, present chemical-physical properties, total polyphenolic content and antioxidant capacity included within commercially acceptable ranges. In particular, the found significant differences, depending on the apple tissue and variety type, are in agreement with data reported by others authors (Hampson et al. 2003, Drogoudi et al. 2008, Henríquez et al. 2010, Thompson-Witrick et al. 2014; etc). Apple peel extract contains the highest total phenolic amount when compared to flesh and whole fruit extract. Peel of the analyzed apples contains about 3 times more polyphenols than whole tissue and about 6 times more polyphenols than flesh tissue. Moreover, a high correlation between phenolic content and antioxidant capacity is evidenced. The highest phenolic content and antioxidant activity are given in peel tissue for 'Fuji' and in flesh tissue for 'Granny Smith'.

The three apples varieties for their qualitative characteristics represent a considerable alternative to traditional Sicilian products and a great deal for the fruit market.

The high total phenolic content and the high antioxidant capacity of peel extract stimulated the interest toward the application of these apple extracts on amyloid aggregation involved in neurodegenerative diseases. For this research, native κ-casein has been used as protein system model. K-casein has specific chemical-physical properties (high mean hydrophobicity, low net charge per residue and

likelihood of forming β-strands) and contains significant regions of natural disordered structure. It exists in a collapsed disordered conformation that renders κ-casein the archetypal model protein of an amyloidogenic intrinsically disordered protein.

Indeed, when incubated under physiological conditions (37 °C, phosphate buffer pH 7.4), κ-casein goes to amyloid formation as demonstrated by increase of the ThT signal and scattered intensity. This propensity of k-casein to form fibers is evidenced by the CD data. Moreover, the AFM images show fibrillar rod-shaped structures and amyloid assembly, confirming the results reported above.

Therefore, native κ-casein results a good model of amyloidogenic protein, suitable to investigate the effect of apple polyphenolic extracts on aggregation process *in vitro*. Ligth Scattering data show a effect of inhibition on the aggregation of κ-casein by peel polyphenolic extracts of the three cultivars, at the concentration of 40μg/ml, but a more strong effect is given by peel polyphenolic extract of cultivar 'Fuji', with a notable reduction of scattered intensity. In fact, the less steep slope in comparison to that of other kinetic profiles suggests that the process of formation of aggregate species in the presence of Fuji peel polyphenolic extract appears slowed.

The inhibitor effect of apple polyphenols is showed by AFM images too. Indeed k-casein incubated with apple polyphenols doesn't form a large number of amyloid assemblies but only some stocky fibrils with prevalence of granular structures.

These results agree with studies performed by Toda et colleagues, which have demonstrated the ability of apple procyanidins to inhibit the aggregation of amyloid-β peptide *in vitro* promoting their potential use as anti-aggregation agents in amyloid aggregation processes. Our work give new suggestions to investigate the action of natural polyphenols as potential therapeutic agents for amyloidosis, supporting the

use of small molecules inhibiting amyloid aggregation, that is one of the most modern strategy searched for the treatment of neurodegenerative diseases.

On the basis of these results an added value can be attribute to the three apple cultivars grown in Sicily.

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7. List of paper and reports

- Guarrasi V., Rappa G.C., Lo Bosco F., Vilasi S., Costa M., Germanà M.A., San Biagio P.L. "Quality control of foods and nutraceutical applications: antioxidant properties of polyphenols". 2nd Italian National Conference on Condenced Matter Physics , Palermo, Italy, 28 September- 2 October 2015

- Rappa G.C., Guarrasi V., Vilasi S., Costa M.., Germanà M.A and San Biagio P.L. "Influenza dell' Acido Gallico e dell'Acido Ferulico, componenti della frazione polifenolica di succhi di pompelmo, sui processi di aggregazione di proteine amiloidi". Agricoltura, Cibo e Salute – Orto Botanico di Palermo, 2014.

- Rappa G.C., Guarrasi V., Vilasi S., Germanà M.A and San Biagio P.L. "Variazioni dei meccanismi di aggregazione e di inibizione di proteine amiloidi da parte di due polifenoli, Procianidina B2 ed (-)-Epicatechina, presenti nelle mele". Agricoltura, Cibo e Salute – Orto Botanico di Palermo, 2014.

- Rappa G.C., Guarrasi V., Vilasi S., Germanà M.A., San Biagio P.L.,."Procianidina B2 ed (-)-Epicatechina presenti nelle mele: studio preliminare del loro effetto sui meccanismi di aggregazione e di inibizione di proteine amiloidi". 86° Congresso della Società Italiana di Biologia Sperimentale, 24 th -25 th October 2013.

~ Rappa G.C., Guarrasi V., Vilasi S., Germanà M.A., San Biagio P.L.,. “Studio preliminare sull’effetto di polifenoli presenti in agrumi sui meccanismi di aggregazione amiloide”. Meeting IBIM/STEBICEF/UNIPA: "Biotecnologie: ricerca di base, interdisciplinare e traslazionale in ambito biomedico", 27th-28th June 2013.

~ Rappa G.C., Vilasi S., Carrotta R., San Biagio P.L., Bulone D.. “Studio del disordine intrinseco di diverse caseine con predittori bioinformatici:implicazioni nel campo delle patologie neurodegenerative”. Meeting IBIM/STEBICEF/UNIPA: "Biotecnologie: ricerca di base, interdisciplinare e traslazionale in ambito biomedico", 27th-28th June 2013.

~ Vilasi S., Carrotta R., Rappa G.C., San Biagio P.L., Bulone D.. “Intrinsic disorder and chaperon-like activity of different caseins”. Italian Society of Biophysics (SIBPA), 57th Annual Meeting, Philadelphia.

8. Seminar participations and courses

~ Epigenetics and hormone dynamics during pollen embryogenesis in crop species and fruit tree. Dr. María-Carmen Risueño. Facoltà di Agraria, Palermo, maggio 2013.

~ Dissecting pollen embryogenesis: a biotechnological tool for crop breeding, potentials and limitations. Dr. Pilar S. Testillano. Facoltà di Agraria, Palermo, maggio 2013.

~ On farm water management for fruit crops in Mediterranean area. Prof. Ahmed El-Araby. Facoltà di Agraria, Palermo, ottobre 2013.

~ Do gibberellins regulate flowering in citrus?. Prof. M. Agustí. Facoltà di Agraria, Palermo, gennaio 2013.

~ Crioconservazione e crioterapia per la salvaguardia della biodiversità vegetale. Dr. M. Lambardi. Facoltà di Agraria, Palermo, aprile 2013.

~ Le malattie del legno della vite e il complesso di funghi associate: sintomi, interazione ospite-patogeno, lotta e diagnosi. Prof. L. Mugnai. Facoltà di Agraria, Palermo, aprile 2013.

~ Fruit growth and root development relationships in loquat. Dr.ssa C. Reig. Facoltà di Agraria, Palermo, gennaio 2013.

~ Evoluzioni metodologiche sull'incapsulamento dell'olivo. Dr. Maurizio Micheli. Facoltà di Agraria, Palermo, marzo 2014.

~ Thermo Scientific – Palermo. Sicurezza alimentare e difesa dell'autenticità delle produzioni nazionali. Orto Botanico, Palermo, 2014

~ Subject: "Interpretation and presentation of scientific results: written and oral" - Università degli Studi di Palermo - Dipartimento di Scienze Agrarie e Forestali.

~ Subject: "Statistical analysis" - Università degli Studi di Palermo - Dipartimento di Scienze Agrarie e Forestali.

9. Website citations

- Borsa Merci Telematica, www.bmti.it
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- Istat 2010-2012. Le principali coltivazioni legnose annata agraria 2011-2012 su http://www.istat.it/it/archivio/124365
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- The World Apple and Pear association, www.wapa-association.org

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