
**UNIVERSITÀ DEGLI STUDI DI MILANO**

**Department of Food, Environmental and Nutritional Sciences (DeFENS)**

**PhD Course in Food Systems**

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# Characterization of Pigmented Cereal Grains and Production of Functional Food: Anthocyanins-Enriched Pasta

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**زندگی، یعنی تحقیق، پژوهش، و فهمیدن چیزی جدید.**

**پروفسور حسابی**

**To my love FARAMARZ**

**Mom and Dad**

**Saba and Ali**

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# Table of contents

# CHAPTER 1

## 1- INTRODUCTION

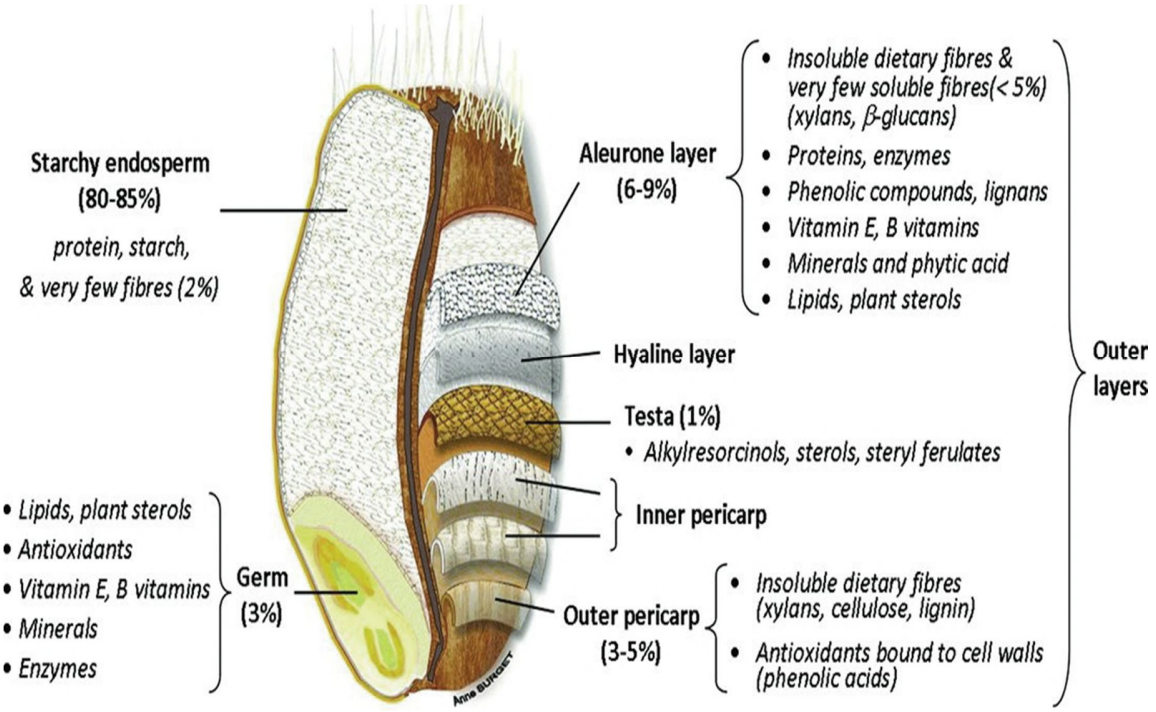
### 1-1 Pigmented cereal grains

Cereals can be defined as the grain or edible seed of the grass family. Botanically, these fruits are called 'caryopsis' and are cultivated in huge quantities as they provide more food energy than any other type of crop, therefore, they are known as staple crops. Cereals are rich sources of minerals, vitamins, carbohydrates, oils, proteins, and fats. They are also very rich in complex carbohydrates. Consuming whole grain products is helpful in preventing cancer, colon disorders, and high blood sugar levels. Cereal grains also enrich individual's overall health with abundant proteins, fats, lipids, minerals, vitamins, and enzymes (Serna-Saldivar 2016).

The structure of whole cereal grain generally is composed of an outer bran coat, a starchy endosperm, and a germ. Bran is the outer layers of the kernel which is made of about 5% of the kernel (Figures 1 & 2). The kernel is rich in fiber and minerals while the bran contains high amounts of thiamine and riboflavin. After refining, the bran layer is removed and the aleurone layer is exposed, which lies just below the bran. This layer is also rich in phosphorous, proteins, fat, and thiamin. In most cases endosperm used for further processing, as this large central part of the kernel has a high percentage of starch and proteins, but has low vitamin or mineral content. The small structure at the rear part of the kernel is known as the germ which is rich in protein, fat, minerals and vitamins (Serna-Saldivar 2016).

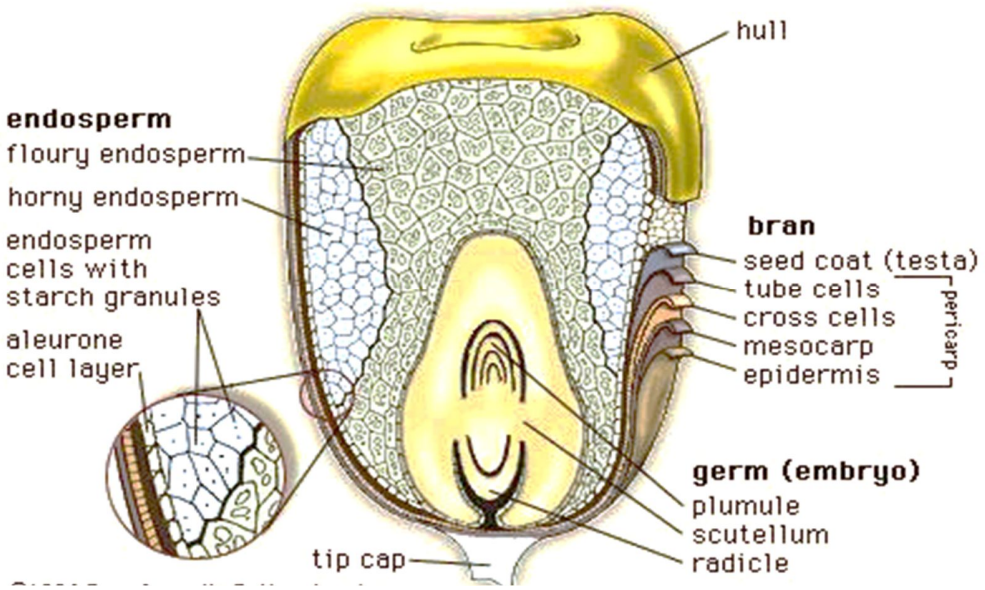
Recent interest in the colorful types of cereal grains relates to the abundance of natural pigments like anthocyanins. Anthocyanins are responsible for the blue, purple, red or black color in different cereals, and they are mostly located in the pericarp or aleurone layers (Liu, Qiu et al. 2010). Typically, these layers are discarded during milling, due do the inconvenience and problems they cause in further processing

Traditionally these pigments were used as natural colorants in food, but lately many health benefits have been attributed to anthocyanins. Today, pigmented cereal grains are treated into two main process; whole-grain products are produced after milling whole kernel aiming at specific color or taste with nutritional values, but anthocyanin-enriched fractions may be obtained after a de-branning steps, and used for further processing. Novel methods of de-branning have improved the yield of anthocyanin-rich fractions, particularly in wheat grains.




Adapted from Barron et al. (2007)

*Figure 1: Wheat grain structure*

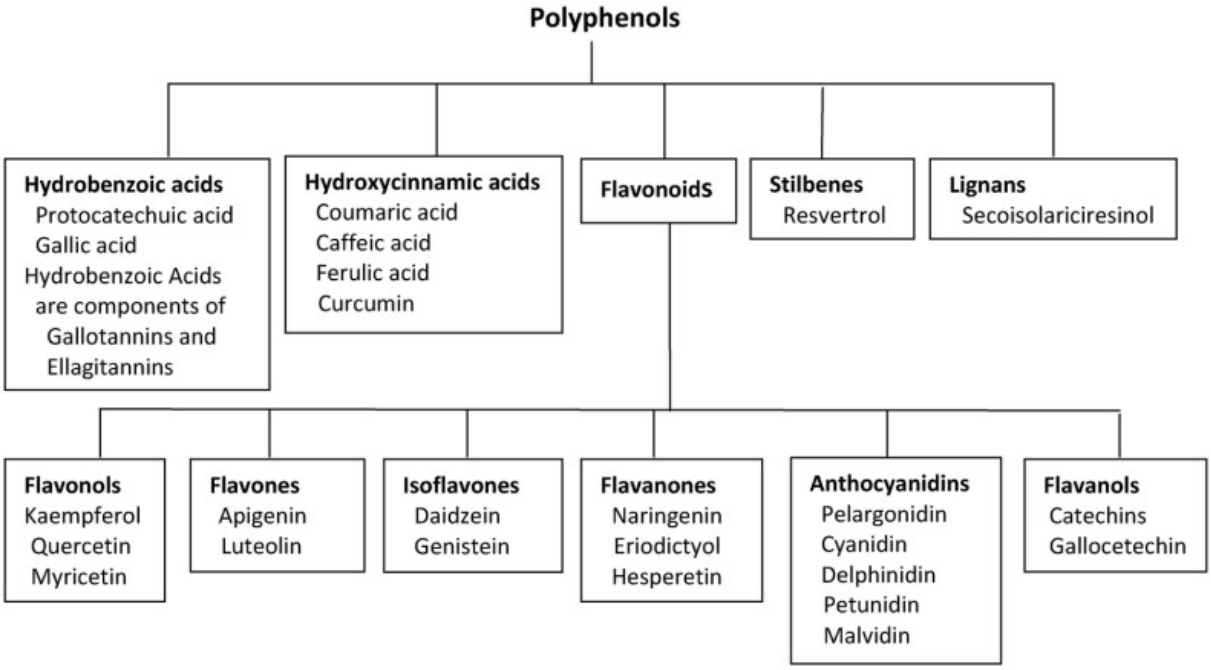



Adapted from Encyclopedia Britannica (2004)

*Figure 2: Corn grain structure*

## 1-2 Polyphenols

Polyphenols are one of the largest groups of phytochemicals in nature, and more than 8000 compounds fall into this category (Figure 3). This class of chemicals includes molecules that present in their structure a phenolic group, that is linked directly to an aromatic ring. These molecules are the secondary metabolites of many plants and are generally involved in their defense against ultraviolet radiation and pathogen attacks (Manach, Scalbert et al. 2004). Being a very large class of compounds, they are divided into different subgroups based on the number of phenolic rings present in their structure, the structural elements that bind these rings to each other, or to the substituents linked to the rings themselves (Tsao 2010). The substances of major biochemical interest within this category are the anthocyanins, belonging to the flavonoid subgroup but in which they are distinguished by their ability to form the flavylium cation that is responsible for numerous bioactive properties (Figure 4).




Adapted from Hardman (2014)

*Figure 3: Classification of polyphenols*

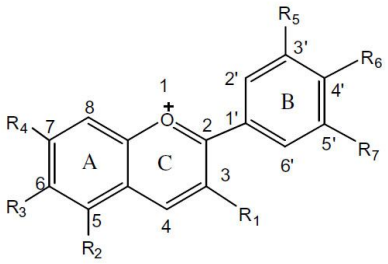



*Figure 4: The basic structure of Anthocyanins*

## 1-3 Anthocyanins

Anthocyanins are water-soluble flavonoids that are responsible for the red, orange, purple and blue colors and are known for their health benefits which include antioxidant activity, anti-

inflammatory activity, regulation of immune responses, anti-cancer, cardiovascular disease protection, enhancement of visual acuity, and hypoglycemic effects (Lila 2004, Abdel-Aal, Young et al. 2006). The basic structure of anthocyanins has two aromatic rings (A and B) and one heterocyclic ring (C) with an oxygen atom. Anthocyanidins, or aglycones, are known as anthocyanins when in their glycoside form, bound to a sugar moiety. There are several hundred known anthocyanins varying in hydroxyl groups (position and amount), methylation of these hydroxyl groups, identity and number of sugars attached to the skeleton, extent of sugar acylation and type of acylating agents (Clifford 2000). In Table 1 the substitution patterns of the six most common anthocyanidins: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin has been listed.

| | |
|---|---|
| Delphinidin | 3,5,7,3’,4’,5’-OH |
| Cyanidin | 3,5,7,3’,4’-OH |
| Pelargonidin | 3,5,7,4’-OH |
| Petunidin | 3,5,7,4’,5’-OH; 3’-OMe |
| Peonidin | 3,5,7,4’-OH; 3’-Ome |
| Malvidin | 3,5,7,4’-OH; 3’,5’-Ome |

*Table 1: Anthocyanidins substitution pattern*

In cereal grains anthocyanins are mainly localized in the bran layers and impart black, purple, blue or reds hue to the grains, based on the amount of accumulated anthocyanins in different layers. For example, the purple color in wheat is mainly because of existence of cyanidin-3-glucoside in pericarp layer, whereas the accumulation of delphinidin in aleuronic layer is responsible for blue color. High concentrations of pelargonidin and catechin-tannins gives the red hue to wheat seeds. (Abdel-Aal, Abou-Arab et al. 2008, Hosseinian, Li et al. 2008). Anthocyanins in corn are located primarily in the aleurone layer of the endosperm.

The anthocyanin profile has been studied in numerous types of colored cereal grains, and ranges from simple ones, with only a few pigments, to very complex, with numerous pigments.

Black rice, red rice, and blue barley have simple composition profiles, blue and purple wheat have intermediate profiles, while the profiles for colored corns are complex with 18-27 different types of anthocyanins. Table 2 summarizes the anthocyanins profile in differently colored grains.

| **Anthocyanin** | **Cereal type and content of individual species** | | | |
|---|---|---|---|---|
| | ***Species*** | ***Source*** | ***mg/kg*** | ***Ref.*** |
| **Cyanidin-3-glucoside** | Corn | Purple (whole meal) | 1135.4 | (Asenstorfer, Wang et al. 2006) |
| | | Purple | 298.9 | (Urias-Lugo, Heredia et al. 2015) |
| | | Blue (whole meal) | 17.4 | (Asenstorfer, Wang et al. 2006) |
| | | Shaman Blue | 110.1 | (Urias-Lugo, Heredia et al. 2015) |
| | | Red | 284.5 | (Urias-Lugo, Heredia et al. 2015) |
| | Wheat | Purple | 4.0 | (Urias-Lugo, Heredia et al. 2015) |
| | | Purple | 10.3 | (Li, Somavat et al. 2017) |
| | | Blue | 3.1 | (Li, Somavat et al. 2017) |
| | | Blue | 20.3 | (Urias-Lugo, Heredia et al. 2015) |
| | | Red | 4.0 | (Li, Somavat et al. 2017) |
| | Rice | Black | 2013.0 | (Urias-Lugo, Heredia et al. 2015) |
| | | Red | 14.0 | (Urias-Lugo, Heredia et al. 2015) |
| | Barley | Blue | 1.2 | (Urias-Lugo, Heredia et al. 2015) |
| | | Purple | 99.1 | (Bellido and Beta 2009) |
| **Cyanidin 3-chloride** | Barley | Purple | n.a. | (Li, Somavat et al. 2017) |
| **Cyanidin-3-arabinoside** | Wheat | Purple | n.a. | (Li, Somavat et al. 2017) |
| **Cyanidin 3-rutinoside** | Rice | Black | 19.9 | (Urias-Lugo, Heredia et al. 2015) |
| | | Red | 1.3 | (Urias-Lugo, Heredia et al. 2015) |
| | Corn | Shaman Blue | 1.1 | (Urias-Lugo, Heredia et al. 2015) |
| | | Red | 284.5 | (Urias-Lugo, Heredia et al. 2015) |
| | Wheat | Blue | 16.8 | (Urias-Lugo, Heredia et al. 2015) |
| **Cyanidin-3-(2G-xylosylrutinoside)** | *Presence reported, no further details* | | | (Li, Somavat et al. 2017) |
| **Cyanidin-3-(6”-succinylglucoside)** | Corn | Purple | n.a. | (Abdel-Aal, Young et al. 2006) |
| | | Blue | n.a. | (Abdel-Aal, Young et al. 2006) |
| | | Red | n.a. | (Abdel-Aal, Young et al. 2006) |
| | Wheat | Purple | 0.6 | (Urias-Lugo, Heredia et al. 2015) |
| | | Purple | 1.1 | (Urias-Lugo, Heredia et al. 2015) |
| | | Purple | 1.2 | (Urias-Lugo, Heredia et al. 2015) |
| **Cyanidin-3-(3”,6”-dimalonylglucoside)** | Corn | Purple (whole meal) | 398.2 | (Asenstorfer, Wang et al. 2006) |
| | | Blue (whole meal) | 40.1 | (Asenstorfer, Wang et al. 2006) |
| | | Shaman Blue | 1.5 | (Urias-Lugo, Heredia et al. 2015) |
| | Rice | Black | 71.8 | (Urias-Lugo, Heredia et al. 2015) |
| | | Red | 1.7 | (Urias-Lugo, Heredia et al. 2015) |
| **Cyanidin-3-rutinoside-3'-glucoside** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) |
| **Cyanidin-3-galactoside** | Wheat | Purple | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | | Blue | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| **Cyanidin-3-(6”-feruloylglucoside)-5-glucoside** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |

| | | | | |
|---|---|---|---|---|
| **Cyanidin-3-(6″-malonylglucoside)** | Corn | Purple | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | | Blue | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | | Red | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | Wheat | Purple | 1.2 | (Urias-Lugo, Heredia et al. 2015) |
| **Delphinidin-3-glucoside** | Wheat | Purple | 65.5 | (Abdel-Aal, Young et al. 2006) |
| | | Purple | 13.7 | (Urias-Lugo, Heredia et al. 2015) |
| | Barley | Purple | 93.0 | (Bellido and Beta 2009) |
| **Delphinidin-3-(6″-malonylglucoside)** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) |
| **Delphinidin-3-rutinoside** | Wheat | Blue | 49.6 | (Urias-Lugo, Heredia et al. 2015) |
| | | Blue | 33.4 | (Garg, Chawla et al. 2016) |
| | Barley | Purple | n.a. | (Garg, Chawla et al. 2016) |
| **Delphinidin-3-sambubioside** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) |
| **Delphinidin-3-caffeoylglucoside** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) |
| **Pelargonidin-3-glucoside** | Corn | Purple | n.a. | (Abdel-Aal, Young et al. 2006) |
| | | Blue | n.a. | |
| | | Red | n.a. | |
| **Pelargonidin-3-(6″-malonylglucoside)** | Corn | Purple | 51.6 | (Asenstorfer, Wang et al. 2006) |
| | | Blue | 6.5 | (Asenstorfer, Wang et al. 2006) |
| | | Red | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | Wheat | Purple | n.a. | |
| **Pelargonidin-3-succinyl-glucoside** | Corn | Purple | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | | Blue | | |
| | | Red | | |
| | Wheat | Purple | | |
| **Pelargonidin-3-rutinoside** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| **Peonidin-3-glucoside** | Corn | Purple | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | | Blue | | |
| | | Red | | |
| | Rice | Black | 162.1 | (Urias-Lugo, Heredia et al. 2015) |
| | | Red | 2.5 | (Urias-Lugo, Heredia et al. 2015) |
| | Wheat | Purple | 2.1 | (Urias-Lugo, Heredia et al. 2015) |
| | | Blue | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| **Peonidin-3,5 diglucoside** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) |
| **Peonidin-3-rutinoside** | Wheat | Black | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | | Blue | 1.2 | (Urias-Lugo, Heredia et al. 2015) |
| **Peonidin-3-arabinoside** | Wheat | Purple | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | | Blue | 2.2 | (Li, Somavat et al. 2017) |
| **Peonidin-3-galactoside** | Wheat | Blue | 1.9 | (Li, Somavat et al. 2017) |
| | | Purple | 0.6 | (Li, Somavat et al. 2017) |
| | | Red | 0.3 | (Li, Somavat et al. 2017) |
| **Peonidin-3-rutinoside-5-glucoside** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) |
| **Peonidin-3--(6″-malonylglucoside)** | Corn | Purple | 145.1 | (Asenstorfer, Wang et al. 2006) |
| | | Blue | 7.4 | (Asenstorfer, Wang et al. 2006) |
| | Wheat | Purple | 0.6 | (Urias-Lugo, Heredia et al. 2015) |
| | | Purple | 0.9 | (Urias-Lugo, Heredia et al. 2015) |

| | | | | |
|---|---|---|---|---|
| | | Purple | 0.5 | (Urias-Lugo, Heredia et al. 2015) |
| **Peonidin-3-succinyl-glucoside** | Wheat | Purple | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | Corn | Red | | |
| **Malvidin-3-glucoside** | Wheat | Purple | 0.5 | (Li, Somavat et al. 2017) |
| | | Blue | 12.4 | (Li, Somavat et al. 2017) |
| | | Red | 0.22 | (Li, Somavat et al. 2017) |
| **Malvidin-3-rutinoside** | Wheat | Blue | 2.0 | (Urias-Lugo, Heredia et al. 2015) |
| **Malvidin-3-(6"-p-caffeoylglucoside)** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) |
| **Malvidin-3-rutinoside-5-glucoside** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) |
| **Petunidin-3-glucoside** | Wheat | Blue | 2.2 | (Urias-Lugo, Heredia et al. 2015) |
| | | Purple | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | | Black | n.a. | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |
| | Barley | Blue | 2.9 | (Urias-Lugo, Heredia et al. 2015) |
| | | Purple | 37.0 | (Bellido and Beta 2009) |
| **Petunidin-3-rutinoside** | Wheat | Blue | 4.5 | (Bellido and Beta 2009) |
| **Petunidin-3-rutinoside-5-glucoside** | *Presence reported, no further details* | | | (Abdel-Aal, Young et al. 2006) , (Garg, Chawla et al. 2016) |

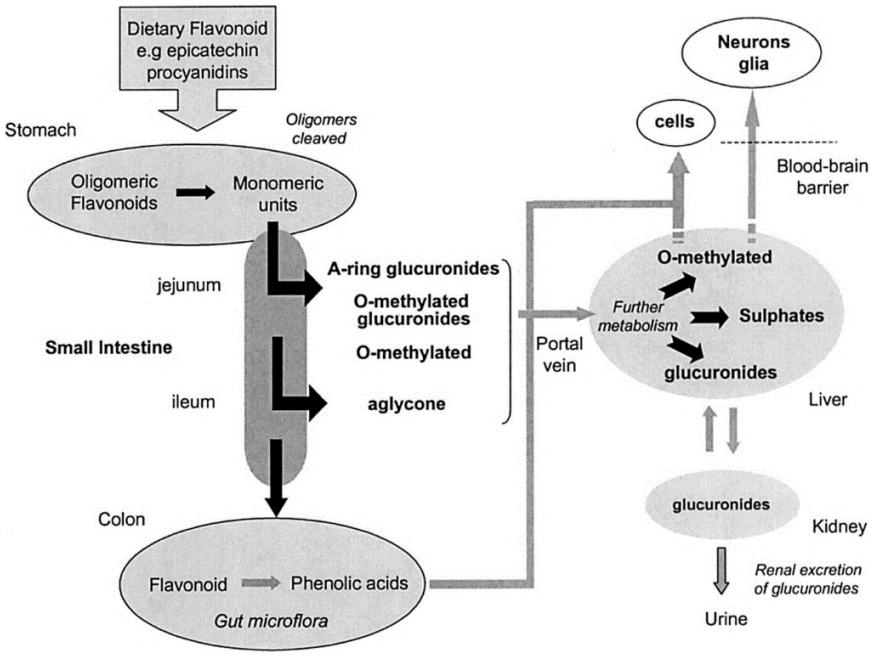
*Table 2: Anthocyanin content and type in different pigmented grain*

There are many sources of anthocyanins: the most common include varieties of fruit and vegetables such as blueberries, raspberries, strawberries, cherries, sour cherries, grapes, oranges and pomegranate, as well as elderberry, mulberry or blackcurrant. A daily intake of between 500 mg and 1 g is estimated, but this value is exceeded if supplements are consumed (Lila 2004). In recent years some varieties of legumes and cereals are however very studied for their content of anthocyanins and polyphenols, such as beans, soybeans, black rice or blue/purple corn and wheat (Olivas-Aguirre, Rodrigo-García et al. 2016).

## 1-4 Bioavailability of Anthocyanins

"Bioavailability" means "the speed and the rate to which an active ingredient or a fraction of that is absorbed and becomes available at the action site" (Chen, Somavat et al. 2017). The methods for determining bioavailability include both human and animal tests and experiments that simulate in vitro performance. Although anthocyanins have shown modest bioavailability, they

have the ability to produce numerous positive effects in both laboratory and in vivo experiments (Kamiloglu, Capanoglu et al. 2015). Therefore, understanding the bioavailability of anthocyanins is critical in validating their health benefits and in developing functional foods that contain anthocyanins (He and Giusti 2010). The absorption, metabolism and elimination of anthocyanins in humans and other in vivo model systems aids in understanding the bioactivity of these pigments.




Adapted from Spencer 2003

*Figure 5: Pathways of dietary flavonoids absorption, metabolism, distribution, and excretion*

Anthocyanins absorption begins in the stomach, where they are released from the food matrix. Indeed, blood plasma levels of anthocyanin were appreciable within minutes of consumption, with excretion occurring within 6-8 hours (McGhie and Walton 2007). However, the main site of anthocyanin absorption is the small intestine, where the P-glucosidases release aglycones from the anthocyanin. Aglycones are smaller and consequently can pass through the

epithelial layer of the small intestine more easily. Intact glycosides may also be absorbed in the small intestine by passive diffusion or through the sodium dependent glucose transporter. Acylated anthocyanins are less efficiently absorbed because of their large molecular size and hydrophobic character. After absorption, enzymes convert them to glucuronides, sulfates, and methylated derivatives in the intestinal epithelium, liver, and kidney (Figure 5). Anthocyanins reaching the large intestine are most likely to be excreted in the feces because of the decreased absorption efficiency of the colon. However, the anthocyanins may be available for microbial fermentation but to date no research has confirmed these events in vivo. Absorbed intact anthocyanins are excreted via urine. Bile secretion may be another means of elimination.

## 1-5 Functional properties of anthocyanins

Anthocyanins, together with the polyphenol group, have been extensively studied in these years in order to investigate its multiple bioactive properties. The traditional and popular medicine of the whole world has always recognized these compounds of therapeutic properties and, in fact, these pigments have been used in the treatment of various diseases because they are considered mediators of a wide range of health benefits, such as 'use of Hibiscus sp. in the treatment of liver disease and hypertension, or the application of blueberry to treat eye disorders, infections, diarrhea and various other diseases (Smith, Marley et al. 2000, Wang, Wang et al. 2000). However, it is only recently that the specific pharmacological properties of the anthocyanins have been verified, measured and quantified, conclusively, in vitro, in vivo, and by clinical trials. In table 3 some therapeutic effects of anthocyanins cyaninidin-3-glucoside in different cell lines has been listed (Olivas-Aguirre, Rodrigo-García et al. 2016).

| **Cell Line** | **Cy3G dose** | **Mechanism** |
|---|---|---|
| Erythrocytes | 10–100 μM | ↓ cholesterol and TBAR in cell membrane |
| Human adherent macrophages (from U937 cells), oral epithelial cells (GMSM-K) and gingival fibroblasts (HGF-1) | 5–25μ g/mL | ↓ IL-6 level (macrophages), cytoprotection (GMSM-K, HGF-1) against nicotine toxicit |
| Colon (Caco2), liver (HepG2), prostate (PC3) | Blue maize ACNs (189–500 μg/g) | ↓ cell proliferation |

| | | |
|---|---|---|
| Gastric cancer (KATO III) | 12.5 µM | ↓ Helicobacter pylori VacA-induced cell death |
| Adipocytes (3T3-L1) | 50 µM | ↓ FoxO -mediated transcription of lipase |
| Hepatome (HepG2) | 1–100 µM | ↑ fatty acid oxidation and AMPK activity |
| Adipocyte | 0.5–50 µM + docosahexanoic acid | ↓ basal lipolysis , inflammatory markers |
| Breast cancer (BT474m MD-MB231, MCF7) | 10 µM | ↓ invasion/increased expression of ErB2 |
| Murine thymoma (EL-4T) | 2.5–5.0 µg/mL | ↓ Il-3 & IL-4 by GATA-3 inhibition |
| Pheochromocytoma (PC-12) | IC50, 15.3 µg/m | ↓ ATP-induced [Ca2+] increase |
| Colon cancer (HT-29) | 25 µM | ↓ IL-8, nitrite, PGE2 |
| human aortic epithelial cells | 0.5–50 µM | ↑ oxiesterol efflux, ↑ABCG /ABCA expressi |
| Heart (isolated mitochondria) | 20 Mm | ↑ phosphorylation, ATP production, ↑$e^-$ carrier |
| Adipocytes (steam cells) | 100 µg/m | ↓ IL-6 level |
| Ovarian cancer (HO-8910PM) | IC50, 13.8 µg/m | ↑ apoptosis, ↓ mucin 4 expression |

*Table 3. Pharmacological properties of Cyanidin-3-glucosid in different cell model lines*

## 1-5-1 Antioxidant capacity of anthocyanins

Oxidative stress is the basis of the most common chronic diseases. Therefore, its modulation is an interesting goal both for their prevention and as a therapeutic approach. Anthocyanins present in many foods and drinks have shown an important role in the prevention of various diseases such as cancer, CVD, type II diabetes, obesity, vision-related diseases, damage to the immune system or degenerative disorders such as Alzheimer's and their antioxidant capacity is undoubtedly the basis of these beneficial effects (Miguel 2011).

Free radicals are molecules that possess an unpaired electron and for this they are very reactive. These are substances that are necessary for both cells and for living organisms in general. In fact, some of them, such as nitric oxide, superoxide anion, the radical species of oxygen (ROS) or nitrogen (RNS) are important mediators within cells. However, it is necessary that the organism is always in a state of equilibrium and that it does not go into a state of oxidative stress that occurs when there is an excessive quantity of radical species, that cannot be disposed of by the internal antioxidant systems, or when there is a malfunction in the disposal activities of such compounds. Excess free radicals can damage various cell structures, such as

DNA, enzymes, proteins and lipids, and are therefore implicated in a growing number of diseases and in the physiological process of aging (Chung, Sung et al. 2006).

Antioxidant substances are molecules that counteract the action of free radicals. Our body has its own, both enzymatic (superoxide dismutase, catalase, glutathione peroxidase and reductase, thioredoxin) and non-enzymatic (glutathione, vitamin E, vitamin C). The defense mechanisms are essentially distinct in primary action systems, which work by converting the radicals into less harmful molecules or by preventing the formation of radicals from other molecules, or secondary ones, that capture the radicals and prevent chain reactions. This second category also includes naturally occurring antioxidants which, if introduced with the diet, thanks to a negative redox potential, provide help to endogenous systems in counteracting the oxidative stress state by promptly yielding electrons to the radical species without converting them in highly reactive species.

Anthocyanins, as well as other polyphenols, possess antioxidant properties thanks to the ability to yield hydrogen and the electrons of their phenolic groups. They also have a chelation potential that can play a role in protecting against the reactions of radicals induced by copper and iron (Miguel 2011). In in-vitro studies the antioxidant capacity of anthocyanins have evaluated for particular types of anthocyanins, among which the Cyanidin-3-O-Glucoside is the most widely used (Olivas-Aguirre, Rodrigo-García et al. 2016, Serra, Almeida et al. 2016).

### 1-5-2 Anti-inflammatory effects of anthocyanins

Inflammation is a multifaceted and complicated process implicated in infiltration and activation of various immune and inflammatory cells, in cytokine production, in signal transduction and in other molecular mechanism that results in a widespread response in cells, organs and tissues. Many drugs and dietary natural compounds are able to exert protective action against inflammation by different mechanisms, acting on several pathological aspects through interference with chronic inflammatory response. The relationship between inflammation and many diseases is well established. Several studies demonstrate that inflammatory pathways are critical targets in the treatment and prevention of these diseases (Kotas and Medzhitov 2015) (Agrawal and Kant 2014, Hartman and Frishman 2014, Siti, Kamisah et al. 2015, Ribatti 2017).

Anthocyanins and polyphenols are reportedly able to control the intracellular signaling cascades as the process of inflammation progresses, by: 1) avoiding the causes of tissue damage, 2) inhibiting signaling pathways and the activation of transcription factors, 3) inhibiting oxidant-generating enzymes and mediators of inflammation, 4) scavenging reactive oxygen and nitrogen species generated by inflammatory cells, and 5) modulating angiogenesis. Figure 6 lists some of the different mechanisms used by anthocyanins in modulating inflammatory pathways, either via gene expression or by affecting enzymatic activities.

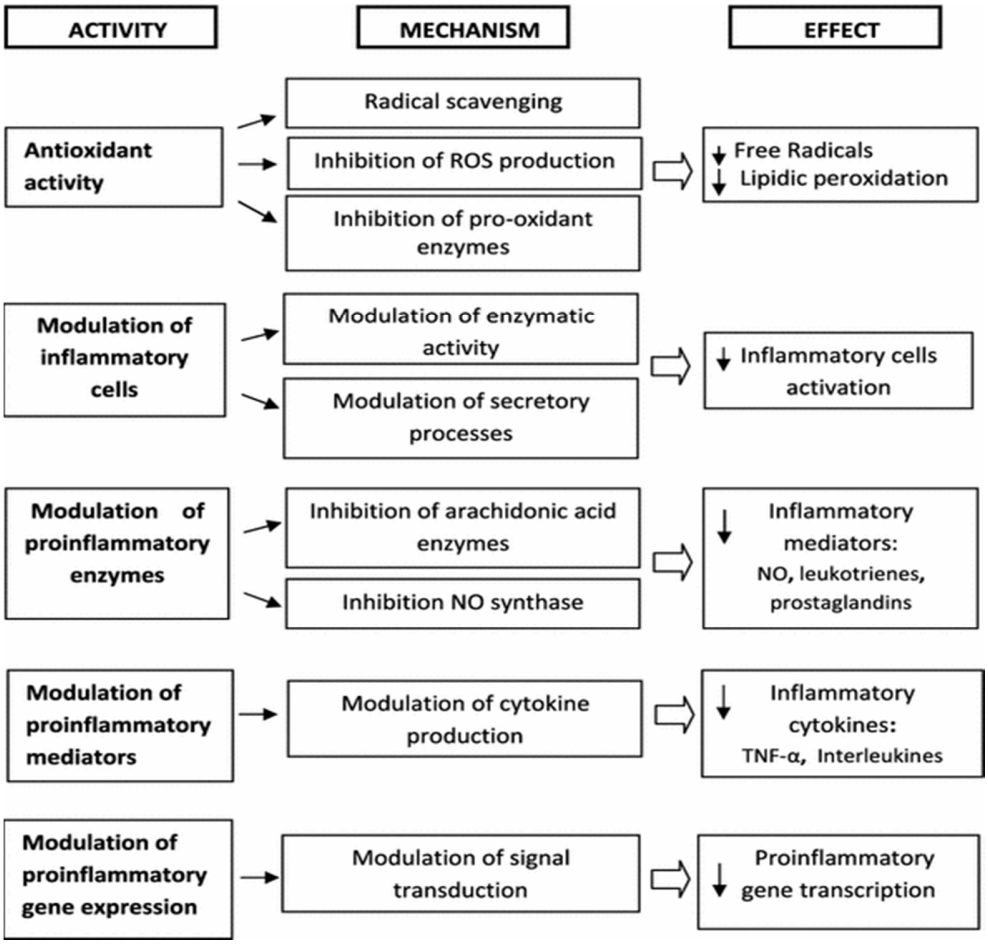



*Figure 6: Different mechanism of modulating inflammation by anthocyanins*

| Biological activities | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Effects** | **Suggested mechanism** | **molecules and derivatives** | | | | | |
| | | **Cyn.** | **Del.** | **Pel.** | **Peo.** | **Mal.** | **Pet.** |
| Anti-oxidant | | (Miguel 2011), (Samyor, Das et al. 2017) | (Miguel 2011), (Huang, Liu et al. 2014) | (Miguel 2011) | (Miguel 2011), (Samyor, Das et al. 2017) | (Miguel 2011) | (Miguel 2011) |
| Anti-inflammatory | Inhibition of proinflammatory cytokines (TNF-α, TGF-β, ILs, PGs, MMPs); inhibition of PDGF-induced VEGF expression through down-regulation of p38 MAPK and JNK signaling in vascular smooth muscle cells.<br><br>Inhibition of COX-2 expression (mRNA and protein levels); suppression of LPS-stimulated activation of transcription factors including C/EBP, AP-1 and NFkB, but not CREB.<br><br>Anti-inflammatory activities of ACNs are reportedly mediated via inhibition of iNOS protein and mRNA expression as well as inhibition of nuclear factor kB (NF-kB) and STAT-1 activation, affecting the expression of several inflammatory genes. | (Hidalgo, Martin-Santamaria et al. 2012), (Pan, Lai et al. 2010) | (Hidalgo et al., 2012 , (Vendrame and Klimis-Zacas 2015) (Jung, Lee et al. 2015), (Sogo, Terahara et al. 2015), (Dayoub, Le Lay et al. 2017) | | Hidalgo et al., 2012 | Hidalgo et al., 2012 | --- |
| Anti-diabetic | inhibition of enzyme activities involved in glucose release and uptake (α-amylase, glucosidase) | (Pan, Lai et al. 2010), (Takahama and Hirota 2018), (Vinayagam and Xu 2015) | (Vinayagam and Xu 2015) | (Huang, Wang et al. 2015), (Vinayagam and Xu 2015) | Huang, Wang et al. 2015) | --- | --- |
| Anti-obesity | ACNs consumption by C57BL/6 mice increases fecal butyrate and hepatic SOD and GPx activities, decreases lipid peroxidation, and down- | (Wu, Guo et al. 2017) | (Wu, Guo et al. 2017) | Wu, Guo et al 2017) | Wu, Guo et al 2017) | --- | (Wu, Guo et al. 2017) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | regulates the expression levels of TNFα, IL-6, iNOS, and NF-κB | | | | | | |
| Anti-carcinogenic | Inhibition of tumor development | (Liang, Guan et al. 2017), (Urias-Lugo, Heredia et al. 2015) | (Urias-Lugo, Heredia et al. 2015), (Lim, Jeong et al. 2016) | --- | --- | --- | --- |
| ACE-inhibitory | Free hydroxyl groups in the structure of ACNs are involved in chelating zinc ions, thus lowering ACE activity. | (Hidalgo et al., 2012 , (Ojeda, Jiménez-Ferrer et al. 2010) | (Hidalgo et al., 2012 | (Hidalgo et al., 2012 | (Hidalgo et al., 2012 | (Hidalgo et al., 2012 | --- |
| Anti-aging | Activation of the DAF-16/FOXO transcription factor | (Chen, Müller et al. 2013) | --- | --- | --- | --- | --- |
| Anti-atherosclerosis | Inhibits MCP-1, TNF-α, ILs and ICAM-1, VCAM-1 (all involved in inflammatory response associated to atherosclerosis development) | (Oak, Chataigneau et al. 2003, Oak, Bedoui et al. 2006, Kuntz, Asseburg et al. 2015, Urias-Lugo, Heredia et al. 2015) | (Oak, Bedoui et al. 2006), (Sikand, Kris-Etherton et al. 2015) | (Sikand, Kris-Etherton et al. 2015) | (Sikand, Kris-Etherton et al. 2015) | (Sikand, Kris-Etherton et al. 2015) | (Sikand, Kris-Etherton et al. 2015) |
| Anti-radical | | (Marković, Pejin et al. 2017) | (Marković, Pejin et al. 2017) | (Marković, Pejin et al. 2017) | --- | (Marković, Pejin et al. 2017) | --- |
| Improved dark adaptation | ACNs consumption could inhibit transient myopia, while reducing eye fatigue, improving dark adaptation, and enhancing retinal blood flow | | --- | --- | --- | --- | --- |

*Table 4: Biological activities of anthocyanins in pigmented grains*

In vivo studies have demonstrated strong protective effects of cyanidin and delphinidin against cardiovascular diseases. These anthocyanins cause reducing PGE2 levels in tissues and

serum TNF-a levels in arthritis. Cyanidin can inhibit TNF-a-induced endothelial cell apoptosis while delphinidin increases eNOS expression by mediating the MAP kinase pathway therefor prevents apoptosis in the aortic endothelial cell. It also acts against ox-LDL-induced damage in HUVECs and regulate apoptotic molecule expression, which prove the effects of delphinidin in preventing both plaque development and atherosclerosis (Vendrame and Klimis-Zacas 2015, Ferrari, Speciale et al. 2016). Table 4 lists some of the suggested mechanism of action of individual anthocyanins and of their derivatives found in pigmented cereals, along with the possible role of anthocyanins in other diseases.

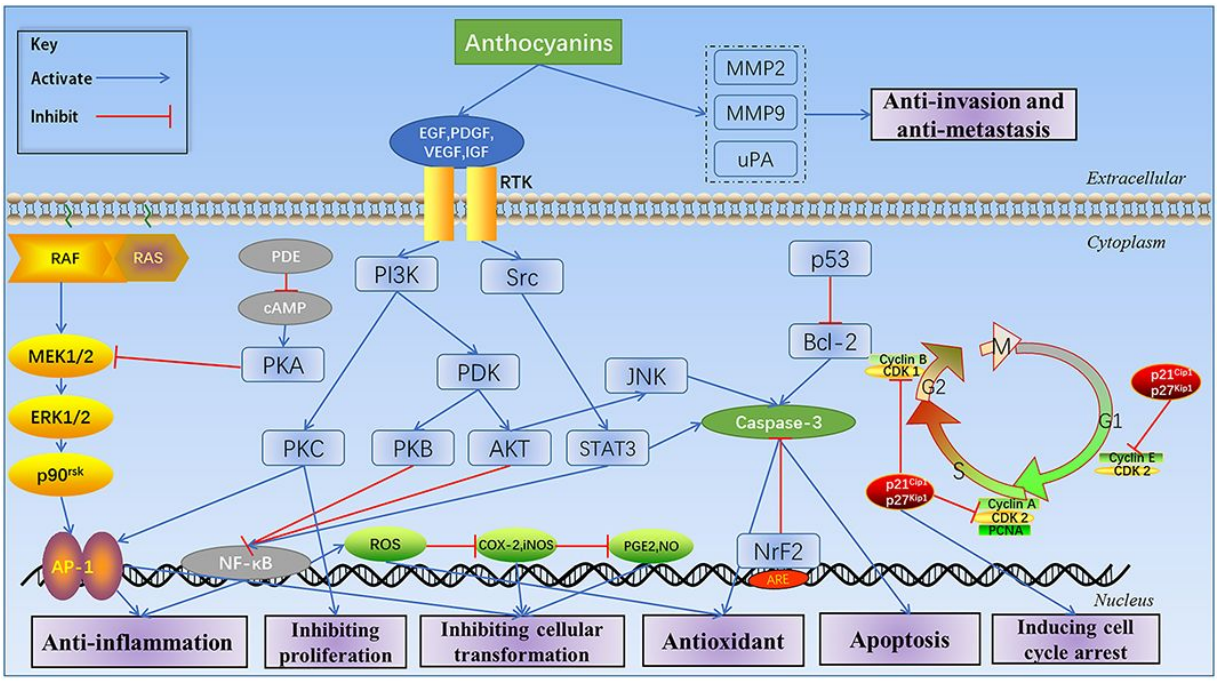
### 1-5-3 Anti-carcinogenic Activity

Inflammation is a necessary process, but, if persisting, it could lead to the development of diseases, including cancer. Tumor development often occurs at sites of inflammation, where an environment ideal for cancer growth is created (Prasad, Ravindran et al. 2010). Anthocyanins have shown great potential as for preventing tumor development. The potential anti-tumor effects of anthocyanins are reportedly based on a wide variety of biological activities, including anti-mutagenesis, induction of differentiation, inhibiting proliferation by modulating signal transduction pathways, inducing cell cycle arrest and stimulating apoptosis or autophagy of cancer cells, anti-invasion, anti-metastasis, reversal of drug resistance and/or increased sensitivity to chemotherapy (Lin, Gong et al. 2017).

Cancer cell growth might be inhibited by anthocyanidins through targeting RTKs (e.g. EGFR, PDGFR and VEGF/VEGFR) and acting on the Ras-MAPK and PI3K/Akt signal cascade pathway. Inflammation might also be inhibited by anthocyanins through acting on the PI3K/Akt and NF-κB pathway to suppress the expression of COX-2 and iNOS and prevent cancer by regulating the expression of phase II antioxidant enzymes to achieve antioxidation through the Nrf2/ARE signalling system.

During cancer initiation, anthocyanins might prevent malignant transformation by targeting the MAPK pathway and AP-1 factor and by inhibiting RTK activity. Anthocyanins can

initiate the expression of p21 and p27, whose products can combine with multiple cyclin-CDKs to down-regulate the expression of CDK-1 and CDK-2, further inhibiting the expression of cyclin-B, cyclin−A and cyclin–E, which promote the expression of CDK inhibitors and cause cancer cells to arrest their growth at the G0/G1 and G2/M stages. During cancer development, anthocyanins can induce apoptosis of cancer cells by activating caspases, mediated by ROS and JNK/p38-MAPK. In addition, anthocyanins might exert their anti-metastatic activities by targeting the VEGF signalling pathway and extracellular matrix degradation (via MMP2, MMP9, uPA) (Lin, Gong et al. 2017).

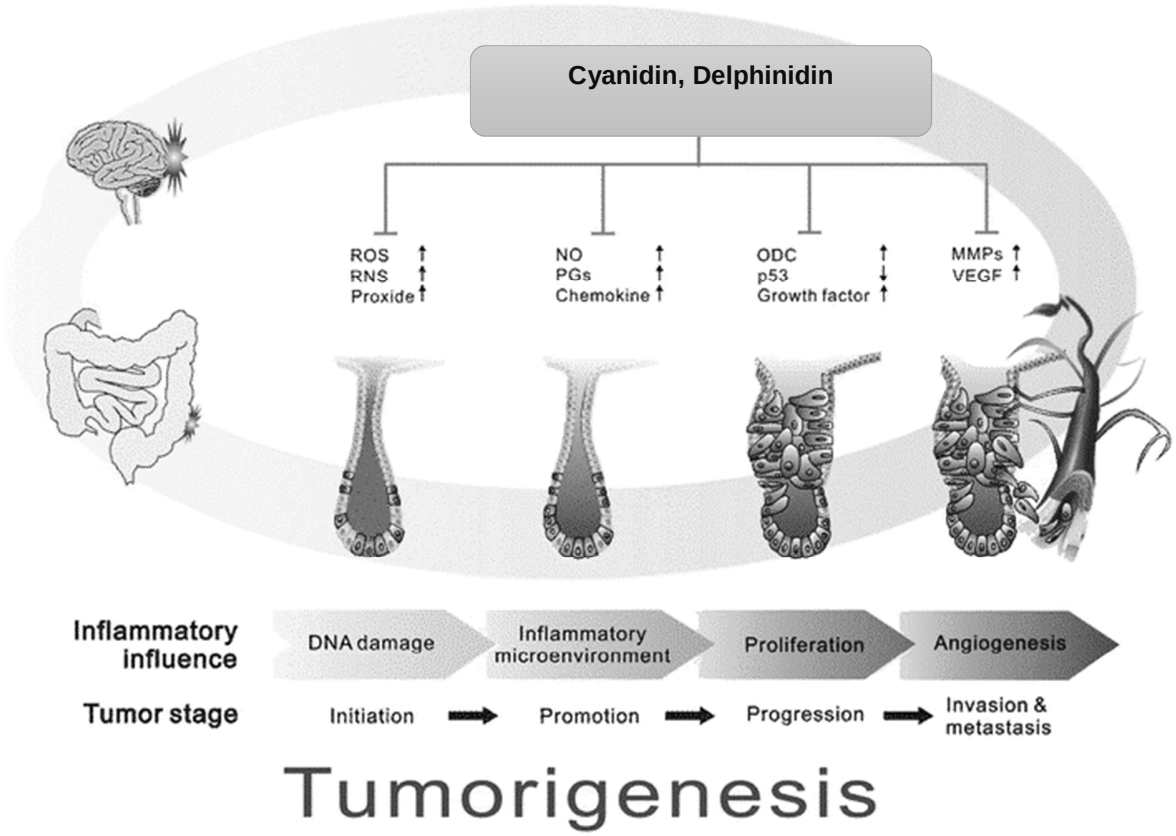



*Adapted from Lin et. al 2017*

*Figure 7: Effects of anthocyanins on the prevention and treatment of cancer*

Many studies have proven the protective effects of anthocyanins in different cancers including colon cancer (Shin, Lu et al. 2011), breast cancer (Li, Xu et al. 2016), prostate cancer (Ha, Bae et al. 2015). Bowen-Forbes and others (2010) evaluated the ability of different Jamaica-

and Michigan-grown blackberry and raspberry anthocyanin extracts to inhibit tumor cell proliferation. The Jamaican fruits exhibited the greatest capacity to inhibit human tumor cell growth, inhibiting lung, colon, gastric, and breast tumor cells by 54%, 50%, 37%, and 24%, respectively (Bowen-Forbes, Zhang et al. 2010). An in vivo study with mice studied the effects of cyanidin-3-glucoside from blackberries on skin tumors. After 20 weeks of treatment, the mice receiving anthocyanin treatments had a significantly lower tumor growth, about a 53% inhibition compared to the control group. These results suggest that cyanidin-3-glucoside has chemotherapeutic activity (Ding, Feng et al. 2006).




Adapted from Pan et. al (2010)

*Figure 8: Anti-carcinogenic effects of some anthocyanins in human cells*

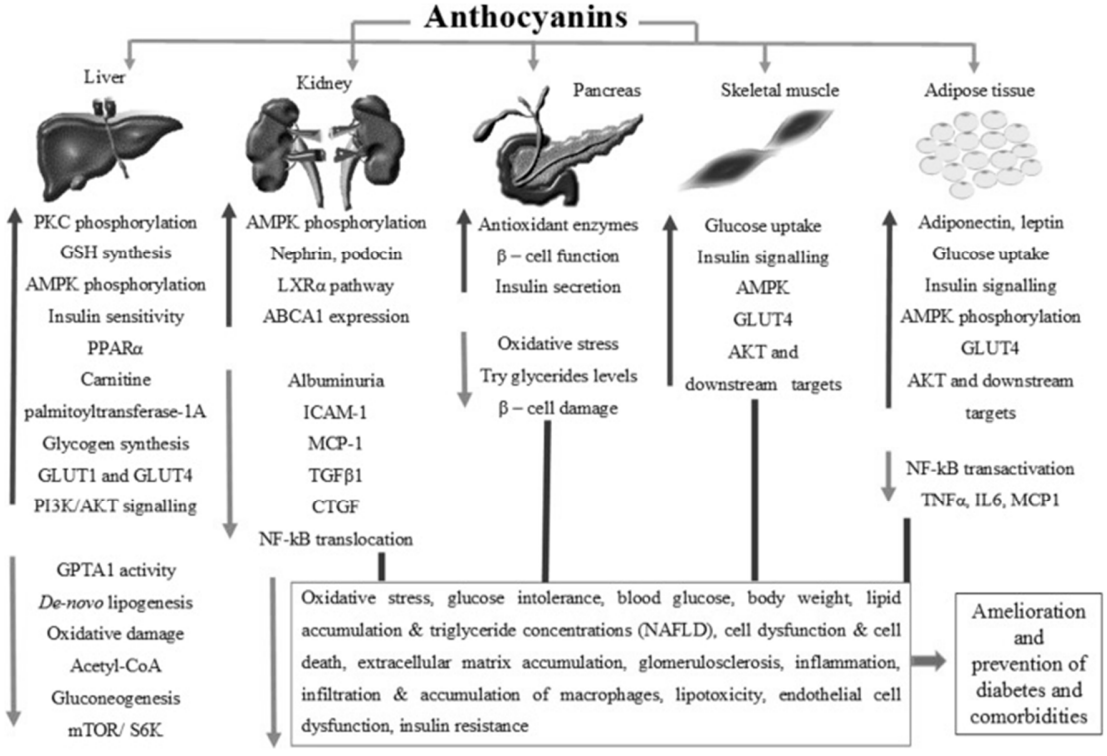
### 1-5-4 Anti-Obesity properties

Obesity results from excessive adipose accumulation caused by an imbalance of energy intake and expenditure. Several metabolic disorders may result from high levels of adipose tissue. Anthocyanins may prevent the accumulation of adipocytes to lessen the risk of metabolic disorders caused by obesity, as indicated by in vivo research on mice and rats (Norberto, Silva et al. 2013) (He and Giusti 2010). Tsuda et al. (2003) studied the effect of varying diets on obesity in rats (Tsuda, Horio et al. 2003). In this study, diets included a control, a purple corn extract, high fat, or high fat and a purple corn extract. After 12 weeks, the rats fed the high fat diet had increased adipocytes. Supplementation with purple corn extract suppressed the weight gain induced by the high fat diet. The suggested mechanism was suppression of mRNA by the purple corn extract to decrease the synthesis of fatty acids and triglycerols.

Other studies also support anthocyanins' ability to help obesity prevention. In a study by Prior et al. (2010), mice were fed either a low fat or high fat diet. Blueberry juice or purified blueberry anthocyanins were combined with drinking water in both the low fat and high fat diets. After 72 days, body weight and percent body fat were higher in the mice fed high fat diets compared to those fed the low-fat diet. However, the mice fed a high fat diet and given blueberry juice or blueberry anthocyanins in their water experienced no change in percent body fat from the low-fat diet mice. However, blueberry juice was not as effective as blueberry anthocyanins in obesity prevention (Prior, E. Wilkes et al. 2010). Wu et al. showed that by adding anthocyanins from Chinese mulberry to the daily food of mice, body weight gain of the male C57BL/6 mice fed with high-fat diet (HFD) was suppressed. In this study three kinds of anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside) was purified from Chinese mulberry *(Morus australis Poir*) and were added to the mic food. Results from a 12-week experiment showed that consumption of purified mulberry anthocyanins (MACN) of 40 or 200 mg/kg could significantly inhibit body weight gain, reduce the resistance to insulin, lower the size of adipocytes, attenuate lipid accumulation and decrease the leptin secretion in the diet-induced obese mice (Wu, Qi et al. 2013).

### 1-5-5 Anti-Diabetic Activity

Type 2 diabetes is a metabolic disorder characterized by insulin resistance and hyperglycemia (Zunino, 2009). Grace and others (2009) researched the efficacy of blueberry anthocyanins to alleviate hyperglycemia in diabetic mice. The mice were fed diets supplemented with phenolic-rich extracts or anthocyanin-rich extracts. Both diets had significant hypoglycemic activity. However, the anthocyanin-enriched diet exhibited greater hypoglycemic effects, decreasing blood glucose levels by 51% compared to 33% with the phenolic-rich diets. These results are comparable to metformin, a widely used anti-diabetic drug. It is important to note that the significant effects of anthocyanin enriched diets were not seen when Labrasol, a bio-enhancing agent, was used in conjunction with anthocyanins.




*Adapted from Gowd et. al (2017)*

*Figure 9: Mechanism of anthocyanins on diabetes prevention*

Numerous studies have demonstrated that anthocyanins can exert the beneficial effects in diabetes by acting on various molecular targets and regulate different signaling pathways in multiple organs and tissues such as liver, pancreas, kidney, adipose, skeletal muscle and brain. Anthocyanins can lower blood glucose levels by protecting β-cells, improving insulin resistance, increasing insulin secretion, improving liver function, and inhibiting carbohydrate hydrolyzing enzymes. The antidiabetic properties of anthocyanins may also attribute to their antioxidant capacity. Taken together, anthocyanins may be a novel small molecule for the prevention and treatment of diabetes.

# 2- MATERIAL AND METHODS

## 2-1 Pigmented grains samples

Three corn varieties and two wheat varieties were used in this study:

Blue Corn (BC T), whole-grain flour

Blue corn (BC MF), whole-grain flour

Red corn (Rostrato), whole-grain flour

Blue wheat (SK), de-branning fraction

Purple wheat, de-branning fraction (F1)

*Figure 10. Pigmented grains; Blue, Red and Purple corn*

*Blue and purple wheat*

## 2-2 Anthocyanin and polyphenol extraction and quantification

### 2-2-1 Extraction method

#### Reagents

- Petroleum ether
- Ethanol (ratio 65:35, v / v 0.3 M HCl)

#### Method

Extraction was performed to optimize the anthocyanin extraction. Two grams of fine flour was weighted, then 15 ml of petroleum ether was added. The tubes were covered by aluminum in order to protect from the light and left them on shaker overnight at room temperature. After centrifugation at 5000 × g for 20 minutes at 10˚ C, the supernatant phase was removed, and the residue dried under a nitrogen flow. Then, 15 ml of ethanol (65:35, v / v) containing 0.3 M HCl was added, and samples left on a shaker at room temperature overnight. The supernatant from centrifugation at 5000 × g for 20 minutes at 10˚ C represents the extract. This procedure was repeated twice and then all the extracts were pooled.

### 2-2-2 Total anthocyanin content (TAC) measurement

#### Reagents

- 0.03 M KCl, pH1 (1.9 g KCl in 980 mL of distilled water)
- 0.4M sodium acetate, pH 4.5, (54.4 g of CH3CO2Na 3H2O in 960 mL of distilled water)

#### Method

Anthocyanins undergo reversible modification of the structure with pH, that results in a variation in their absorbance spectrum. Extracts from each type of grains were used and two dilutions were prepared for each of them: one with a potassium chloride buffer pH 0.03 solution and the

other with 0.4 M sodium acetate buffer pH 4.5. Solutions, consisting of 2 ml of buffer solution and 0.5 ml of sample, diluted to a final volume of 2.5 ml were centrifuged (20 minutes at 5000 X g and 10 ° C). The absorbance was then determined at 520 nm and 700 nm, using distilled water as blank. It is important to read the absorbance within a time range of 20-60 minutes after preparation of the samples (Giusti and Wrolstad 2001) to obtain a reliable measure. The anthocyanin content was calculated as cyanidine-3-O-glucoside (PM = 449.2 and ε = 26900) and the result expressed as micrograms of cyanidin 3-O-glucoside equivalents per gram of dry sample flour. The analyzes were performed in triplicate. The absorbance (A) of the diluted sample was calculated as follows:

$$A = (A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}$$

$$TA\left(\frac{mg}{l}\right) = \left(A \times MW \times DF \times 1000\right) / \varepsilon \times l$$

$$TA\left(\frac{mg}{g_{ex}}\right) = \left[TA\left(\frac{mg}{l}\right) / 100\right] / m_{ex}(g)$$

$$TA\left(\frac{mg}{gFW}\right) = TA\left(\frac{mg}{g_{ex}}\right) \times re\,\mathrm{cov}\,ery\,(\%) / 100$$

*Equation 1: Total anthocyanins content calculation*

**2-2-3 Total polyphenols content (TPC) measurement**

**Reagents**

- Folin-Ciocalteu reagent
- 15% sodium carbonate (w / v)

**Method**

For the determination of the total content of polyphenols (TPCs) the Folin-Ciocalteu method was used (Singleton, Orthofer et al. 1999). Briefly, 100 µL of extract was mixed with 500 µL of Folin-

Ciocalteu reagent and 2 ml of sodium carbonate at 15% (w / v) was added, then distilled water was added to a final volume of 10 ml. The mixture was placed at room temperature for 1 hour, in darkness. It was then centrifuged at 12,000 rpm for 10 min. The absorbance of the supernatant was read at 765 nm against distilled water. The results were expressed as mg gallic acid equivalents per gram of dry sample.

### 2-2-4 Qualitative and quantitative characterization by HPLC

HPLC analyzes were performed on a WATER 717 self-sampler instrument equipped with a binary pump with Rheodyne injector and a UV-VIS detector operated by the Chemstation Galaxy. This apparatus was used for analysis of anthocyanins and phenolic compounds in order to separate the different components and their quantitative evaluation. RP-HPLC profiling was performed on a Waters 600 E HPLC, equipped with a Waters 717 auto-sampler and a Waters 996 PDA detector (Waters, Milan, IT), by using a C18 column (5 µm, 4.6 × 250 mm, Waters, Milan, Italy). Typically, 0.1-0.2 mL of individual ethanol/HCl extracts were loaded on the column. Elution was carried out at 0.8 mL/min, using a linear gradient from 100% A (0.1% trifluoroacetic acid in water) to 100% B (0.1% trifluoracetic acid in acetonitrile) in 48 min. The gradient program was: 0 to 5 % B in 5 min; 5 to 40 % B from 5 to 40 min; 40 to 70% B from 40 to 48 min (Table 5).

| **CROMATOGRAPH CONDITION** | | **ELUTION CONDITION** | | |
|---|---|---|---|---|
| **Sample** | As such | **t (min)** | **A (0.1% TFA in $H_2O$)** | **B (0.1% TFA in AcN)** |
| **Flux** | 0.8 ml/min | 0 | 100% | 0% |
| **Primary pressure** | 63-65 bar | 5 | 95% | 5% |
| | | 40 | 60% | 40% |
| | | 48 | 0% | 100% |

*Table 5: The gradient used for HPLC*

The absorbance was monitored at different wavelengths: quantification of the anthocyanins present in the extracts was obtained using cyanidin 3-O-glucoside, cyanidin chloride and delphinidin chloride standards. Instead, for the quantification of polyphenols the reference standards included ferulic acid, rutin, quercitin, catechin and epicatechin.

| **Standard** | **λ (nm)** |
|---|---|
| Cyanidin chloride | 520 |
| Delphinidin | 520 |
| Ferulic Acid | 320 |
| Rutin | 350 |
| Quercetin | 350 |
| Catechin | 280 |
| Epicatechin | 280 |

*Table 6: Standard used for HPLC analysis and relative reference wavelengths*

**2-3 Antioxidant capacity with FRAP method (Ferric Reducing Antioxidant Power)**

**Reagents**

- acetate buffer 300 mM;
- TPTZ 10 mM;
- FeCl3 20 mM

**Method**

The antioxidant activity was evaluated using the FRAP assay which allows to measure the ability of the extracts to reduce ferric iron (Fe $^{3+}$) to ferrous iron (Fe $^{2+}$). In certain pH conditions (3.6) and in the presence of TPTZ (2,4,6-tris (2-pyridyl)-s-triazine), these ions form complexes with different characteristics, in particular the reduced derivative (Fe2 + -TPTZ) assumes a blue color with a maximum absorption of 593 nm which can be measured spectrophotometrically. The working FRAP reagent was obtained from the mixture of 50 ml of acetate buffer 300 mM, 5 ml of TPTZ 10 mM and 5 ml of FeCl3 20 mM. The extracts were diluted with 65% ethanol since the reading range is optimal between 0.200 and 0.900 OD. Appropriate amounts of 3 ml of working FRAP reagent solution were preheated in tubes at 37 ° C. Following addition of the diluted extracts, samples were warmed again for 4 minutes at 37 ° C, and then read at 593 nm.

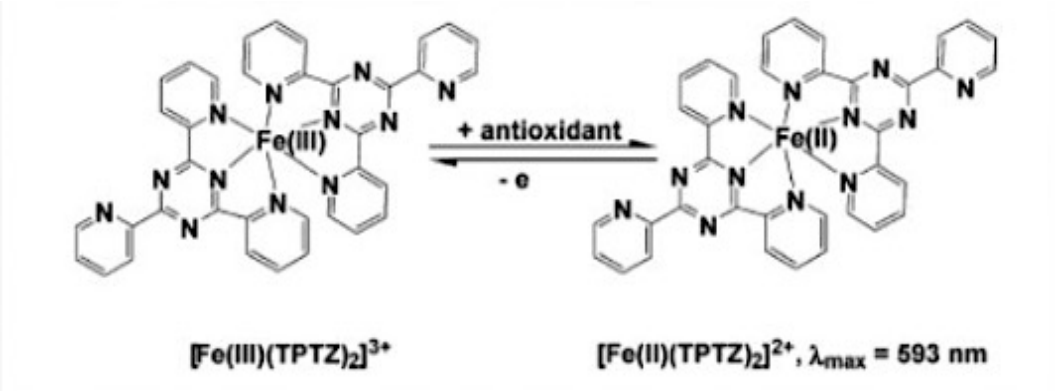



*Figure 11: FRAP Reaction*

A calibration curve was constructed by using a solution of $FeSO_4 \cdot 7H_2O$ in methanol. Results are expressed as millimoles of Fe(II) sulfate equivalents per gram of dry sample.

**2-4 Anti-diabetic effects**

**2-4-1 α-glucosidase & α-amylase enzymes inhibitory activities**

**Reagents**

- Intestinal rat acetone powder (N1377-5G)
- p-nitrophenyl a-D-glucoside (p-NPG)

- Porcine pancreatic α-amylase
- p-nitrophenyl α-D-maltopentaoside (p-NPGP)
- 50 mM phosphate buffer (pH 6.8)
- acarbose

**Method**

To measure and quantify the enzymatic inhibition of alpha-glucosidase, a solution was prepared by weighing 200 mg of rat intestinal acetone powder dissolving in 4 ml of 50 mM cold phosphate buffer (pH 6.8) and sonicating for 15 minutes at 4 ° C. The suspension was then vortexed for 20 minutes, centrifuged at 10000 x g for 30 minutes at 4 ° C and then the supernatant containing enzyme was used. For this assay we combined 0.65 mL of 50 mM phosphate buffer pH 6.8, 0.1 mL of the previously prepared enzyme solution (rat intestinal acetone powder) and 0.05 mL of extract. After 5 minutes at 37 ° C, 0.2 ml of 1 mM pNPG was added, and samples incubated in a shaker at 37 ° C for 25 minutes. The alpha-amylase activity assay was conducted by mixing 0.55 mL of 50 mM phosphate buffer, 0.2 mL of the enzymatic solution of pancreatic porcine α-amylase and 0.05 mL of our extracts. After pre-incubation for 5 minutes at 37 ° C., the reaction was started by adding 0.2 mL of 1 mM pNPGP and samples incubated in a shaker at 37 ° C for 55 minutes.

For both enzymatic reactions, the solution was then centrifuged at 10000 x g for 3 minutes and read the absorbance of the supernatant at 405 nm. Blanks were prepared in the absence of enzyme (in both enzymatic assay) and of the ethanol/HCl mixture, and controls were otherwise complete reaction mixtures containing appropriate volumes of ethanol/HCl, but no bioactive-rich extracts. Tests were performed in triplicate for each dilution of the original extract. Results were compared with those obtained by using known amounts of acarbose (from a stock solution in ethanol/HCl) as the reference inhibitor (from 5 mg/L to 50 mg/L).

**2-5 Anti-inflammatory properties**

**Materials and reagents**

Dulbecco's Modified Eagle's Medium, trypsin, d-luciferin, adenosine triphosphate (ATP), Phosphate buffered saline (PBS), Fetal Bovine Serum (FBS), penicillin, streptomycin, zeocin, non-essential amino acids, l-glutamine, interleukin βLuciferase, plasmid pNiFty2-Luc (Invivogen, Labogen Rho, Italy).

**2-5-1 In vitro Cultivation of Caco-2 cells**

Caco-2 cells are a cell line derived from adenocarcinoma of the human colon-rectal epithelium and represent an excellent model of the intestinal human barrier. For cultivation, FBS, 100 U ml-1 of penicillin, 0.1 mg ml-1 streptomycin, 0.1 mM non-essential amino acids (NEAA) were used for 10% (v / v) heat inactivation (30 min at 56 ° C) and 2 mM L-glutamine and incubated at 37 ° C in a water incubator incubated in a 95% air atmosphere and 5% carbon dioxide.

**2-5-2 NF-kb activation study**

Stable recombinant Caco-2 cell line was generated by transfecting cells with plasmid pNiFty2-Luc (Taverniti, Fracassetti et al. 2014). This plasmid contains a promoter with 5 sites for binding NF-kb followed by luciferase firefly with a Luc reporter gene. The stimuli that activate NF-kb promote the binding to the promoter by generating the gene expression of luciferase. Briefly, Caco-2 cells were transfected with STOS kit (GeneSpin, Milan, Italy) in accordance with the manufacturer's protocol. Subsequently, the cells were resuspended in fresh DMEM, disseminated in 24-well plates and incubated for 48 h to obtain the expression of resistance to antibiotics. Finally, stable recombinant clones were selected by adding 50 μg of mL-1 zeocin into the culture medium.

**2-5-3 Pacing protocol and NF-kb-Luc assay protocol**

After growth in the presence of 50 μg mL-1 of zeocin, monolayers of differentiated cells (approximately 3 x 105 cell well-1 cells) were carefully washed with 0.1 M Tris-HCl (pH 8.0). Subsequently, fresh DMEM containing 100 mM HEPES (pH 7.4) was added to THE cells. The extracts used for the assay were prepared at different concentrations (calculated considering the concentration of total anthocyanins (in range of 25-100 μM). Only small pH modifications were recorded (pH 7.6 ± 0.1) when the cells were supplemented with concentrate doses corresponding

to the maximum anthocyanin concentration. Recombinant Caco-2 cells were simultaneously stimulated with interleukin- β (IL β, 20 ng mL-1), used as a pro-inflammatory stimulus. After incubating at 37 ° C for 4 h, the plates were placed in ice for 15 minutes. The Caco-2 cells were then mechanically detached from the bottom of the multiwells and then were transferred to a eppendorf tube and sonicated for 10 seconds using a Bandelin SONOPLUS ultrasonic homogenizer (Bandelin Electronic GmbH & Co., Berlin, Germany). The insoluble particles were removed by centrifugation (5 minutes at 10000 x g) and the supernatant transferred to a new test tube. Appropriate aliquots (0.1 ml) of supernatant were placed in duplicate in the wells of the 96-well microtiter well (PerkinElmer, Monza, Italy). Then, 0.012 mL of a 10 mM ATP solution and 0.012 mL of 0.1 mM D-luciferin solution were added, and the bioluminescence emitted was immediately measured at a frequency of 120 s with a MULTILABEL Vector3 1420 counter (PerkinElmer). The maximum of the light production curve was considered, in duplicate experiments. Figure 12 describes the principle of this assay.

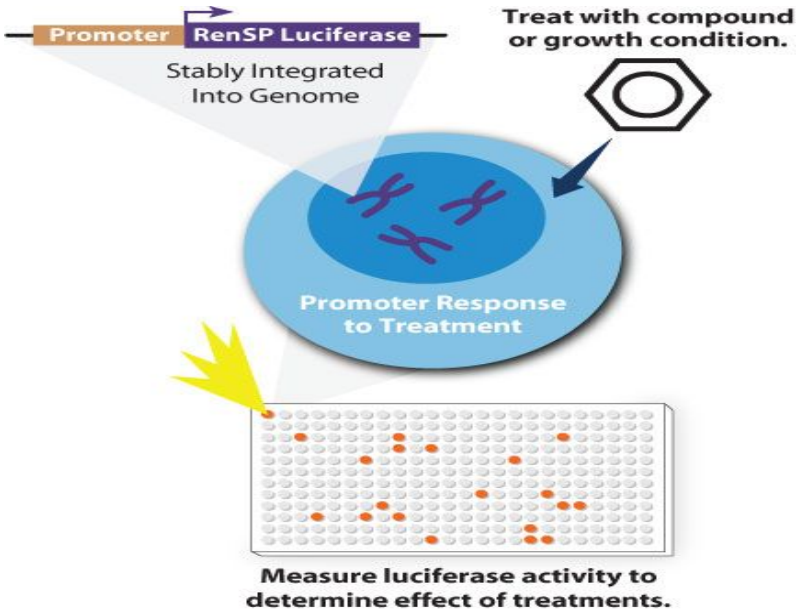



*Figure 12: The LightSwitch transfection-ready, promoter reporter constructs that can be used to quantify repression in response to induction of the NFκB / Inflammation pathway*

## 3- RESULTS

### 3-1 Quantification of total anthocyanin contents

The first part of the characterization of the pigmented grains obtained from the ethanolic extracts of the different samples of cereals for quantification of the total anthocyanin content using the common differential pH method. The results are expressed as mg of cyanidine-3-o-glucoside equivalent per gram of dry flour for each sample (Table 7).

| Grains | TAC (mg Cyn-3-Glc eq/kg) |
|---|---|
| **Purple wheat F1** | **690 ± 20$^{a}$** |
| **Blue Wheat (SK DEC)** | **170 ± 10$^{c}$** |
| **Blue Corn (T)** | **660 ± 60$^{a}$** |
| **Blue Corn (MF)** | **530 ± 10$^{b}$** |
| **Red Corn (Rostrato)** | **140 ± 10$^{c}$** |

*Table 7: Total anthocyanins content in pigmented cereal grain samples*

The results presented in Table 7 indicate that the de-branning fraction of purple wheat had the highest content of total anthocyanins. Blue corn (T) had the highest content of total anthocyanins among corn varieties, following by blue corn (BCMF).

### 3-2 Determination of the total content of polyphenols

For the determination of the total content of polyphenols (TPCs) the Folin-Ciocalteu method was used, and results are expressed as mg equivalent gallic acid (GA), as presented in Table 8.

| Grains | TPC (g GAE/kg) |
|---|---|
| **Purple wheat F1** | **73.6 ± 0.44[a]** |
| **Blue Wheat (SK DEC)** | **33.04 ± 0.24[c]** |
| **Blue Corn (T)** | **30.86 ± 0.65[c]** |
| **Blue Corn (MF)** | **33.7 ± 0.50 [c]** |
| **Red Corn (Rostrato)** | **63.7 ± 0.79[b]** |

*Table 8: Total polyphenol content in pigmented cereal grain samples*

Data in Table 8 indicate that the total phenolic component in the de-branning fraction from purple wheat is higher than in all the other pigmented grains tested here. The red variety of corn (Rostrato) showed the highest content of total polyphenol among corn varieties.

### 3-3 Qualitative and Quantitative Characterization by HPLC

In order to allow the qualitative evaluation of anthocyanins and polyphenols, preliminary analyses were necessary, and involved use of standard compounds, considered to be significant in the various extracts. The standards analyzed were: cyanidine chloride, delphinidin, cyanidine-3O-glucoside, ferulic acid, rutin, quercetin, catechin, epicatechin. Results obtained with various standards, with detection at the appropriate wavelengths as listed under methods, are reported in Table 9.

| Standards | Area of peak/µg | Time of exit (minute) |
|---|---|---|
| **Cyn-3-O-Glc** | 384362 | 23.27 |
| **Cyn-Cl** | 788873 | 27.77 |
| **Delphinidin** | 1048608 | 25.66 |
| **Ferulic Acid** | 634442 | 29.04 |
| **Rutin** | 412837 | 27.25 |
| **Quercetin** | 442573 | 37.05 |
| **Catechin** | 12551103 | 22.6 |
| **Epicatechin** | 15084033 | 24.5 |

*Table 9: Results related to different standards performed by HPLC*

### 3-3-1 Quantitative analysis on extracted samples of pigmented grains samples

The actual chromatograms obtained from extracts of individual grains are presented in the following figures, being grouped in terms of the wavelength used for detection and, therefore, of the class of chemicals being revealed. Results of these profiling attempts are shown in Tables 10 and 11, that presents a quantitative analysis of those compounds that could be identified unequivocally.

Indeed, caution should be used when interpreting these data, as even very extensive phenolics profiling carried out by most updated methodologies (Siebenhandl, Grausgruber et al 2007; Ficco, Mastrangelo et al 2014) did not allow straightforward structural identification of 40-70 % of the total anthocyanins present in grains and grain-derived foods.

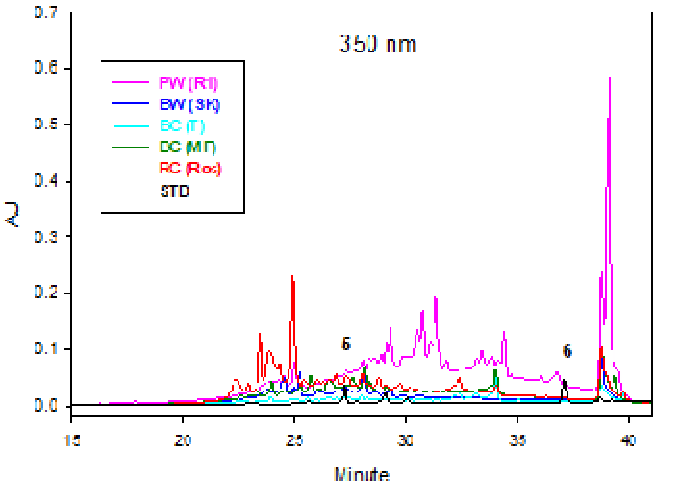
With these limitations, more than 90% of the total anthocyanin content in extracts from the debranning of purple wheat was accounted for by non-glicosylated cyanidin (≈ 35 mg/kg of

the original material), whereas cyanidin-3-glucoside (≈9.5 mg/kg of the original material) made up about 60% of total anthocyanins in extracts from a debranning fraction of blue wheat (BWSK). The remainder of the colored materials in BWSK was mostly accounted for by non-glycosylated delphinidin. Consistent with the color traits, extracts from blue corn MF had a very high content of cyanidin and delphinidin (both at ≈ 60 mg/kg), whereas cyanidin-3-glucoside (≈ 37 mg/kg) accounted for less than 10% of the colored materials in this material. Despite the use of different extraction protocols and solvents, the values obtained in the current study are reasonably close to those reported for similar - although not identical – grains (Abdel-Aal, Young and Rabalski 2006).

| | **Identified anthocyanins, mg/kg sample** | | |
|---|---|---|---|
| **Material** | **Cyanidin** | **Delphinidin** | **Cyanidin-3-O-glucoside** |
| **PWD1** | **36.2 ± 4.1$^{c}$** | **52.4 ± 7.1$^{c}$** | **32.1 ± 2.1$^{a}$** |
| **BWSK** | **19.5 ± 2.2$^{d}$** | **90.5 ± 8.2$^{b}$** | **19.4 ± 1.4$^{b}$** |
| **BCT** | **201.1 ± 9.0$^{a}$** | **97.0 ± 2.8$^{b}$** | **23.2 ± 0.9$^{b}$** |
| **BCMF** | **27.2 ± 1.7$^{b}$** | **114.4 ± 6.4$^{a}$** | **35.2 ± 1.9$^{a}$** |
| **Rostrato** | **16.1 ± 1.1$^{d}$** | **5.2 ± 0.5$^{d}$** | **3.1 ± 0.5$^{c}$** |

*Data are mean values ± standard deviation. Different superscripts in a given column indicate statistically different values (p=0.01).*

*Table 10: Quantitative analysis of identified anthocyanins by HPLC*


350 nm
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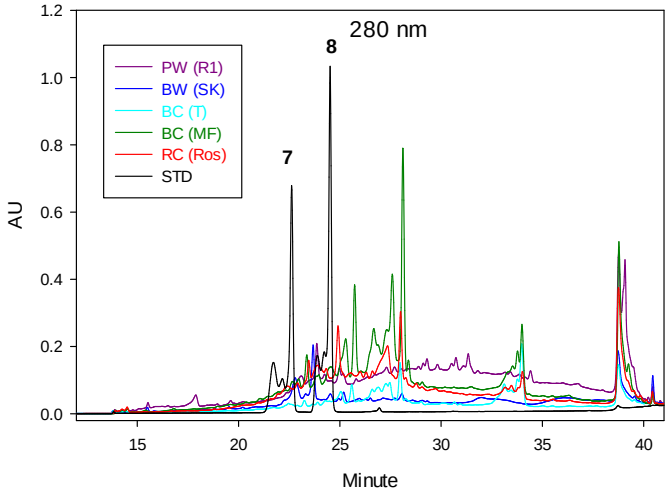
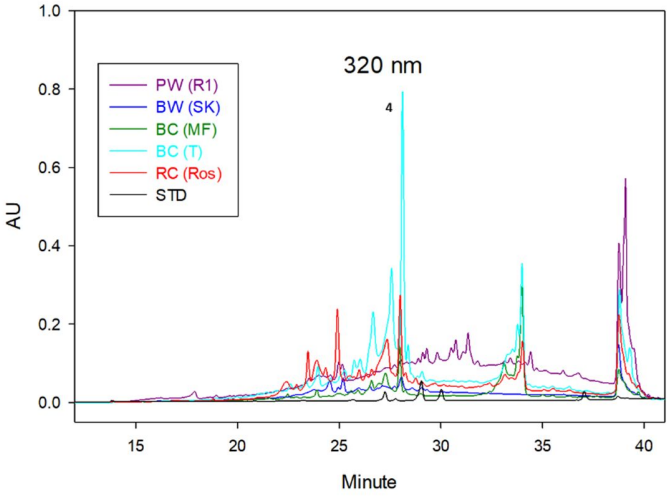
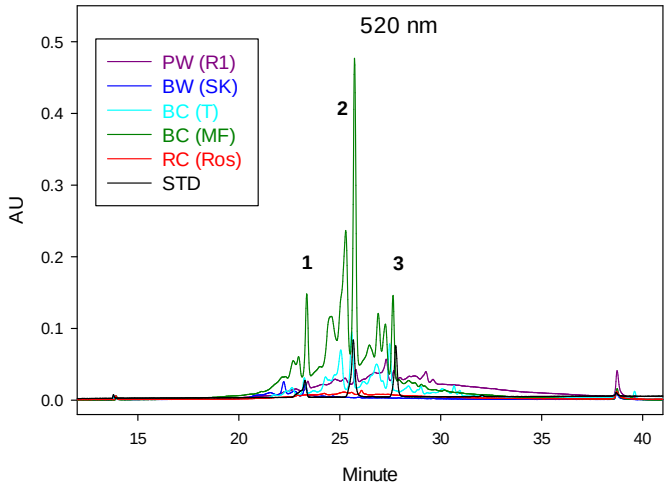
BW (SK)
BC (T)
BC (MF)
RC (Ros)
STD
AU
Minute
6
6


280 nm
PW (R1)
BW (SK)
BC (T)
BC (MF)
RC (Ros)
STD
AU
Minute
7
8


320 nm
PW (R1)
BW (SK)
BC (MF)
BC (T)
RC (Ros)
STD
AU
Minute
4




For other phenolics identified in HPLC profiling, we found anthocyanin-poor extracts from BCT to be the richest in ferulic acid, whereas extracts from blue wheat variety Skorpion and red corn Rostrato contained the highest amounts of rutin and catechin. The first fraction after debranning in purple wheat extracts had by far the highest content in quercetin and the lowest in rutin, as well as high epicatechin levels. However, epicatechin levels in extracts from the red Rostrato corn wholemeal were 4-fold higher than in purple wheat F1 extracts (and 10 to 15-fold higher than those in extracts from wholemeal from both the blue corn varieties considered here) (Table 11).

These findings may not provide a comprehensive analytical profiling of each and every compound in these extracts. Rather, these data confirm that the absolute amounts of individual components (and their ratios) are greatly different among the various grains and their varieties. Such variability should be taken into account when trying to correlate compositional data of individual extracts with properties measured by assessing their functionality by using either chemical or enzymatic assays or on highly sensitive cellular models.

| | Identified phenolics, mg/kg of sample | | | | |
|---|---|---|---|---|---|
| **Material** | **Ferulic acid** | **Rutin** | **Quercetin** | **Catechin** | **Epicatechin** |
| **PWF1** | **18.4 ± 1.8$^{d}$** | **2.0 ± 0.5$^{d}$** | **18.7 ± 1.6$^{a}$** | **76.2 ± 5.3$^{d}$** | **121.1 ± 8.7$^{b}$** |
| **BWSK** | **71.8 ± 3.1$^{b}$** | **63.0 ± 2.0$^{a}$** | **1.9 ± 0.3$^{c}$** | **385.6 ± 5.5$^{a}$** | **63.8 ± 3.4$^{c}$** |
| **BCT** | **113.9 ± 3.4$^{a}$** | **10.7 ± 1.5$^{c}$** | **0.6 ± 0.1$^{d}$** | **112.8 ±2.9$^{c}$** | **31.7 ± 3.1$^{e}$** |
| **BCMF** | **73.0 ± 2.8$^{b}$** | **26.2 ± 2.8$^{b}$** | **0.8 ± 0.3$^{d}$** | **252.5 ± 32.6$^{b}$** | **48.8 ± 3.1$^{d}$** |
| **Rostrato** | **28.5 ± 1.5$^{c}$** | **62.8 ± 2.6$^{a}$** | **3.8 ± 0.7$^{b}$** | **284.2 ± 4.2$^{b}$** | **465.1 ± 7.9$^{a}$** |

*Data are mean values ± standard deviation. Different superscripts in a given column indicate statistically different values (p=0.01).*

*Table 11: Quantitative analysis of identified polyphenols by HPLC*

### 3-4 Assessment of the antioxidant capacity

The evaluation of the antioxidant capacity was carried out only on ethanolic extracts using the FRAP method. This procedure is widely used for determining in vitro antioxidant capacity since it is fast, simple and inexpensive. Results are expressed as micromoles of ferrous ion product (Table 12).

| **Grains** | **FRAP (mmolFe(II)eq/kg)** |
|---|---|
| **Purple wheat F1** | **47.5 ± 10.26$^{a}$** |
| **Blue Wheat (SK DEC)** | **17.9 ± 1.68$^{b}$** |
| **Blue Corn (T)** | **22.08 ± 2.82$^{b}$** |
| **Blue Corn (MF)** | **18.9 ± 1.83$^{b}$** |
| **Red Corn (Rostrato)** | **47.2 ± 3.28$^{a}$** |

*Table 12: Antioxidant capacity in pigmented cereal grain sample extracts*

The Rostrato corn had the lowest TAC among all the grains characterized in this study. However, the total polyphenol content and the overall antioxidant activity in extracts from the whole flour of Rostrato corn were much higher than those found in equivalent extracts from other pigmented corn varieties. TPC and FRAP values for Rostrato red corn were indeed comparable to those measured in the bioactives-enriched fraction obtained from debranning of purple wheat. Statistical analysis of the data in Table 1 shows a positive correlation ($R^2$ = 0.970; $P$ < 0.001) between TPC and antioxidant activity (expressed as FRAP) in the various samples. However, the antioxidant activity of individual phenolic compounds in biological systems is reportedly dependent on their chemical structure (Marko, Puppel, et al 2004), and a thorough chemical profiling of the various extracts is required to assess the chemical basis for their different antioxidant capacity.

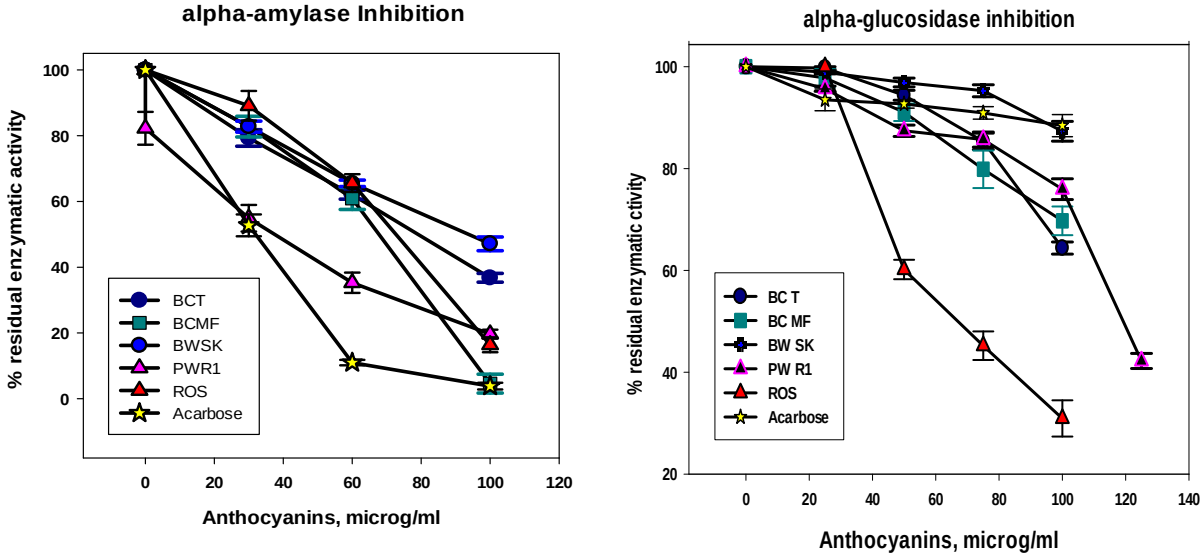
**3-5 Evaluation of enzymatic inhibition capacity**

There are many studies attesting to an interesting inhibitory effect of anthocyanins against two enzymes: alpha-glucosidase and alpha-amylase. Alpha-amylase acts in particular on starch and glycogen by cutting its internal bonds, releasing more readily absorbable oligosaccharides. Alpha-glucosidase hydrolyses maltose, a disaccharide composed of two glucose molecules. Both enzymes are involved in the digestion and absorption of carbohydrates, and considered responsible for the post-prandial glycemic peak, pursued to decrease in diabetic subjects. By limiting their activity, therefore, there will be less bioavailability of oligosaccharides and absorbable sugars and, consequently, a better glycemic control.

In this study, the activities of both enzymes were measured, comparing inhibition by grain extracts with the inhibitory activity of acarbose, a molecule used to treat diabetic and obese subjects. As shown in the two panels of graph 2, all extracts gave dose-dependent inhibition of both enzymes. Please note that the actual amount of extracts used in these assays was variable, as the concentration of bioactive species in assays was normalized for the total anthocyanin content of individual extracts. All extracts but the one from BWSK inhibited brush-border alpha-glucosidase more effectively than equivalent concentrations of acarbose, and extracts from the Rostrato red corn whole meal proved to be - by far - the most efficient. Acarbose was the most efficient inhibitor of pancreatic alpha-amylase, at least at concentrations below 0.1 mg/ml. In the

case of alpha-amylase, the inhibitory efficacy of extracts was found to increase in the order: BWSK≤BCT<Rostrato≤BCMF<PWF1.

A comparison of our data with literature reports also indicates that the inhibitory effects observed with the cereal extracts used here (on a comparable anthocyanidin content) is sensibly higher that that reported on similar extracts from a variety of pigmented plant materials. On an anthocyanin content basis, the values of $I_{50}$ measured in this study are in the 40-100 microgram/ml range, that is, about half those reported in previous studies with purified anthocyanins (see, for instance, Akkarachiyasit, Charoenlertkul et al 2010). This may be taken as an indication of synergistic effects between anthocyanidins and phenolics in the extracts used in this study, as already hypothesized for phenolics and anthocyanins from other sources (McDougall and Stewart 2005; Boath, Stewart and McDougall 2012). However, getting some molecular clues on the nature of the involved compounds involved in this hypothetical synergy and on the specific concentration ranges required for optimum activity will only be possible - in the case of pigmented grains - when complete compositional profiles will become available.



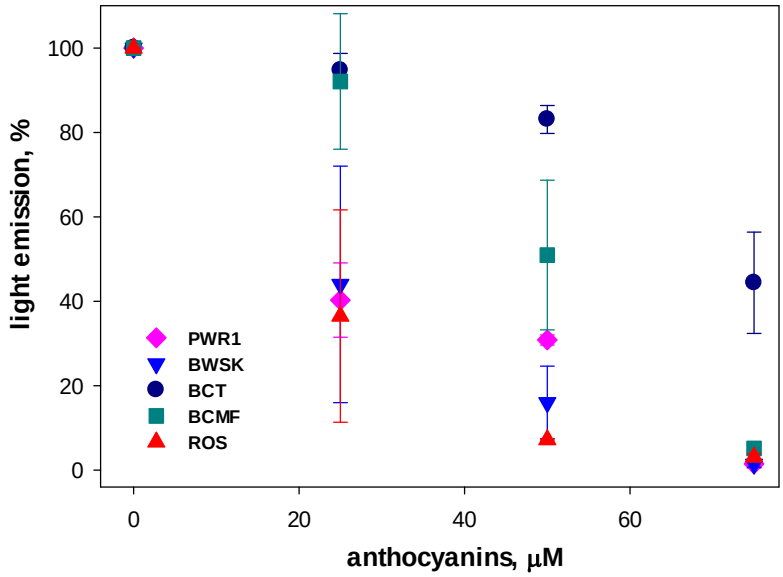

*Graph 2: Inhibition of enzymes involved in glucose metabolism and uptake by extracts from different pigmented grains*

A comparison of our data with literature reports also indicates that the inhibitory effects observed with the cereal extracts used here (on a comparable anthocyanidin content) is sensibly

higher that that reported on similar extracts from a variety of pigmented plant materials. On an anthocyanin content basis, the values of $I_{50}$ measured in this study are in the 40-100 microgram/ml range, that is, about half those reported in previous studies with purified anthocyanins (see, for instance, Akkarachiyasit, Charoenlertkul et al 2010). This may be taken as an indication of synergistic effects between anthocyanidins and phenolics in the extracts used in this study, as already hypothesized for phenolics and anthocyanins from other sources (McDougall and Stewart 2005; Boath, Stewart and McDougall 2012). However, getting some molecular clues on the nature of the involved compounds involved in this hypothetical synergy and on the specific concentration ranges required for optimum activity will only be possible - in the case of pigmented grains - when complete compositional profiles will become available.

**3-6 In vitro study of anti-inflammatory activity on Caco-2 cells**

The purpose of this experiment was to evaluate the immunomodulatory capacity of the ethanolic extracts from pigmented grain samples on Caco-2 modified cells. In these cells, by inserting a plasmid containing a promoter with 5 binding sites for NF-kb followed by luciferase of firefly, if stimulated with interleukin, a high luminous emission should be recorded. However, if they have been contacted with anti-inflammatory substances such as anthocyanins or polyphenols present in the extracts of grains flour, such light emission should be suppressed proportionally with the reduction of the inflammatory status in the cells. As evident from graph 3, for all pigmented grain extracts had dose-responsive anti-inflammatory properties.




*Graph 3: Immunosuppressive effects of the various extracts. Data are presented as percent inhibition of IL-1β-stimulated expression of NF-κB.*

One of the important points to consider is that at lower concentrations, in particular 0.025 mM, the responses to inflammatory stimulation were dissimilar for the samples. By increasing concentration, however, the effect becomes more and more overlapping. This behavior is explained by emphasizing the importance of the synergistic effect of the various bioactive compounds present in the extracts: the anti-inflammatory properties of these samples are not only attributable to the presence of anthocyanin but also to other compounds like phenolic compounds. The presence of relatively high amount of quercetin in purple wheat and rutin and epicatechin in Rostrato can be verified by the 99% suppression of inflammatory status within the cells.

**4- Discussion**

As shown in Table 7, the total anthocyanin content (TAC) varied significantly among the various grains, confirming previous reports (Abdel-Aal, Young et al. 2006). The fraction derived from the debranning of purple wheat (PWF1) had the highest TAC, closely followed by whole meals from corn varieties BCT and BMF. The fraction obtained from debranning of Blue Wheat SK (BWSK) and the Rostrato corn whole meal had the lowest TAC values.

A comparison with literature data (Wu, Beecher et al. 2006) indicates that even the highest TAC values found in the materials under scrutiny here are far below those reported for some grapes (1.2 mg/g whole fruit in Concord grapes) or in cultivated blueberries (about 4 mg/g whole fruit), but it is of interest that the richest fractions - at least in the case of purple wheat - correspond to materials that are typically considered byproducts, and therefore are not used for direct human consumption.

A study on anthocyanin profile in various grain tissues indicated that TAC in purple wheat bran was in the 0.45 mg/g range, roughly twice what measured by the same Authors in blue wheat bran (Abdel-Aal, Young et al. 2006). In addition, TAC in the combined whole bran and milling shorts from purple wheat was in the 0.17 mg/g range, whereas TAC blue wheat middlings was in the 0.03 mg/g range. Total anthocyanins content in the aleuronic layer from blue wheat did not exceed 0.25 mg/g in previous studies (Jaafar, Sharifah et al. 2013). By comparing these values with those in the specific debranning fractions used in this study, the significant advantage of introducing an appropriately designed debranning step as for obtaining anthocyanin-rich materials from grains that contain anthocyanins only in their outer layer may be seen as self-evident.

The total anthocyanin contents of the blue corn varieties investigated here (both BCT and BMF) was almost twice that reported in previous studies on similar grains (Nankar, Dungan et al. 2016). Whether this relates to the use of different solvents for anthocyanidin extraction in these studies, to the remarkable cultivar-related variability, or to the effects of breeding conditions (Harakotr, Suriharn et al. 2014) (Giordano, Beta et al. 2017) remains to be ascertained.

The Rostrato corn had the lowest TAC among all the grains characterized in this study. However, the total polyphenol content and the overall antioxidant activity in extracts from the

whole flour of Rostrato corn were much higher than those found in equivalent extracts from other pigmented corn varieties. TPC and FRAP values for Rostrato red corn were indeed comparable to those measured in the bioactives-enriched fraction obtained from debranning of purple wheat. Statistical analysis of the data in Table 8 shows a positive correlation ($R^2 = 0.970$; $P < 0.001$) between TPC and antioxidant activity (expressed as FRAP) in the various samples. However, the antioxidant activity of individual phenolic compounds in biological systems is reportedly dependent on their chemical structure (Marko, Puppel et al. 2004).

Abdel-Al showed that the average content of anthocyanins in purple wheat bran was 452.9 ± 32.7 mg/kg and in blue wheat bran was 235.9 ± 127.8 mg/kg, while in the purple pericarp TAC was 2.56 ± 0.06 mg/100g and in PPW (purple pericarp wheat) bran + shorts were 16.86 ± 0.68, and in blue aleurone wheat TAC was 22.58 ± 0.05 and 5.29 ± 0.44 in bran and middlings respectively (Abdel-Aal and Hucl 2003). In this study Purple wheat (F1) also exhibited the highest total phenolic content and antioxidant activity amongst all grains extracts. Significant differences in the concentrations of total anthocyanins were previously reported between blue, purple and red corn or wheat. (Abdel-Aal, Young et al. 2006). The concentration of anthocyanins was ranged between 0.14 mg/g-0.69 mg/g for different verities of debranned fractions or wholegrain wheat and corn (Table 7). The average concentration of anthocyanins in blue wheat was reported as 0.21 mg/g while in purple wheat and purple corn were 0.096 mg/ g and 1.3 mg/g respectively (Abdel-Aal, Young et al. 2006). In our study blue wheat debranned-fraction had an average TAC of 0.17 mg/g (Table 7) which is close to that of reported one in previous study (2.12 ± 0.003 mg/g) (Abdel-Aal, Young et al. 2006), it is remarkable to consider that the main part of total anthocyanins in the blue wheat grains is accumulated in the debran parts which usually remove from the kernel. Earlier studies stated that pigments in purple wheat was found mainly in the pericarp of the grain (0.05 – 0.171 mg/g) while for blue wheat the TAC was highest in the aleuronic layer (0.111 – 0.251 mg/g) (Jaafar, Sharifah et al. 2013).

The total anthocyanins content in blue corn (T) (0.66 mg/g) was higher than that of reported by Nankar et al which might be related to use of different solvents for extractions (Nankar, Dungan et al. 2016). It was found that TAC of four different varieties of Mexican blue

corn was ranged from 54 to 115 mg/100g where cyanidin-3-glucoside was counted for 75.7% of TAC (Yang and Zhai 2010).

The total polyphenol content in purple corn kernel is reported as 6.9 ± 0.4 mg GAE/g, also TAC is 0.75 ± 0.06 mg/g, where the predominant anthocyanins was cyanidin-3-Glu (121.6 ± 6.8 μg/g), and predominant phenolic compound was Ferulic acid (23.1 ± 0.6 μg/g) in that study.
Total polyphenol content in purple wheat debran was 73.6 ± 0.44 (mg GAE/g) (Table 8) and ferulic acid was 18.4 ± 1.8 μg/g, where the amount reported for purple wheat pericarp in middlings was 145.15 ± 9.45 mg/100g and ferulic acid was calculated for 53.18 ± 1.8 mg/100g, while in blue aleurone wheat TPC was 761.64 ± 18.19 in bran and 183.41 ± 19.51 mg/100g in middlings and the amount of ferulic acid was 350.33 ± 3.75 and 71.43 ± 0.83 mg/100g respectively in bran and middlings (Siebenhandl, Grausgruber et al. 2007). In Mexican and American blue corn total polyphenol content was 451 ± 18.1 mg/kg DW, 1310 ± 52 respectively, and ferulic acid was identified as the main phenolic acids in Mexican blue corn (202 ± 4.6 mg/kg DW), whereas free ferulic acid in American blue corn was calculated as 927 ± 15 mg/kg DW, and the catechin amount was counted for 21.4 ± 1.5 and 13.9 ± 0.8 mg/kg DW in Mexican and American blue corn respectively. Therefore, Mexican blue corn displayed a higher antioxidant capacity (29.0 μmol TE/g DW) respect to the American blue corn (25.6 μmol TE/g DW) (Del Pozo-Insfran, Brenes et al. 2006).

In this study the total polyphenol content for blue corn (T) was 30.86 ± 0.65 mg GAE/kg and ferulic acid was the main phenolic compounds (113.9±3.4 μg/g) in cooperation with catechin (112.8±2.9 μg/g) while for blue corn (MF) variety the TPC was 33.7± 0.50 mg GAE /kg which showed higher concentration of rutin and catechin contents than blue corn MF (26.2 ± 2.8 μg/g, 252.5 ±32.6 μg/g) (Table 10). The values of 142.1 mg GAE/100 g DW for blue corn and 140.7 mg GAE/100 g DW for red corn has been reported previously (Mora-Rochin, Gutiérrez-Uribe et al. 2010).

Ferulic acid has been reported as the most abundant phenolic acid in all varieties of colored wheat samples (74-87 mg/100 g), sum up to 79% of the total phenolic acids (Liu, Qiu et al. 2010). It was also identified as the main phenolic acids in the Mexican blue corn (202 mg/kg

DW) (Del Pozo-Insfran, Brenes et al. 2006). In Pisankalla (red kernel) corn ferulic acid content was reported as 269.5 mg/100g DW (González-Muñoz, Quesille-Villalobos et al. 2013).

It has been proved that generally buckwheat has high content of rutin (Kreft, Fabjan et al. 2006), although in this study, blue wheat (SK) showed the highest content of rutin amongst all grains tested here (63.0 ± 2.0 μg/g) (Table 10). In fact, it should be noted that profiling of anthocyanins and phenolic compounds by HPLC was presented as a quantitative analysis of those compounds that could be identified unequivocally. Indeed, caution should be used when interpreting these data, as even very extensive phenolics profiling carried out by most updated methodologies (Siebenhandl, Grausgruber et al 2007; Ficco, Mastrangelo et al 2014) did not allow straightforward structural identification of 40-70 % of the total anthocyanins present in grains and grain-derived foods. With these limitations, the data in Tables 10 & 11 indicate point out to some obvious and some unexpected differences in the anthocyanin profile. Despite the use of different extraction protocols and solvents, the values presented in Tables 10 & 11 of the current study are reasonably close to those reported for similar - although not identical – grains (Abdel-Aal, Young and Rabalski 2006; Giordano et al. 2017). Taking into account only blue colored grains, the cyanidin/delphinidin ratio (considering both the glycosylated and non-glycosylated forms of cyaniding) ranged from 0.5 (BCMF) to 1.7 (BCT) in corn flours, and from 1.3 (PWD1) to 5.4 (BWSK) in wheat samples. This may be of some relevance, in consideration of the structural and reactivity differences among the two classes of anthocyanins.

Kroon et al showed that pure catechin has approximately 1.5 higher antioxidant capacity than ferulic acid on an equal molar basis (Kroon and Williamson 1999). Therefor the high FRAP in red corn (ROS) can be explained by the overall effectiveness of relatively high content of rutin (62.8 ± 2.6 μg/g) combined with other phenolic compounds in this grain. Red corn (Rostrato) also had the highest concentration of epicatechin, whereas the higher amount of catechin was seen in blue wheat than other samples. The total phenolic content in blue maize was detected as 164 ± 14 mg gallic acid/g and the ferric reducing power (FRAP) was determined as 15.5± 0.1 mmol FeSo4 /g (Camelo-Méndez, Agama-Acevedo et al. 2017).

In a study light purple wheat variety Shandongzimai showed the lowest antioxidant activity (FRAP= 28.1 mmol FeSo4 /g DW) whereas Black wheat variety Heibaoshi had the highest

total phenolic content (659.8 μg GAE/g) and antioxidant activity (FRAP: 35.9 mmol FeSo4 /g), however in deep purple wheat Jizi variety (JZ) TPC and FRAP amounts were 0.54 mg GAE/g and 28.2 mmol FeSo4 /g respectively (Li, Ma et al. 2015). Whole meal flour of purple wheat showed higher TAC, TPC and antioxidant activity compare to refined purple wheat flour (30.84 ± 0.81 mg/kgC3G, 4.19± 0.03 mg/g F.A, 4.98 ± 0.03 micromol /g Trolox) (Yu and Nanguet 2013).

**Identification of Anthocyanins and Polyphenols by HPLC**

Anthocyanins and phenolic compounds in the extracts were separated and quantified. Blue corn (BC MF) exhibited the highest content of cyanidin chloride, delphinidin chloride and cyanidin-3-O-glucoside between all grains. In purple wheat (F1) more than 90% of total anthocyanin content was made by cyanidin chloride while in blue wheat (BWSK) the average content of cyanidin-3-glucoside was counted for 58.7% over all. Blue corn (MF) displayed the highest content of delphinidin-chloride with 160 ±6.4 (μg/g). The highest content of ferulic acid was observed in blue corn (T) (Table 10). Blue wheat debranned fraction (BWSK)and red corn (Rostarto) had the highest amounts of rutin (63.00±2.0, 62.8±2.6 (μg/g)). These two grains contain also higher contents of catechin (385.6± 5.5 (μg/g) in BWSK) and epicatechin (465.1±7.9 (μg/g) in ROS). Purple wheat showed higher epicatechin than blue wheat (121.1 ± 8.7(μg/g)) variety.

HPLC profiling of total anthocyanins and polyphenols in this study showed that more than 90% of purple wheat (F1) anthocyanins are composed by cyanidin and its derivatives, while in blue varieties like blue wheat and blue corn delphinidin is the second main anthocyanins. Cyanidin was also the main aglycon in Rostrato which can be responsible for the red color of this variety of corn. Abdel-Al has reported cyanidin-3-glucoside as the main anthocyanins in purple wheat which was the second main anthocyanin in blue wheat while the major anthocyanin in blue wheat was composed by delphinidin-3-glucoside. Delphinidin glycosides has been indicated as the main anthocyanins in blue wheat (56.5 ± 4.6 μg/g) which is counted for 37% of total anthocyanins in blue wheat grain, in fact delphinidin is considered as the main aglycone in blue wheat grains, being ~ 69% of the total anthocyanidins (Escribano-Bailón, Santos-Buelga et al. 2004), while

Cyanidin-3-glucoside has been identified as the major anthocyanin in purple wheat. Anthocyanins characterization by Abdel-al revealed the different anthocyanins in blue wheat and purple wheat which the amount of cyanidin-3-Glucoside was 20.3 ± 1.5 μg/g and 4.0 ± 0.1 μg/g respectively, while for blue corn and purple corn cyanidin-3-Glucoside was 110.2 ± 5.1μg/g and 298.9 ±14.4 μg/g (Abdel-Aal, Young et al. 2006).

**Anti-inflammatory properties of pigmented grains extracts**

Anti-inflammatory effects of anthocyanins and polyphenols from different fruits and cereal grains have been studied both in vitro and in vivo (Min, Ryu et al. 2010, Zhang, Ravipati et al. 2011, Li, Lim et al. 2012, Lee, Kim et al. 2014, Chen, Somavat et al. 2017, Luna-Vital, Weiss et al. 20 7,) and it has been reported that these compounds are able to decrease inflammation by modulating NF-κB signaling pathways at several steps (Youdim, Martin et al. 2000, Metzger, Barnes et al. 2008, Romier-Crouzet, Van De Walle et al. 2009, Zhang, Kang et al. 2011, Asgary, Sahebkar et al. 2014, Pérez, del Castillo et al. 2014). In this study, anti-inflammatory properties of the anthocyanins-rich extracts of the pigmented grains were compared by determining the production of NF-κB in the inflamed Caco-2 cells by interleukin β: three concentrations were considered for each extract. As shown in graph. 1, the anthocyanin-rich fractions grains were effective to reduce the inflammatory response in a dose dependent manner. The concentration of 75μM in BC T and PW (F1) decreased NF-κB activation by 99.9% and 99.8% respectively, while these value for ROS, BW SK, BC MF were about 94.5%, 92.1% and 66.2% respectively at the same concentration.

Studies on blueberry show that anthocyanin rich fractions reduced NF-κB activation, induced by IL- β in intestinal epithelial Caco2 cells at concentrations of 50 and 100 μg mL− by 68.9% and 85.2%, respectively ($p \leq 0.05$). (Taverniti, Fracassetti et al. 2014) Although it is noteworthy that blueberry anthocyanins can be transported through the Caco-2 cell monolayers with different transport efficiency or absorption efficacy depending on their structures. (Yi, Akoh et al. 2006). Vitaglione has reported that after whole grain wheat consumption for 8 weeks plasma tumor necrosis factor-α (TNF-α) reduced and after 4 weeks interleukin (IL)-10 increased (P= 0.04) (Vitaglione, Mennella et al. 2015)

In this study, we have observed anti-inflammatory effects of anthocyanins and polyphenols from pigmented grains extracts on the inflamed Caco-2 cells showing that extractions from purple wheat and blue corn are much more efficient to decreasing inflammation caused by interleukin- β. In fact, it can be elucidated by the presence of cyanidin and ferulic acid as two well-known anti-inflammatory compounds in these grains.

Studies on the purple corn bioactives has been revealed that this pigmented grain can debilitate high-glucose-induced mesangial inflammation, expansion, and hyperplasia by disturbing the inflammatory action of interleukin-8 (IL-8) (Li and others 2012a). When the renal mesangial cells of db/db mice were exposed to high-glucose to induce diabetes, the production of IL-8, a chemokine that is linked to inflammatory processes in glomeruli, was markedly elevated (Li and others 2012a). In contrast, cells that received purple corn anthocyanin-rich extract treatments showed mitigation of IL-8 secretion in a dose-dependent manner (Li and others 2012a). Another study has also demonstrated that purple corn pigments antagonized diabetic kidney problems through control of the IL-8 Tyk2-STAT-signaling pathway (Kang and others 2012). Cyanidin-3-glucoside (C3G) is the most abundant anthocyanin throughout the plant kingdom as well as in purple corn (Kong and others 2003; Lao and Giusti 2016). Numerous research studies have shown that C3G has strong anti-inflammatory properties, although not all sources of C3G were purple corn (Reddy and others2005; Min and others 2010; Serra and others 2013;). Cyclo-oxygenase (COX) is a well-known inflammatory protein whose abnormal up-regulation is commonly found in many cancers (Martin and others 2003; Wang and Stoner 2008). C3G from Prunus Cerasus exhibited strong anti-inflammatory property by efficiently inhibiting COX-1 and COX-2 enzyme activity (Reddy and others 2005). Additionally, Tsuda and others (2002) demonstrated C3G may play a role in the prevention of the NO-mediated inflammatory diseases. C3G was found to suppress zymosan-induce In clinical studies, it has been demonstrated that purple corn consumption provoked diabetic kidney problems produced by inflammation in db/db mice through control of the IL-8-Tyk2-STAT-signaling pathway (Kang, Li et al. 2012). Using Cyanidin-3-G from black rice and its metabolites on RAW 264.7 cells inflamed by LPS, could suppress the production of the proinflammatory cytokines like TNF-α, interleukin- β (IL β), and inflammatory mediators like nitroxide and PGE2. (Min, Ryu et al. 2010) Additional of pigmented

wheat bran extract into RAW264.7 macrophage cells resulted in the oxidation suppression significantly. The IC50 value calculated for the pigmented wheat extract towards intracellular oxidation after 2 hours was 87 µg/ml which this amount is similar to the values needed to suppress intracellular oxidation by anthocyanin yielded from Saskatoon berries or blackberries (Hu, Kwok et al. 2005, Elisia, Hu et al. 2007)

In vitro studies showed that proanthocyanins in purple, blue, and red corn have anti-inflammatory effects demonstrated by inhibiting 66% of inducible nitric oxide synthase and 89% of cyclooxygenase-2 activities (Chen, Somavat et al. 2017). In an interesting study on the bioavailability of phenolic compounds Anson. N. et al., have investigated the antioxidant and anti-inflammatory capacity compounds from different wheat fractions after gastrointestinal digestion. In that study, the bio-accessible compounds from aleurone, bran and flour were obtained from the upper gastrointestinal tract by a dynamic in vitro model. Bio-accessible compounds from aleurone showed the highest antioxidant capacity and these bioactives could provide a prolonged anti-inflammatory effect comparing to bran and flour (Anson, Havenaar et al. 2010). Epidemiological evidences have indicated that consumption of anthocyanin-rich foods reduces the prevalence of chronic diseases. It has been proved that by feeding C57BL/6 mice by purple corn anthocyanin (doses of 200 mg kg− in the -week trial) bodyweight was reduced by 16.6%, and the levels of fecal butyric acid were effectively increased, also hepatic enzymes like SOD and GPx activities were increased whereas lipid peroxidation decreased, and the gene expression levels of TNFα, IL-6, iNOS, and NF-κB was downregulated. In fact, purple corn anthocyanins could decreased the production of inflammatory cytokines in this type of mice presenting pathophysiological condition of inflammation and obesity which is indicated by high expression levels of inflammatory markers like TNFα, IL-6, iNOS, and NF-κB genes (Wu, Guo et al. 2017).Purple corn feeding in rats also could reduce the visceral adiposity index, total body fat mass, systolic blood pressure, plasma triacylglycerol and total cholesterol. It also could improve the glucose tolerance as well as liver and cardiovascular structure and function. Inflammatory cell infiltration also was reduced in rat heart and liver. Therefor Purple corn consumption having bioactive molecules can be suggested to control metabolic syndrome symptoms via controlling the damages induced by inflammation (Bhaswant, Shafie et al. 2017).

Studding on cyanidin-3-glucoside and resveratrol revealed their anti-inflammatory properties even higher than that of 5-aminosalicylic acid (5-ASA) known as an anti-inflammatory drug which is pharmaceutically used in the treatment of IBD, and the mechanism of its action has been explained through decreasing the production of inflammatory markers by down-regulation of cytokine-induced Janus kinase/signal transducer and activation of transcription (JAK-STAT) pathway, in caco-2 cell lines (Serra, Almeida et al. 2016). The effects of consuming Purple Konini wheat with the total anthocyanin content (TAC) of 41.70 mg/kg by rats was studied and the analysis on blood and liver enzyme activities tissues of these animals showed significant antioxidant activity (measured by FRAP) ($P < 0.05$), which suggested the effect of consumption nutrients containing higher levels of anthocyanins can improve the antioxidant activity and functionality of the liver tissues (Mrkvicová, Pavlata et al. 2017).

Therefore, more studies should be carried out as for assessing the mechanisms involved in the anti-inflammatory effects of pigmented grains consumption in humans. Our study indicated the possible role of anthocyanins and phenolic compounds in cereals as good sources with notable anti-inflammatory effects. Therefore, these grains can be considered as ingredients for making foods like bread, noodle or pasta, or as additives in dairy products or beverages with high nutritional values. Consequently, it is necessary to provide more evidenced of their health benefits by human consumption and investigated pigmented grains consumption on the possible induction or repression of genes coding for antioxidants compounds in further studies. By using metabolomics, it can be investigated how antioxidant-rich cereal grains can or may modify metabolism and the related metabolic pathways can be elucidated. Their effects on the intestinal microbiota can be addressed to investigate the level of absorption and the nature of metabolites formed after consumption of anthocyanin-rich pigmented grains products. These findings will provide novel information on the health benefits of cereal grains and the development of healthy functional foods.

**Inhibition of intestinal α-Glucosidase and pancreatic α-amylase activities**

The α-glucosidase and α-amylase inhibitor activity of colored grains in vitro was evaluated. Purple wheat (F1) and red corn (ROS) showed much stronger potency to inhibit α-glucosidase enzyme

than acarbose. Though the other grain extracts had slight effects on inhibiting the activity of enzyme, but it was not meaningful (Graph 2). Blue corn (MF) (IC 50: 60.15 µg/mL) and red corn (ROS) (IC 50: 68.36 µg/mL) showed the higher α-amylase inhibitor activity than the other grains extracts, whereas purple wheat (F1) fraction had the highest α-amylase inhibitor activity between wheat samples (IC 50: 81.25 µg/mL).

Anti-hyperglycemia property of blue corn flour extract was displayed a-amylase inhibitory activity by 93.4±1.3% in a study by Camelo (Camelo-Méndez, Agama-Acevedo et al. 2017). The inhibition of these enzymes mainly results in delayed breakdown of carbohydrates and consequent uptake of glucose in blood stream, which is crucial to sustain the level of post-prandial glucose. Acarbose which is considered as the drug in the management of T2D inhibit maltase and sucrase was used as the standard in this study. Doses suggested by European regulations for Acarbose is between 25 and 200 mg three times daily of course depending on severity of diabetes disease (Castro-Acosta, Hall et al. 2016). Therefor administration of certain polyphenols in the diet may contribute to the synergistic effects on sucrase and maltase inhibitory activity. In different studies pure anthocyanins or flavonoids has been tested for their α-amylase inhibitory activity: IC 50 values for cyanidin, cyanidin-3-glucoside and cyanidin-3-galactoside were 0.38 ± 0.01 mM, 0.30± 0.01 mM and >1.00 mM respectively (Akkarachiyasit, Charoenlertkul et al. 2010) while the IC 50 value of cyanidin-3-rutinoside against pancreatic α-amylase was 24.4 ± 0. µM and the kinetic analysis have revealed that pancreatic œamylase inhibition by cyanidin-3-rutinoside is in a non-competitive manner (Akkarachiyasit, Yibchok-Anun et al. 2011). The inhibitory effects observed with the cereal extracts in this study is sensibly higher that that reported before from a variety of pigmented plant materials. On an anthocyanin content basis, the values of I50 measured here are in the 40-100 microgram/ml range, that is, about half those reported in previous studies with purified anthocyanins (Akkarachiyasit, Charoenlertkul et al. 2010). This may be taken as an indication of synergistic effects between anthocyanidins and phenolics in the extracts used in this study, as already hypothesized for phenolics and anthocyanins from other sources (Boath, Stewart et al. 2012).

Like acarbose, anthocyanin can act as a competitive α-glucosidase inhibitor because of the structural similarity between the normal substrate maltose and the glucosyl group with β-linked connection (Mcdougall and Stewart 2005).

It has been demonstrated that the inhibitory effects on α-amylase are largely arbitrated by proanthocyanidins, whereas α-glucosidase activity is modulated by the anthocyanin-containing portion. The inhibition of individual polyphenolic compounds isolated from Maize (Zea mays L.) on the activity of a-glucosidase was found much higher than standard acarbose, indicating that these bioactives can be used as strong inhibitors against a-glucosidase enzyme (Nile and Park 2014). In vivo studies using Caco-2 cells showed sugar uptake inhibition by anthocyanin extracts and phenolic compounds. Cyanidin-3-rutinoside is much stronger than cyanidin-3- galactoside to inhibit sucrase rather than maltase which is maybe related to the disaccharide structure of rutinose resulting in the higher α-amylase inhibitory activity (Akkarachiyasit, Charoenlertkul et al. 2010, Akkarachiyasit, Charoenlertkul et al. 2010). It has been shown that cyanidin aglycone itself also can inhibit sucrase activity. In fact, cyanidin glycosides can have principally potent to make lower postprandial glycaemia levels (Grussu, Stewart et al. 2011, Boath, Stewart et al. 2012).

Qualitative SAR (structure–activity relationship) has revealed the mechanism of α-amylase and α-glucosidase inhibition by polyphenols in particular by flavonoids: (1) Hydroxyl groups in particular at positions of C5 and C7 in the A–C ring and C30 and C40 -positions of the B ring can increase the inhibitory activity toward a-glucosidase and a-amylase;(2) Methylation and methoxylation can ‘‘blocks’’ the free hydroxyl groups therefor decrease the inhibition;(3) Though glycosylation evidently increases the number of free hydroxyl groups but make lower the inhibitory activity of polyphenols glycosides than that of their aglycone counterparts; (4) Flavonoids planarity which is due to the unsaturation of the C2–C3 bonds might increases a-glucosidase and a-amylase inhibitory activities (Gonzales, Smagghe et al. 2015). Copeland showed that the type of α-amylase inhibition is competitive although in another study the inhibition type of cyanidin-3-rutinoside is indicated as non-competitive (Akkarachiyasit, Yibchok-Anun et al. 2011, Jariyapamornkoon, Yibchok-anun et al. 2013).

Red rice polyphenols showed strong inhibition against pancreatic a-amylase activity (IC 50: 3.61 mg/mL), and the inhibition type was reported as a combination of competitive and noncompetitive inhibition (Liu, Hu et al. 2017). It also has been showed that the combination of ferulic acid and feruloylated arabinoxylan mono- and oligosaccharides from corn bran and wheat aleurone could inhibit sucrase activity by 18.70 ± 5.25, 23.28 ± 4.51% respectively and the IC50 values for ferulic acid was 0.09 mg/mL in wheat aleuron and 0.10 mg/mL in corn bran (Malunga and Eck 2016).

Clinical studies investigated the effect of polyphenols on glucose transport in the Caco-2 cell line, and the results showed that following the release of glucose from sucrose and starch by the activity of a-amylase and a-glucosidase enzymes, its absorption can be disrupted by interactions between anthocyanins and intestinal sugar transporters like GLUT2 and SGLT1. It has been reported that flavonoids can inhibit only GLUT2. In particular, quercetin is 4 times more potent (78%) than Ferulic Acid at the same concentration to inhibit GLUT2. Considering that corn has 4 times more ferulic acid content than wheat, it can be an appropriate ingredient in food making, while data has shown that by daily consumption of 77 mg of ferulic acid the levels of postprandial plasma glucose levels can be effectively suppressed, therefore it implies the importance of cereal grains incorporation into food materials although it should be noticed that its uptake largely depends on the type of cereal grains and the level of its bioavailability (Malunga and Eck 2016).

## 5- Conclusions

Pigmented cereal grains (purple/blue wheat and corn) were characterized for their bioactive compounds. This study confirms that anthocyanins and phenolic compounds in pigmented cereals are present in concentrations high enough for considering these materials as good sources of bioactives. Although to a different extent, all the grains extracts characterized in this study showed anti-inflammatory and enzyme inhibiting properties. Thus, these grains appear suitable as ingredients for the direct transformation into foods like bread, noodles, or pasta, or as possible additives in dairy products or beverages, in particular when association with high levels of other bioactives (such as dietary fiber) is of relevance. In this frame, it is of interest that both anti-inflammatory and enzyme inhibiting activities were high in materials (such as the fractions obtained from de-hulling of pigmented wheat) that are rich in fiber and are not commonly used for human consumption.

However, the molecular reasons for the different efficacy of the various extracts characterized in this study remain somehow elusive, as remains elusive the reason for several of the extracts studied here being more active (at equivalent concentration of bioactives) than extracts from other sources. Indeed, the data presented here suggest that neither the enzymatic inhibition nor the anti-inflammatory effects could be attributed to a specific component in the extracts. On the contrary, our data highlight that both effects may be ascribed to some type of synergy among components in a given extract.

# CHAPTER 2

## 6. PRODUCING A TYPICAL FOOD INCORPORATING PURPLE WHEAT GRAIN

### 6-1 Preparing pasta samples

The pasta samples used for this study were produced using pilot plants located at C.R.A. in Sant'Angelo Lodigiano (LO). Four different types of pasta were then prepared, each made with a different composition: pasta 1 was made with a mixture of 0.15 kg of common wheat flour and 0.5 kg of Fraction 1; pasta 2 with was made with 1.5 kg of 100% soft wheat flour and 0.5 kg of Fraction 2; pasta 3 by mixing 1.5 kg of 100% hard wheat semolina 0.5 kg of Fraction 1; pasta 4 with mixing 1.5 kg of 100% hard wheat semolina and 0.5 kg of Fraction 2 (Table 13). As depicted in figure 13, F 1 and F2 fractions are respectively correspond to the first fraction of decortication, consisting only of external grain coatings, and the second fraction of decortication, produced following a second debranning phase.

| IDENTIFICATION | NAME | DESCRIPTION |
|---|---|---|
| Pasta 1 | **F + F1** | **Flour 75% soft wheat + 25% Fraction 1** |
| Pasta 2 | **F + F2** | **Flour 75% soft wheat + 25% Fraction 2** |
| Pasta 3 | **S + F1** | **Semolina 75% hard wheat + 25% Fraction 1** |
| Pasta 4 | **S + F2** | **Semolina 75% hard wheat + 25% Fraction 2** |

*Table 13: Pasta sample types description*

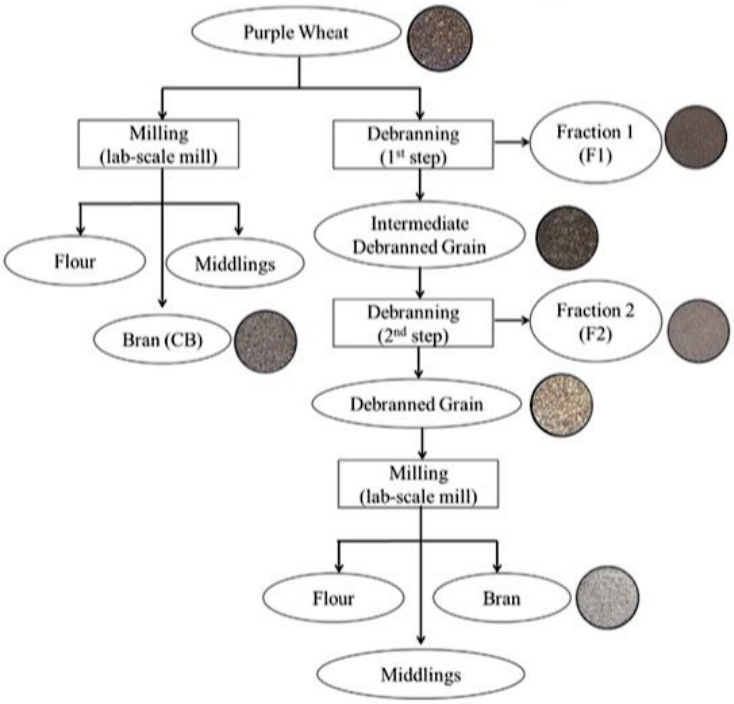



*Figure 13: Decortication process of Purple Wheat and production of Fraction 1 and Fraction 2*





**6-2 Cooking and Freeze-drying of Samples**

A five grams aliquot of each pasta sample was weighted and cooked in 50 mL of distilled water. Pasta samples were cooked at different time intervals, following instructions set forth in previous experiments (Table 14):

| Pasta Sample | TIME OF COOKING |
|---|---|
| **F + F1** | **3' and 30''** |
| **F + F2** | **3' and 30''** |
| **S + F1** | **3'** |
| **S + F2** | **3'** |

*Table 14: Time of cooking for each type of pasta*

After cooling, the samples were placed in refrigerator and then in a lyophilizier for at least 1 day allowing to complete lyophilizing; then they were milled and stored in a dark place for further analysis. The ethanolic extraction was then performed on all types of pasta. The characterization of bioactives like anthocyanins and phenolic compounds in pasta sample were obtained both before and after cooking (using the same methods previously described in PART I). For anti-diabetic properties and anti-inflammatory activities of the pasta sample extracts, analysis was completed only on the extracts after cooking pasta samples.

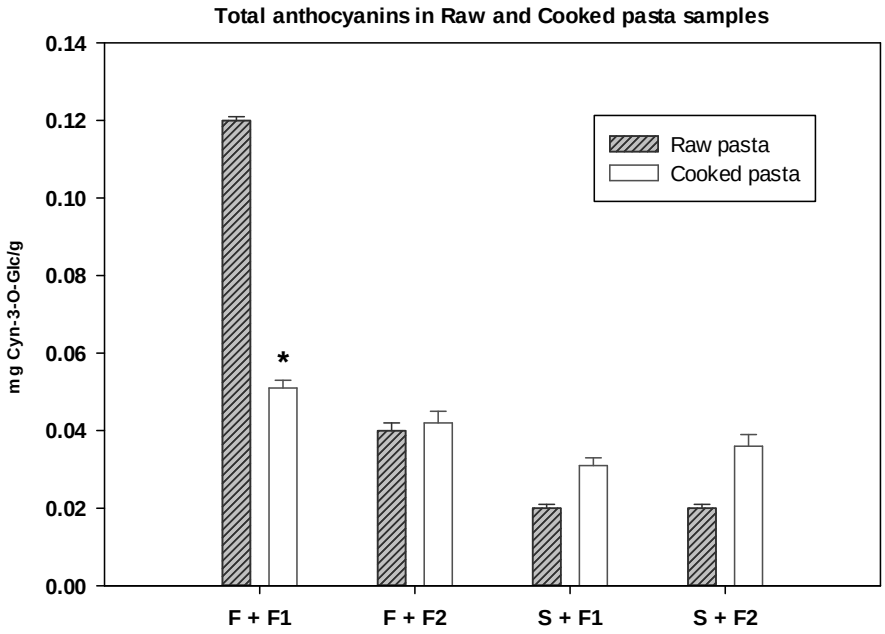
## 7- RESULTS & DISCUSSION

### 7-1 Quantification of total anthocyanin contents

The first part of the characterization of the pastes obtained from the processing of the different fractions of Purple Wheat provided for quantification of the total anthocyanin content. The results are expressed as mg of cyanidine-3-o-glucoside equivalent per gram of flour; in the case of pasta before cooking, the flour refers to the flour obtained directly from the grinding of the pasta, while in the case of the cooked pasta is the flour obtained after cooking, lyophilization and subsequent grinding of the same pasta [Table 15]. Representing the same data within a chart, it can be appreciated the changes that have took place after cooking on pasta samples (Graph 4):

| Sample | Total Anthocyanin Raw pasta (mg/g) | Total Anthocyanin Cooked pasta (mg/g) |
|---|---|---|
| **F + F1** | 0.12 ± 0.001 [a] | 0.051 ± 0.002 [a] |
| **F + F2** | 0.04 ± 0.002 [c] | 0.042 ± 0.003 [c] |
| **S + F1** | 0.02 ± 0.001 [b] | 0.031 ± 0.002 [a] |
| **S + F2** | 0.02 ± 0.001 [a] | 0.036 ± 0.003 [b] |

*Table 15: Total anthocyanin content in pasta samples before and after cooking*




*Graph 4: Total anthocyanins in pasta samples before and after cooking*

Therefore, we can identify two different effects: in the sample of pasta made from flour added to the first fraction (F + F1) there is a marked reduction in the anthocyanin content while in all the other three samples the quantitative value is almost unchanged and even increased. The cooking step is a very delicate phase for these samples, since anthocyanins, and more generally phenolic compounds, are extremely sensitive to light and heat. Moreover, being water-soluble compounds, boiling in water is certainly a passage that affects the final product. These reasons, explain the visible effects on the first sample. In fact, it was extremely rich in anthocyanin before cooking, as fraction 1 is richest in bioactive compounds. Even after cooking and losing much of the content of these substances, this still remained the richest anthocyanin sample among the four analyzed pasta samples.

As for the other extracts, and in particular those derived from pasta samples made from hard wheat flour, the explanation of their behavior might be different. There is also a loss of anthocyanin in these cases as a result of exposure to heat, but another phenomenon occurs at

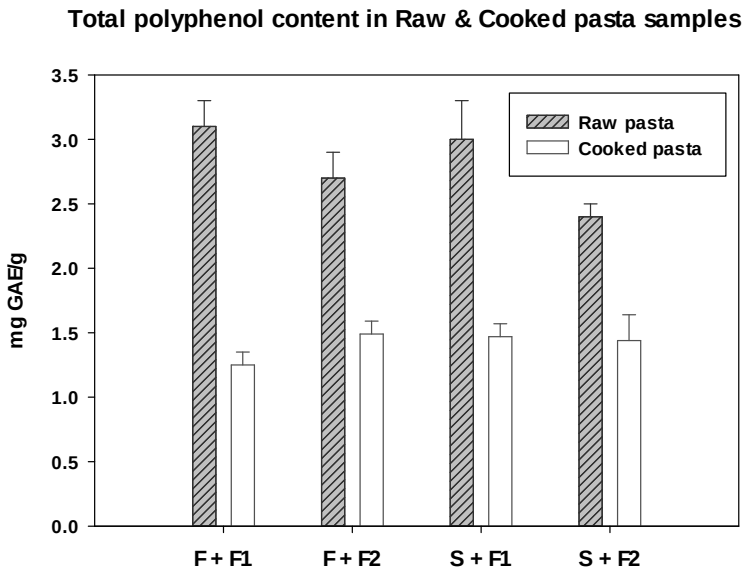
the same time: denaturing, that takes place under the pasta structure during cooking phase, predominantly to the gluten fraction, to expose and release bioactive compounds and, in particular, anthocyanins which were otherwise unavailable during the extraction process on the raw pasta samples. Consequently, simultaneous loss of compounds due to cooking and exposure of others following denaturation of the structure is certainly occurring in all samples, but most significantly in F + F2, S + F1 and S + F2.

### 7-2 Determination of the total polyphenols content

For the determination of the total content of polyphenols (TPCs) the Folin-Ciocalteu method was used as described previously. Again, as previously stated for the quantification of total anthocyanins, the quantities of polyphenols in the samples before and after cooking were compared, so that the variation can be detected (Table 16 & Graph 5).

| Sample | Total Polyphenols in Raw pasta (mg/g) | Total Polyphenols in Cooked pasta (mg/g) |
|---|---|---|
| **F + F1** | $3.1 \pm 0.2^{a}$ | $1.25 \pm 0.1$ |
| **F + F2** | $2.7 \pm 0.2^{c}$ | $1.49 \pm 0.1$ |
| **S + F1** | $3.0 \pm 0.3^{b}$ | $1.47 \pm 0.1$ |
| **S + F2** | $2.4 \pm 0.1^{ab}$ | $1.44 \pm 0.2$ |

*Table 16: Total Polyphenol contents in pasta sample before and after cooking*




*Graph 5: Total polyphenol content in pasta sample before and after cooking*

### 7-3 Qualitative and Quantitative characterization by HPLC

The qualitative and quantitative characterization technique considered most suitable was HPLC. The differences on anthocyanins and some of phenolic compounds in pasta samples can be appreciated from the HPLC chromatograms before and after cooking, (the presence of the different compounds selected in these samples; Cyanidin chloride, Delphinidin, Ferulic acid, Rutin and Quercetin).

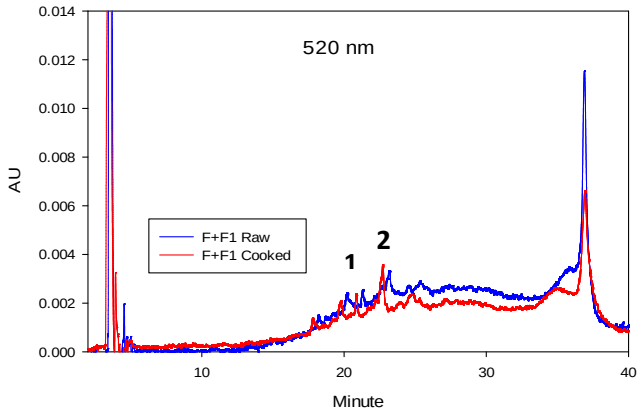
| Standards | Time of exit (minute) | Area of peak/µg |
|---|---|---|
| **Cyanidin chloride** | 22.7 | 6380826.6 |
| **Delphinidin** | 20.3 | 5238520.0 |
| **Ferulic Acid** | 22.9 | 4564785.7 |
| **Rutin** | 22.5 | 2002447.0 |
| **Quercitin** | 32.2 | 3667017.6 |

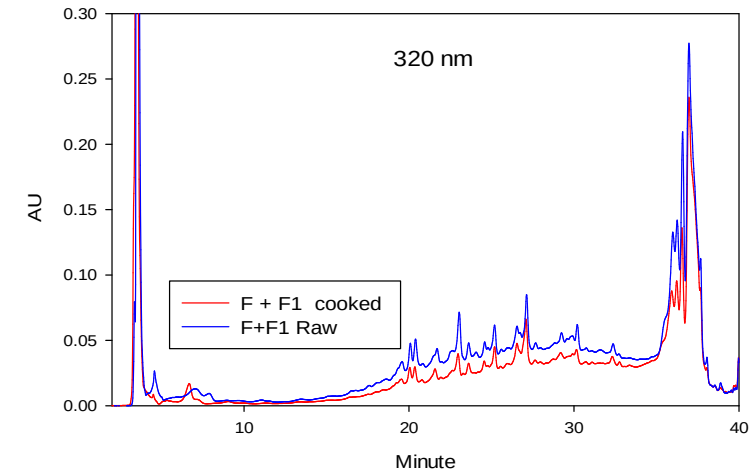
*Table 17: Results of different standards performed by HPLC*

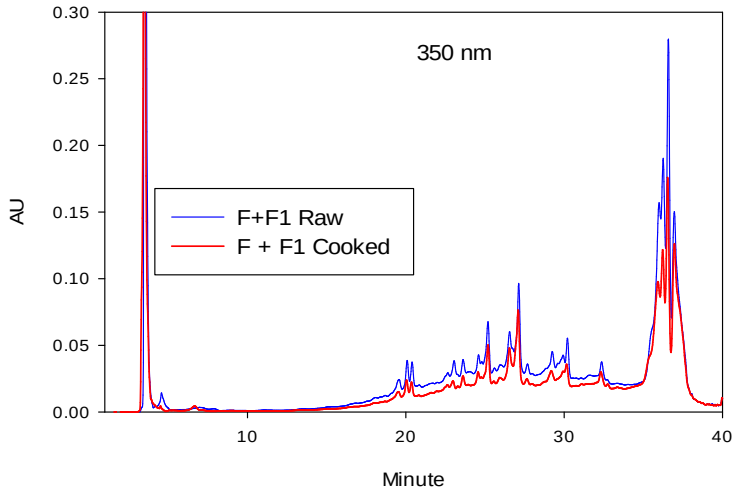
### 7-4 Qualitative analysis on extracts of cooked pasta samples

The chromatograms of the extracts derived from the samples of cooked pasta, therefore, observed at the correct wavelengths, were compared with the peaks obtained from the standards and the various bioactive compounds contained within were identified.

Observing them you can highlight some aspects. First of all, for each wavelength, all the chromatograms for the four samples of analyzed samples were reported. They are almost overlapping with each other, where two anthocyanins cyanidin chloride and delphinidin as numbers 1 & 2, ferulic acid by number 3, rutin and quercetin by numbers 4 and 5 are labeled on the related chromatograms in graph 6.



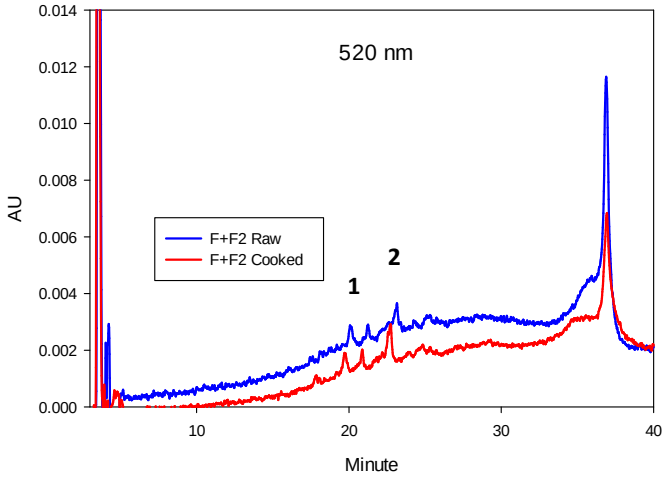


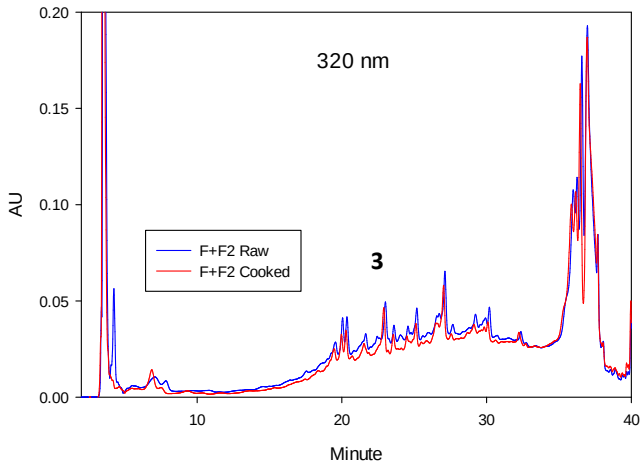



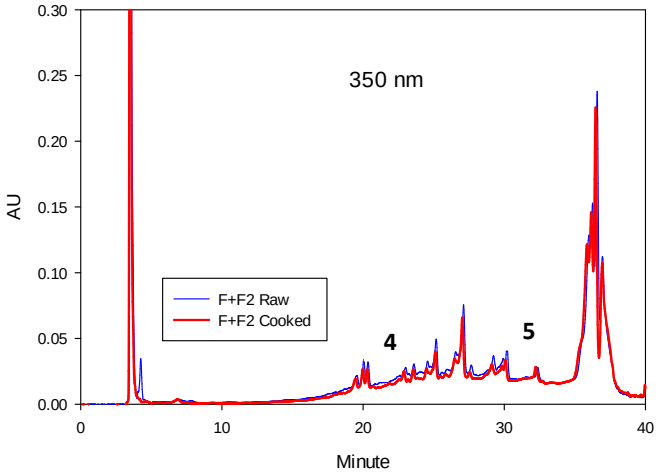

*Graph 6: Chromatograms of anthocyanins and polyphenols in raw and cooked pasta extracts*

Moreover, it can be emphasized that the chromatograms are so similar to each other with respect to peak output times but have differences in terms of quantity of compounds, which are

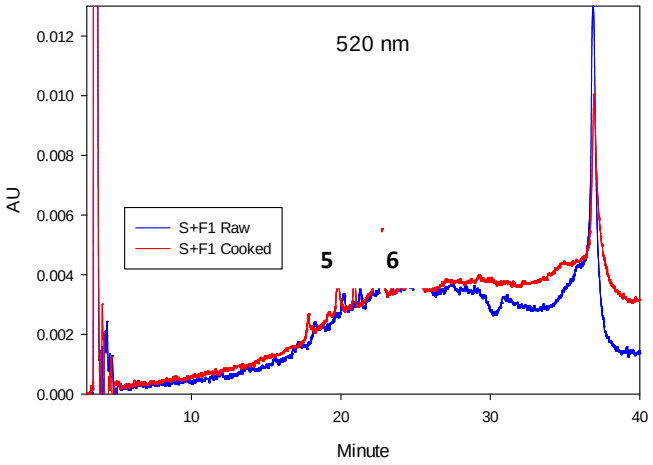
already visible from this analysis system: F + F2 will in fact certainly have a quantity of cyanidin, delphinidin, rutin and quercetin lower than S + F1.

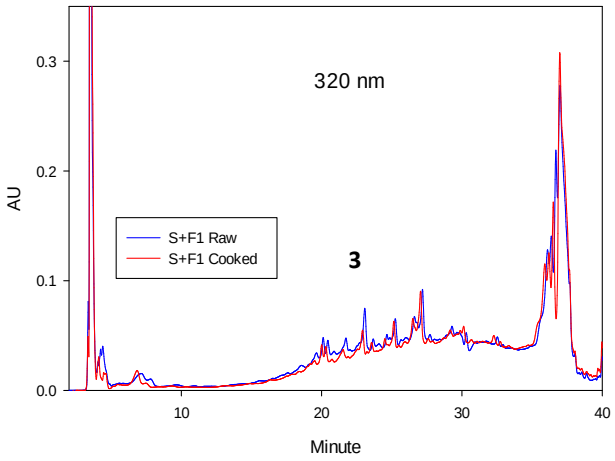


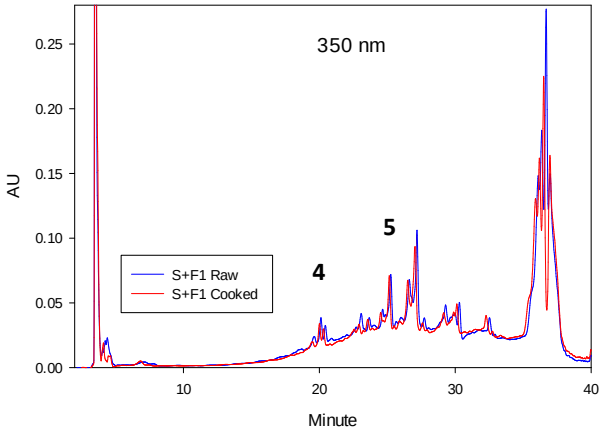



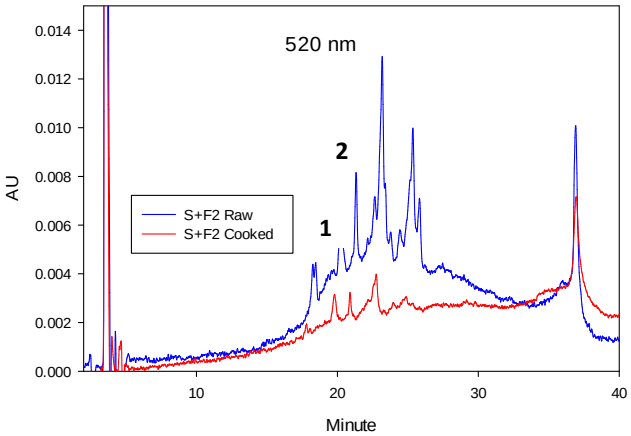


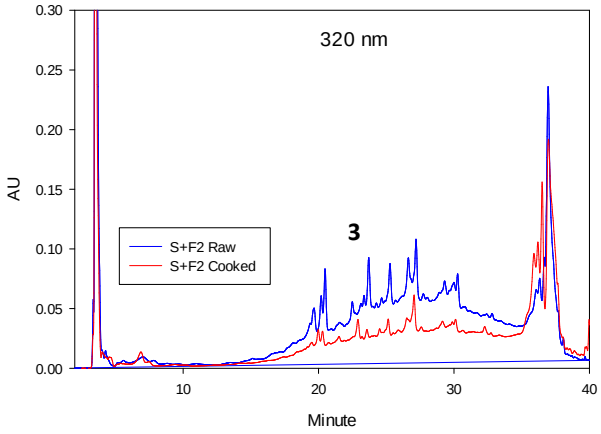

Finally, it is important to emphasize how more unidentified peaks emerge within these chromatograms. These, in fact, relate to other compounds, other than those identified by the use of standards, but which presumably may in some cases also have a bioactive and synergistic function. As can be seen by observing these data, the most present compound within the cooking pasta samples is undoubtedly ferulic acid. Anthocyanins, however, confirming what was already visible at a glance from the chromatograms are present in small quantities.

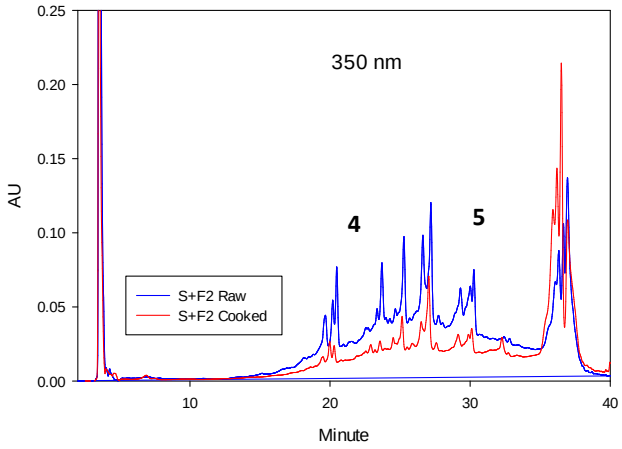



320 nm
AU
Minute
S+F1 Raw
S+F1 Cooked
3
0.0
0.1
0.2
0.3
10
20
30
40


350 nm
AU
Minute
S+F1 Raw
S+F1 Cooked
4
5
0.00
0.05
0.10
0.15
0.20
0.25
10
20
30
40


520 nm
AU
Minute
S+F2 Raw
S+F2 Cooked
1
2
0.000
0.002
0.004
0.006
0.008
0.010
0.012
0.014
10
20
30
40



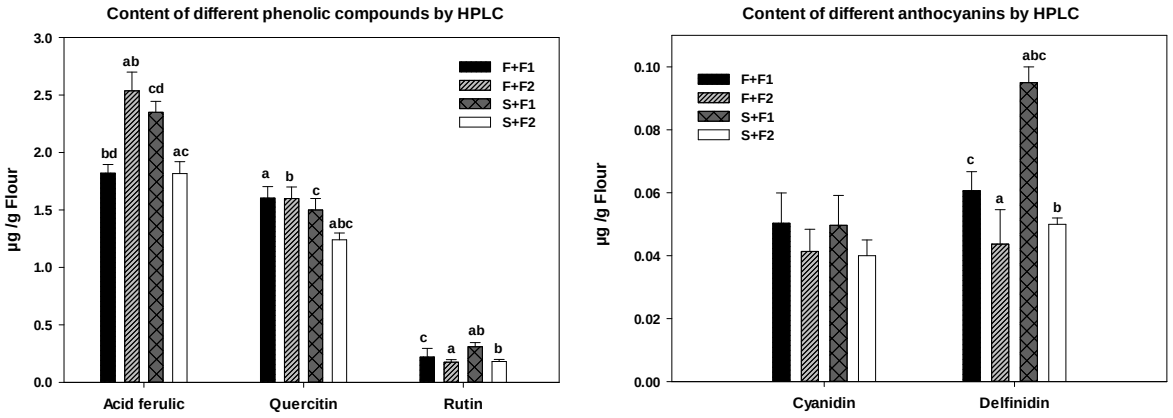

By comparing the different extracts, it is noticeable that each extract presents all the desired compounds, but in different proportions. The richest anthocyanin samples are F + F1 and S + F1: these data confirm preliminary analyzes that showed that fraction 1, was itself richer in these bioactive compounds than fraction 2.

### 7-5 Quantitative analysis on extracted samples of cooked pasta

Reporting the area of the peaks formed by the standards with those of the chromatograms of the different samples, it was possible to trace the quantitative determination of these same compounds. The results are thus expressed as micrograms of each molecule contained in a gram of flour derived from the cooking, lyophilization and grinding of the analyzed pasta (Table 18).

| (µg /g Flour) | F+F1 | F+F2 | S+F1 | S+F2 |
|---|---|---|---|---|
| **Cyanidin chloride** | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.05 ± 0.01 | 0.04 ± 0.01 |
| **Delphinidin** | 0.06 ± 0.006 | 0.04 ± 0.01 | 0.10 ± 0.01 | 0.05 ± 0.0 |
| **Ferulic Acid** | 1.82 ± 0.08 | 2.54 ± 0.16 | 2.35 ± 0.1 | 1.82 ± 0.1 |
| **Rutin** | 0.22 ± 0.08 | 0.18 ± 0.02 | 0.31 ± 0.04 | 0.18 ± 0.02 |
| **Quercitin** | 1.6 ± 0.1 | 1.6 ± 0.1 | 1.50 ± 0.1 | 1.24 ± 0.06 |

*Table 18: Quantification of the content of different compounds by HPLC chromatogram analysis*




As can be seen by observing these data, the most present compound within the cooked samples is undoubtedly ferulic acid. Anthocyanins, however, as it was already visible from the chromatograms are present in small quantities.

In addition, for some molecules, particularly for delphinidin and cyanidin chloride, in most samples the quantification has given rise to truly irresistible amounts.

Comparing the different extracts, one finds that each extract has its own composition, i.e. present all the desired compounds, but in different proportions. However, one can observe that the richest anthocyanin samples are F + F1 and S + F1: these data confirm preliminary analyzes which showed that fraction 1, the one obtained from the outer layer of the grains, was itself richer in these bioactive compounds than the fraction 2.

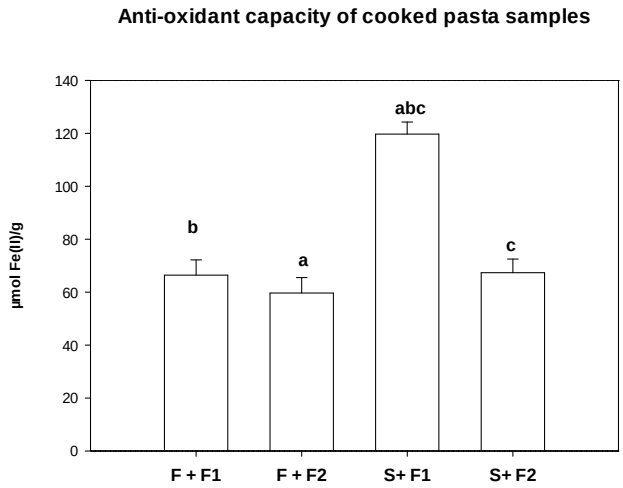
Another interesting evaluation was that of comparing the chromatogram data obtained from raw pasta extracts with those of pasta cooked, previously shown. Considering also the data on the quantification of total anthocyanins and polyphenols, cooking lowers the levels of such compounds due to their water solubility and their high sensitivity to light and heat. However, in some cases this phenomenon does not occur, indeed, the amount of bioactive components seems to increase: this is probably explained by the fact that cooking may induce changes in the structure of the pasta that will release and make it more accessible to extracting molecules otherwise imprisoned in gluten-free mesh, making them to some extent more available.

### 7-6 Assessment of the antioxidant capacity

The evaluation of the antioxidant capacity was carried out only on cooked pasta samples and, for this purpose, the FRAP method was used as was described previously, expressing the results as micromoles of ferrous ion produced (Table 19).

| **Pasta** | **Antioxidant activity (µmol Fe(II)/g)** |
|---|---|
| **F + F1** | 66.45 ± 5.76 |
| **F + F2** | 59.64 ± 5.9 |
| **S+ F1** | 119.71 ± 4.5 |
| **S+ F2** | 67.36 ± 5.1 |

*Table 19: Quantification of antioxidant capacity in cooked pasta samples*




The pasta sample with the highest antioxidant capacity is S + F1, i.e., the one obtained by mixing hard wheat flour and fraction 1. If we compare this property with the total content of anthocyanin and polyphenols, it can be deduced that there is no quantitative correspondence between them: in fact, the sample with an increased amount of anthocyanin was F + F1 and that with more polyphenols was F + F2. Hence, it can be assumed that the relationship is of a qualitative nature: depending on the composition of the samples, in terms of bioactive compounds, it will have a greater or lesser antioxidant effect. Then, the sample which, if cooked, manages to maintain a composition that ensures a greater antioxidant effect, is S + F1.

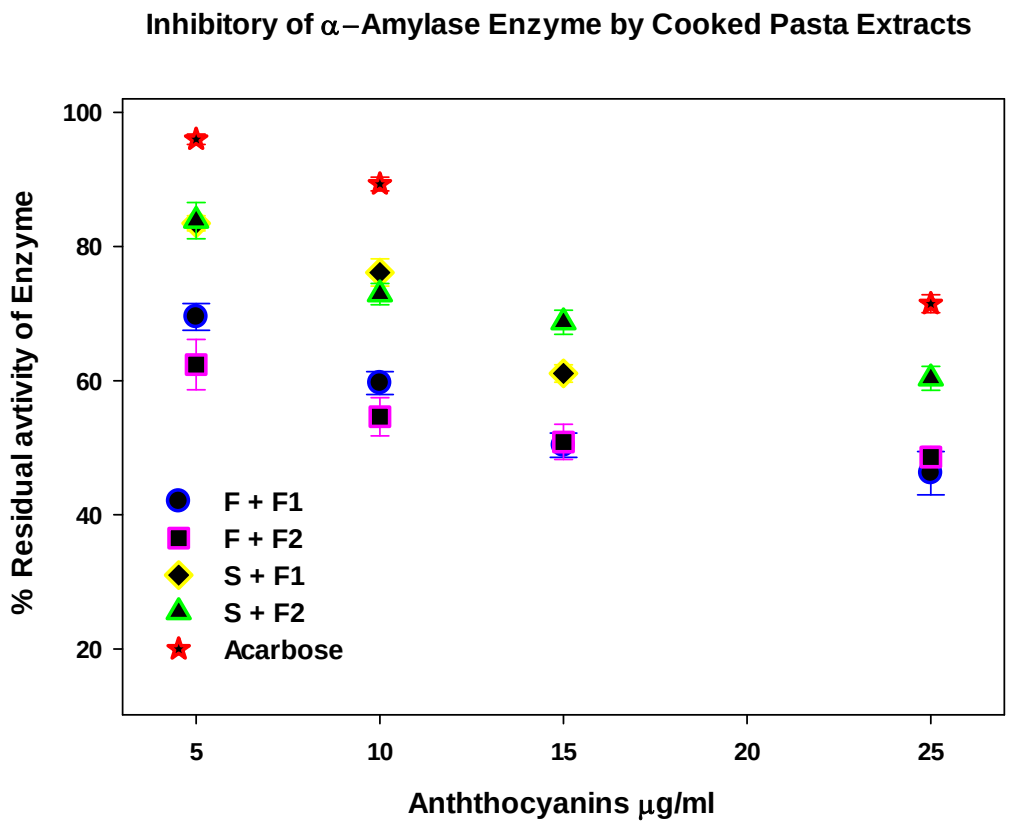
### 7.7 Evaluation of enzymatic inhibition capacity

In the extracts from cooked pasta sample, the activities of both enzymes were measured, comparing them with the inhibitory activity of acarbose.

#### 7-7-1 Enzymatic inhibition of alpha-amylase

The assay provided the data in graph 7. As can be seen, in all four samples of cooked pasta gave dose-dependent inhibition of the enzyme activity. The samples that have an inhibitory activity greater than 25 µg / mL are F + F1 and F + F2, most likely due to their particular composition. An

inhibitory activity of 50% at the concentration of about 10 µg / mL of anthocyanin is observed for these two types of pasta.

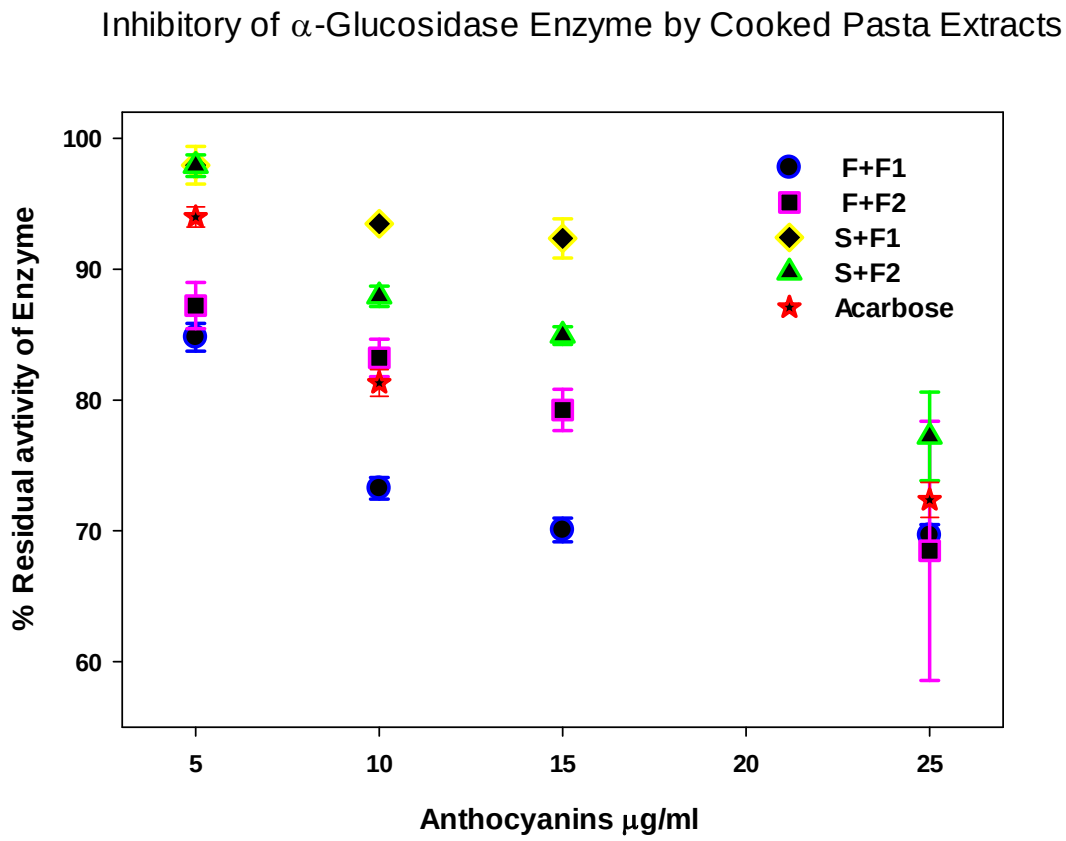



*Graph 7: Enzymatic inhibition of alpha-amylase*

**7-7-2 Enzymatic inhibition of alpha-glucosidase**

The assay provided the data in graph 8. Unlike alpha-amylase, alpha-glucosidase inhibitory activity is significantly lower. In general, however, there is a fair linearity between increased anthocyanin concentration and the decrease in residual enzymatic activity. Even in this case, the sample that exhibited this property more markedly was F + F2, which at the concentration of 25 µg / mL had a residual enzymatic activity of 68%, whereas in case of F + F1 the residual enzymatic activity is 70%. Therefore, at the same concentration, the anthocyanins contained in the F + F1

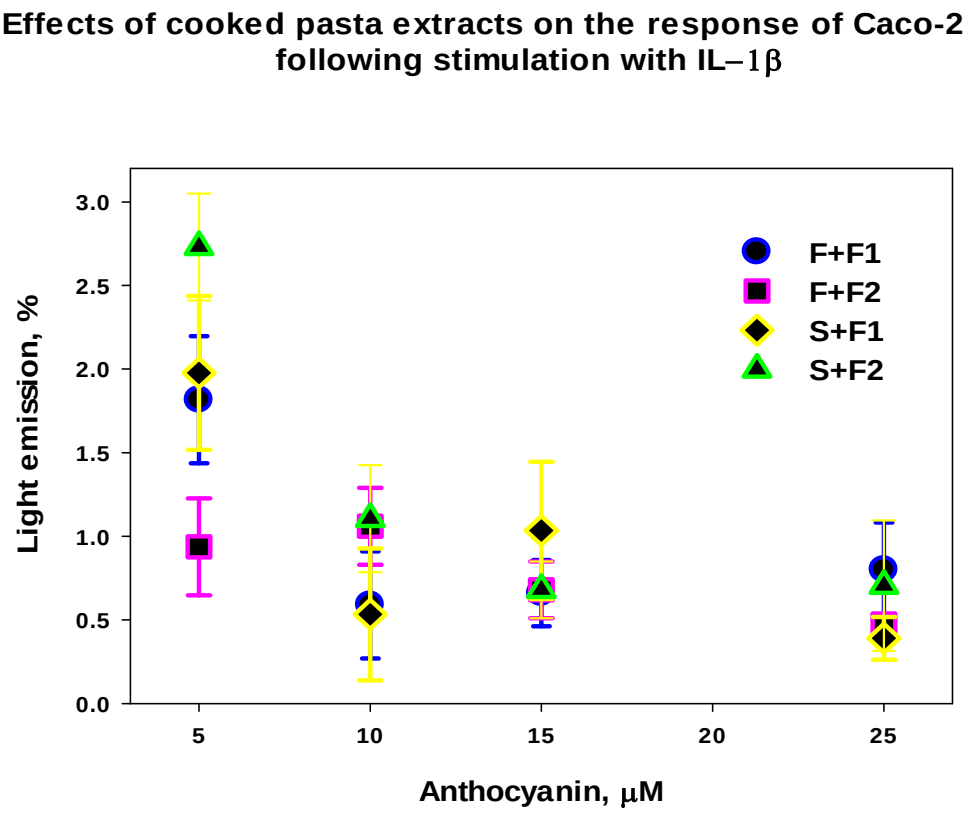
and F + F2 samples, due to the synergistic effect of other substances, have a greater effect on the reduction of enzymatic activity.




*Graph 8: Enzymatic inhibition of alpha-glucosidase*

**7-8 In vitro study of anti-inflammatory activity of cooked pasta extracts on Caco-2 cells**

The purpose of this experiment was to evaluate the immunomodulatory capacity of cooked pasta samples on Caco-2 modified cells. Ethanolic extracts (without acid) were prepared from cooked pasta samples, and the defined concentrations were considered. The results obtained in different repetitions were normalized using the positive control values of interlucine- β as referenceAs seen from graph 9, all of four samples the retained the anti-inflammatory properties observed on grain extracts, including the dependence on bioactive concentration.




*Graph 9: Immunosuppressive effects of the 4 type of pasta extracts. Data are presented as percent inhibition of IL-1ϐ-stimulated expression of NF-κB.*

One of the important points to consider is that at lower concentrations, in particular 5 mM, the responses to inflammatory stimulation were distinct for the four samples. By increasing concentration, however, the effect becomes more and more overlapping. This behavior is explained by emphasizing the importance of the synergistic effect of the various bioactive compounds present in the extracts: the anti-inflammatory properties of these samples are not only attributable to the presence of anthocyanin but also to other compounds contained within as all phenolic compounds which were previously identified.

## 8- CONCLUSIONS

Pasta samples consisting of different fractions of purple wheat were characterized both before and after cooking, so that any modification induced by this process could be observed. The incorporation of anthocyanin-rich fraction into pasta showed very interesting data: although there was a reduction in this component in F + F1, due to the high sensitivity of heating, in the case of other samples the content has remained roughly unchanged. The most plausible explanation is that the denaturing which takes place on pasta structure during the cooking phase, predominantly to the gluten fraction, leads to the release both protein and bioactive compounds and in particular anthocyanins that would otherwise not be accessible in extraction phase on the raw product. However, in order to provide this explanation, more in-depth studies and analysis of the molecular structure of such samples would be needed.

Subsequently, the content of total polyphenols was quantified. In this case, pre- and post-cooking data were all compliant and showed a drastic decreased in these components when pasta was cooked. The sample with more residual polyphenols was F + F2.

In addition, a qualitative and quantitative evaluation of such extracts was carried out by HPLC analysis to look for the presence of certain compounds: cyanidin chloride, delphinidin chloride, rutin, quercitin and ferulic acid. The most abundant compound in extracted from cooked paste was ferulic acid, particularly in F + F2. Anti oxidant properties were retainned in all four samples, and to a greater extent in the S + F1 sample case. All the samples were capable of significantly lowering cellular response to an inflammatory stimulus in our model system. Finally, experimentation on enzymatic inhibitory activity gave two distinct results: the samples were more efficient on alpha-amylase, than on alpha-glucosidase. In both cases, the samples with greater inhibitory activity were F + F1 and F + F2.

According to the International Food Information Council (IFIC), functional foods are foods or dietary components that may provide a health benefit beyond basic nutrition (Wildman 2016). The *Nutrition Business Journal* classified functional foods as "food fortified with added or concentrated ingredients to functional levels", which improves health or performance.

Functional foods include enriched cereals, bread, sport drinks, bars, fortified snack foods, baby foods, prepared meals and more. In this area the term "functional" implies the food that has some identified value leading to health benefits, including reduced risk of disease. Functional foods may include everything from natural foods, such as orange juice with added calcium or additional carotenoids, to formulated ready-to-drink beverages containing antioxidants and immune-supporting factors. Recent progress in food manufacturing aimed to produce more functional food from natural ingredient to control different common disease including against obesity, diabetes, hypertension and dyslipidemia and cardiovascular disease (Mohamed 2014).

Nutraceuticals with antioxidant effects are through the most common additives into functional foods. In the introduction of this thesis we have seen that the most important effects of anthocyanins and polyphenols are their antioxidant activity that is attributed to many other of their benefits including anti-inflammatory properties. Therefore, most of the functional foods are including those foods containing remarkedly high contents of natural polyphenols and anthocyanins (Shahidi and Ambigaipalan 2015). In this regard new kinds of naturally colorful bread and pasta have been introduced to the market in recent years (Pasqualone, Bianco et al. 2015, Cárdenas-Hernández, Beta et al. 2016, Pasqualone, Gambacorta et al. 2016, Aiello, Di Bona et al. 2017, Zanoletti, Parizad et al. 2017, Abdel-Aal, Hucl et al. 2018). The antioxidant properties and diabetes alleviation have been well investigated in these products but there is rare study on the anti-inflammatory effects of the anthocyanins-enriched naturally products up to date.

Purple pasta enriched with anthocyanins rich fractions from purple wheat produced in this PhD project showed significant antioxidant activity after cooking. It also was very effective to inhibit the α-amylase enzyme activity and it possess immunomodulating effects on the inflamed cell models. Therefore, purple pasta may be considered as anew functional food with anti-inflammatory properties although further studies should address the level of absorption and the bioavailability of natural bioactives after consumption in individuals and their possible role in regulating the intestinal microbiota.

9- **References**:


Abdel-Aal, E.-S. M., A. A. Abou-Arab, T. H. Gamel, P. Hucl, J. C. Young and I. Rabalski (2008). "Fractionation of blue wheat anthocyanin compounds and their contribution to antioxidant properties." Journal of Agricultural and Food Chemistry **56**(23): 11171-11177.

Abdel-Aal, E.-S. M. and P. Hucl (2003). "Composition and stability of anthocyanins in blue-grained wheat." Journal of Agricultural and Food Chemistry **51**(8): 2174-2180.

Abdel-Aal, E.-S. M., P. Hucl and I. Rabalski (2018). "Compositional and antioxidant properties of anthocyanin-rich products prepared from purple wheat." Food Chemistry.

Abdel-Aal, E.-S. M., J. C. Young and I. Rabalski (2006). "Anthocyanin composition in black, blue, pink, purple, and red cereal grains." Journal of Agricultural and Food Chemistry **54**(13): 4696-4704.

Agrawal, N. K. and S. Kant (2014). "Targeting inflammation in diabetes: Newer therapeutic options." World Journal of Diabetes **5**(5): 697.

Aiello, A., D. Di Bona, G. Candore, C. Carru, A. Zinellu, G. Di Miceli, A. Nicosia, C. M. Gambino, P. Ruisi and C. Caruso (2017). "Targeting Aging with Functional Food: Pasta with Opuntia Single-Arm Pilot Study." Rejuvenation Research.

Akkarachiyasit, S., P. Charoenlertkul, S. Yibchok-anun and S. Adisakwattana (2010). "Inhibitory activities of cyanidin and its glycosides and synergistic effect with acarbose against intestinal α-glucosidase and pancreatic α-amylase." International Journal of Molecular Sciences **11**(9): 3387-3396.

Akkarachiyasit, S., P. Charoenlertkul, S. Yibchok-anun and S. Adisakwattana (2010). "Inhibitory Activities of Cyanidin and Its Glycosides and Synergistic Effect with Acarbose against Intestinal α-Glucosidase and Pancreatic α-Amylase." International Journal of Molecular Sciences **11**(9): 3387.

Akkarachiyasit, S., S. Yibchok-Anun, S. Wacharasindhu and S. Adisakwattana (2011). "In Vitro Inhibitory Effects of Cyandin-3-rutinoside on Pancreatic α-Amylase and Its Combined Effect with Acarbose." Molecules **16**(3): 2075.

Anson, N. M., R. Havenaar, A. Bast and G. R. Haenen (2010). "Antioxidant and anti-inflammatory capacity of bioaccessible compounds from wheat fractions after gastrointestinal digestion." Journal of Cereal Science **51**(1): 110-114.

Asenstorfer, R., Y. Wang and D. Mares (2006). "Chemical structure of flavonoid compounds in wheat (Triticum aestivum L.) flour that contribute to the yellow colour of Asian alkaline noodles." Journal of Cereal Science **43**(1): 108-119.

Asgary, S., A. Sahebkar, M. R. Afshani, M. Keshvari, S. Haghjooyjavanmard and M. Rafieian-Kopaei (20 4). "Clinical evaluation of blood pressure lowering, endothelial function improving, hypolipidemic and anti-inflammatory effects of pomegranate juice in hypertensive subjects." Phytotherapy Research **28**(2): 193-199.

Bellido, G. G. and T. Beta (2009). "Anthocyanin composition and oxygen radical scavenging capacity (ORAC) of milled and pearled purple, black, and common barley." Journal of Agricultural and Food Chemistry **57**(3): 1022-1028.

Bhaswant, M., S. R. Shafie, M. L. Mathai, P. Mouatt and L. Brown (2017). "Anthocyanins in chokeberry and purple maize attenuate diet-induced metabolic syndrome in rats." Nutrition **41**: 24-31.

Boath, A. S., D. Stewart and G. J. McDougall (20 2). "Berry components inhibit α-glucosidase in vitro: Synergies between acarbose and polyphenols from black currant and rowanberry." Food Chemistry **135**(3): 929-936.

Bowen-Forbes, C. S., Y. Zhang and M. G. Nair (2010). "Anthocyanin content, antioxidant, anti-inflammatory and anticancer properties of blackberry and raspberry fruits." Journal of Food Composition and Analysis **23**(6): 554-560.

Camelo-Méndez, G. A., E. Agama-Acevedo, J. Tovar and L. A. Bello-Pérez (2017). "Functional study of raw and cooked blue maize flour: Starch digestibility, total phenolic content and antioxidant activity." Journal of Cereal Science **76**: 179-185.

Cárdenas-Hernández, A., T. Beta, G. Loarca-Piña, E. Castaño-Tostado, J. O. Nieto-Barrera and S. Mendoza (2016). "Improved functional properties of pasta: Enrichment with amaranth seed flour and dried amaranth leaves." Journal of Cereal Science **72**: 84-90.

Castro-Acosta, M., W. Hall and C. Corpe (2016). "Polyphenol-rich blackcurrant and apple extracts inhibit glucose uptake in in vitro models of intestinal sugar transport, but individual anthocyanins have no effect." The Proceedings of the Nutrition Society **75**(OCE3).

Chen, C., P. Somavat, V. Singh and E. G. de Mejia (2017). "Chemical characterization of proanthocyanidins in purple, blue, and red maize coproducts from different milling processes and their anti-inflammatory properties." Industrial Crops and Products **109**: 464-475.

Chen, W., D. Müller, E. Richling and M. Wink (2013). "Anthocyanin-rich purple wheat prolongs the life span of Caenorhabditis elegans probably by activating the DAF-16/FOXO transcription factor." Journal of Agricultural and Food Chemistry **61**(12): 3047-3053.

Chung, H. Y., B. Sung, K. J. Jung, Y. Zou and B. P. Yu (2006). "The molecular inflammatory process in aging." Antioxidants & redox signaling **8**(3-4): 572-581.

Clifford, M. N. (2000). "Anthocyanins–nature, occurrence and dietary burden." Journal of the Science of Food and Agriculture **80**(7): 1063-1072.

Dayoub, O., S. Le Lay, R. Soleti, N. Clere, G. Hilairet, S. Dubois, F. Gagnadoux, J. Boursier, M. C. Martínez and R. Andriantsitohaina (20 7). "Estrogen receptor α/HDAC/NFAT axis for delphinidin effects on proliferation and differentiation of T lymphocytes from patients with cardiovascular risks." Scientific Reports **7**(1): 9378.

Del Pozo-Insfran, D., C. H. Brenes, S. O. S. Saldivar and S. T. Talcott (2006). "Polyphenolic and antioxidant content of white and blue corn (Zea mays L.) products." Food Research International **39**(6): 696-703.

Ding, M., R. Feng, S. Y. Wang, L. Bowman, Y. Lu, Y. Qian, V. Castranova, B.-H. Jiang and X. Shi (2006). "Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity." Journal of Biological Chemistry **281**(25): 17359-17368.

Elisia, I., C. Hu, D. G. Popovich and D. D. Kitts (2007). "Antioxidant assessment of an anthocyanin-enriched blackberry extract." Food Chemistry **101**(3): 1052-1058.

Escribano-Bailón, M. T., C. Santos-Buelga and J. C. Rivas-Gonzalo (2004). "Anthocyanins in cereals." Journal of Chromatography A **1054**(1): 129-141.

Ferrari, D., A. Speciale, M. Cristani, D. Fratantonio, M. S. Molonia, G. Ranaldi, A. Saija and F. Cimino (2016). "Cyanidin-3-O-glucoside inhibits NF-kB signalling in intestinal epithelial cells exposed to TNF-α and exerts protective effects via Nrf2 pathway activation." Toxicology Letters **264**: 51-58.

Garg, M., M. Chawla, V. Chunduri, R. Kumar, S. Sharma, N. K. Sharma, N. Kaur, A. Kumar, J. K. Mundey and M. K. Saini (2016). "Transfer of grain colors to elite wheat cultivars and their characterization." Journal of Cereal Science **71**: 138-144.

Giordano, D., T. Beta, A. Reyneri and M. Blandino (2017). "Changes in the Phenolic Acid Content and Antioxidant Activity During Kernel Development of Corn (Zea mays L.) and Relationship with Mycotoxin Contamination." Cereal Chemistry **94**(2): 315-324.

Giusti, M. M. and R. E. Wrolstad (2001). "Characterization and measurement of anthocyanins by UV-visible spectroscopy." Current Protocols in Food Analytical Chemistry.

Gonzales, G. B., G. Smagghe, C. Grootaert, M. Zotti, K. Raes and J. V. Camp (2015). "Flavonoid interactions during digestion, absorption, distribution and metabolism: a sequential structure–activity/property

relationship-based approach in the study of bioavailability and bioactivity." Drug Metabolism Reviews **47**(2): 175-190.

González-Muñoz, A., A. M. Quesille-Villalobos, C. Fuentealba, K. Shetty and L. Gálvez Ranilla (2013). "Potential of Chilean native corn (Zea mays L.) accessions as natural sources of phenolic antioxidants and in vitro bioactivity for hyperglycemia and hypertension management." Journal of Agricultural and Food Chemistry **61**(46): 10995-11007.

Grussu, D., D. Stewart and G. J. McDougall (20 ). "Berry polyphenols inhibit-amylase in vitro: identifying active components in rowanberry and raspberry." Journal of Agricultural and Food Chemistry **59**(6): 2324-2331.

Ha, U., W. J. Bae, S. J. Kim, B. I. Yoon, S. H. Hong, J. Y. Lee, T.-K. Hwang, S. Y. Hwang, Z. Wang and S. W. Kim (2015). "Anthocyanin induces apoptosis of DU-145 cells in vitro and inhibits xenograft growth of prostate cancer." Yonsei Medical Journal **56**(1): 16-23.

Harakotr, B., B. Suriharn, R. Tangwongchai, M. P. Scott and K. Lertrat (2014). "Anthocyanin, phenolics and antioxidant activity changes in purple waxy corn as affected by traditional cooking." Food Chemistry **164**: 510-517.

Hartman, J. and W. H. Frishman (2014). "Inflammation and atherosclerosis: a review of the role of interleukin-6 in the development of atherosclerosis and the potential for targeted drug therapy." Cardiology in review **22**(3): 147-151.

He, J. and M. M. Giusti (2010). "Anthocyanins: natural colorants with health-promoting properties." Annual review of food science and technology **1**: 163-187.

Hidalgo, M., S. Martin-Santamaria, I. Recio, C. Sanchez-Moreno, B. Pascual-Teresa, G. Rimbach and S. Pascual-Teresa (2012). "Potential anti-inflammatory, anti-adhesive, anti/estrogenic, and angiotensin-converting enzyme inhibitory activities of anthocyanins and their gut metabolites." Genes & Nutrition **7**(2): 295.

Hosseinian, F. S., W. Li and T. Beta (2008). "Measurement of anthocyanins and other phytochemicals in purple wheat." Food Chemistry **109**(4): 916-924.

Hu, C., B. Kwok and D. Kitts (2005). "Saskatoon berries (Amelanchier alnifolia Nutt.) scavenge free radicals and inhibit intracellular oxidation." Food Research International **38**(8): 1079-1085.

Huang, B., Z. Wang, J. H. Park, O. H. Ryu, M. K. Choi, J.-Y. Lee, Y.-H. Kang and S. S. Lim (2015). "Anti-diabetic effect of purple corn extract on C57BL/KsJ db/db mice." Nutrition Research and Practice **9**(1): 22-29.

Huang, W.-Y., Y.-M. Liu, J. Wang, X.-N. Wang and C.-Y. Li (2014). "Anti-inflammatory effect of the blueberry anthocyanins malvidin-3-glucoside and malvidin-3-galactoside in endothelial cells." Molecules **19**(8): 12827-12841.

Jaafar, S., N. Sharifah, J. Baron, S. Siebenhandl-Ehn, T. Rosenau, S. Böhmdorfer and H. Grausgruber (2013). "Increased anthocyanin content in purple pericarp× blue aleurone wheat crosses." Plant Breeding **132**(6): 546-552.

Jariyapamornkoon, N., S. Yibchok-anun and S. Adisakwattana (2013). "Inhibition of advanced glycation end products by red grape skin extract and its antioxidant activity." BMC Complementary and Alternative Medicine **13**(1): 171.

Jung, H., H. J. Lee, H. Cho and K. T. Hwang (2015). "Anti-Inflammatory Activities of Rubus Fruit Anthocyanins in Inflamed Human Intestinal Epithelial Cells." Journal of Food Biochemistry **39**(3): 300-309.

Kamiloglu, S., E. Capanoglu, C. Grootaert and J. Van Camp (2015). "Anthocyanin absorption and metabolism by human intestinal Caco-2 cells—A review." International Journal of Molecular Sciences **16**(9): 21555-21574.

Kang, M.-K., J. Li, J.-L. Kim, J.-H. Gong, S.-N. Kwak, J. H. Y. Park, J.-Y. Lee, S. S. Lim and Y.-H. Kang (2012). "Purple corn anthocyanins inhibit diabetes-associated glomerular monocyte activation and macrophage infiltration." American Journal of Physiology-Renal Physiology **303**(7): F1060-F1069.

Kotas, M. E. and R. Medzhitov (2015). "Homeostasis, inflammation, and disease susceptibility." Cell **160**(5): 816-827.
Kreft, I., N. Fabjan and K. Yasumoto (2006). "Rutin content in buckwheat (Fagopyrum esculentum Moench) food materials and products." Food Chemistry **98**(3): 508-512.
Kroon, P. A. and G. Williamson (1999). "Hydroxycinnamates in plants and food: current and future perspectives." Journal of the Science of Food and Agriculture **79**(3): 355-361.
Kuntz, S., H. Asseburg, S. Dold, A. Römpp, B. Fröhling, C. Kunz and S. Rudloff (2015). "Inhibition of low-grade inflammation by anthocyanins from grape extract in an in vitro epithelial-endothelial co-culture model." Food & function **6**(4): 1136-1149.
Lee, S. G., B. Kim, Y. Yang, T. X. Pham, Y.-K. Park, J. Manatou, S. I. Koo, O. K. Chun and J.-Y. Lee (2014). "Berry anthocyanins suppress the expression and secretion of proinflammatory mediators in macrophages by inhibiting nuclear translocation of NF-κB independent of NRF2-mediated mechanism." The Journal of Nutritional Biochemistry **25**(4): 404-411.
Li, J., S. S. Lim, J.-Y. Lee, J.-K. Kim, S.-W. Kang, J.-L. Kim and Y.-H. Kang (2012). "Purple corn anthocyanins dampened high-glucose-induced mesangial fibrosis and inflammation: possible renoprotective role in diabetic nephropathy." The Journal of Nutritional Biochemistry **23**(4): 320-331.
Li, Q., P. Somavat, V. Singh, L. Chatham and E. G. de Mejia (2017). "A comparative study of anthocyanin distribution in purple and blue corn coproducts from three conventional fractionation processes." Food Chemistry **231**: 332-339.
Li, X., J. Xu, X. Tang, Y. Liu, X. Yu, Z. Wang and W. Liu (2016). "Anthocyanins inhibit trastuzumab-resistant breast cancer in vitro and in vivo." Molecular medicine reports **13**(5): 4007-4013.
Li, Y., D. Ma, D. Sun, C. Wang, J. Zhang, Y. Xie and T. Guo (2015). "Total phenolic, flavonoid content, and antioxidant activity of flour, noodles, and steamed bread made from different colored wheat grains by three milling methods." The Crop Journal **3**(4): 328-334.
Liang, T., R. Guan, Z. Wang, H. Shen, Q. Xia and M. Liu (2017). "Comparison of anticancer activity and antioxidant activity between cyanidin-3-O-glucoside liposomes and cyanidin-3-O-glucoside in Caco-2 cells in vitro." RSC Advances **7**(59): 37359-37368.
Lila, M. A. (2004). "Anthocyanins and human health: an in vitro investigative approach." BioMed Research International **2004**(5): 306-313.
Lim, W., W. Jeong and G. Song (2016). "Delphinidin suppresses proliferation and migration of human ovarian clear cell carcinoma cells through blocking AKT and ERK1/2 MAPK signaling pathways." Molecular and cellular endocrinology **422**: 172-181.
Lin, B. W., C. C. Gong, H. F. Song and Y. Y. Cui (2017). "Effects of anthocyanins on the prevention and treatment of cancer." British Journal of Pharmacology **174**(11): 1226-1243.
Liu, M., B. Hu, H. Zhang, Y. Zhang, L. Wang, H. Qian and X. Qi (2017). "Inhibition study of red rice polyphenols on pancreatic α-amylase activity by kinetic analysis and molecular docking." Journal of Cereal Science **76**: 186-192.
Liu, Q., Y. Qiu and T. Beta (2010). "Comparison of antioxidant activities of different colored wheat grains and analysis of phenolic compounds." Journal of agricultural and food chemistry **58**(16): 9235-9241.
Luna-Vital, D., M. Weiss and E. G. Mejia (20 7). "Anthocyanins from Purple Corn Ameliorated TNF-α-Induced Inflammation and Insulin Resistance in 3T3-L Adipocytes via Activation of Insulin Signaling and Enhanced GLUT4 Translocation." Molecular Nutrition & Food Research **61**, 1700362.
Malunga, L. N. and P. Eck (2016). "Inhibition of intestinal α-glucosidase and glucose absorption by feruloylated arabinoxylan mono-and oligosaccharides from corn bran and wheat aleurone." Journal of Nutrition and Metabolism article id 1932532.
Manach, C., A. Scalbert, C. Morand, C. Rémésy and L. Jiménez (2004). "Polyphenols: food sources and bioavailability." Am J Clin Nutr **79**, 727-747.

Marko, D., N. Puppel, Z. Tjaden, S. Jakobs and G. Pahlke (2004). "The substitution pattern of anthocyanidins affects different cellular signaling cascades regulating cell proliferation." Molecular Nutrition & Food Research **48**(4): 318-325.

Marković, J. M. D., B. Pejin, D. Milenković, D. Amić, N. Begović, M. Mojović and Z. S. Marković (20 7). "Antiradical activity of delphinidin, pelargonidin and malvin towards hydroxyl and nitric oxide radicals: The energy requirements calculations as a prediction of the possible antiradical mechanisms." Food Chemistry **218**: 440-446.

Mcdougall, G. J. and D. Stewart (2005). "The inhibitory effects of berry polyphenols on digestive enzymes." Biofactors **23**(4): 189-195.

McGhie, T. K. and M. C. Walton (2007). "The bioavailability and absorption of anthocyanins: towards a better understanding." Molecular Nutrition & Food Research **51**(6): 702-713.

Metzger, B. T., D. M. Barnes and J. D. Reed (2008). "Purple carrot (Daucus carota L.) polyacetylenes decrease lipopolysaccharide-induced expression of inflammatory proteins in macrophage and endothelial cells." Journal of Agricultural and Food Chemistry **56**(10): 3554-3560.

Miguel, M. G. (2011). "Anthocyanins: Antioxidant and/or anti-inflammatory activities."

Min, S.-W., S.-N. Ryu and D.-H. Kim (2010). "Anti-inflammatory effects of black rice, cyanidin-3-O-β-D-glycoside, and its metabolites, cyanidin and protocatechuic acid." International Immunopharmacology **10**(8): 959-966.

Mohamed, S. (2014). "Functional foods against metabolic syndrome (obesity, diabetes, hypertension and dyslipidemia) and cardiovasular disease." Trends in Food Science & Technology **35**(2): 114-128.

Mora-Rochin, S., J. A. Gutiérrez-Uribe, S. O. Serna-Saldivar, P. Sánchez-Peña, C. Reyes-Moreno and J. Milán-Carrillo (2010). "Phenolic content and antioxidant activity of tortillas produced from pigmented maize processed by conventional nixtamalization or extrusion cooking." Journal of Cereal Science **52**(3): 502-508.

Mrkvicová, E., L. Pavlata, F. Karásek, O. Šťastník, E. Doležalová, V. Trojan, T. Vyhnánek, L. Hřivna, V. Holeksová and J. Mareš (2017). "The influence of feeding purple wheat with higher content of anthocyanins on antioxidant status and selected enzyme activity of animals." Acta Veterinaria Brno **85**(4): 371-376.

Nankar, A. N., B. Dungan, N. Paz, N. Sudasinghe, T. Schaub, F. O. Holguin and R. C. Pratt (2016). "Quantitative and qualitative evaluation of kernel anthocyanins from southwestern United States blue corn." Journal of the Science of Food and Agriculture **96**(13): 4542-4552.

Nile, S. H. and S. W. Park (20 4). "Antioxidant, α-Glucosidase and Xanthine Oxidase Inhibitory Activity of Bioactive Compounds From Maize (Zea mays L.)." Chemical Biology & Drug Design **83**(1): 119-125.

Norberto, S., S. Silva, M. Meireles, A. Faria, M. Pintado and C. Calhau (2013). "Blueberry anthocyanins in health promotion: A metabolic overview." Journal of Functional Foods **5**(4): 1518-1528.

Oak, M. H., J. Bedoui, S. F. Madeira, K. Chalupsky and V. Schini-Kerth (2006). "Delphinidin and cyanidin inhibit PDGFAB-induced VEGF release in vascular smooth muscle cells by preventing activation of p38 MAPK and JNK." British Journal of Pharmacology **149**(3): 283-290.

Oak, M. H., M. Chataigneau, T. Keravis, T. Chataigneau, A. Beretz and R. Andriantsitohaina (2003). "Red wine polyphenolic compounds inhibit vascular endothelial growth factor expression in vascular smooth muscle cells by preventing the activation of the p38 mitogen-activated protein kinase pathway." Arterioscler Thromb Vasc Biol **23**.

Ojeda, D., E. Jiménez-Ferrer, A. Zamilpa, A. Herrera-Arellano, J. Tortoriello and L. Alvarez (2010). "Inhibition of angiotensin convertin enzyme (ACE) activity by the anthocyanins delphinidin-and cyanidin-3-O-sambubiosides from Hibiscus sabdariffa." Journal of Ethnopharmacology **127**(1): 7-10.

Olivas-Aguirre, F. J., J. Rodrigo-García, N. d. R. Martínez-Ruiz, A. I. Cárdenas-Robles, S. O. Mendoza-Díaz, E. Álvarez-Parrilla, G. A. González-Aguilar, L. A. de la Rosa, A. Ramos-Jiménez and A. Wall-Medrano

(2016). "Cyanidin-3-O-glucoside: physical-chemistry, foodomics and health effects." Molecules **21**(9): 1264.

Pan, M.-H., C.-S. Lai and C.-T. Ho (2010). "Anti-inflammatory activity of natural dietary flavonoids." Food & Function **1**(1): 15-31.

Pasqualone, A., A. M. Bianco, V. M. Paradiso, C. Summo, G. Gambacorta, F. Caponio and A. Blanco (2015). "Production and characterization of functional biscuits obtained from purple wheat." Food Chemistry **180**: 64-70.

Pasqualone, A., G. Gambacorta, C. Summo, F. Caponio, G. Di Miceli, Z. Flagella, P. P. Marrese, G. Piro, C. Perrotta and L. De Bellis (2016). "Functional, textural and sensory properties of dry pasta supplemented with lyophilized tomato matrix or with durum wheat bran extracts produced by supercritical carbon dioxide or ultrasound." Food Chemistry **213**: 545-553.

Prasad, S., J. Ravindran and B. B. Aggarwal (2010). "NF-κB and cancer: how intimate is this relationship." Molecular and Cellular Biochemistry **336**(1-2): 25-37.

Prior, R. L., S. E. Wilkes, T. R. Rogers, R. C. Khanal, X. Wu and L. R. Howard (2010). "Purified blueberry anthocyanins and blueberry juice alter development of obesity in mice fed an obesogenic high-fat diet." Journal of Agricultural and Food Chemistry **58**(7): 3970-3976.

Ribatti, D. (2017). Inflammation and Cancer. Inflammation and Angiogenesis, Springer**:** 17-24.

Romier-Crouzet, B., J. Van De Walle, A. During, A. Joly, C. Rousseau, O. Henry, Y. Larondelle and Y.-J. Schneider (2009). "Inhibition of inflammatory mediators by polyphenolic plant extracts in human intestinal Caco-2 cells." Food and Chemical Toxicology **47**(6): 1221-1230.

Samyor, D., A. B. Das and S. C. Deka (2017). "Pigmented rice a potential source of bioactive compounds: a review." International Journal of Food Science & Technology.

Serna-Saldivar, S. O. (2016). Cereal grains: properties, processing, and nutritional attributes, CRC Press.

Serra, D., L. M. Almeida and T. C. Dinis (2016). "Anti-inflammatory protection afforded by cyanidin-3-glucoside and resveratrol in human intestinal cells via Nrf2 and PPAR-γ: Comparison with 5-aminosalicylic acid." Chemico-biological interactions **260**: 102-109.

Shahidi, F. and P. Ambigaipalan (2015). "Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects – A review." Journal of Functional Foods **18**: 820-897.

Shin, D. Y., J. N. Lu, G.-Y. Kim, J. M. Jung, H. S. Kang, W. S. Lee and Y. H. Choi (2011). "Anti-invasive activities of anthocyanins through modulation of tight junctions and suppression of matrix metalloproteinase activities in HCT-116 human colon carcinoma cells." Oncology Reports **25**(2): 567-572.

Siebenhandl, S., H. Grausgruber, N. Pellegrini, D. Del Rio, V. Fogliano, R. Pernice and E. Berghofer (2007). "Phytochemical profile of main antioxidants in different fractions of purple and blue wheat, and black barley." Journal of Agricultural and Food Chemistry **55**(21): 8541-8547.

Sikand, G., P. Kris-Etherton and N. M. Boulos (2015). "Impact of functional foods on prevention of cardiovascular disease and diabetes." Current Cardiology Reports **17**(6): 39.

Singleton, V. L., R. Orthofer and R. M. Lamuela-Raventós (1999). "[14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent." Methods in Enzymology **299**: 152-178.

Siti, H. N., Y. Kamisah and J. Kamsiah (2015). "The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease (a review)." Vascular Pharmacology **71**: 40-56.

Smith, M., K. Marley, D. Seigler, K. Singletary and B. Meline (2000). "Bioactive properties of wild blueberry fruits." Journal of Food Science **65**(2): 352-356.

Sogo, T., N. Terahara, A. Hisanaga, T. Kumamoto, T. Yamashiro, S. Wu, K. Sakao and D. X. Hou (2015). "Anti-inflammatory activity and molecular mechanism of delphinidin 3-sambubioside, a Hibiscus anthocyanin." Biofactors **41**(1): 58-65.

Takahama, U. and S. Hirota (20 8). "Interactions of flavonoids with αamylase and starch slowing down its digestion." Food & Function.

Taverniti, V., D. Fracassetti, C. Del Bo', C. Lanti, M. Minuzzo, D. Klimis-Zacas, P. Riso and S. Guglielmetti (2014). "Immunomodulatory effect of a wild blueberry anthocyanin-rich extract in human Caco-2 intestinal cells." Journal of Agricultural and Food Chemistry **62**(33): 8346-8351.

Tsao, R. (2010). "Chemistry and biochemistry of dietary polyphenols." Nutrients **2**(12): 1231-1246.

Tsuda, T., F. Horio, K. Uchida, H. Aoki and T. Osawa (2003). "Dietary cyanidin 3-O-β-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice." The Journal of Nutrition **133**(7): 2125-2130.

Urias-Lugo, D., J. Heredia, M. Muy-Rangel, J. Valdez-Torres, S. Serna-Saldívar and J. Gutiérrez-Uribe (2015). "Anthocyanins and phenolic acids of hybrid and native blue maize (Zea mays L.) extracts and their antiproliferative activity in mammary (MCF7), liver (HepG2), colon (Caco2 and HT29) and prostate (PC3) cancer cells." Plant Foods for Human Nutrition **70**(2): 193-199.

Vendrame, S. and D. Klimis-Zacas (2015). "Anti-inflammatory effect of anthocyanins via modulation of nuclear factor-κB and mitogen-activated protein kinase signaling cascades." Nutrition Reviews **73**(6): 348-358.

Vinayagam, R. and B. Xu (2015). "Antidiabetic properties of dietary flavonoids: a cellular mechanism review." Nutrition & Metabolism **12**(1): 60.

Vitaglione, P., I. Mennella, R. Ferracane, A. A. Rivellese, R. Giacco, D. Ercolini, S. M. Gibbons, A. La Storia, J. A. Gilbert and S. Jonnalagadda (2015). "Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: role of polyphenols bound to cereal dietary fiber." The American Journal of Clinical Nutrition **101**(2): 251-261.

Wang, C.-J., J.-M. Wang, W.-L. Lin, C.-Y. Chu, F.-P. Chou and T.-H. Tseng (2000). "Protective effect of Hibiscus anthocyanins against tert-butyl hydroperoxide-induced hepatic toxicity in rats." Food and Chemical Toxicology **38**(5): 411-416.

Wildman, R. E. (2016). Handbook of nutraceuticals and functional foods, CRC press.

Wu, T., X. Guo, M. Zhang, L. Yang, R. Liu and J. Yin (2017). "Anthocyanins in black rice, soybean and purple corn increase fecal butyric acid and prevent liver inflammation in high fat diet-induced obese mice." Food & function **8**(9): 3178-3186.

Wu, T., X. Qi, Y. Liu, J. Guo, R. Zhu, W. Chen, X. Zheng and T. Yu (2013). "Dietary supplementation with purified mulberry (Morus australis Poir) anthocyanins suppresses body weight gain in high-fat diet fed C57BL/6 mice." Food Chemistry **141**(1): 482-487.

Wu, X., G. R. Beecher, J. M. Holden, D. B. Haytowitz, S. E. Gebhardt and R. L. Prior (2006). "Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption." Journal of Agricultural and Food Chemistry**5 4**(11): 4069-4075.

Yang, Z. and W. Zhai (2010). "Identification and antioxidant activity of anthocyanins extracted from the seed and cob of purple corn (Zea mays L.)." Innovative Food Science & Emerging Technologies **11**(1): 169-176.

Yi, W., C. C. Akoh, J. Fischer and G. Krewer (2006). "Absorption of Anthocyanins from Blueberry Extracts by Caco-2 Human Intestinal Cell Monolayers." Journal of Agricultural and Food Chemistry **54**(15): 5651-5658.

Youdim, K. A., A. Martin and J. A. Joseph (2000). "Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress11Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee by the US Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable." Free Radical Biology and Medicine **29**(1): 51-60.

Yu, L. and A.-L. Nanguet (2013). "Comparison of antioxidant properties of refined and whole wheat flour and bread." Antioxidants **2**(4): 370-383.

Zanoletti, M., P. A. Parizad, V. Lavelli, C. Cecchini, P. Menesatti, A. Marti and M. A. Pagani (2017). "Debranning of purple wheat: recovery of anthocyanin-rich fractions and their use in pasta production." LWT-Food Science and Technology **75**: 663-669.

Zhang, B., M. Kang, Q. Xie, B. Xu, C. Sun, K. Chen and Y. Wu (2011). "Anthocyanins from Chinese bayberry extract protect β cells from oxidative stress-mediated injury via HO-1 upregulation." J Agric Food Chem **59**.

Zhang, L., A. S. Ravipati, S. R. Koyyalamudi, S. C. Jeong, N. Reddy, P. T. Smith, J. Bartlett, K. Shanmugam, G. Münch and M. J. Wu (2011). "Antioxidant and anti-inflammatory activities of selected medicinal plants containing phenolic and flavonoid compounds." Journal of Agricultural and Food Chemistry**5 9**(23): 12361-12367.

## Appendix I

**ABSTRACT**: This study was aimed at characterizing the anthocyanin profile in different varieties of pigmented corn and wheat and in some of their milling fractions, at assessing the anthocyanins and polyphenol profiles, and at investigating the anti-inflammatory and enzyme-inhibiting activities of these materials. Acid/ethanol extracts were used to assess total anthocyanins, overall antioxidant activity, the overall polyphenol profile, and for evaluating the inhibition of pancreatic a-amylase and of intestinal alpha-glucosidase. Dose-dependent inhibition of both enzymes was evident in all extracts within the same range of bioactives concentration, although with a different efficiency of individual extracts towards each enzyme. Anti-inflammatory response was evaluated by using acid-free extracts and a cellular model based on Caco-2 cells transiently transfected with a luciferase reporter gene responding to cytokine stimulation. The response of interleukin-stimulated cells decreased significantly in a dose-responsive manner in the presence of 20-50 micromol/liter anthocyanins from all grains extracts, again with a different efficiency. By comparing the different inhibitory ability of extracts from the various sources, it appears that the observed effects are in most cases higher than what observed in similar extracts from other sources, and that the activity in each extract may be related to specific synergies between anthocyanins and polyphenols. Therefore, we attempted to incorporate these materials into staple food. Purple pasta was prepared by introducing anthocyanin-rich fractions from purple wheat (PWF1 & PWF2), into soft wheat flour and durum wheat semolina. Bioactive levels were assessed in the different types of pasta after production and after cooking. Acid/ethanol extracts from cooked pasta retained high levels of TAC and TPC, as well as significant anti-oxidant activity, anti-inflammatory properties on cell models, and enzyme inhibitory activities. Thus, in conclusion, it can be stated that foods incorporating a naturally rich source of bioactive compounds. retain beneficial properties for the organism also after cooking.

## Appendix II

**SOMMARIO**: Lo scopo di questo studio è caratterizzare il profilo di antocianine in diverse varietà di mais e grano pigmentato e in alcune delle loro frazioni di macinazione, e di indagare le attività antinfiammatorie e di inibizione di enzimi di questi materiali. Estratti acido/etanolici sono stati usati per valutare gli antociani totali, l'attività antiossidante complessiva, il profilo globale dei polifenoli e per valutare l'inibizione dell'amilasi pancreatica e dell'alfa-glucosidasi intestinale. L'inibizione dose-dipendente di entrambi gli enzimi era evidente in tutti gli estratti all'interno della stessa gamma di concentrazione di bioattivi, sebbene con una diversa efficienza dei singoli estratti verso ciascun enzima. La risposta anti-infiammatoria è stata valutata utilizzando estratti privi di acido e un modello cellulare basato su cellule Caco-2 trasfettate transitoriamente con un gene reporter della luciferasi che risponde alla stimolazione con citochine. La risposta immunitaria delle cellule stimolate con interleuchina è diminuita significativamente in modo dose-reattivo in presenza di antociani da 50-50 micromol/l da tutti gli estratti di grani, sempre con una diversa efficienza. Confrontando la diversa capacità inibitoria degli estratti dalle varie fonti, sembra che gli effetti osservati siano nella maggior parte dei casi superiori a quelli osservati in estratti simili da altre fonti e che l'attività in ciascun estratto possa essere correlata a specifiche sinergie tra antocianine e polifenoli.

Pertanto, è parso interessante incorporare questi materiali in alimenti di largo consumo. La pasta viola è stata preparata introducendo frazioni ricche di antocianine dal grano viola (PWF1 e PWF2), farina di grano tenero e farina di grano duro. Sono stati preparati quattro diversi tipi di pasta, e il contenuto di bioattivi è stato determinato dopo la produzione e la cottura. Estratti acido/etanolici da campioni di pasta cotta hanno dimostrato la presenza di una notevole quantità di TAC e TPC, con ritenzione di una significativa attività antiossidante, delle proprietà antinfiammatorie su modelli cellulari e dell'attività inibitoria. Quindi, in conclusione, si può affermare che tali alimenti, costituiti da una materia prima naturalmente ricca di composti bioattivi, conservano proprietà benefiche per l'organismo anche dopo la cottura. Tuttavia, sono necessari ulteriori studi per consentire una maggiore caratterizzazione dei prodotti, e per studiare la possibile biodisponibilità in vivo di questi composti dopo il consumo.

## Appendix III

**Abbreviations:**

ACN: Anthocyanins

TAC: Total Anthocyanins Content

TPC: Total Polyphenols Content

FRAP: Ferric Reducing Antioxidant Power

PWF1: Purple Wheat first fraction after debranning

PWF2: Purple Wheat second fraction after debranning

BWSK: Blue Wheat variety Skorpion (Debranned fraction)

BCT: Blue Corn Variety T (Whole meal)

BCMF: Blue Corn Variety MF (Whole meal)

ROS: Red corn variety Rostrato (Whole meal)

Cyn-Cl: Cyanidin Chloride

Cyn-3-O-Glc: Cyanidin-3-O-Glucoside

Pet: Petunidine

Mal: Malvinidine

Peo: Peonidin

Pel: Pelargonidin

Del: Delphinidin

AcN: Acetonitrile

TFA: Trifluoroacetic Acid

IL-1β: Interleukin 1 beta

Nf-kb: Nuclear factor kappa-B

## Appendix IV

## List of Figures

# Appendix V

## List of Tables

# Appendix VI

## List of Graphs

# Appendix VII

## Scientific production

**Papers in peer-reviewed Journals:**

**Abbasi Parizad, P.**, Capraro, J., Scarafoni, A., Bonomi, F., Blandino, M., Giordano, D., Carpen, A., Iametti, S. Molecular aspects of the bio-functional properties of selected pigmented cereals, *(Submitted to Food & Function)*

Zanoletti, M., **Abbasi Parizad, P**., Lavelli, V., Cecchini, C., Menesatti, P., Marti, A., & Pagani, M. A. (2017) Debranning of purple wheat: Recovery of anthocyanin-rich fractions and their use in pasta production. *LWT-Food Science and Technology*,*75*, 663-669.

Marti, A., **Abbasi Parizad, P**., Marengo, M., Erba, D., Pagani, M. A., & Casiraghi, M. C. (2017) In Vitro Starch Digestibility of Commercial Gluten-Free Pasta: The Role of Ingredients and Origin. *Journal of Food Science*, *82*(4), 1012-1019.

Marti, A., Cattaneo, S., Benedetti, S., Buratti, S., **Abbasi Parizad, P.,** Masotti, F., Iametti, S., Pagani, M. A. (2017). Characterization of Whole Grain Pasta: Integrating Physical, Chemical, Molecular, and Instrumental Sensory Approaches. *Journal of Food Science*, *82*(11), 2583-2590.

**International meeting and conferences:**

**Oral presentations:**

Pigmented grains as a source of immunomodulating bioactives. *15th European. Young Cereal Scientists and Technologists Workshop. Milan/Bergamo, Italy, April 26$^{th}$-29$^{th}$, 2016*

Pigmented cereals have remarkable functional activities. *IUBMB advanced school, Spetses, Greece. May 15$^{th}$-19$^{th}$ 2017*

Anti-inflammatory and enzyme inhibitory effects of anthocyanins from colored cereals. *11th ISANH World Congress on Polyphenols Applications, Vienna, June 20$^{th}$ -21$^{st}$ 2017*

Molecular aspects of the functional activities of pigmented grains. *22nd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Free University of Bozen, September 20th-22nd, 2017*

**Poster Presentations:**

Phenolics from pigmented grains have remarkable immunomodulating properties ***Poster prize*** *for the presentation in Miami Winter Symposium, January 24th-27th, 2016*

Molecular characterization of pasta enriched in fiber and antioxidants: investigation of their functional role by in vitro and in vivo approaches. *21th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Naples Federico II, Portici, September 14th-16th, 2016*

Incorporating bioactives into functional foods: Pasta from pigmented grains. *The 1st NLRCS Congress, Mashhad, Iran. September 6th- 8th 2017*

Pigmented cereals: functional properties of the bioactive components. *11˚ Convegno dell'Associazione Italiana di Scienza e Tecnologia dei Cereali (AISTEC) ,Centro Congressi Frentani, Roma. Novembre 22th-24th 2017*