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CICLO XXX

CHEMOPREVENTIVE POTENTIAL OF OAT DERIVED PHYTOCHEMICALS.
The role of naturally isolated and chemically synthesized avenanthramides.

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RELATORE

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Contents

Abstract

The present research project was designed to evaluate the main parameters which affect the nutrient level during the formulation of oat-based food products. The focus was on avenanthramides (AVNs), a group of more than 30 phenol compounds found exclusively in oats. The three main AVNs (2c, 2p and 2f) were isolated by us, as a mixture (n-MIX), from oat sprouts, as well as chemically synthesized, in order to test their health protective effects, namely antioxidant, anti-proliferative and anti-inflammatory activities.

First, we described the morphology of oat caryopsis, in order to localize nutrients within its layers, with particular attention to phenol compounds. Conventional optical microscopy coupled with specific staining techniques, as well as Environmental Scanning Electron Microscopy-Energy Dispersive Spectroscopy enabled us to identify macronutrients, whereas autofluorescence allowed us to observe phenol compounds in the outer layers of oat caryopsis. Second, we evaluated the effects of genotype/environment interactions and oat processing, on phenol concentration. Our results indicated that genotype was the main determinant on AVN levels, as naked oats showed about three-fold higher values than dehulled oats. Nevertheless, also the interaction soil *x* genotype was relevant on AVN content. Regarding the mechanical processing, the greatest loss of phenol antioxidants was observed after dehulling, as AVNs were also found in hulls; milling reduced bound polyphenols only, due to their covalent bound with the fibrous matrix.

Third, we evaluated the increase of AVN levels during malting, in order to obtain functional products, namely oat-based cookies, which underwent to simulated digestion process to evaluate the recovery of antioxidants. Minor AVNs, namely 2s, $2p_d$, $2f_d$, in addition to the three main forms, were identified by us in malted oats and in their derived cookies. Total AVNs showed a ten-fold increase after five days of malting, probably due to enzymatically catalyzed *de novo* biosynthesis. Cooking did not affect the AVN content of our bakery products, whereas the *in vitro* digestion provided a relative bioaccessibility, ranging from 10 to 55%, depending on the individual AVN form.

Finally, we evaluated the main biological effects exerted by the n-MIX or synthetic AVNs s-2c, s-2p, s-2f. Among the three synthetic forms, the s-2c showed the highest antioxidant capacity, detected by three different methods (DPPH, ABTS, ORAC), due to the presence of two hydroxyl groups in the molecule.

The study of their anti-proliferative effect was carried out on one normal cell line (NCTC 2544) and three different cancer cell lines (CaCo-2, HepG2, Hep3B). On NCTC 2544 no cytotoxic effect was observed in the range 0-120 µM, whereas on cancer cell lines the n-MIX and the s-2c showed the greatest citotoxicity. The anti-tumor activity was exploited by AVNs by targeting the extrinsic apoptotic pathway, as demonstrated by the activation of caspases 3, 8, 2. The strong pro-apoptotic effect of AVNs was linked to: high membrane permeability, as ascertained by their cellular antioxidant capacity; downregulation of the pro-survival factors, like the hypoxia inducible factor (HIF1A) and the vascular endothelial growth factor (VEGFA), as well as the cyclooxigenase (COX-2).
The overall conclusion is that malting can be a good method to increase AVN levels in oats. The choice of the cultivar, as well as the mechanical processing and storage conditions are key factors to ensure the maintenance of high levels of AVNs in the final oat-based functional products.

Keywords: antioxidant; anti-inflammatory; anti-proliferative; avenanthramides; *Avena sativa* L.; malting; oat-based cookies; polyphenols.

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The present thesis is based on the following papers:

I. Panato A, Antonini E, Bortolotti F, Ninfali P. 2017. The histology of grain caryopses for nutrient location: a comparative study of six cereals. *International Journal of Food Science and Technology* **52**, 1238-1245.

II. Antonini E, Zara C, Valentini L, Gobbi P, Ninfali P, Menotta M. Novel insights into globoids of aleurone, starch granules and the outer layers of three cereals gained using atomic force microscopy and environmental scanning electronic microscopy. *Submitted for publication.*

III. Antonini E, Lombardi F, Alfieri M, Diamantini G, Redaelli R, Ninfali P. 2016. Nutritional characterization of naked and dehulled oat cultivar samples at harvest and after storage. *Journal of Cereal Science* **72**, 46-53.

IV. Antonini E, Diamantini G, Ninfali P. 2017. The effect of mechanical processing on avenanthramide and phenol levels in two organically grown Italian oat cultivars. *Journal of Food Science and Technology* **54**, 2279-2287.

V. Oat-based food products. *Manuscript to be submitted.*

VI. Scarpa ES, Antonini E, Palma F, Mari M, Ninfali P. 2017. Antiproliferative Activity of Vitexin-2-O-Xyloside and Avenanthramides on CaCo-2 and HepG2 Cancer Cells Occurs Through Apoptosis Induction and Reduction of Pro-Survival Mechanisms. *European Journal of Nutrition*. DOI: 10.1007/s00394-017-1418-y.

VII. Scarpa ES, Mari M, Antonini E, Palma F, Ninfali P. Natural and synthetic avenanthramides: a comparative study of their antioxidant, anti-proliferative and anti-inflammatory effects. *Submitted for publication.*

List of abbreviations

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AVNs, avenanthramides

BPs, bound polyphenols

cv., cultivar

CAA, cellular antioxidant capacity

COX, cyclooxygenase

DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate

DPPH, 2,2-Diphenyl-1-picrylhydrazyl

EDS, energy dispersive spectroscopy

ESEM, environmental scanning electron microscopy

FPs, free polyphenols

HIF1A, hypoxia inducible factor 1A

HPLC, high performance liquid chromatography

MS, mass spectrometry

n-AVN, natural avenanthramide

ORAC, oxygen radical absorbance capacity

PDA, photo diode array

ROS, reactive oxygen species

s-AVN, synthetic avenanthramide

SRB, sulforhodamine B

TPs, total polyphenols

VEGF, vascular endothelial growth factor

WGF, whole grain flour

2p, N-(4'hydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid

2c, N-(3', 4'-dihydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid

2f, N-(4'-hydroxy-3'-methoxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid

2s, N-(4'-hydroxy-3',5'-dimethoxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid

Introduction

Oats (*Avena sativa* L.) are important crops with a lot of potential for improvement by breeding based on a wealth of European genetic resources (Redaelli et al., 2014). They differ from other cereals in that they are consumed almost exclusively in wholegrain products (van der Kamp et al., 2014). The importance of wholegrain consumption has long been investigated for reduction of the risk of chronic diseases, such as coronary heart disease (Mellen et al., 2008), type II diabetes (Liu et al., 2000) and certain cancers (Haas et al., 2009; Kasum et al., 2002). In this regard, oat-based food products have gained considerable attention in recent years as they are rich in β-glucan, belonging to the soluble fiber, with positive effects on blood glucose and cholesterol levels (Daou and Zhang, 2012; EU, 2012). The mechanisms by which oat β-glucan reduce blood glucose and cholesterol levels are shown in Fig. 1.

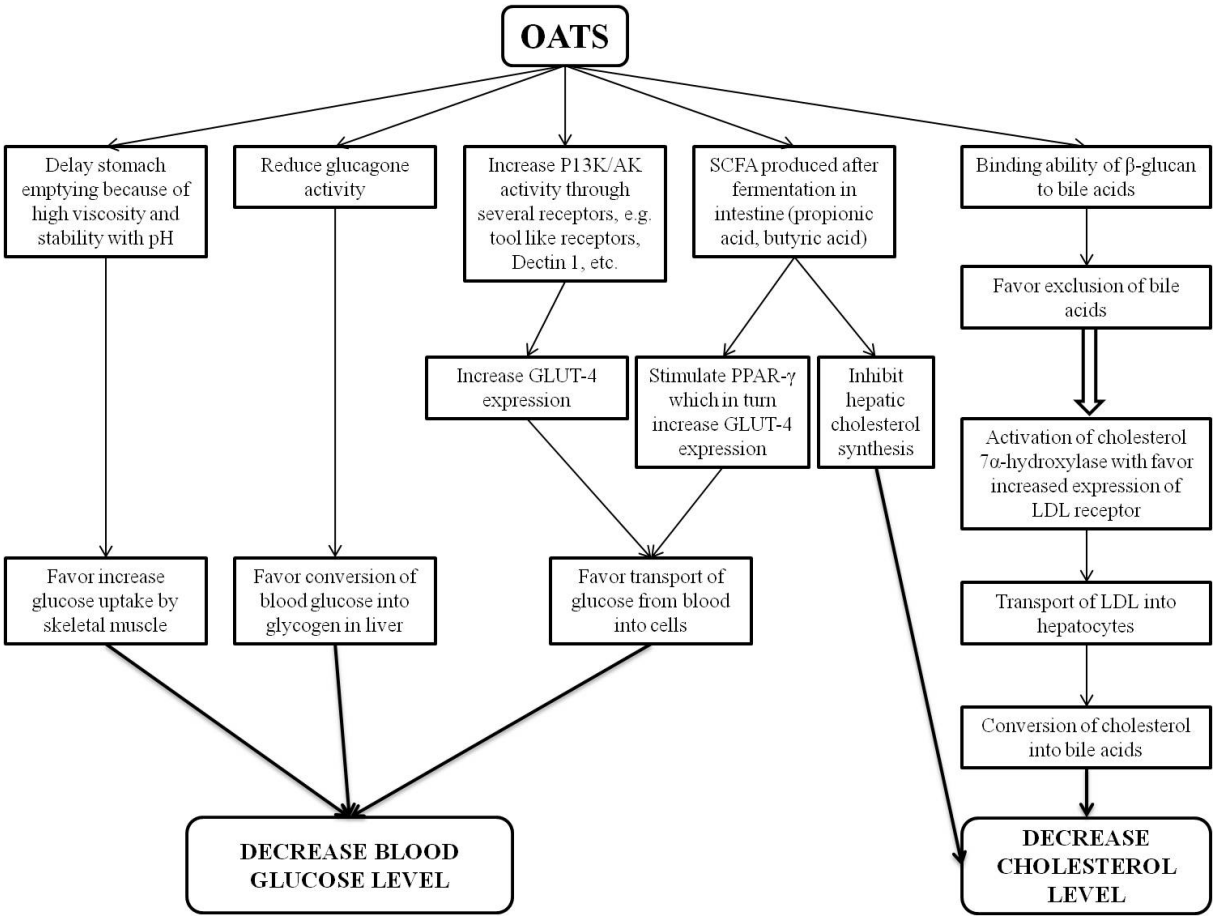


Figure 1. Mechanisms of oat β-glucan against diabetes and dyslipidemia (Varma et al., 2016).

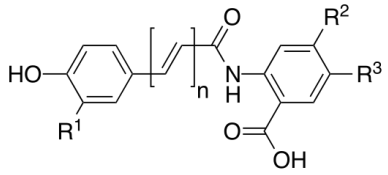
The European Commission (EU, 2009) has included oats among permitted ingredients for celiac patients, if the gluten content does not exceed 20 mg/kg. This is an attractive task for the industry, due to lack of micronutrients in gluten free flours. In recent years, the demand of oat-based products has increased. Therefore, the industry is providing technology for new and diversified oat-based functional foods, like pasta or cakes, in order to favor the consumption of this cereal, reduce the cost of production and cover meals of all day times (Rasane et al., 2015).

Specific oat phenol compounds have received increasing interests along the last decades (Peterson, 2001). In particular, oats biosynthesize, in response to pathogen infections, a group of unique secondary metabolites, termed avenanthramides (AVNs) (Collins, 1989).
Chemically, AVNs consist of an anthranilic acid derivative, linked to a cinnamic acid derivative; the substitution on one of the two moieties allows to distinguish them among a group of about 30 forms. The most abundant AVNs in oats are reported in Table 1.

To describe AVNs, two nomenclature systems have been used in the literature. Collins (1989) used alphabetic descriptors (Fig. 2), whereas Dimberg et al. (1993) assigned numbers to indicate the substitution on the anthranilic acid moiety (*1* for anthranilic, *2* for 5-hydroxy, *3* for 5-hydroxy-4-methoxy anthranilic acids) and letters to indicate the substitution on the cinnamic acid moiety (*c* for caffeic, *f* for ferulic, *p* for *p*-coumaric, *s* for synapic acid).

Table 1. Main AVN forms, as named by Collins and Dimberg.

Collins (1989)	**Dimberg *et al.* (1993)**	***n***	**R^1**	**R^2**	**R^3**
A	2p	1	H	H	OH
B	2f	1	OCH_3	H	OH
C	2c	1	OH	H	OH
O	$2p_d$	2	H	H	OH
P	$2f_d$	2	OCH_3	H	OH



AVN $2p_d$ and $2f_d$ differs from 2p and 2f in their additional double bond; R1, R2, R3, main substituents.

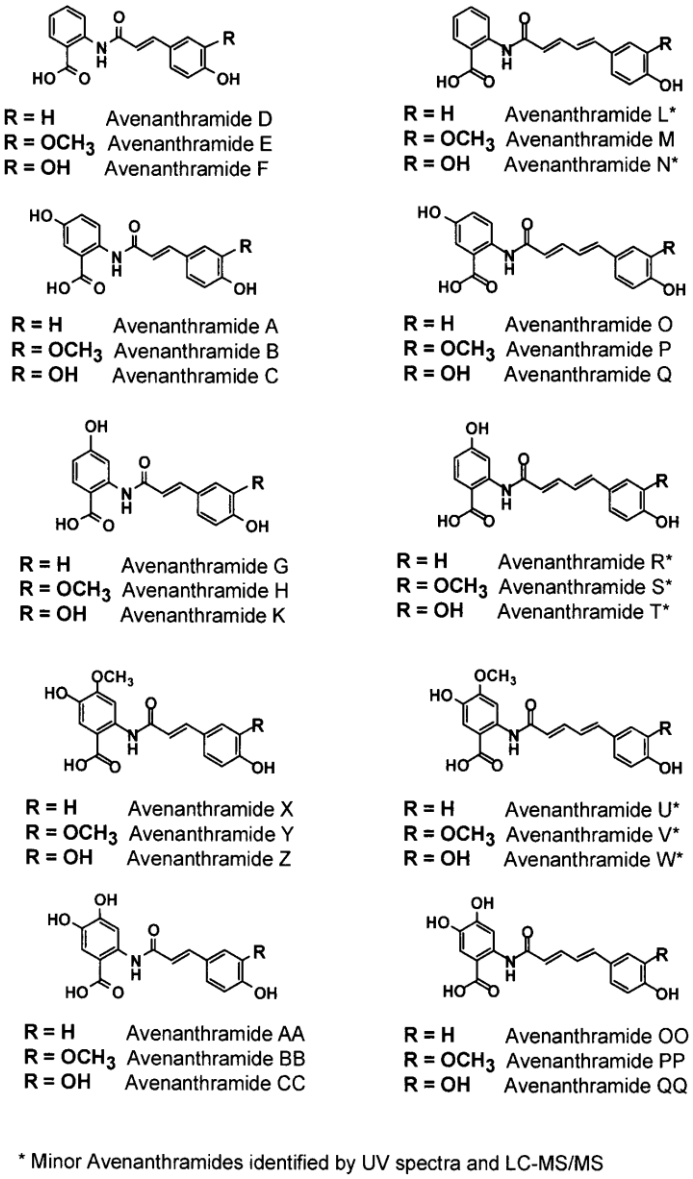


Figure 2. Structural formula of 30 AVNs described by Collins and Burrows (2012).

Regarding genotype/environment interactions, AVN concentrations in oat kernel vary from 2 to 300 mg/kg, probably due to both genetically and environmental factors (Bratt et al., 2003; Bryngelsson et al., 2002; Emmons and Peterson, 2001; Mattila et al., 2005; Yang et al., 2014). In a recent report, the total AVN content in 137 genotypes, distributed over Europe, was found to reach levels up to 4081 mg/kg (Redaelli et al., 2016).

Concerning the localization of AVNs in oat caryopsis, their content is always higher in the outer layers of the grain than in the starchy endosperm, indicating that AVNs are localized primarily in the bran fraction (Emmons et al., 1999; Mattila et al., 2005).

The AVN levels in oat kernels can be increased through physiological processes. Malting is a process of soaking and germinating cereal grains, practiced for millennia, to change the composition of the caryopsis for a variety of end purposes (Hübner and Arendt, 2013).
Several studies demonstrated that AVNs increased in concentration by steeping and germination processes, under controlled times and temperatures (Bryngelsson et al., 2002; Skoglund et al., 2008; Xu et al., 2009), due to the activation of the enzyme hydroxycinnamoyl-CoA:hydroxyanthranilate-*N*-hydroxycinnamoyltransferase, involved in their biosynthesis (Bryngelsson et al., 2003; Matsukawa et al., 2000; Skoglund et al., 2008). Moreover, Collins and Burrows (2012) developed a method termed "false malting" able to increase the AVN level of about 7.5-fold more than the starting level, by reducing the germination rate to less than 1%. In this way, after drying, malted oats can be used for most commercial food processing procedures.

Several authors studied the chemical stability of AVNs as a result of different technological processes or storage conditions. In particular, Dimberg et al. (1996) assessed that heating and drying oat grains did not significantly influence the 2c or 2f concentrations, while the AVN 2p was greatly reduced. The AVN 2p and 2f were stable at pH variations, from acid to alkaline condition, while 2c was sensitive to alkaline pH, especially in combination with thermal treatments (Dimberg et al., 2001).
AVN concentration was also studied before and after the processing of oat foods (bread, fresh pasta, muffins), demonstrating that the total content of the 3 most common forms (2c, 2p and 2f) increased in all tested products (Dimberg et al., 2001). This increase was tentatively explained by new synthesis, release of linked forms, increased extraction after processing or combination of all these factors (Dimberg et al., 2001).
Moreover, Bryngelsson et al. (2002) investigated the effect of commercial heat processing on AVN content. They found that the AVN 2p was reduced by steaming, the AVNs 2c and 2p decreased during autoclaving of oats and all three AVNs were reduced in drum-dried rolled oats (Bryngelsson et al., 2002).

Oat AVNs have been shown to be bio-available in humans, hamsters and rats (Chen et al., 2004; Chen et al., 2007; Koenig et al., 2011). In comparison to human and hamster, AVN bioavailability in rats is low, but the ranking order of plasma concentration by AVN type (2p >> 2f > 2c) is the same (Koenig et al., 2011).

Biological effects of AVNs have been studied for their antioxidant, anti-itching and anti-inflammatory effects, *in vitro* and *in vivo*, as well as for their anti-proliferative activity, *in vitro* (Meydani, 2009).

Antioxidant activity. The AVN antioxidant activity is 10 to 30 times higher than that of other phenols present in oat kernel, such as caffeic and vanillic acids (Dimberg et al., 1993). In particular, AVNs 2c exhibits the highest *in vitro* antioxidant capacity (Peterson, 2001).
In vivo studies demonstrated that a supplementation of rat diet with AVNs increases the activity of antioxidant enzymes, like superoxide dismutase in skeletal muscle, liver and kidney, as well as glutathione peroxidase in heart and skeletal muscles and attenuates the Reactive Oxygen Species (ROS) production (Ji et al., 2003). In addition, a synergistic action of AVNs with vitamin C was found in hamsters, in the protection of LDL oxidation (Chen et al., 2004).

Anti-itching activity. Oatmeal has been used for centuries as a soothing agent to relieve itch and irritation associated with various xerotic dermatoses (Sur et al., 2008). A low concentration of AVNs (1 ppb) would be able to inhibit the degradation of the Inhibitor of Nuclear Factor kappa B-alpha (I*k*B-α) in keratinocytes, which correlated with decreased phosphorylation of p65 subunit of Nuclear Factor kappa B (NF-*k*B) (Sur et al., 2008). Furthermore, cells treated with AVNs showed a significant inhibition of tumor necrosis factor-alpha (TNF-alpha) induced NF-*k*B luciferase activity and subsequent reduction of interleukin-8 (IL-8) release. Additionally, topical application of 1-3 ppm AVNs mitigated inflammation in murine models of contact hypersensitivity and neurogenic inflammation and reduced pruritogen-induced scratching in a murine itch model (Sur et al., 2008). Recently, Fowler (2014) showed that oat extract containing AVNs has antihistaminic and anti-irritant activities.
Finally, dihydroavenanthramide D, a synthetic analog of AVNs, acts as a Neurokinin-1 Receptor (NK1R) inhibitor, reduces the secretion of the cytokine IL-6 and could be a promising candidate for topical treatments of chronic pruritus (Heuschkel et al., 2008).

Anti-inflammatory activity. AVNs exert anti-inflammatory activity by inhibiting the proliferation of smooth muscle cells in humans and increasing the nitric oxide production, which are considered the two key factors in the prevention of atherosclerosis (Nie et al., 2006a). The mechanism underlying this protective effect is that AVNs may arrest the cell cycle at the G1 phase, by up-regulating the p53-p21cip1 pathway and inhibiting the phosphorylation of retinoblastoma protein (Loden et al., 1999). Moreover, the methyl esters of AVNs 2c was

shown to inhibit the proteasome activity and increase the overall levels of high mass, ubiquitin-conjugated protein in endothelial cells (Guo et al., 2008).

Furthermore, AVNs are structurally similar to Tranilast™ (Fig. 3), a synthetic compound used like antihistaminic drug, which exerts also an anti-proliferative effect on smooth muscle cells, preventing restenosis after angioplasty (Nie et al., 2006b).

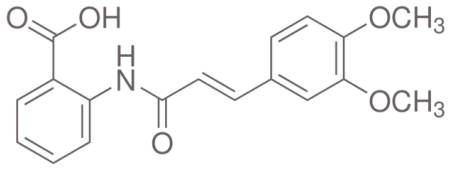


Figure 3. Structural formula of Tranilast™

Another study demonstrated that AVN 1f acts as an *in vitro* inhibitor of lipoxygenase, a key enzyme of leukotriene biosynthesis, which participates in allergic and inflammatory responses (Liu et al., 2004).

Anti-proliferative activity. Nie et al. (2007) and Guo et al. (2010) showed the anti-proliferative effect of the natural AVNs-enriched extract of oats (AvExO), isolated from oat bran, as well as the AVN 2c and the methylated form of AVN 2c (CH3-AVN 2c), synthetically prepared, on several colon cancer cell lines. These unique polyphenols significantly inhibited the proliferation of colonic cancer cell lines in the magnitude order of HCT116 > CaCo2 > LS174 > HT29. In addition, they had no cytotoxic effects on differentiated colon cancer cells, which represent the characteristics of normal colon epithelial cells (Guo et al., 2010). CH3-AVN 2c was the most potent AVN in the inhibition of colon cancer cell proliferation, probably due to the presence of a single methyl ester group in the chemical structure of AVNs, which might increase its lipid solubility and bioavailability, making it readily incorporated into the cell membrane and allowing it to hinder the molecular pathways that are involved in cell proliferation.

A plausible mechanism for AVNs' inhibition of colon cancer cell proliferation may be the inhibition of proteasome activity, the subsequent stabilization of p53 protein and cell cycle arrest (Guo et al., 2010).

Objectives

Taken together, this information makes AVNs interesting candidates in the search for new oat-based food products, which may be significant contributors to the health benefits. As the AVN concentration in the final product is critical for their biological effects, the objectives of the present study were:

- To describe the morphological structures of oat caryopsis, with particular attention to the presence of phenol compounds.
- To check the effect of genetic and environmental factors on the modulation of the antioxidant and AVN levels, in order to take into consideration the retention of nutrients after storage or mechanical processing.
- To standardize an oat malting method able to increase the AVN concentration, in order to realize functional oat-based bakery products able to exert healthy effects in humans.
- To evaluate the antioxidant, anti-proliferative and anti-inflammatory activities of AVNs isolated from oat sprouts, as well as chemically synthesizes forms.

Materials and Methods

Table 2 gives a brief description of the samples and analyses performed in the studies.

Table 2. Summary of samples and analyses performed in this thesis.

Paper	Samples	Analysis
I	Italian husked oat [1]	• Morphological analysis and nutrient localization [2]
II	Italian naked oat [3]	• Morphological analysis and elemental composition [4]
III	15 husked and 15 naked oats [5]	• Total polyphenols (free + bound) • Total AVNs (2c + 2p + 2f) • Retention of antioxidants after storage [6]
IV	Two Italian husked oats [7]	• Total AVNs (2c + 2p + 2f) • Total polyphenols (free + bound) • Total ORAC (free + bound) • Retention of antioxidants after dehulling and milling [8]
V	Six formulations of cookies obtained from malted oats [9]	• Free polyphenols • ORAC of free polyphenols • Total AVNs (2c + 2p + 2f) • *In vitro* digestion
VII	Natural AVNs isolated from oat sprouts [10]	• AVN purification • Cytotoxicity test (CaCo-2, HepG2) • Caspase 3, 8, 9 activities • Cellular antioxidant activity (CAA) • RTqPCR assays of HIF1A, VEGFA
VII	Synthetic AVNs	• Chemical antioxidant capacity (ORAC, ABTS, DPPH) • Cellular antioxidant activity (CAA) • Cytotoxicity test (CaCo-2, Hep3B) • Caspase 3, 8, 2 activities • RTqPCR assay of COX-2 and COX-2 activity assay

[1] Husked oat (cv. Flavia) was provided by Terra Bio Soc. Coop., Schieti di Urbino (PU, Italy) and dehulled by hand before cross-sections.

[2] Oat sections (5µm) were stained with the Azan Trichrome kit according to Heidenhain and Periodic acid-Shiff staining and examined using a a Nikon Coolscope Digital Microscope (Tokyo, Japan) to identify macronutrients. Unstained sections were examined using a fluorescence microscope (Axioskop 2 from Carl Zeiss, Oberkochen, Germany), with an excitation wavelength of 365 nm and an emission wavelength of 418 nm to identify the autofluorescence of phenol compounds.

[3] Naked oat (cv. Leda) was provided by Terra Bio Soc. Coop., Schieti di Urbino (PU, Italy).

[4] Oat was cut in perpendicular slices with a sharp stainless steel razor. The oat slices were deposited into the aluminum specimen stubs, previously covered with a conductive carbon adhesive disk (TAAB Ltd, Berks, England) and analyzed by Environmental Scanning Electron Microscope (FEI, Hillsboro, Oregon, USA), equipped with an energy dispersive X-ray spectrometer (EDAX inc., Mahwah, NJ, USA).

[5] Samples (15 husked and 15 naked oats) were provided by the Maize Research Unit of Bergamo (Italy) and sown in Montanaso L. (LO, Italy). Husked oats were dehulled by hand before analysis.

[6] Samples (six husked and six naked) were selected among the 30 oat cultivars and analyzed for their antioxidant content over 12 months of storage (T12), in a cold room at + 7°C and 55% rh. The former were stored with hulls and dehulled by hand before analysis.

[7] Husked oats (cv. Donata and Flavia) were provided by Terra Bio Soc. Coop., Schieti di Urbino (PU, Italy) and organically grown in two different soils: a loamy soil, located in Urbino (Italy, 43°43′34″ N; 12°38′10″ E) and a medium texture soil, located in Siena (Italy, 43°20′27″ N; 11°2′38″ E).

[8] Conditioned oat grains were dehulled with an industrial dehuller operating at an appropriate speed to yield an acceptable percentage of unbroken groats. The hulls accounted for 30 ± 2% of the groats. Milling was performed at 131 rpm, with a final flour yield of 75 ± 1%.

[9] Naked oats (cv. Luna) were malted for five days (M0→M5) by COBI, Consorzio Italiano di Produttori dell'Orzo e della Birra (AN, Italy), using the procedure for brewing. In particular, oats were steeped at 20°C for 24 h, then drained and kept in the dark, at 15°C, for five days in the same containers used for barley malting.

Six formulations of malted oat-based cookies were realized by « Il mulino di Nino », Azienda Agricola Roncarati (AN, Italy) and labeled as: 3A, 4A, 5A (A triplet); 3B, 4B, 5B (B triplet). Fig. 4 shows the flow-chart summarizing the main approach to obtain malted oat-based cookies.

A triplet (3A, 4A, 5A): cookies were realized with 35% of flour obtained from oat malted for 3, 4, 5 days, respectively.

B triplet (3B, 4B, 5B): cookies were realized with 45% of flour obtained from oat malted for 3, 4, 5 days, respectively.

The other ingredients were: wheat flour (65% in the A triplet, 55% in the B triplet); sugar (300 g); 4 eggs; baking powder (12 g). Cookies were cooked in ventilated oven for 30 min at 180°C.

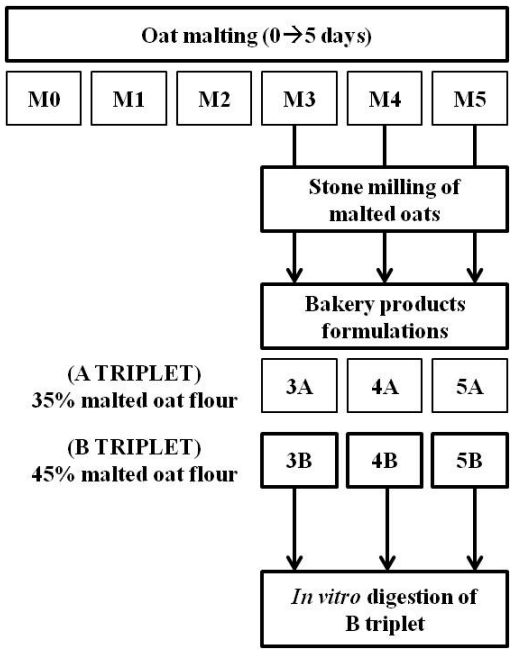


Figure 4. Flow chart summarizing the main approach to obtain malted oat-based cookies.

[10] Oat grains, provided by Terra Bio Soc. Coop. (Urbino, Italy), were steeped and germinated for 14 and 72 hours, respectively, using an automatic sprouter (Fresh Life 2000, Corrupad Korea Co. Ltd., Korea).

Extraction of free polyphenols (FPs)

FPs were extracted following the procedure of Wise (2011), with some modifications. Briefly, 1 g of flour was treated with 3 x 10 mL of 80% EtOH in a 10 mM H_3PO_4 buffer, pH 2.8, in a water bath at 50°C, under shaking. After each extraction, the samples were centrifuged at 3000 rpm for 10 min. The three supernatants were collected and stored at -20°C until analysis.

Extraction of bound polyphenols (BPs)

BPs were extracted following the procedure of Verardo et al. (2011), with some modifications. Briefly, the solid residue of FP extraction was digested with 100 ml of 2 M sodium hydroxide at room temperature for 20 h by shaking under nitrogen gas. The mixture was acidified to pH 2-3 by adding 10 M HCl in a cooling ice bath. Five ml of the acidified mixture was taken and treated twice with 10 ml of hexane to remove lipids. The aqueous solution was extracted twice with 5 ml of ethyl acetate. The polar fractions were pooled and evaporated to dryness. The BPs were reconstituted with 1 ml of 1/1 acetone/water (v/v).

Determination of total phenols (TPs)

FPs and BPs were determined using the Folin-Ciocalteu method (Singleton et al., 1999). A calibration curve was made each time with the standard caffeic acid (from 50 to 400 μg/mL). Total content of polyphenols (TPs) is reported as the sum of FPs and BPs.

Isolation of AVNs from oat sprouts

AVNs were extracted from milled oat following the procedure reported in "*Extraction of FPs*", with the exception that ethanol was acidified with 0.1% glacial acetic acid, instead of 10 mM H_3PO_4 buffer.

The raw extract was concentrated by rotary evaporation to 1/10 of the initial volume. To remove lipophilic components from the extract, we followed the procedure previously reported (Liu et al., 2004), with some modifications. Briefly, Octyl Sepharose^{TM} CL 4-B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was added to the extract (0.5 ml per g extracted). The mixture was completely dried, re-suspended in acidified 50% ethanol and transferred to a glass chromatography column containing Octyl Sepharose^{TM} CL 4-B, previously pre-equilibrated in acidified 50% ethanol. The column was then eluted with 3 x bed volume (V_b) of acidified 50% ethanol. The eluate was concentrated under vacuum, at 40°C, by rotary evaporation.

The defatted dried extract was dissolved in acidified 30% ethanol and purified on a Sephadex LH-20 column (50 x 150 mm; Sigma-Aldrich Inc., St. Louis, USA), equilibrated in acidified 30% ethanol, and controlled by the LC-Chromatograph AKTA Purifier 10 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The mobile phase was changed as follows: 2 x Vb of acidified 30% ethanol in isocratic mode to remove amino acids, free sugars, proteins, etc. (Liu et al., 2004); 2 x Vb of acidified 50% ethanol in isocratic mode to remove the flavonoids and phenolic acids (Liu et al., 2004); 2 x Vb of acidified ethanol from 50 to 95% in gradient mode, to recover the absorbed AVNs. The flow rate was 5 ml/min and absorbance values were obtained at 330 nm.

Syntheses of AVNs

AVNs were synthesized according to previous synthetic strategies (Günther-Jordanland et al., 2016; Wise, 2011), with some modifications. Briefly, 2 ml of a 5 mM solution of the appropriate phenylpropanoid (*p*-coumaric, ferulic or caffeic acid) in pyridine were added to 10 ml of acetic anhydride. The mixture was then stirred for 5 h at room temperature. Cold water (50 mL) was added and the solution was left to stand at 5°C for one hour. The precipitate was collected by filtration and washed several times with cold water. The compound was dried overnight at 45°C under vacuum. The acetylated phenylpropanoid was then dissolved in 15 ml of dimethylformamide with 2.1 mL of triethylamine. The solution was stirred at 0°C and an equimolar amount of (Benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate in solution in CH_2Cl_2 (36 mL) was added dropwise. Next, a solution of 5-hydroxyanthranilic acid (equimolar to the protected phenylpropanoid) in 15 mL of dimethylformamide was added dropwise to the solution. The solution was stirred at 0°C for 30 min and then for 2 h at room temperature. Subsequently, 0.5 M HCl was added (80 mL) and the mixture was stored overnight at 4°C. The acetoxy avenantramides were extracted with ethyl acetate and the solvent was removed under reduced pressure. Finally, the protecting acetyl groups were removed by dissolving the avenanthramides in MeOH: Water (1:1) 80 mL with the addition of 4 equivalents of ammonium acetate, and stirred over night at room temperature. The mixture was then acidified with 2N HCl and extracted with ethyl acetate. The solvent was removed under reduced pressure and the final compounds were purified by crystallization in MeOH and water (2p and 2f) or CH_2Cl_2 and hexane (2c).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 Ultrashield spectrometer, using CD_3OD as a solvent. Chemical shifts (δ scale) were reported in parts per

million (ppm) relative to the central peak of the solvent. Coupling constants (*J* values) were given in hertz (Hz).

HPLC-PDA-MS analysis of AVNs

The ethanolic extract was filtered before analysis (Iso-Disc Filters, PFTE-4-4; 4 mm x 0.45 µm; Supelco Inc., Bellefonte, USA) and directly analyzed in a Waters instrument equipped with Alliance HT 2795 High Performance Liquid Chromatography (HPLC), 2996 Photo Diode Array (PDA) and Micromass LC/MS ZQ 2000 detector, following the known procedure (Jastrebova et al., 2006; Wise, 2011), with some modifications. A C18 column, LiChroCART® (250 x 4 mm), with a particle size of 5 µm, was used. The mobile phase used consisted of acetonitrile (solvent A) and 0.1 % aqueous formic acid (solvent B). The gradient was changed as follows: 0-5 min 2.4 % A (isocratic), 5-20 min to 24 % A, 20-38 min to 40 % A, 38-50 min to 75 % A. The total running time was 50 min. The injected sample volume was 50 µL and the flow rate was 0.8 mL/min. UV spectra were recorded from 220 to 420 nm, whereas the chromatograms were registered at 330 nm. Electrospray ionization (ESI) was operated in positive and negative ion mode in a range of 150-370 amu. Capillary voltage was set at 3 kV, source temperature at 100°C and desolvation temperature at 300°C. The cone and desolvation nitrogen gas flows were 50 and 500 L/h, respectively. Data were processed using MassLynx 4.1 (Waters, Milford, USA). To identity AVNs in the HPLC chromatograms, retention time, UV spectra, MS ESI(+) and ESI(-) spectra were compared with those of commercially available external standards (AVN 2p, 2f, 2c, Sigma-Aldrich, St. Louis, USA), and the peak areas were used for quantification.

Chemical antioxidant capacity of synthetic AVNs

DPPH assay

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was performed following the method previously reported (Brand-Williams et al., 1995), with same modifications. Briefly, 150 µl of AVN solution (50 µM in methanol), was mixed with 850 µl of freshly made DPPH solution (100 µM methanol). The absorbance of the mixture was recorded at 515 nm after incubation for ten minutes in the dark, at room temperature. Initial absorbance readings for DPPH, used as control, were 0.95±0.05. Antioxidant activity was expressed as percentage of inhibition calculated by the following equation:

(1- $\frac{Abs\ sample}{Abs\ control}$) × 100.

ABTS assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity was measured as previously reported (Ferri et al., 2013), with slight modifications. ABTS working solution was prepared by dissolving 7 mM ABTS in water, then treating with 140 mM potassium persulfate in water. The solution was allowed to stand for 12-16 hours in the dark, at room temperature, to produce the ABTS radical. Immediately before the analysis, the ABTS radical solution was diluted 1:90 in ethanol to reach the absorbance value of 0.70±0.02 at 734 nm (control). The assay was performed as follows: 1000 µl of diluted ABTS radical solution was mixed with 10 µl of AVN solution (10 µM in ethanol). After four minutes incubation, the absorbance was read at 734 nm. Antioxidant activity was expressed as percentage of inhibition calculated as reported for the DPPH method.

ORAC assay

The Oxigen Radical Absorbance Capacity (ORAC) assay was performed using a Fluostar Optima plate reader fluorimeter (BMG Labtech, Offenburgh, Germany), equipped with a temperature-controlled incubation chamber and automatic injection pump, as previously reported (Ninfali et al., 2009). Fluorescence was read at 485 nm ex. and 520 nm em. until complete extinction. A calibration curve was made each time with the standard Trolox (from 25 to 500 µM).

***In vitro* digestion of malted oat-based food products**

Simulation of a complete digestion was studied according to procedure suggested by Oomen et al. (2003) and occured in three steps (Table 3): 1) 2 g of sample was mixed with 30 ml of artificial saliva, incubated at 37°C, and rotated for 5 min (oral phase); 2) 45 ml of a gastric juice was added at pH 1 and the solution rotated for 120 min (gastric phase); and 3) the suspension resulting from the second stage was mixed with 90 ml of a synthetic duodenal solution and with 30 ml bile (intestinal phase). The two suspensions obtained (gastric and intestinal) were centrifuged for 30 min at 12,000 g to separate digestive juice from digested sample.

An aliquot (50 ml) of the final solution (≈ 200 ml) was extracted with 300 ml ethyl acetate, dried under nitrogen, redisolved in 1.5 ml of 80% ethanol and analyzed for polyphenols, ORAC

and AVN concentration. A blank sample was also prepared, in order to take into account possible impurity of reagents and release from containers.

Relative bioaccessibility (%) was calculated as follows = phenols of digested fraction/phenols of cookies (d.w.) x100.

Absolute bioaccessibility (mg per serving) was calculated as follows = phenols of cookies (d.w.) x (relative bioaccessibility, %) x serving size (50 g).

Table 3. Three-stage *in vitro* digestion procedure.

Digestion	Sample	Extraction reagent	Temperature (°C)	Time (min)
Oral phase	2g	30 ml of salivary	37	5’
Gastric phase	The suspension resulting after the first phase	45ml of gastric solution	37	120’
Intestinal phase		90 ml of duodenal solution and 30 ml of biliar solution	37	120’

Table 4 shows the digestive juices and their relevant concentrations used to simulate a complete digestion (oral + gastric + intestinal).

Table 4. Composition of 1L of salivary, gastric, duodenal and biliar juices.

Constituent	**Concentration (g/L)**	**Salivary juice (ml)**	**Gastric (ml)**	**Duodenal (ml)**	**Biliar (ml)**
NaCl	175.3	1.7	15.7	40	30
KCl	89.6	10	9.2	6.3	4.2
KSCN	20.0	10			
NaH_2PO_4	88.8	10	3.0		
Na_2SO_4	57.0	10			
KH_2PO_4	8.0			10	
$NaHCO_3$	84.7			40	68.3
NaOH	40.0	1.8			
$CaCl_2$	22.2		18	9.0	10
$MgCl_2$	5.0			10	
NH_4Cl	30.6		10		
HCl	440.3		8.3	0.5	0.5
Urea	25.0	8	3.4	4.0	10
Glucose	65.0		10		
Glucoronic acid	2.0		10		
Glucoseamin Hydrocloride	33.0		10		
Mucin		50 mg	3000 mg		
α-Amylase		145 mg			
Uric acid		15 mg			
BSA			1000 mg	1000 mg	1000 mg
Pepsin			1000 mg		
Pancreatin				3000 mg	
Lipase				500 mg	
Bile					6000 mg

Cell cultures

HepG2 and Hep3B liver cancer cell lines and CaCo-2 colon cancer cell line were purchased from the American Type Culture Collection (ATCC, Rockville, USA).

CaCo-2 and HepG2 were maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µM MEM non-essential amino acid solution. Hep3B cancer cells were kept in the same medium, but with the addition of 200 µM MEM non-essential amino acid solution. NCTC 2544 normal human keratinocytes were obtained from Interlab Cell Line Collection (ICLC, Genova, Italy) and maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. Cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

Sulforhodamine B (SRB) assay

Cells (10^4/well for CaCo-2, Hep3B and NCTC 2544; $2x10^4$/well for HepG2) were plated in 96-well plates and treated with AVNs, for 24, 48 and 72 hours. At the end of treatment, cells were incubated with SRB as previously reported (Farabegoli et al., 2017). The results were expressed as the percentage of cell viability, compared to control untreated cells (100% viability).

Cellular Antioxidant Capacity (CAA)

Cells were seeded ($2x10^5$ for CaCo-2 and $3x10^5$ for HepG2) in 6-well plates and treated for 24h with AVNs. After removing of cell medium, the H_2O_2-mediated production of ROS in CaCo-2 and HepG2 cells was monitored following the procedure previously reported (Farabegoli et al., 2017), with the substitution of PBS with $HBSS^{--}$ only.

After the incubation with 10 µM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min in the dark, the cells were observed at microscope with a green FITC filter (OLYMPUS IX51). Cells showing a bright and intense green fluorescence were counted as positive, whereas cells with no or low fluorescence were counted as negative. Three fields per sample were analyzed and each time at least 100 cells were counted. Three independent experiments were performed for both CaCo-2 and HepG2 cells and results were expressed as % DCF fluorescent positive cells versus control. The images were obtained using the software OLYMPUS Ps-blm1.

Caspase 3, 9, 8 and 2 activities

CaCo-2, HepG2, Hep3B cells were untreated, or treated with AVNs for 24 or 48 h, then the Petri dishes were put in ice and the cytosols were extracted following the procedure previously reported (Scarpa et al., 2016).

Caspase 3, 9, 8 and 2 activities were assessed through the Caspase Colorimetric Assay Kit (BioVision, Milpitas, USA) according to the manufacturer's instructions. First, 100 µg of cytosol from untreated or treated CaCo-2, HepG2 and Hep3B cells was incubated with the caspase reaction buffer (25 mM Hepes pH 7.4, 50 mM NaCl, 0.05% CHAPS, 0.5 mM EDTA, 5% glycerol, 5 mM DTT), then the substrate (200 µM final concentration) DMQD-pNA specific for caspase 3, or IETD-pNA specific for caspase 8, or LEHD-pNA specific for caspase 9, or VDVAD-pNA specific for caspase 2, was added. The samples were then transferred to a 96-well microplate and incubated at 37ºC for 2h. The absorbance was measured at λ=405 nm in a microplate reader (BioRad Laboratories, Hercules, USA).

RNA extraction, cDNA production and RTqPCR experiments

Total RNA was extracted from CaCo-2, HepG2 and Hep3B cells using the TriReagent (Invitrogen, Carlsbad, USA), following the manufacturer's protocol. cDNA production and RTqPCR assays were performed as previously reported (Scarpa et al., 2016), using the KAPA SYBR Fast (2x) ABI PRISM qPCR Kit (KAPA Biosystems Inc., Cape Town, South Africa) and the ABI PRISM 7700 Sequence Detector (Applied Biosystems Inc., Foster City, USA). Thermal cycling conditions were as follows: 95ºC for 10', followed by 40 cycles of 15'' at 95ºC, 15'' at 60ºC, 15'' at 72ºC. The $2^{-\Delta\Delta Ct}$ method (Livak method) was used for relative quantification of gene expression. β-Actin was utilized as the housekeeping gene.

The following primers were used: β-Actin F: 5'-GCGAGAAGATGACCCAGATC-3' , R: 5'-GGATAGCACAGCCTGGATAG-3'; COX-2 F: 5'-CACCCATGTCAAAACCGAGG-3' , R: 5'-CCGGTGTTGAGCAGTTTTCTC-3'; HIF1A F: 5'-TCTGGGTTGAAACTCAAGCAACTG-3', R: 5'-CAACCGGTTTAAGGACACATTCTG-3' (150 bp); VEGFA F: 5'-TCACAGGTACAGGGATGAGGACAC-3', R: 5'-CAAAGCACAGCAATGTCCTGAAG-3' (72 bp).

COX-2 activity assay

CaCo-2 and Hep3B cytosols were extracted as previously described (Guo et al., 2010), but using the COX lysis buffer (100 mM Tris/HCl pH 7.8, 1 mM EDTA). The protein concentration was assessed by the Bradford assay (Biorad Laboratories, Hercules, USA), measuring the absorbance values at λ=595 nm. COX-2 activity was assessed using the COX Activity Assay Kit (Cayman chemical, USA) according to the manufacturer's instructions. Subsequently, 150 µg of proteins were incubated for 10 min at 25 ºC with either the COX assay buffer (100 mM Tris/HCl pH 8.0) (CTRL), COX-1 inhibitor SC-560, SC-560 and 110 µM AVNs. The samples were then incubated with 210 µM arachidonic acid and the colorimetric substrate TMPD. The absorbance of the samples was measured at λ=590 nm, using a spectrophotometer. The following formula was used to calculate the enzymatic activity:
COX Activity = ($\Delta A_{590\ nm}$/10 min/0.00826 μM^{-1}) x 1.0 ml/ml of cell cytosol / 2.

Statistical analysis

Chemical analyses (FPs, BPs, AVNs, DPPH, ABTS assays) were performed in triplicate, and the results were reported as the mean value ± standard deviations (SD). ORAC assay was established by eight independent determinations for each sample; each value was the mean ± SD. Linear regression analysis and Pearson's correlation coefficient were performed using Microsoft Excel® software; statistical differences were calculated using the Student's t-test and one-way ANOVA with the SPSS® 17.0 software (SPSS Inc., IBM, Chicago, USA).

Results and discussion

Morphology of oat caryopsis

The caryopsis of cereals contains specific nutrients (Fardet, 2010; Liu, 2007; van der Kamp et al., 2014), unevenly distributed within its botanical fractions, i.e. the outer layers, including the aleurone, the starchy endosperm and the germ (Fardet, 2010; Zielinski and Kozlowska, 2000). Nutrient concentration in grains is generally assessed by analytical destructive techniques (i.e. spectrophotometer, HPLC). However, the knowledge of the specific localization of nutrients in the caryopsis is considered of importance for two main reasons: *a)* to understand the nutritional value of whole grain flour (WGF); *b)* to regulate the industrial processes of grain transformation into WGF, in order to save nutrients (Rosa-Sibakov et al., 2015).

Regarding the localization on nutrients, we performed an imaging analysis of oat caryopsis based on light microscopy (Fig. 5A) and environmental scanning electron microscopy–energy dispersive spectroscopy (ESEM–EDS) (Fig. 5B), in order to characterize the main structural fraction.

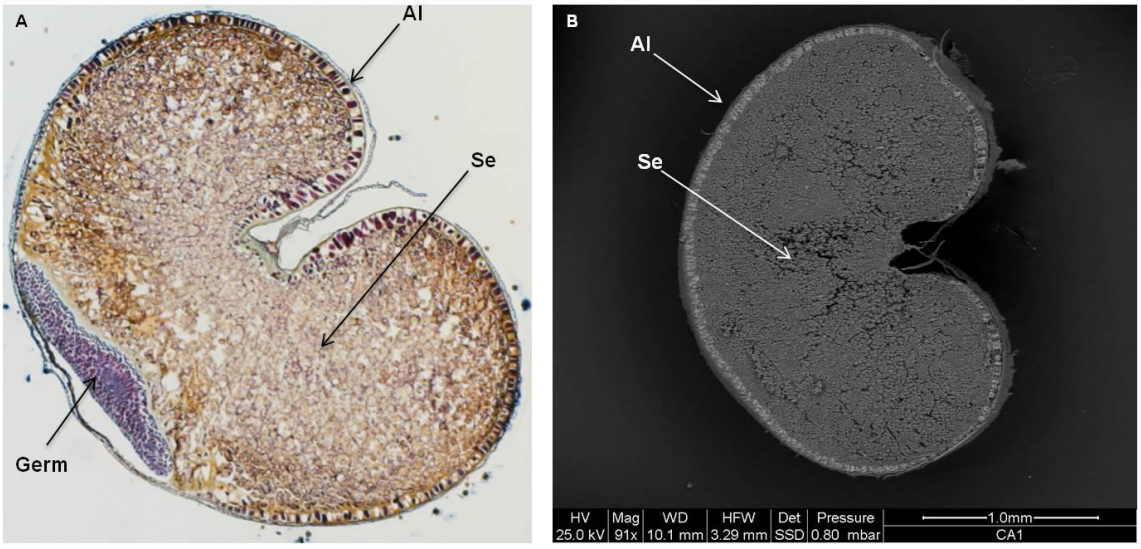


Figure 5. Light microscopy (A) and ESEM-EDS (B) micrographs of oat caryopsis. Al, aleurone; Se, starchy endosperm.

Fig. 6 shows the aleurone *(Al)* and starch endosperm *(Se)* of oat caryopsis at higher magnification.

Examining the morphology, the aleurone is characterized by one line of cells divided by thick walls (Fig. 6 A1, B1). The ESEM-EDS technique revealed the presence of several globoids *(Gl)* within each aleurone cell, 1-2 µm in diameter (Fig. 6 B3), constituted by a remarkable concentration of P, related to the presence of phytic acid, as well as Mg, K and Ca (Fig. 6 B5). Depending on the refining level, part of the aleurone is discarded as bran during milling, along with phytic acid, which is interesting for its health protective functions (Abebe et al., 2007; Ockenden et al., 2004; Silva and Bracarense, 2016).

Oat starch endosperm was characterized by granules *(Sg)* of different shapes and sizes, appearing as if they had formed through the aggregation of several smaller granules (Fig. 6 A2, B2). According to the literature, the structure and properties of these granules are influenced by the amount of lipids associated with the starch (Gudmundsson and Eliasson, 1989; Morrison, 1988). The elemental composition of starch granules (Fig. 6 B4), detected by ESEM-EDS, revealed the presence of C and O, accounting for about 99.6% of starch polysaccharides. The chemical elements P, S, and K constituted the remaining 0.4% (Fig. 6 B6).

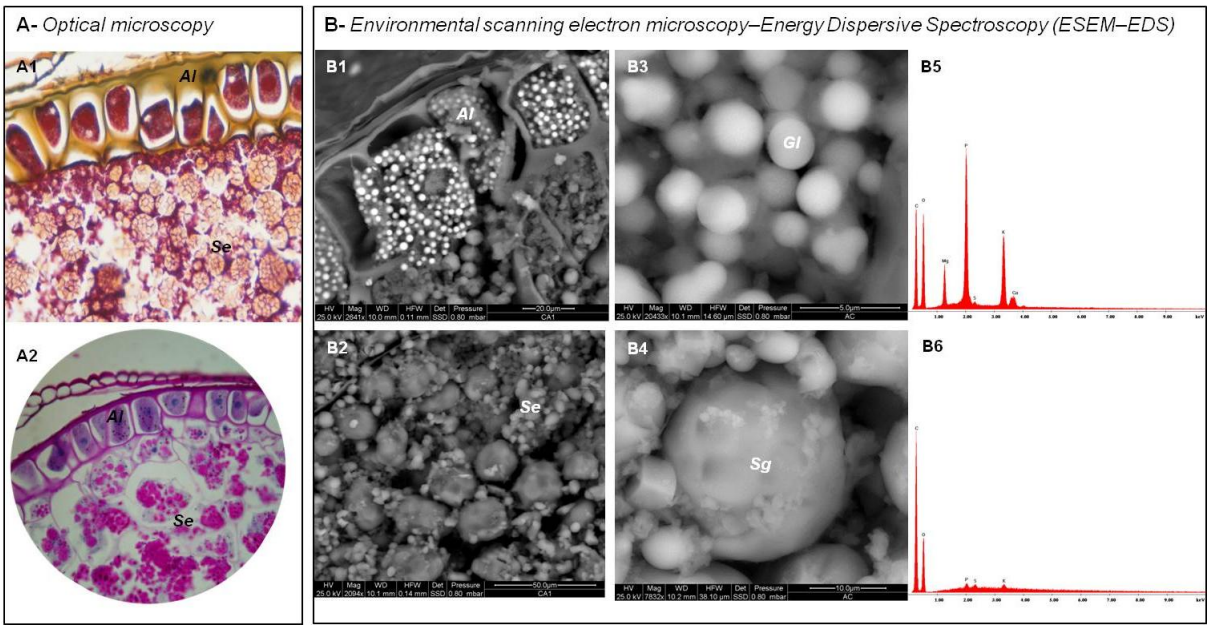


Figure 6. Higher magnification images of aleurone and starchy endosperm of oat caryopsis revealed by light microscopy (A) and ESEM-EDS (B).

Sections were stained using the Azan Trichrome kit according to Heidenhain staining (A1, x20) and Periodic acid-Shiff staining (A2, x60). ESEM-EDS micrographs of aleurone (*Al*, B1), starch endosperm (*Se*, B2), globoids of aleurone (*Gl*, B3), starch granules of endosperm (*Sg*, B4). ESEM-EDS spectra of globoids of aleurone (B5) and starch granules of endosperm (B6).

Effect of genotype on antioxidant levels

The AVN content is strictly influenced by genetic and environmental factors (Redaelli et al., 2016; Yang et al., 2014). Among oats, naked oat, a variety where the hulls are genetically removed, has been receiving growing interest due to its higher protein and lipid levels than husked oat (Biel et al., 2014). In addition, naked oat allows food industries to reduce the production costs, as the dehulling step is avoided and moreover it is easily utilized for malting processes. As the hulls must be removed from husked oat because they are unpalatable (Singh et al., 2013), a comparison of the non-caloric nutrient content between naked and dehulled oat cultivars has been considered by us a point of interest for health professionals and technologists.

For these reasons, 15 naked and 15 dehulled oat cultivar samples (cvs.) were analyzed by us for their antioxidant molecules, including free (FPs) and bound (BPs) polyphenols and total AVNs (2c + 2p + 2f).

Data at harvest allowed us interesting comparative considerations among cvs. (Table 5). In fact, total polyphenols (TPs) of the 30 oat genotypes did not show any differences between the mean values of the two groups (naked and dehulled).

Despite this, when individual free (FPs) and bound (BPs) polyphenols were compared, it was observed that inside to each group, BP values were higher than FP values (Table 5), thus confirming data reported in literature (Adom and Liu, 2002; Verardo et al., 2011). Moreover, FPs were significantly higher in naked than in dehulled oats (Table 5).

The individual AVNs (2c, 2p, 2f) and their sum were found to be roughly three times higher in naked than in dehulled oats (Table 5), thus suggesting that genotype is the main determinant, as previously reported (Redaelli et al., 2016). In naked oats, the 2c form, containing caffeic acid, is the most important representative, whereas in dehulled oats, the 2f form, composed by ferulic acid, is the dominant. Our AVNs data, related to naked oats, agree with data from other authors (Tong et al., 2014).

Table 5. Content of polyphenols and AVNs in 30 oat cultivars.

Genotypes	Nation	Polyphenols (g/kg d.m.)			AVNs (mg/kg d.m.)			
		FPs	BPs	TPs	2c	2p	2f	Total
***A. sativa ssp. nudisativa* L.**								
BD114	Italy	0.75±0.01^{ef}	1.02±0.07^{bcd}	1.77±0.04	49±2^{g}	35±2^{fg}	42±2^{e}	127±8
BD124	Italy	0.81±0.05^{ef}	1.15±0.01^{bc}	1.93±0.03	54±3^{fg}	49±2^{f}	56±3de	159±11
BULLION	UK	0.77±0.05^{ef}	1.16±0.02^{bc}	1.93±0.04	52±3^{g}	45±2^{fg}	52±3de	149±11
HJA72095N	Finland	0.89±0.03^{def}	1.05±0.07^{bcd}	1.94±0.04	39±2^{g}	39±2^{fg}	47±2^{e}	125±8
HJA76037N	Finland	0.73±0.03^{f}	0.69±0.04^{d}	1.42±0.03	19±1^{h}	10±1^{h}	15±1^{fg}	44±3
ICON	UK	1.10±0.08^{bc}	1.35±0.09^{abc}	2.45±0.09	181±10^{b}	129±8^{b}	221±10^{a}	531±37
IRINA	Italy	0.82±0.08^{def}	1.14±0.10^{bc}	1.96±0.09	73±4^{ef}	47±2^{fg}	25±1^{f}	145±10
KRYPTON	UK	0.99±0.09^{cde}	1.08±0.04^{bcd}	2.07±0.07	155±8^{c}	95±5^{d}	85±4^{c}	335±23
KYNON	UK	0.80±0.04^{ef}	0.97±0.02^{cd}	1.77±0.03	40±2^{g}	35±2^{g}	51±3^{e}	126±8
LEXICON	UK	1.25±0.07^{ab}	1.06±0.07^{bcd}	2.31±0.03	182±9^{b}	107±5^{cd}	196±10^{b}	485±35
LUNA	Italy	0.90±0.07^{cdef}	1.26±0.12^{abc}	2.16±0.10	14±1^{h}	12±1^{h}	9±0^{g}	35±2
RHEA x PADARN542	France	1.06±0.06^{bcd}	1.24±0.08^{abc}	2.30±0.09	104±5^{d}	114±6^{c}	93±5^{c}	312±30
TERRA	Canada	0.89±0.08^{def}	0.99±0.05^{bcd}	1.88±0.07	78±4^{e}	44±2^{fg}	86±4^{c}	208±15
USPEH	Russia	1.41±0.10^{a}	1.63±0.03^{a}	3.04±0.07	308±15^{a}	237±12^{a}	205±10^{b}	750±39
VIR2301	Denmark	0.94±0.08^{cdef}	1.41±0.09^{ab}	2.35±0.08	54±3^{fg}	76±4^{e}	67±3^{d}	197±11
Mean±SD		**0.94±0.19***	**1.14±0.21***	**2.09±0.36***	**94±78***	**72±57***	**83±66***	**249±194***
***A. sativa* L.**								
BORUTA	Poland	0.79±0.07^{bc}	0.88±0.07^{f}	1.67±0.08	4±0^{g}	7±0^{hi}	11±1^{gh}	21±1
COACH	Germany	0.75±0.08^{bc}	0.89±0.07^{f}	1.64±0.08	12±1^{ef}	14±1^{efg}	37±2^{d}	63±4
DOLPHIN	Australia	0.82±0.03^{bc}	1.23±0.04^{bcd}	2.05±0.04	28±1^{c}	45±2^{c}	76±4^{b}	149±11
EWALD	Austria	0.78±0.03^{bc}	1.21±0.05^{cd}	1.99±0.05	7±0^{fg}	8±0^{hi}	11±1^{h}	26±1
MARTIN	Norway	0.81±0.06^{bc}	1.11±0.02^{de}	1.92±0.04	23±1^{cd}	38±2^{d}	46±2^{c}	107±8
MILLENIUM	UK	0.92±0.03^{ab}	1.44±0.09^{ab}	2.36±0.06	132±7^{a}	67±3^{b}	94±5^{a}	293±21
MONIDA	USA	0.71±0.04^{c}	1.38±0.10^{bc}	2.09±0.07	24±1^{cd}	18±1^{e}	22±1^{f}	64±4
MUTINE	France	1.03±0.01^{a}	1.62±0.02^{a}	2.65±0.05	7±0^{fg}	9±0^{ghi}	18±1^{f}	34±2
NEKLAN	Czech Rep.	0.83±0.02^{bc}	0.94±0.04^{ef}	1.77±0.04	113±6^{b}	83±4^{a}	98±5^{a}	294±20
NY79084-1	USA	0.81±0.07^{bc}	0.96±0.06^{ef}	1.77±0.07	8±0^{fg}	8±0^{hi}	4±0^{h}	20±1
OWARE	New Zealand	0.82±0.08^{bc}	0.93±0.02^{ef}	1.75±0.05	15±1^{e}	18±1^{e}	30±1^{e}	63±4
SISKO	Finland	0.68±0.06^{c}	1.10±0.05^{de}	1.78±0.07	5±0^{g}	5±0^{i}	5±0^{h}	15±0
STO. ALEIXO	Portugal	0.78±0.03^{bc}	1.25±0.03^{bcd}	2.03±0.04	19±1de	12±1^{fgh}	17±1^{fg}	48±3
TADZHIKSKII	Russia	0.92±0.02^{ab}	0.85±0.07^{f}	1.77±0.07	13±1^{ef}	7±0^{hi}	7±0^{h}	27±1
TIPPECANOE	USA	0.65±0.02^{c}	1.13±0.04^{de}	1.78±0.03	18±1de	15±1^{ef}	17±1^{fg}	50±3
Mean±SD		**0.81±0.09**†	**1.13±0.22***	**1.93±0.27***	**28±38**†	**24±23**†	**33±31**†	**85±89**†

FPs, free polyphenols; BPs, bound polyphenols; TPs, total polyphenol (TPs = FPs + BPs); AVNs, avenanthramides. Different letters $^{(a,b)}$ indicate a statistically significant difference among cvs. of each group (ANOVA test); different symbols $^{(*,\dagger)}$ indicate a statistically significant difference between the mean values of each group (Student's *t* test).

Effect of storage conditions of antioxidant levels

The caryopsis of cereals is constituted by living cells in the aleurone and germ, which slowly metabolize nutrients during storage. Although several reports analyzed the variability ranges of the health protective nutrients in naked and husked cultivars at harvest (Bratt et al., 2003; Bryngelsson et al., 2002; Emmons and Peterson, 2001; Mattila et al., 2005; Redaelli et al., 2016; Yang et al., 2014), the extent of their changes during storage have never been investigated, particularly those regarding the free and bound phenols and their antioxidant capacities.

For these reasons, we measured the stability of antioxidant molecules, over 12 months of storage, in a cold room at + 7°C and 55% relative humidity, in a group of six naked and six dehulled cvs., selected among the 30 oat genotypes described in the previous paragraph.

After 12 months of storage, BPs (Fig. 7B) were significantly reduced compared to FPs (Fig. 7A), which seemed to be more preserved.

Regarding the antioxidant capacity, the ORAC values of FPs showed a significant decay from T0 to T12, paralleling that observed in the Folin-Ciocalteu assay of polyphenols. Nevertheless, FPs (Fig. 7C) showed ORAC values about five fold higher than those of BPs (Fig. 7D), due to the relevant presence of soluble phenols, including AVNs, which better scavenge peroxyl radicals than BPs (Adom and Liu, 2002).

Considering the average values of naked and dehulled oats, in terms of antioxidant capacity, our data show that FPs contribute with 11,300 μmolTE/100 g of wholegrain flour (WGF), whereas the antioxidant activity of BPs was around 2,000 μmolTE/100 g, which brings the total ORAC (FPs+BPs) to 13,300 μmolTE/100 g of WGF.

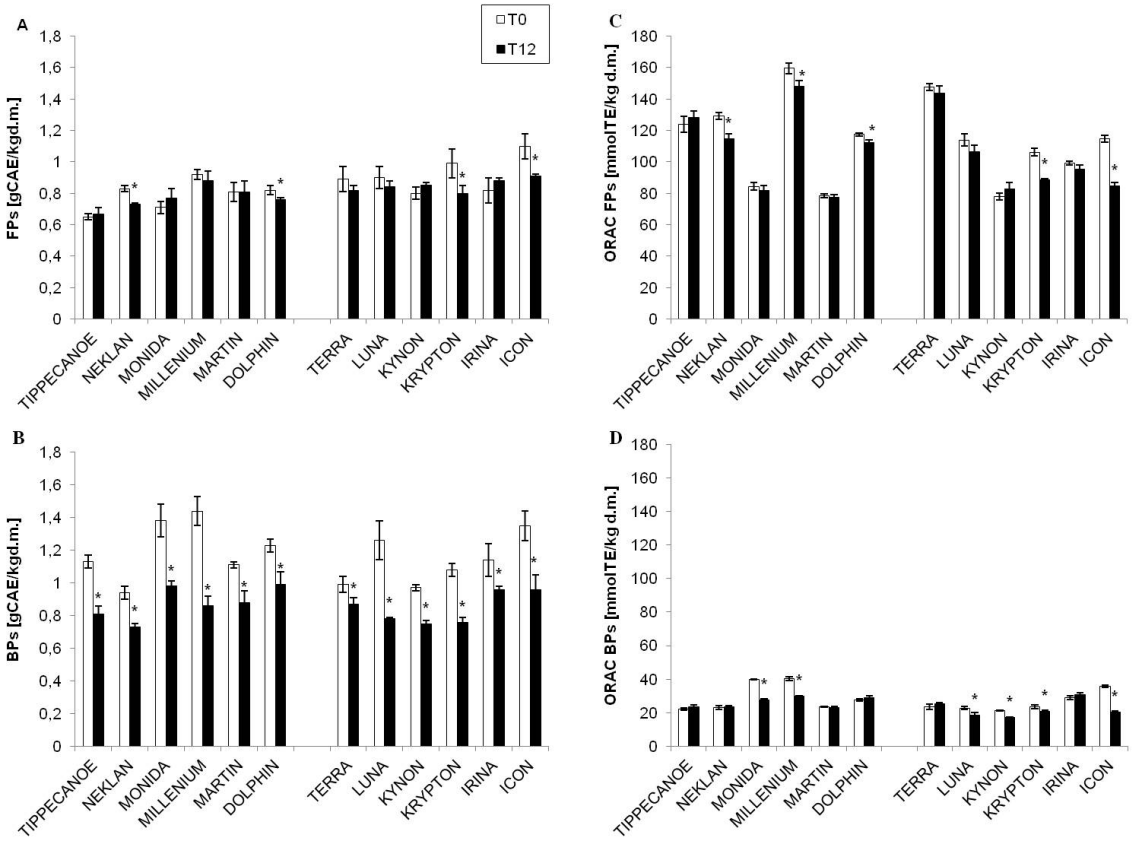


Figure 7. Changes of free (FP, A) and bound (BP, B) phenol contents and their antioxidant capacities, detected by the ORAC method (ORAC of FPs, C; ORAC of BPs, D), at time zero (T0) and after 12 months of storage (T12), in 12 oat cultivars (six dehulled and six naked).

* indicating the cultivar which showed a statistically significant reduction at T12, with respect to T0, by Student's *t*-test with $p < 0.05$. FP and BP values are expressed as g of caffeic acid equivalents (CAE) kg^{-1} dry matter (d.m.). ORAC values are expressed as mmol of Trolox equivalents (TE) kg^{-1} d.m.

After 12 months of storage, individual AVNs (2c, 2p, 2f) showed a reduction in their concentration, ranging from 15 to 90 %, in most of the analyzed cultivars (Fig. 8). Only two samples, LUNA among naked and MONIDA among dehulled, showed no significant reduction in any AVN form (Fig. 8).

Among the three AVNs, the 2c (Fig. 8A) appeared to be the most preserved form after 12 months of storage, as compared to 2p (Fig. 8B) and 2f (Fig. 8C), though other authors found to be the most sensitive one when subjected to different treatments (Dimberg et al., 2001). Interestingly, it seemed that the AVN decrease was higher in those samples which showed the highest levels at T0. Indeed, those cultivars with lower AVN values at T0 showed a very small or no decay. This occurs very likely because the metabolite synthetic machinery is always balanced by a similar expression of the degradative machinery, in order to adapt the vegetable to different environmental conditions (Frati et al., 2016).

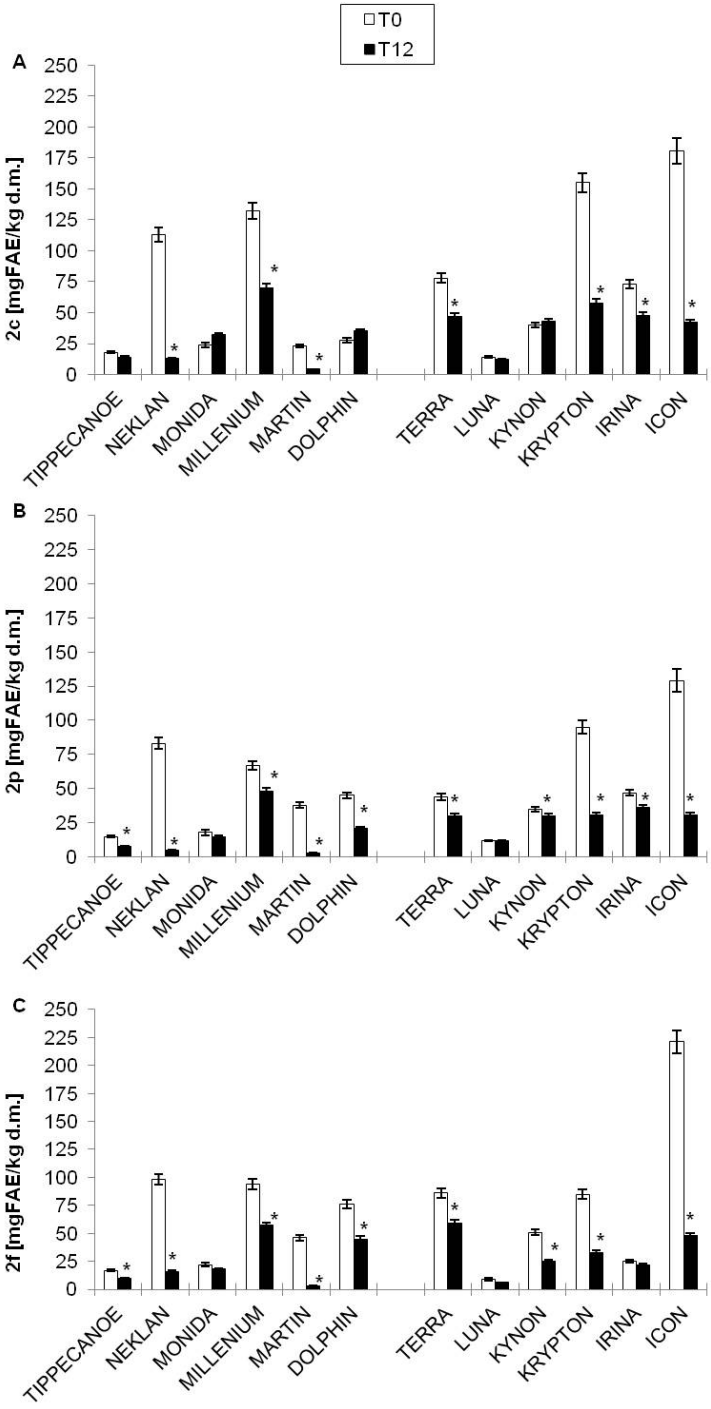


Figure 8. Changes of the three main avenanthramide (AVN 2c, 2p, 2f) contents, at time zero (T0) and after 12 months of storage (T12), in 12 oat cultivars (six dehulled and six naked).

* indicating the cultivar which showed a statistically significant reduction at T12, with respect to T0, by Student's *t*-test with $p < 0.05$. AVN values are expressed as mg of ferulic acid equivalent (FAE) kg^{-1} dry matter (d.m.).

Effect of mechanical processing of antioxidant levels

The two main processes involved in grain transformation are dehulling and milling, both influenced by the mechanical applied pressure and moisture of the grains (Kaur et al., 2014). When grains are processed into flours, nutrients inside the endosperm caryopsis are conserved, whereas those linked to the aleurone are partially lost in the bran fraction. In fact, the milling process breaks the outer layers of the caryopsis (Fig. 9A) into large plaques, covered by starch (Fig. 9B); whereas the oat flour is primarily constituted by disrupted starchy granules and traces of residual bran fractions (Fig. 9C).

Concerning the outer layers, they are rich of phenolic compounds. The fluorescent light (Fig. 9D) highlighted the polyhedral structures associated with ferulic acid (Fulcher, 1982), which was found to be abundant in the bran (Fig. 9E) and in traces in the flour (Fig. 9F). Although autofluorescence does not allow the identification of the nature of phenol compounds, it can be useful to understand that it is better to mill as mild and prolonged as possible, till when the bran is grounded into fine particles, to avoid excessive separation of the bran from the flour and loss of phenols.

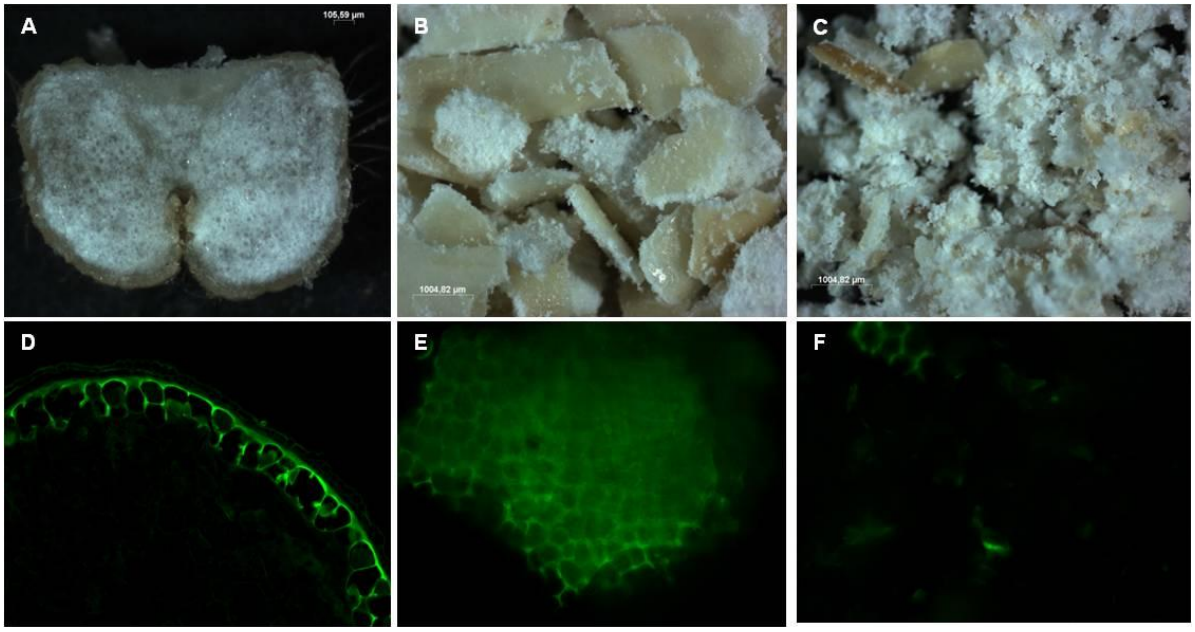


Figure 9. Intact oat caryopsis (A), oat bran (B) and flour (C) observed by optical microscopy after milling. Autofluorescence of the outer layers of unstained oat section (D), oat bran (E) and flour (F).

The effect of industrial dehulling and milling on AVNs, total polyphenols, and their antioxidant capacities, was observed by us in two husked oat cultivars, grown in two different soils, labeled as: D1, cv. Donata in loamy soil; D2, cv. Donata in a medium texture soil; F1, cv. Flavia in loamy soil; F2, cv. Flavia in a medium texture soil.

Both mechanical processes reduced AVNs, polyphenols and ORAC values, excluding the original differences stemming from genotype or soil, which were evident in the raw grains (Table 6). The genotype played an important role on AVN content, as cv. Donata showed about three fold higher AVN values than cv. Flavia, as well as the interaction soil x genotype, with D1>D2 and F2>F1 (Table 6). Moreover, in the raw Donata grains, free (FPs) and bound (BPs) polyphenols were in equal concentration (Table 6), whereas in the raw Flavia grains, BPs were significantly higher than FPs. Nevertheless, the ORAC values of FPs were significantly higher than those of BPs, in all samples (Table 6), due to the relevant presence of AVNs, which provide a higher number of hydroxyl groups able to better scavenge peroxyl radicals than BPs, in the ORAC test (Prior, 2015).

Dehulling markedly decreased the three AVN forms (2c, 2p, 2f), bound polyphenols (BPs) and their corresponding ORAC values, in all samples (Table 6). Free polyphenols (FPs) remained stable in both Flavia groats, whereas they were reduced in both Donata groats (Table 6).
The reduction of AVNs after dehulling can be explained by the fact that they are primarily located in hulls and in the outer layers of the caryopsis, including the aleurone (Yang et al., 2014).

Milling did not affect AVNs, FPs and their antioxidant capacities in any of the samples (Table 6). However, it further reduced BPs in both cvs. with a consequent drop in the ORAC values of BPs to one half of the values found in the dehulled groats (Table 6), due to the removal of the fiber to which BPs are covalently bound (Verardo et al., 2011).

Table 6. Effect of mechanical processing on antioxidant levels.

Parameters	Processing	D1	D2	F1	F2
AVNs (mg/kg d.w.)					
2c	**Raw grain**	**282 ± 21** ^{a*}	**246 ± 11** ^{a**}	**68 ± 6** ^{a†}	**100 ± 6** ^{a‡}
	Dehulling	55 ± 6 ^{b}	38 ± 6 ^{b}	46 ± 5 ^{b}	45 ± 6 ^{b}
	Milling	63 ± 5 ^{b}	40 ± 5 ^{b}	59 ± 7 ^{b}	51 ± 7 ^{b}
2p	**Raw grain**	**404 ± 36** ^{a*}	**333 ± 14** ^{a**}	**123 ± 14** ^{a†}	**172 ± 12** ^{a‡}
	Dehulling	106 ± 11 ^{b}	93 ± 10 ^{b}	85 ± 5 ^{b}	96 ± 8 ^{b}
	Milling	114 ± 11 ^{b}	112 ± 10 ^{b}	86 ± 7 ^{b}	114 ± 11 ^{b}
2f	**Raw grain**	**417 ± 47** ^{a*}	**340 ± 7** ^{a**}	**113 ±16** ^{a†}	**168 ± 17** ^{a‡}
	Dehulling	101 ± 6 ^{b}	82 ± 7 ^{b}	83 ± 2 ^{b}	97 ± 8 ^{b}
	Milling	113 ± 11 ^{b}	96 ± 1 ^{b}	93 ± 12 ^{b}	116 ± 11 ^{b}
Polyphenols (g/kg d.w.)					
FPs	**Raw grain**	**1.89 ± 0.10** ^{a*}	**1.99 ± 0.11** ^{a*}	**1.27 ± 0.10** ^{a**}	**1.49 ± 0.13** ^{a**}
	Dehulling	1.05 ± 0.08 ^{b}	1.29 ± 0.10 ^{b}	1.24 ± 0.09 ^{a}	1.27 ± 0.10 ^{a}
	Milling	1.11 ± 0.06 ^{b}	1.30 ± 0.03 ^{b}	1.23 ± 0.03 ^{a}	1.28 ± 0.05 ^{a}
BPs	**Raw grain**	**2.02 ± 0.15** ^{a*}	**2.24 ± 0.28** ^{a*}	**2.64 ± 0.18** ^{a**}	**2.71 ± 0.32** ^{a**}
	Dehulling	0.73 ± 0.06 ^{b}	0.77 ± 0.05 ^{b}	0.71 ± 0.06 ^{b}	0.76 ± 0.05 ^{b}
	Milling	0.55 ± 0.03 ^{c}	0.48 ± 0.07 ^{c}	0.43 ± 0.09 ^{c}	0.56 ± 0.07 ^{c}
Antioxidant capacity (mmolTE/kg d.w.)					
ORAC of FPs	**Raw grain**	**195.3 ± 13.0** ^{a*}	**186.2 ± 13.6** ^{a*}	**122.7 ± 9.3** ^{a**}	**100.0 ± 9.4** ^{a**}
	Dehulling	122.9 ± 8.8 ^{b}	128.5 ± 11.7 ^{b}	114.8 ± 10.6 ^{a}	111.2 ± 3.0 ^{a}
	Milling	135.2 ± 5.4 ^{b}	129.7 ± 7.5 ^{b}	129.3 ± 8.7 ^{a}	120.7 ± 8.4 ^{a}
ORAC of BPs	**Raw grain**	**60.8 ± 2.6** ^{a*}	**46.5 ± 4.8** ^{a**}	**46.3 ± 1.7** ^{a**}	**66.2 ± 2.8** ^{a*}
	Dehulling	31.9 ± 7.9 ^{b}	35.3 ± 3.1 ^{b}	38.1 ± 2.8 ^{b}	36.6 ± 3.0 ^{b}
	Milling	15.4 ± 1.1 ^{c}	17.8 ± 1.0 ^{c}	13.7 ± 1.5 ^{c}	12.0 ± 1.1 ^{c}

Each value is shown as mean ± standard deviation and is expressed on dry weight (d.w.).

AVNs, avenanthramides; D1, cv. Donata in loamy soil; D2, cv. Donata in medium texture soil; F1, cv. Flavia in loamy soil; F2, cv. Flavia in medium texture soil; FPs, free phenols; BPs, bound phenols; ORAC, Oxygen Radical Absorbance Capacity. $^{a-c}$ Values with different lower case letters in the same column, for each parameter, indicate significant differences ($p < 0.05$) along the processing chain, from raw grain to flour. *,† Different symbol in the same row indicate significant differences ($p < 0.05$) stemming from genotype or soil type.

Overall, these studies highlight that: *(a)* biodiversity in oats is mainly expressed by their content of micronutrients, and in particular by AVNs, whose levels are strictly related to genotype; *(b)* mechanical processing and storage conditions also have an impact on AVN content. Therefore, the choice of the cultivar, storage conditions and the technology of transformation are important tools to guarantee the maintenance of the highest AVN levels, which are the major healthy molecules considered in this thesis.

Effect of malting and cooking on antioxidant levels

Steeping and germination are the most common physiological ways to increase polyphenol and AVN concentrations (Bryngelsson et al., 2002; Skoglund et al., 2008; Xu et al., 2009). Nevertheless, a malting method able to prevent the seeds from germinating has been studied, in order to make the malted oats suitable for most commercial food processing procedures (Collins and Burrows, 2012).

In this study, we used the procedure which is generally performed in the brewing industry, in order to keep low the germination percentage of oats and to increase the AVN concentration for the formulation of cookies fortified with AVNs to be used for breakfast or snack.

First, we evaluate the change of free polyphenol (FP), antioxidant capacity and AVN levels in the Italian naked oat genotype (cv. Luna), during five days of malting (M0→M5).
Interestingly, FP values tripled from M0 to M5, as well as their antioxidant capacities, measured by the ORAC method, thus confirming the link between the two parameters (Fig. 10).

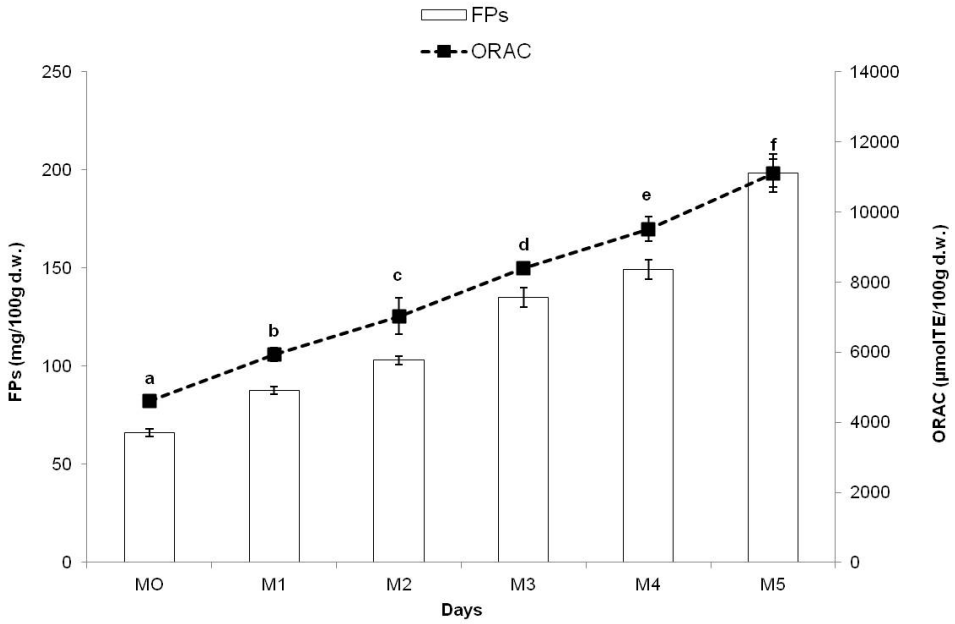


Figure 10. Free polyphenols (FPs) and ORAC after five days of malting (M0-M5).

Regarding AVNs, we measured the concentration of the three main forms (2c, 2p, 2f) as well as other minor forms (2s, $2p_d$, $2f_d$) and their total, during five days of malting (Fig. 11). Total AVNs showed about 10-fold higher values at M5 with respect to M0, although they seemed to level off in the range M3-M5.

Concerning the concentration of specific AVN forms, the 2c was the most representative, reaching values of 12 mg/100g d.w. at M5 (Fig. 11). Nevertheless, Lp and Lf showed the greatest increases during 5 days of malting, i.e. about 20-fold and 40-fold more than the starting levels, respectively.

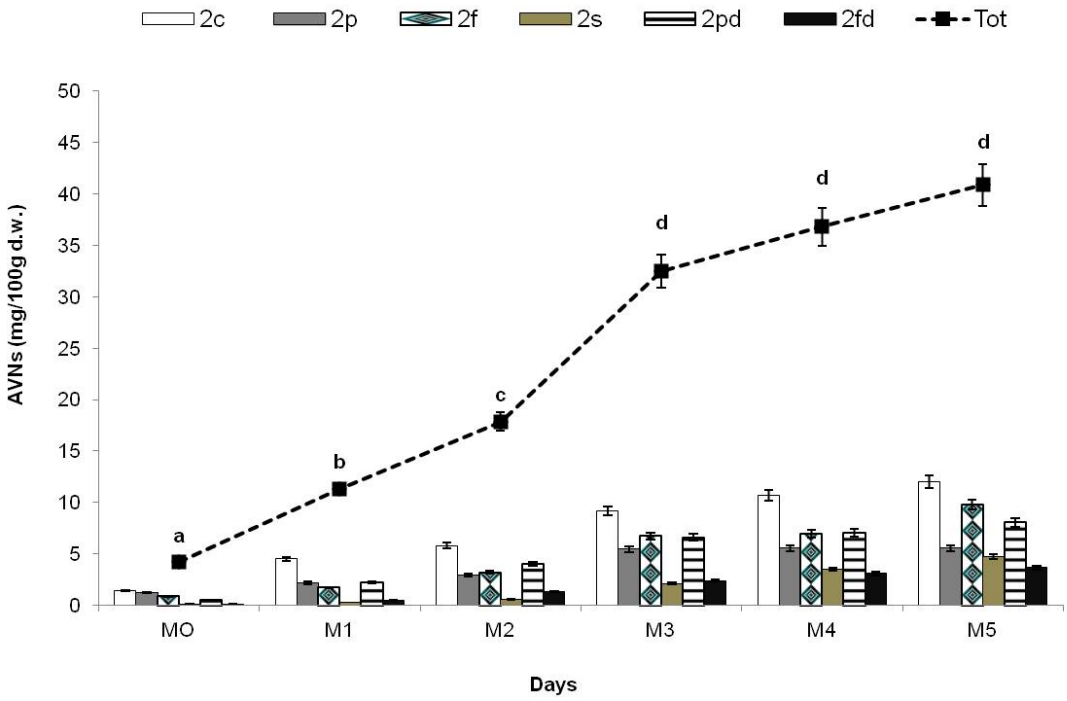


Figure 11. Individual and total avenanthramides (AVNs) after five days of malting.

Second, we focused on the optimal ratios between the two main ingredients which constituted the food matrix of malted oat-based cookies: the wheat flour and the flour obtained from malted oat. As the total AVN concentration showed a leveling in the last three days of malting (Fig. 11), we decided to use the malted oat flour, obtained from M3, M4 and M5, in two different percentages, i.e. 35 % in the A triplet of cookies (3A, 4A, 5A), 45% in the B triplet of cookies (3B, 4B, 5B). *For more details see note n. 9 of Material & Methods section.*

Fig. 12 shows the FP and ORAC values of the six malted-oat based cookies. The best performances in terms of FPs and ORAC values were obtained in the 5A and 5B formulations (Fig. 12).

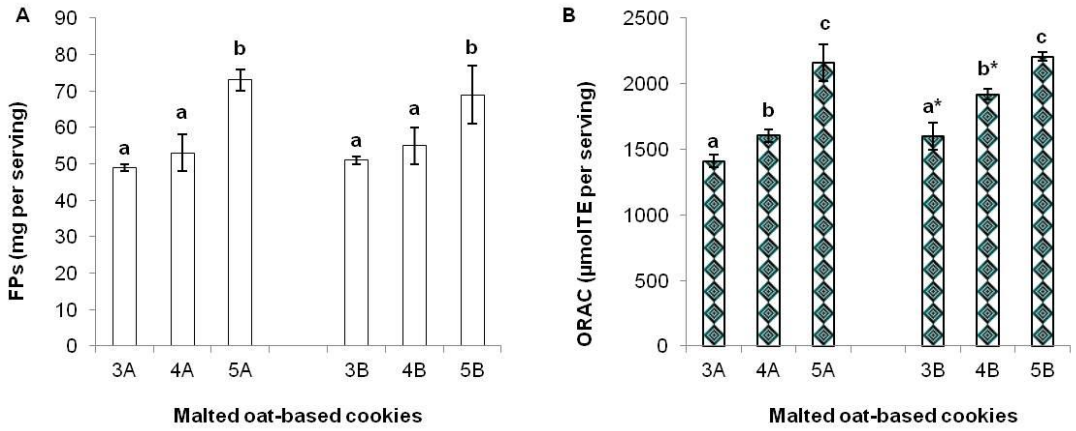


Figure 12. Free polyphenols (FPs, A) and antioxidant capacity (ORAC, B) in malted oat-based cookies.

FP_s, values are expressed as mg per serving; ORAC, values are expressed as µmolTE per serving. Per serving = 50 g of malted oat-based cookies, on dry weight. [a,b] Different letters indicate statistically significant differences ($p<0.05$ one-way ANOVA) among each triplet of cookies (3A, 4A, 5A; 3B, 4B, 5B). [*]$p<0.05$ (Student's *t* test) compared to the corresponding ORAC value of 3A or 4A formulation.

Table 7 shows the individual and total AVN forms in malted oat-based cookies. In the A triplet of cookies, 2c, 2f and total AVN concentrations increased, with 5A > 4A > 3A, whereas all other forms showed a remarkable increase in the 5A formulation only (Table 7). In the B triplet, only the AVN 2f showed a gradual increase from 3B to 5B formulation, whereas all other forms remained rather stable. Comparing the same malting day, the differences were more evident, with 3B>3A, 4B>4A in all individual and total AVN forms (Table 7).

Table 7. Individual and total AVNs in malted oat-based cookies.

Cookies	AVNs						
	2c	**2p**	**2f**	**2s**	**$2p_d$**	**$2f_d$**	**Total**
3A	0.53±0.03[a]	0.45±0.02[a]	0.63±0.03[a]	0.21±0.03[a]	0.75±0.05[a]	0.29±0.02[a]	2.88±0.14[a]
4A	0.99±0.05[b]	0.47±0.03[a]	0.72±0.04[b]	0.25±0.01[a]	0.75±0.02[a]	0.30±0.05[a]	3.48±0.17[b]
5A	1.16±0.05[c]	0.62±0.03[b]	1.08±0.05[c]	0.55±0.05[b]	1.07±0.03[b]	0.47±0.02[b]	4.93±0.25[c]
3B	1.08±0.05[a*]	0.57±0.03[a*]	0.82±0.04[a*]	0.29±0.01[a*]	1.00±0.05[a*]	0.36±0.02[a*]	4.12±0.20[a*]
4B	1.43±0.08[b*]	0.65±0.05[a*]	1.01±0.05[b*]	0.35±0.05[a*]	1.05±0.05[a*]	0.43±0.02[b*]	4.92±0.25[b*]
5B	1.15±0.06[a]	0.59±0.02[a]	1.11±0.06[c]	0.47±0.03[b]	1.04±0.05[a]	0.44±0.02[b]	4.80±0.24[b]

AVNs, avenanthramides, values are expressed as mg per serving. Per serving = 50 g of cookies, on dry weight.

[a,b] Different letters indicate, in each column, statistically significant differences ($p<0.05$ one-way ANOVA) among each triplet of cookies (3A, 4A, 5A; 3B, 4B, 5B). [*]$p<0.05$ (Student's *t* test) compared to the corresponding AVN value of 3A or 4A or 5A.

Recovery of antioxidants after *in vitro* digestion

Taking into account the formulations which showed the best performance in terms of AVNs, i.e. 3B, 4B and 5B cookies, we carried out the *in vitro* gastrointestinal digestion, according to the procedure suggested by Oomen et al. (2003), in order to evaluate the bioaccessibility of polyphenols (FPs and AVNs) and their antioxidant capacity (ORAC). Bioaccessibility, i.e. the fraction of elements that is released from food matrix by the digestive juice (Versantvoort et al., 2005), allows to assess the fraction of elements that arrives into systemic circulation (bioavailability), which exerts a biological effect. The application of this *in vitro* digestion model, based on oral and gastric digestions followed by bile–pancreas solution, provides a useful alternative to animal and human models by screening food ingredients in a short time (Hur et al., 2011).

Fig. 13 shows the relative bioaccessibility (Fig. 13A), as well as the absolute bioaccessibility of FPs (Fig. 13B) and ORAC (Fig. 13C). Surprisingly, the recovery of FPs and ORAC, decreased in the following order: 3B > 4B > 5B. These results suggest that some phenol compounds, developed in the last days of malting, are lost during the transfer from the food matrix (starting formulation) to the bioaccessible fraction of the final digestion product, probably due to their instability under neutral or alkaline conditions applied in the *in vitro* digestion (Friedman and Jürgens, 2000).

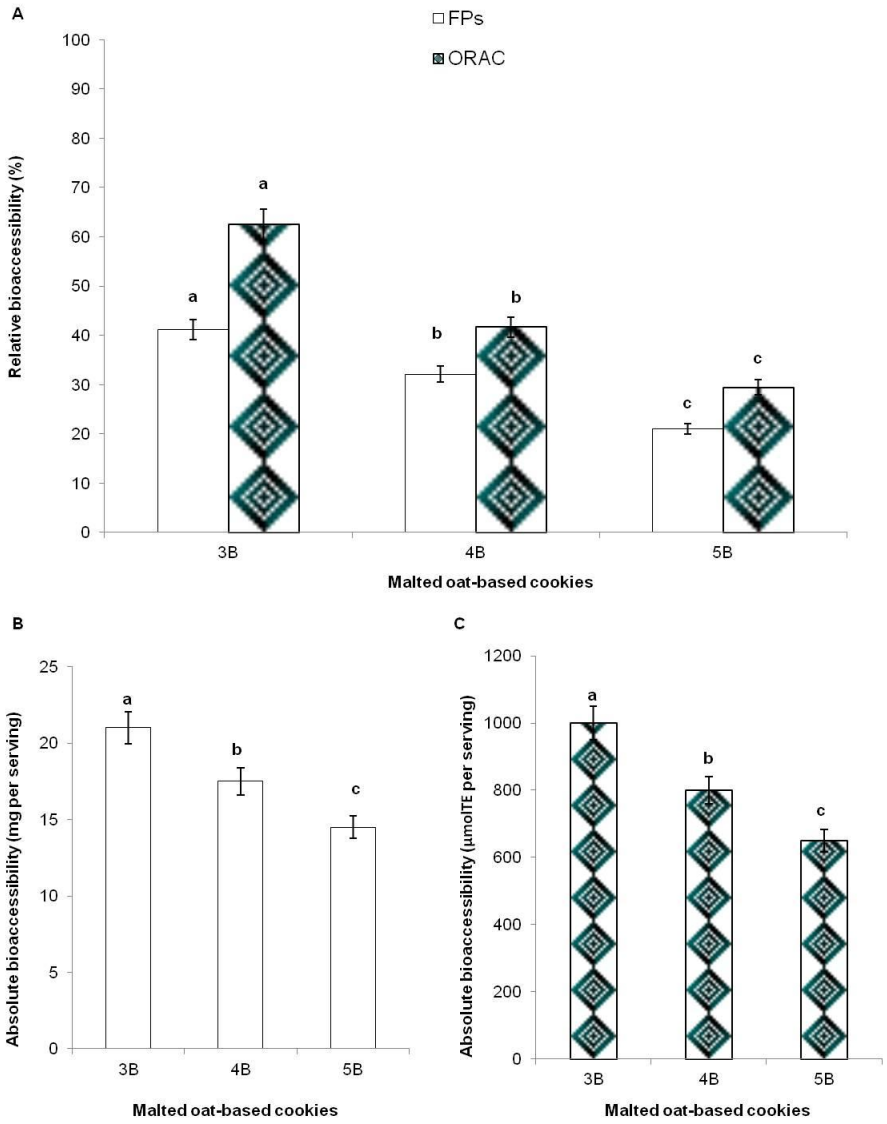


Figure 13. Absolute and relative bioaccessibility of free polyphenols (A) and their antioxidant capacity (B) from malted oat-based cookies.

Per serving = 50 g of malted oat-based cookies. [a,b] Different letters indicate, for absolute bioaccessibility, statistically significant differences among cookies ($p<0.05$ one-way ANOVA). *,† Different symbols indicate, for relative bioaccessibility, statistically significant differences among cookies ($p<0.05$ one-way ANOVA).

Subsequently, we evaluated the effect of the simulated gastrointestinal digestion on individual AVN level.

Fig. 14 shows the HPLC chromatograms of 5B cookies before and after *in vitro* digestion.

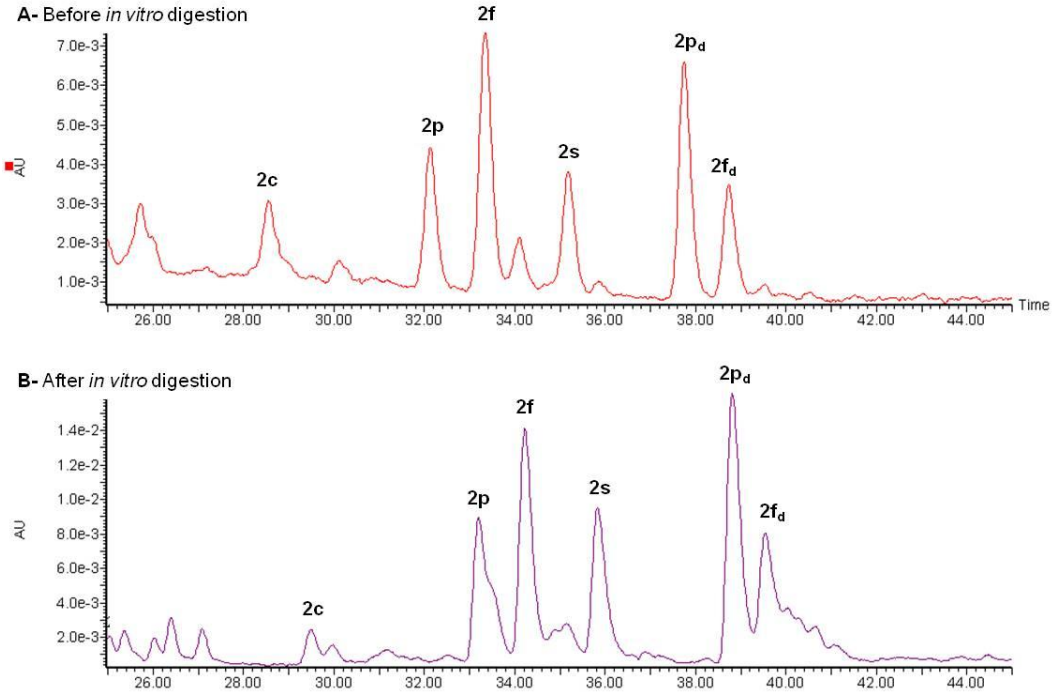


Figure 14. HPLC chromatograms of 5B malted oat-based cookies before (A) and after (B) *in vitro* digestion.

Fig. 15 shows relative (A) and absolute (B) bioaccessibilities of individual and total AVNs from malted oat-based cookies. In this case, we observed a reverse situation with respect to that found for FPs and ORAC values, as the bioaccessibility increased progressively from 3B to 5B, for each individual and total AVNs, with the exception of the 2c form. In fact, the AVN 2c did not show any significant difference among 3B, 4B, 5B formulations and was characterized by the lowest recovery, although it was the most important representative form in malted oat (Fig. 11). This result is in agreement with other authors, under similar experimental conditions (Li et al., 2016).

Indeed, from our results, the absolute bioaccessibility of 5B formulation reached the value of 1.6 mg of total AVNs considering 50 g of serving size (Fig. 15B).

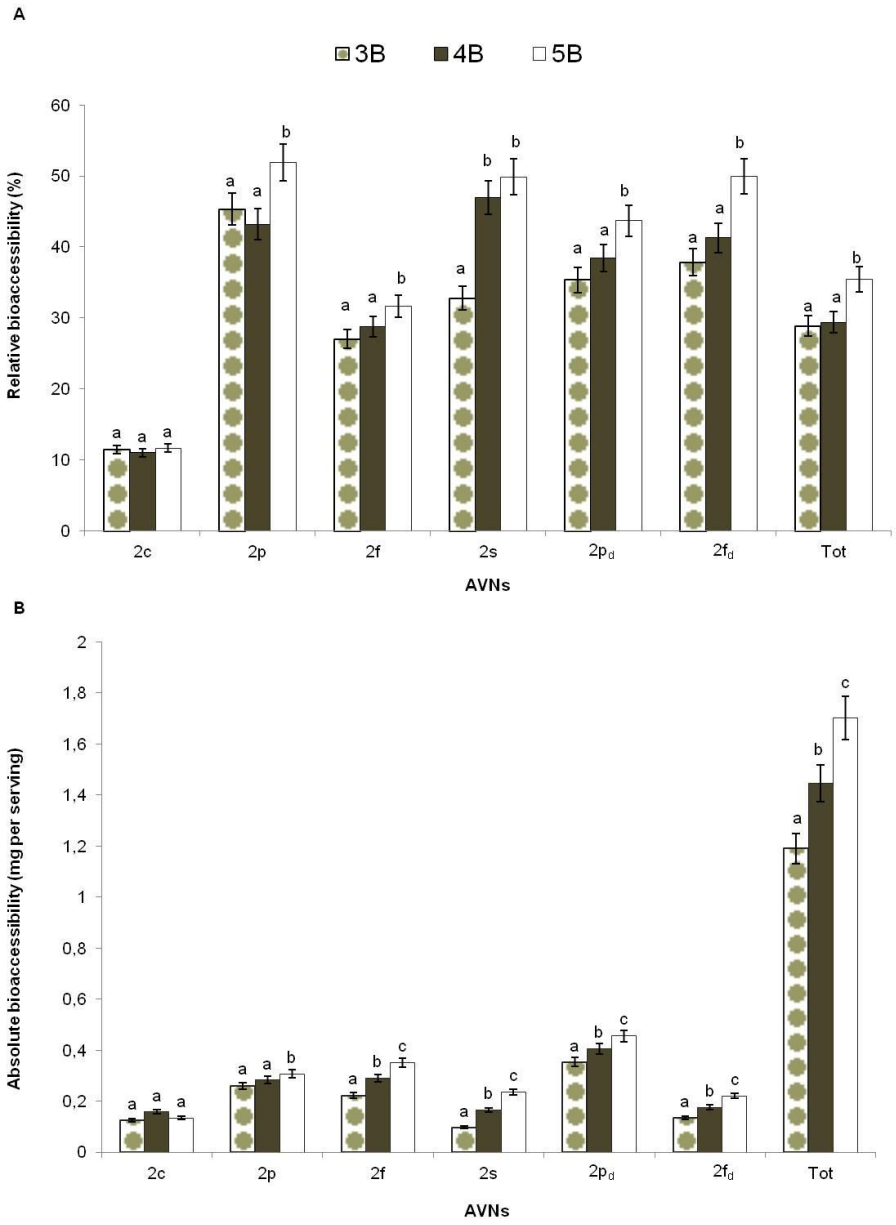


Figure 15. Relative bioaccessibility (A) and absolute bioaccessibility (B) of AVNs from malted oat-based cookies.

Per serving = 50 g of malted oat-based cookies. [a,b] Different letters indicate, for each AVN form, statistically significant differences among cookiess ($p<0.05$ one-way ANOVA).

Overall, this study highlighted that our malting procedure can be a good method to increase the AVN concentration, keeping low the germination rate of oats. Cooking does not affect the AVN level in malted-oat based cookies, thus confirming data present in the literature (Dimberg et al., 2001). The recovery of AVNs after the *in vitro* digestion could be sufficient to exploit physiological effects, as deduced from studies in humans after acute consumption of AVN enriched mixture from oats (Chen et al., 2004).

Biological effects of natural AVNs isolated from oat sprouts

To document the AVN healthy effect, a deep investigation was carried out by considering their chemo-preventive effects as anticancer and anti-inflammatory agents.

First, we evaluated the anti-proliferative activity of AVNs in their natural forms, extracted from oat sprouts, on normal cells (NCTC 2544), as well as on colon cancer (CaCo-2) and hepatocarcinoma (HepG2) cell lines.

To extract AVN from oat sprouts, we set up a purification protocol able to obtain the AVN 2f, at 95% purity level (Fig. 16A) and a mixture of AVNs**,** constituted by the three main forms (2c, 2p, 2f), at 80% purity, and other minor AVN forms (peaks 1-4), accounting for the remaining 20% (Fig. 16B). In the presentation of the following results, we used the label **n-2f** for the natural AVN 2f and **n-MIX** for the natural AVN mixture (2c, 2p, 2f).

The protocol used for the chromatographic purification of AVNs was reproducible in several repeated experiments and gave a profile which slightly differs from those of other authors (Liu et al., 2004).

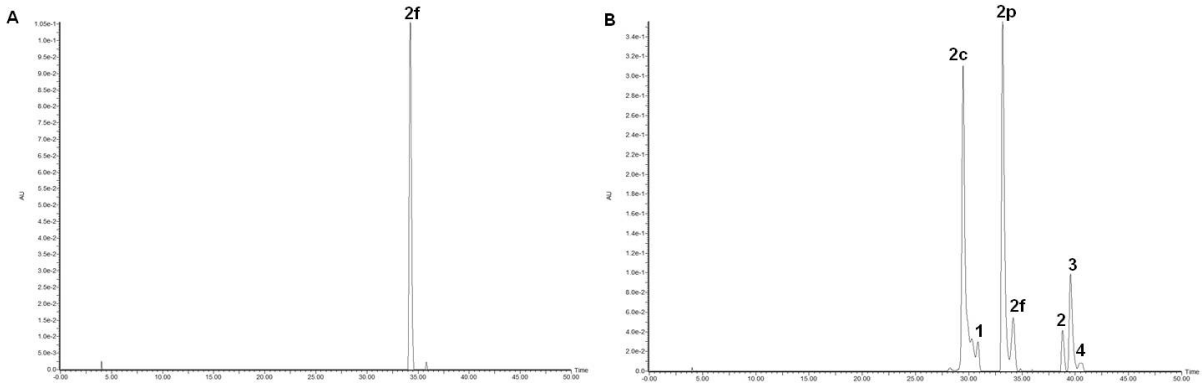


Figure 16. HPLC chromatogram of pure AVNs. (A) n-2f contains the AVN 2f at 95% purity level; (B) n-MIX contains a mixture of AVNs 2c, 2p, 2f at 37:35:8 % purity level.

Studies on normal human cells allowed us to choose the concentration range of n-2f and n-MIX to be used in the dose response experiments of anti-proliferative activity. As no cytotoxic effect was detected by us when AVNs were used in the range 0-120 µM on normal NCTC 2544 cells (Fig. 17), we planned to evaluate anti-proliferative effects on CaCo-2 and HepG2 cancer cells in this concentration range. As AVNs are absorbed in the gut and reach the liver through the entero-hepatic recirculation (Chen et al., 2007), the study of their cytotoxicity on colon cancer and hepatocarcinoma cells is justified by a pathophysiological point of view. In fact, colorectal

carcinoma and hepatocarcinoma are linked, because the colorectal cancer is able to invade the primary tissue and generate liver metastases (Ferlay et al., 2007). Therefore a drug, which shows remarkable activity against both cancer types, is relevant for chemoprevention.

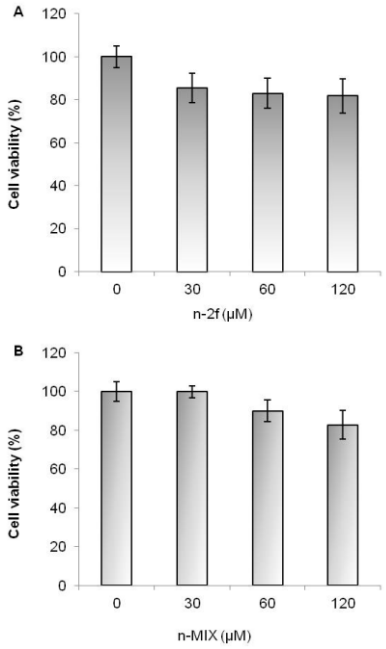


Figure 17. Effects of n-2f (A) and n-MIX (B) on NCTC 2544 normal human keratinocytes, after 48h treatment.

Data were expressed as percentage of cell viability (%) relative to the control, represented by corresponding untreated cells. Two replicate experiments with three samples analyzed for each replicate (n=6) were performed.

In order to test the anticancer effect of AVNs, we performed the cytotoxicity test of n-2f and n-MIX, on CaCo-2 and HepG2 cells, using the SRB assay. The hepatocarcinoma HepG2 cells were never tested for the AVN anticancer effect. Time courses of 24, 48 and 72h were evaluated, but the maximum antiproliferative effect was observed at 48h, so we measured the IC_{50} values at this time (Table 8).

The n-MIX was more cytotoxic than n-2f in HepG2 cancer cells, as indicated by its lower IC_{50} value, whereas in CaCo-2 cells they showed similar IC_{50} values (Table 8). Moreover, the IC_{50} value of n-MIX in the HepG2 was lower than the corresponding IC_{50} value in CaCo-2 cells. On the contrary, the IC_{50} value of n-2f in the HepG2 was higher than the corresponding IC_{50} value in CaCo-2 cells.

Table 8. IC_{50} cytotoxicity values of n-2f and n-MIX on CaCo-2 and HepG2 cancer cell lines.

	IC_{50} values at 48 h treatment	
	n-2f	**n-MIX**
CaCo-2	126.5 ± 12.5^{a}	114.6 ± 5.5^{a}
HepG2	$182.7 \pm 18.1^{a**}$	$39.9 \pm 4.1^{b***}$

Two replicate experiments with four samples analyzed for each replicate (n = 8) were performed; a,b Different letters in the same row indicate statistically significant differences among the IC_{50} values of AVNs ($p<0.05$, one-way ANOVA). $^{**}p<0.01$; $^{***}p<0.001$ compared to the corresponding IC_{50} value in CaCo-2 cells.

We further investigated if AVNs exploit their anti-tumor activities by targeting apoptosis pathways (Li-Weber, 2013; Wang et al., 2012). Apoptosis is a specific mechanism of cell death, which regulates tissue homeostasis, through the elimination of potentially deleterious cells (Holdenrieder and Stieber, 2004) and is controlled by two pathways: the intrinsic and extrinsic pathway. The former is regulated by the initiator caspase 9, leading to activation of executioner caspase 3; the latter is triggered by the interaction of the death ligand with its death receptor, leading to activation of initiator caspase 8 and then of caspase 3 (Samali and Jager, 2014).

The ability of AVNs to activate both the initiator caspases 8 and 9 and the effector caspase 3 was investigated by us through the direct assay of the caspases at 48h, in CaCo-2 and HepG2 cells.

Our results demonstrated that AVNs were able to activate the extrinsic apoptotic pathway, through the increase in caspase 3 and 8 activity levels, with the n-MIX showing a stronger pro-apoptotic effect than n-2f (Fig. 18).

The differences in the response to the cytotoxic and pro-apoptotic effects in the two cancer cell lines could be linked to the different genetic background of CaCo-2 and HepG2 cells. In fact, CaCo-2 colon cancer cells are characterized by a mutated TP53 gene and overexpression of anti-apoptotic factors, which confer resistance to anticancer drugs (Piccirillo et al., 2009). HepG2 liver cancer cells are characterized by a wild type TP53 gene and mutations in the tumor suppressor gene Cyclin-Dependent Kinase inhibitor 2A (Heo et al., 2015), which confer high resistance to apoptosis induction (Ye and Chen, 2016). However, the discrepancy in the cytotoxic and pro-apoptotic effects could be linked to the intracellular antioxidant capacity of n-MIX when compared to AVN 2f (Chen et al., 2007).

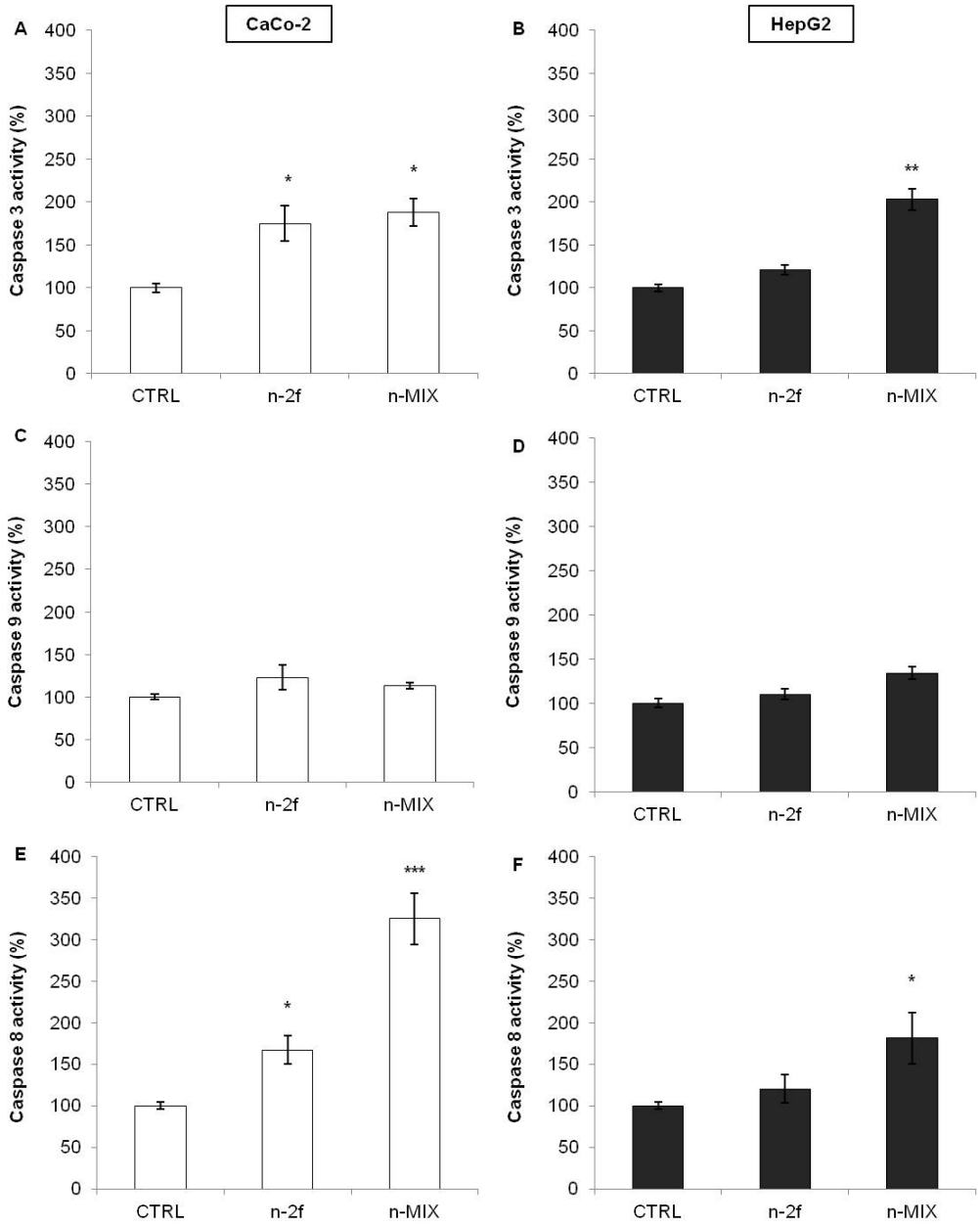


Figure 18. Evaluation of caspase 3, caspase 9 and caspase 8 activity levels, induced by n-2f and n-MIX in CaCo-2 (A, C, E, respectively) and HepG2 (B, D, F, respectively) cancer cells.

Cells were untreated (CTRL), or treated for 48h with n-2f (120 μM), or n-MIX (120 μM). Data were expressed as % caspase activity levels. Three replicate experiments with two samples analyzed for each replicate (n=6) were performed. *p<0.05, **p<0.01, ***p<0.001

To have an indication of the intracellular antioxidant capacity of AVNs through the cell membranes, we tested their cellular antioxidant capacity (CAA), through the DCFH-DA assay, by evaluating the ability of n-2f and n-MIX to modulate the production of exogenous ROS, generated by H_2O_2. CaCo-2 and HepG2 cells were treated for 24h with the lowest concentration of n-2f and n-MIX able to induce a significant anti-proliferative effect on those cancer cells (Fig. 19).

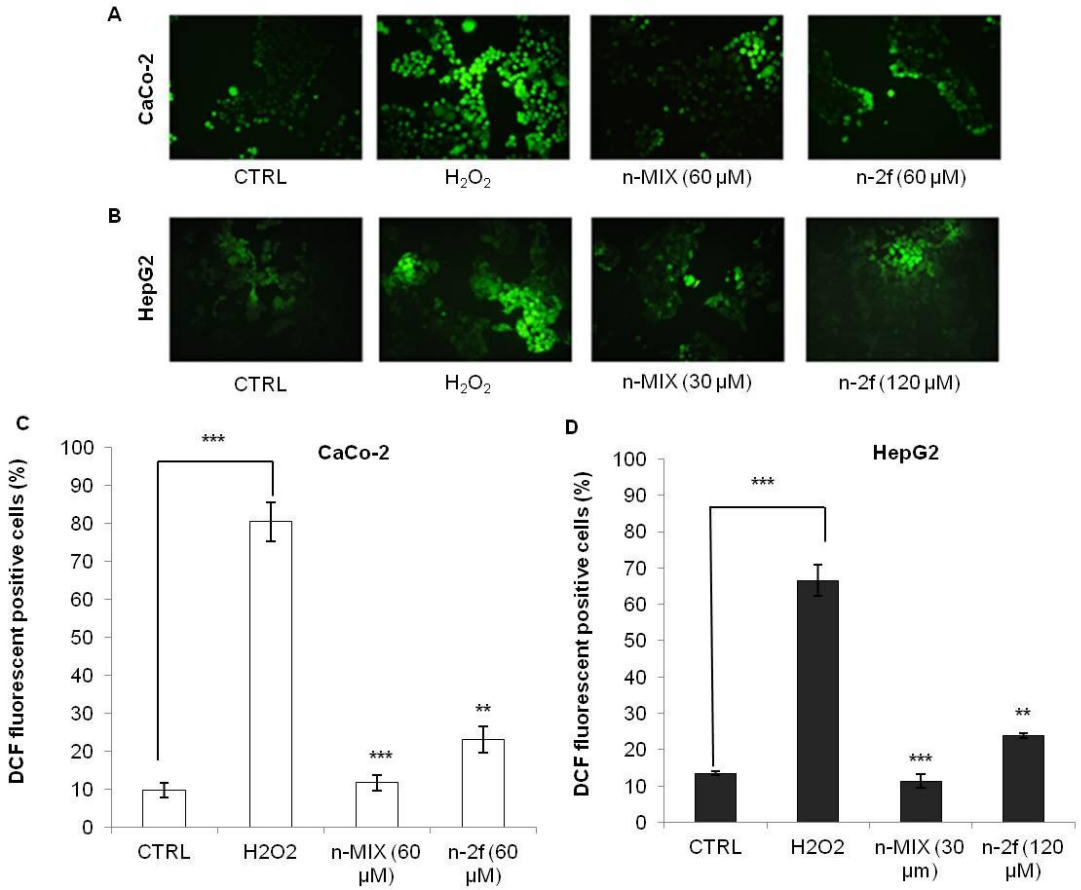


Figure 19. Representative images of DCFH-DA assay in CaCo-2 (A) and in HepG2 (B) cells treated with phytochemicals before H2O2 treatment.

CTRL, untreated cells. H_2O_2, cells treated with 1 mM H_2O_2 for 1 h. (A, C) n-MIX (60 µM), cells treated with 60 µM n-MIX for 24h and then with 1 mM H_2O_2 for 1 h; n-2f (60 µM), cells treated with 60 µM n-2f for 24h and then with 1 mM H_2O_2 for 1 h. (B, D) n-MIX (30 µM), cells treated with 30 µM n-MIX for 24h and then with 1 mM H_2O_2 for 1 h; n-2f (120 µM), cells treated with 120 µM n-2f for 24h and then with 1 mM H_2O_2 for 1 h.

Three fields per sample were analyzed and each time at least 100 cells were counted; three independent experiments were performed for both CaCo-2 and HepG2 cancer cells (n=9) and results were expressed as % DCF fluorescent positive cells versus control; **p<0.01, ***p<0.001

Our results indicated that AVNs behave as ROS suppressor with the n-MIX showing a stronger CAA than n-2f in both CaCo-2 (Fig. 19A, 19C) and HepG2 (Fig. 19B, 19D) cells, remarkably reducing the number of green fluorescent cells, after treatment with H_2O_2. Therefore, the stronger pro-apoptotic effect of n-MIX could be linked to the higher membrane permeability of AVN 2p and 2c present in the n-MIX than the AVN 2f.

Although the role of antioxidant molecules, which detoxify ROS, has been controversial in the field of cancer research because of the dichotomous ability of certain antioxidants to promote or suppress ROS, our data are in line with the ascertained effect that the tumor growth and metastasis can be reversed by decreasing the ROS concentration below the level needed to sustain the proliferative capacity of specific cancer cells (Zhang et al., 2002). In other words, at low concentrations, ROS help cancer cell proliferation, but at higher level they hampers the proliferation.

Indeed, we further investigated whether the apoptotic program is controlled by other important factors, in the protein signaling network, which control the expression of genes involved in the pro-survival mechanisms, like Hypoxia inducible factor (HIF1A) and Vascular Endothelial Growth Factor (VEGFA) (Hanahan and Weinberg, 2011). In particular, HIF1A plays an oncogenic role in colorectal cancer, since its overexpression has been associated with higher mortality rate and shorter survival (Baba et al., 2010), whereas VEGFA is the principal regulator of angiogenesis during tumor growth (Ellis et al., 2000; Ferrara, 2005). The upregulation of HIF1A and VEGFA is functional to provide more oxygen and increase reduced glutathione (GSH) to the cancer cells for their adaptation to the oxidative stress (Gorrini et al., 2013; Harris and Brugge, 2015).

Through RTqPCR assays, we assessed whether n-MIX was able to reduce the expression levels of the pro-survival genes HIF1A and VEGFA in CaCo-2 and HepG2 cancer cells (Fig. 20). We choose to test n-MIX only due to its lower IC_{50} value, to the greater induction in caspase 8 and caspase 3 activity levels and to the higher CAA exerted in both cancer cell lines, when compared to n-2f.

Our data showed that n-MIX remarkably reduced the expression levels of HIF1A and VEGFA, in both cancer cell lines (Fig. 20), thus confirming data obtained with other flavonoids (Huang et al., 2015).

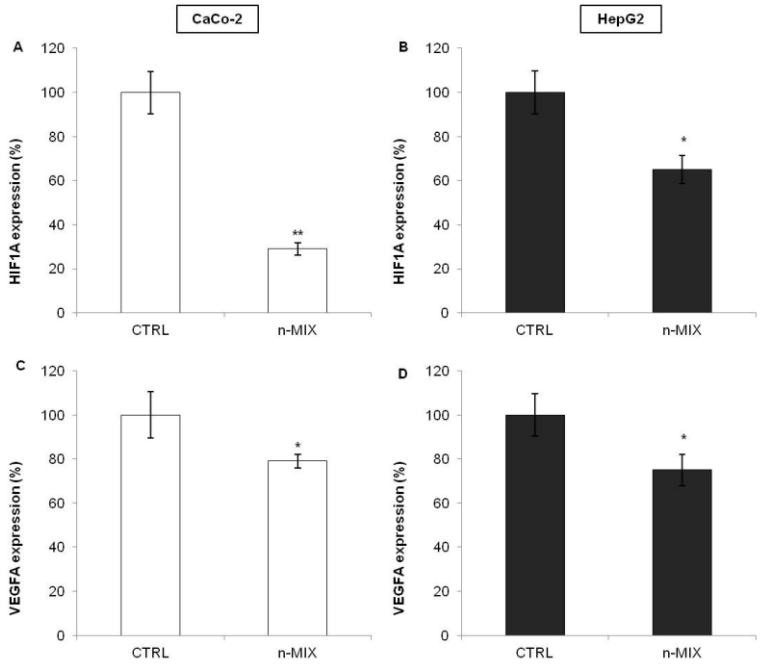


Figure 20. Evaluation, through RTqPCR assay, of the effects of n-MIX on HIF1A and VEGFA expression levels in CaCo-2 (A, C) and HepG2 (B, D) cancer cells. HIF1A and VEGFA mRNA levels were measured and normalized to the mRNA levels of the housekeeping gene β-actin.

Results obtained from untreated cells (CTRL) and from CaCo-2 and HepG2 cells treated with n-MIX (120 μM) for 24h, are shown. Three replicate experiments with three samples analyzed for each replicate (n=9) were performed. *$p<0.05$, **$p<0.01$.

Biological effects of synthetic AVNs

As a consequence of these evidences, we considered of importance to synthesize the three main AVNs in order to explore which form exploits greater biological effects, in comparison with the AVN mixture purified from oat sprouts.

The synthesis of s-2c, s-2f, s-2p was performed in our laboratory following a published protocol (Günther-Jordanland et al., 2016; Wise, 2011) with few modifications (Fig. 21). In particular, with respect to Wise (2011), we used the PyBOP instead of BOP as capling reagent, the ammonium acetate in methanol-water instead of pyrrolidine in dichloromethane for the final deprotection.

The molecules were characterized by HPLC-MS and NMR analysis (see Appendix, NMR spectra) and the yield was 45% in all synthetic processes, with respect to 40% of Wise (2011).

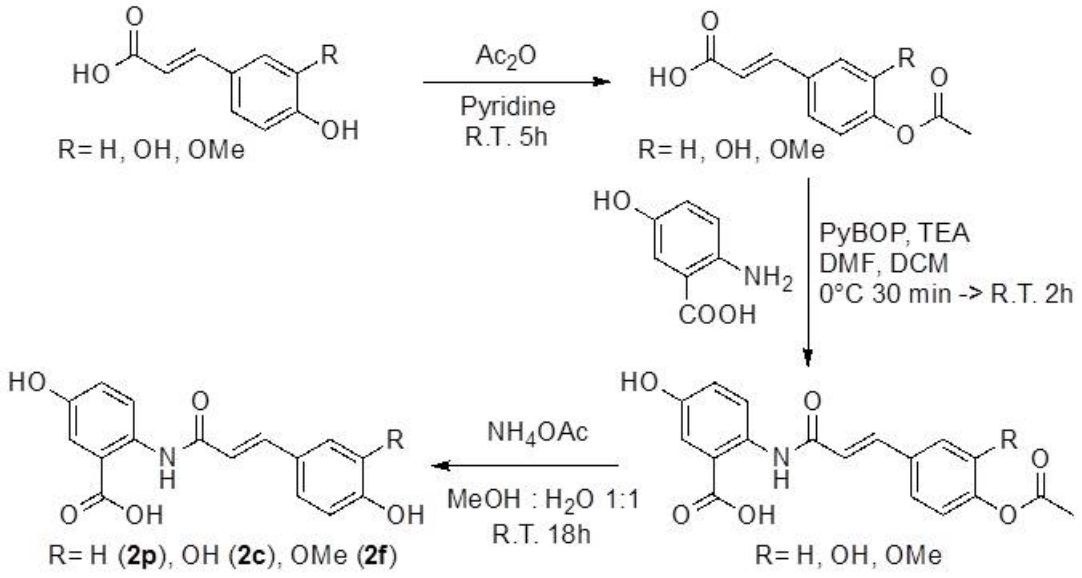


Figure 21. Scheme of the reactions occurring in the AVN syntheses.

AC_2O, acetic anyhidride; DCM, diclorometano; DMF, dimetilformammide; NH_4OAc, ammonium acetate; PyBOP, (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; TEA, Trietilammina

Antioxidant activity of synthetic AVNs

The chemical characterization of synthetic AVNs was also performed by measuring the antioxidant activities by means of three different methods: DPPH, ABTS, ORAC.

The DPPH assay gave similar results to the ABTS assay with AVN s-2c showing the highest antioxidant activity, followed by the s-2f and then by the s-2p (Table 9). Regarding the ORAC method, the s-2c was the most active antioxidant against the peroxyl radical, followed by the s-2p and s-2f, thus confirming data reported in the literature (Yang et al., 2014).

Table 9. Antioxidant activities of synthetic AVNs determined by three different tests.

	DPPH†	**ABTS**‡	**ORAC**††
s-2c	80.67±1.29[a]	50.79±2.54[a]	30.05±0.20[a]
s-2p	12.19±0.91[c]	14.17±2.16[c]	28.28±0.42[b]
s-2f	46.62±2.33[b]	45.17±3.73[b]	19.33±0.16[c]

† Percent (%) inhibition of DPPH radical calculated after 10 min incubation of 50 µM AVN solution with DPPH radical solution; values are the mean ± SD of three independent determinations. ‡ Percent (%) inhibition of ABTS radical calculated after 4 min incubation of 10 µM AVN solution with ABTS radical solution; values are the mean ± SD of three independent determinations. †† ORAC values are expressed as mmolTE/g and are the mean ± SD of six independent measures on 20 µM solution. a,c Different letters, in the same column, indicate statistically significant differences among AVNs (one-way ANOVA, $p<0.05$).

The s-2c was the most potent antioxidant by means of the three assays, due to the presence of the two hydroxyl groups in the cinnamic acid part of the molecule, which has the major influence on reactivity (Bratt et al., 2003).

The inversion of the order between the s-2f and s-2p in the DPPH and ABTS assays, with respect to the ORAC, could be due to the different mechanisms of action of the test. In fact, in the ORAC assay, the peroxyl radical, is quickly reduced by hydrogen atom transfer reaction, brought to completion (Prior, 2015).

The DPPH and ABTS radicals are long lived hydrogen radicals, which react slowly with many antioxidants, with a mechanism based on electron transfer reaction (Huang et al., 2005). In both assays (DPPH and ABTS), the presence of the methoxyl group makes more favorable the reducing activity of the AVN s-2f with respect to the s-2p. Indeed, as suggested by others (Bratt et al., 2003), the production of new compounds able to donate additional hydrogen atoms may occur during the course of the assay, thus contributing to favor the AVN s-2f with respect to the s-2p.

Cellular antioxidant activity of synthetic AVNs

To evaluate the ability of synthetic AVNs to modulate the production of exogenous ROS, generated by H_2O_2, and compare their ability with those of n-MIX, we performed the DCFH-DA assay using the n-MIX and synthetic AVNs in CaCo-2 cells.

The assay showed that s-2c, s-2f and s-2p were all able to enter the cancer cells and deplete H_2O_2-mediated oxidative stress (Fig. 22). Quantitative data analysis did not reveal any difference between the n-MIX and s-2c, s-2f, s-2p after 24h treatment (Fig. 22).

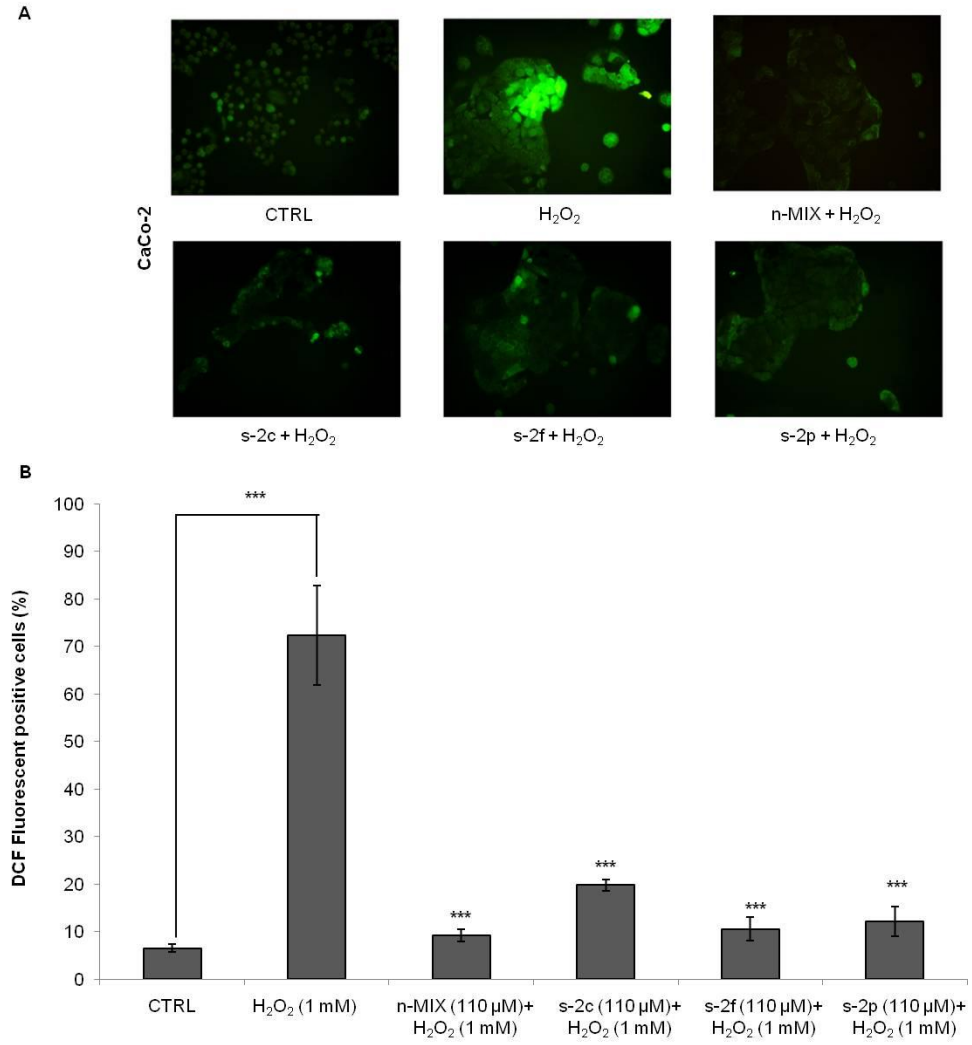


Figure 22. Representative images of DCFH-DA assay in CaCo-2 cells (A) treated with natural and synthetic AVNs before H2O2 treatment.

CTRL, untreated cells; H_2O_2, cells treated with 1 mM H_2O_2 for 1h. AVN treatment: Cells were treated with 110 µM of n-MIX or s-2c or s-2f or s-2p for 24h and then with 1 mM H_2O_2 for 1h (B).

Three fields per sample were analyzed and each time at least 100 cells were counted; two independent experiments were performed (n = 6) and results were expressed as % DCF fluorescent positive cells versus control; ***$p < 0.001$.

Anti-proliferative activity of synthetic AVNs

The cytotoxic effects of the synthetically prepared AVN forms (s-2c, s-2f, s-2p) and the n-MIX (purified from oat sprouts) was evaluated on CaCo-2 and Hep3B cancer cell lines at 24h. A synthetic AVN mix (s-MIX) was set up in order to simulate the relative percentage of AVN forms found in the natural AVN mix, i.e. 35% 2c, 37% 2p, 8% 2f.

CaCo-2 cells are characterized by a mutated TP53 gene and overexpression of anti-apoptotic factors (Piccirillo et al., 2009), while Hep3B cells produce an inactive p53 protein and show a high resistance to conventional anticancer drugs (Gambari et al., 2014). The 24h treatment was sufficient to detect the IC_{50} values, as the extrinsic pathway of apoptosis occurs in a short time, comprised in the range 6-24h (Lin et al., 2004).

In CaCo-2 cancer cells, the s-2c was found to be more cytotoxic than s-2f and s-2p, as indicated by its lower IC_{50} value; in Hep3B cells, all the synthetic AVNs showed similar cytotoxic effects (Table 10). Moreover, the IC_{50} values of the s-MIX were not significantly different from the IC_{50} values of the n-MIX both in CaCo-2 ($p=0.36$) and Hep3B ($p=0.84$) cancer cell lines (Table 10). This shows that the antiproliferative effect of the n-MIX was induced by the main forms, i.e. AVN 2c, 2f and 2p, with a negligible contribution from the minor AVN forms.

The IC_{50} values of all AVN forms in the Hep3B were lower than the corresponding IC_{50} values in CaCo-2 cells (Table 10).

Table 10. IC_{50} cytotoxicity values of n-MIX and synthetic AVN on CaCo-2 and Hep3B cancer cell lines.

	s-2c	**s-2p**	**s-2f**	**s-MIX**	**n-MIX**
CaCo-2	110 ± 6^{a}	230 ± 16^{c}	216 ± 17^{c}	148 ± 15^{b}	159 ± 11^{b}
Hep3B	$128\pm9^{a*}$	$137\pm10^{a***}$	$129\pm9^{a***}$	$120\pm14^{a**}$	$118\pm8^{a**}$

Two replicate experiments with four samples analyzed for each replicate (n=8) were performed; ^{a,b} Different letters in the same row indicate statistically significant differences among the IC_{50} values of AVNs ($p<0.05$, one-way ANOVA). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ compared to the corresponding IC_{50} value in CaCo-2 cells.

Differences in the genetic background and efficiency of anti-apoptotic mechanisms between the two cancer cell lines probably account for this discrepancy (Gambari et al., 2014; Liu et al., 2014). Hep3B cells, which possess a strong genetic resistance to anticancer agents (Gambari et al., 2014), were found to be very sensitive to the anti-proliferative effects induced by the AVNs.

Therefore, molecules such as AVNs, are of great value, especially when they selectively target cancer cells with no damage to normal human cells.

A deep investigation was performed on the pro-apoptotic effect of synthetic AVNs in comparison with the natural AVN mixture (n-MIX).
Previously, we showed that the n-MIX was able to activate the extrinsic apoptotic pathway, which is regulated by initiator caspase 8 and effector caspase 3 (Samali and Jager, 2014). As caspase 2 can cooperate with caspase 8 to increase the activity levels of caspase 3 (Lin et al., 2004), we evaluated the ability of the n-MIX and the synthetic AVNs to activate caspases 3, 8 and 2 after 24h treatments, on the basis of a previous report (Berger et al., 2011).

Table 11 shows that the n-MIX and the individual synthetic AVNs induced a significant increase in the activity levels of caspases 3 and 8 in both CaCo-2 and Hep3B cancer cells.
In addition, the n-MIX and s-2c treatments were able to significantly activate the caspase 2 in both CaCo-2 and Hep3B cells (Table 11).
The caspase 2 activation can be explained by the interaction of the phytochemical with the intracellular network, consisting of the proteins of the PIDDosome, the molecular complex that leads to the maturation and activation of caspase 2 (Puccini et al., 2013).

Table 11. Caspase 3, 8 and 2 activity levels, after 24h AVN treatments, on CaCo-2 and Hep3B cancer cells

	CTRL	**n-MIX**	**s-2c**	**s-2p**	**s-2f**
CaCo-2					
Caspase 3	100±5	170±12^{b*}	237±36^{a**}	138±13^{c*}	140±4^{c*}
Caspase 8	100±3	199±6^{b**}	218±10^{a**}	145±5^{c*}	140±17^{c*}
Caspase 2	100±4	137±15^{b*}	160±10^{a*}	128±4^{b}	114±2^{c}
Hep3B					
Caspase 3	100±4	235±39^{a**}	219±27^{a**}	230±39^{a**}	244±45^{a**}
Caspase 8	100±5	230±45^{a**}	245±40^{a**}	267±28^{a**}	276±13^{a**}
Caspase 2	100±3	130±9^{a*}	140±2^{a*}	124±6^{ab}	121±2^{ab}

The 110 μM AVN concentration, i.e. the lowest IC_{50} value after 24h of treatment, was chosen to test the pro-apoptotic effects of s-2c, s-2p, s-2f and the n-MIX. Two replicate experiments with two samples analyzed for each replicate (n=4) were performed. ^{a,d} Different letters in the same row indicate statistically significant differences among the % of caspase activity level induced by the different AVNs ($p<0.05$, one-way ANOVA). *$p<0.05$ compared to CTRL; **$p<0.01$ compared to CTRL.

Anti-inflammatory activity of the s-2c versus the n-MIX

The activation of the network of caspases 2, 8 and 3 is only one aspect accounting for the cytotoxicity of the synthetic and natural AVNs in the two cancer cell lines.

In fact, the pro-apoptotic effect of the AVNs is also boosted by the downregulation of several pro-survival markers, as well as by factors linked to the tumor microenvironment (Hanahan and Weinberg, 2011).

For example, the survival and over-proliferation of cancer cells could also be linked to the cyclooxygenase (COX) isoforms, that catalyze the production of inflammatory prostaglandins from arachidonic acid. Constitutively expressed COX-1 maintains the homeostatic level of prostaglandins, whereas inducible COX-2 is highly expressed in many types of solid cancers and contributes to tumor growth and invasiveness (Smith et al., 2000; Wang and DuBois, 2010) via inhibition of apoptosis and augmentation of angiogenesis (Zha et al., 2004).

Therefore, in our study we investigated the anti-inflammatory effect of s-2c and n-MIX by measuring the COX-2 expression levels on CaCo-2 and Hep3B cancer cell lines (Fig. 23). We choose to test the s-2c only, due to its lower IC_{50} value and to the greater induction in caspase 8, caspase 3 and caspase 2 activity levels, when compared to s-2f and s-2p.

Our data indicated that both the n-MIX and s-2c were able to reduce COX-2 mRNA levels in CaCo-2 cancer cells (Fig. 23A), whereas only the n-MIX was able to downregulate COX-2 expression in Hep3B cells (Fig. 23B).

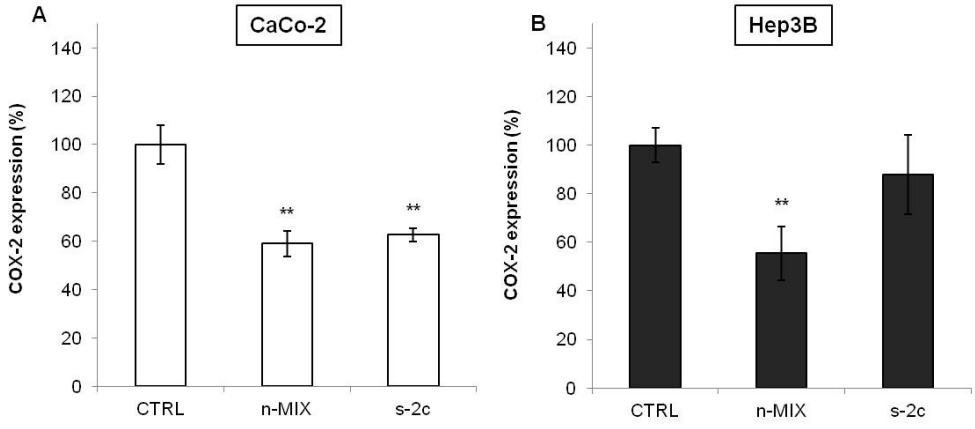


Figure 23. Evaluation of the effects of n-MIX and s-2c on COX-2 expression levels in CaCo-2 (A) and Hep3B (B) cancer cells.

The COX-2 mRNA levels were measured and normalized to the mRNA levels of the housekeeping gene β-actin. Results obtained from untreated cells (CTRL) and from CaCo-2 and Hep3B cells treated with n-MIX (110 µM) or s-2c (110 µM) for 24h are shown. Two replicate experiments with three samples analyzed for each replicate (n = 6) were performed. $^{**}p < 0.01$.

In the light of previous findings (Sun et al., 2017), we evaluated the AVN-mediated modulation of the activity levels of COX-2 (Fig. 24). COX-2 isoform accounted for 92.0±4.2 % and 94.5±1.5 % of the COX activity in CaCo-2 and Hep3B cells, respectively. The n-MIX and s-2c form were able to reduce COX-2 activity by 50.1±10.3 % and 19.5±4.9 %, respectively (Fig. 24A), whereas in Hep3B cells, the n-MIX reduced COX-2 activity by 27.0±5.5 %, while s-2c did not induce any significant activity inhibition (Fig. 24B).

Elevated COX-2 expression and activity levels are correlated with a poor response to therapy in several forms of cancer, including adenocarcinomas and hepatocarcinomas (Diab et al., 2015). Hence, the reduction of COX-2 activity levels in CaCo-2 and Hep3B cancer cells, induced by the n-MIX, points to the potential role of AVNs as effective chemopreventive agents.

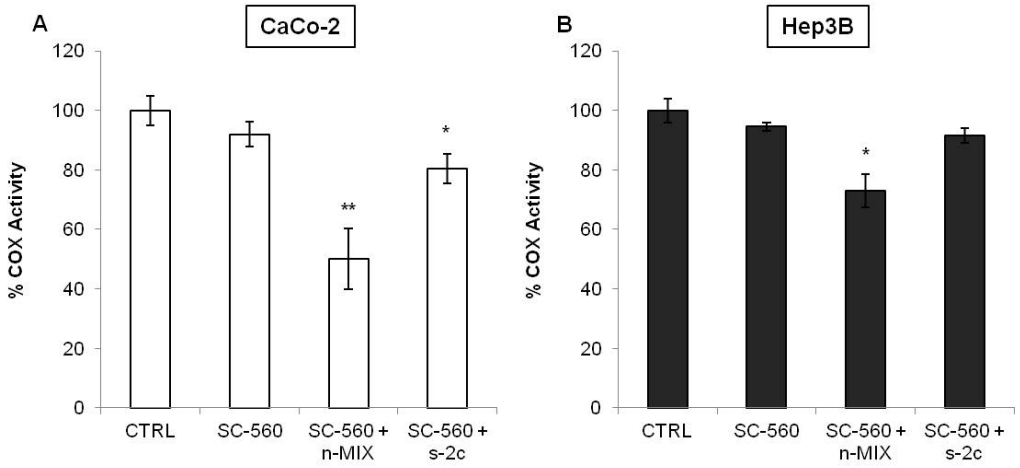


Figure 24. Evaluation of COX activity levels, modulated by n-MIX and s-2c in the cytosols extracted from CaCo-2 (A) and from Hep3B (B) cancer cells.

Cytosols were untreated (CTRL), incubated with COX-1 inhibitor SC-560 alone, with COX-1 inhibitor and 110 µM n-MIX or with COX-1 inhibitor and 110 µM s-2c for 10 min at RT. Three replicate experiments (n = 3) were performed. *$p < 0.05$, **$p < 0.01$.

In conclusion, the synthetic AVNs 2p, 2f and 2c and the natural AVN mixture all possess pleiotropic anticancer properties, but showed variable levels of effectiveness in the two neoplastic cell lines under study, which are characterized by different histopathologies.

The n-MIX appeared to be the strongest anticancer cocktail, while among the synthetic AVNs, s-2c was the most effective compound.

Individual synthesized AVNs pave the way for further investigations to assess various mixtures of AVNs, changing the relative proportions of the three AVN forms to obtain a complete characterization of their molecular targets.

A mixture of synthetic AVNs, similar to that which is found in oats, can be more effective in chemoprevention than a single AVN.

The anti-inflammatory effect of AVNs (Meydani, 2009) was confirmed by us through the ability of AVNs to both inhibit COX-2 enzyme activity and reduce its mRNA levels.

Our *in vitro* study creates the basis for their possible use for chemoprevention against the insurgence of both colon and liver cancers also *in vivo*.

Important outcomes of the thesis

The first outcome of this study was given by our ability to isolate natural AVNs from selected oat cultivars. Our purification protocol has been improved by us and it was reliable and reproducible and it can be scaled up to obtain gram quantities of the molecules.

The second outcome was the ability to synthesize AVNs with a remarkable recovery and purity. This allowed the third important outcome, i.e. the demonstration of the chemopreventive effect of both natural and synthetic AVN mixture, in comparison with individual synthetic molecules. The chemiopreventive activity of the AVN mixture resulted higher than the individual AVNs, a frequent but not obvious effect which is linked to the synergy of molecules in their biological effects.

The fourth outcome regarded the malting conditions which were satisfactory, as they brought the AVN level to ten-fold higher values than the basal level. However, the applied conditions can be ameliorated by searching the finest times and temperatures ranges.

The fifth outcome regarded the demonstration that from dried malted oats we obtained a flour with a good taste and suitability for being mixed with wheat flours and other ingredients, without any loss of AVNs in the final products.

Finally, the digestion of the bakery, in a simulated *in vitro* process, showed the bio-accessibility of AVNs which can be absorbed through the enterocytes with a percentage higher than 30%, a concentration able to exploit preventive effects against diseases in humans.

Concluding remarks and future research

This thesis demonstrated the importance to select oat genotypes, as well as storage conditions and processing, in order to save nutrients, particularly AVNs.

We referred to the AVN concentration of few oat genotypes, but there are thousands of cultivars, which have never been explored. Most of them are seeded on large scale and merit to be investigated by applying the procedures presented in this study.

Regarding the malting process, it was done following the standard procedure for brewing, but the protocol can be improved by standardizing times and temperatures and adapting them to specific oat cultivars.

Indeed, cleaning and kilning procedures, which regulate the production of Maillard compounds, should be ameliorated.

The isolation of AVNs from malted oat can be improved by scaling up, possibly by utilizing batch wise methods, which allows the production of higher amounts of concentrated food grade AVN mixture to be used for the preparation of fortified foods and for *in vivo* studies.

The chemopreventive effects of AVNs must be more deeply studied in the anti-itching effects, which have not been explored in the present thesis, but it is considered relevant in the pharmacological activity of oat-derived products.

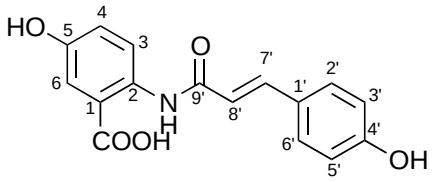
The role of the tricine (4,5,7-trihydroxil-3'-5' dimeytoxiflavone), the anti-inflammatory and anticancer agent present in oat, must be studied in combination with AVNs, due to its significant contribute to the biological activity of oat-derived products (Verschoyle et al., 2006).

The formulation of our functional cookies may be ameliorated with the addition of technological adjuvants, as well as with an optimal ratio of oat and wheat flours. The use of pseudocereals, like buckwheat, amaranth or quinoa, together with oat flour could be the right solution to produce functional foods for celiac patients and patients with intestinal inflammatory diseases.

The formulation of foods containing natural molecules, able to counteract the disease insurgence, is positively accepted by the community. Therefore, on the basis of our results, innovative foods fortified with AVNs can be developed to be used for chemoprevention and treatment of chronic inflammatory diseases.

Appendix

NMR Spectra compound 2p



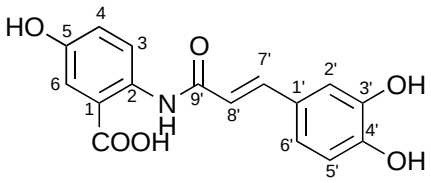
^{1}H NMR (400 MHz, CD_3OD) δ 6.52 (d, *J* = 15.6 Hz, 1H), 6.82 (d, *J* = 8.6 Hz, 2H), 7.02 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.48 (d, *J* = 8.6 Hz, 2H), 7.51 (d, *J* = 3.0 Hz, 1H), 7.57 (d, *J* = 15.6 Hz, 1H), 8.45 (d, *J* = 9.0 Hz, 1H).

Hydrogen	**^{1}H from Wise (2011) 400 MHz in CD_3OD**	**^{1}H from Günther-Jordanland (2016) 500 MHz in CD_3OD**	**Our Proton ^{1}H 400 MHz in CD_3OD**
H-8’	6.53 (d, *J* = 15.6 Hz, 1H)	6.53 (d, *J* = 15.6 Hz, 1H)	6.52 (d, *J* = 15.6 Hz, 1H)
H-3’,5’	6.82 (d, *J* = 8.6 Hz, 2H)	6.82 (d, *J* = 8.6 Hz, 2H)	6.82 (d, *J* = 8.6 Hz, 2H)
H-4	7.03 (dd, *J* = 9.0, 3.0 Hz, 1H)	6.98 (dd, *J* = 9.0, 3.0 Hz, 1H)	7.02 (dd, *J* = 9.0, 3.0 Hz, 1H)
H-2’,6’	7.49 (d, *J* = 8.6 Hz, 2H)	7.48 (d, *J* = 8.6 Hz, 2H)	7.48 (d, *J* = 8.6 Hz, 2H)
H-6	7.51 (d, *J* = 3.0 Hz, 1H)	7.51 (d, *J* = 3.0 Hz, 1H)	7.51 (d, *J* = 3.0 Hz, 1H)
H-7’	7.57 (d, *J* = 15.6 Hz, 1H)	7.56 (d, *J* = 15.6 Hz, 1H)	7.57 (d, *J* = 15.6 Hz, 1H)
H-3	8.45 (d, *J* = 9.0 Hz, 1H)	8.45 (d, *J* = 9.0 Hz, 1H)	8.45 (d, *J* = 9.0 Hz, 1H)

^{13}C NMR (100 MHz, CD_3OD) δ 116.8, 118.2, 119.3, 120.6, 122.0, 123.5, 127.5, 130.9, 134.8, 143.0, 154.2, 160.9, 166.8, 171.2.

Carbon	**^{13}C from Günther-Jordanland (2016) 125 MHz in CD_3OD**	**Our Carbon ^{13}C 100 MHz in CD_3OD**
C-3’/5’	116.8	116.8
C-6	118.3	118.2
C-8’	119.6	119.3
C-1	120.5	120.6
C-4	121.3	122.0
C-3	123.2	123.5
C-1’	127.6	127.5
C-2’/6’	130.8	130.9
C-2	134.8	134.8
C-7’	142.8	143.0
C-5	154.2	154.2
C-4’	160.9	160.9
C-9’	166.5	166.8
C-7	171.1	171.2

NMR Spectra compound 2c



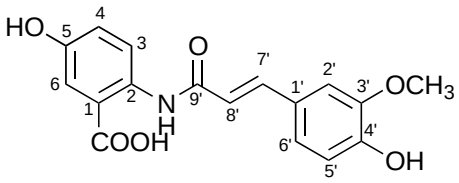
^{1}H NMR (400 MHz, CD_3OD) δ 6.45 (d, *J* = 15.6 Hz, 1H), 6.79 (d, *J* = 8.1 Hz, 1H), 6.97 (dd, *J* = 8.2, 2.1 Hz, 1H), 7.02 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.07 (d, *J* = 2.1 Hz, 1H), 7.50 (d, *J* = 15.6 Hz, 1H), 7.51 (d, *J* = 3.0 Hz, 1H), 8.45 (d, *J* = 9.0 Hz, 1H).

Hydrogen	**^{1}H from Wise (2011) 400 MHz in CD_3OD**	**^{1}H from Günther-Jordanland (2016) 500 MHz in CD_3OD**	**Our Proton ^{1}H 400 MHz in CD_3OD**
H-8’	6.46 (d, *J* = 15.5 Hz, 1H)	6.46 (d, *J* = 15.6 Hz, 1H)	6.45 (d, *J* = 15.6 Hz, 1H)
H-5’	6.79 (d, *J* = 8.2 Hz, 1H)	6.79 (d, *J* = 8.3 Hz, 1H)	6.79 (d, *J* = 8.1 Hz, 1H)
H-6’	6.97 (dd, *J* = 8.2, 1.7 Hz, 1H)	6.97 (dd, *J* = 8.3, 2.1 Hz, 1H)	6.97 (dd, *J* = 8.2, 2.1 Hz, 1H)
H-4	7.03 (dd, *J* = 9.0, 3.0 Hz, 1H)	7.00 (dd, *J* = 9.0, 2.9 Hz, 1H)	7.02 (dd, *J* = 9.0, 3.0 Hz, 1H)
H-2’	7.07 (d, *J* = 1.7 Hz, 1H)	7.07 (d, *J* = 2.0 Hz, 1H)	7.07 (d, *J* = 2.1 Hz, 1H)
H-7’	7.50 (d, *J* = 15.5 Hz, 1H)	7.50 (d, *J* = 15.7 Hz, 1H)	7.50 (d, *J* = 15.6 Hz, 1H)
H-6	7.51 (d, *J* = 3.0 Hz, 1H)	7.51 (d, *J* = 2.9 Hz, 1H)	7.51 (d, *J* = 3.0 Hz, 1H)
H-3	8.45 (d, *J* = 9.0 Hz, 1H)	8.44 (d, *J* = 9.0 Hz, 1H)	8.45 (d, *J* = 9.0 Hz, 1H)

^{13}C NMR (100 MHz, CD_3OD) δ 115.1, 116.5, 118.2, 119.3, 119.4, 122.0, 122.5, 123.4, 128.1, 134.8, 143.4, 146.8, 149.1, 154.1, 166.7, 171.3.

Carbon	**^{13}C from Günther-Jordanland (2016) 125 MHz in CD_3OD**	**Our Carbon ^{13}C 100 MHz in CD_3OD**
C-2’	115.1	115.1
C-5’	116.5	116.5
C-6	118.2	118.2
C-8’	119.5	119.3
C-1	120.4	119.4
C-4	121.6	122.0
C-6’	122.5	122.5
C-3	123.3	123.4
C-1’	128.1	128.1
C-2	134.7	134.8
C-7’	143.3	143.4
C-3’	146.8	146.8
C-4’	149.1	149.1
C-5	154.1	154.1
C-9’	166.8	166.7
C-7	171.7	171.3

NMR Spectra compound 2f



^{1}H NMR (400 MHz, CD_3OD) δ 3.92 (s, 3H), 6.56 (d, *J* = 15.6 Hz, 1H), 6.82 (d, *J* = 8.1 Hz, 1H), 7.03 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.09 (dd, *J* = 8.2, 1.9 Hz, 1H), 7.23 (d, *J* = 1.9 Hz, 1H), 7.51 (d, *J* = 3.0 Hz, 1H), 7.56 (d, *J* = 15.6 Hz, 1H), 8.46 (d, *J* = 9.0 Hz, 1H).

Hydrogen	**^{1}H from Wise (2011) 400 MHz in CD_3OD**	**^{1}H from Günther-Jordanland (2016) 400 MHz in CD_3OD**	**Our Proton ^{1}H 400 MHz in CD_3OD**
H-OCH_3	3.92 (s, 3H)	3.92 (s, 3H)	3.92 (s, 3H)
H-8’	6.57 (d, *J* = 15.6 Hz, 1H)	6.56 (d, *J* = 15.6 Hz, 1H)	6.56 (d, *J* = 15.6 Hz, 1H)
H-5’	6.82 (d, *J* = 8.2 Hz, 1H)	6.82 (d, *J* = 8.2 Hz, 1H)	6.82 (d, *J* = 8.1 Hz, 1H)
H-4	7.03 (dd, *J* = 9.0, 3.0 Hz, 1H)	6.99 (dd, *J* = 9.0, 3.0 Hz, 1H)	7.03 (dd, *J* = 9.0, 3.0 Hz, 1H)
H-6’	7.10 (dd, *J* = 8.2, 1.6 Hz, 1H)	7.09 (dd, *J* = 8.1, 2.0 Hz, 1H)	7.09 (dd, *J* = 8.2, 1.9 Hz, 1H)
H-2’	7.23 (d, *J* = 1.6 Hz, 1H)	7.22 (d, *J* = 1.9 Hz, 1H)	7.23 (d, *J* = 1.9 Hz, 1H)
H-6	7.51 (d, *J* = 3.0 Hz, 1H)	7.51 (d, *J* = 3.0 Hz, 1H)	7.51 (d, *J* = 3.0 Hz, 1H)
H-7’	7.57 (d, *J* = 15.6 Hz, 1H)	7.56 (d, *J* = 15.6 Hz, 1H)	7.56 (d, *J* = 15.6 Hz, 1H)
H-3	8.46 (d, *J* = 9.0 Hz, 1H)	8.45 (d, *J* = 9.0 Hz, 1H)	8.46 (d, *J* = 9.0 Hz, 1H)

^{13}C NMR (100 MHz, CD_3OD) δ 56.5, 111.6, 116.5, 118.2, 119.3, 119.6, 122.0, 123.5, 123.8, 128.1, 134.8, 143.3, 149.4, 150.2, 154.2, 166.7, 171.2.

Carbon	**^{13}C from Günther-Jordanland (2016) 100 MHz in CD_3OD**	**Our Carbon ^{13}C 100 MHz in CD_3OD**
C-10’	56.5	56.5
C-2’	111.6	111.6
C-5’	116.5	116.5
C-6	118.2	118.2
C-8’	119.3	119.3
C-1	119.6	119.6
C-4	122.0	122.0
C-3	123.5	123.5
C-6’	123.8	123.8
C-1’	128.1	128.1
C-2’	134.8	134.8
C-7’	143.4	143.3
C-3’	149.4	149.4
C-4’	150.2	150.2
C-5	154.2	154.2
C-9’	166.8	166.7
C-7	171.2	171.2

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