Antioksidacijska aktivnost zelene salate

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GRADUATE THESIS

Zagreb, June 2017.

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ANTIOXIDANT CAPACITY OF LETTUCE

Experimental part of Graduate thesis was done at the Department of Nutrition and Food Sciences, Faculty of Pharmacy, University of Granada under the supervision of Jose Angel Rufian-Henares, Full Professor and the assistance of Sergio Pérez Burillo, PhD Student, University of Granada.

Theoretical part of Graduate thesis was done in the Laboratory of Food process Engineering at the Department of Food Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb under the supervision of Suzana Rimac Brnčić, Full Professor, University of Zagreb.

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor, professor Jose Angel Rufian Henares, for giving me the opportunity to perform Master's thesis in his laboratory, allowing me access to all equipment and chemicals needed for performing the experiments; and also for his time and help during the whole experiment.

I want to thank Sergio Pérez Burillo for all the help, patience and time dedicated to students in laboratory and assistance during every step of the research.

Many thanks also to my colleagues and co-workers Miguel Lopez and Ellisabetta Serena for help and commitment to this research.

I also want to thank to my mentor Suzana Rimac Brnčić for opportunity and help to go abroad; her help, support and patience through all steps of doing my Master thesis.

Many thanks to my family (especially my parents), my fiancé, and to all my friends who were helping me and supporting me through all this time.

TEMELJNA DOKUMENTACIJSKA KARTICA

Diplomski rad

Sveučilište u Zagrebu
Prehrambeno-biotehnološki fakultet
Zavod za prehrambeno-tehnološko inženjerstvo
Laboratorij za procesno-prehrambeno inženjerstvo

Znanstveno područje: Biotehničke znanosti
Znanstveno polje: Nutricionizam

ANTIOKSIDACIJSKA AKTIVNOST ZELENE SALATE

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Sažetak:
Zelena salata je jedna od najčešće konzumiranih vrsta povrća u svijetu te predstavlja značajan izvor bioaktivnih tvari u prehrani. Talog kave u posljednje se vrijeme istažuje kao potencijalno iskoristiv u poljoprivredi, budući da obiluje hranjivim i bioaktivnim spojevima. U ovom radu antioksidacijska aktivnost zelene salate uzgajane na tlu obogaćenim talogom kave određena je nakon in vitro fiziološke probave i fermentacije. Antioksidacijska aktivnost određena je sljedećim metodama: FRAP metoda (Ferric Reducing Ability of Plasma), ABTS metoda (2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonic acid) radical), IC-AAPH metoda (Indigo Carmine- 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride), IC-OH metoda (Indigo Carmine Hidroxiles) te IC RED metoda (Indigo Carmine Reduction). Utvrđen je pozitivan učinak dodatka taloga kave u tlo za uzgoj zelene salate na antioksidacijsku aktivnost zelene salate.

Ključne riječi: talog kave, zelena salata, antioksidacijske metode, in vitro probava

Rad sadrži: 56 stranice, 21 slika, 12 tablica, 94 literaturna navoda

Jezik izvornika: engleski

Rad je u tiskanom i elektroničkom (pdf format) obliku pohranjen u: Knjižnica Prehrambeno-biotehnološkog fakulteta, Kačićeva 23, Zagreb

Mentor: Prof. dr.sc. Suzana Rimac Brnčić

Stručno vodstvo: prof. Jose Angel Rufian Henares

Pomoć pri izradi: Sergio Pérez Burillo, doktorand

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Datum obrane: 20. srpnja 2017.

BASIC DOCUMENTATION CARD

Graduate Thesis

University of Zagreb
Faculty of Food Technology and Biotechnology
Department of Food Technology Engineering
Laboratory for Food Process Engineering

Scientific area: Biotechnical Sciences
Scientific field: Nutrition

ANTIOXIDANT CAPACITY OF LETTUCE

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Abstract:
Lettuce represents one of vegetables most frequently consumed in world, representing therefore an important source of bioactive compounds in the diet. Spent coffee grounds have been investigated as potential by-products for recycling in agriculture, since they have huge re-use potential due to their richness in nutritive and bioactive compounds, In this work, antioxidant capacity of lettuce grown on soil enriched with spent coffee grounds was evaluated after in vitro physiological digestion and fermentation. Antioxidant capacity has been measured by FRAP (Ferric Reducing Ability of Plasma), ABTS (2, 2′-azinobis(3-ethylbenzothiazoline 6-sulfonic acid) radical), IC-AAPH (Indigo Carmine-2,2'-azobis-2-methyl-propanimidamide, dihydrochloride), IC-OH (Indigo Carmine Hidroxiles) and IC RED (Indigo Carmine Reduction)methods. Positive impact of spent coffee grounds in soil on antioxidant capacity of lettuce has been shown.

Keywords: Spent Coffee Grounds, Lettuce, Antioxidant methods, in vitro digestion
Thesis contains: 56 pages, 21 figures, 12 tables, 94 references
Original in: English
Graduate Thesis in printed and electronic (pdf format**) version is deposited in:** Library of the Faculty of Food Technology and Biotechnology**,** Kačićeva 23, Zagreb.

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Thesis defended: 20 July 2017

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1. INTRODUCTION

Lettuce represents a major and low-cost green leafy vegetable, being one of the most frequently consumed vegetables in the world. Although lettuce reportedly provides relatively low levels of antioxidative phytochemicals, its high per capita consumption makes it a considerable contributor to the amount of antioxidants in the diet. On the other hand, studies have highlighted the importance of variation in factors such as cultivar, agronomic practices, climatic conditions, and storage conditions, as a key tool to obtain healthful and more nutritious food crops.

Coffee is one of the most significant commodities in the world trade. Regarding to some sources, there are countries, like Finland, for example, that yearly consume 9,6 kilograms of coffee per capita). Statistics for Croatia says that an average Croat consumes about 3,5 kilograms in a year. Worldwide it results in huge amounts of spent coffee grounds (SCG) in consuming countries. Lately, these residues have been investigated as potential by-products for recycling in agriculture, since they have a huge re-use potential due to their richness in nutritive and bioactive compounds, and the majority of researches has been done with lettuce.

Many investigations have been done about reusing SCG for enhancing plant growing, and many of them have tried to assess antioxidant capacity of lettuce treated with spent coffee ground by applying different methods of chemical extractions. Anyway, those methods do not extract all the antioxidants both from solid and liquid fractions of samples. Nevertheless, the chemical extraction is not what naturally happens in a human digestive system (chemical extraction is being done with solvents like ether, methanol and chloroform, which must not exist in a human organism); and, when talking about antioxidants, we are usually thinking of their benefits on human health. In this research, a method used to estimate the effect of SCG on antioxidant capacity of lettuce is a simulated *in vitro* physiological digestion and fermentation; and samples were grown on soil treated, besides SCG, with different minerals (nitrogen, phosphorus, potassium) and their mixtures. Antioxidant capacity has been measured by five assays: FRAP, ABTS, IC AAPH, IC OH and IC RED, measuring after simulated digestion and fermentation (of both liquid and solid phase) and summing up these values. Values were compared with those of corresponding samples (treated with same mineral mixtures, but no SCG) and with the control (sample not treated with minerals nor SCG).

Still, more researches are required on this field to confirm and explain benefits of spent coffee grounds on antioxidant capacity of lettuce and other vegetables and, hopefully, to provide best combination of minerals and fertilizers for growing soil. Thus, the aim of this study is to examine if spent coffee grounds amendment to the soil has an impact on antioxidant enhancement of lettuce.

2. THEORETICAL PART

2.1. Lettuce

Diets containing a high proportion of fruits and vegetables have been shown to reduce the incidence of chronic diseases in Western countries (Nicolle et al., 2004 according to Block et al., 1992 and Block, 1992). In fruits and vegetables, especially in lettuce, antioxidant micronutrients such as polyphenols and carotenoids could have an important role in preventive nutrition, but they are dependent on high variation among cultivars and growth conditions (Nicolle et al., 2004). According to Mou (2008), there are six main lettuce types based upon leaf shape, size, texture, head formation, and stem type. They are (1) crisphead lettuce (*var. capitata L. nidus jaggeri Helm*), (2) butterhead lettuce (*var. capitata L. nidus tenerrima Helm*), (3) romaine or cos lettuce (*var. longifolia Lam., var. romana Hort. in Bailey*), (4) leaf or cutting lettuce (*var. acephala Alef., syn. var. secalina Alef., syn. var. crispa L.*), (5) stem or stalk (Asparagus) lettuce (*var. angustana Irish ex Bremer, syn. var. asparagine Bailey, syn. L. angustana Hort. In Vilm.*), and (6) Latin lettuce (no scientific name). Growing conditions, like soil properties, fertilization and irrigation regime, growing season, quality and quantity of light, but also postharvest handling and storage influence nutrient contents.

Lettuce is an important agricultural commodity worldwide which is, unlikely to many other vegetables, available over the whole year. Many green leafy vegetables are rich in micronutrients such as carotenoids and folic acid, but their consumption is often insufficient to provide a good intake of these compounds. Lettuce is commonly consumed in salad mixes and consumption of salads is increasing. Therefore, lettuce can contribute considerably to the nutritional content of diets (Kenny and O'Beirne, 2009). Additionaly, more nutrients are retained compared to other vegetables that are cooked or processed, such as potatoes, since lettuce is generally eaten raw.

The potential beneficial effect of lettuce depends on its composition, especially its micronutrient content. Lettuce is high in water content (95%) and low in calories. It has nutritional benefits due to its contribution to dietary fiber, presence of several important dietary minerals (natrium, potassium, calcium, iron), and variety of vitamins (folate, vitamin C and E) and bioactive compounds (e.g., carotenoids, and phenolic compounds). In vitro and in vivo studies have shown anti-inflammatory, cholesterol-lowering, and anti-diabetic activities attributed to the bioactive compounds in lettuce (Kim et al., 2016). Rice-Evans et al.

(1995) reported that flavonoids (flavonols and anthocyanins) have greater antioxidant activity than either vitamin C or E. Epidemiological studies indicated that carotenoids and vitamins E and C are among the molecules in the diet which turn out to play a preventive role in cancer and heart diseases (Nicolle et al., 2004).

Lettuce, although low in fat, contains polyunsaturated fatty acids (PUFA) which are important for health. Essential fatty acids, such as omega-6 PUFA, linoleic acid (LA) and omega-3 PUFA, α-linolenic acid (ALA) must be obtained from the diet (Kaur et al., 2014).
Lettuce also provides carbohydrates with lower digestibility and, therefore, lower caloric content such as sugar alcohols. Lettuce also provides dietary fiber (USDA, 2015).
Similar to most vegetables, lettuce is not an important source of protein. However, it contains various non-caloric nutrients that can contribute to nutritional status and benefit health.
Lettuce contains low amount of natrium and provides a relatively good amount of iron, depending on the types and cultivars. Depending on lettuce type, potassium content was comparable to that in spinach which is considered a good vegetable source of potassium (USDA, 2015). Fresh lettuce (100g) provides 4–8% of recommended potassium intake of 4.7g/day for adults (Institute of Medicine, 2005).

Depending on lettuce type, calcium content in lettuce is either lower or comparable to other rich plant sources of calcium such as spinach (USDA, 2015). Despite potentially higher bioavailability due to absence of oxalate, fresh lettuce (100g) provides only 2–6% of the recommended calcium intake of 1000–1200mg/day for adults (Institute of Medicine, 1997). Comparisons of mineral content of lettuce among studies were limited by the large variation in reported mineral contents. This may be due to factors such as different soil mineral composition (Pinto et al., 2014) and type of lettuce head (Mou and Ryder, 2004). Overall, mineral analysis showed lettuce was a relatively good source of iron and low in natrium.

Leafy vegetables are considered as a rich source of folate (vitamin B9). Compared to several other vegetables, lettuce is an excellent source of folate (according to USDA, 2015 even better than spinach). Fresh lettuce (100g) depending on lettuce type can provide up to 18% of RDA for folate.

Vitamin C content in lettuce is lower compared to other leafy vegetables such as spinach and kale (USDA, 2015). Among popularly consumed lettuce in the US, green leaf is a particularly good source of vitamin C (USDA, 2015) meeting 22% of the RDA. Additionally, subtypes of leaf lettuce, such as continental, red oak, and lollo rosso lettuces were reported to provide high levels of vitamin C (Llorach et al., 2008).

Vitamin E content differs widely among lettuce types and it is difficult to explain the large variation, due to lack of cultivation and genetic information on lettuces types. Based on studies reporting carotenoid contents, lettuce is a valuable source of carotenoids, specifically β-carotene and lutein (USDA, 2015) (Kim et al., 2016).

It is regarded as an important source of phytonutrients. The chemical composition of the plant revealed the presence of various classes of secondary metabolites, such as terpenoids, flavonoids, and phenols which should be responsible for its biological activities (Noumedem et al., 2017). Phenolic subgroups reported in lettuce are phenolic acids and flavonoids. Common phenolic acids in lettuce are caffeic acid, chlorogenic acid, and their derivatives (Ferreres et al., 1997; Llorach et al., 2008; Zhao et al., 2007). Quercetin and kaempferol derivatives, anthocyanins, and flavone luteolin are identified flavonoids in lettuce (Llorach et al., 2008). However, these compounds were not consistently detected in all cultivars and content varies with lettuce types.

Overall, studies showed that lettuce provides not only a variety of bioactive compounds but also different amounts of these compounds among lettuce types. Obtaining higher amounts of these bioactive phytochemicals can be achieved by either choosing lettuces with red pigmented leaves or through manipulating cultivar conditions (Kim et al., 2016).

2.2. Coffee – origin, production and consumption

Coffee has been consumed for over 1,000 years and nowadays it is the most consumed drink in the world (more than 400 billion cups yearly) (Mussato et al. according to Sobésa Café 2008). coffee, grown in about 80 countries, is one of the world's most popular beverage and second largest traded commodity after petroleum (Murthy & Naidu, 2012). Botanically, coffee belongs to genus *Coffea* of a family *Rubiaceae* and is present in more than 80 species (Bridson and Verdcourt, 1988). However, only two species are of commercial interest: *Coffea arabica* (Arabica), which is considered as the most noble coffee plant and therefore represents 75% of world coffee production; and *Coffea canephora* (Robusta), considered as the sourest, but also most resistant on pests, which represents the rest 25% of the world production (Belitz et al. 2009; Etienne 2005). The coffee tree cultivation takes places in countries of South, with tropical clime; largest coffee producers in the world are Brazil, Vietnam, Colombia, Indonesia and Ethiopia (ECF, 2014). However, 77% of coffee is consumed by North countries, and only the rest 23% by producing countries (FAO, 2013). Global production of coffee has increased 21% since 2005, with total exports equalling 8 million tons (ICO, 2010). Europe has the

highest per capita coffee consumption in the world. The EU consumes 2.5 million tons of coffee per year, which equates to 4 kilos of roasted coffee per EU inhabitant per year, and Croatia is amongst largest consumers, as shown in Figure 1 (ECF, 2014). According to national statistics, per capita coffee consumption in Croatia in last 10 years is about 3,5 kg (3,44 kg-3,70 kg) (CBS, 2016).

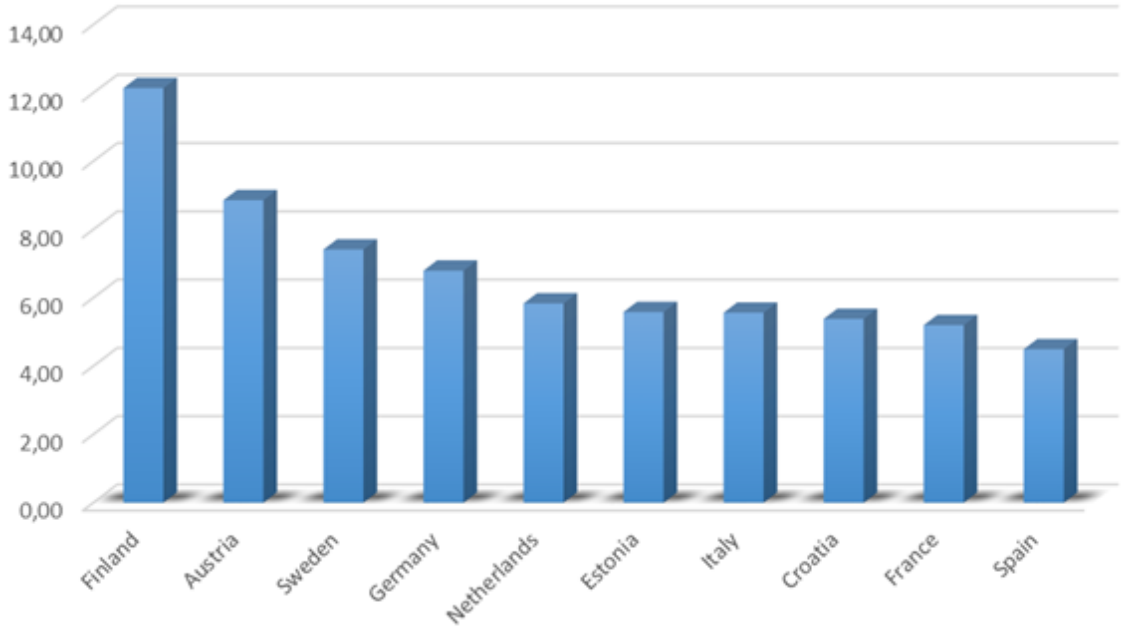

Figure 1. Per capita coffee consumption in selected EU member states in 2013 (kg green coffee equivalent) (http://www.ecf-coffee.org/about-coffee/coffee-consumption-in-europe)

Coffee cherries are the raw fruit of the coffee plant, which are composed of two coffee beans covered by a thin parchment like hull and further surrounded by pulp (Figure 2.) (Mussato et al., 2011 according to Arya and Rao, 2007). The fruit or berry has a green outer skin which, when ripe, turns red-violet or deep red and encloses the sweet mesocarp or the pulp and the stone-fruit bean. The latter consists of two elliptical hemispheres with flattened adjacent sides. Silverskin (a yellowish transparent spermoderm) covers each hemisphere. Covering both hemispheres and separating them from each other is the strong fibrous endocarp, called the “parchment” (Belitz et al. 2009).

Caffeine is the most known component of coffee beans. In raw Arabica coffee, caffeine can be found in values between 0.8% and 1.4% (w/w), while for the Robusta variety these values vary from 1.7% to 4.0% (w/w) (Mussato et al., 2011 according to Belitz et al. 2009).

However, coffee bean is constituted by several other components, including cellulose, minerals, sugars, lipids, tannins, and polyphenols.

a) **b)**

Figure 2. a) Longitudinal section of coffee cherry (Mussato et al., 2011) and b) transversal section (Geromel et al., 2006).

Minerals include potassium, magnesium, calcium, sodium, iron, manganese, rubidium, zinc, copper, strontium, chromium, vanadium, barium, nickel, cobalt, lead, molybdenum, titanium, and cadmium. Among the sugars, sucrose, glucose, fructose, arabinose, galactose, and mannose are present. Several amino acids such as alanine, arginine, asparagine, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine can also be found in these beans (Mussato et al., 2011 according to Belitz et al. 2009; Grembecka et al. 2007; Santos and Oliveira 2001). Additionally, coffee beans contain vitamin of complex B, the niacin (vitamin B3 and PP), and chlorogenic acid in proportions that may vary from 7% to 12%, three to five times more than the caffeine (Mussato et al., 2011 according to Belitz et al. 2009; Lima 2003; Trugo 2003; Trugo and Macrae 1984). Among the substances present in the chemical composition of coffee, only caffeine is thermostable, i.e., it is not destroyed by excessive roasting. Other substances like proteins, sugars, chlorogenic acid, trigonelline, and fat may be preserved or even destroyed and transformed into reactive products during the coffee roasting process (Mussato et al., 2011 according to Ginz et al. 2000; Lima 2003; Rawel and Kulling 2007; Trugo, 2003; Trugo and Macrae 1984).

The processing of coffee is initiated with the conversion of coffee cherries into green coffee beans, and starts with the removal of both the pulp and hull using either a wet or dry method. Depending on the method of coffee cherries processing, i.e., wet or dry process, the solid residues obtained are differently named: pulp or husk (Mussato et al., 2011 according to

Pandey et al. 2000). The dry method, commonly used for Robusta, is technologically simpler comparing with the wet method, which is generally used for Arabica coffee beans. In wet coffee process, the pulp and hull are removed while the cherry is still fresh. The roasting of coffee beans is another very important stage in coffee processing, since specific organoleptic compounds (flavours, aromas, and color) are developed and affect the quality of the coffee and the excellence of the coffee beverage, as a consequence (Mussato et al., 2011 according to Hernández et al. 2008; Franca et al. 2005a; Fujioka and Shibamoto 2008). This process is time–temperature dependent and leads to several changes in the chemical composition and biological activities of coffee as a result of the transformation of naturally occurring polyphenolic constituents into a complex mixture of Maillard reaction products (Mussato et al., 2011 according to Czerny et al. 1999; Sacchetti et al. 2009), as well as the formation of organic compounds resulting from pyrolysis (Mussato et al., 2011 according to Daglia et al. 2000). Besides the chemical reactions during coffee roasting, moisture loss and other major changes (color, volume, mass, form, pH, density, and volatile components) occur, while CO_2 is generated (Mussato et al., 2011 according to Hernández et al. 2008). Therefore, coffee roasting is extremely complex process considering the importance of the heat transferred to the bean (Mussato et al. according to Franca et al. 2009). Additionally, the roasted beans are ground, usually by multi-stage grinders. Some roasted beans are packaged and shipped as whole beans. Finally, the ground coffee is vacuum sealed and shipped. Roasting is probably the most important factor in the development of the complex flavours that make coffee enjoyable. (Wei et al., 2012 according to Illy et al., 1995). Composition of coffee beans after roasting process varies greatly, depending on variety and extent of roasting (Belitz et al., 2009). During the roasting process, the beans are subjected to many chemical reactions, leading to important physical changes and to the formation of the substances responsible for the sensory qualities of the beverage (Wei et al., 2012 according to Clifford, 1985). The most important changes in chemical composition of coffee bean occurred during roasting are given in Table 1. (Clarke and Macrae, 1985). Protein is exposed to extensive changes when heated in the presence of carbohydrates. There is a shift of the amino acid composition of coffee protein acid hydrolysates before and after bean roasting. The total amino acid content of the hydrolysate drops by about 30% because of considerable degradation. Arginine, aspartic acid, cystine, histidine, lysine, serine, threonine and methionine, being especially reactive amino acids, are somewhat decreased in roasted coffee, while the stable amino acids, particularly alanine, glutamic acid and leucine, are relatively increased. Free amino acids occur only in traces in roasted coffee.

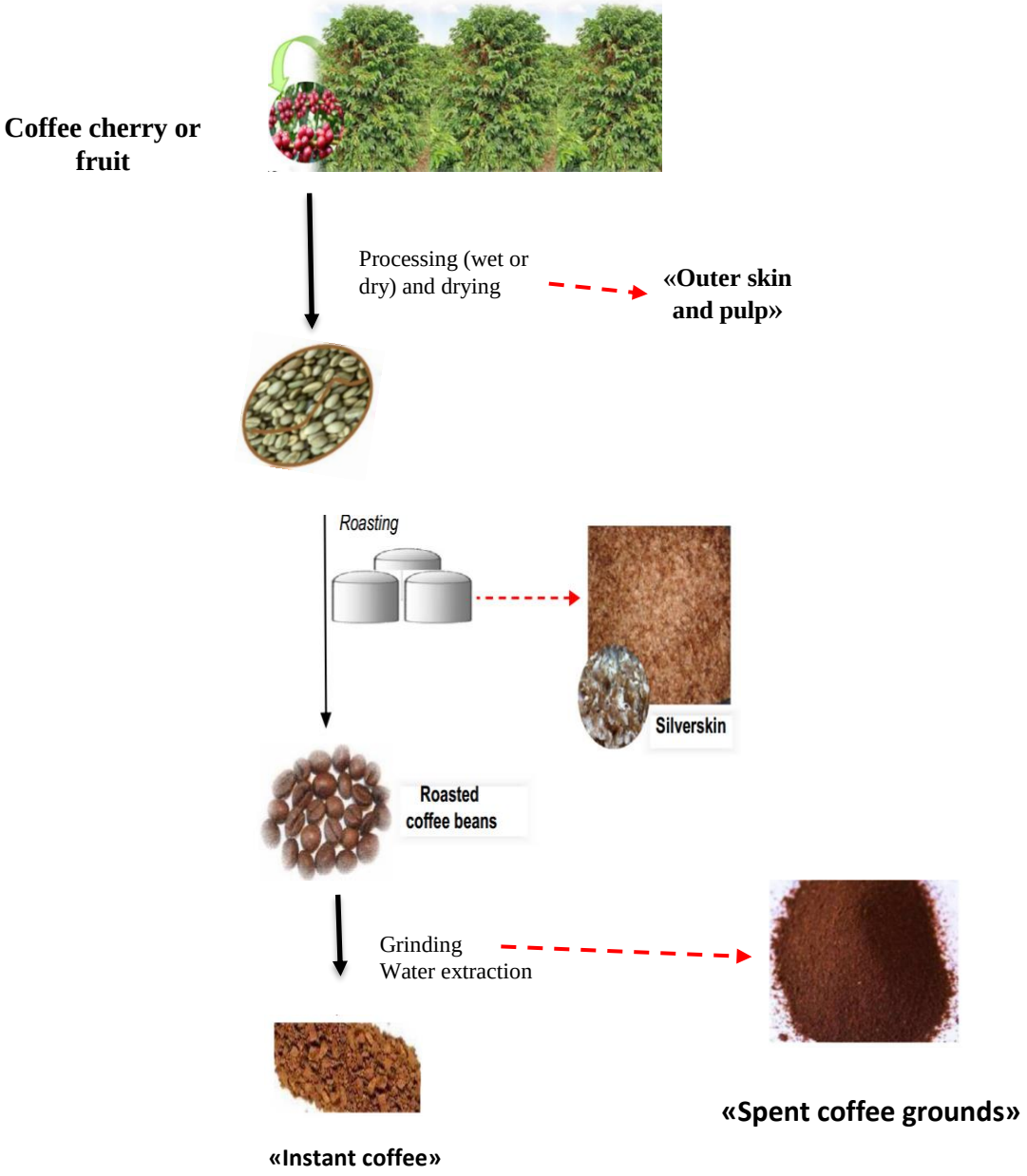

Figure 3. Coffee processing scheme (Mussato et al., 2011)

Most of the carbohydrates present are insoluble, like cellulose and polysaccharides consisting of mannose, galactose and arabinose. During roasting a proportion of the polysaccharides are degraded into fragments which are soluble. Sucrose present in raw coffee is decomposed in roasted coffee, while monosaccharides also occur in a lesser extent.

The lipid fraction seems to be very stable and survives the roasting process with only small changes. Predominant fatty acid is linoleic acid is, followed by palmitic acid. The

diterpenes present are cafestol and kahweol. Cafestol and kahweol are degraded by the roasting process. Major compounds of the sterol fraction are sitosterol and stigmasterol.
The caffeine level in beans is during roasting only slightly decreased. Caffeine obtained by the decaffeination process and synthetic caffeine are often used by the pharmaceutical and soft drink industries.

In the green coffee bean aroma 300 volatile compounds have been found (Clarke and Vitzthum, 2001); and majority corresponds to pyridines, furans, amines, aldehydes, ketones, alcohols, acids and different sulfuric compounds. A large quantity of volatile compounds is being formed during roasting and, as a result, coffee bean contains about 850 volatile compounds: pyrazines, pyrroles, hydrocarbons, acids, aldehydes, esters, alcohols and thiophenes, among others (Clarke and Vitzthum, 2001; Flament and Bessière, 2002). It has been reported that there are at least 30 chlorogenic acids in coffee beans (Wei et al., 2012 according to Clifford et al., 2003; Clifford et al., 2006a; Clifford et al., 2006b), of which the three most abundant kinds (3-CQA, 4-CQA, and 5-CQA) have been detected and assigned by NMR of green coffee bean extracts (Wei et al., 2012 according to Wei et al., 2010) and roasted coffee bean extracts. (Wei et al., 2012 according to Wei et al., 2011). The major polyphenols in green coffee beans are chlorogenic acids, and their degradation leads to the release of quinic acid, which is known as the dominant acid in roasted coffee, and cinnamic acid.

In previous studies, importance of trigonelline has been well-documented: not only as a precursor of flavour and aroma compounds but also as a beneficial nutritional factor. (Wei et al., 2012 according to Taguchi et al., 1985 and Viani and Horman, 1974). Reports on the temperature-based degradation of trigonelline have revealed nicotinic acid and nicotinamide, as well as their *O*- and *N*-methyl derivatives, as reaction products (Wei et al., 2012 according to Viani and Horman, 1974). The decrease in the *N*-methylpyridinium level after 7-min-roasting was probably attributable to further decomposition and/or interaction with other thermolytic products. Nicotinic acid, which is an important vitamin, but also the second major thermal degradation product of trigonelline, is positively correlated with the roasting degree (Wei et al., 2012).

Brown compounds (melanoidins) are present in the soluble fraction of roasted coffee. melanoidins can be derived from Maillard reactions or from carbohydrate caramelization. The structures of these compounds have not yet been clarified. Apparently, chlorogenic acid is also involved in such browning reactions, since caffeic acid has been identified in alkali hydrolysates of melanoidins.

The only temperature-resistant components of coffee bean seem to be proteins, even though their concentration changes with water-content decrease.

As with all plant materials, potassium is predominant in coffee ash (1.1%), followed by calcium (0.2%) and magnesium (0.2%). The predominant anions are phosphate (0.2%) and sulphate (0.1%). Many other elements are also present, although in trace amounts (Belitz et al., 2009).

Table 1. Chemical composition of green and roasted coffee bean; varieties Arabica and Robusta (Clarke et al., 1985).

Components	ARÁBICA		ROBUSTA	
	Green coffee bean	Roasted coffee bean	Green coffee bean	Roasted coffee bean
Polysaccharides	50.0-55.0	24.0-39.0	37.0-47.0	-
Oligosaccharides	6.0-8.0	0-3.5	5.0-7.0	0-3.5
Lipids	12.0-18.0	14.5-20.0	9.0-13.0	11.0-16.0
Free amino acids	2.0	0	2.0	0
Proteins	11.0-13.0	13.0-15.0	11.0-13.0	13.0-15.0
Chlorogenic acids	5.5-8.0	1.2-2.3	7.0-10.0	3.9-4.6
Caffeine	0.9-1.2	0-1.0	1.6-2.4	0-2.0
Trigonelline	1.0-1.2	0.5-1.0	0.6-0.8	0.3-0.6
Fatty acids	1.5-2.0	1.0-1.5	1.5-2.0	1.0-1.5
Minerals	3.0-4.2	3.5-4.5	4.0-4.5	4.6-5.6
Melanoidins	-	16.0-17.0	-	16.0-17.0

After roasting, coffee beans are being ground in different ways depending on desired preparation mode, since various modes require appropriate grinding stage. The three most used techniques of coffee brew preparation are preparation in Moka pot (which requires medium-fine grinding stage), filter machine (for which is required medium ground coffee) or in espresso machine (here the extra fine grinding stage is required).

Depending on preparation way, i.e. on grinding stage, different residues are obtained. In this work, focus will be on the residues obtained by espresso coffee preparation – espresso spent coffee grounds.

In the coffee brew preparation, soluble compounds of the roasted and ground coffee beans are being extracted with hot water. The soluble solids can be extracted in various ways.

Soluble coffee industries use extraction batteries, that work on a system of high temperatures and pressures to achieve the highest possible extraction of solids (Schwartzber, 1980). In public places, such as cafeterias, or at home, different extraction type coffee makers are used, like dripping or percolating coffee machines, or espresso machines. Every system extracts different soluble solids percentage, providing beverages with diverse concentrations, depending on what every consumer prefers (Davids, 1991).

In the present study, a special attention will be payed to those beverages obtained by an espresso coffee makers, commonly used in restaurants and cafeterias. The espresso coffee is consumed daily by millions of people in the world, due to its quick preparation on one hand, and very concentrated aroma on the other hand. To obtain this type of coffee, water under high pressure (between 5 and 15 bars) is let to pass through a ground coffee blend. Besides pressure, obtaining an espresso requires also a temperature of passing water to be between 88°C and 92°C, since higher temperature would destroy the delicate compounds, and lower temperature wouldn't provide their extraction; and the coffee beans should be finely grind (Caprioli et al., 2012).

Such preparation process results in well-known coffee beverage on one side, and an important part of solid residue, named "Spent Coffee Grounds" (SCG) on the other side.
Although there are plenty of studies analysing the parameters of pressure and temperature of passing water to extract the largest number of soluble solids, significant quantities of insoluble compounds, which cannot be extracted, remain in spent coffee. Even though the process of roasting improves the hot water extracting capacity, only 6-12% (depending on extent roasting) of coffee polysaccharides are possible to extract during beverage preparation (Nunes et al., 2002). Post-extraction analyses of spent coffee grounds showed that mannose is the main residue among sugars (57%), followed by galactose (26%), glucose (11%) and arabinose (6%) (Simões et al., 2009). On the other part, roasted coffee contains approximately 23% of melanoidins (Belitz, 2009), of which nearly 3% is being solubilized in coffee beverage, while the rest is remaining in SCG.

2.3. Residues generated in the coffee industry

Coffee processing produces significant amounts of undervalued residues, which are toxic since they are highly rich in organic and inorganic compounds; so they can cause high environmental pollution if released into the ecosystem without adequate pre-treatment (Mussatto et al., 2011).

Almost all developed, but also underdeveloped countries are trying to adapt to this reality by modifying their processes so that their residues can be recycled. Therefore, most large companies no longer consider residues as waste, but as a raw material for other processes (Mussatto et al. 2006). That way, they are acting in accordance with global policies, such as the one highlighted by World Health Organization: Sustainability is also of growing prominence and there is increasing interest in further promoting circular economy, in line with the European Union (EU) waste hierarchy, which gives priority to reduced production and re-use or recycling of waste over incineration and landfilling (WHO, 2015).

The agro-industrial production, but also consumption of coffee worldwide is responsible for the production of large amounts of residues which include pulp, bark, mucilage, residual water, and in the process of extracting the coffee drink, the espresso coffee grounds (SCG) (Mou, 2009). These residues can be divided in two categories: those generated in the producing countries, representing more than 50% of the coffee fruit mass, and those produced in the consuming countries after beverage preparation (Cruz et al., 2012a).

In the first place, producing countries generate, during the process of drying the green coffee, a huge quantity of mucilage that wraps a ripe coffee fruit. This by-product is valorised in cultivating countries. Secondly, in the coffee industry, during the roasting and processing, and later in coffee brew elaboration, two main residue types are being generated: coffee silver skin (CS) and spent coffee grounds (SCG). The CS is an integument that overcoats the coffee bean (Figure 4.A, B) and is obtained as a by-product resulting after roasting process. This residue contains high concentration of soluble dietary fibre (86% of total dietary fibre) (Table 2.) and high antioxidant capacity, probably due to phenolic compounds concentration of coffee bean, and to presence of other compounds formed in Maillard reactions during the roasting process, like melanoidins (Borrelli et al., 2004). The SCG is a residue with small-size particles (Figure 4.C, D), high humidity (80 to 85%), organic load and acidity; obtained during treatment of coffee blend with hot water or steam for preparation of instant coffee. Almost 50% of coffee produced in world is processed for soluble coffee preparation (Ramalakshmi et al., 2009), for which the SCG is generated in huge quantities, about 6 million tons yearly (Tokimoto et al. 2005). In numbers, 1 ton of green coffee generates about 650kg of SCG, and for every 1kg of soluble coffee produced, approximately 2kg of humid SCG are obtained (Pfluger 1975).

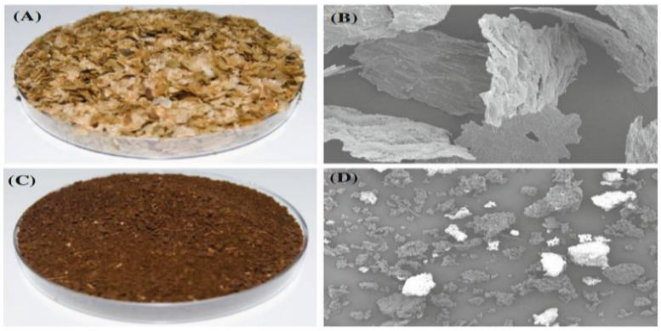

Figure 4. Appearance of coffee silver skin (A, B) and spent coffee grounds (C, D). B and D represent electronic microscope scanning of their particles with 50X increasing.

Chemical composition of these residues is shown in Table 2. It can be noted that SCG are richer in sugars then CS, of which mannose and galactose are outstanding, and proteins also represent an important fraction in SCG (Mussato et al., 2011).

Table 2. Chemical composition (g/100g) of coffee silver skin (CS) and spent coffee grounds (SCG) (Mussato et al., 2011).

	CS	**SCG**
Cellulose (Glucose)	17.8	8.6
Hemicellulose	13.1	36.7
Xylose	4.7	0.0
Arabinose	2.0	1.7
Galactose	3.8	13.8
Mannose	2.6	21.2
Proteins	17.4	13.6
Fats	2.2	Nd
Ashes	5.9	1.6
Total fibres	62.4	Nd
Soluble	53.7	Nd
Insoluble	8.8	Nd
Organic matter	Nd	90.5
Nitrogen	Nd	2.3
Carbon/Nitrogen (C/N)	Nd	22/1

The environmental impacts of coffee are enormous, with large quantities of solid and liquid wastes generated worldwide (Roussos et al., 1998 and Hue et al., 2006). In a life cycle analysis of coffee, Salomone (2003) reported cultivation and consumption of coffee as the two largest contributors to negative environmental impacts.

Spent coffee grounds represent an under-utilized high nutrient and energy material with potential as a niche market compost for horticultural use (Barreto et al., 2008 and Kondamudi et al., 2008). Nowadays, coffee residues in general are recycled by composting, vermicomposting, bio-gas production, mushroom production, animal feed and, more recently, for extracting value-added fractions for bio-diesel and ethanol production. However, most of these processes are not technologically efficient, or may cause secondary pollution or are not economically viable. On the other hand, coffee residues may have potential as suitable liming material, as NPK fertilizer and as promoters of water and nutrient retention in soils. Even though SCG have been used for a long time as soil amendment at domestic levels, the efficiency of this practice is still not scientifically supported, and safety for fertilization use still lacks confirmation (Cruz et al., 2015). Supporting an increased application of this residue in domestic agriculture, apparently with benefits on plant protection and appearance, recent studies highlighted the possibility of vegetable enrichment in bioactive compounds, specificaly carotenoids, when cultivated in the presence of reduced amounts of fresh-SCG (Cruz et al., 2012b). However, many other antioxidant substances may benefit from this preharvest treatment, requiring detailed evaluation (Cruz et al., 2013).

2.4. SCG as a valuable source of antioxidant compounds

SCG contain several human health related compounds, such as phenolics, with demonstrated antioxidant, anti-bacterial, antiviral, anti-inflammatory and anti-carcinogenic activities (Campos-Vega et al (2015) according to de Souza et al. (2004)).

In a research of Yen et al. (2005) antioxidant activity of roasted coffee residues was evaluated. Results obtained from HPLC quantification of phenolic acids and nonphenolic compounds remained in roasted coffee residues indicate that they have excellent potential for use as a natural antioxidant source.

Bravo et al. (2012) after evaluating the main hydrophilic antioxidant compounds (3-, 4-, and 5-monocaffeoylquinic and 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids, caffeine, and browned compounds, including melanoidins) and the antioxidant capacity (Folin–Ciocalteu, ABTS, DPPH, Fremy's salt, and TEMPO) in Arabica and Robusta spent coffee obtained from

the preparation of coffee brews with the most common coffeemakers (filter, espresso, plunger, and mocha) concluded that spent coffee obtained from the most common coffeemakers used at domestic and cafeteria levels (filter and espresso), and in less proportion from plunger ones, could be considered as a good potential source of hydrophilic bioactive compounds.

Also, Ramalakshmi et al. (2009) claimed that the presence of phenolics and chlorogenic acids in appreciable quantities along with brown pigments makes these coffee by-products a source for natural antioxidants.

Extracts from spent coffee have been evaluated for biological activity also in research of Ramalakshmi, Kubra, & Rao (2008) and Ramalakshmi et al. (2009). They have shown strong radical-scavenging, antioxidant and anti-tumor activity, although only limited anti-inflammatory and anti-allergic action. Their antioxidative properties could be the consequence of the presence of caffeine, trigonelline and chlorogenic acids (Esquivel and Jimenez, 2012 according to Franca et al., 2005b; Ramalakshmi et al., 2007).

In a research made by Cruz, Cardoso, et al. (2012a) espresso spent coffee grounds were chemically characterized to predict their potential, as a source of bioactive compounds, by comparison with the ones from the soluble coffee industry. Espresso spent coffee, for its greater richness in highly pursued natural compounds, such as caffeine and CGA, revealed a similar or even greater reuse potential than the one expected from spent grounds obtained from the soluble coffee industry, exhausted of most of its soluble components.

3. EXPERIMENTAL PART

3.1.MATERIALS

3.1.1. Selection of plant material: Lettuce

As a plant material, for this experiment lettuce has been chosen, due to its rapid growth and for being one of the world's most produced vegetable species. Its high per capita consumption makes it a considerable contributor to the amount of antioxidants in the diet.

This essay was carried out with a variety *Lactuca sativa var longifolia* (Little Gem Duende), commonly known as "Romaine lettuce". It grows with a long head and, as observed in Figure 5., has robust, extended leaves and central nerve.

Figure 5. *L. sativa var longifolia* (Organic Seeds, 2012);

Unlikely to other lettuce varieties, it is more heat tolerant. Seedlings were procured in south Spain (Granada, Motril, Saliplant S.L.), after germination and cultivation during 35 days. Then 72 seedlings were collected and sent to Department of Vegetal Physiology of Science Faculty, University of Granada.

a)

b)

Figure 6. a) some of samples in laboratory; and **b)** samples in cultivation room

3.1.2. Collection of SCG

As described previously, the aim of this study is to utilize the residues generated as result of an everyday activity, such as having a coffee in a cafeteria or restaurant. In this context, the coffee grounds used in this study were procured from cafeteria of Faculty of Pharmacy, University of Granada. Being a habitual break-time place to have a cup of coffee for number of students, professors and workers, this object is a significant SCG-producer. That for, it seemed logical to try to recycle those SCG at the same institute.

The staff was asked to save the espresso spent coffee grounds (Figure 7.) generated during one work day; then, spread to form a thin layer, it was subjected to drying until become dry to the touch.

Figure 7. Spent coffee grounds obtained from espresso coffee.

3.1.3. Soil preparation

3.1.3.1. NPK experiment

For this part of experiment, Vega soil was sieved to particles of 5 mm and approximately 400 g mixtures of the soil and two different percentages of spent coffee grounds (0 and 7.5%) were put into 0,3 L pots.

The experiment was conducted during 40 days under controlled conditions:

Temperature: 22°C day /18°C night

Humidity: 50% day/ 60% night

Humidity of samples has been maintained between field capacity and wilting point during whole experiment.

Table 4. describes compositions of fertilizers every sample was treated with.

Nitrogen has been added as mixture of urea and HNO_3: for 0.105 g of N→0.155 g of urea + 0.194 g of HNO_3 (0.139 ml).

Potassium has been added in form of KCl: for 0.087 g of K→0.166 g of KCl.

Phosphorus has been added in form of PO_4H_3: 0.046 g of P→0.209 g of PO_4H_3 (0.123 ml).

Compost 15:15:15 (15% N, 15% P, 15% K) has been added in quantity 0.7 g (which consists of 0.105 g of N+0.087 g of K+0.046 g of P).

Note that, when adding NPK directly, it is being added as 15:15:15 compost. Otherwise, when adding, for example, NP, it is being added N in form of urea and nitrate and phosphorus as phosphoric acid.

Table 4. Composition of fertilizers in soil for NPK experiment

Sample	**Treatment**
0-SCG - NPK	0% SCG + NPK (Compost 15:15:15)
0-SCG - N	0% SCG + N
0-SCG - P	0% SCG + P
0-SCG - K	0% SCG + K
0-SCG - NP	0% SCG + NP
0-SCG - NK	0% SCG + NK
0-SCG - PK	0% SCG + PK
0-SCG	0% SCG - without N/P/K
7.5-SCG - NPK	7.5% SCG + NPK (Compost 15:15:15)
7.5-SCG - N	7.5% SCG + N
7.5-SCG - P	7.5% SCG + P
7.5-SCG - K	7.5% SCG + K
7.5-SCG - NP	7.5% SCG + NP
7.5-SCG - NK	7.5% SCG + NK
7.5-SCG - PK	7.5% SCG + PK

3.1.3.2. Nitrogen experiment

For this part of experiment, Vega soil was sieved to particles of 5 mm and approximately 400 g mixtures of the soil and different percentages of spent coffee grounds (0; 1; 7,5 and 15%) were put into 0,3 L pots.

Experiment was being conducted during 40 days under controlled conditions:

Temperature: 22°C day /18°C night

Humidity: 50% day/ 60% night

Humidity of samples has been maintained between field capacity and wilting point during whole experiment.

Content of added nitrogen in three different quantities and SCG in soil for each sample treated are presented in Table 5.

Table 5. Content of nitrogen and SCG in soil for nitrogen experiment.

control	**0% SCG, without N**
L-N -0-SCG	**0% SCG, low dosage N** (0.02 g of nitrogen →0.057 g of NO_3NH_4)
L- N- 1- SCG	**1% SCG, low dosage N** (0.02 g of nitrogen →0.057 g of NO_3NH_4)
L-N -7.5- SCG	**7.5% SCG, low dosage N** (0.02 g of nitrogen →0.057 g of NO_3NH_4)
L-N -15-SCG	**15% SCG, low dosage N** (0.02 g of nitrogen →0.057 g of NO_3NH_4)
M- N- 0- SCG	**0% SCG, medium dosage N** (0.10 g of nitrogen →0.286 g of NO_3NH_4)
M- N 1- SCG	**1% SCG, medium dosage N** (0.10 g of nitrogen →0.286 g of NO_3NH_4)
M-N-7.5- SCG	**7.5% SCG, medium dosage N** (0.10 g of nitrogen →0.286 g of NO_3NH_4)
M- N- 15- SCG	**15% SCG, medium dosage N** (0.10 g of nitrogen →0.286 g of NO_3NH_4)
H- N- 0- SCG	**0% SCG, high dosage N** (0.58 g of nitrogen →1.657 g of NO_3NH_4)
H- N- 1- SCG	**1% SCG, high dosage N** (0.58 g of nitrogen →1.657 g of NO_3NH_4)
H- N- 7.5-SCG	**7.5% SCG, high dosage N** (0.58 g of nitrogen →1.657 g of NO_3NH_4)
H- N- 15-SCG	**15% SCG, high dosage N** (0.58 g of nitrogen →1.657 g of NO_3NH_4)

3.2. METHODS

3.2.1. *In vitro* digestion

Since the aim of this study is investigating how polyphenols from SCG contribute to the quantity of polyphenols in human body, a method used to enable such quantification is *in vitro* simulated gastrointestinal digestion. Therefore, although all parts of this experiment were done in laboratory, the digestion simulates, as much as possible, the physiological processes occurring in the human organism. In Table 6. there are listed solutions and enzymes used for simulated digestion.

Table 6. List of solutions and enzymes used for simulated *in vitro* digestion.

Solutions	Enzymes
- 0.3 M $CaCl_2(H_2O)_2$ - 0.5 M KCl - 0.5 M KH_2PO_4 - 1 M $NaHCO_3$ - 2 M NaCl - 0.15 M $MgCl_2(H_2O)_6$ - 0.5 M $(NH_4)_2CO_3$ - 4 M NaOH and 6M HCl: for pH adjustment of stock solutions of simulated digestion fluids	- Alpha-amylase from - Porcine pepsin - Pancreatin - Bile

Following text sections describe digestion phases, which are also showed on Figure 8.

Oral Phase (the ratio food: SSF is 50:50, for example 5 g of food in 5 mL of SSF)

First, the desired amount of food has to be weighed inside the tubes. It is wise to save some sample to measure its humidity. Due to the very short time that this phase lasts, it is advisable to prepare the pepsin solution at the same time of the salivary solution. Thus, quantity of alpha amylase should be weighed as to reach a concentration of 150 U/mL in the SSF and a quantity of pepsin as to reach a concentration of 4000 U/mL in the SGF (since the pepsin concentration on the final mix has to be 2000 U/mL). Both enzymes should be dissolved in their respective volumes of fluids. Secondly, alpha-amylase solution should be prepared dissolving the corresponding amount of enzyme in the corresponding volume of SSF. The enzyme solution has to be prepared as to reach a concentration of 150 U/mL since

after mixing it with food it will be diluted to half reaching then the aim concentration of 75 U/mL in the final mix.
After that, 25 μL of 0.3 M $CaCl_2$ is added in each tube taking into account that this volume is for 5 g of food. Once the mix is done, it is kept at 37 ºC for 2 minutes with shaking.

Gastric Phase (the ratio SSF: SGF is 50:50, for example 10 mL from the oral phase in 10 mL of SGF)

As the pepsin solution is already prepared, it is ought just to add the corresponding volume of SGF to the tube and then to add 5 μL of 0.3 M $CaCl_2$ to each tube. After that, 6 M HCl should be added to adjust to pH 3 each tube. Finally, it has to be incubated with shaking for 2 h at 37 ºC.

Intestinal Phase (the ratio SSG: SIF is 50:50, for example 20 mL from the gastric phase in 20 mL of SIF)

Before the gastric phase finishes, the SIF solution should be prepared. To do that, bile has to be weighed as to reach a concentration of 20 mM in SIF and 10 mM in the final mix. The corresponding volume has to be added to the bile and dissolved with help of a magnetic stirrer and heated (37 ºC). This might last some time, 20-30 minutes. Then the quantity of pancreatin, needed to reach a concentration of 26.74 mg/mL in the SIF, has to be weighed and thus, the aimed concentration of 13.369 mg/mL should be reached in the final mix.
Next step is adding carefully, bit by bit, the pancreatin to the SIF and bile solution while stirring. The SIF will then be ready (20 mM bile and 67.2 mg/mL pancreatin). Once the gastric phase is done, the corresponding volume should be added to each tube and also 40 μL of 0.3 M $CaCl_2$ to each tube. Finally, needed volume of 4M NaOH in order to reach a pH of 7 should be added. Tubes should then be incubated with shaking for 2 h at 37 ºC.

Storing the samples

When the intestinal phase had finished, the tubes have to be buried in ice in order to stop the digestion reaction. Once they are cold enough, it is ought to centrifuge them at 6000 rpm for 10 minutes. Then, the digested soluble fraction (DSF) can be separated from the

insoluble (solid) fraction, aliquots taken of the liquid phase, and the solid phase stored. Since physiologically a 10% of the soluble fraction goes to the small intestine, it is ought to add this 10% of the soluble fraction to the solid. Before adding this 10% of the soluble fraction, it is important to weigh the solid to know the percentage of food digested.

They have to be stored at -80 ºC for further analysis. If the solid phase is lyophilized, it should be weighed before and after drying in order to know the humidity. Such humidity will be useful later on when calculating the % of fresh "food" fermented.

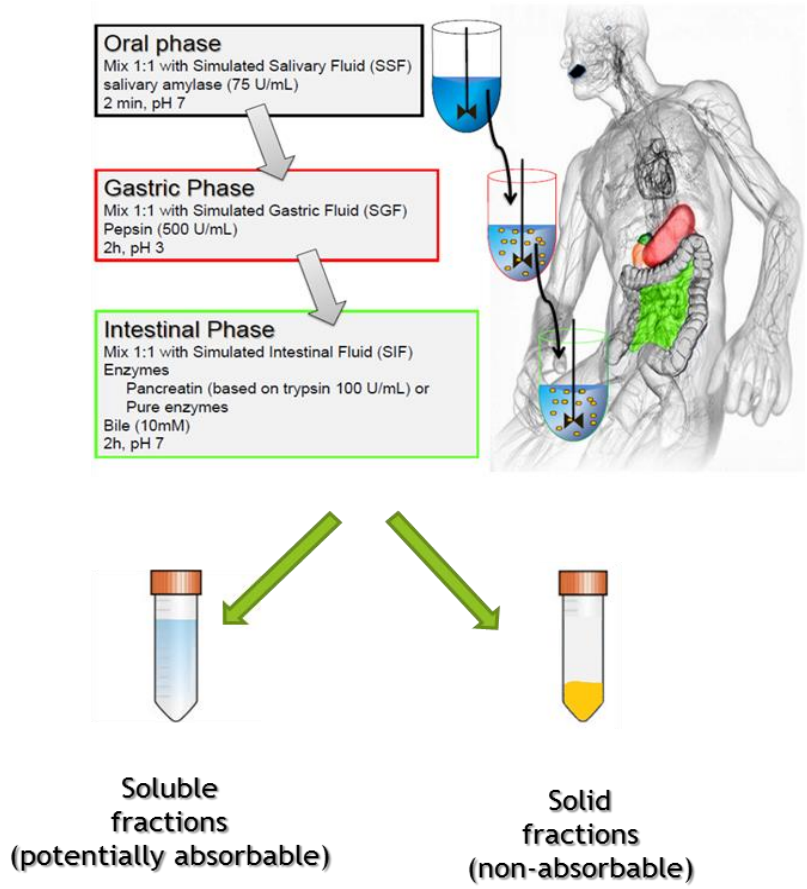

Figure 8. Scheme of *in vitro* digestion

3.2.2. *In vitro* fermentation

After the digestion, the following process occurring in the human organism is fermentation in large intestine. To carry out this experiment in most physiological way, next process to manage was simulated *in vitro* fermentation. In Table 7. there are listed reactants and solutions used for this experimental stage.

Table 7. List of reactants and solutions used for simulated *in vitro* fermentation.

Reactants	***Solutions***
- Sodium phosphate monobasic dehydrate ($NaH_2PO_4 \cdot 2H_2O$) - Tryptone - Cysteine - Sodium sulphide (Na_2S) - Resazurin HCl and NaOH to adjust buffer pH	- Fermentation medium - Reductive solution - Resazurin - Fermentation final solution

Fermentation medium: peptone water 15 g/L. 15 g of tryptone is added to almost a litre of distilled water and the pH adjusted to 7. Then, the volume is complemented to 1 L.

Reductive solution: 312 mg of cysteine and 312 mg of sodium sulphide are weighed in the same flask. After that 2 mL of 1 M NaOH is added and the volume is complemented to 50 mL.

Resazurin: resazurin solution at a concentration of 0.1 % (w/v).

Fermentation final solution: for each 10mL of fermentation medium 0.5 mL of reductive solution is added. For each 800 mL of mix 1 mL of resazurin solution is added. Finally, the solution is sterilized and nitrogen bubbled to make sure the medium is reduced. If the color of the solution is pink, the medium is reduced. Shaking might be needed to notice the color.

Fecal samples from healthy donors were obtained in the morning in sterile containers and stored at 4 ^{o}C till the inoculum was prepared. Briefly, 500 mg of digested wet-solid residue was added into a screw-cap tube, making sure that enough digested residue is left in order to determine its water content. Then, a 10 % of soluble fraction is added but only in proportion to the digested residue to be fermented (i.e. if the soluble fraction weigh was 36 mL, and the total digested residue weigh was 5g, the 10 % would be 3.6 mL to be added to 5 g of residue, but as the amount to be fermented is only 500 mg, the volume to be added is 0.36 mL). Then, 7.5 mL of fermentation final solution and 2 mL of inoculum (consisting of a solution of 32 % feces in phosphate buffer 100 mM, pH = 7.0) were added, reaching a final volume of 10 mL + soluble fraction volume.

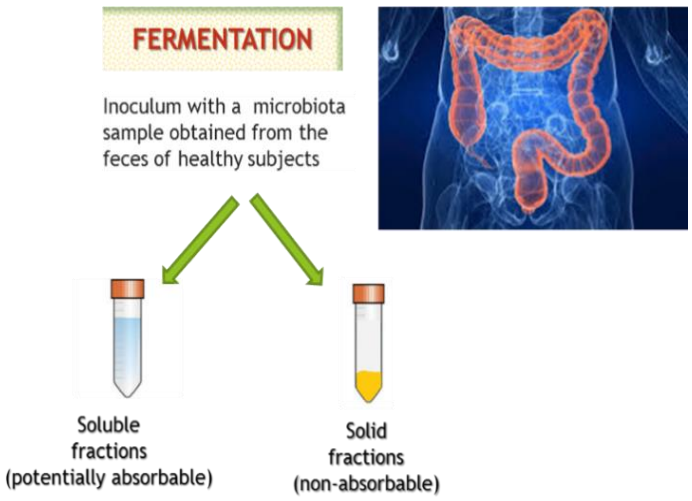

Figure 9. Scheme of *in vitro* fermentation

Nitrogen was bubbled in order to reach an anaerobic atmosphere and the mix was incubated at 37 ºC for 20 hours under oscillation. Right after, the samples were buried in ice to stop microbial activity and centrifuged. The supernatant was collected as fermented soluble fraction (FSF) potentially absorbable after fermentation and stored at -80 ºC. The solid residue, representing the unabsorbed fraction after fermentation, was also stored for direct antioxidant activity measuring.

3.2.3. Measurement of antioxidant capacity

The antioxidant capacity was measured in the fresh soluble fractions deriving from the digestion (DSF) and from the fermentation (FSF), and the lyophilized insoluble fractions obtained after fermentation. In particular, five different approaches were applied: FRAP assay, ABTS assay, Indigo Carmine AAPH method, Indigo Carmine OH method, Indigo Carmine RED method. The Figure 10. shows a scheme of the fractions analyzed by the five methods.

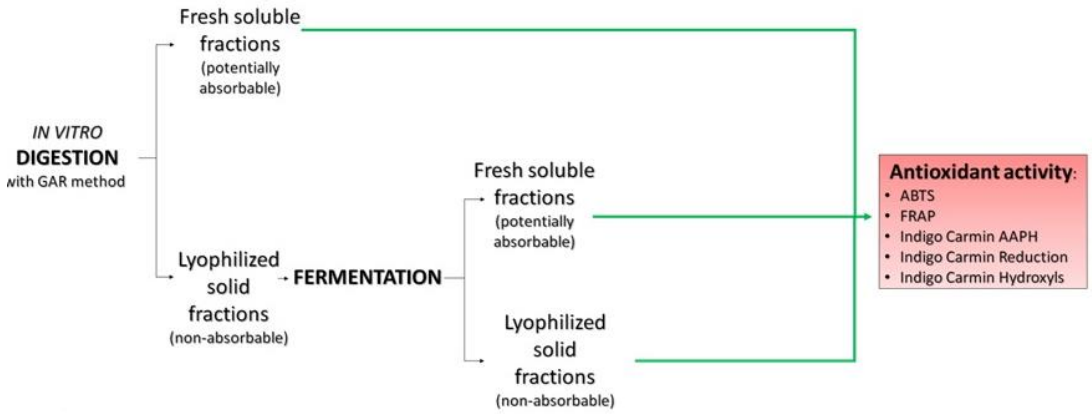

Figure 10. Scheme of the fractions analyzed

3.2.3.1. FRAP assay

To measure sample's reduction potential, one of the method used was the FRAP (Ferric-reducing ability power) assay. In Table 8. there are listed reagents and materials used for this method.

Table 8. Reagents and materials used for FRAP assay

Reagents	Materials
- **2,4,6-Tri(2-pyridyl)-s-triazyne (TPTZ)** - **6–Hydroxy–2,5,7,8–tetramethylchromane–2-carboxylic acid (Trolox)** - **Iron (III) chloride** - **Sodium acetate** - **NaOH and HCl 1M for pH adjustment**	- 96-well transparent polystyrene microplate (Biogen Científica, Spain) - FLUOStar Omega microplate reader (BMG Labtech, Germany)

Solutions preparation

40 mM HCl solution: 330 µL of HCL 37% is dissolved in 100 ml of distilled water.

10 mM TPTZ solution: 0.0312 g is weighed and dissolved in 10mL of 40 mM HCl.

20 mM Iron (III) chloride solution: 0.1352 g is weighed and dissolved in 25 mL of distilled water.

0.3 mM pH 3.6 acetate buffer: 0.0061 g is weighed and dissolved in 200 mL of distilled water. The pH is adjusted to 3.6 and the volume complemented to 250 mL.

Daily FRAP solution: mixed in this order: 25 mL of the buffer, 2.5 mL of Iron (III) solution and 2.5 mL of TPTZ solution.

The ferric reducing ability of each sample solution was estimated according to the procedure described by Benzie & Strain (Benzie & Strain, 1996) and adapted to a microplate reader. Briefly, 280 µl of FRAP reagent, prepared freshly and warmed at 37 ºC, was mixed in each well of a transparent 96-well polystyrene microplate with 20 µl of sample or water to provide appropriate blank reagent. Readings of maximum absorbance (595 nm) were taken every 60 s using a FLUOStar Omega microplate reader. Temperature was maintained at 37 ºC and the reaction was monitored for 30 minutes. Trolox stock solutions were used to perform the calibration curves. Results were also expressed as mmol equivalents of Trolox per mL of sample.

3.2.3.2. ABTS assay

ABTS method was conducted to further measure radical scavenging activity. Reagents and materials used for this assay are given in Table 9.

Table 9. Reagents and materials used for ABTS assay

Reagents	Materials
- **2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)** - **Potassium persulphate** - **6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox)** - **Ethanol** - **Water**	- 96-well transparent polystyrene microplate (Biogen Científica, Spain) - FLUOStar Omega microplate reader (BMG Labtech, Germany)

Solutions preparation

7 mM ABTS solution: 0.0384 g is weighed and dissolved in 10 mL of distilled water.

2.45 mM potassium persulphate: 0.0662 g is weighed and dissolved in 100 mL of distilled water.

$ABTS^{\cdot+}$ solution: solution 1 and 2 are mixed at 50:50. This solution is left in darkness for 16 hours before using it. This solution can be used during a week.

Water-ethanol (50:50).

Daily $ABTS^{\cdot+}$ solution: $ABTS^{\cdot+}$ solution is diluted with ethanol-water solution until obtain an absorbance of 0.70 ± 0.02 at 730 nm. The spectrophotometer is used for the measurement.

The antioxidant capacity was estimated in terms of radical scavenging activity following the procedure described in Re *et al.* (Re *et al.*, 1999). Briefly, $ABTS^{+\cdot}$ was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in a dark at room temperature for 12-16 h before use. The $ABTS^{\cdot+}$–solution was diluted with an ethanol:water (50:50) mixture to an absorbance of 0.70 ± 0.02 at 730 nm. After placing 20 µl of sample or Trolox standard, 280 µL of diluted $ABTS^{\cdot+}$–solution was added on a transparent 96-well polystyrene microplate. Absorbance readings were taken every 60 s for 20 min on a FLUOStar Omega microplate reader with temperature control (37 ºC). Calibration was performed, as described previously, with a Trolox stock solution. Results were expressed as mmol equivalents of Trolox per mL of sample.

3.2.3.3. Indigo Carmine AAPH method

The first assay using Indigo Carmine as indicator is the one with AAPH radical. Reagents and materials used for this method are listed in Table 10.

Table 10. Reagents and materials used for Indigo Carmine AAPH method

Reagents	Materials
- **Sodium hydrogenophosphate (NaH_2PO_4)** - **Indigo Carmine** - **6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid(Trolox)** - **2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH)** - **NaOH and HCl 1M for pH adjustment**	- 96-well transparent polystyrene microplate (Biogen Científica, Spain) - FLUOStar Omega microplate reader (BMG Labtech, Germany)

Solutions preparation

Phosphate buffer 0.075 M pH 7.24: for 1 L 11.700 g of sodium hydrogenophosphate is weighed and dissolved in almost a liter of distilled water. Then the pH is adjusted up to 7.24 and the volume complemented to 1 L.

Indigo Carmine solution 1 mM: for 100 mL 0.04663g is weighed and dissolved in 100 mL of distilled water.

AAPH solution (1.5 g/10 mL): 1.5 g of AAPH is weighed and dissolved in 10 mL of distilled water.

Daily solution: 4mL of Indigo Carmine solution and 22 mL of phosphate buffer are mixed.

The assay was carried out using a FLUOStar Omega microplate reader with temperature control (37 ºC) and measuring the absorbance at 610 nm. Each well of the transparent 96-well polystyrene microplate used contained 40 µL of Indigo Carmine indicator (1 mM), 220 µL of phosphate buffer (75 mM; pH 7.24), 20 µL of AAPH solution and 20 µL of sample or standard. Each assay was carried out with two blanks: one containing indicator and buffer and the other with indicator, buffer and the AAPH solution. Absorbance was measured every 60

seconds in kinetic mode and the absorbance values used were those obtained after 65 minutes of reaction. The calibration curve was obtained using Trolox, ranging from 1.00 mg/mL to 0.01 mg/mL, and the results are expressed as μmol Trolox equivalent per mL of sample.

This method uses the AAPH radical which is a synthetic molecule aiming to simulate peroxyl radicals (produced during fat oxidation). AAPH radical will oxidize the indicator turning from blue to transparent. The standard or the sample due to their activity against this radical will reduce the indicator oxidation.

3.2.3..4. Indigo Carmine OH method

Another method conducted using Indigo Carmine indicator was the one with hydrogen peroxide. Reagents and materials used for its assessment are listed in Table 11.

Table 11. Reagents and materials used for Indigo Carmine OH method

Reagents	Materials
- **Sodium hydrogenphosphate (NaH_2PO_4)** - **Indigo Carmine** - **Iron (III) chloride ($FeCl_3 \cdot 6H_2O$)** - **Hydrogen peroxide 30% (8.82M) (H_2O_2)** - **Sodium phosphotungstate tribasic hydrate ($Na_3[P(W_3O_{10})_4] \cdot aq$)** - **Catechin** - **NaOH and HCl 1M for pH adjustment**	- 96-well transparent polystyrene microplate (Biogen Científica, Spain) - FLUOStar Omega microplate reader (BMG Labtech, Germany)

Solutions preparation

Phosphate buffer 0.5 M pH 7.24: for 1 L 78.005 g of sodium hydrogenphosphate is weighed and dissolved in almost a liter of distilled water. Then the pH is adjusted up to 7.24 and the volume complemented to 1 L.

Indigo Carmine solution 1 mM: for 100 mL 0.04663g is weighed and dissolved in 100 mL of distilled water.

100 μM Iron (III) solution: to obtain a 10 mM solution 0.0027 g is weighed and dissolved in 1 mL of distilled water. To obtain the 100 μM Iron (III) solution, the 1/100 dilution should be made.

100 μM phosphotungstate solution: to obtain a 1mM solution, 0.2946 g is weighed and dissolved in 100 mL of distilled water. The 1/10 dilution should be made.

Iron III+phosphotungstate solution: solution of iron (III) solution and phosphotungstate solution (50:50) are mixed together.

Hydrogen peroxide solution (0.88 M): 1 mL of hydrogen peroxide is taken from the reagent bottle and the volume is complemented to 10 mL.

Daily solution: 14.5 mL of phosphate buffer, 3 mL of Iron III+phosphotungstate solution and 1.5 mL of Indigo Carmine solution are mixed together.

The assay was carried out using a FLUOStar Omega microplate reader with temperature control (37 ºC) and measuring the absorbance at 610 nm. A transparent 96-well polystyrene microplate was used, in which each well contained 190 μL of daily solution, 20 μL of freshly prepared H_2O_2 (4 M) and 90 μL of sample or standard. Each assay was carried out with two blanks: one containing indicator and buffer and the other with indicator, buffer and the Fenton reagents. Absorbance was measured every 60 seconds in kinetic mode and the absorbance values used were those obtained at 80 minutes. The calibration curve was obtained using catechin, ranging from 0.1 mg/mL to 10 mg/ml, and the results are expressed as μmol catechin equivalent per mL of sample.

3.2.3.5. Indigo Carmine RED method

The last conducted method of those using Indigo Carmine indicator in reactions is this one, measuring capacity of sample to reduce indicator. Reagents and materials needed for this method are given in Table 12.

Table 12. Reagents and materials used for Indigo Carmine RED method

Reagents	Materials
- **Sodium hydrogenophosphate (NaH2PO4)** - **Indigo Carmine** - **Catechin** - **NaOH and HCl 1 M for pH adjustment**	- 96-well transparent polystyrene microplate (Biogen Científica, Spain) - FLUOStar Omega microplate reader (BMG Labtech, Germany)

Solutions preparation

Phosphate buffer 0.5 M pH 7.24: for 1 L 78.005 g of sodium hydrogenophosphate is weighed and dissolved in almost a liter of distilled water. Then the pH is adjusted up to 7.24 and the volume complemented to 1 L.

Indigo Carmine solution 1 mM: for 100 mL 0.04663 g is weighed and dissolved in 100 mL of distilled water.

Daily solution: 1.5 mL of Indigo Carmine solution and 18.5 mL of phosphate buffer are mixed together.

The assay was carried out using a FLUOStar Omega microplate reader with temperature control (37 ºC) and measuring the absorbance at 400 nm. Each well of the transparent 96-well polystyrene microplate used contained 200 µL of daily solution and 100 µL of sample. Absorbance was measured every 60 seconds in kinetic mode and the absorbance values used were those obtained after 60 minutes of reaction. The calibration curve was obtained using catechin, ranging from 0.1 mg/mL to 50.0 mg/ml, and the results are expressed as µmol catechin equivalent per mL of sample.

In this method, we measure the global reduction capacity of the sample due to the ability of the indicator to turn green when it is being reduced.

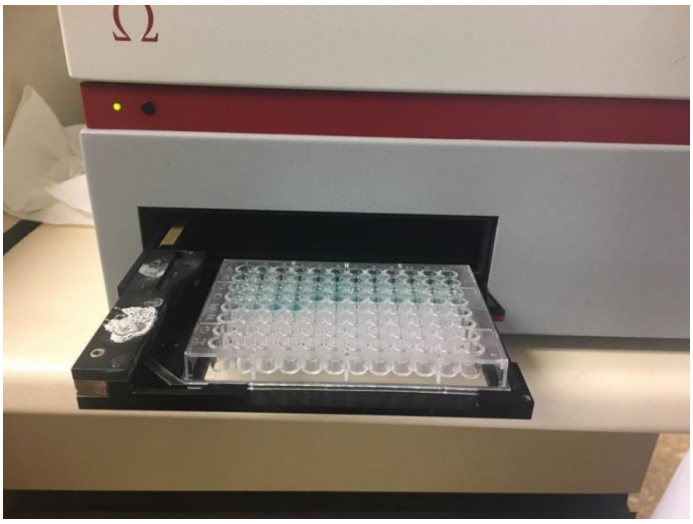

Figure 11. Microplate with samples in microplate reader

4. RESULTS AND DISCUSSION

In this study it has been investigated whether addition of spent coffee grounds to soil has impact on antioxidant capacity enhancement of lettuce. Antioxidant capacity of lettuce grown on soil enriched with SCG was evaluated after simulated *in vitro* digestion and fermentation. The antioxidant capacity was measured in the fresh soluble fractions deriving from the digestion (DSF) and from the fermentation (FSF), and the lyophilized insoluble fractions obtained after fermentation. In particular, five different approaches were applied: FRAP assay, ABTS assay, Indigo Carmine AAPH method, Indigo Carmine OH method, Indigo Carmine RED method. In this part results of all five methods will be shown and briefly discussed.

4.1. FRAP assay

Results of FRAP assay are shown in Figures 12. and 13. The NPK results show higher antioxidant capacity of lettuce grown on soil treated with SCG, compared with samples grown on soil treated with same fertilizer without SCG (the largest increase is notable at samples grown on soil treated with potassium and phosphorus and their mixture; while almost no difference is noted at samples grown on soil treated with nitrogen and potassium-nitrogen mixture), neither compared with control (sample not treated with SCG nor with fertilizers). Furthermore, lettuce grown on soil treated only with fertilizers generally doesn’t have a higher antioxidant capacity in comparison with the control.

In addition, results of the N experiment show that antioxidant capacity of majority of samples is slightly increasing with SCG addition in soil; and the increment is being less for samples with more nitrogen added. Besides, all samples grown on soil treated with nitrogen have lower antioxidant capacity than the control one (when soil wasn't treated with nitrogen nor SCG).

According to these results, nitrogen treatment might be the reason of lower antioxidant capacity of samples grown on soil treated with SCG and nitrogen alone and/or combined with potassium and phosphorus. On the other hand, according to Cruz and dos Santos (2015), SCG may cause nitrogen immobilization and consequently decrease of antioxidant capacity. Mixture of nitrogen, phosphorus and potassium may not cause such decrease because it

contains nitrogen in other form (directly added to compost), but also because of presence of phosphorus.

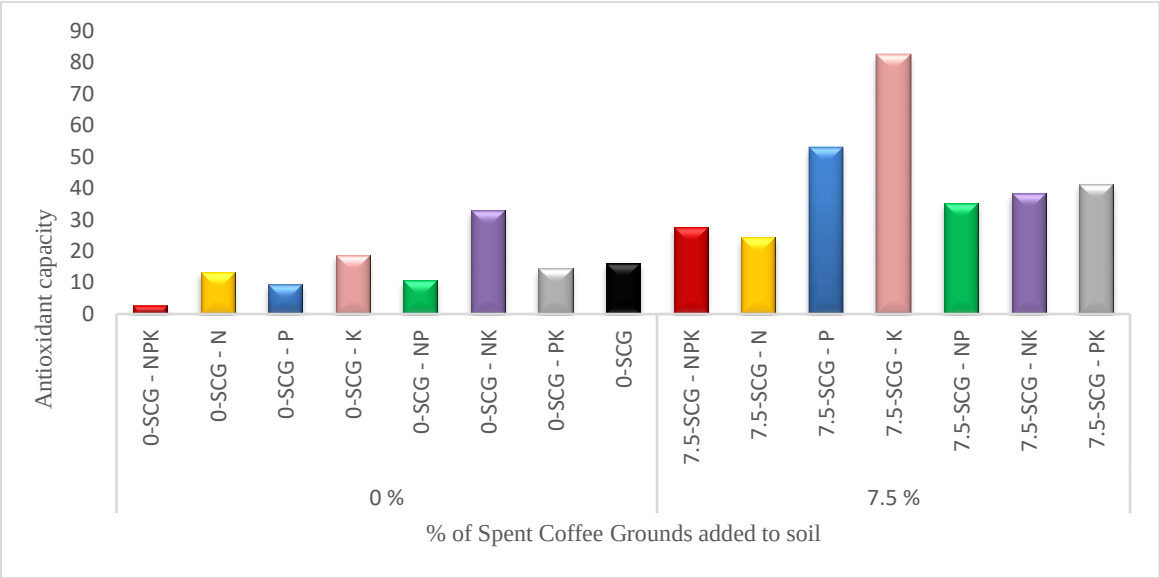

Figure 12. Antioxidant capacity of lettuce grown on soil treated with N/P/K measured by FRAP method after *in vitro* digestion and fermentation depending on SCG addition. Results are means of 3 values of each sample.

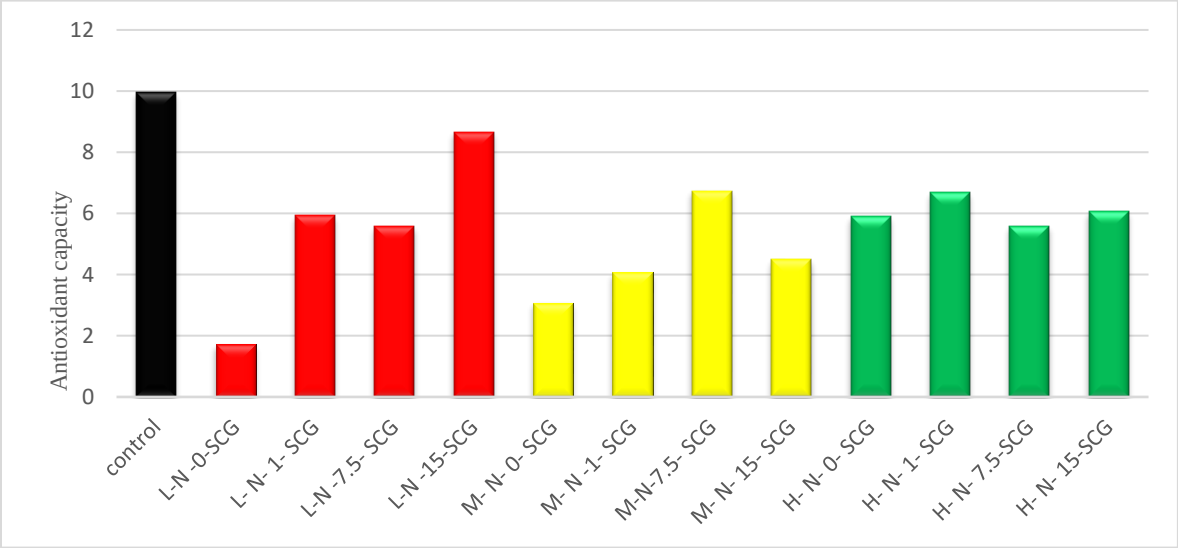

Figure 13. Antioxidant capacity of lettuce grown on soil treated with N measured by FRAP method after *in vitro* digestion depending on SCG addition. Results are means of 3 values of each sample.

4.2. ABTS assay

Results of ABTS assay are shown in Figures 14. and 15. The NPK experiment results show a trend of increasing antioxidant capacity with addition of SCG in soil for all samples compared with those without SCG amendment. The largest increment is noted for samples grown on soil treated with K and P and their mixture, while the samples grown on soil treated with N and NK mixture have the least increment. Compared with the control, all samples grown on soil treated with SCG have higher antioxidant capacities, while those grown on soil not treated with SCG don't, in general, show increased antioxidant capacities.

The N experiment results show remarkable progressive antioxidant capacity increase with addition of SCG for low N–treated samples; unlikely to medium N- and especially high N-treated samples that show progressive antioxidant capacity decrease with SCG addition.
In comparison with control, no trend of increase or decrease of antioxidant capacity is noted.
Nitrogen alone and in combination with potassium seems to allow only a slight increase of antioxidant capacity of samples although SCG amendment (unlikely to NP and NPK mixture); or SCG may cause N immobilization and therefore decrease of antioxidant capacity.

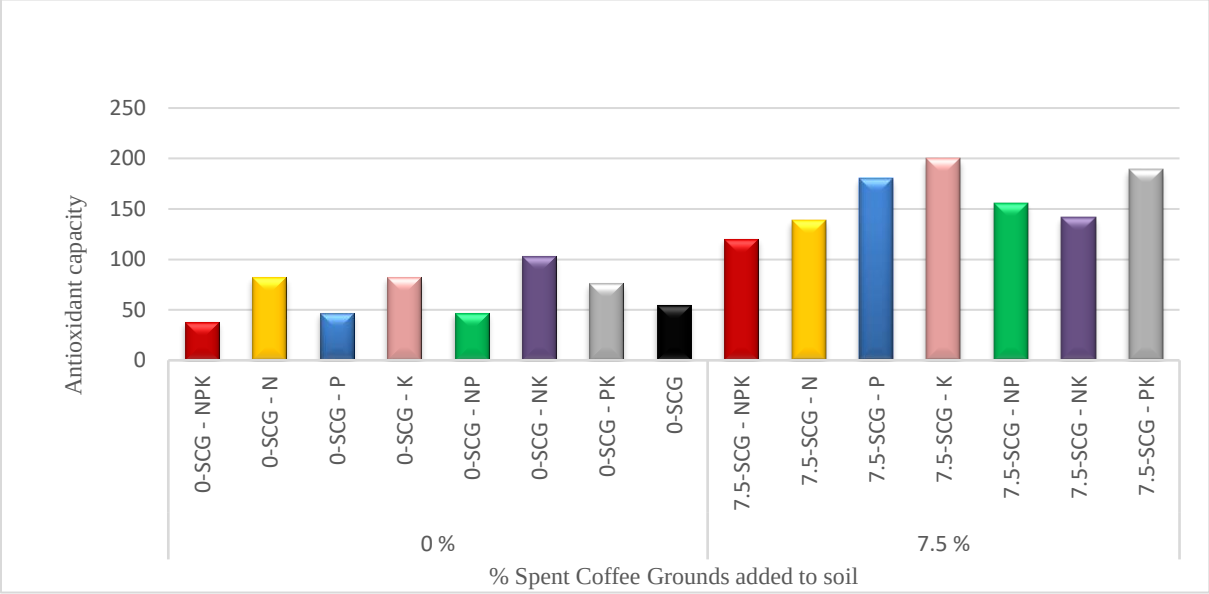

Figure 14. Antioxidant capacity of lettuce grown on soil treated with N/P/K measured by ABTS method after in vitro digestion and fermentation depending on SCG addition. Results are means of 3 values of each sample.

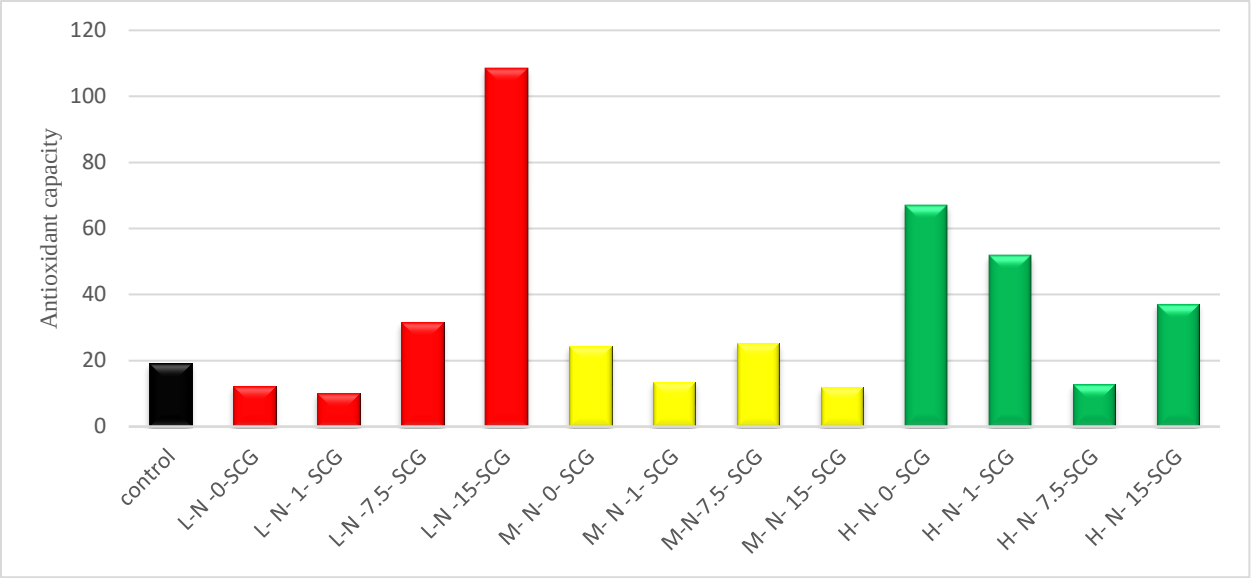

Figure 15. Antioxidant capacity of lettuce grown on soil treated with N measured by ABTS method after digestion depending on SCG addition. Results are means of 3 values of each sample.

4.3. Indigo Carmine AAPH method

Results of Indigo Carmine AAPH method are shown in Figures 16. and 17. From the results of NPK experiment it can be noted a significant enhancement of antioxidant capacity of samples grown on soil treated with K, NP and PK when adding SCG. Samples grown on soil treated with NPK, N, NK and P don't, on the contrary, have an insignificant antioxidant capacity enhancement despite adding the same quantity of SCG. Comparing with control, all samples, whether grown on soil with or without SCG, have anitoxidant capacity values similar to the control sample, except K, NP and PK treated with SCG.

Regarding to results of the N experiment, a trend of antioxidant capacity enhancement can be noted when augmenting SCG addition; however, that trend is going to a lesser extent as the N level is growing. In comparison with control, all samples have much higher antioxidant capacity levels, especially those treated with higher SCG percentages.

To sum up, it can be noted that most samples, both in N and NPK experiment, grown on soil treated with nitrogen (alone or mixed with other minerals) reveal antioxidant capacity enhancement in lesser extent than other samples, especially when comparing to samples grown on soil treated with potassium.

Still, in comparison to other samples grown on soil not treated with SCG, samples grown on soil treated with nitrogen and its mixtures don't show lower antioxidant capacity values.

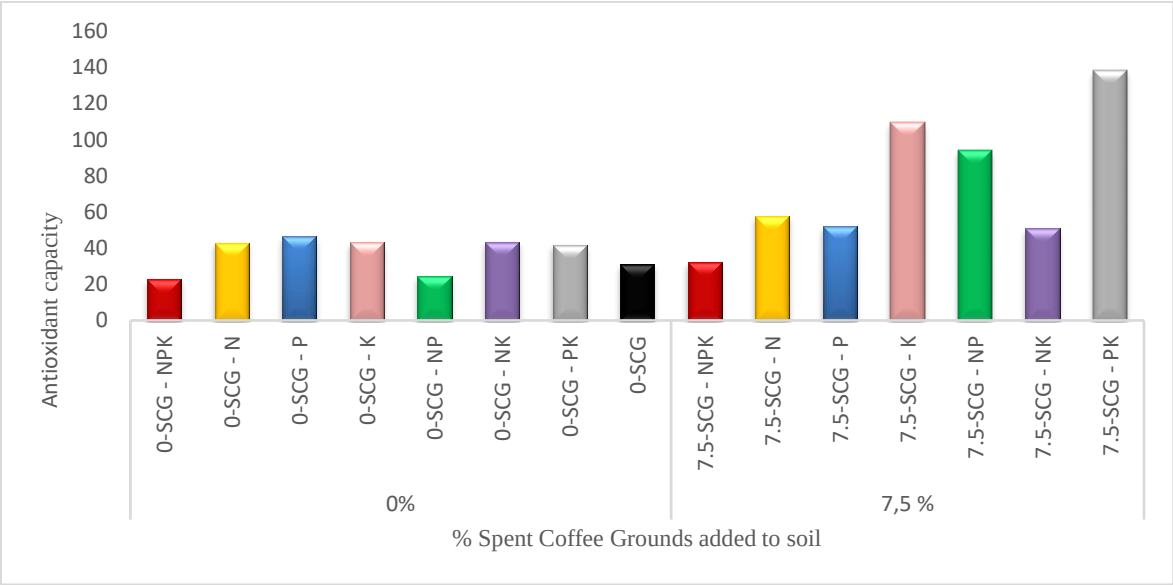

Figure 16. Antioxidant capacity of lettuce grown on soil treated with N/P/K measured by indigo Carmine AAPH method after digestion and fermentation depending on SCG addition. Results are means of 3 values of each sample.

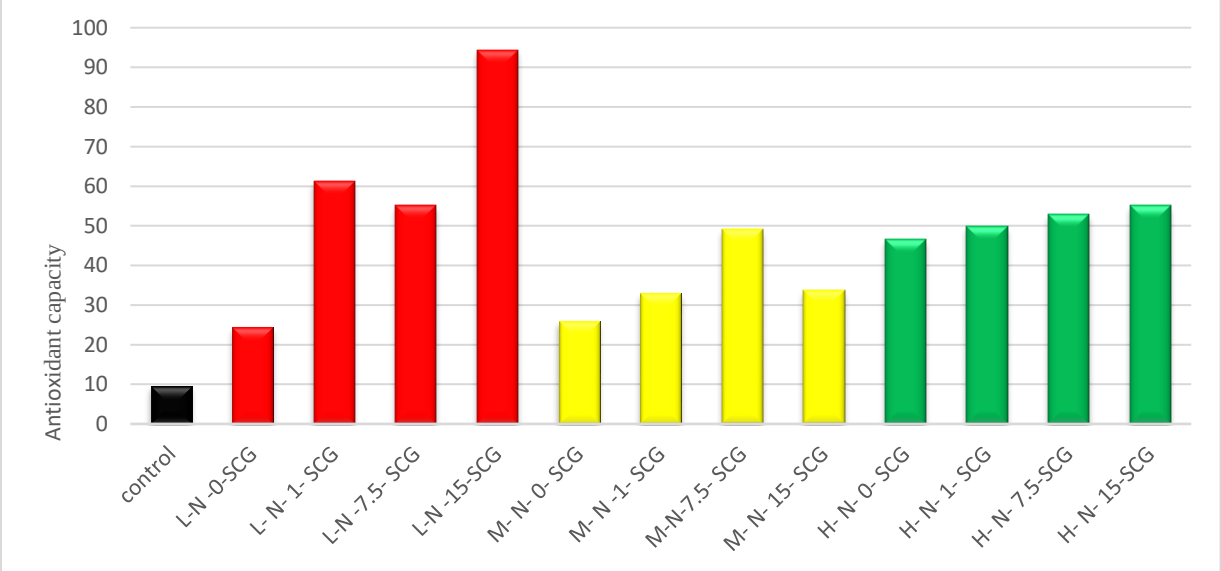

Figure 17. Antioxidant capacity of lettuce grown on soil treated with N measured by indigo Carmine AAPH method after digestion depending on SCG addition . Results are means of 3 values of each sample.

4.4. Indigo Carmine OH method

Figures 18. and 19. show results of Indigo Carmine OH method. Unlikely to results measured by other methods, these results show quite higher antioxidant capacity of control sample comparing to samples grown on soil treated with minerals (without SCG), but also with some of those grown on soil treated with SCG. Samples grown on soil treated with K and PK, anyway, are showing similar AOH increase after SCG amendment to soil as in previous methods; while N and NPK treated samples show a bit higher antioxidant capacity increase after SCG addition to soil than previously seen. The antioxidant capacities of NP and NK treated samples are still constant in not appearing affected by SCG amendment to soil; and antioxidant capacity of P-treated sample measured by this method is even decreased under SCG influence.

Results of the N experiment are not showing a specific trend of increased or decreased antioxidant capacities related to SCG amendment; although values of samples grown on soil treated with less nitrogen are still higher and growing in a bit greater extent. An exception is the sample grown on soil treated with high N level and no SCG, with unexplainable quite high antioxidant capacity (what might be applied also for L-N-1-SCG sample). Regarding to control, most samples have very similar, and some of them much higher antioxidant capacities. Despite these exceptions, which might have been caused by some measuring or calculating mistake, or simply by sample difference; mutual impact of K, PK and SCG on antioxidant capacity increase is remaining constant.

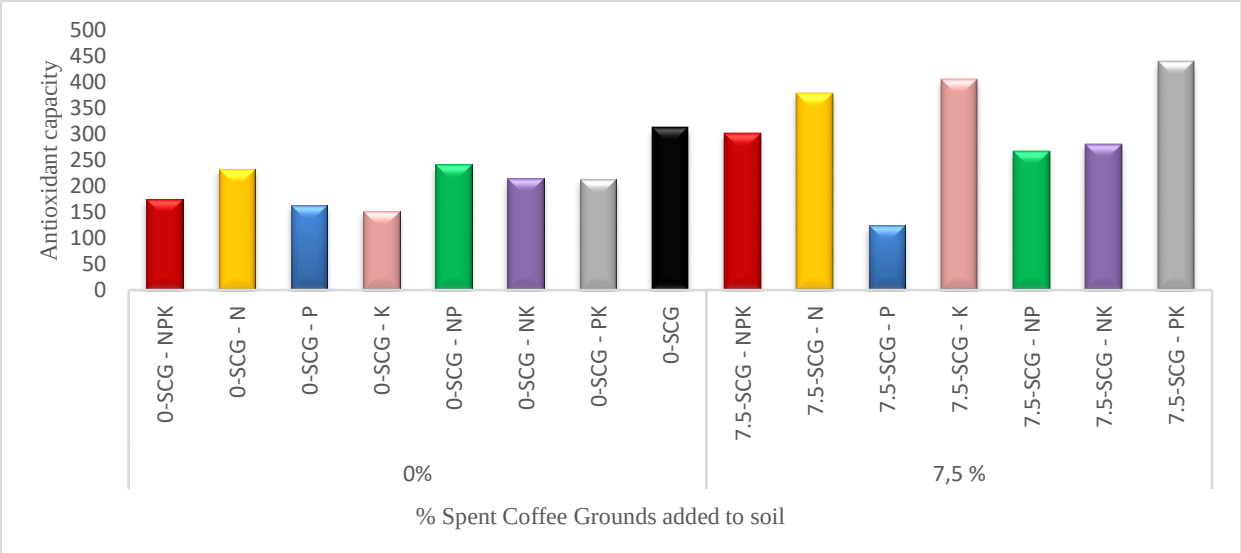

Figure 18. Antioxidant capacity of lettuce grown on soil treated with N/P/K measured by indigo Carmine OH method afer *in vitro* digestion and fermentation depending on SCG addition. Results are means of 3 values of each sample.

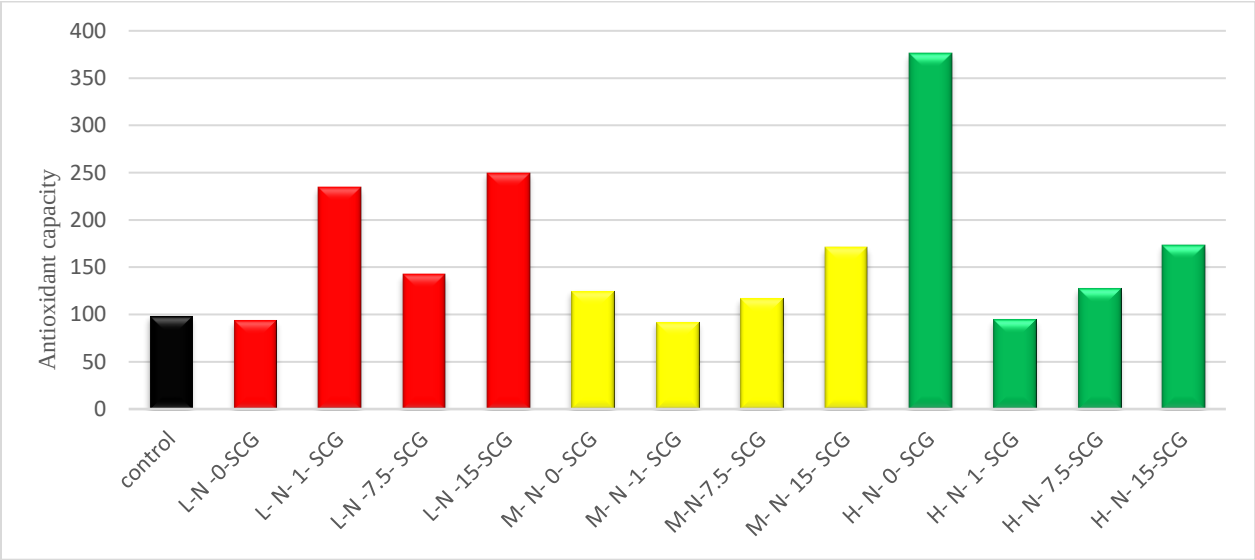

Figure 19. Antioxidant capacity of lettuce grown on soil treated with N measured by Indigo Carmine OH method after digestion depending on SCG addition . Results are means of 3 values of each sample.

4.5. Indigo Carmine RED method

Indigo Carmine RED method results of NPK experiment show increased antioxidant capacity values for all samples grown on soil treated with SCG, especially the K, P and PK treated samples. N and NPK treated samples have, unlikely to previous results, extensively increased antioxidant capacities under SCG-treatment; while NP and NK samples seem to follow results of former methods. As about the control, all SCG-treated samples (except the NP sample) have higher antioxidant capacities, and samples grown on soil without SCG have lower antioxidant capacities.

N experiment results show a trend of increasing antioxidant capacity values proportionally to SCG addition and N-treatment levels. Also, all samples have similar (L-N-0-SCG and M-N-0-SCG) or quite higher antioxidant capacities than the control. Compared with others, this methods' results seem to be an exception looking at nitrogen effect on antioxidant capacity increment; and the only with similar effect on SCG treated samples are K and PK treatment.

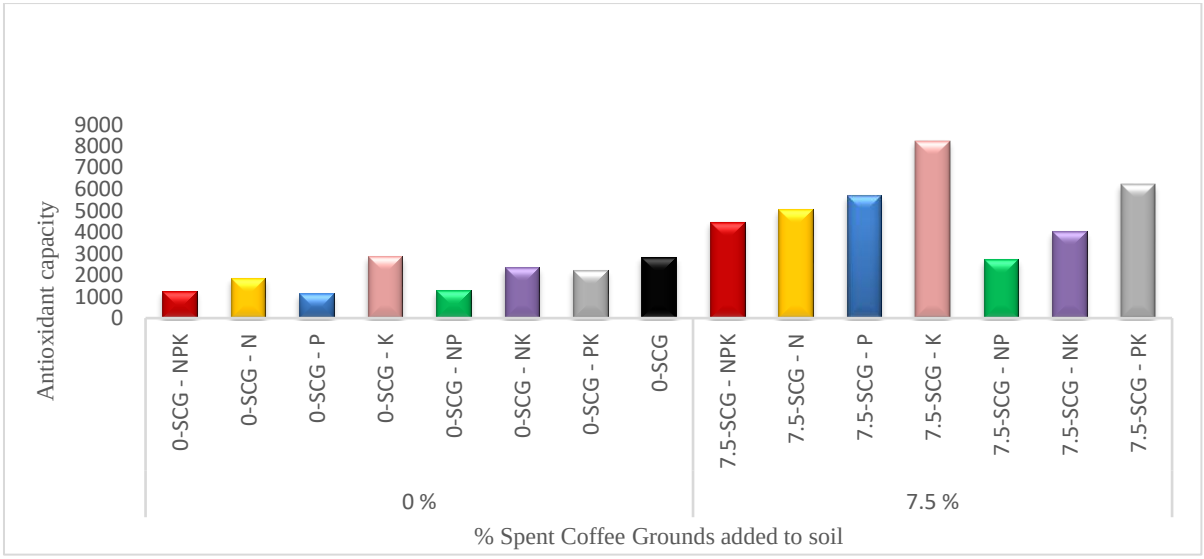

Figure 20. Antioxidant capacity of lettuce grown on soil treated with N/P/K measured by indigo Carmine RED method after *in vitro* digestion and fementation depending on SCG addition. Results are means of 3 values of each sample.

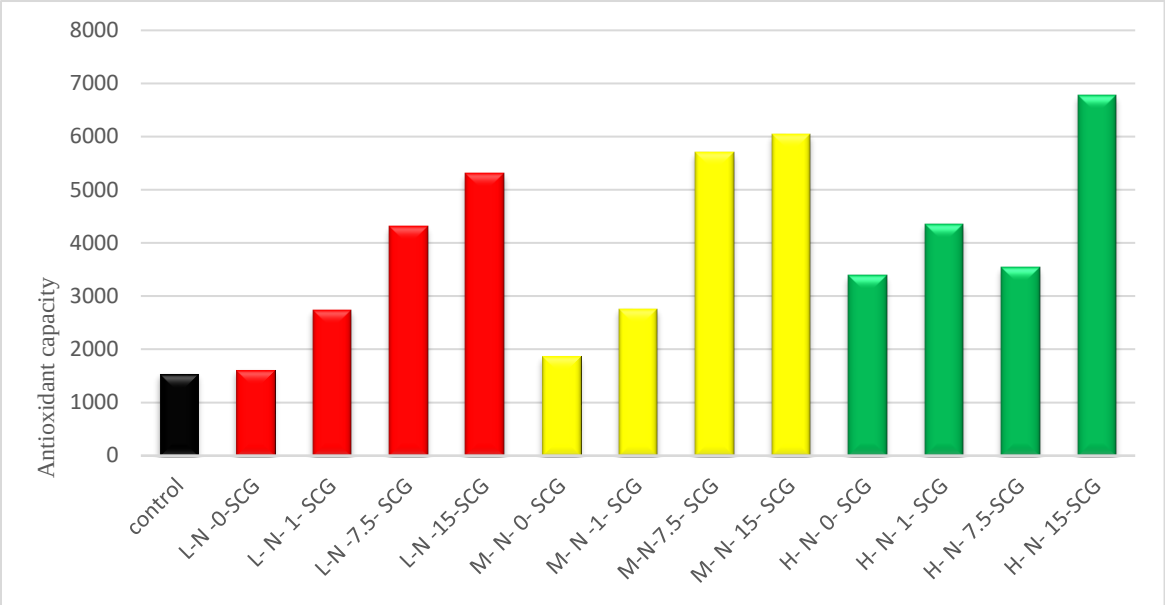

Figure 21. Antioxidant capacity of lettuce grown on soil treated with N measured by Indigo Carmine RED method after *in vitro* digestion depending on SCG addition. Results are means of 3 values of each sample.

5. CONCLUSIONS

Based on the results presented in this Thesis following conclusions can be made:

1. According to FRAP assay results, spent coffee grounds have remarkable impact on increased antioxidant capacity of lettuce, especially in presence of potassium. Spent coffee grounds in combination with nitrogen have influence on a slight increase in antioxidant capacity values.
2. ABTS assay results lead to conclusion that spent coffee grounds have a positive impact on lettuce's antioxidant capacity increment, and the best results are achieved when combined with potassium and phosphorus. Nitrogen is neither in this case related to antioxidant capacity increment.
3. Conclusion that can be made from Indigo Carmine AAPH method results is antioxidant capacity enhancement related with spent coffee grounds addition to soil. That enhancement is going to lesser extent with addition of nitrogen, oppositely of potassium and potassium-phosphorus and -nitrogen mixture.
4. According to IC OH assay results, antioxidant capacity of lettuce is increasing regarding to spent coffee grounds amendment to soil; that impact is higher when potassium and potassium-phosphorus mixture is added. All in all, nitrogen is in this assay, too, related to lower antioxidant capacity than other minerals.
5. Indigo Carmine RED method results show expected increment of antioxidant capacity by spent coffee grounds amendment; especially in combination with potassium and phosphorus and their mixture. In this case, nitrogen amendment is related to a large enhancement of antioxidant capacity of lettuce.
6. To sum up, antioxidant capacity has, in general, been increased for all samples grown on soil treated with spent coffee grounds. According to available literature, there are two possible reasons: antioxidants from spent coffee grounds have been absorbed by lettuce tissue through the soil; or spent coffee grounds amendment to soil has caused stress for a plant and, consequently, the increase of antioxidant capacity, since plants produce more polyphenols in order to compete the stress.

7. Addition of minerals (except from nitrogen) seems to have contributing impact on that enhancement, while added alone do not have an effect. The best results are being achieved with combined addition of spent coffee grounds and potassium. Phosphorus has similar effects, although not constant in all assays.
8. In the nitrogen case, real reason and causal-consequent relationship can't be confirmed so far.

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