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COFFEE BY-PRODUCTS

Sustainable Agro-Industrial Recovery and Impact on Vegetables Quality

Dissertation of 2th Cycle of Studies Leading to the Master's Degree in Quality Control
Specialization in Food and Water

Performed under the supervision of:
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SUB-PRODUTOS DO CAFÉ

Recuperação Sustentável da Agro-Indústria e Impacto na Qualidade de Vegetais

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ABSTRACT

Food wastage has reached alarming levels, with almost one-third of food produced for human consumption lost or wasted every year. Therefore, several food by-products have been studied in order to assess their potential to be totally or partially reused in food industry or others. Coffee industry is a classical model of food waste generation during the whole production process, from the field to the cup. Many attempts have been made with the purpose of giving a new life to these residues, though very few information is available for coffee remains produced in cafeteria/household environments, as espresso spent coffee grounds (SCG).

Due to its recognised richness in bioactive compounds, the effect of SCG on lettuce nutritional composition was assessed. At first, a randomised greenhouse pot experiment was conducted with different amounts of fresh and composted SCG, allowing to conclude that low amounts of composted-SCG (up to 15%, v/v) led to highly significant increment of essential mineral elements in Butterhead lettuce cultivar “Four Seasons”, as potassium, manganese, magnesium and sodium. Furthermore, a slight enhancement of antioxidant compounds was also verified for low amounts of composted-SCG (up to 20%, v/v). All these effects were accompanied by increased yield, proportionally to the amounts of composted-SCG, of utmost importance for producers. In opposition, fresh-SCG induced a lower mineral content and low yield, but higher carotenoids, chlorophylls and tocochromanols amounts, which was attributed to the oxidative stress induced by bioactive compounds (such as caffeine) that are still present in this by-product and low mineral availability. Based on the positive outcomes of composted-SCG treatments, a final studied was then carried out in a field experiment with Batavia lettuce cultivar “Rolina” using SCG composted directly in the soil. Again, low amounts of composted-SCG (up to 10%, v/v) lead to an evident enhancement of vegetables nutritional profile, without yield loss.

Hence, SCG composting seems to be a practical approach that offers an alternative and direct reuse for this by-product, extendable to other crops, providing value-added vegetable products.

Keywords: Bioactive compounds; Food chemistry; Lettuce; Spent coffee grounds; Waste reuse

RESUMO

O desperdício alimentar atingiu níveis preocupantes, com cerca de um terço dos alimentos produzidos para consumo humano desperdiçado anualmente. Assim, diversos sub-produtos alimentares têm sido estudados de forma a avaliar o seu potencial para serem total ou parcialmente reutilizados na indústria alimentar, ou outras. A indústria do café é um exemplo clássico da formação de resíduos alimentares durante todo o processo de produção, desde o campo até à chávena. Têm vindo a ser feitas várias tentativas com o intuito de dar uma nova vida a estes resíduos, apesar de ainda estar disponível pouca informação para os resíduos produzidos em cafetarias/ambientes domésticos, em particular as borras de café expresso.

Devido à riqueza da borra de café em compostos bioactivos, foi investigado o seu efeito na composição nutricional da alface. Em primeiro lugar, foi conduzido um estudo aleatório em vaso e estufa com diferentes quantidades de borra de café espresso fresca e compostada, permitindo concluir que pequenas porções de borra de café compostada (até 15%, v/v) levaram a um aumento altamente significativo de minerais essenciais em alface Bola de Manteiga cultivar "Quatro Estações", como o potássio, manganês, magnésio e sódio. Para além disso, foi verificada uma ligeira melhoria dos compostos antioxidantes para quantidades de borra de café compostada até 20%, v/v. Estes efeitos foram acompanhados por um aumento do rendimento produtivo, proporcional à quantidade de borra de café, de elevada importância para os produtores. O cultivo com borra de café fresca originou um baixo conteúdo em minerais e baixo rendimento, mas um elevado em carotenóides, clorofilas e tococromanóis, tendo sido atribuído ao stress oxidativo induzido pelos compostos bioactivos (nomeadamente a cafeína) ainda presentes neste sub-produto e a baixa disponibilidade em minerais. Baseado nos resultados positivos dos tratamentos com borra de café compostada, foi executado um estudo final em campo com alface Batavia cultivar "Rolina" utilizando borra de café compostada directamente no solo. Os resultados foram coerentes com os anteriormente obtidos, i.e. reduzidas quantidades de borra de café compostada (até 10%, v/v) levando a uma evidente melhoria do perfil nutricional dos vegetais, sem perda de rendimento.

Em conclusão, a compostagem de borra de café parece ser uma abordagem prática que oferece uma reutilização alternativa e directa deste sub-produto, extensível a outras culturas, providenciando produtos vegetais de valor acrescentado.

Palavras-chave: Compostos bioactivos; Química alimentar; Alface; Borra de café; Reutilização de resíduos

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- **Cruz R**, Gomes T, Ferreira A, Mendes E, Baptista P, Cunha S, Pereira JA, Ramalhosa E, Casal S. Antioxidant activity and bioactive compounds of lettuce improved by espresso coffee residues. Food Chem 2014. 145: 95-101.

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ABBREVIATIONS

a*	–	Redness
ADPPH	–	Absorbance of DPPH Control Solution
ANOVA	–	Analysis of Variance
AOAC	–	Association of Official Analytical Chemists
AS	–	Absorbance of Solution
b*	–	Yellowness
BHT	–	Butylated Hydroxytoluene
C_{ab}*	–	Chroma
CI*	–	Colour Index
CV	–	Canonical Variate
DPPH	–	2,2-Diphenyl-1-picrylhydrazyl
DW	–	Dry Weight
EC_{50}	–	Half Maximal Effective Concentration
ECR	–	European Commission Regulation
EPA	–	U.S. Environmental Protection Agency
FAO	–	Food And Agriculture Organization of the United Nations
FCR	–	Folin-Ciocalteu Reagent
FLD	–	Fluorescence Detector
FW	–	Fresh Weight
GAE	–	Gallic Acid Equivalents
h_{ab}	–	Hue Angle
HPLC	–	High-Performance Liquid Chromatography
HR-CS-FAAS	–	High-Resolution Continuum Source Flame Atomic Absorption Spectrometry
IS	–	Internal Standard
L*	–	Lightness
LDA	–	Linear Discriminant Analysis
MANOVA	–	Multivariate Analysis Of Variance
n.d.	–	Not Detected
n.s.	–	Not Significant
PC	–	Principal Component Factor
PCA	–	Principal Component Analysis
PDA	–	Photodiode Array Detector
rpm	–	Rotations Per Minute
SCG	–	Spent Coffee Grounds
AA	–	Total Ascorbic Acid
TCEP	–	Tris(2-carboxyethyl)phosphine
TOC	–	Total Organic Carbon

TON	–	Total Organic Nitrogen
TPC	–	Total Phenolic Content
V	–	Volume

THEORETICAL PART

1. FOOD MANAGEMENT AND WASTE REUSE

1.1. Introduction

1.2. Major Food Waste Sources And Target Elements

1.2.1. *Food wastage chain and environmental impact*

1.2.2. *Food by-products and their recovery*

1.1. Introduction

Food losses represent a major negative issue in the efforts to balance food resources, with severe economic and environmental impacts. Despite the frequent media statements on the urgent need to increase food production to meet present and future global demands, it seems that insufficient attention is paid to current losses in global food supply chain, which are probably substantial, including its resulting carbon and water footprint.

Approximately one-third of food produced for human consumption is lost or wasted worldwide, which amounts to about 1.3 billion tons/year (FAO, 2011). According to Food and Agriculture Organization of the United Nations (FAO), “food loss” refers to a decrease in mass (dry matter) or nutritional value (quality) of food that was originally intended for human consumption (FAO, 2013a). These losses are mainly caused by inefficiencies in the food supply chains, lack of access to markets or eventual natural disasters (FAO, 2013a). Furthermore, the same document also defines “food waste” as the food, appropriate for human consumption, which is discarded, whether or not after it is kept beyond its expiry date or left to spoil, being in line with the European legislation (ECD, 2008) definition of “waste”, though “bio-waste” better defines these food remains. On the other hand, the term “food wastage” embraces both food loss and food waste (FAO, 2013a).

In general, food is either lost or wasted throughout their supply chain, from initial production down to final household consumption. In fact, in medium- and high-income countries, food is significantly wasted at the consumption stage, though substantial losses also occur early in pre-consumption phases. For instance, *per capita* food wastage in Europe and North-America is estimated to be near 280-300 kg/year, in sub-Saharan Africa and South/Southeast Asia it is reduced to 120-170 kg/year, while in low-income countries only a small part of this wastage is observed at the consumer level (FAO, 2011). FAO estimated that the *per capita* food waste by consumers in Europe and North-America is around 95-115 kg/year, while this figure in sub-Saharan Africa and South/Southeast Asia is only 6-11 kg/year (FAO, 2011).

A combination of consumers’ behaviour and lack of communication in the supply chain underlies the previously described high levels of food waste in affluent societies. Indeed, the causes of food wastage in low-income countries are mainly related to financial or technical limitations in harvesting techniques, storage and cooling facilities (FAO, 2011). On the other hand, in medium/high-income countries, these are mainly connected to

consumer behaviour, including over-supply, as well as to a lack of coordination between different actors in the supply chain (FAO, 2011).

Therefore, FAO has elaborated a toolkit detailing three general levels where action is needed in order to divert food from landfills, being in agreement with U.S. Environmental Protection Agency recommendations (EPA) (FAO, 2013b; EPA, 2014):

1. Food wastage reduction: Beyond improving losses of crops on farms due to poor practices, doing more to better balance production with demand;
2. Re-usage within the human food chain: Finding secondary markets or donating extra food to feed vulnerable members of society. If the food is not fit for human consumption, it should be diverted for livestock feed;
3. Recycling and recovery: Waste recycling, anaerobic digestion, composting, and incineration with energy recovery allow energy and nutrients to be recovered from food waste.

With the effort to evaluate and diminish food wastage, several action programmes and campaigns have been initiated all over the world: Australian Research Council Food Waste Project (in Australia), Food Waste Reduction Programme (in Japan), U.S. Food Recovery Challenge (a partnership between U.S. Department of Agriculture and EPA, in the United States of America), Waste and Resources Action Programme (in United Kingdom), and Food Use for Social Innovation by Optimizing Waste Prevention Strategies (in European Union), among others (ARC, 2010; FUSIONS, 2012; EPA, 2014; EPD, 2014; WRAP, 2014). Moreover, the new European Union research and innovation programme, Horizon 2020, presents a section focused on food security matter and sustainable agriculture. For the purpose, €3.7 billion investments will be made in the renewable bio-based economy to use Europe's unexploited wastes as feedstock to make fossil-free and greener everyday products, between 2014 and 2024 (EC, 2014a).

It is now clear that there is a global consciousness on the subject of food wastage reduction. As a consequence, scientific community started to realize that by discharging food wastes, there is also a loss in their potential reuse inside the food chain itself. Hence, the concept of “food by-products” has been increasingly explored aiming to find new technologies to recapture new uses and functional substances from this new type of raw materials and to develop new and safe products with an attractive market value. Still, one should be aware that, according to the European legislation, the term “by-product” defines a substance or object, resulting from a production process, not being regarded as waste, since it would be further used directly without any further processing other than normal

industrial practice (ECD, 2008). However, it must fulfil all relevant product, environmental and health protection requirements for the specific use with no adverse environmental or human health impacts (ECD, 2008).

Hence, throughout this primary review, more specific and recent attempts to recover food wastes or even high added-value components derived from them will be described. In the following sections, the major sources of food wastes production and recent pratical attempts of its reuse will be defined, with a special emphasis to coffee industry.

1.2. Major Food Waste Sources And Target Elements

1.2.1. Food wastage chain and environmental impact

The environmental assessment and loss/waste estimation of all food commodities is based on a life cycle approach that encompasses the entire “food cycle” (Fig. 1.1).

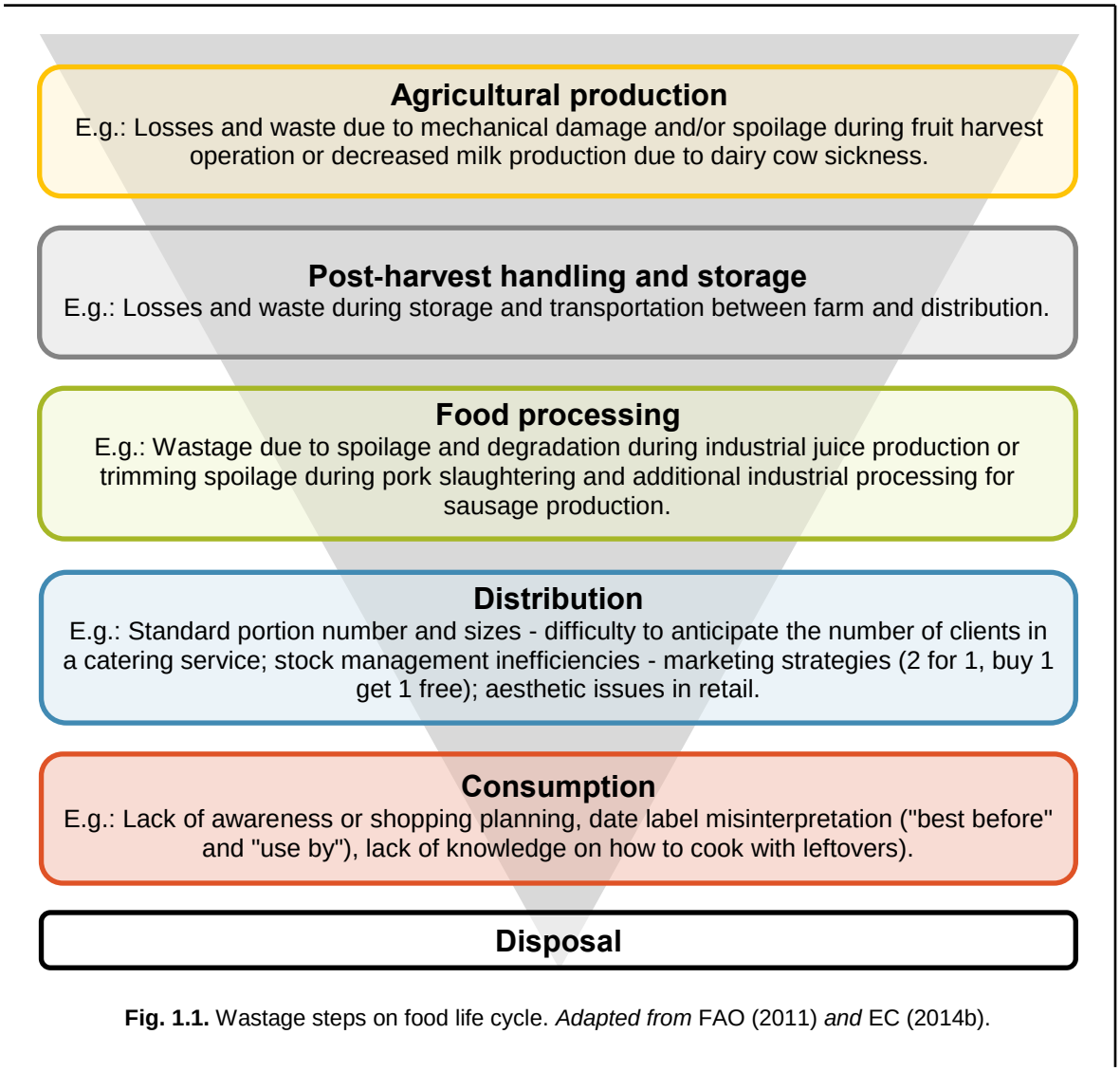

Fig. 1.1. Wastage steps on food life cycle. *Adapted from* FAO (2011) *and* EC (2014b).

Fifty-four percent of the world's food wastage occurs "upstream" during production, post-harvest handling and storage (FAO, 2013a). This fact is frequently observed for highly perishable food products, making them more susceptible do crop losses and/or easily damaged during harvest and post-harvest activities. In addition to the waste of resources itself, one cannot disregard its resulting environmental impacts. The

environmental footprint of food wastage is often assessed through their carbon footprint, water footprint, land occupation/degradation impact, and potential biodiversity impact (FAO, 2013a). Hence, the main stages of food life cycle as well as the major environmental impacts of each of the seven food commodity groups defined by FAO, will be further detailed.

Regarding (i) cereals, wheat is the major crop in medium- and high-income regions, being highly wasted (40-50%) at the consumer phase (FAO, 2011). However, rice is the dominant crop in low-income regions, mostly lost during agricultural production and post-harvest handling and storage (FAO, 2011). Wastage of cereals in Asia represents also a significant problem, with major impacts on carbon emissions and water and land use. Rice's profile is particularly noticeable, given its high methane emissions combined with a large level of wastage mentioned (FAO, 2013a).

In the (ii) roots and tubers group, potato is the dominating crop supply in medium- and high-income countries. Despite occurring mostly during agricultural production, food waste at the consumer level is also high (FAO, 2011). Although experiencing high volumes of wastage in Sub-Saharan Africa, Europe and industrialized Asia, this commodity has low carbon, water and land intensity, mostly because yields are high, thus limiting the impacts per kg (FAO, 2013a).

In the (iii) oil crops and pulses subcategory, dominant crops vary according to world regions. This is a particular case where the highest losses occur mainly at agriculture and post-harvest stages, since they are generally consumed as vegetable oils (FAO, 2011). Contribution of oil crops and pulses wastage to the carbon footprint is low in all regions (1 to 6% of the carbon footprint of the region), though they may have considerable impacts on biodiversity due to its high production volumes and intensities (FAO, 2013a).

Concerning (iv) fruits and vegetables commodity group, losses in post-harvesting are exceptionally high, mostly due to quality standards set by retailers (FAO, 2011). Fruit wastage contributes significantly to water waste in Asia, Latin America, and Europe, mainly as a result of extremely high wastage volumes (FAO, 2013a). A large volume of vegetable wastage in industrialized Asia, Europe, and South and South East Asia translates into a large carbon footprint for that sector (FAO, 2013a).

In the case of (v) meat products, losses in all developing regions are equally distributed throughout the food supply chain. However, relatively high losses are verified in agricultural production in sub-Saharan Africa, owing to high animal mortality during livestock breeding (FAO, 2011). Worldwide wastage volumes of meat are relatively low, though their land occupation and carbon footprint levels are particularly pronounced in

high-income countries and Latin America, which, in combination, account for 80% of all meat wastage (FAO, 2013a).

Regarding (vi) fish and seafood, a large proportion of food products are wasted by deterioration occurring during fresh fish and seafood distribution or wasted in household environments (FAO, 2011). Contribution of fish and seafood wastage to the carbon footprint is low in all regions, although they are a considerable source of biodiversity (mainly of marine origin) decline (FAO, 2013a).

Finally, as regards to (vii) dairy products, waste at the consumption level makes up approximately 40-65% of total food waste (FAO, 2011). Land occupation intensity is much higher in North Africa, Western Asia & Central Asia than in other regions for milk production (FAO, 2013a).

Globally, the later a product is lost or wasted along the supply chain, the higher the environmental cost, as "downstream" impacts will be added to the initial production impact (FAO, 2013a).

1.2.2. Food by-products and their recovery

If one intends to recover nutritional and/or functional compounds derived from agricultural and food processing by-products, "upstream" wastes are in general more appealing. These sources are abundant, confined in few locations and less susceptible to deterioration compared to the wastes produced at the end of the food supply chain (Galanakis, 2012). On the other hand, "downstream" food wastes are widely distributed, so they require an additional collection stage, which compromises their valorization as sources for components recovery.

Food wastes originated along the food supply chain can be divided in two main groups according to their plant or animal origin and 7 subcategories, as previously mentioned, with target ingredients for recovery, detailed in Table 1.1. As observed, most sources are available at the industrial level, while only few are created at consumer's stages.

According to the European Commission Regulation (ECR, 2009), "animal by-products" are defined as the "entire bodies or parts of animals, products of animal origin or other products obtained from animals, which are not intended for human consumption, including oocytes, embryos and semen". These are mostly produced in slaughterhouses during the production of foodstuffs of animal origin such as dairy products, and in the course of the disposal of dead animals and during disease control measures (ECR, 2009). Unfortunately, animal by-products may pose a potential risk to wildlife, public health and

Table 1.1
Food wastes sources and corresponding target ingredients for recovery

Waste origin	Selected sources	Target ingredients
Plant		
(i) Cereals	Rice bran [a]	Albumin, globulin, hemicellulose B, insoluble dietary fiber
	Wheat middling	Arabinoxylans
	Wheat straw	Hemicellulose
	Wheat bran	Glucuronoarabinoxylans
	Oat mill waste	β-Glucan
	Malt dust	Glucose, arabinose, galactose
	Brewery's spent grains	Arabinoxylans
(ii) Root & tubers	Potato peel	Phenols
	Sugar beet molasses	Organic acids
(iii) Oil crops & pulses	Sunflower seed	Phytosterols
	Soybean seed	Phytosterols
	Soybean oil waste	Phytosterols
	Soybean wastewater	Albumin
	Olive pomace	Phenols
	Olive mill wastewater	Phenols & pectin
(iv) Fruits & vegetables	Cold hardy mandarin peel	Narirutin
	Orange peel	Hesperidin, apocarotenoid, limonene
	Lemon by-product	Pectin
	Apple pomace	Pectin
	Apple skin	Phenols
	Peach pomace	Pectin
	Apricot kernel	Protein
	Grape pomace	Dietary fiber
	Grape skin	Phenols
	Wine lees	Calcium tartate, enocyanin
	Banana peel	Cyanidin-3-rutinoside
	Kiwifruits remains	Soluble and insoluble dietary fiber
	Coffee pulp	Phenols
	Coffee husk	Cyanidin-3-rutinoside, dietary fiber
	Coffee silverskin	Phenols, dietary fiber
	Spent coffee grounds [a]	Phenols, dietary fiber, aroma compounds
	Carrot peel	β-carotene, phenols
	Tomato pomace	Lycopene
	Tomato skin	Carotenoids
	Cauliflower floret & curd	Pectin
Animal		
(v) Meat products	Chicken by-products [a]	Proteins
	Slaughterhouse by-products	Proteins
	Bovine blood	Proteins
	Beef lung	Protein concentrates
	Sheep visceral mass	Protein hydrolyzates
(vi) Fish & seafood	Fish leftovers (skin, head, bones)	Proteins, lipids
	Shrimp & crab shells	Chitosan/chitin
	Surimi wastewater	Proteins
(vii) Dairy products	Cheese whey [a]	Lactose, β-lactoglobulin, α-lactalbumin

[a] Food wastes produced at least at consumer phase.
Adapted from Galanakis (2012) *and* Murthy and Naidu (2012).

the environment. Past crises related to disease or contaminants outbreaks, such as bovine spongiform encephalopathy and the presence of dioxins in feeding stuffs, have shown the consequences of the improper use of some animal by-products not only from a health perspective but also on a socioeconomic level (Hahn, 1999; Sapkota et al., 2007; ECR; 2009).

Still, these food wastes present a high protein load, which is too valuable to be discharged on the environment (Galanakis, 2012). Regarding this kind of wastes, the most extracted substances are, in fact, proteins (Table 1.1), which could be considered as functional ingredients in meat products, for instance as emulsifying, gelling or foaming agents comparable to those of commercial ingredients from milk (whey proteins, Na-caseinates), egg white, blood (beef plasma) and soy (Selmane et al., 2008).

Nevertheless, plant processing wastes comprise the major group (FAO, 2011) and thus, these are the most widely studied substrates. The most successfully recovered compounds include the selective extraction of phenols, pectin and phytosterols (Table 1.1), easily used in the food and pharmaceutical industries.

Beer industry uses barley as a common starch source, malted to dissolve starch in the grains before brewing. Due to the increase volume of beer production worldwide, large amounts of by-products are generated, which has encouraged the development of new reuse applications of these specific residues, such as the high yield extraction of arabinoxylans, glucose, arabinose, galactose and even proteins (Fischer and Bipp, 2005; Roos et al., 2009; Vieira et al., 2014).

Soybean and sunflower seed remains after oil extraction have been studied for the recovery of phytosterols (Copeland and Belcher, 2001) though more direct approaches have been used for many years, such as their use as animal feed (Rizzi et al., 2002). Being a very popular oil crop in Mediterranean countries, olive oil production gives rise to large volumes of wastes, including olive mill pomace and wastewaters, which have been widely and tentatively valorised as a source of bioactive phenols and more recently, pectin (Obied et al., 2005; de Marco et al., 2007; Galanakis et al., 2010).

In general, fruit and vegetable by-products are the most widely and historically investigated group. Once these by-products are constituted of soft tissues rich in antioxidants and dietary fibres, the simultaneous extraction in two separate streams is easily performed (Galanakis, 2012). In particular, citrus by-products have been frequently applied for the extraction of pectin and dietary fibre (Lario et al., 2004; Masmoudi et al., 2008). Several patented methodologies aiming a commercial application (mainly as food antioxidants and supplement) of reused food wastes are available for these by-products

(Bonnell, 1983; Lavecchia and Zuorro, 2008; Anming et al., 2010; Guangyu and Zhang, 2011).

In resume, low-cost and environmentally friendly recovery of valuable substances from food wastes is an important and daily challenge for food scientists. Distinct food by-products have been investigated over the years in order to find potential applications, though most of these studied wastes are generated before consumption, being those produced afterwards frequently neglected.

It has also been phrased that the largest food wastes volumes occur in the fruit and vegetable group, where coffee is included. Indeed, the coffee industry has a great and worldwide representativeness and it is responsible for huge amounts of wastes all along the enteire productive chain, from the field to the cup. Hence, the following chapter aims to elucidate the current status of coffee waste volumes and its promessing reuses, with a special emphasis on post-consumer residues – spent coffee grounds.

2. COFFEE RESIDUES AND PROSPECTIVE TRENDS

2.1. Coffee And Its Wastes: From Bean To Cup

2.1.1. *Pre-roasting coffee by-products*

2.1.1.1. Dry processing

2.1.1.2. Semi-dry and wet processing

2.1.2. *Post-roasting coffee by-products*

2.1.2.1. Coffee silverskin

2.1.2.2. Spent coffee grounds

2.2. Espresso Spent Coffee: The Grounds Revival

2.2.1. *Chemical composition and reuse approaches*

2.2.2. *Sustainability initiatives*

2.1. Coffee And Its Wastes: From Bean To Cup

Coffee belongs to the botanical family *Rubiaceae*, being a widespread and largely traded commodity. Two coffee species attain higher commercial relevance – *Coffea arabica*, with greater representativeness, and *Coffea canephora*, usually known as Arabica and Robusta, respectively. Despite being indigenous to Ethiopia, coffee production has spread worldwide, where adequate environmental conditions were gathered, as well as its consumption (Clarke, 1989).

Coffee beverage, as we know it, is the result of a complex sequence of technological processes, starting in the field and finishing in the cup. After being harvest, coffee cherries undertake a primary processing, of which three distinct methods are available – dry, wet or semi-dried. In the former, the whole cherries (beans, mucilage, pulp, and shell) are dried under the sun or in mechanical dryers, until the dry shell (husk) is easily mechanically removed. Regarding the wet process, the cherries are mechanically pulped and the mucilage remains (pectin and sugars) are removed by microbial fermentation, washed and dried similarly to the dry method. Finally, in the more recent semi-dry method, the cherries are pulped, but the fermentation step is omitted, being dried with the mucilage remains (Vincent, 1987). A summary diagram of these coffee cherries post-harvest handling techniques is represented in Fig. 2.1, where the steps contributing to the major generation of solid residues are highlighted. Huge amounts of contaminated waters are also produced in several washing steps, with a high carbon load and therefore with high impact on the environment. Other minor wastes may comprise defective green coffee beans and coffee tree leaves during harvest.

As depicted from the diagram, most of these residues affect the coffee producing countries, as Brazil, Vietnam, Colombia, Indonesia, Ethiopia, India, etc., in a total of more than 70 countries, most of them with low- to medium-income. In opposition, high-income countries are usually just coffee consumers, with local roasting and extractive industries based on green coffee imports. Therefore, two major classes of coffee by-products maybe distinguished, i.e. those derived from green coffee production, and therefore restricted to the producing countries, and those obtained after roasting, with a wider distribution.

In the last decade, the tentative use of these agro-industrial wastes in diverse sectors has been the focus of several studies. Each class of coffee residues will be further detailed in the following subsections as well as its potential novel applications.

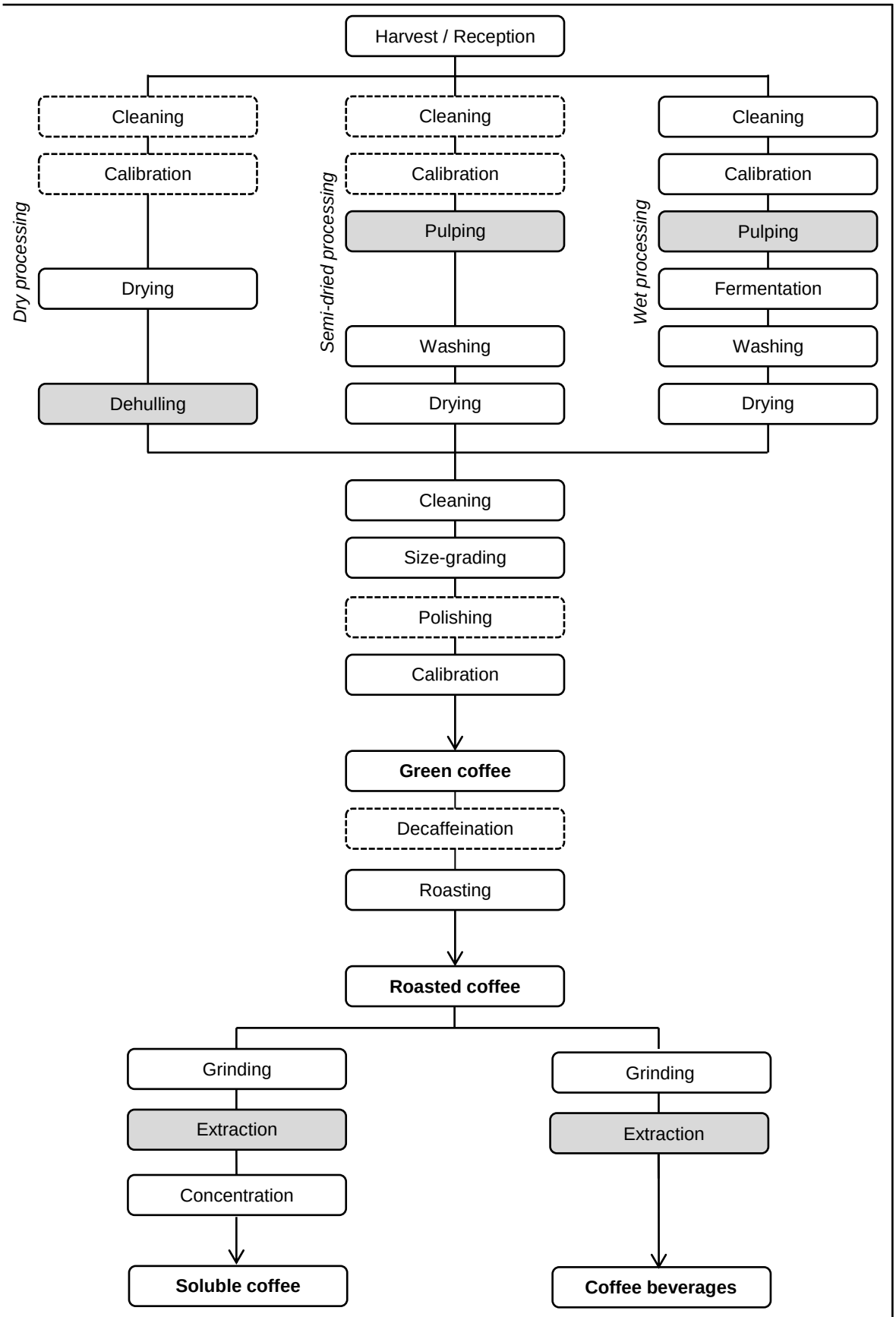

Fig. 2.1. The life cycle of coffee products and residues generation steps. Grey boxes indicate major steps of coffee solid residues production. *Adapted from* Cruz et al. (2015).

2.1.1. Pre-roasting coffee by-products

2.1.1.1. Dry processing

Depending on the method chosen for coffee processing, different residues may be produced. Dry processing generates only one type of coffee residue – coffee cherry husks, comprising the coffee cherry outer skin, pulp and parchment (Fig. 2.2A), and representing about 12% of the berry on dry-weight basis. About 0.18 tons of husks are released from 1 ton of fresh coffee fruits, producing around 150 to 200 kg of commercial green coffee (Murthy and Naidu, 2012).

Fig. 2.2. Major coffee by-products. A – Husks; B – Pulp; C – Silverskin; D – Spent coffee grounds.

Coffee husks are composed by 58-85% total carbohydrates, 8-11% protein, and 0.5-3% lipids (Franca et al., 2009; Murthy and Naidu, 2012). Minor amounts of bioactive compounds, as caffeine and chlorogenic acids, are also present in this residue (Franca et al., 2009; Murthy and Naidu, 2012).

Many application approaches have been studied for coffee cherry husks re-utilization, as substrate for biogas and alcohol production, biosorbents in the removal of cationic dyes from aqueous solutions, converted into fuel pellets or extracted for bioactive substances

recovery (Oliveira et al., 2008; Gouvea et al., 2009; Jayachandra et al., 2011; Murthy and Naidu, 2012). Besides, coffee husks demonstrated to be suitable candidates for a more direct use as substrate for edible mushrooms production or composting (Fan et al., 2000; Kassa et al., 2011; da Silva et al., 2012).

2.1.1.2. Semi-dry and wet processing

Coffee pulp is the first by-product obtained during wet or semi-dry processing and represents 29% dry-weight of the whole cherry (Murthy and Naidu, 2012). For every 2 tons of commercial green coffee produced, 1 ton of coffee pulp is obtained (Murthy and Naidu, 2012). Coffee pulp comprises the exocarp (outerskin) as well as the mesocarp (fleshy portion) (Fig. 2.2B). It is essentially rich in carbohydrates (32%), proteins (5-13%) and minerals (9%) and contains also considerable amounts of tannins, polyphenols and caffeine (Ulloa Rojas et al., 2003). Similarly to coffee husks, coffee pulp has been studied to be reused for mushroom production, composting, biosorbent, bioactive compounds extraction purposes (as soluble and bound hydroxycinnamates) (Murthy and Naidu, 2012; Rodriguez-Duran et al., 2014).

2.1.2. Post-roasting coffee by-products

After undergoing the primary processing, green coffee is now available for roasting, which will alter completely its physical and chemical composition, and consequently, the by-products produced. These last coffee remains are produced immediately after roasting – coffee silverskin – and after beverage preparation, in industrial or at cafeteria/household environments.

2.1.2.1. Coffee silverskin

Coffee silverskin (Fig. 2.2C), frequently known as “chaff”, is the first coffee industry residue produced in consuming countries, since it is released during roasting (Fig. 2.1), if the leaves were not polished before shiping. It consists on the tegument of coffee beans and thus have a very low mass, comprising 4.2% (w/w) of the green coffee bean (Ballesteros et al., *in Press*), with reduced environmental impact It is highly rich in soluble dietary fiber (54% of total dietary fiber) and compounds with antioxidant capacity, particularly phenolic compounds (Mussatto et al., 2011a; Ballesteros et al., *in Press*).

Published data on coffee silverskin reuse are scarce. Aiming the extraction value-added compounds, some authors have showed that this coffee residue constitutes a fine source of antioxidants and dietary fibre and may be considered as a new potential functional ingredient (Borrelli et al. 2004; Machado et al., 2012; Narita and Inouye, 2012; Pourfarzad et al., 2013). Furthermore, coffee silverskin maybe used as support and nutrient source during fructooligosaccharides and β-furctofuranosidase production by *Aspergillus japonicus* under solid-state fermentation conditions, used as raw material to produce fuel ethanol or as an ingredient in anti-aging cosmetics and functional foods (Mussatto and Teixeira, 2010; Mussatto et al., 2012; Bilbao et al., 2014).

2.1.2.2. Spent coffee grounds

Coffee brews are usually prepared with an Arabica coffee or Arabica/Robusta blends, from single or different geographical origins, being available to consumers as roasted beans, whole or ground, or even as instant/soluble coffee. Thus, under the "spent coffee grounds" terminology, one can include those obtained from the soluble coffee industry as well as those produced after brewing at cafeterias or at home.

As stated by the European Coffee Report (2014), soluble coffee trade in Europe to and from non-European destinations represents currently around 49 and 45 thousand tons per year, respectively. This fact is the result of the common preference for this easy and fast beverage preparation mode by coffee consumers. Consequently, high consumption is also accompanied by high waste generation in the soluble coffee industries. In fact, industrial spent coffee grounds have an estimated woldwide annual production of 6 million tons, since about 2 kg of wet spent coffee grounds are obtained to each 1 kg of soluble coffee produced (Mussatto et al., 2011a).

These industrial spent coffee grounds, i.e. generated from soluble coffee production, are a coffee by-product with fine particle size, high moisture (80-85%), high organic load, and acidity (Mussatto et al., 2011a).

Due to its chemical features, direct disposal into the environment is thus unadvisable. Therefore, plenty of environmentally friendly approaches have been proposed in recent years. For instance, industrial spent coffee may be used as burning fuel in the industrial soluble industry directly, as a source to produce low-cost CO_2 adsorbents, dyes or heavy metals adsorbents, to produce biodiesel and fuel pellets or, other value-added products as H_2 and ethanol, and as a substract for edible fungus production (Leifa et al., 2001; Kondamudi et al., 2008; Franca et al., 2009; Machado et al., 2012; Plaza et al., 2012;

Limousy et al., 2013). Recently, by using *Saccharomyces cerevisiae*, Sampaio et al. (2013) described a process for the production of a spirit beverage, including the chemical composition and sensory profile of this distillate, revealing an organoleptic quality acceptable for human consumption.

Besides all the aformentioned potential uses for industrial spent coffee grounds, several studies describing its bioactivity, amino acids and sugars contents have also been performed aiming to find alternatives for the reuse of this residue, as cited by Mussatto et al. (2011a).

Still, industrial spent coffee grounds are chemically different from those obtained in cafeterias/household environments. In fact, industrial spent coffee constituents are much more effectively extracted, thus resulting in more chemically exhausted remains, in comparison to spent coffee obtained after brewing. Hence, the following section will focus on the latter residues production process, chemical composition and recent attempts on its reuse, thus creating a lauching pad into the main goal of this dissertation – the espresso spent coffee grounds reuse.

2.2. Espresso Spent Coffee: The Grounds Revival

In general, beverage preparation results from the direct contact of ground roasted coffee with hot water, during which the soluble substances are partially extracted, while the oils become partially emulsified. Hence, it is clear that the beverage chemical composition is dependent on the extractive efficiency, which relies on diverse factors, including the coffee species, roasting degree, grinding grade, coffee/water ratio, water quality, temperature, pressure and percolation time (Illy and Viani, 2005). Therefore, different extraction processes will lead to sensorial and chemically distinct brews and, thus, distinct spent coffee grounds.

Three major groups of extraction methods are usually distinguished (Pictet, 1989):

1. Decoction methods, embracing boiled coffee, Turkish coffee, percolator coffee, and vacuum coffee;
2. Infusion methods, such as filter coffee and Napoletana coffee;
3. Pressure methods, which include plunger, moka, and espresso coffee.

Decoction methods have a limitative extraction yield, since its rate decreases with brew concentration, though higher temperatures favour increased extraction yields and rates. As regards to infusion methods, sensorial milder beverages are obtained, though a much more effective extraction of water-soluble substances is observed, due to the prolonged contact time between clean water and ground coffee. Finally, pressure methods, by being subjected to higher pressures during extraction, give rise to highly concentrated brews with distinct chemical, and thus, sensorial properties (Petracco, 2001).

Despite its Italian roots, the popularity of espresso coffee has spread all around the world, in particular in Latin European countries, but recently North-American and Japanese markets have also become very fond of this product (Petracco, 2001). Hence, as it has been stated all along the current dissertation, high consumption patterns are also responsible for relevant waste volumes, and espresso coffee is not an exception. As a result of preparing an espresso, a new kind of by-product is produced – espresso spent coffee grounds (Fig. 2.2D). Being a small beverage (30−50 mL), extracted in a reduced time (30 s), the ground residue is still rich in soluble components, opening an array of potential applications, side by side with an equally increased ecotoxicological concern if disposed as is (Petracco, 2001; Buerge et al., 2003).

In order to predict the feasibility of espresso spent coffee grounds reuse in new industrial sectors, its chemical composition should be known, detailed in the following subsections, as well as its already proposed applications.

2.2.1. Chemical composition and reuse approaches

The first study regarding the chemical charaterization of espresso spent coffee grounds was performed by Cruz et al. (2012a) aiming to predict their potential as a source of bioactive compounds, by comparison with the spent grounds from the soluble coffee industry. The study involved a total of 50 samples from 14 trademarks, collected in several coffee shops and prepared with distinct coffee machines. The average chemical composition of the analysed spent grounds is presented in Table 2.1.

Table 2.1
Chemical composition of espresso spent coffee grounds

Component	Average content [a] (± SD)
Moisture (g/100 g)	63.0 ± 3.6
Total soluble solids (g/100 g)	19.7 ± 3.2
pH	5.7 ± 0.2
Nitrogen (g/100 g)	2.3 ± 0.1
Crude protein (g/100 g)	14.2 ± 0.7
Total fat (g/100 g)	12.5 ± 1.3
Caffeine (mg/100 g)	453 ± 134
Total chlorogenic acids (mg/100 g)	479 ± 139
5-Cafeoylquinic acid (mg/100 g)	141 ± 50
Total ashes (g/100 g)	1.9 ± 0.7
K (mg/100 g)	882 ± 466
Mg (mg/100 g)	220 ± 134
P (mg/100 g)	153 ± 50
Ca (mg/100 g)	35 ± 12
Na (mg/100 g)	20 ± 15
Fe (mg/100 g)	4.6 ± 2.1
Mn (mg/100 g)	2.7 ± 1.0
Cu (mg/100 g)	2.5 ± 1.2

[a] All components are expressed on a dry basis, except for moisture.
Adapted from Cruz et al., 2012a.

Despite the fact that lipid (12.5%) and nitrogen (2.3%) contents were similar to those of industrial spent coffee (14% and 2.3%, respectively) (Calixto et al., 2011; Mussatto et al., 2011a; Ballesteros et al., *in Press*), major differences were verified for other analysed components. The high extraction efficiency imposed by the industrial process results in spent grounds with reduced water-soluble components (6.6%) (Yen et al., 2005) in relation to espresso spent grounds (19.7%). Consequently, there is still a significant pool of bioactivity retained in the espresso spent grounds, better represented by its high caffeine and total chlorogenic acids content, the later being ten times higher than those

reported for soluble spent coffee (479 *vs* 57 mg/100g, dry weight) (Mussatto et al., 2011b), therefore with higher potential for its recovery and further use by the food or pharmacutical industries.

Based on the features of their lipid content, spent coffee grounds have also been targeted as a potencial source for biodiesel production by using enzymatic, acid or akaline catalysis (Burton et al., 2010; Al-Hamamre et al., 2012; Jenkins et al., 2014). Couto et al. (2009) have also analysed extracted lipids by supercritical fluid extraction from SCG to assess its potential reuse.

Furthermore, its richness in N, K, P, and Mg, among others, makes espresso spent grounds a potential soil amendment product (Kondamundi et al., 2008), although requiring special attention to the potential environmental widespread of its bioactive constituents, as caffeine, and implication on plant development. Its lower moisture levels (63.0%) in comparison to industrial ones (80-85%), make this residue less prone to microbial growth, while implying also lower transportation costs (Mussatto et al., 2011a; Cruz et al., 2012a).

In conclusion, espresso coffee residues reveal greater potential to be used in the food and pharmaceutical industries than industrial spent coffee, justifying their selective assembly.

2.2.2. Sustainability initiatives

Coffee beverage by-products have only become the focus of scientific research in the last few of years.

Several authors have attempt to recover natural antioxidants from spent coffee grounds for further use in food and pharmaceutical products (Bravo et al., 2012; Cruz et al., 2012a; Panusa et al., 2013). Petrik et al. (2014) have demonstrated the suitability of espresso spent grounds as a substracte for carotenogenic yeast strains cultivation aiming the production of vitamin-enriched biomass, of which *Sporobolomyces roseus* showed the highest potential. It has also been used as substrate for the production of enzymes, namely fructosyl transferase, the enzyme responsible for the fructooligosaccharides production from sucrose, by using *Aspergillus oryzae* (Sangeetha et al., 2004).

Despite being innovative and interesting alternatives, the aforementioned studies imply the use of expensive technologies and sometimes even chemical components, while generating new residues after the process. With the efforts to find a more direct and easy application approach for this coffee by-product, using the residue as a whole, and based

on the knowledge of its use in domestic agriculture without any scientific background, Cruz et al. (2012b) evaluated the effect of direct addition fresh espresso spent grounds to the soil for lettuce production in a pot experiment. Despite the low productive yields obtained, all evaluated pigments increased proportionally to spent coffee amounts, particularly carotenoids, increasing the nuttritional value of the vegetable produced.

This evidence opened a new array of possibilities to reuse this high-volume food waste in a cost-effective manner. Besides, conscious that Liu and Price (2011) demonstrated that spent coffee remains could be used as a slow-release source of nitrogen for fertilising after composting, more studies should be perform in order to optimize conditions in order to ascertain espresso spent grounds effectiveness in horticulture cultivation.

3. OBJECTIVES AND METHODOLOGICAL OVERVIEW

The main objective of this dissertation was to study the effect of espresso spent coffee as soil amendment, fresh or after composting, on the nutritional quality of leafy vegetables, in particular lettuce. Specific objectives included:

- The development and validation of analytical methodologies required for the inherent analysis of both soil and lettuce;
- The nutritional and physical characterization of plants produced;
- The establishment of optimal treatment conditions for higher crop yield and increased quality.

Some crucial steps of this research are resumed in Fig. 3.1.

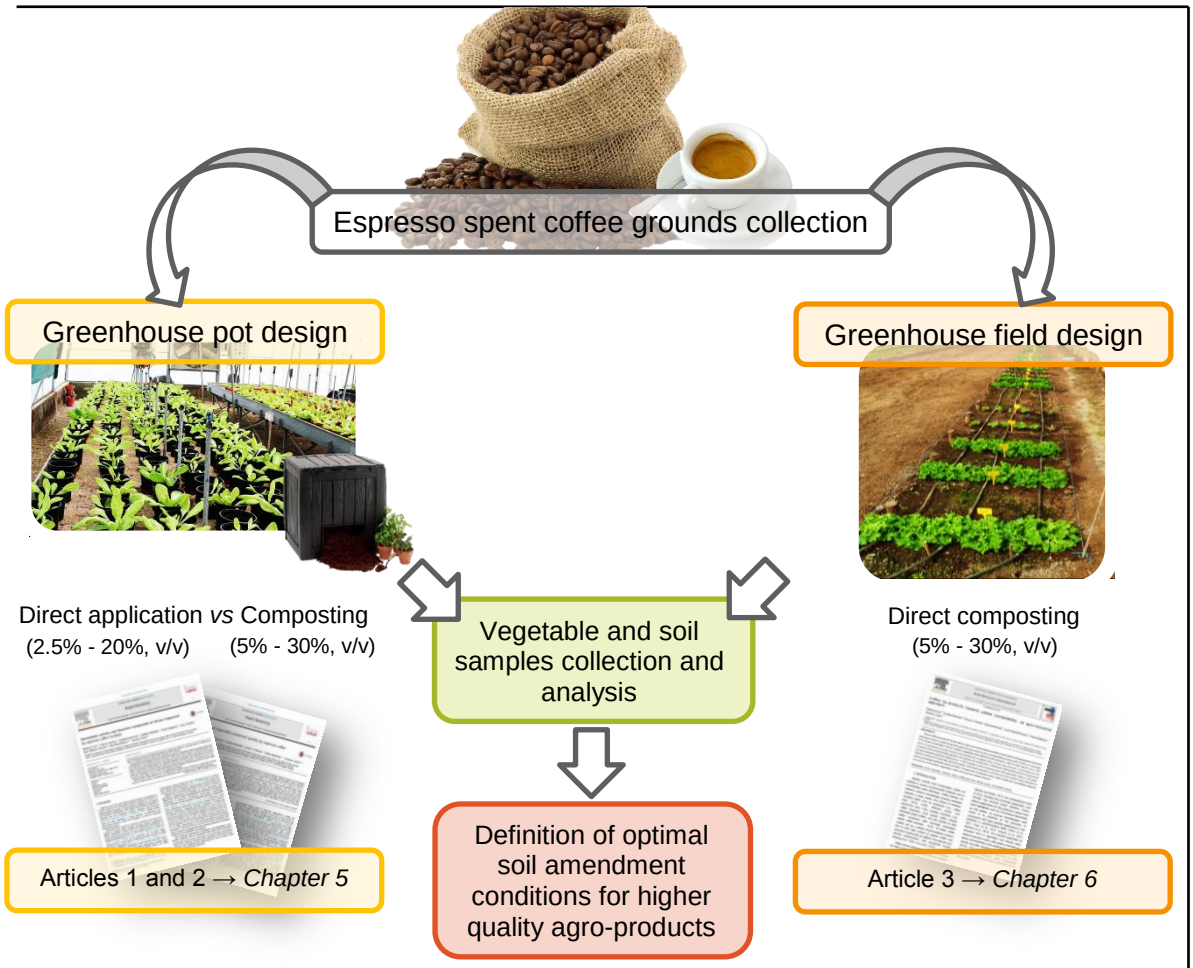

Fig. 3.1. Main steps in the research process.

EXPERIMENTAL PART

4. MATERIALS AND METHODS

4.1. Sampling

4.2. Analytical Methods

4.2.1. *Lettuce analysis*

4.2.2. *Soil analysis*

4.2.3. Statistical analysis

4.1. Sampling

Two independent experiments were conducted. The first consisted in a pot experiment using fresh and composted espresso spent coffee grounds, hereafter designated as SCG, and the second was a field experiment using direct composting, generally known as pit or trench composting. The experiments occurred at different periods of time, i.e. years 2012 and 2013. With the purpose of accomplishing the objectives defined for this work, plant, soil and SCG (as is or composted) were collected.

Espresso coffee remains were collected from several coffee shops, serving espresso coffee on a regular basis. For the first experiment (see *Chapter 5*, p. 40 and 52), two assays were performed in parallel, one using fresh-SCG and another using composted-SCG, both in different volume ratios with plain vegetable soil, the latter used as control (0%) for both experiments. The composted SCG were prepared by LIPOR, using equal volumes of fresh SCG, fresh garden grass and straw, and left to compost for a period of 6 months. In the second experiment (see *Chapter 6*, p. 66), the fresh SCG were let to compost in the field, mixed with soil in isolated holes, for a period of 4 months prior to crop cultivation. Pre- and post-harvest soil samples were collected in both experiments, preserved by drying at 60ºC in a forced-air oven (WTC-Binder, Germany).

In the first experiment, *Lactuca sativa* var. *capitata* cv. ‘‘Four Seasons’’ were cultivated in a greenhouse pot design (for more details, see *Chapter 5*, p. 41), being randomly divided according to the treatment group (n = 2 × 5 × 15 + 15). For the second experiment, *Lactuca sativa* var. *capitata* cv. ‘‘Rolina” were cultivated in a greenhouse field design (for more details, see *Chapter 6*, p. 66), being again randomly split into different treatment groups (n = 6 × 15). After harvest, edible parts were carefully washed, dried, stored in polyethylene bags and preserved according to the subsequent analysis requirements. The use of different cultivars was imposed by their availability and adaptability to the pot/field experiments.

4.2. Analytical Methods

4.2.1. Lettuce analysis

Color evaluation was determined on fresh whole lettuce leaves from the second assay using a Minolta colorimeter in the CIELAB scale, using L* (lightness), a* (redness), b* (yellowness), h_{ab} (hue angle), C_{ab}* (chroma), and CI* (color index) (see *Chapter 6*, p. 67).

The moisture content of plant samples were determined by drying 3 g of sample in an air oven at 103 ± 2 ºC until constant weight (16 – 18 h), according to to AOAC method 923.03 (AOAC, 2000).

As regards to lettuce chlorophylls and carotenoids, in the first experiment, a chromatographic determination was carried out using a reverse-phase HPLC-PDA methodology after a Folch-like method extraction as detailled in *Chapter 5*, p. 42, allowing the quantification of individual compounds, previously validated (Cruz et al., 2012b). For the second experiment, a spectophotometric method was chosen for total pigments estimation, using a solid-liquid extraction with methanol based on the method of Lichtenthaler and Buschmann (2001a, 2001b), as described in *Chapter 6*, p. 67.

In both experiments, vitamin E was extracted using a Folch-like method, specifically developed for the purpose (Cruz and Casal, 2013). Briefly, fresh ground sample (<1.5g) was extracted twice with methanol:dichloromethane (1:2) and NaCl 0.9% (w/v), in the presence of ascorbic acid, butylated hydroxytoluene (BHT) and internal standard (IS). Organic phases were combined, vacuum-dried and the extract was recovered with n-hexane. Quantification was performed by internal standard method after a normal-phase HPLC-FLD separation (see *Chapter 5*, p. 43 and *Chapter 6*, p. 68).

Total ascorbic acid (AA, ascorbic acid and dehydroascorbic acid), quantified in the second assay, was extracted from freeze-dried leaves by a solid-liquid extraction procedure, using an acidic preservative solution of tris(2-carboxyethyl)phosphine, based on the method of Chebrolu et al. (2012) and de Velde et al. (2012). Finally, the chromatographic analysis was performed by a reverse-phase HPLC-PDA method, validated for linearity, limits of detection and quantification, precision, and recovery, as detailled in *Chapter 6*, p. 68).

For antioxidant activity assays (radical scavenging activity, reducing power, total reducing capacity), an extractive step was performed prior to specific reactive processes by maceration with aqueous methanolic solution (and further aqueous acetonic solution, in

the second experiment), as described in *Chapter 5,* p. 41 – 42 and *Chapter 6*, p. 68. Afterwards, spectrophotometric absorbances of samples and standard solutions were determined in a microplate reader.

After dry ashing, for total ashes estimation, and nitric acid digestion, high-resolution continuum source atomic absorption spectrometry with flame atomization was selected for all mineral analyses (potassium, magnesium, calcium, sodium, manganese, zinc, copper, iron), except for phosphorous which was analyzed by a standard vanadomolybdophosphoric acid colorimetric method (see *Chapter 5*, p. 52 – 53).

4.2.2. Soil analysis

Soil pH (H_2O) and specific electric conductivity were determined, following standard procedures, in the upper layer of aqueous soil extract. This solution was then freeze-dried for later caffeine quantification by reverse-phase HPLC-PDA (see *Chapter 6*, p. 67). Chromatographic separation was accomplished by reverse-phase HPLC-PDA, as described by Cruz et al. (2012a) and quantidied by the external standard method.

Total organic carbon (TOC) content was estimated by the oxidation of organic matter in a acidic sodium dichromate solution at 135 °C (see *Chapter 6*, p. 67), based on Skjemstad and Baldock (2008) method.

Total ashes and mineral content of post-harvest soil samples were determined by dry ashing followed by nitric acid/hydrochloric acid digestion on a hot plate. Quantification was then performed as described for lettuce analysis.

4.2.3. Statistical analysis

Statistical analyses included analysis of variance, regression and linear correlation analysis, as well as principal component analysis (PCA) or linear discriminant analysis (LDA). Such were performed at a 5% significance level, using SPSS software, version 21.0 or 22.0 (IBM Corporation, New York, USA).

5. FRESH VS. COMPOSTED SPENT COFFEE GROUNDS

“Antioxidant activity and bioactive compounds of lettuce improved by espresso coffee residues” in *Food Chem* 2014; 145: 95-101.

“Improvement of vegetables elemental quality by espresso coffee residues” in *Food Chem* 2014; 148: 294-299.

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Antioxidant activity and bioactive compounds of lettuce improved by espresso coffee residues

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ABSTRACT

The antioxidant activity and individual bioactive compounds of lettuce, cultivated with 2.5–30% (v/v) of fresh or composted espresso spent coffee grounds, were assessed.

A progressive enhancement of lettuce's antioxidant capacity, evaluated by radical scavenging effect and reducing power, was exhibited with the increment of fresh spent coffee amounts, while this pattern was not so clear with composted treatments. Total reducing capacity also improved, particularly for low spent coffee concentrations. Additionally, very significant positive correlations were observed for all carotenoids in plants from fresh spent coffee treatments, particularly for violaxanthin, evaluated by HPLC. Furthermore, chlorophyll a was a good discriminating factor between control group and all spent coffee treated samples, while vitamin E was not significantly affected.

Espresso spent coffee grounds are a recognized and valuable source of bioactive compounds, proving herein, for the first time, to potentiate the antioxidant pool and quality of the vegetables produced.

Keywords: *Lettuce; Antioxidant activity; Bioactive compounds; Carotenoids; Vitamin E; Spent coffee grounds*

1. INTRODUCTION

Coffee is one of the most important agricultural commodities in world's trade but its processing produces significant amounts of undervalued by-products. These residues are highly rich in organic and inorganic compounds, with high environmental pollution if released into the ecosystem without adequate pre-treatment (Mussatto et al., 2011a). Coffee wastes reuse has been therefore a major priority of producing and consuming countries, both for ecological, as well as economic and social reasons.

Following these concerns, numerous attempts have been considered to recycle coffee residues through compost preparation, biogas production, animal feed and mushrooms production, or more recently by extraction of value-added fractions, as lipids for biodiesel production (Mussatto et al., 2011a; Oliveira et al., 2008). In the particular case of spent coffee grounds (SCG), recent reports emphasised their richness in coffee bioactive compounds (Bravo et al., 2012; Cruz et al., 2012a), as caffeine or chlorogenic acids, derived from the incomplete extraction process of beverage preparation. Indeed, these are the same compounds receiving health claims on coffee consumption, being among the main contributors for its antioxidant capacity and bioactivity (Bravo et al., 2012). Thus, their presence in SCG makes it senseless to depose them without further use.

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, particularly carotenoids, when cultivated in the presence of reduced amounts of fresh-SCG (Cruz et al., 2012b). However, many other antioxidant substances may receive benefits from this preharvest treatment, requiring detailed evaluation. Furthermore, the influence of other coffee-based

amendments, such as the composted ones which are traditionally applied at domestic levels, has not been studied so far. In order to support an effective and sustained reuse of this coffee waste, this study aimed to clarify the effect of espresso SCG as soil amendment for agricultural purposes, as is or after composting, on the nutritional quality of green leafy vegetables. A particular focus was devoted to their antioxidant features as these are amongst the most important bioactive attributes resulting from vegetable consumption.

The combined assessment of lipophilic and hydrophilic compounds, together with an estimation of the plant total antioxidant activity by *in vitro* assays will allow a first and better elucidation about SCG's influence on green leafy vegetables nutritional characteristics, thus exploring and supporting the reuse of these coffee residues for agriculture purposes.

2. MATERIALS AND METHODS

2.1. Standards and reagents

Authentic standard *trans*-β-carotene, and the internal standard (IS) *trans*-β-apo-8'-carotenal were purchased from Fluka Chemie (Germany), while gallic acid, lutein, and chlorophyll *a* were obtained from Sigma–Aldrich (Germany), and pheophytin *a* was obtained from an acidified chlorophyll *a* solution (Sievers & Hynninem, 1977). Tocopherols (α-, and γ-) were acquired from Supelco (USA) and tocol, used as IS for tocopherol quantification, was from Matreya Inc. (USA). The concentration of the individual carotenoids (lutein and *trans*-β-carotene), chlorophyll *a* (≈ 0.1 mg/mL), and tocopherols (≈ 5 mg/mL) were evaluated by UV/VIS spectrophotometry (UV-1800, Shimadzu, Japan), using published absorption coefficients (Britton, 1995; Nesaretnam et al., 2007). A stock solution of trans-β-apo-8'-carotenal (0.65 mg/mL) was prepared in ethyl acetate while tocol was prepared in n-hexane (1.0 mg/mL).

Ethyl acetate, n-hexane and methanol, all HPLC grade, were from Sigma–Aldrich. All the remaining reagents were analytical grade from several suppliers and included: butylated hydroxytoluene (BHT), chloroform, 1,4-dioxane, sodium chloride, triethylamine, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and iron (III) chloride hexahydrate, Folin and Ciocalteu's reagent, sodium carbonate and trichloroacetic acid. Phosphate buffer (pH 6.6) was prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate.

2.2. Experimental design

2.2.1. Soil and spent coffee material

Two different assays were developed simultaneously, one using fresh-SCG and another using composted-SCG. Each SCG batch was assembled with coffee wastes collected from several coffee shops, in Porto metropolitan area (NW Portugal), serving espresso coffee.

Regarding the study using fresh-SCG, five mixtures with plain vegetable soil (Siro® Germe, Portugal) were prepared: 2.5%, 5%, 10%, 15% and 20%, all on a volume basis, and distributed by 1 L plastic pots. For the second assay, SCG were previously composted for 6 months at a specialised municipal solid waste treatment facility (Lipor, Porto, NW Portugal). Fresh-SCG were collected and composted on the following day. Equivalent volumes of fresh grass, straw and fresh-SCG were piled in a 100 L composter, at ambient temperature, from July to January. Temperature and moisture were controlled weekly with a digital thermo-hygrometer, being revolved and watered when compost's moisture dropped below 60%. After a six-month period, the compost was completely stabilized, being transported to the greenhouse where five final mixtures were prepared with plain soil: 5%, 10%, 15%, 20% and 30%, also on a volume basis. Plain vegetable soil was used as control (0%) for both experiments.

2.2.2. Plant material and growth conditions

The experimental setup for this study was a randomized greenhouse pot design performed at the School of Agriculture, Polytechnic Institute of Bragança (NE Portugal), under controlled conditions (day/night thermal regime of 23/18 ± 2 ºC, 70 ± 10% relative humidity, natural light).

Butterhead lettuce seeds (*Lactuca sativa* L. var. *capitata* cv. ''Four Seasons'') were germinated with organic substrate (Siro® Germe, Portugal), during spring season in the Northern hemisphere. After 4 weeks, the healthy plantlets were transferred to the plastic pots containing the mixtures prepared. For each SCG percentage (5 levels) and assay (fresh and composted), plus control, thirty pots were prepared, giving a total of 330 plants. All pots were watered after transplanting, and distributed randomly through the greenhouse longitudinal extension (Fig. 5.1). Every two days all plants were irrigated (50 mL) and, at the 7th and 21st days, irrigation water was supplemented with 0.2% (v/v) of Complesal 12-4-6 (N/P/K) nutritive solution (Bayer, Portugal).

Fig. 5.1. Distribution of lettuce samples in the greenhouse during the experiment.

Lettuce plants were harvested after 5 weeks, carefully washed with deionized water, and the edible part was separated and weight. Due to the reduced individual plants weight for all the assays, the 30 plants from each group were divided into 10 subgroups of 3 plants, named hereafter as samples.

For the antioxidant capacity assays, five samples from each treatment were dried in a ventilated oven at 60 ºC (Memmert GmbH & Co. KG, Germany) until constant weight, whose procedure was already been verified not to have any effect on the antioxidant capacity results (Gomes et al., 2013). For quantification of lipophilic compounds, the remaining samples were frozen at -18 ºC in polyethylene bags. Before analysis, plants were carefully homogenised in food processors (Silvercrest, Germany), and immediately sampled for the chemical analyses. Moisture was evaluated by oven drying at 103 ± 2 ºC (WTC Binder, Germany).

2.3. Chemical analysis

2.3.1. Antioxidant activity

2.3.1.1. Extraction procedure. Triplicate amounts of dried lettuce samples (1 g) were macerated (1 h; at 60 ºC), under stirring (25 mL; 30% methanol, v/v), as previously described by Gomes and coworkers (2013). The extracts were filtered through Whatman No. 42 paper and stored at -18 ºC.

2.3.1.2. Radical scavenging activity. Lettuce samples were analysed for their capacity to scavenge the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, according to the method described by Hatano, Kagawa, Yasuhara, and Okuda (1988). Briefly, extract solution (0.3 mL) was mixed with DPPH radical methanolic solution (2.7 mL; 6×10^{-5} M), kept in the dark for 60 min and the absorbance measured at 517 nm (Genesys 10S UV–Vis Thermo Scientific, USA). DPPH radical scavenging effect was evaluated by the following equation:

DPPH Radical Scavenging Effect (%) = [(ADPPH - AS)/ADPPH] × 100, where AS was the absorbance of the lettuce solution and ADPPH the absorbance of DPPH control solution. Different concentrations of lettuce extract were prepared, allowing the determination of half maximal effective concentration (EC_{50}) values, which corresponded

to the extract concentration that originates a DPPH scavenging effect equal to 50%.

2.3.1.3. Reducing power. An estimation of the extracts reducing power was determined by the procedure described by Oyaizu (1986). Briefly, several extract concentrations extracts (1 mL) were mixed with phosphate buffer (2.5 mL; 0.2 M; pH 6.6) and potassium ferricyanide solution (2.5 mL; 1% w/v), incubated at 50 ºC on a water bath for 20 min. After cooling, trichloroacetic acid (2.5 mL; 10% w/v) was added and the solution. An aliquot of 2.5 mL was withdrawn and iron (III) chloride hexahydrate solution (0.5 mL; 0.1% w/v) was added. The absorbance of the resulting Prussian blue solution was measured at 700 nm after 2 min against a reagent blank. The EC_{50} values were calculated, corresponding to the extract concentration that gives an absorbance equal to 0.5.

2.3.1.4. Total reducing capacity. Total reducing capacity of lettuce extracts was determined by the colorimetric Folin–Ciocalteu method as described by Singleton and Rossi (1965). Briefly, the extract solution (1 mL) was mixed with Folin–Ciocalteu reagent (1 mL). After 3 min, sodium carbonate saturated solution (1 mL) was added and the final volume was adjusted to 10 mL with distilled water. The reaction was kept in the dark during 90 min, and afterwards the spectrophotometric absorbance was determined at 725 nm. Gallic acid solutions (0.01–0.4 mM) were used as standards, being the results expressed as mg of gallic acid equivalents per g of lettuce (mg GAE/g fresh weight; FW).

2.3.2. Chlorophylls, carotenoids and tocochromanols quantification

2.3.2.1. Extraction procedure. Analytes extraction was performed according to the method reported by Zhou et al. (2011), with minor adjustments. Triplicate amounts of lettuce samples (0.5 g) were macerated (1 h), under stirring, with cold chloroform:methanol (5 mL; 2:1, v/v), containing BHT (0.1%, w/v) and both IS. Subsequently, 0.9% (w/v) sodium chloride (1 mL) was added, the mixture was homogenised (1 min), centrifuged (5000 rpm, 3 min) and the clear lower layer was transferred to an amber flask. The extractive procedure was repeated twice with chloroform. The combined extracts were concentrated under a gentle nitrogen stream and recovered to a total volume of 1 mL of ethyl acetate. After a further centrifugation at 13,000 rpm (5 min), half supernatant was separated and kept refrigerated until the chlorophyll and carotenoid HPLC analysis, performed within the same working day. The remaining half of the extract was used for vitamin E assessment by normal-phase HPLC.

2.3.2.2. HPLC equipment and chromatographic separation chlorophylls and carotenoids. Chlorophylls and carotenoids. The analytical method was duly validated in a previous study (Cruz et al., 2012b). Chromatographic analyses were performed using a HPLC system (Gilson, France), with a photodiode array detector (Varian Prostar, USA) controlled by a data processor software (Varian Star Workstation, USA). Chromatographic separation was achieved by injecting, in duplicate, 20 µL of the extract, into a 250 × 4.6 mm Phenomenex Luna ODS-2 (5 µm particle size) column (USA) and eluted with a 30 min linear gradient from 20% ethyl acetate and 80% aqueous methanol (80%, v/v) to 100% ethyl acetate, always with 0.05% (v/v) triethylamine, as described by Caldwell and Britz (2006). The flow rate was 1 mL/min with the room temperature maintained at 22 ± 2 ºC.

For compound identification, spectra were registered at the 350–550 nm band and compared with authentic standards (lutein, β-carotene, chlorophyll *a* and pheophytin *a* and literature data (Britton, 1995). All analytes were quantified on the basis of the chromatograms obtained at 440 nm,

except for chlorophyll *a* and pheophytin *a*, which were quantified at 412 nm. Quantification was processed by the internal standard method, using calibration curves with at least six concentrations for each standard, subjected to the entire extraction procedure. Lutein calibration curve was selected for neoxanthin, violaxanthin and lactucaxanthin determination, while chlorophyll *a*, recorded at 440 nm, was used for chlorophyll *b* estimation. Pheophytin *a* and pheophytin *b*, present in low and variable amounts were calculated and reported as chlorophyll *a* and chlorophyll *b*, respectively.

Vitamin E. The vitamin E analytical method was properly validated in a previous study (Cruz & Casal, 2013) and was accomplished by an integrated system with a data transmitter (Jasco LC-NetII/ADC, Japan), pump (Jasco PU-980, Japan), a refrigerated auto-sampler (Jasco AS – 2057 Plus, Japan) and a fluorescence detector (Jasco FP-2020 Plus, Japan) programmed for excitation at 290 nm and emission at 330 nm. Data were analysed using ChromNAV Control Center – JASCO Chromatography Data Station. The chromatographic separation was achieved with a Supelcosil™ LC-SI column (7.5 cm × 3 mm; 3 µm) (Supelco, Germany).

The mobile phase used was a mixture of 1,4-dioxane in n-hexane (2.5%, v/v) at a flow rate of 0.70 mL/min, operating at constant room temperature (22 ± 2 ºC), and the injection volume was 20 µL. The compounds were identified by chromatographic comparisons with authentic standards and by their UV spectra, using a DAD detector (Jasco MD-2015 Plus, Japan) connected in series. Quantification was based on the fluorescence signal response, using 6-level calibration curves with internal standard.

2.4. Caffeine quantification

Extraction and chromatographic analysis of caffeine from freshand composted-SCG was performed according to Cruz and colleagues (2012a).

2.5. Statistical analyses

2.5.1. Analysis of variance

The results are presented as mean values and standard deviation from triplicate analysis of each sample. The outcomes of variance analysis, comparing control samples and those cultivated with different amounts of SCG, are discussed. Hence, normal distribution of the residuals and the homogeneity of variances were evaluated through the Kolmogorov–Smirnov test (sample size >50) and the Levene's test, respectively. Afterwards, all dependent variables were studied using a one-way ANOVA, subjected or not to Welch correction, depending if the requirement of the homogeneity of variances was verified or not. Furthermore, if a statistical significant effect was verified, post hoc tests, Duncan's or Dunnett's T3 test, were also applied for means comparison, depending if equal variances were assumed or not.

Statistical analyses were performed at a 5% significance level, using SPSS software, version 21.0 (IBM Corporation, New York, USA).

2.5.2. Linear correlation analysis

Pearson's correlation, using SPSS software, version 21.0 (IBM Corporation, New York, USA) was established between the different amounts of SCG and each bioactive parameter analysed.

2.5.3. Principal components analysis

Principal components analysis (PCA) was applied for reducing the number of bioactive components that adequately summarise the effect of the coffee grounds on the lettuce nutritional composition.

PCA was performed by using SPSS software, version 21.0 (IBM Corporation, New York, USA).

3. RESULTS AND DISCUSSION

3.1. Effect of espresso spent coffee on lettuce antioxidant activity

Lettuce was chosen as model plant in this study due to its rapid growth, and also for being one of the vegetable species with higher worldwide production (Rodriguez-Amaya, 2010).

The influence of fresh-SCG on lettuce radical scavenging capacity and reducing power is reported on Figs. 5.2 and 5.3, respectively.

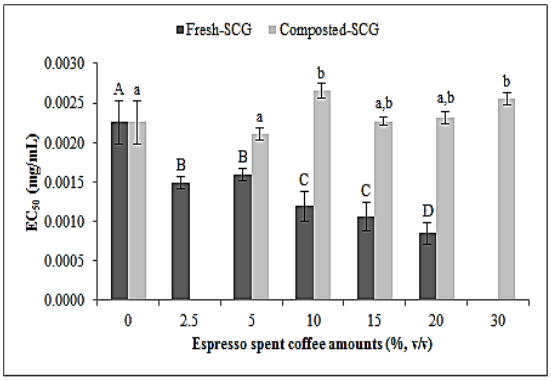

Fig. 5.2. Changes in the DPPH radical scavenging effect of fresh lettuce grown in soil with fresh-SCG (dark bars; mean ± standard deviation; n = 15) or with composted-SCG (light bars; mean ± standard deviation; n = 15) at different percentages. Values with the same letter (upper-case letter for fresh-SCG treatments and lower-case letters for composted-SCG treatments) do not differ significantly ($p < 0.05$) from the given mean. SCG, spent coffee grounds.

All means were compared by Dunnett's T3 test, since homogeneity of variances was not confirmed by Levene's test ($p < 0.05$), excepting for composted-SCG reducing power outcomes whose means were compared by Duncan's test, since homogeneity of variances was confirmed by Levene's test ($p > 0.05$).

Regarding DPPH radical scavenging effect, a gradual decrease in the EC_{50} value was observed in lettuces grown in substrate with increasing fresh-SCG percentage (Fig. 5.2), indicating an improved radical scavenging capacity. All samples groups were significantly different from the control. In parallel, a strong linear correlation between DPPH radical scavenging effect and fresh-SCG percentage was found ($r = -0.911$, $p < 0.001$). The lowest EC_{50} values were determined for the 15% and 20% fresh-SCG amounts. On the other hand, results of the lettuce extracts cultivated in the presence of composted-SCG were statistically similar or slightly higher than the control, independently of the amounts of added SCG (Fig. 5.2). This fact was confirmed by a weak linear correlation between DPPH radical scavenging effect and the composted-SCG percentage ($r = 0.331$, $p = 0.034$). Generally, extracts of lettuce grown in fresh-SCG showed higher antioxidant activity, demonstrating that the use of this type of coffee waste may result in the production of vegetables with increased biological activity.

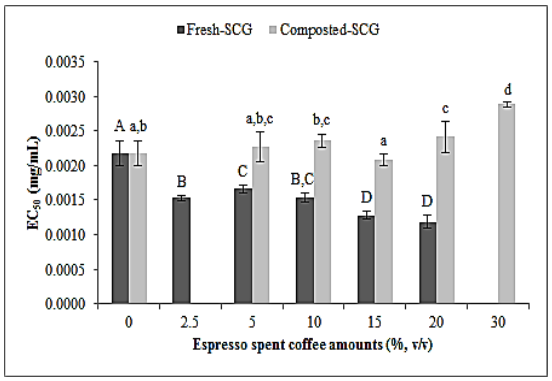

Fig. 5.3. Changes in the reducing power of fresh lettuce grown in soil with fresh-SCG (dark bars; mean ± standard deviation; n = 15) or with composted-SCG (light bars; mean ± standard deviation; n = 15) at different percentages. Values with the same letter (upper-case letter for fresh-SCG treatments and lower-case letters for composted-SCG treatments) do not differ significantly ($p < 0.05$) from the given mean. SCG, spent coffee grounds.

The reducing power outcomes are shown in Fig. 5.3 and an identical pattern to radical scavenging activity was observed for both fresh- and composted-SCG treatments. Therefore, EC_{50} values of lettuces grown in the presence of composted-SCG were statistically similar to the control, with the exception of 20% and 30% concentrations which were definitely higher. This tendency was also verified by Pearson's correlation analysis, showing a positive linear correlation between reducing power effect and composted-SCG percentage ($r = 0.588$, $p < 0.001$). The progressively lower figures achieved with increasing fresh-SCG amounts indicated again a higher antioxidant activity for these plants, especially at 15% and 20% amendment levels

which revealed the lowest EC_{50} values. Strong negative linear correlation between reducing power effect and the fresh-SCG percentage was also confirmed by the Pearson's correlation test (r = -0.876, p < 0.001).

The abovementioned assays showed that if lettuces with high antioxidant activity are desired, fresh-SCG can be used for their production. However, it would be of great interest to ascertain which compounds are truly responsible for such antioxidant enhancement. For the purpose, some major hydrophilic and lipophilic antioxidants, as their related compounds, were evaluated.

3.2. Effect of espresso spent coffee on lettuce hydrophilic antioxidants

Phenolic compounds are abundantly present in plants and play a fundamental role in human diet, mainly due to their antioxidant properties. Despite knowing that the Folin–Ciocalteu method is not specific for these substances, this assay is regularly used to estimate total phenols content indirectly in several vegetable matrices, allowing the estimation of total reducing capacity of the extracts. Thus, it was selected to investigate if changes in lettuce antioxidant profile with pre-harvest SCG treatments are related to their phenolic fraction.

It was observed that, when using fresh-SCG, higher total reducing capacities were observed, mainly in lettuces grown in low spent coffee concentrations (Fig. 5.4). All means were compared by Dunnett's T3 test, since homogeneity of variances was not confirmed by Levene's test (p < 0.05). However, no linear correlation was confirmed between total reducing capacity and the fresh-SCG percentage (r = 0.177, p = 0.059), possibly due to their unregular behaviour. On the contrary, when using composted-SCG, total reducing capacity of lettuce extracts were slightly lower than the control and statistical differences were observed, though any treatment was entirely distinct from another. Thus, a weak negative correlation was accomplished by Pearson's correlation test between total reducing capacity and the composted-SCG percentage (r = -0.186, p = 0.023).

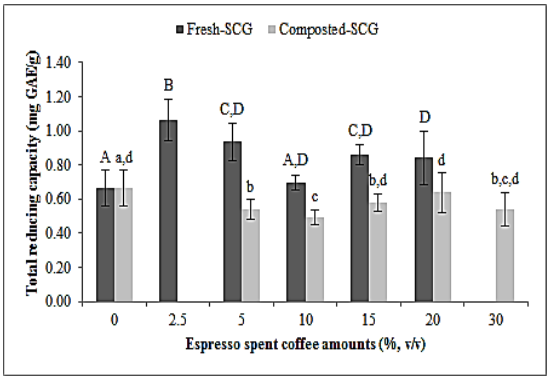

Fig. 5.4. Changes in the total reducing capacity of fresh lettuce grown in soil with fresh-SCG (dark bars; mean ± standard deviation; n = 15) or with composted-SCG (light bars; mean ± standard deviation; n = 15) at different percentages. Values with the same letter (upper-case letter for fresh-SCG treatments and lower-case letters for composted-SCG treatments) do not differ significantly (p < 0.05) from the given mean. SCG, spent coffee grounds.

These figures were consistent with the previous antioxidant assays performed, supporting that the phenolic compounds are important contributors for the plant potential antioxidant capacity. Knowing that plants produce phenolic compounds as a plant-defense mechanism in response to stress (Hossain et al., 2010), the use of high percentages of fresh-SCG may have been a stress factor, resulting in an improved production of compounds with antioxidant activity. The correlation factors for estimated phenolic compounds were, however, comparatively lower than those achieved with the other antioxidant assays, supporting that other compounds might be involved.

3.3. Effect of espresso spent coffee on lettuce lipophilic antioxidants and related substances

The present work also intended to provide a detailed evaluation on bioactive lipophilic compounds, thus enabling a better understanding of the fresh-SCG or composted-SCG impact on overall nutritive and antioxidant capacity.

Considering that all variables, from agricultural practices to handling cares, were identical to all samples, the differences observed herein are strictly related to the cultivation medium composition. Thus, the outcomes obtained for the control group will be compared with literature data, but the influence of SCG will be discussed by comparison with samples grown under the same conditions, in the absence of SCG (0%, control group).

Table 5.1 specifies the amounts of all aforementioned substances for each SCG group, being the results presented on a FW basis, as consumed. The two major lettuce's carotenoids (lutein and β-carotene) were individually quantified, as well as its typical minor carotenoids (neoxanthin, violaxanthin and lactucaxanthin). As described by other authors (Caldwell & Britz, 2006; Kimura & Rodriguez-Amaya, 2003), all samples revealed a similar chlorophyll and carotenoid profile, with higher amounts of chlorophyll *a* than chlorophyll *b*, as well as a higher content of lutein or β-carotene than neoxanthin, violaxanthin and lactucaxanthin.

It should be taken into consideration that there is a high variability among lettuce varieties and cultivars, growth conditions (light exposure, temperature, humidity, and nutrient supply), planting season, and particularly analytical methodologies (Britz et al., 2008; Lampi et al., 2010; Mou, 2005; Shin et al., 2009), hindering literature comparison. Still, in comparison with published results for Butterhead lettuce, our control group presented slightly higher lutein amounts (Table 5.1) than those reported by Kimura and Rodriguez- Amaya (2003) (2.14 ± 0.14 mg/100 g, FW) and Mou (2005) (0.54–2.54 mg/100 g, FW). As regards to β-carotene's content (Table 5.1), Cardoso et al. (2009) reported similar values, ranging from 2.10 to 3.58 mg/100 g, FW. The amounts of minor carotenoids (Table 5.1) found in the present work are within the same range as those found in previous studies (Baslam et al., 2013; Kimura & Rodriguez-Amaya, 2003) as well as individual and total chlorophylls (Table 5.1) (Agüero et al., 2008; Mou, 2005). α-Tocopherol is the most biologically active form of vitamin E and, together with γ-tocopherol, comprises the main tocochromanols in lettuce. Even so, it is known that the vitamin E amounts are highly variable among cultivars, growth conditions and particularly analytical methodologies (Britz et al., 2008; Lampi et al., 2010; Shin et al., 2009). α-Tocopherol amounts found (Table 5.1) are in accordance with Szymanska and Kruk (2008), which varied from 0.51 to 1.62 mg/100 g (FW) though total vitamin E is slightly higher in our samples.

Nevertheless, the main goal of the present study is to establish a straight connection between the aforementioned compounds and the increase of fresh-SCG or composted-SCG, knowing that all samples were cultivated under the same conditions, which suppresses such variations within this study.

3.3.1. Fresh espresso spent coffee influence

The employment of fresh-SCG as soil amendment proved to be a determinant factor for lettuce carotenoids amounts (Table 5.1). In fact, lutein revealed an increment up to 98% and β-carotene, the second major carotenoid in lettuce, increased its content up to 90%, when 15% (v/v) of fresh-SCG was applied. Regarding minor carotenoids, neoxanthin content revealed a great enhancement up to 95% if 5% (v/v) to 15% (v/v) of fresh-SCG were utilised. An outstanding improvement was observed for violaxanthin and lactucaxanthin, up to 240% and 155%, respectively. In general, all fresh- SCG groups were statistically different ($p < 0.05$) from control group for all minor compounds. Besides, a very significant and strong positive correlation was observed for all carotenoids, particularly for violaxanthin.

Table 5.1
Carotenoids, chlorophylls, and tocochromanols composition (mg/100g) of lettuce samples after cultivation with espresso spent coffee (mean ± SD, fresh weight basis)

Compounds	Fresh espresso spent coffee amounts (v/v)						*p*	Pearson's correlation coefficient
	0%	2.5%	5%	10%	15%	20%		
Lutein	4.00±1.16 a	5.34±1.57 a,b	6.57±1.12 b,c	6.03±1.99 a	7.85±1.41 c	7.06±1.86 b,c	< 0.001**	0.610**
β-Carotene	3.68±1.11 a	4.25±1.27 a	4.42±0.65 a	4.58±1.15 a	6.64±1.37 b	6.18±1.47 b	< 0.001*	0.617**
Neoxanthin	1.32±0.55 a	1.62±0.58 a,c	2.50±0.46 b,c	2.50±0.81 b,c	2.51±0.47 b	2.28±0.85 c	< 0.001**	0.542**
Violaxanthin	0.76±0.59 a	1.76±0.65 b	3.06±0.55 c	3.59±0.90 c	3.49±0.81 c	3.05±0.70 c	< 0.001*	0.748**
Lactucaxanthin	0.20 ±0.08 a	0.32±0.11 b	0.47±0.12 c	0.58±0.22 c	0.62±0.24 c	0.62±0.26 c	< 0.001**	0.694**
Chlorophyll *a*	8.53±5.16 a	15.79±5.62 b	15.15±2.55 b	13.71±5.57 a,b	17.92±8.25 b	8.70±4.01 a	< 0.001**	0.169
Chlorophyll *b*	4.61±1.57 a	5.63±1.87 a.b	6.07±1.10 b	5.88±1.84 a,b	8.21±2.20 c	6.28±1.40 b	< 0.001*	0.435**
α-Tocopherol	1.73±0.50 a,c	1.85±0.24 a.c	2.16±0.15 b,c	2.13±0.45 c	1.80±0.51 c	2.08±0.36 c	0.001**	0.231*
γ-Tocopherol	1.40±0.35 a,b	1.76±0.62 a,b	1.59±0.20 b	0.97±0.33 c	1.17±0.35 a,c	1.21±0.29 a,c	< 0.001**	-0.323**
Compounds	Composted espresso spent coffee amounts (v/v)						*p*	Pearson's correlation coefficient
	0%	5%	10%	15%	20%	30%		
Lutein	4.00±1.16 a	6.68±1.70 b	6.83±1.15 b	6.29±0.71 b	5.99±1.44 b	6.26±1.31 b	< 0.001**	0.387**
β-Carotene	3.68±1.11 a	5.26±1.34 b	5.24±0.81 b	5.26±0.73 b	4.75±1.00 b	4.75±1.05 b	< 0.001*	0.257**
Neoxanthin	1.32±0.55 a	3.35±1.09 b	4.02±0.84 b,c	4.03±0.78 b,c	3.74±0.92 b,c	5.06±1.81 c	< 0.001**	0.701**
Violaxanthin	0.76±0.59 a,c	0.28±0.09 b	0.36±0.08 b,c	0.33±0.05 b,c	0.33±0.08 b,c	0.40±0.09 c	0.001**	-0.318**
Lactucaxanthin	0.20±0.08 a	0.28±0.09 b	0.36±0.07 c,d	0.34±0.05 b,c	0.34±0.08 b,c	0.40±0.10 d	< 0.001*	0.621**
Chlorophyll *a*	8.53±5.16 a	18.92±5.20 b	19.04±3.06 b	18.51±2.30 b	18.26±3.65 b	16.62±3.32 b	< 0.001**	0.447**
Chlorophyll *b*	4.61±1.57 a	7.86±2.03 b	8.01±1.53 b	7.79±0.89 b	7.62±1.68 b	7.02±1.29 b	< 0.001*	0.392**
α-Tocopherol	1.73±0.50	1.66±0.42	1.51±0.38	1.75±0.30	1.95±0.44	1.54±0.18	0.038**	-0.022
γ-Tocopherol	1.40±0.35 a	1.21±0.32 a,c	0.83±0.09 b,c	1.25±0.41 a,c	1.27±0.40 a,c	1.06±0.29 c	< 0.001**	-0.224*

Different letters in a row show statistically significant differences ($p < 0.05$) from the given mean.

*p values from one-way ANOVA analysis. Means were compared by Duncan's test, since homogeneity of variances was confirmed by Levene's test ($p > 0.05$).

**p values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since homogeneity of variances was not confirmed by Levene's test ($p < 0.05$).

Pearson's correlation is significant at the 0.01 level (**p) or at the 0.05 level (*p).

Concerning lettuce chlorophylls, chlorophyll *a* showed a high variation coefficient in intra-group samples, which might be related to each individual lettuce biomass. Nevertheless, there is a notable increment of their amounts when using fresh-SCG, though for 20% (v/v) group a reduction is verified, thus justifying no statistically significant Pearson's correlation for chlorophyll *a*. Notwithstanding, as regards to chlorophyll *b*, its amounts improved up to 89%, showing a very significant positive correlation between its amounts and the employment of fresh-SCG. This tendency has already been verified by Cruz and colleagues (2012), where the carotenoid and chlorophyll concentrations in lettuce were significantly improved by the presence of fresh-SCG in cultivation medium.

With reference to α- and γ-tocopherols, a significant positive Pearson's correlation was confirmed for the former with the increase of fresh-SCG. However, a significant negative Pearson's correlation was verified for the latter, though for low amounts of fresh-SCG (2.5% and 5%, v/v) a slight enhancement was perceived.

By developing a parallelism between these individual substances with the preceding antioxidant evaluations performed herein (Figs. 5.2 and 5.3) a favourable correspondence with the increment of fresh-SCG is obtained. Therefore, lettuce carotenoids, chlorophylls and tocochromanols might also be involved in the improvement of the overall antioxidant activity.

3.3.2. Composted espresso spent coffee influence

Knowing that fresh-SCG still presents bioactive compounds, caffeine included (320 mg/100 g, dry weight), and that these might negatively influence vegetable development, we have attempted to verify the effect of composting on SCG, which revealed an absence of caffeine. Focusing on composted-SCG experiment, both chlorophylls and carotenoids in all treated samples were again statistically different ($p < 0.05$) from control group (Table 5.1). For low amounts of composted-SCG, and comparing to control group, lutein's content presented an 85% increment and β-carotene increased up to 71%. Nevertheless, there is not a strong positive correlation with the composted-SCG amounts, since their content stabilizes despite the initial rise. Concerning minor carotenoids, both neoxanthin and lactucaxanthin revealed very significant and strong positive Pearson's correlation with the increase of composted-SCG, while violaxanthin showed a different trend, since its content decreased down to 74%, which was not verified for fresh-SCG pre-harvest treatments. In general, total carotenoids amounts were higher for the 10% (v/v) group.

On the other hand, for chlorophylls *a* and *b*, a 111% and an 87% rise was respectively verified once again for the 10% (v/v) group, showing a slightly better photosynthetic capacity, rather than an overall antioxidant profile.

With reference to composted-SCG effect on lettuce's vitamin E, there were no significant statistical differences with regard to α-tocopherol amounts between control group and further treatments; though a minor reduction is perceived (Table 5.1). Furthermore, γ-tocopherol was occasionally but significantly lower in the 10% (v/v) group in comparison to control. However, a significant negative correlation ($r = -0.224$, $p < 0.05$) was obtained with the increase of composted-SCG.

In brief, a small enhancement of antioxidant compounds was observed for low amounts of composted-SCG, which tend to reduce for higher percentages, as 20% and 30% (v/v). This observation is coherent with those found in the antioxidant assays (Figs. 5.2 and 5.3).

3.4. Espresso spent coffee type and lettuce overall and detailed antioxidant activity

With the data acquired from the lettuce plants treated with fresh- or composted-SCG, a PCA test was executed in order to classify the plants based on its overall hydrophilic and lipophilic antioxidant profile and activity. It allowed explaining 81% of total data variance by using two principal components based on the two antioxidant capacity assays, neoxanthin, violaxanthin and chlorophyll *a*, as represented in Fig. 5.5.

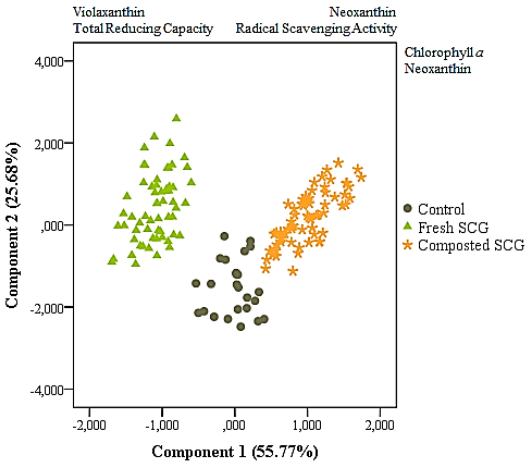

Fig. 5.5. Principal components analysis using overall and detailed antioxidant activity parameters of lettuce cultivated with different percentages of espresso spent coffee grounds, composted or not.

Herein, three distinct lettuce groups are represented, corresponding to control, fresh-SCG and composted-SCG groups. The first principal component factor (PC1), which comprises 56% of the total variance, is able to separate the three groups, being those subjected to the fresh-SCG treatment located in the negative region, while composted-SCG samples are positioned in the positive region and control samples in the center. This observation may be justified by the fact that fresh-SCG lettuce samples reported the highest figures for violaxanthin (PC1 loading = -0.825) and total reducing capacity (PC1 loading = -0.816), while composted-SCG lettuce samples revealed higher amounts of neoxanthin (PC1 loading = 0.673) as well as higher EC_{50} values for radical scavenging activity (PC1 loading = 0.899), equivalent to lower antiradical activities. The second component factor (PC2), which justifies 26% of the total variance observed, was able to separate all the samples from the control group which may be explain by the higher chlorophyll *a* (PC2 loading = 0.808) and neoxanthin (PC1 loading = 0.587) content of the formers. Therefore, despite the higher amounts of neoxanthin in composted-SCG treatment, fresh-SCG treatments showed a remarkable ability to improve lettuce antioxidant activity in comparison to non-treated samples.

Environmental stresses trigger a wide variety of plant responses, ranging from altered gene expression to changes in cellular metabolism and growth (Munné-Bosch, 2005). During potentially harmful stress (such as those caused by light, drought, salinity, extreme temperatures, pathogen infections, etc.), breakdown of chlorophyll qualifies as a detoxification mechanism during senescence, which is vitally important for plant development and survival (Hörtensteiner, 2006; Munné-Bosch, 2005). When photosynthesis is compromised, carotenoids and tocopherols are produced in greater quantity in order to protect membranes from oxidative damage. Once fresh-SCG presents higher bioactive potential (e.g. caffeine) than composted-SCG, an increase of carotenoids, chlorophylls and tocochromanols might be expected in order to protect membranes from oxidative damage (Cruz, Baptista, et al., 2012).

Furthermore, there are also plenty of factors that may alter the total phenolic compounds content in vegetables, such as those of genetic character, environmental conditions, post-harvest settings or processing particularities (Tomás-Barberán & Espín, 2001). In our particular case, only one environmental condition varied among our samples, i.e. SCG presence and amounts. According to Tomás-Barberán and Espín (2001) and Cartea et al. (2011), soil composition (mineral and organic nutrients) has a marked influence on the phenolic content of plants.

Since these substances have beneficial biological properties, the presence of espresso coffee residues can be regarded as favourable in a nutritional point of view. However, and from the results obtained, the potential benefits are maximised for low spent coffee amounts.

4. CONCLUSIONS

The current study confirms the effect of fresh-SCG and composted-SCG in lettuce's antioxidant capacity and bioactive compounds when exposed to different pre-treatments conditions. An enhancement of plant's overall and specific antioxidants was perceived, both hydrophilic and lipophilic, likely due to plant induced-stress by typical SCG bioactive compounds. The utilisation espresso spent coffee grounds constitutes a practical and costeffective approach to obtain vegetable products of higher nutritional value.

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Improvement of vegetables elemental quality by espresso coffee residues

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ABSTRACT

Spent coffee grounds (SCG) are usually disposed as common garbage, without specific reuse strategies implemented so far. Due to its recognized richness in bioactive compounds, the effect of SCG on lettuce's macro- and micro-elements was assessed to define its effectiveness for agro industrial reuse. A greenhouse pot experiment was conducted with different amounts of fresh and composted spent coffee, and potassium, magnesium, phosphorous, calcium, sodium, iron, manganese, zinc and copper were analyzed. A progressive decrease on all lettuce mineral elements was verified with the increase of fresh spent coffee, except for potassium. In opposition, an increment of lettuce's essential macro-elements was verified when low amounts of composted spent coffee were applied (5%, v/v), increasing potassium content by 40%, manganese by 30%, magnesium by 20%, and sodium by 10%, of nutritional relevance This practical approach offers an alternative reuse for this by-product, extendable to other crops, providing value-added vegetable products.

Keywords: *Coffee by-products; Lettuce; Composting; Minerals; Macro-elements; Micro-elements.*

1. INTRODUCTION

Coffee wastes management has been the focus of recent developments, aiming to implement ecological disposal approaches and develop value-added alternatives. These by-products are generated in the producing countries (e.g., coffee pulp, cherry husks, and parchment skin), by the roasting industries (e.g., coffee silverskin), in the soluble coffee industry and after brewing, these last designated generally as ‘‘spent coffee’’.

Industrial spent coffee has been recycled through compost preparation, biogas production, animal feed and mushrooms production, or more recently by extraction of value-added fractions, as lipids for biodiesel production or ethanol (Mussatto et al., 2011a; Oliveira et al., 2008). Additionally, the spent coffee grounds, obtained after beverage preparation, represent tons of residues produced worldwide in coffee shops, cafeterias, restaurants, or at home. In particular, spent coffee grounds (SCG) has already proven to have a huge reuse potential for diverse applications, even greater than the one expected from industrial spent coffee due to its increased richness in nutritive and bioactive constituents (Bravo et al., 2012; Cruz et al., 2012). Among others, coffee grounds are increasingly being used in domestic agriculture, usually by disposal of small amounts directly in the soil, or by mixing in composting piles, still with unsupported claims of efficiency and safety for human consumption. Indeed, Kasongo et al. (2011) have shown that coffee wastes may be used as liming material and NPK fertilizer, and they promote increased water and nutrient retention. Also, some authors showed the feasibility of directly using spent coffee as substrates for edible mushroom cultivation without any pre-treatment (Murthy & Naidu, 2012). On the other hand, Liu and Price (2011) demonstrated that these remains can be successfully composted using in-vessel, static aerated pile or vermicomposting systems, all with C:N ratios within the optimum range of <25:1,

making them interesting for use as a slow-release source of nitrogen for fertilising.

In order to support a safe employment of SCG as soil amendment for horticultural purposes, it is mandatory to evaluate the vegetables nutritional and bioactive features. Following a previous work on chlorophylls and carotenoids in lettuce (*Lactuca sativa* L.) grown with diversified amounts of fresh SCG (Cruz et al., 2012b), this work focused on the detailed assessment of lettuce mineral composition of nutritional relevance. Lettuce represents a major and low-cost green leafy vegetable, being one of the most frequently consumed vegetables in the world, representing therefore a major source of phytonutrients, including minerals, in the diet (Mou, 2009). In particular, one intended to verify if the elemental composition of lettuce plants was altered by the use of SCG, while providing also a sustained comparison between fresh and composted coffee grounds.

In detail, the contents of five biologically essential macroelements were estimated: potassium (K), magnesium (Mg), phosphorous (P), calcium (Ca) and sodium (Na). Additionally, four essential micro-elements were also determined: iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) in lettuce samples subjected to several pre-harvest treatments.

2. MATERIALS AND METHODS

2.1. Spent coffee grounds and soil material

Espresso spent coffee grounds were collected from several coffee shops, in Oporto metropolitan area (NW Portugal). Two separate assays were performed, using fresh-SCG alongside with composted-SCG. Regarding the former study, five fresh mixtures were assembled: 2.5%, 5%, 10%, 15% and 20%, all on a volume basis, using plain vegetable soil (Siro® Germe, Leal e Soares, Lda.; Portugal) as control (0%), and distributed by 1 L plastic pots. Concerning the latter, SCG was previously composted for a period of 6 months at a specialised municipal solid waste treatment facility (Lipor, Oporto, NW Portugal) with equal volumes of fresh grass and straw/sawdust, being regularly revolved and watered when moisture was below 60%. Another five mixtures were prepared: 5%, 10%, 15%, 20% and 30%, again on a volume basis, and distributed in 1 L plastic pots.

2.2. Plant material and growth conditions

The experiments were conducted with *L. sativa* var. *capitata* cv. ‘‘Four Seasons’’ plants, in the greenhouses of the School of Agriculture of the Polytechnic Institute of Bragança (NE Portugal), under proper ventilation, controlled temperature (25 ± 2ºC) conditions, and adequate irrigation (for details please consult Cruz et al., 2012b). Fifteen plants from each fresh and composted treatment, including the control group, were harvested after 39 days, and assembled in 5 groups of three plants, in a total 55 samples (control + 5 fresh treatments + 5 composted treatments = 11 treatments × 5 plant groups). After careful wash with ultra-pure water, samples were stored in polyethylene bags at -18ºC for subsequent freeze-drying (Telstar Cryodos-80, Spain), preserved in the dark at 4ºC, and finely homogenised in a food processor (Silvercrest, Germany) for chemical analyses. Post-harvest soil samples were also collected from 33 pots (3 per treatment), dried in a forced-air oven (WTC-Binder, Germany) and preserved for chemical analysis. All analyses were performed in triplicate.

2.3. Total ashes and mineral composition

Dry ashing of both lettuce and soil samples was performed according to AOAC method 920.93 (AOAC, 2000) at 500 ºC in a muffle furnace (48000 Furnace, Thermolyne, USA), until white ashes were achieved. After cooling in a desiccator, ashes were weighed for total mineral content estimation. For lettuce samples, a portion of ashes (100 mg) was dissolved in water acidified with 1% nitric acid (65% v/v, Suprapur®, Merck, Germany) (5 mL) for

mineral composition assessment. Spent coffee and soil ashes were subjected to nitric acid/hydrochloric acid digestion on a hot plate as described by Newman and Zhao (2005). For all samples and depending on the analysed element, appropriate dilutions were conducted with ultra-pure water (18.2 MΩ/cm, Millipore, France).

The elements Ca, Mg, Na, K, Fe, Cu and Mn were quantified as described by Oliveira et al. (2012), and accomplished by high-resolution continuum source flame atomic absorption spectrometry (HR-CS-FAAS; ContrAA 700, Analytik jena, Germany). This equipment is operate with a xenon short-arc lamp XBO 301 (GLE, Germany) with a nominal power of 300 W, functioning in a hotspot mode as a continuum radiation source, with a spectral band ranging from 190 to 900 nm, being coupled to an auto-sampler (AS 52 S, Analytik Jena, Germany). Reconstituted air-acetylene (Air Liquid, Portugal) oxidising flame was used for atomization.

Phosphorous was estimated by a dual beam UV visible spectrophotometer (Evolution 300, Thermo Scientific, USA) at 420 nm according to Greenberg et al. (1992).

Standard solutions of Ca, Mg, Fe, Cu and Mn, were prepared from the correspondent 1000 mg/L stock solutions (Panreac, Spain). P, K and Na standard solutions were obtained by potassium dihydrogen phosphate (99.5%, Riedel-de Haën), potassium chloride (99.5%, Riedel-de Haën) and sodium chloride (99.8%, Riedel-de Haën) dissolution in ultrapure water, respectively. A 1% caesium chloride (p.a., Sigma–Aldrich) solution was added as ionisation suppressor for flame atomization analysis.

2.4. Statistical analyses

2.4.1. Analysis of variance

The results obtained from analysis of variance, comparing control samples and those grown with different amounts of SCG for all the groups, are discussed. Thus, normal distribution of the residuals and the homogeneity of variances were assessed by means of the Kolmogorov–Smirnov test (sample size >50) and the Levene's test, respectively. Then, all dependent variables were analysed using a one-way ANOVA, subjected or not to Welch correction, depending if the requirement of the homogeneity of variances was accomplished or not. Also, post hoc tests were also applied, if a statistical significant effect was verified, using Tukey's or Dunnett's T3 test for means comparison, depending if equal variances were assumed or not.

A multivariate analysis of variance (MANOVA) was also performed in order to assess the effects of SCG percentages and SCG type (fresh or composted) on lettuce's mineral composition. Statistical analyses were performed at a 5% significance level, using SPSS software, version 21.0 (IBM Corporation, New York, USA).

2.4.2. Regression analysis and linear correlation

Regression analysis and Pearson's correlation, using SPSS software, version 21.0 (IBM Corporation, New York, USA) and Excel from Microsoft Corporation, were established between the different amounts of SCG and each element analysed.

2.4.3. Linear discriminant analysis

A linear discriminant analysis (LDA) was performed by using SPSS software, version 21.0 (IBM Corporation, New York, USA). It was used as a supervised learning technique to classify the different samples of lettuce cultivated with different percentages of SCG, composted or not, according to their mineral composition and total ashes. A stepwise technique, using the Wilk's lambda method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection. To verify which canonical discriminant

functions were significant, the Wilk's lambda test was applied.

To avoid overoptimistic data modulation, a leaving-one-out cross-validation procedure was carried out to assess the model performance. Moreover, the sensitivity and specificity of the discriminant model were computed from the number of individuals correctly predicted as belonging to an assigned group (López et al., 2008; Rencher, 1995).

3. RESULTS AND DISCUSSION

3.1. Mineral composition of lettuce plants

High variability regarding lettuce mineral data in the literature is observed, mainly due to agricultural (e.g., cultivar, location, agricultural practices, rainfall/irrigation, salinity and temperature) (Kawashima & Soares, 2003), technological (e.g., washed/unwashed) (Nali et al., 2009), and analytical differences. Therefore, the results obtained in the present study for control group will be compared with literature data, but the influence of spent coffee will be discussed by comparison with samples grown under the same condition, in the absence of SCG (0%, control group).

From a global point of view, our results for lettuce mineral composition (Table 5.2) are comparable with those presented in the literature, independently from the variations observed with the tested soil treatments that will be further discussed. Specifying the macro-elements in lettuce, K notoriously represents the most abundant mineral, with amounts similar to those reported by Nali et al. (2009) (7850.9 ± 673.3 mg/100g, dry weight, DW) and Kelly and Bateman (2010) (approximately, 3500–7200 mg/100g, DW) for conventional cultivation, but higher than those found by Mou (2009) (5409.1 mg/100g, DW). Concerning Mg amounts, the results are higher than those reported by Mou (2009) (295.4 mg/100g, DW), Nali et al. (2009) (321.2 ± 19.4 mg/100g, DW) and Kelly and Bateman (2010) (approximately, 190–380 mg/100g, DW) for conventional cultivation. Ca content is in accordance to Mou (2009) (795.4 mg/100g, DW), to Nali et al. (2009) (1060.0 ± 69.6 mg/100g, DW) for washed samples, as to Kelly and Bateman (2010) (approximately, 400–1000 mg/100g, DW) for conventional cultivation. The attained values for P in lettuce were higher than the ones described by Mou (2009) (750.0 mg/100g, DW). The Na outcomes were in accordance to those defined by Nali et al. (2009) (349.1 ± 48.9 mg/100g, DW) and to Kelly and Bateman (2010) (approximately, 0–400 mg/100g, DW) for conventional cultivation, and slightly higher than those reported by Mou (2009) (113.6 mg/100g, DW).

Regarding minor elements, Fe amounts are in accordance with the values presented by Mou (2009) (28.1 mg/100g, DW) and Kelly and Bateman (2010) (approximately, 0–57 mg/100g, DW) for conventional cultivation, but lower than those reported by Nali et al. (2009) (56.0 ± 8.8 mg/100g, DW). Furthermore, Mn contents are slightly higher than the amounts defined by Mou (2009) (4.1 mg/100g, DW) and Kelly and Bateman (2010) (approximately, 0.5–9.0 mg/100g, DW) for conventional cultivation. The Zn figures are similar to the ones described by Nali et al. (2009) (8.1 ± 0.6 mg/100g, DW), though slightly above the results presented by Kelly and Bateman (2010) (3.5–7.5 mg/100g, DW). Finally, Cu contents are similar to the reported by Kelly and Bateman (2010) (0.5–1.0 mg/100g, DW) for conventional cultivation, but lower than those described by Nali et al. (2009) (3.8 ± 8.0 mg/100g, DW) and higher than those reported by Mou (2009) (0.36 mg/100g, DW). No literature data were found for Butterhead lettuce regarding total ashes content, and so its particular discussion will be made by single comparison to control group.

Deviations of element amounts in lettuce among literature data are very common, since metal bioavailability as well as plant-uptake is strictly related to its form of entering the soil, the chemical and physical properties of the soil around

Table 5.2
Total ashes (g/100g) and mineral composition (mg/100g) of lettuce and soil samples after cultivation with fresh spent coffee (mean ± SD, dry weight basis)

Sample	Element	Fresh spent coffee amounts (v/v)						*p*
		0%	2.5%	5%	10%	15%	20%	
Lettuce	K	7363.8 ± 343.6 a	8108 ± 632.9 b	8097.9 ± 246.5 b	8289.5 ± 379.6 b	6882 ± 1233.8 a	6697.1 ± 1485.9 a	< 0.001**
	Mg	1158.4 ± 108.1 a	816.4 ± 95.8 b	642.7 ± 95.1 c	540.5 ± 37.8 c,d	528.8 ± 162.3 c,d	492.3 ± 100.9 d	< 0.001**
	Ca	937.0 ± 56.2 a	641.3 ± 58.6 b	646.1 ± 51.7 c	476.2 ± 63.1 d	486.7 ± 84.8 b,c,d	392.8 ± 60.7 c	< 0.001**
	P	893.4 ± 118.6 a	756.5 ± 26.5 b	608.5 ± 59.6 b	689.5 ± 61.2 c	618.8 ± 171.6 c	548.5 ± 107.3 d	< 0.001*
	Na	336.7 ± 12.7 a	283.8 ± 7.9 b	241.2 ± 20.5 c	214.8 ± 24.3 c	228.9 ± 19.3 c	230.2 ± 56.4 c	< 0.001**
	Fe	25.2 ± 1.3 a	11.9 ± 1.8 b,c	15.8 ± 3.8 c,d	16.7 ± 4.7 d	10.2 ± 4.1 b,e	7.6 ± 2.9 e	< 0.001**
	Mn	20.0 ± 2.6 a	9.4 ± 1.7 b	8.0 ± 1.3 b	8.8 ± 1.6 b	12.8 ± 0.7 c	18.7 ± 3.6 a	< 0.001**
	Zn	8.7 ± 1.3 a	8.3 ± 0.7 a	6.7 ± 0.6 b	6.7 ± 0.9 b,c	5.5 ± 1.3 c	5.2 ± 1.8 b,c	< 0.001**
	Cu	0.6 ± 0.1 a	0.2 ± 0.1 b	0.5 ± 0.2 a	1.0 ± 0.1 c	1.0 ± 0.4 c	0.9 ± 0.2 c	< 0.001**
	Total ashes	21.5 ± 1 a	22.3 ± 1.6 a	22.6 ± 1.5 a	21.8 ± 0.3 a	18.5 ± 3.2 b	16.6 ± 3.4 b	< 0.001**
Soil	K	203.1 ± 17.6 a	125.2 ± 6.0 b	202.1 ± 17.4 a	230.3 ± 0.4 c	253.1 ± 34.3 c,d	298.2 ± 26.5 d	< 0.001**
	Mg	4.7 ± 0.5 a	4.9 ± 0.6 a,b	5.6 ± 0.4 b	6.4 ± 0.2 c	6.6 ± 0.6 c	6.8 ± 1.1 b,c	< 0.001**
	Ca	5670.8 ± 1351.5 a,c	4767.9 ± 541.8 a,c	5372.7 ± 1157.5 a	4446.7 ± 123.6 b	5005.3 ± 1082.6 a,c	5262.3 ± 369.8 c	< 0.001**
	P	240.9 ± 56.7 a	199.6 ± 25.6 a,b	191.4 ± 12.4 a,b	137.2 ± 7.6 b	219.1 ± 20.5 a,b	241.1 ± 12.4 a	< 0.001**
	Na	44.9 ± 10.0 a	34.0 ± 0.8 b	30.4 ± 6.0 b,c	28.4 ± 1.3 c	28.3 ± 4.3 c	29.7 ± 1.3 c	< 0.001**
	Fe	161.3 ± 6.2 a	137.3 ± 9.9 b,d	159.3 ± 19.3 a,b	122.0 ± 1.4 c,d	151.4 ± 19.4 a,b,d	128.7 ± 16.9 d	< 0.001**
	Mn	5.9 ± 0.3 a	5.2 ± 0.2 b	5.7 ± 0.9 a,b	5.4 ± 0.1 b	5.3 ± 0.3 b	5.3 ± 0.3 b	< 0.001**
	Zn	2.6 ± 0.6 a	3.0 ± 0.7 a,b	3.6 ± 1.1 a,b	3.5 ± 0.3 b	2.0 ± 0.4 c	2.1 ± 0.2 c	< 0.001**
	Cu	1.0 ± 0.2 a	1.0 ± 0.1 a	1.1 ± 0.2 a,c	1.5 ± 0.0 b	1.3 ± 0.2 b,c,d	1.5 ± 0.1 d	< 0.001**
	Total ashes	18.8 ± 3.8 a,c	18.1 ± 0.1 a,c	14.1 ± 1.4 b,c	12.8 ± 0.9 b	16.1 ± 1.8 c	15.8 ± 2.2 c	< 0.001**

Different letters in a row show statistically significant differences ($p < 0.05$) between means.

*p values from one-way ANOVA analysis. Means were compared by Tukey's test, since homogeneity of variances was confirmed by Levene's test ($p > 0.05$).

**p values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since homogeneity of variances was not confirmed by Levene's test ($p < 0.05$).

the plant roots and the crop type (Alloway, 1995; Morikawa & Saigusa, 2008). According to Kabata-Pendias (2004), plants reveal a great adaptation to the variable composition of growth media, developing uptake/exclusion mechanisms for a given nutrient, either under deficiency or excessive conditions in soil. Another interesting deviation factor may be the complex relationship between arbuscular mycorrhizal fungi and plant nutrient uptake from soil, creating an inconsistent effect on different nutrients (Gosling et al., 2006). Nevertheless, this study main goal is to ascertain if there is an effective change of lettuce mineral profile when SCG is used as soil amendment, as it will be discussed hereafter.

3.2. Effect of fresh espresso spent coffee on lettuce's mineral profile

The results achieved for total ashes and individual elements in lettuce, cultivated in soil containing different percentages of fresh-SCG, and the soil sampled after harvest, are detailed in Table 5.2, on a DW basis.

Statistically significant differences were observed for all elements when compared to control group, though regarding total ashes, a progressive decline with the fresh-SCG increase was detected, particularly for the 15% and 20% treatments. This can be interpreted as a sign of plant's privation for some elements. Hence, by comparison to the control group, excepting K, there is a consistent decrease of all mineral elements with the increase of SCG percentage, being though more perceptible for Mg, Ca and P, right from the lowest 2.5% supplementation level.

In particular, Mg and P decrease their amounts up to 60% in the plant, while Na and Ca are apparently less affected, with a reduction of only up to 40%. Furthermore, all minor elements reveal a high variability as well as a superior reduction of their contents, all up to 70%. Nevertheless, the reduction of Fe amounts may become of main concern from the nutritional point of view, as a high prevalence of Fe deficiency anaemia is common, especially in vegetarian women (Chiplonkar et al., 1999). Yet, some compounds may increase the micronutrient bioavailability in diets, such as the combination of Vitamin C and Fe intake (Flyman & Afolayan, 2006). Hence, it would be interesting to evaluate if there an increase of Vitamin C in lettuce pre-treated with SCG in order to ascertain if Fe absorption may be potentiate despite its content reduction. Indeed, only K revealed a momentary increase in its content in comparison to control samples, being more visible up to 10%, decreasing from this point further. This fact is coherent with SCG elemental composition, in which K accounts for 40% of its mineral content, on a dry basis (Cruz et al., 2012a).

Additionally, regression analysis and Pearson's correlation were performed between the added SCG amounts and the mineral parameters both for fresh- and composted-SCG (Table 5.3). It was verified that the employment of fresh-SCG as pre-harvest treatment was highly and negatively correlated with the results obtained in almost all the analysed minerals, except with the Mn (R^2 = 0.0013, p = 0.037) and Cu (R^2 = 0.3554, p = 0.596) amounts. Thus, it may be stated that, despite few exceptions, fresh-SCG influences the composition of the lettuce in a percentage dependent manner.

Interestingly, an inverse pattern is observed in the post-harvest soil samples (Table 5.2), where an underlying increment of such minerals is observed. This increase may be the result of the actual SCG mineral composition, particularly due to its richness in K (Cruz et al., 2012a) or a retention of minerals by the coffee matrix through the presence of potentially metal-chelating substances (Morikawa & Saigusa, 2008), making them less available for the plant. Still, all quantified mineral amounts correspond to their total content, thus such cannot be directly explored as available for plant nutrition, justifying the reduction in the plant mineral status despite the increased amounts in the soil.

Table 5.3
Regression analysis and correlations between mineral composition and total ashes of lettuce samples with the percentage of spent coffee added as soil amendment

Element	Fresh spent coffee			Composted spent coffee		
	Equation	R^2	Pearson´s correlation coefficient	Equation	R^2	Pearson´s correlation coefficient
K	y = -0.001x + 7.447	0.1000	-0.316*	y = 0.001x - 5.165	0.4737	0.609*
Mg	y = -0.006x + 7.416	0.6889	-0.830*	y = -0.005x + 9.276	0.4150	-0.663*
Ca	y = -0.008x + 8.279	0.7652	-0.875*	y = -0.008x + 9.162	0.8540	-0.926*
P	y = -0.008x + 8.820	0.4576	-0.676*	y = -0.008x + 9.612	0.6398	-0.803*
Na	y = -0.024x + 9.705	0.5021	-0.709*	y = -0.004x + 4.869	0.0048	n.s.
Fe	y = -0.182x + 6.156	0.4775	-0.691*	y = -0.253x + 7.829	0.6292	-0.808*
Mn	y = 0.012x + 3.350	0.0013	n.s.	y = -0.119x + 6.018	0.1083	-0.307*
Zn	y = -0.733x + 8.538	0.5528	-0.744*	y = -0.530x + 7.761	0.2172	-0.433*
Cu	y = 3.013x + 1.381	0.3554	0.596*	y = 4.488x + 0.896	0.2430	0.475*
Total ashes	y = -0.338x + 10.436	0.3484	-0.590*	y = 0.383x - 5.722	0.1470	0.351*

*Pearson's correlations are significant at the 0.01 level; n.s., not significant.

3.3. Effect of composted espresso spent coffee on lettuce's mineral profile

During composting, most organic compounds are gradually degraded by natural microflora, resulting in an increased proportion of mineral amounts and availability. In the particular case of spent coffee, the reduction in the carbon structure can decrease its metal-binding capacity, of potential interest for plant nutrition. However, as previously stated, total mineral amounts of compost do not allow a prediction of its bioavailability.

Aware of the increased and broad practice of adding SCG to compost piles, particularly in the domestic ones, a simulation of this custom, using a simplified scheme for easier standardization, was conducted. Therefore, we used only fresh grass to simulate common domestic vegetable wastes, increasing carbon sources with straw and some sawdust to absorb humidity. The final compost was sampled for analysis and mixed, on a volume basis, with plain soil, as described above. However, knowing that compost initially contained only about a half of its volume in fresh-SCG, the amounts of compost added were doubled in comparison with the fresh-SCG for a more consistent discussion.

Regarding composted-SCG treatment (Table 5.4), a general improvement is observed for total mineral amounts as well as for some elements. In particular, the presence of 5% of composted-SCG enhanced lettuces K content by 40%, Mn by 30%, Mg by 20%, and Na by 10%. This K increment may be valuable, since with the increasing consumption of processed food, with lower K amounts, combined with a reduction in the ingestion of fruits and vegetables, led to a decline of K intake (He & MacGregor, 2006). A blood pressure decrease in hypertensive individuals is observed by increasing K intake, as well as a cardiovascular disease mortality reduction (not only due to blood pressure diminution), a slow progression of renal disease and an urinary calcium excretion decrease (He & MacGregor, 2006). Still for this supplementation level (Table 5.4), the remaining elements were generally not affected, except for the micro-elements Fe and Cu, which were significantly reduced. From the 10% supplementation level forward, only K kept its increasing tendency, while all the other elements remained similar to the control group or were progressively reduced (Mg, Ca, P, Fe and Zn).

These observations indicate that the composting of the SCG was only effective in increasing the elements availability when in very low amounts (5%). Still, when the elemental composition of the composted soil samples is observed (Table 5.4), a generalised consistency is observed for all the elements through the different supplementation levels, including Fe, in opposition to the previous study with fresh grounds, while K, Mg and Na remained increased.

Such better outcomes, in comparison to fresh-SCG pre-treatment may be related to a minor induced stress due to caffeine degradation (Mohanpuria & Yadav, 2009), as well as a better phytoavailability of such elements for plant uptake during composting (Hsu & Lo, 2001).

One could expect that if the results were improved at the 5% supplementation level, a proportional benefit could be expected at the 10% levels but that was not the case. Since the composting process of organic wastes results in a net loss of total organic matter and an increase of inorganic elements (He et al., 1995), it is predictable that the main products of such practice are fully mineralised materials such as CO_2, H_2O, mineral ions, stabilized organic matter and ash (Hsu & Lo, 2001), thus allowing a good nutrient uptake by plants. However, other elements present in the SCG are probably responsible for the reduced mineral amounts in the lettuce plants.

Table 5.4
Total ashes (g/100g) and mineral composition (mg/100g) of lettuce and soil samples after cultivation with composted spent coffee (mean ± SD, dry weight basis)

Sample	Element	Fresh spent coffee amounts (v/v)						*p*
		0%	2.5%	5%	10%	15%	20%	
Lettuce	K	7363.8 ± 343.6 a	9040.9 ± 471.3 b,c	8537.3 ± 799.6 c	10198.2 ± 727.9 d	9624.9 ± 747.6 b,d	9715.6 ± 1338.3 b,c,d	< 0.001**
	Mg	1158.4 ± 108.1 a,c	1384.4 ± 116.3 b	1232.9 ± 124.7 c	1002.1 ± 175.5 d	1030.1 ± 127.8 a,d	839.9 ± 108.8 e	< 0.001*
	Ca	937.0 ± 56.2 a	886.0 ± 31.8 a	759.3 ± 95.4 a	548.1 ± 76 b	525.0 ± 20.1 b,c	468.4 ± 44.3 c	< 0.001*
	P	893.4 ± 118.6 a	882.2 ± 92.6 a	819.8 ± 74.9 b	673.2 ± 106.9 c	614.5 ± 83.7 c	549.6 ± 95.8 d	< 0.001**
	Na	336.7 ± 12.7 a	373.6 ± 18.6 b	354.6 ± 43.8 a,b	326.9 ± 38.5 a	333.1 ± 23.9 a	354.2 ± 26.7 a,b	< 0.001**
	Fe	25.2 ± 1.3 a	19.3 ± 1.9 b	17.3 ± 2.3 b,d	13.3 ± 3.3 c	15.0 ± 4.2 c,d	10.8 ± 3.5 c	< 0.001**
	Mn	20.0 ± 2.6 a	26.2 ± 5.4 b	22.7 ± 2.2 a,b	17.0 ± 2.5 c	22.1 ± 5.6 a,b,c	17.5 ± 2.7 a,c	< 0.001**
	Zn	8.7 ± 1.3 a,c	9.4 ± 0.7 a	8.2 ± 1.3 a,c	6.8 ± 1.2 b	7.4 ± 1.6 b,c	7.4 ± 1.3 b,c	< 0.001*
	Cu	0.6 ± 0.1 a,c,d	0.2 ± 0.0 b	0.5 ± 0.1 c	0.7 ± 0.0 d	0.7 ± 0.2 d	0.7 ± 0.1 d	< 0.001**
	Total ashes	21.5 ± 1.0 a	25.2 ± 1.0 b	24.8 ± 1.1 b,c	24.5 ± 1.6 b,c	23.9 ± 0.6 c	24.5 ± 1.5 b,c	< 0.001**
Soil	K	203.1 ± 17.6 a	286.8 ± 11.1 b	354.2 ± 76.9 b,c	463.0 ± 69.4 c	560.1 ± 156.2 c	603.5 ± 322.7 a,b,c	< 0.001**
	Mg	4.7 ± 0.5 a	6.6 ± 0.8 b	8.9 ± 2.4 b,c	9.8 ± 0.7 c	7.3 ± 2.2 a,b,c	10.6 ± 4.8 a,b,c	< 0.001**
	Ca	5670.8 ± 1351.5 a	4874.0 ± 127.2 b	4552.0 ± 385.4 b	5192.2 ± 1041.0 a,b	4187.2 ± 1149.3 a,b	4735.4 ± 2544.3 a,b	0.01**
	P	241.0 ± 56.7	173.4 ± 17.0	168.0 ± 37.3	190.8 ± 13.7	189.1 ± 35.9	196.1 ± 85.3	0.059**
	Na	44.9 ± 10.0 a	70.1 ± 31.6 a,b	62.3 ± 13.9 a,b	87.7 ± 21.6 b	85.7 ± 42.1 a,b	73.8 ± 33.7 a,b	0.001**
	Fe	161.3 ± 6.2 a,b	152.1 ± 16.5 a	164.4 ± 31.8 a,b	182.1 ± 11.9 b	164.8 ± 29.3 a,b	186.7 ± 77.1 a,b	0.017**
	Mn	5.9 ± 0.3 a,b	5.5 ± 1.0 a	6.0 ± 1.5 a,b	9.4 ± 2.0 b	5.5 ± 0.6 a	7.6 ± 3.9 a,b	0.006**
	Zn	2.6 ± 0.6 a	2.5 ± 0.4 a	3.2 ± 1.0 a	3.7 ± 0.9 a	2.6 ± 0.7 a	2.2 ± 0.4 a	0.017**
	Cu	1.0 ± 0.2 a	1.0 ± 0.2 a	1.4 ± 0.1 b	1.4 ± 0.2 b	1.8 ± 0.3 b	1.6 ± 0.7 a,b	< 0.001**
	Total ashes	18.8 ± 3.8 a,b	16.7 ± 1.8 a	15.8 ± 2.2 a	16.1 ± 0.5 a	21.0 ± 0.4 b	20.3 ± 5.2 a,b	< 0.001**

Different letters in a row show statistically significant differences ($p < 0.05$) between means.

*p values from one-way ANOVA analysis. Means were compared by Tukey's test, since homogeneity of variances was confirmed by Levene's test ($p > 0.05$).

**p values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since homogeneity of variances was not confirmed by Levene's test ($p < 0.05$).

3.4. Comparison of fresh and composted espresso spent coffee amendment on lettuce mineral profile

With the data acquired from the lettuce plants treated with fresh- or composted-SCG, two different statistical tools were applied in order to tentatively investigate the effect of SCG type and/or percentage on mineral composition by a MANOVA test (Table 5.5) and to discriminate the plants based on its mineral profile by a LDA test (Fig. 5.6).

First, the MANOVA outcomes revealed, in general, significant effects of percentage and type, as well as significant interaction between both factors for individual elements contents in lettuce (Table 5.5). The only exception was observed for Na content which was not significantly influenced by the interaction of SCG percentage and type ($F = 1.315$, $p = 0.269$). F values corresponding to SCG type were higher than the F values of the SCG percentage effect for K ($F = 133.193$, $p < 0.001$), Mg ($F = 596.035$, $p < 0.001$), Na ($F = 347.058$, $p < 0.001$), Fe ($F = 21.117$, $p < 0.001$), Mn ($F = 295.452$, $p < 0.001$), Zn ($F = 28.801$, $p < 0.001$), and total ashes ($F = 150.073$, $p < 0.001$), showing greater importance of the former factor than the latter. On the other hand, the effect of SCG percentage was superior to the effect of SCG type for P ($F = 31.760$, $p < 0.001$), Ca ($F = 142.612$, $p < 0.001$) and Cu ($F = 55.663$, $p < 0.001$). Hence, for the majority of the analysed elements, the employment of fresh- or composted-SCG as soil amendment will be determinant for the variability of lettuce mineral composition.

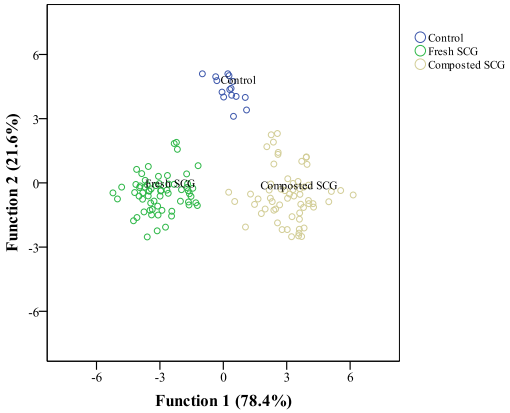

Fig. 5.6. Linear discriminant analysis using individual mineral and total ashes contents of lettuce cultivated with different percentages of spent coffee grounds (SCG), composted or not.

Second, a stepwise LDA was applied to all mineral elements content, resulting in a discriminant model with two discriminant functions that explained 100% of the variance, explaining the first discriminant function 78.4% of the variance and the second discriminant function 21.6% (Fig. 5.6). The model obtained was based on the ten variables originally used, which demonstrate the discriminant power of the mineral elements of the samples. Control samples and lettuce cultivated

Table 5.5
Multivariate analysis of variance applied to lettuce's mineral profile and total ashes results

Element	Spent coffee percentage		Spent coffee type		Interaction	
	F	*p*	F	*p*	F	*p*
K	6.318	< 0.001	133.193	< 0.001	9.880	< 0.001
Mg	55.907	< 0.001	596.035	< 0.001	4.331	0.003
Ca	142.612	< 0.001	105.095	< 0.001	10.506	< 0.001
P	31.760	< 0.001	15.870	< 0.001	7.418	< 0.001
Na	13.073	< 0.001	347.058	< 0.001	1.315	0.269
Fe	16.246	< 0.001	21.117	< 0.001	10.086	< 0.001
Mn	13.300	< 0.001	295.452	< 0.001	42.435	< 0.001
Zn	20.150	< 0.001	28.801	< 0.001	2.701	0.034
Cu	55.663	< 0.001	24.689	< 0.001	6.336	< 0.001
Total ashes	13.063	< 0.001	150.073	< 0.001	11.360	< 0.001

with fresh- and composted-SCG are completely separated from each other, creating independent groups of samples. This observation transmits clearly that, according to the cultivation treatment applied, the mineral composition of lettuces is severely affected.

Furthermore, the ten variables used in the discriminant model allowed to correctly classify 100% of the samples for the original groups as well as for the cross validation procedure (sensitivities and specificities of 100%).

4. CONCLUSION

This study demonstrated the effect of fresh-SCG and composted-SCG in lettuce mineral content. A significant reduction of most of plant elements was perceived with fresh-SCG, likely due to reduced mineral availability and plant stress induced by typical coffee bioactive residues, as caffeine. Nevertheless, more studies are needed in order to clarify this issue. In opposition, low amounts of composted-SCG (up to 15% v/v) induced a major increment of essential macro-elements in lettuce, enhancing its quality features.

From the food industry point of view, the possibility of reutilizing coffee by-products, as an easy and economically feasible soil amendment, represents an exciting opportunity to obtain products of high nutritional value.

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6. DIRECTLY COMPOSTED SPENT COFFEE GROUNDS

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In submission process

Coffee by-products towards global sustainability: an agro-industrial approach

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ABSTRACT

Spent coffee grounds (SCG) represent a high volume food waste worldwide, and several reuse approaches have been attempted. Herein, a greenhouse field experiment was carried out by cultivating Batavia lettuce with 5-30% (v/v) espresso SCG directly composted in the soil. Healthy vegetables were obtained for all treatments, without yield loss for up to 10% SCG. A progressive increment of green color intensity with increasing SCG content was observed, corroborated by the increase of their photosynthetic pigments (chlorophylls and carotenoids). Furthermore, total ascorbic acid and tocopherols showed statistical significant differences ($p < 0.001$) between control and test groups, being generally higher in the latter. Marked variations of nutritionally relevant minerals, particularly potassium, phosphorous and sodium were revealed at higher percentage treatments (20% and 30%). This approach constitutes a clean, direct, simple and cost-effective measure to produce value-added vegetables, while reducing food waste disposal.

Keywords: *Food chemistry; Lettuce; Spent coffee grounds; Waste reuse; Antioxidant activity.*

1. INTRODUCTION

Modern society food consumption habits are leading to severe environmental problems due to uncontrolled waste discharge. Coffee industry, for instance, which is increasingly growing worldwide, releases a huge variety and volume of residues, resulting either from its production (e.g., coffee pulp, cherry husks, defective beans, and parchment skin), from roasting industries (e.g., coffee silverskin), from soluble coffee industry (industrial spent coffee), and also directly by daily coffee consumers after beverage preparation (spent coffee grounds - SCG). All these coffee wastes set up a global ecotoxicological concern due to their high content of organic matter and bioactive components as caffeine, free phenols and tannins (polyphenols), with recognized toxicity (Buerge et al., 2003; Cruz et al., 2012a).

While several applications have been proposed for many of the aforementioned coffee by-products (Murthy and Naidu, 2012), the compositional worth of SCG, resulting from beverage preparation, only recently has received proper attention. Hence, distinct applications for this particular residue have been highlighted, such as CO_2/mineral adsorbents, as fuel pellets, as enzyme immobilization solid carrier or as substrate for the extraction of compounds with huge potential applications in the industrial or pharmaceutical fields (Plaza et al., 2012; Chen et al., 2013; Panusa et al, 2013; Barbosa et al., 2014). The potential application of espresso SCG in agriculture has already been tested in previous works, with positive outcomes on plenty micronutrients in pot experiments using SCG directly and after classical composting (Cruz et al., 2014a; Cruz et al., 2014b). However, no field experiments on industrial scale horticultural production simulation have been performed yet on this subject.

Therefore, aiming to support a cost-effective, simple, direct and sustained reuse of this coffee by-product, the current work was developed using direct composting of espresso SCG on the soil (pit or trench composting), for lettuce cultivation, being the overall performance evaluated through vegetables physical and nutritional quality, including yield, color, pigments, vitamins, total phenols and minerals.

2. MATERIALS AND METHODS

2.1. Reagents

L-Ascorbic acid, caffeine, gallic acid, D-(+)-glucose and tocopherols (α-, and γ-) were purchased from Sigma-Aldrich (Germany). Tocol, used as internal standard for tocopherol quantification, was from Matreya Inc. (USA).

Methanol and n-hexane, both HPLC grade, were from Sigma-Aldrich. All the remaining reagents were analytical grade from several suppliers and included: acetone, anhydrous sodium sulfate, butylated hydroxytoluene (BHT), caesium chloride, chloroform, 1,4-dioxane, Folin and Ciocalteu's reagent (FCR), glacial acetic acid, magnesium oxide, metaphosphoric acid, methanol, nitric acid, sodium acetate, sodium dichromate dihydrated, sodium carbonate, sulfuric acid, tris(2-carboxyethyl)phosphine (TCEP), this last used for ascorbic acid extraction as a 2.5 mM aqueous solution with 3% (w/v) metaphosphoric acid and 8% (v/v) glacial acetic acid.

2.2. Experimental design

The experimental setup for this study was a randomized greenhouse field design performed at the School of Agriculture, Polytechnic Institute of Bragança (NE Portugal), under controlled conditions (day/night thermal regime of 23/18 ± 2 ºC, 70 ± 10% relative humidity, and natural sunlight).

A total of approximately 100 kg of espresso SCG were collected from several coffee shops in January of 2013. The greenhouse ground was prepared to receive five × five random distributions of 40 L holes (100 cm length x 20 cm width), covered with a plastic film. After homogenization of the coffee batch, five mixtures with plain vegetable soil were prepared: 0, 5, 10, 20, and 30%, all on a volume basis, in a total of 200 L each. The holes were filled with the mixtures, watered, and left to rest for a 4 month period. Samples were collected at this initial stage for analysis, being preserved in plastic bags at -18ºC (pre-composting soil). After this direct composting period, the soil was revolved and homogenized, soil samples were collected again (pre-harvest soil), and Batavia lettuce plantlets (*Lactuca sativa* L. var. *capitata* cv. "Rolina") were planted, five on each plot with a 50 x 20 cm compass, in a total of 125 plants.

Lettuce plants were harvested after five weeks, carefully washed with deionized water to remove soil contamination, and the edible part was separated and weighted (Fig. 6.1). Post-harvest soil samples were also collected at this stage. Afterwards, 15 plants from each test group were assembled into five composites of three plants, named hereafter as samples and transported immediately to the lab under refrigeration. After color evaluation, whole leaves were immediately frozen at -80ºC in polyethylene bags and then freeze-dried (Telstar Cryodos-80, Spain). Dried samples were carefully homogenized by grinding in a blender (Grindomix, Retsch GmbH, Germany) and further sieved (150 μm mesh size). Vegetable samples were stored at 4 ± 2 ºC, protected from light. Pre-freezing and post-lyophilization moisture was evaluated by oven drying at 103±2 °C (WTC Binder, Germany).

Fig. 6.1. Example of plants collected at harvest.

All soil samples from the three sampling dates were spread on plastic trays (~10 mm layer) and dried in a forced-air oven (WTC-Binder, Germany) at 40ºC, for a 24 hours period (ISO, 11464:2006). Later, samples were crushed and sieved (2 mm mesh size).

All analyses were performed at least in duplicate.

2.3. Chemical Analysis

2.3.1. Soil samples

2.3.1.1. Physicochemical characteristics. Soil pH (H_2O) was determined in the upper layer of 1:5 (w/v) prepared with deionized water, according to ISO 10390:2005. Specific electric conductivity was performed as described in ISO 11265:1994. Total organic carbon (TOC) content was estimated by the oxidation of organic matter in a mixture of sodium dichromate (0.17 M) solution and sulfuric acid (98 %, v/v) at a temperature of 135 °C (Skjemstad and Baldock, 2008).

2.3.1.2. Caffeine. For caffeine analysis in soil, the extracts previously obtained for pH (H_2O) were filtered through a Whatman® filter Nº42 and 10 mL were freeze-dried. The extracts were further recovered with 1 mL of deionized water for HPLC analysis. Chromatographic conditions were previously described in Cruz et al. (2012a).

2.3.2. Lettuce samples

2.3.2.1. Color evaluation. Instrumental color was measured across the surface of fresh whole lettuce leaves using a Minolta CR-400 colorimeter (Konica Minolta Optics Inc., Japan). The color coordinates were computed in the CIELAB scale in a CIE C/2° illuminant/observer condition and a 2.5 cm port/viewing area. Color results were expressed as tristimulus parameters: lightness (L*), redness (a*), yellowness (b*), hue angle (hab), chroma (Cab*), and color index (CI*), determined as CI* = (a* × 1000) ÷ (L* × b*) in accordance with Goñi et al. (2010). Color evaluation was performed in 15 leaves from each composite sample, with the final results being the average of 75 measurements per treatment.

2.3.2.2. Chlorophylls and carotenoids. Analytes extraction was performed according to the method reported by Lichtenthaler and Buschmann (2001a), with minor adjustments. Duplicate amounts of freeze-dried lettuce samples (20 mg) were extracted with methanol (1 mL), after addition of MgO (50 mg), and the mixture was homogenized (5 min.), centrifuged (13 000 rpm, 0 ºC, 5 min.) and the clear upper layer was transferred to an amber flask. The extractive procedure was repeated twice with methanol (500 µL). The combined extracts were diluted (1:5) with methanol and dehydrated with anhydrous Na_2SO_4. After new centrifugation (13 000 rpm, 0 ºC, 2 min.), absorbance was recorded at 470, 520, 652.4, 665.2 and 750 nm for chlorophyll *a*, chlorophyll *b* and total carotenoids quantification, as described by Lichtenthaler and Buschmann (2001b).

Proper measures for pigments preservation were taken during extraction, such as absence of light, low temperature operating conditions and the presence of an acid neutralizing agent (MgO), for pheophytins formation avoidance (Lichtenthaler and Buschmann, 2001b).

2.3.2.3. Total phenolic content. The Folin-Ciocalteau method, despite knowing to give an estimation of total reducing capacity rather than a true phenolic content, is the mostly commonly reported in lettuce, being therefore chosen. Based on the guidelines proposed by Pérez-Jiménez et al. (2008), duplicate amounts of freeze-dried sample (500 mg) were macerated with 20 mL methanol:water (50:50, v/v, pH = 2), under stirring for 1 h. After centrifugation (5 000 rpm, 4 ºC, 10 min.), the residue was re-extracted with 20 mL acetone:water (70:30, v/v), under stirring for 1 h.

After a second centrifugation (5 000 rpm, 4 ºC, 10 min.), the supernatants were combined and filtered (nylon filter, 0.22 µm) for subsequent analysis.

Hence, total phenolic content (TPC) of lettuce extracts was determined by the colorimetric Folin-Ciocalteu method as described by Singleton and Rossi (1965). Briefly, the extract solution (100 µL) was mixed with FCR (100 µL). After 3 min., Na2CO3 saturated solution (100 µL) was added and the final volume was adjusted to 1000 µL with deionized water. The reaction was kept in the dark during 90 min, and afterwards the spectrophotometric absorbance was determined at 725 nm. Gallic acid solutions (0.016 to 0.068 mM) were used as standards, being the results expressed as mg of gallic acid equivalents per 100 g of fresh lettuce (GAE/100 g fresh weight; FW).

2.3.2.4. Total ascorbic acid. Extraction procedure and further analysis of total ascorbic acid (AA) was based on the methods previously described by Chebrolu et al. (2012) and de Velde et al. (2012), with minor adjustments. Briefly, freeze-dried lettuce samples (60 mg) were extracted with 500 µL + 400 µL of extractive solution, vortex mixed (2 min.) and centrifuged (13 000 rpm, 0 ºC, 3 min.), always protected from light. Supernatants were combined, 100 µL of methanol were added, and the final extract was analyzed immediately.

Chromatographic analyses were performed using a HPLC system (Gilson, France), with a photodiode array detector (Varian Prostar, USA) controlled by a data processor software (Varian Star Workstation, USA). Chromatographic separation was achieved by injecting, in duplicate, 20 µL of the extract, into a 150×4.6 mm C18 Spherisorb ODS-2 (3 µm particle size) column (Waters, Ireland) and eluted with a 10 min. gradient from 98% acetate buffer (sodium acetate and glacial acetic acid, 0.03 M) and 2% methanol (v/v) to 80% of aqueous methanol (70%, v/v). Before returning to starting conditions, the column was equilibrated for 10 min. before the next sample injection. The flow rate was 0.6 mL.min^{-1} with the room temperature maintained at 22 ± 2 ºC.

For compound identification, spectra were registered at the 200 – 550 nm bands and compared with standard (L-ascorbic acid), being then quantified at 266 nm by the external standard method, using calibration curves with at least six concentrations, subjected to the entire extraction procedure.

The current analytical method was validated according to IUPAC guidelines (Thompson et al., 2002) for linearity (3 – 76 µg/mL, R^2 = 0.9998), limits of detection (1.6 µg/mL) and quantification (5.2 µg/mL), precision (n = 6, intra-day, RSD = 2.7%; n = 6 × 2, inter-day, RSD = 6.3%), and recovery (n = 3 × 3, 103.5 ± 5.7%).

2.3.2.5. Tocochromanols. Vitamin E extraction method based on the Folch method was used, as previously validated (Cruz & Casal, 2013). Separation was accomplished with a JASCO HPLC system equipped with a refrigerated auto-sampler (Jasco AS – 950, Japan) and a fluorescence detector (Jasco FP-2020 Plus, Japan) programmed for excitation at 290 nm and emission at 330 nm. Data were analyzed using Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France). The chromatographic separation was achieved by normal-phase using a Supelcosil™ LC-SI column (7.5cm x 3mm; 3µm) (Supelco, Germany) and 1,4-dioxane in n-hexane (2.5 %, v/v) as mobile phase at a flow rate of 0.70 mL/min, operating at constant room temperature (22 ± 2 ºC). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using 6-level calibration curves with internal standard (tocol).

2.3.2.6. Total ashes and mineral composition. Extraction procedures and analysis of plants

elemental quality have been already described in a previous study (Cruz et al., 2014b). Briefly, after dry-ashing and further nitric acid (1%, v/v) dissolution of the white ashes obtained, the elements Ca, Mg, Na, K, Fe, Cu and Mn were analyzed by high-resolution continuum source flame atomic absorption spectrometry, while P was quantified by the vanadomolybdate method and spectrophotometrically detected at 420 nm.

2.4. Statistical Analyses

All analyses were performed using SPSS software, version 22.0 (IBM Corporation, New York, USA), as detailed below.

2.4.1. Analysis of variance

The results are presented as mean values and standard deviation from duplicate analysis of each sample. The outcomes of variance analysis, comparing control samples and those cultivated with several percentages of SCG, are discussed. For the purpose, normal distribution of the residuals and the homogeneity of variances were evaluated through the Kolmogorov–Smirnov test (sample size > 50) and the Levene's test, respectively. Afterwards, all dependent variables were studied using a one-way ANOVA, subjected or not to Welch correction, depending if the requirement of the homogeneity of variances was verified or not. Furthermore, if a statistical significant effect was verified, post hoc tests, Duncan's or Dunnett's T3 test, were also applied for means comparison, depending if equal variances were assumed or not.

2.4.2. Correlation analysis

Pearson's correlation was established between the different amounts of SCG and each parameter analyzed.

2.4.3. Linear discriminant analysis

A supervised learning technique was used to classify the lettuce samples cultivated with different percentages of SCG, according to all parameters analyzed, after variable standardization to mean zero and unit variance. A stepwise technique, using the Wilk's lambda method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection. To verify which canonical discriminant functions were significant, the Wilk's lambda test was applied. To avoid overoptimistic data modulation, a leaving-one-out cross-validation procedure was carried out to assess the model performance. Moreover, the sensitivity and specificity of the discriminant model were computed from the number of individuals correctly predicted as belonging to an assigned group (Rencher, 1995).

3. RESULTS AND DISCUSSION

3.1. Soil analysis

Physicochemical analyses were carried out to characterize the soil initial status, after the direct composting period (corresponding to the cultivation moment) as well as at the harvest date. For the purpose, pH, conductivity, TOC and caffeine residues were determined (Table 6.1) One of the SCG features is their acidity, usually with pH values ranging from 5.2 to 5.9 (Cruz et al., 2012a). Indeed, the soil pH was initially significantly lower than the control ($p < 0.001$) for all the mixtures, with a strong negative Pearson's correlation ($r = 0.957$) with the SCG amount. After five months of direct composting, soil pH increased on all treatments, despite remaining significantly lower than the control in the 10% to 30% mixtures. After harvest, a transversal pH increase was observed, with all soil mixtures above 7.0. The magnitude of the differences observed in the post-harvest were reduced, with only significant differences observed for the 20% and 30% mixtures, and the correlation significance was also reduced ($r = 0.697$).

Table 6.1
Soil analysis before and after lettuce cultivation with fresh spent coffee grounds (mean ± SD, dry weight basis)

Pre-composting							
Analyte	0%	5%	10%	20%	30%	*p*	*r*
pH	7.03 ± 0.01 A	6.87 ± 0.06 B	6.73 ± 0.16 B,C,D	6.49 ± 0.08 C	6.16 ± 0.05 D	<0.001	-0.957*
Conductivity (mS/cm)	0.72 ± 0.12 A	0.83 ± 0.07 A	0.73 ± 0.06 A,B	0.65 ± 0.02 B	0.64 ± 0.07 A,B	0.005	-0.303
TOC (g/100g)	2.46 ± 0.09 A	3.54 ± 0.21 A	3.85 ± 0.07 A,B	4.62 ± 0.50 B	3.99 ± 0.18 C	<0.001	0.782*
Caffeine (mg/100g)	n.d.	n.d.	0.07 ± 0.02 A	0.14 ± 0.16 B	2.16 ± 0.34 B	<0.001	0.863*
Pre-harvest							
Analyte	0%	5%	10%	20%	30%	*p*	*r*
pH	7.19 ± 0.18 A	7.13 ± 0.10 A	7.08 ± 0.18 B	6.68 ± 0.07 B	6.42 ± 0.17 B	<0.001	-0.858*
Conductivity (mS/cm)	0.74 ± 0.13 A	0.74 ± 0.15 A	0.58 ± 0.03 B	0.41 ± 0.07 B	0.46 ± 0.05 B	<0.001	-0.777*
TOC (g/100g)	2.34 ± 0.07 A	3.47 ± 0.20 A,B	4.46 ± 0.58 B	5.28 ± 0.37 C	5.85 ± 0.63 D	<0.001	0.948*
Caffeine (mg/100g)	n.d.	n.d.	n.d.	n.d.	n.d.	-	-
Post-harvest							
Analyte	0%	5%	10%	20%	30%	*p*	*r*
pH	7.54 ± 0.17 A	7.46 ± 0.03 A,B	7.31 ± 7.09 A,B	7.37 ± 0.16 B	7.09 ± 0.20 B	0.007	-0.679*
Conductivity (mS/cm)	0.31 ± 0.14	0.32 ± 0.03	0.29 ± 0.11	0.30 ± 0.09	0.32 ± 0.04	0.921	0.010
TOC (g/100g)	2.14 ± 0.21 A	2.80 ± 0.15 A	3.29 ± 0.13 B	3.86 ± 0.27 C	5.61 ± 1.09 D	<0.001	0.889*
Caffeine (mg/100g)	n.d.	n.d.	n.d.	n.d.	n.d.	-	-

TOC, total organic carbon; n.d., not detected.

Different upper case letters (soil, n = 3 × 5) in a row show statistically significant differences ($p < 0.05$) from the given mean.

P values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since homogeneity of variances was not confirmed by Levene test ($p < 0.05$).

Pearson's correlation (*r*) is significant at the 0.01 level (**p*).

In general, the pH values were within those acceptable for plant growth (Coria-Cayupán et al., 2009), as this parameter is crucial for controlling elements bioavailability; in particular, if soil pH decreases there is a superior availability of metal cations for plant uptake (Pauget et al., 2012).

As regards to electrical conductivity, all samples revealed suitable figures according to maximum recommended limit of 4 mS/cm (Coria-Cayupán et al., 2009), decreasing with the addition of SCG, and particularly after the cultivation period (Table 6.1) with similar values on all the treatments at harvest time.

Total organic carbon (or organic matter content) also plays a major role in elemental availability, once soils with high TOC content may retain both anionic and cationic species in an exchangeable form, enhancing plant roots uptake (Zeng et al., 2011). As a result of higher amounts of SCG, TOC content also increases along groups (highly significant and strong positive Pearson's correlation) before and after the direct composting period, being reduced during plant development (Table 6.1), except for the 30% treatment.

Finally, being potentially a major bioactive component in SCG, caffeine was also quantified in soil samples (Table 6.1). Residual amounts were only found at the pre-composting samples, with a strong correlation with the SCG amounts (p < 0.863). Caffeine was not detected after composting or at harvest.

3.2. Lettuce physicochemical features and photosynthetic capacity

Being a highly cultivated green leafy vegetable worldwide, mostly consumed by its high nutritional value (e.g., vitamins, essential minerals and fiber) and low price, lettuce also presents a fast growing rate, thus becoming an excellent crop model for the present study. Due to the huge diversity of lettuce cultivars, literature comparison regarding a particular one is hard to accomplish, thus this task will be performed by comparing the results from control group to the ones published on the same variety, i.e. *L. sativa L.* var. *capitata*, despite being from a different cultivar.

Production yield (Table 6.2) was not affected with the presence of up to 10% SCG. However, a 36% reduction in the mean head mass was observed in the 20% group, increasing to 60% in the 30% group. Nevertheless, all plants had a healthy appearance and moisture was not affected, with a global average of 95.5%.

In addition to overall visual quality and texture features, leaves color stands as a major selection factor for consumers, since it indirectly represents the vegetable freshness. Hence, Table 6.2 shows the mean values of the color parameters for all tested treatment groups. The results of color coordinates (L*, a*, b* and CI*) for control plants are in accordance to those obtained by Goñi et al. (2010). Statistically significant differences were found on all parameters. For higher amounts of SCG (20% and 30%), lettuce revealed lower L* values, corresponding to less "lightness", while for 5% and 10% the L* was superior to the control samples. Furthermore, with the increase of SCG as soil amendment, lettuce revealed lower a* values in comparison with control, thus presenting themselves greener, while the 5% and 10% samples were less green than the control. This color behavior was confirmed by the total color intensity in the chroma value. The CI* presented values always within the "dark green" (Goñi et al. 2010), but again more dark in the 20% and 30% samples and lighter in the 5% and 10%, in comparison to the control. This diverse behavior reduced the significance of the correlation between the color attributes and the SCG amounts but the color differences were still statistically relevant.

These colorimetric results are in accordance with the observed chemical behavior (r = -0.592, p = 0.01, between total chlorophylls and CI*), where again highly significant and strong positive Pearson's correlations were obtained for chlorophyll *a*, chlorophyll *b* and total carotenoids in

Table 6.2
Fresh weight (mean ± SD, g) and color parameters of lettuce samples after cultivation with espresso spent coffee grounds (mean ± SD, CIELAB units)

Parameter	0%	5%	10%	20%	30%	*p*	*r*
Head weight	246.9 ± 83.1 A	245.3 ± 50.8 A	253.3 ± 57.2 A	158.7 ± 67.2 B	95.0 ± 51.9 C	< 0.001*	-0.635**
*L**	55.9 ± 6.6 A	61.1 ± 3.1 A	60.2 ± 4.8 A	48.2 ± 3.2 B	47.7 ± 1.8 B	< 0.001**	-0.595**
*a**	-17.2 ± 1.1 A,C	-15.6 ± 1.8 B,C	-16.2 ± 1.6C	-18.2 ± 1.1 A,D	-18.9 ± 1.1 D	< 0.001*	-0.506**
*b**	32.5 ± 1.1 A,B	30.7 ± 3.5 A,B	31.5 ± 1.8 A	33.1 ± 2.3 A,B	33.7 ± 1.4 B	0.031**	0.322*
C_{ab}^*	36.8 ± 1.3 A,B	34.5 ± 3.8 A,B	35.5 ± 2.3 A	37.8 ± 2.5 A,B	38.7 ± 1.6 B	0.007**	0.385**
h_{ab}	117.5 ± 1.3 A,B	116.5 ± 0.5 A	116.7 ± 1.3 A	118.6 ± 0.6 B,C	119.4 ± 0.9 C	< 0.001**	0.572**
CI*	-9.6 ± 1.8 A	-8.7 ± 0.7 A	-8.9 ± 1.3 A	-11.8 ± 1.1 B	-12.1 ± 0.6 B	< 0.001**	-0.630**

*a**, redness; *b**, yellowness; C_{ab}*, chroma; CI*, color index; h_{ab}, hue angle; *L**, lightness.
Different upper case letters (lettuce, n = 15 × 5) in a row show statistically significant differences ($p < 0.05$) from the given mean.
**P* values from one-way ANOVA analysis. Means were compared by Duncan's test, since homogeneity of variances was confirmed by Levene test ($p > 0.05$).
***P* values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since homogeneity of variances was not confirmed by Levene test ($p < 0.05$).
Pearson's correlation (*r*) is significant at the 0.01 level (***p*) or at a 0.05 level (**p*).

Table 6.3
Bioactive compounds of lettuce samples after cultivation with fresh spent coffee grounds (mean ± SD, fresh weight basis)

Analyte (mg.100g^{-1})	0%	5%	10%	20%	30%	*p*	*r*
Chlorophyll a	10.6 ± 1.8 A	10.6 ± 1.4 A	10.5 ± 0.8A	12.7 ± 1.8 B	23.3 ± 1.9 C	< 0.001*	0.753
Chlorophyll b	7.3 ± 1.2 A,B	7.0 ± 1.0 A,B	6.9 ± 0.6 B	8.0 ± 0.9 A	14.3 ± 0.7 C	< 0.001**	0.707
Total carotenoids	1.8 ± 0.3 A	1.9 ± 0.3 A	1.9 ± 0.2 A	2.6 ± 0.5 B	4.6 ± 0.5 C	< 0.001*	0.797
Total ascorbic acid	3.2 ± 0.7 A	4.0 ± 0.6 B	5.2 ± 1.1 C	5.2 ± 0.4 C	9.8 ± 1.2 D	< 0.001*	0.817
Total phenolic content[a]	24.8 ± 2.5 A	21.6 ± 2.6 A	17.7 ± 2.1 B	22.0 ± 0.6 A	39.0 ± 5.3 C	< 0.001**	0.536
α-Tocopherol	0.44 ± 0.06 A	0.46 ± 0.07 A	0.42 ± 0.05 A	0.45 ± 0.05 A	0.74 ± 0.07 B	< 0.001*	0.631
γ-Tocopherol	0.36 ± 0.04 A,B	0.38 ± 0.04 B,C	0.34 ± 0.02 A	0.40 ± 0.03 B,C	0.61 ± 0.03 D	< 0.001*	0.700

[a] Results are expressed as mg GAE/100g. GAE, gallic acid equivalents.
Different upper case letters (lettuce, n = 10 × 5) in a row show statistically significant differences ($p < 0.05$) from the given mean.
**P* values from one-way ANOVA analysis. Means were compared by Duncan's test, since homogeneity of variances was confirmed by Levene test ($p > 0.05$).
***P* values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since homogeneity of variances was not confirmed by Levene test ($p < 0.05$).
Pearson's correlation (*r*) is significant at the 0.01 level.

comparison to the amounts of SCG tested (Table 6.3).

In our study, chlorophyll *a* varied from 7.6 mg/100g to 26.9 mg/100g, while chlorophyll *b* ranged from 5.4 mg/100g to 16.9 mg/100g, and total carotenoids accounted with 1.3 mg/100g up to 5.2 mg/100g. There was a 110%, 100% and 130% average increment of chlorophyll *a*, chlorophyll *b* and total carotenoids, respectively, from control group to 30% group (Table 6.3). Aware that carotenoids biosynthesis is highly dependent on mineral nitrogen supply, the adequate total organic carbon/total organic nitrogen (TOC/TON) ratio of espresso SCG favors the mineralization rate of organic nitrogen in the soil, therefore promoting carotenoids formation (Mozafar, 1993; Cruz et al., 2012b). As already verified in a previous study, β-carotene, known for its provitaminic characteristics, is a major component of total carotenoid content in lettuce (Cruz et al., 2014a), thus SCG may also contribute to lettuce vitamin A enhancement.

In comparison with literature data, the amounts of both chlorophylls in control group are in accordance to our previous study in *L. sativa* var. *capitata* cv. “Four Seasons” and several others on the literature (Lin et al., 2013; Cruz et al., 2014a, Pinto et al., 2014), yet total carotenoids were lower in the present study (Patras et al., 2011; Baslam et al., 2013; Lin et al., 2013; Cruz et al., 2014a; Pinto et al., 2014), though similar to the figures reported by Mou (2009). Although it could be a specificity of this lettuce variety, the solvents used for extraction and the quantification methodology (spectrophotometry and HPLC) might impose some differences. Still, the main goal of the current work of a direct comparison between control group and the SCG treatments was achieved.

3.3. Antioxidants and antioxidant activity

Ascorbic acid is the major biologically active form of vitamin C, which eventually may be oxidized to dehydroascorbic acid, with less biological activity, both ubiquitously found in several vegetables. Conscious that vitamin C, i.e. total AA (the sum of ascorbic acid and dehydroascorbic acid) cannot be synthesized by humans, vegetables constitute a main source of this nutrient.

From all the bioactive molecules analyzed in lettuce, vitamin C was the one with a greater potentiation due to the presence of espresso SCG in soil (Table 6.3). In general, this parameter varied from 3.2 mg/100g (control) up to 9.8 mg/100g (30% SCG). As observed by Pearson’s correlation coefficient, there was a linear increase of its content with the rise of SCG percentages. In particular, total AA was 155% more concentrated in the 30% SCG group than in the control. Total AA in control group was within literature values (Mou, 2009, Aćamović-Djoković et al., 2011), being slightly lower than the ones quantified by Selma et al. (2012), Baslam et al. (2013) and Bonasia et al. (2013), though higher than the results achieved by Patras et al. (2011). This compound is known to be highly dependent on the soil conditions and lettuce variety (Sorensen et al., 1994, Aćamović-Djoković et al., 2011).

Another nutritionally relevant antioxidant, often synthesized by photosynthetic individuals, occurring mainly in leaves and seeds is vitamin E (Munné-Bosch and Falk, 2004). It comprises tocopherols and tocotrienols (α-, β-, γ-, and δ-), being α-tocopherol the most biologically active and usually the most prevalent in vegetables. In the analyzed samples, α-tocopherol was the predominant isomer, ranging from 0.44 mg/100g (control) to 0.74 mg/100g (30% SCG), followed by γ-tocopherol, which varied between 0.36 mg/100g (control) and 0.61 mg/100g (30% SCG) (Table 6.3). Regardless of the very statistically significant correlation with the presence of SCG, there were almost only notorious statistical differences for the 30% group, especially for α-tocopherol (Table 6.3). α-Tocopherol and total vitamin E contents in the control samples were in accordance to Szymanska and Kruk (2008).

Other minor compounds have also a relevant contribution to the global antioxidant capacity exhibited by green vegetables, namely the phenolic compounds. In order to have a global idea on the variation in these compounds, the Folin-Ciocalteau method was applied. No statistically significant differences have been observed for this parameter regarding most lettuce groups (Table 6.3), except for the 30% group with an outstanding and statistically significant increase ($p < 0.001$). In this particular case, TPC increased almost 80% in comparison to control group. Regardless of minor fluctuations between groups, Pearson's correlation test also revealed a highly significant and positive correlation between TPC of lettuce and the amount of SCG used (Table 6.3). As to the values quantified, a huge variability on TPC levels in lettuce is found in the literature. The control group presented TPC figures within those found in the literature (Table 6.3), slightly lower than those determined by Bonasia et al. (2013) and Złotek et al. (2014), while higher than the ones obtained by Patras et al. (2011) and by Baslam et al. (2013).

The aforementioned outcomes of antioxidant activity and individual antioxidants presented equivalent performance to color and pigment amounts, i.e. the presence of medium-high percentages of espresso SCG seems to improve green leafy vegetables bioactive quality.

3.4. Multi-elemental composition

The results obtained for total mineral content and individual elemental analysis are presented in Table 6.4. In general, the figures obtained for control group are in accordance to others reported in the literature (Mou, 2009; Baslam et al., 2013; Cruz et al., 2014b). As regards to total ashes, there are few statistical differences among test groups, though it is possible to highlight that 20% and 30% groups present slightly higher contents in comparison to lower SCG percentage groups. The macro-elements analyzed (potassium, phosphorous, magnesium, and calcium) comprised 50.13 ± 0.88% of total ashes. Among these, and as expected for lettuce, potassium is the element with higher prevalence in all samples, ranging from 337 mg/100g (FW) in the control to 571 mg/100g (FW) with 30% SCG.

It is interesting to notice that there are no evident statistical differences between macro-elements composition of control group and the lower SCG percentage groups (up to 10-20%). However, in the 30% SCG there is a remarkable increment of all elements, where they at least double up their amounts in comparison with control lettuce. This rise in elemental composition occurs in a linear tendency, which is supported by highly significant and strong positive Pearson's correlations (Table 6.4).

It is also worth mentioning that sodium content had an unforeseen increment of 250% at 20% and 615% at 30%, in comparison to control group. Nonetheless, assuming an average serving size of lettuce per day of 25 g, the amount of sodium provided remains insignificant (0.001%) considering World Health Organization guidelines on sodium consumption for adults of 2 g sodium/day (WHO, 2007). Besides, the higher potassium content in these samples, provide a favorable sodium/potassium ratio for reducing blood pressure, cardiovascular mortality (Chang et al., 2006). Moreover, iron content increased up to 205% which, together with total AA increment for the 30% SCG group, has its bioavailability potentiated (Flyman and Afolayan, 2006).

3.5. Overall effect of espresso spent coffee grounds in lettuce: multivariate analysis

A stepwise LDA was applied to all variables under study, resulting in a discriminant model with two discriminant functions that explained 98.8% of the variance, justifying the first discriminant function 93.5% of the variance and the second discriminant function 5.3%, such being displayed in a canonical variate (CV) scatterplot (Fig. 6.2). The obtained model included the following variables, by

Table 6.4
Total ashes mineral composition (mg/100g) of lettuce after cultivation with fresh spent coffee grounds (mean ± SD, fresh weight basis)

Analyte	0%	5%	10%	20%	30%	*p*	*r*
Total ashes	801± 62 A,B,C	798 ± 35 A	743 ± 29 B	850 ± 36 C	1382 ± 72 D	< 0.001**	0.714**
K	338 ± 38 A,B	3522 ± 26 A	321± 15B	360± 20 A	572 ± 66 C	< 0.001**	0.677*
P	26.0 ± 2.7 A,C	31.1 ± 2.0 B	24.5 ± 0.9 C	30.8 ± 4.3 A,B	63.7 ± 5.1 D	< 0.001**	0.717*
Mg	23.9 ± 2.7 A,B,C	22.6 ± 1.4 B	22.8 ± 1.4 B	26.4 ± 1.9 C	40.9 ± 5.4 D	< 0.001**	0.711*
Ca	7.6 ± 2.2 A,B,C	5.3 ± 0.7 A	5.2 ± 0.9 A	7.0 ± 0.9 B	8.6 ± 1.2 C	< 0.001**	0.274
Na	1.4 ± 0.6 A	1.2 ± 0.3 A	1.7 ± 1.1 A	3.7 ± 2.3 A	8.8 ± 1.6 B	< 0.001**	0.756*
Fe	0.35 ± 0.04 A	0.29 ± 0.04 B	0.31 ± 0.02 A,B	0.51 ± 0.04 C	0.72 ± 0.14 D	< 0.001**	0.778*
Zn	0.08 ± 0.01 A	0.08 ± 0.01 A	0.08 ± 0.01 A	0.09 ± 0.01 A	0.15 ± 0.02 B	< 0.001*	0.719*
Mn	0.06 ± 0.00 A	0.06 ± 0.00 A,B	0.05 ± 0.00 B	0.06 ± 0.00 C	0.11 ± 0.00 D	< 0.001*	0.697*
Cu	0.02 ± 0.00 A	0.02 ± 0.00 A	0.02 ± 0.00 A	0.02 ± 0.00 A	0.04 ± 0.01 B	< 0.001**	0.694*

Different upper case letters (lettuce, n = 10 × 5) in a row show statistically significant differences ($p < 0.05$) from the given mean.

**P* values from one-way ANOVA analysis. Means were compared by Duncan's test, since homogeneity of variances was confirmed by Levene test ($p > 0.05$).

***P* values from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since homogeneity of variances was not confirmed by Levene test ($p < 0.05$).

Pearson's correlation (*r*) is significant at the 0.01 level (**p*).

selection order: total ashes, manganese, iron, total AA, calcium, and L*. The relation between the selected standardized variables is represented by:

CV_1 = 1.233 × [Total ashes] + 0.541 × [Mg] + 0.412 × [Fe] + 0.469 × [Total AA] - 0.620 × [Ca] - 0.158 × {L*}

CV_2 = - 0.798 × [Fe] + 0.761 × {L*} - 0.663 × [Ca] + 0.410 × [Mg] + 0.335 × [Total AA] + 0.260 × [Total ashes]

Furthermore, the seven variables used in the discriminant model allowed to correctly classifying 93.2% of the samples for the original groups as well as for the cross validation procedure (sensitivities and specificities of 100%).

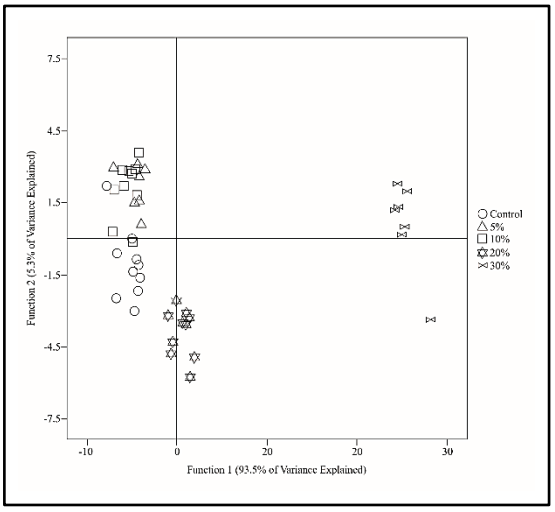

Fig. 6.2. Two dimension linear discriminant analysis applied to lettuce cultivated with different percentages of espresso spent coffee grounds.

Accordingly, control samples and lettuce cultivated with higher SCG percentages are completely separated from each other, creating independent groups of samples. This observation transmits clearly that, according to the amounts of SCG applied, the nutritional composition and general appearance of lettuces is clearly altered. In particular, control group is slightly separated from groups 5% and 10%, hardly distinguished between them, mainly due to an improvement of L* and a decrease of iron content in the latters. Secondly, 20% group is undoubtedly detached from the lower percentage groups, mostly owing to an increase of iron and total AA contents, plus a decrease in L*, even more noticeable for the 30% SCG samples.

Therefore, despite the minor decrease in fresh plants lightness, a worthy potentiation of major nutritional and bioactive aspects of its composition was observed for the 20% and particularly for the 30% group. Still, one cannot disregard the fact that these treatments led to a significant reduction of growth yield for the 20% and 30% groups (up to 60%, Table 6.2), despite of revealing an overall improvement of lettuce nutritional quality.

4. CONCLUSIONS

The present study confirms the positive impact of SCG as soil amendment in lettuce physical and nutritional features. When present in higher percentages (20 to 30%), SCG lead to an improvement of leaves color intensity, thus becoming more appealing to the consumers. Furthermore, all analyzed overall and individual antioxidants, as well as mineral elements, revealed an enhancement with the increment of SCG amounts in soil. For up to 10% no changes in yield were observed but a reduction in crop's yield was observed for the higher SCG treatment groups (20% and 30%), requiring a balanced choice by domestic and industrial producers between productivity and bioactivity.

This is the first study endorsing the potential of SCG use in agroindustry, on a real field experiment, and it allowed concluding that espresso spent coffee constitutes a direct, environmental friendly and profitable reuse approach to produce nutritionally valuable vegetables.

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7. GENERAL DISCUSSION

Coffee industry is responsible for the production of several and distinct by-products, which may be generated in both producing and consuming countries, all presenting environmental drawbacks if not properly treated. Aiming to lessen this problem and to find new sustainable alternatives for coffee beverage by-products, a low-cost and direct horticultural approach has been assessed. For the purpose, experiments were carried out using lettuce as a model plant due to its fast growth rate, higher worldwide production and consumption, and recognised knowledge on its chemical composition for literature comparison.

In order to determine the feasibility of this application, greenhouse experiments were conducted in two separate phases. The first intended to evaluate the impact of using SCG as is or after composting on nutritional features of lettuce grown in plastic pots, while the second, based on the results previously achieved, was tested in a greenhouse field design.

Aiming to study the effect of direct fresh-SCG (2.5% to 20%, v/v), the antioxidant activity, individual bioactive compounds and mineral composition of green lettuce were investigated. Radical scavenging effect and reducing power assays translated into favorable outcomes regarding lettuce antioxidant activity (Fig. 5.2 and 5.3) (see *Chapter 5*, p. 44). In addition, phenolic compounds, estimated by Folin-Ciocalteau method, also revealed a slight increment for low amounts (up to 5%) of fresh-SCG (Fig. 5.4). Nevertheless, clear variations of individual antioxidant compounds were observed (Table 5.1). It was verified that regardless of the SCG percentage used, fresh-SCG treated plants were characterized by high violaxanthin and total reducing capacity figures, which increased up to 240% and 79%, respectively, in comparison to control group (Table 5.1).

These same samples were further studied in terms of their elemental composition. A progressive decline of total ashes and individual elements, excepting K, with the fresh-SCG increase was verified, particularly for the 15% and 20% treatments (Table 5.2). This reduction was more evident for Mg and P, though Fe decrease may become of main concern from the nutritional point of view. Post-harvest soil analysis reveal an inverse pattern of mineral content, which can be a result of plant's privation for some elements.

The evolution of some major analytical parameters with the increase of fresh-SCG amounts is resumed in Fig. 7.1A.

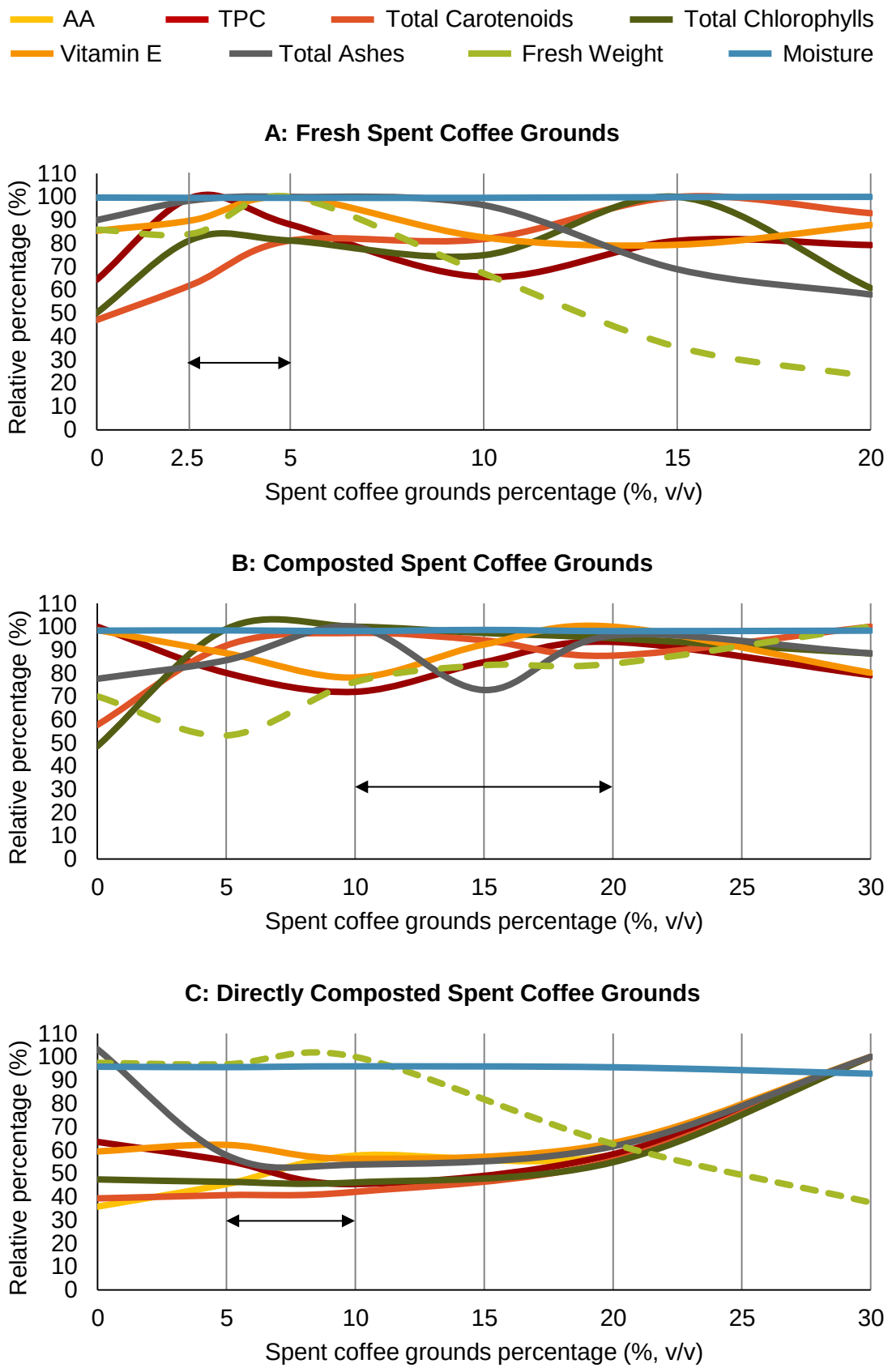

Fig. 7.1. Evolution of some analytical parameters with the increase of spent coffee grounds. AA, ascorbic acid; TPC, total phenolic content. Moisture content is presented as g/100g.

Simultaneously to the previsouly discussed parameters, yield is an important feature form the producers point of view and, therefore, was included in the comparison Generally, there was a slight increase in the 5% group but a noticeable reduction of crop's yield was observed from this point forward, despite of the increment of most bioactive substances and stable moisture content, which compromises its application as soil amendment on an industrial scale. Therefore, in order not to compromise crop's yield or the minerals content, while taking advantage of the incresed richeness in antioxidants, only as to 5% is advised.

In parallel, the impact of composted-SCG (5% to 30%, v/v) in produced vegetables was also assessed. As regards to antioxidant activity assays, with some minor differences, the majority of treatments were similar to control group (Fig. 5.2, 5.3 and 5.4). Concerning lipophilic antioxidants, composted-SCG treated vegetables, in general, presented higher neoxanthin and chlorophyll *a* than the control, verifying a 191% and 111% increment (Table 5.1). The elemental profile of these plants was also evaluated and an inverse pattern in relation to the previous study was verfied, i.e. composted-SCG treatments induced a compositional enhancement of lettuce, in particular for low amounts applied (Table 5.3). In fact, the presence of 5% of composted-SCG increased lettuces K content by 40%, Mn by 30%, Mg by 20%, and Na by 10%. Herein, Fig. 7.1B shows not only the aforementioned improvements on a nutritional level with the use of composted-SCG, but also that this treatment increases the vegetables fresh weight in comparison to control group and proportionally to the amounts added. Indeed, the major advantages of this process are visible between 15 and 20%.

Multivariate satistical analyses confirmed the aforementioned differences between main tested groups (control, fresh-SCG and composted-SCG) (Fig. 5.5 and 5.6). Besides, it was defined that the type of SCG (fresh or composted) was much more relevant in terms of final outcomes, rather than the SCG percentage used (Table 5.3).

Therefore, by combining the results achieved from both studies, it was possible to declare that some bioactive components of fresh-SCG, such as caffeine, might constitute a stress factor for adequate plant development and metabolism. This statement was actually supported by the evidence of carotenoids and tocopherols increment in the lettuces under fresh-SCG treatment, which usually occurs in order to protect membranes from oxidative damage (Munné-Bosch, 2005; Hörtensteiner, 2006). Furthemore, during composting, most organic compounds are gradually degraded by natural microflora, resulting in an increased proportion of mineral amounts and availability, as observed by the element potentiation in composted-SCG treated plants.

Even though the feasibility of composted-SCG for agricultural purposes was proved effective, the classic composting process itself may constitute a barrier to the espresso SCG reuse, due to the need of standardizing its composition by carefully selecting the wasted food in order to assure a well-balanced final product. In this context, a final experiment was carried out with more complementary analyses, a different cultivar and with direct composting (more simple to perform and easing large scale application) of SCG in a field cultivation design. Composting (5-30%) and cultivation was performed directly on the field. Firstly, soil analyses after composting with soil for 4 months revealed an absence of caffeine at the moment of plantlets transplantation (Table 6.1), thus reducing this possible stressing factor. Secondly, concerning vegetables features, the presence of high SCG amounts (20% and 30%) induced a statistically significant ($p < 0.001$) fresh weight reduction of lettuces produced, though such presented more intense and vivid green color (Table 6.2).

As regards to their individual antioxidants, the low SCG percentage groups (5% and 10%) were generally not statistically different from the control group, despite of a particular exception to this tendency – the total ascorbic acid content. Total ascorbic acid content in the samples under study was statistically different between all groups ($p < 0.001$), with a linear increasing tendency with the SCG amounts (Table 6.3). The elemental composition of lettuce followed the same incremental pattern as total ascorbic acid (Table 6.4), being even more evident for macroelements, such as K, P and Mg. Since all studied variables presented significant statistical differences ($p < 0.05$) between grouped samples, a linear discriminant analysis was carried out, thus revealing that the values of total ashes, Mg, Fe, total ascorbic acid, Ca, and lightness (L^*) certified the separation of all group samples from the control group (Fig. 6.2). The field experiment revealed a more clear pattern of nutritional enhancement of produced green vegetables, with all selected parameters increasing with the SCG content in the soil (Fig. 7.1C). Despite of the decrease in plants weight for some treatments, samples revealed constant moisture showing that they were actually more rich in these healthy components. Nevertheless, up to 10% of directly composted-SCG one can benefit from this enrichment, alongside with a more industrially profitable product. It should be taken into acount that the effects on a second crop could be different, requiring effective field studies to verify if the nutritional effects are kept while crop yield potentially improves due to the increased availability of spent coffee components over time.

Hence, this final study allowed to conclude that in order to improve both antioxidant and elemental composition, while preserving the crop's yield, on a real scale level and regardless of the target cultivar, low amounts of composted SCG (up to 10%) shall be

used as soil amendment. The use of espresso coffee by-products revealed to be a practical and economically feasible alternative to reduce food waste and to generate value-added agro-industrial products, in particular green leafy vegetables.

FINAL REMARKS

The generation of coffee by-products is continuously increasing as a result of high consumption demand for this beverage. The present dissertation explored the chemical features of the main residues produced by this industry, as well as the latest reuse approaches studied by field researchers. Particular attention was paid for espresso coffee remains due to its prominent consumption pattern in the region and thus high volume waste production, besides the fact that there is still insufficient knowledge on possible alternatives for this food residue.

Aware that the use of SCG in domestic agriculture is an increasing popular practice, despite the absence of a scientific background on this matter, this work intended to evaluate the effect of SCG as soil amendment for horticultural purposes. Therefore, different lettuce cultivars were studied and several chemical analyses were performed in order to support the feasibility of this approach, which was endorsed by the achieved outcomes. Optimal crop features were accomplished with low amounts (up to 10%, v/v) of directly composted-SCG, or up to 20% (v/v) of classical composting, while only up to 5% of fresh-SCG is advised. Hence, this coffee by-product seems to be adequate for a innovative agricultural practice providing nutritionally enhanced vegetable products.

Therefore, some future trends on this subject may be suggested. This research should definetly be extended to other crops, in order to verify if there is an equal or even greater potentiation of nutritional quality. Other physical analyses could be considered to complement the overall evaluation, such as texture measurement and sensorial analysis. Also, the effects on second crops would also be studied.

Global environmental consciousness on the urgent need to reduce food waste is settled, thus it only requires a selective collection and transport of SCG from producing establishments to agro-industries to turn this practice into a sustainable reality.

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