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***"Espresso Coffee preparation: analytical study to improve the quality of the product"***


**PhD Student**
*Dr. Simone Angeloni*

**Supervisor**
*Prof. Sauro Vittori*

# Contents

# Preface

The present PhD thesis has been the work of three years of research on coffee and is based on three published papers, one submitted works under review, and some not published yet results. The research project has been divided into three parts strictly connected to each other. The first part focuses on the development of new quantitative methods for phytoestrogens analysis in coffee. This yielded the content characterization of these molecules, with healthy effects, in the whole coffee chain, from green been to cup. Thank to our results, other bioactive compounds can be monitored for studying the quality of coffee. The second part is more oriented on applied investigation for studying the influence of different variables on espresso coffee extraction, aiming at lowering the amount of coffee used. Lowering the amount of R&G coffee can lead, in the long run, to a more sustainable consumption, by reducing the amount of the raw material and, in the end, producing less amount of waste while maintaining the same quality of beverage. The last part of my research has been more closely associated to this concept: adding value to coffee silverskin, a coffee industrial residue, by studying its odor-active compounds. Thus, the research on aroma compound characterization can be interesting for food industry leading to innovative applications and original reuses, while generating less amount of residues to disposal.

# Abstract

The PhD project was divided into three main parts. The first part aimed to develop and optimize efficient and simply methods for the quantification of nine phytoestrogens in green coffee, and three lignans in ground and espresso coffee (EC), using HPLC-MS/MS. For this purpose, different extraction processes, i.e., acid and base hydrolysis, enzymatic hydrolysis, organic solvent extraction, and a combination of these, have been studied. The best performing procedures, in term of recovery and quantitative data, were chosen, validated and finally applied to different samples coming from diverse geographical origins. The final objective was to characterize the content of these compounds in the whole coffee chain, from green beans to cup. The second part concerned the optimization of espresso coffee extraction, by modifying some machine variables aiming to produce a good quality espresso coffee, lowering the amount of coffee powder used for espresso preparation. The variables under study were the particle size distribution (PSD) of roast and ground (R&G) coffee, the design of the filter basket and the height of perforated disc. The EC quality was investigated from chemical point of view and it concerned the total solids (TS) analysis and the quantification of caffeine, chlorogenic acids and trigonelline. In addition, some analysis on volatile fraction were also performed. The third part of the project focused on promoting and adding value to coffee silverskin (CS) in the perspective of its application in the food industry. The work aimed to characterize the odor-active compounds in CS and compare them with those present in coffee beans. The characterization of aroma fraction was carried out by gas chromatography-olfactometry/flame ionization detector (GC-O/FID) and by comparing the odors and odorants with those found in coffee beans. The identification was carried out by comparing with reference compounds the retention index and the odor quality. Finally, for unequivocal identification, after fractionation, the samples were analysed into comprehensive two-dimensional gas chromatography-mass spectrometry (GCxGC-MS). Moreover, the Aroma Extract Dilution Analysis (AEDA) was performed to assess the potency of each odors/odorants.

Regarding the phytoestrogen characterization, we found two enzymatic digestions, i.e., clara-diastase (Method 3.10-EC) and taka-diastase (Method 5-R&G), as the most performing processes for lignan extraction from espresso and R&G coffee, respectively. Secoisolariciresinol was the lignans present at the highest concentration in espresso (27.9-52.0 µg L-1) and in R&G coffee (84.4-257.8 µg kg-1). Moreover, the extraction yield of lignans revealed that they are almost completely extracted during coffee percolation. For green coffee matrix, the best performing process was Method 7-GC, a double extraction composed of base hydrolysis in MeOH and enzymatic digestion with clara-diastase and we found that lignans (total content: 286.5-8131.8 µg kg-1) were more abundant than isoflavones (total content: 3.4-300.0 µg kg-1). Our studies on espresso coffee optimization showed higher levels of TS, caffeine, trigonelline and chlorogenic acids for reference samples (ECs extracted with mixed particles) and for 200-300 µm mesh size, when standard filter (A) was set in the machine. By decreasing the amount of R&G coffee, similar TS levels and contents of bioactive compounds to standard condition, were obtained for reference compounds. The best filter baskets, in term of quantitative data, were A and B and the study on perforated discs suggested that using lower amount of ground coffee permits to obtain the same extraction yield increasing the height of perforated disc. From our investigations on CS, four volatile compounds were identified for the first time in coffee. In detail, 4-methyloctanoic acid and trans-4,5-epoxy-(E)-2-decenal were characterized in coffee beans and silverskin, while 6-acetyl-2,3,4,5-tetrahydropyridine and 5-methyl-2-methoxyphenol only in coffee beans. The highest flavour dilution (FD) factors in CS were obtained for furaneol, 2-methoxy-4-vinylphenol and 2-methoxyphenol and, moreover, other typical coffee aroma volatiles were found, e.g., 2-furfurylthiol, 2,3-butanedione, vanillin, 2-isobutyl-3-methoxypyrazine, etc. Some aromas, such as furaneol, 2-methoxy-4-vinylphenol, vanillin, trans-4,5-epoxy-(E)-2-decenal, 2-acetyl-1-pyrroline and 2-acetylpyrazine, occurred with similar FD factors in CS and coffee beans. Our studies demonstrated that CS contains an interesting odor-active compound fraction with high similarity to coffee beans.

# 1. Introduction

## 1.1 Coffee and coffee word

Coffee is a daily part of our food habits and our social life. Drinking habits, together with authentic processing and preparation methods, represent and characterize a typical coffee culture. Nowadays, with a more and more increasing demand of a high-quality food products, coffee should be considered, and sometimes it is considered, a high-quality artisanal food like wine. This aspect has been strongly developed with the advent of specialty coffee, also called gourmet coffee, that has coincided with both an increase in urban growth and the regular appearance of affordable luxuries (Lannigan, 2019). How the "coffee" word is borned and become popular is still not clear. The early forms of the word in English (coffee), in French (café), in German (kaffe) and in Italian (caffè) indicate a derivation from Arabic and/or Turkish (Online Etymology Dictionary, n.d.). Various theories have been formulated but no written evidence has been found on who and where people have started to use this word. Often, the word dated back to Arabic and other times to African language. In the 15$^{th}$ Century, the Arabic word "bunn" (bun) was used for both the coffee tree and its fruits. Coffee fruit is called "kahva" in Arabic. It has been claimed that the word "kahva" in Arabic is an altered version of "Kaffa". The word coffee (kahve) in Turkish is used for the drink that is brewed by boiling this plant's beans. From African language coffee was named "Kaffa", a city in the Ethiopian region of Soha, which is considered the motherland of coffee plant and the first centre of coffee production (Yılmaz, Acar-Tek, & Sözlü, 2017).

## 1.2 Coffee trade

Coffee is one of the most consumed beverages in the world and it is also an important agricultural product of the international trade (Risticevic, Carasek, & Pawliszyn, 2008). According to recent data of the International Coffee Organization (ICO), the global coffee consumption from 2018 to 2019 has reached 165 million of 60 kg bags. The American countries have been the largest consumers with about 62 million of 60 kg bags, with the North America on the top (30 million of 60 kg bags). Europe came after America with a consumption of about 54 million of 60 kg bags. In the last year, the main producing countries have been the Brazil with about 63 million, followed by Vietnam (31 million),

Colombia (14 million) and Indonesia (9 million). The production of arabica coffee slightly increased from 2017 to 2018 while the robusta showed a marked increment (International Coffee Organization, 2019b). In fact, some recent projections on coffee market have revealed a reduction of arabica production but an increment of robusta for the next year, 2019-2020 (International Coffee Organization, 2019a).

### 1.3 Coffee plant

The coffee plants belong to *Rubiaceae* family, genus *Coffea*. More than 100 species are part of *Coffea* genus (The Plant List 2013, 2019) but only two, i.e., *Coffea arabica* L. (arabica) and *Coffea canephora* Pierre ex A. Froehner (robusta) are responsible of 99% of global coffee production (Pham, Reardon-Smith, Mushtaq, & Cockfield, 2019). The original geographical origins of *Coffea* genus is restricted to tropical humid regions of Africa and islands in the West Indian Ocean. In detail, originally arabica was a shrub living in the undergrow of the forests of the southwest of Ethiopia and north of Kenya at elevation between 1300 and 2000 m. Robusta originated from the humid lowland forests of tropical Africa (Folmer, 2017). Coffee is a perennial plant and evergreen in nature. It has a prominent vertical stem with shallow root system, the feeder roots of arabica coffee penetrate relatively deeper into the soil whereas robusta has feeder roots concentrated very close to the soil surface. Coffee leaves are opposite decussate on suckers. The leaves appear shiny, wavy, and dark green in color with conspicuous veins. The inflorescence is a condensed cymose type subtended by bracts. Coffee is a short day plant, hence, the floral initiation takes place in short day conditions of 8-11 h of day light. Pollination takes place within 6 h after flowering. The fruit is a drupe, called cherry or berry, usually fleshy, containing generally two seeds (coffee beans) but sometimes only one and, in that case, the fruit assumes a rounder shape and it is known as pea-berry (Ghosh & Venkatachalapathy, 2014). It varies in size but very little in shape. Its colour varies from yellow to black, though it is mostly orange to red. Seeds are elliptical or egg-shaped and the seed coat is represented by the silverskin (Murthy & Madhava Naidu, 2012; Teketay, 1999).

### 1.4 Harvesting processes

Several factors, related to the types of land, economic reasons, types of coffee, distribution of cherry maturities and size of farm, influence the way of picking. The harvesting processes can be generally classified under manual and mechanical. The manual method is the oldest and the gatherers have to handly pick the cherries from the coffee branches. This manual technique is required in difficult lands with steep slope where it is impossible the use of large machines and it is preferred also when a selective harvesting is a requirement, since the gatherers can select, for instance, only the ripe cherries. Multiple batches of picking are necessary to pick all fruits of farm and this makes the process laborious and expensive. Mechanical harvesting is preferred by large company to take advantage of flat land but needs big investments for equipment. However, growers can share the costs and, later on can amortise the expenses by higher efficiency and reduction of labour cost. With this method, a sorting of ripe and unripe cherries is requested to avoid the negative influence on resulting coffee. There are other ways of picking, such as stripping and the use of vibrating mechanical fingers. The stripping is characterized by hand pulling out the berries, which can be at various stages of ripeness, from each branch, and the resulting crops can contain unripe, ripe and dried fruits. For this reason, a series of separation steps are necessary to increase the quality of the production systems. The picking with the vibrating mechanical fingers is a mechanical stripping that employs a small machine equipped with an engine for finger movements. Eventually, there are some new developments to facilitate the picking like as by using vibration rings applied to the trunk, which lightly shake the plants, and using vacuum pump to select ripe cherries and pull it out from the branch. These machines still need to be miniaturized and their cost reduced to be affordable by growers and smallholders. (Folmer, 2017).

### 1.5 Separation and processing methods

The processing methods contemplate a set of procedures to be used after the coffee harvest, which transform berry into green coffee, which is the processed raw seed of coffee. After harvesting fruits are transported to company in hoppers for winnowing and separation. Just after coffee harvesting, the berries should be immediately taken for processing to avoid storing wet fruit and to reduce the risk

of quality loss caused by fungi and mycotoxins. The winnowing is an important step for removing light impurities, i.e., leaves, sticks, and other debris, from fruit. Small producers still use the manual winnowing, while the mechanical one is preferred from large companies. Impurities are then removed by winnowing, so the coffee crop is ready for the hydraulic separator. The hydraulic separation selects the fruits on the base of their density, more dense fruit (unripe and ripe) and less dense fruit (dry, overripe, etc, known as floaters) and, at the same time, cleans the crop. Moreover, it removes contaminates that were not taken away in the previous stages. A size separation can be also performed with cylindrical sieve for floaters as well as for denser fruits. Finally, the selection of ripe fruits can be done using an electronic separator with an optical reader that ejects or selects the fruits on the base of their colour per comparison with the standard one programmed into machine. After these separation and selection phases, the coffee is submitted to processing methods. The method choice depends on many factors, i.e., climatic condition, consumer demand, water usage rights and the availability of technology for treating residue water. For example, in equatorial region where constant rain coincides with the coffee harvest, the natural drying of coffee is not possible, and the wet methods should be adopted. The two basic processing methods are dry and wet (Borém & Shuler, 2014; Folmer, 2017) and are described in the following chapters.

### 1.5.1 Dry method

The dry method, also called natural, is the oldest processing used to handle berry and is largely carried out in tropical regions where the dry season coincides with the coffee crop period. The process consists in drying the whole coffee fruit without removing the external layers. Hence, it is the simplest method and generates minimal environmental impact, since it produces only a small quantity of residual solids and liquids and does not produce wastewater with elevated levels of organic material as the wet method does (Borém & Shuler, 2014). The traditional drying process is performed immediately after the harvesting by sun drying berries on a clean dry floor or on mats. The bed depth should be less than 40 mm and the cherries should be raked frequently to assure homogeneous drying and prevent fermentation or discoloration (Ghosh & Venkatachalapathy, 2014). For this method, it is

essential a previous fruits sorting on the base of their density to separate floaters from unripe and ripe drupes in order to form groups with similar humidity. This will generate a homogeneous resulting product with a uniform moisture level. The drying process can be also done by mechanical dryer and by a combination of sun and mechanical exsiccation (Illy, Viani, Liverani, & Petracco, 2009). The main problems associated with the dry process are dust and dirt blown onto the coffee bed, and unexpected rainstorms that can soak the coffee batch very quickly. After the drying process the product, so-called fruit pod, should contain a moisture level up to the limit of 12% and can be stored or directly processed by hulling and cleaning phases. In the hulling process the fruit pods are hulled to remove the pericarp; this is generally done by mechanical hullers, which are equipped with an air flow system, namely catador, for the pericarp and bean separation. The cleaning phase consists in a separation of green beans on the base of their size, shape, density and defect presence, aimed to remove defective beans and form homogeneous clusters. The sorting process is performed by a preliminary sifting through size and shape and then the mechanical and optical instruments determine a separation according to bean density and defect presence (Ghosh & Venkatachalapathy, 2014; Illy et al., 2009).

### 1.5.2 Wet methods

The wet methods are generally adopted in those equatorial regions where the raining season coincides with the coffee harvest, a condition that is not appropriate for natural drying. An important requirement is the availability of fresh water used for coffee processing. The wet methods can be classified under three main types: fully washed; pulped natural; semi-washed. The fully washed method is the most common and the traditional one. It consists in the mechanical removing of skin and pulp of the fruit and in a further stage of mucilage degradation through biological fermentation. The resulting coffee, called parchment coffee, is then cleaned and dried. The pulped natural is a wet method where the skin and pulp, and part of mucilage are removed mechanically. However, the remain mucilage is not removed by fermentation or other manners but is dried intact with the parchment coffee. The resulting coffee is called honey coffee for its resemblance with dry honey and

caramel. Semi-washed method is similar to fully wet method but instead of biological fermentation, aimed at removing the mucilage layer, a mechanical removal is performed. The main advantage of this method is the removing of all mucilage parts without the use of fermentation tanks, therefore diminishing the overall amount of wastewater (Borém & Shuler, 2014). Independently of the chosen method, coffee must be dried to 10-12% of moisture content. Drying should take place immediately after processing to prevent off-flavour development. The dried products will be then submitted to hulling and cleaning phases like natural method (Ghosh & Venkatachalapathy, 2014). Several studies reported that post-harvesting methods have a direct influence on the final quality of coffee beverage (de Melo Pereira et al., 2019; Gonzalez-Rios et al., 2007b; Joët et al., 2010), and among the different steps of coffee processing, the microbial mucilage removal has a major influence on the volatile composition and can affect the quality of the final coffee drink (de Melo Pereira et al., 2019).

### 1.6 Coffee roasting

The roasting process is one of the most important stages of coffee processing and consists of giving energy, in the way of heat, to green beans. It is generally defined as a dry heat treatment and is a basic step for coffee flavour development and for the acquisition of certain physical properties of beans. In fact, during roasting process several aromas are generated from different chemical reactions and some physical properties of beans are modified by simplifying the further steps of the coffee preparation, such as grinding and brewing. The main chemical processes involved in this thermic treatment are the Strecker degradation, the Maillard reaction and pyrolysis reactions (Fadai, Melrose, Please, Schulman, & Van Gorder, 2017; Flament, 2002). Also, several physical modifications, such as volume increment, mass and density decrement, shape and colour changes, oil migration and dehydration occur in coffee beans during roasting process. The temperature adopted for coffee roasting is generally higher than those for other roasted foods (nuts, cocoa, etc) and, normally requires to exceed 180 °C up to 200-250 °C for a minimal duration to trigger the characteristic chemical reaction of roasting. A typical roasting time may be from 3 to 20 min (Folmer, 2017). Briefly, the roasting process can be divided in two phases: the drying phase and the roasting phase which follows.

In the first, that occurs at temperature below 160 °C, the coffee bean is dehydrated, releasing steam and initiating the expansion of the solid matrix. In the second phase, the coffee bean reaches temperatures above 180 °C, and exothermic reactions, involving polysaccharides, proteins, chlorogenic acid, and trigonelline, begin to form the compounds responsible for the colour, flavour, and aroma of roasted beans. In this stage, $CO_2$ is released as the product of the reaction contributing to the matrix expansion (Bottazzi, Farina, Milani, & Montorsi, 2012; Bustos-Vanegas et al., 2018). When the desired roasting degree is reached, the hot mass of beans must be rapidly cooled down through water quenching or air jet to stop the roasting process and prevent excessive roasting. Different industrial technologies based on specific mechanism of heating, such as hot air, infrared, microwave, superheated steam and so on, have been applied in industrial operations of roasting. The widespread technology is still the hot air system. These roasting machines operate moving constantly the coffee beans inside the roasting chamber to assure homogeneous heat transfer from the hot air to coffee. Another heating mechanism occurs when heat is transferred from the hot walls of roasting chamber to the beans. For large systems it is important to control the whole process and, for this purpose, big roasting machines are equipped with sophisticated process control systems, which assure to set up and control the roasting profile (Folmer, 2017).

### 1.7 Coffee grinding and influence of particle sizes on EC extraction

The grinding process is an essential step for transforming the inedible coffee beans into coffee powder, called roast and ground (R&G) coffee, ready for brewing. The grinding consists in converting roasted beans through the application of mechanical forces into R&G coffee aimed to increase the specific extraction surface, or rather to increase the extent of the interface between water and coffee. In this manner the extraction of soluble and emulsifiable substances is facilitated and, at the same time, the rupture of coffee bean tissues and cells accelerates the release of carbon dioxide ($CO_2$) gas and volatile aroma, especially highly volatile compounds, and allows an easier extraction of the remaining aroma (Akiyama et al., 2003; Susana Andueza, Paz De Peña, & Cid, 2003; Folmer, 2017). The grinding of natural products like coffee beans always produces lots of particles of different sizes

and shapes. The spread over the range of all sizes gives the so-called particle size distribution (PSD) (Folmer, 2017). Generally, the grinding process is empirically optimized by setting a specific distance between the grinding tools, aimed to produce an adequate average of particle size for the respective coffee preparation. In fact, several studies have reported that particle sizes have an influence in coffee preparation, especially in espresso coffee. (Susana Andueza et al., 2003; Derossi, Ricci, Caporizzi, Fiore, & Severini, 2018; Kuhn, Lang, Bezold, Minceva, & Briesen, 2017; Severini, Ricci, Marone, Derossi, & De Pilli, 2015; Severini, Derossi, Ricci, Caporizzi, & Fiore, 2018). In detail, Andueza et al., (2003) studied the influence of grinding and torrefacto roasting on the chemical and sensorial characteristics of espresso coffee. They found that total solids (TS), caffeine, trigonelline and chlorogenic acids increased inversely with particle size, but not significant differences on sensory attributes was reported. Instead, Severini et al., (2015) demonstrated that extraction time and grinding level significantly affect the overall aromatic profiles of EC, which was measured by using an electronic nose system. Another work (Kuhn et al., 2017) showed that particle size significantly affects the extraction kinetics and confirmed that the smaller particles leading to a higher extracted amount of caffeine and trigonelline. Last year, Derossi et al., (2018) reported that the use of different particle sizes for Turkish, American and espresso coffee brewing has the major impact on espresso extraction and in particular on pH changes of beverage. Although studies about the influence of PSD on EC extraction are reported, a lack of investigations occurs for other variables, i.e., filter basket, amount of R&G coffee and perforated disc, which influence the extraction process too. The filter basket is the conical support, drilled on the bottom, where the coffee powder is placed and tamped (coffee cake) (Illy et al., 2009). The perforated disc is a metal plate, assembled under the shower of each serving group, that adjusts the distance between the coffee cake and the shower and assures an adequate empty space for cake swelling. To the best of our knowledge, only one paper studied the influence of filter basket and different particle sizes on espresso extraction. Specifically, this work investigated the effects of 1-cup and 2-cups filter basket on EC extraction at three grinding sizes (Severini, Derossi, Fiore, Ricci, & Marone, 2016). Per each range of particle sizes, when 2-cups filter

was installed onto machine, they found higher contents of total solids and caffeine in the coffee portions generating from the first 8 s of percolation.

### 1.8 Coffee preparation methods

The brewing process is the last transformation of coffee, which must transfer the unique and pleasant flavour from R&G coffee into beverage. From a chemical point of view, it is a solid-liquid extraction that uses water as extraction solvent, to take out several volatile and non-volatile molecules from coffee powder. The resulting cup should be characterized by a subtle equilibrium of aroma, taste and mouthfeel (Folmer, 2017). Depending on geographic, cultural and social context, as well as on personal preferences, numerous preparation methods and extraction processes have been developed and then introduced in the society since the discovery of coffee as a beverage (Gloess et al., 2013). The huge variety of preparation, such as Turkish coffee, drip coffee, French press, espresso coffee, Moka pot, cold brew, etc, can be characterized by extraction tools but can also be grouped by various key parameters influencing the final flavour profile, e.g., pressure, brewing time, compaction, etc (Folmer, 2017). Turkish coffee is prepared by grinding beans to fine particles and then adding water and coffee powder in a pot (e.g., cesve). The water is brought to boil for no more than an instant, and then the heating is usually stopped. This results in a strong coffee with a layer of foam on the top and sediments that settle on the bottom of the cup. Drip coffee, also called filter coffee or pour over brew, consists in brewing coffee by pouring hot water onto coffee powder, generally milled as coarse particles. The water passes into coffee by gravity and the powder remains into a holder containing a filtering device. Various filter sizes, shapes and materials are commercially available. The water can be applied manually or by automatic drip filter machine. This method produces a coffee milder than Turkish's one and with enhanced acidity and flavour. Rather coarse particles are used for French press as well. In this preparation technique, the water and R&G coffee are placed into a vessel known as French press, *cafetière*, coffee press or coffee plunger, and the steeping is performed for a specific time (2-5 min), depending on the intensity of extraction the *barista* prefers. The liquid part is separated from the solid phase by using a plunger containing a filter device. Because inefficient metal-

mesh filtration, higher sediment levels are generally obtained rather than drip coffee. The most popular household for brewing coffee in Italy is moka pot. This tool is composed of three-chamber design. The bottom chamber is filled with water and the coffee powder is placed on the middle chamber. By heating, the hot water and steam pass through coffee bed extracting soluble and emulsifiable substances. The air-vapor pressure generated in the bottom chamber drives the extraction process. The beverage will be then collected in the upper section (Caprioli, Cortese, Sagratini, & Vittori, 2015; Folmer, 2017; Gloess et al., 2013; Sunarharum, Williams, & Smyth, 2014). Another example of technique having experienced a recent surge in popularity is cold brew coffee. The cold brew consists in preparing coffee with cold water, usually at room temperature or lower, over a period longer than other coffee preparation methods. In fact, the steeping time ranges from 8 to 24 h. Therefore, the main differences with the other techniques are the extraction temperature and brewing time. Temperature often significantly influences the aqueous solubility of compounds, hence, brewing temperatures significantly modify the composition of hot and cold brews. In addition, the longer brewing times of cold brew coffee may affect the content of numerous substances (Fuller & Rao, 2017; Lane, Palmer, Christie, Ehlting, & Le, 2017). The most popular method that exploits the pressured and hot water for preparing a pleasant and short beverage is the espresso coffee.

### 1.8.1 Espresso coffee

The espresso coffee is one of the most widely-consumed beverage in the word and mainly in southern Europe and Central America (Caprioli et al., 2015). The Italian word *espresso* suggests that this beverage must be quickly prepared on customer demands or rather extemporaneously prepared after the consumer orders. Another important characteristic of espresso coffee is the use of pressure. The beverage must be prepared not only with hot water, as the main coffee brewing, but also under pressure. Therefore, for the espresso definition three essential features are required: extemporaneous preparation, fast brewing and use of pressure. A typical definition of espresso is the following: a concentrated polyphasic beverage with a characteristic foam layer (crema) on the surface, prepared by forcing hot water (90 ± 5 °C) under pressure (9 ± 2 bar) into a tamped R&G coffee (called coffee

cake) for a short period of time (30 ± 5 s). The cup volume can change from 15 to 50 ml on consumer preferences (Illy et al., 2009). The preparation conditions, such as amount of R&G coffee to brew, design of filter basket, time of extraction, volume in cup, etc, change from country to country. To obtain a Certified Italian Espresso Coffee (EC) the beverage and the preparation have to conform to strict production specifications. These specifications are issued by the Italian Espresso National Institute and approved by a Third-Party Body, and it is safeguarded and promoted through a product certification (certificate of product conformity Csqa n. 214: 24 September 1999, DTP 008 Ed.1). Some important conditions to be applied for the production of a Certified Italian Espresso are: R&G coffee: 7 ± 0.5 g, exit temperature of water from the unit 90 ± 2 °C, temperature of the drink in the cup 67 ± 3 °C, entry water pressure 9 ± 1 bar, percolation time 25 ± 2.5 s, viscosity at 45 °C > 1.5 mPa, total fat > 2 mg/ml, caffeine < 100 mg/cup, volume in the cup (including foam) 25 ± 2.5 ml (Caprioli et al., 2015; Odello & Odello, 2006). For the EC preparation, numerous machines are commercially available, with different design and technologies; almost all are composed by three essential parts: the pump, the extraction chamber and the heat exchanger. The extraction occurs when water is brought to the desired pressure and then forced through a heat exchanger, which brings water to the chosen temperature. Then, hot water proceeds to the extraction chamber, which is composed of heated and fixed part in which the filter holder is fitted snugly into. The filter holder contains a filter basket where the coffee cake is settled. In the extraction chamber, water crosses the perforated disc and is sprayed, through the shower, evenly over the coffee cake surface. The pre-infusion takes place at first seconds where the coffee absorbs some millilitres of water and swells. This allows the coffee surface to reach the required permeability (Caprioli et al., 2015), and then the extraction phase begins. The extraction phase is a complex mechanism where several phenomena occur, i.e., dissolution of aqueous soluble compounds, forced extraction of some less soluble compounds and physically entrapped molecules (e.g., arabinogalactans), degradation reactions due to heating that can affect the solubility of many substances (e.g., galactomannans), migration of fine particles and coffee oil through the water flow, etc (Chen Zhang, Linforth, & Fisk, 2012).Compounds with different

chemical-physical properties will be extracted at the end of the process and this process will result in a complex flavour beverage. Many variables related to EC preparation, such as amount of R&G coffee, PSD, filter basket, perforated disc, water quality, temperature and pressure of water, percolation time, cake porosity, etc, can act an important role in coffee extraction (Illy et al., 2009). In the last years, the majority of works have focused on studying how water temperature, water pressure and particle sizes can influence the EC in cup (Andueza et al., 2002; Andueza et al., 2003; Caprioli et al., 2014, 2013; Derossi et al., 2018; Kuhn et al., 2017; Salamanca, Fiol, González, Saez, & Villaescusa, 2017; Severini et al., 2015; Severini et al., 2018). Instead, to the best of our knowledge none has investigated the importance of R&G coffee amount and the possibility to reach good quality in EC lowering the amount of powder. So far, the mass of R&G coffee is empirically chosen on the base of filter basket design and other conditions dictated from the experience. Moreover, the extraction with different filter baskets could have an impact on coffee quality, but it has not been studied yet. Considering that these extraction variables are strictly connected to each other, one of our aim has been to investigate the quality of EC prepared by changing particle sizes, perforated disk heights and filter baskets, aimed at lowering the amount of ground coffee used. For further details on project goals please refer to chapter 2 (2. Aims of the project).

### 1.9 Bioactive compounds in coffee

Bioactive compounds are molecules generally produced by plants which possess pharmacology and/or toxicology effects in man and animals. They are usually produced as secondary metabolites; vitamins are not part of the term “bioactive plant compounds”. Hence, a complete definition of bioactive compounds is secondary plant metabolites eliciting pharmacological and/or toxicological effect in humans and animals. The secondary metabolites are substances derived from a side pathway beside the primary biosynthetic and metabolic routes of important compounds for plant growth and development (sugars, carbohydrates, amino acids, proteins and lipids) and are not necessary for daily functioning of plants. Several aspects and criteria, i.e., biological effects, botanical categorization based on family and genera of plants, chemical classes, biochemical pathway, etc, are used for

bioactive compounds classification (BERNHOFT, 2010). Considering the biochemical pathway and the chemical classes, the main bioactive compounds in coffee can be generally divided in alkaloids (caffeine and trigonelline), derivatives of phenolic acids (chlorogenic acids), diterpenoids (cafestol and kahweol), flavonoids (isoflavones) and phenylpropanoids (lignans). Cafestol and kahweol are the two most abundant unsaponifiable pentacyclic diterpene alcohols of the kaurene family occurring in coffee oil (Rafael et al., 2010). Diterpenes are present in the most important coffee species (arabica and robusta) and are more abundant in *Coffea arabica* L.; other two substances of the family, i.e., 16-O-methylcafestol and 16-O-methylkahweol, specifically occur in robusta coffee; kahweol is reported to be specific in arabica. Therefore, the differences on diterpene contents can be exploited for coffee species identification (Illy et al., 2009; Novaes, Bayan, Neto, & Rezende, 2019; Rafael et al., 2010). These molecules are extensively studied also for their association with health issues. For example, cafestol and kahweol can induce the degradation of toxic compounds and provide hepatoprotective effects against some toxicants, such as aflatoxin B1 and acrolein. Other studies reported the antioxidant, anti-inflammatory and anticarcinogenic activities of these lipids (Preedy, 2014).

### 1.9.1 Alkaloids: caffeine and trigonelline

Alkaloids are a huge groups of nitrogen compounds defined by IUPAC as "basic nitrogen compounds (mostly heterocyclic) occurring mostly in the plant kingdom (but not excluding those of animal origin). Amino acids, peptides, proteins, nucleotides, nucleic acids, amino sugars and antibiotics are not normally regarded as alkaloids. By extension, certain neutral compounds biogenetically related to basic alkaloids are included" (McNaught & Wilkinson, 2014). Caffeine is a secondary metabolite of the alkaloid family, belonging to the purine alkaloid group. The purine alkaloids derive from purine nucleotides and are widespread in plant kingdom occurring in nearly 100 species. The methylxanthines, e.g., caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine), together with methyl uric acids, such as liberine and methylliberine, are the most common purine alkaloids (Ashihara, Sano, & Crozier, 2008). Caffeine is the best-known compounds in coffee, and it is one of the most widely consumed active food ingredient in the world. It is present in beverages like

coffee, tea, soft drinks, energy drinks as well as in products containing cocoa, chocolate and in a variety of dietary supplements and medications. The attractiveness and recognition of this molecule is due to the effect that it produces on the body and mind. In fact, caffeine stimulates the central nervous system aiding to stay awake and improving mental alertness after fatigue (Heckman, Weil, & de Mejia, 2010). Moreover, other properties are attributed to caffeine, such as raising the blood pressure as a result of increases in total peripheral resistance, relaxation of bronchial muscle, increase of gastric acid secretion and diuresis, etc (Caprioli et al., 2014; Grosso, Godos, Galvano, & Giovannucci, 2017). It is known that robusta coffee contains an higher amount of caffeine than arabica; in fact, levels in green beans are from 1.6 to 2.4% and from 0.9 to 1.2% of the dry weights, respectively. (Illy et al., 2009; Preedy, 2014). During the roasting process the content of caffeine does not dramatically change but it has been reported that dark roasted beans can contain lower caffeine amount than light roasted beans (Hečimović, Belščak-Cvitanović, Horžić, & Komes, 2011). On the other hand, trigonelline level is intensely affected by thermal processes; after roasting the content of this molecule noticeably decreases, due to a series of degradation mechanisms, which form volatile compounds, responsible for flavour formation, and non-volatile molecules. (Casal, Beatriz Oliveira, & Ferreira, 2000; Illy et al., 2009). Trigonelline is a pyridine alkaloid, firstly isolated from *Trigonella foenum-graecum* L., which has been found in many plant and animal species (Zheng & Ashihara, 2004). In green beans, this pyridine is present in higher concentration in arabica (1-1.2% of dry weights) than robusta (0.6-0.8% of dry weights); during roasting process trigonelline can decompose via two major routes, i.e., decarboxylation and methyl rearrangement, to afford pyridines, and N-demethylation to give nicotinic acid. Trigonelline is also degraded by decarboxylation, generating the *N*-methylpyridinium cation, inductor of enzyme systems involved in detoxification of xenobiotics, activator of the Nrf2/ARE pathway, inducing cellular defence mechanisms, and novel phytoestrogen (Jeszka-Skowron, Zgoła-Grześkowiak, & Grześkowiak, 2014; Stadler, Varga, Hau, Arce Vera, & Welti, 2002). In coffee beverages, the levels of caffeine, trigonelline and other bioactive compounds, such as chlorogenic acids, are influenced by the process used for preparation (Gloess et al., 2013)

and, in addition and mainly in espresso coffee, by the chosen variables for the extraction, such as temperature and pressure of water, particle sizes, ratio of water and powder, etc. (Andueza et al., 2002; Caprioli et al., 2014; Severini et al., 2015; Severini et al., 2018). The analytical methods used for caffeine and trigonelline quantification are characterized by several extraction procedures and different analytical instruments chosen and optimized according to target matrix (green beans, espresso coffee…). A common quantitative approach is characterized by water extraction and quantitative analysis by high-performance liquid chromatography coupled with diode-array detection or mass spectrometry (HPLC-DAD or HPLC-MS) (Caprioli et al., 2014; Casal, Oliveira, Alves, & Ferreira, 2000; Jeszka-Skowron et al., 2014; Perrone, Donangelo, & Farah, 2008).

### 1.9.2 Derivates of phenolic acids: chlorogenic acids

Chlorogenic acids (CGAs) are a large group of compounds derived from the esterification between certain *trans*-cinnamic acids, phenolic compounds, and (-)-quinic acid. Chlorogenic acids do not contain chlorine despite the "chloro" prefix. Their name probably comes from Greek which means light green. This is likely because their green colour after oxidation reactions (Kremr, Bajer, Bajerová, Surmová, & Ventura, 2016). Chlorogenic acids occur in several plants; coffee is an important source of these conjugated compounds. They can be formed by the binding of three different *trans*-phenyl-3-propenoic acids (cinnamic acids), differing in their ring substitution, with (-)-quinic acid. The most common *trans*-cinnamic acids are caffeic (3,4-dihydroxycinnamic acid); ferulic (3-methoxy, 4-hydroxy), sinapic (3,5-dimethoxy, 4-hydroxy) and *p*-coumaric (4-hydroxy). Chlorogenic acids can be classified by the identity, number and position of the acyl residues in the following subgroups: the relatively widespread mono-esters of caffeic acid (caffeoylquinic acids or CQA), *p*-coumaric acid (*p*-coumaroylquinic acids or *p*CoQA) and ferulic acid (feruloylquinic acids or FQA); di-esters (diCQA), tri-esters (triCQA) and the single tetra-ester of caffeic acid (tetraCQA); mixed di-esters of caffeic and ferulic acid (caffeoylferuloylquinic acids or CFQA) which are characteristic of robusta coffee and other minor groups (Clifford, 2000). The 99% of the total CGAs in coffee is represented by mono-acyl (CQA, FQA and *p*CoQA) and di-acyl (di-caffeoylquinic acids and caffeoylferuloylquinic acids)

compounds with at least three isomers per class (De Rosso, Colomban, Flamini, & Navarini, 2018). In green beans, the content of chlorogenic acids is superior in robusta than arabica; it can vary from 7 to 10% and from 5.5 to 8% of dry weights, respectively (Preedy, 2014). During roasting process several chemical changes occur in these molecules leading to important aroma formation (Moon & Shibamoto, 2010; Müller, Lang, & Hofmann, 2006); in dehydrating conditions, occurring in the latter roasting stages, there is the formation of chlorogenic lactones (CGL), characterized by a bitter taste (Farah, De Paulis, Moreira, Trugo, & Martin, 2006; Farah, De Paulis, Trugo, & Martin, 2005). The most common individual chlorogenic acid is 5-*O*-caffeoyiquinic acid (5-CQA), which sometimes is still called 3-*O*-caffeoylquinic acid (3-CQA) or chlorogenic acid. This because the IUPAC assigned the correct rules for nomenclature in 1976 and the current 5-CQA was currently 3-CQA. The use of these two old names should be discouraged (Clifford, 2000; Kremr et al., 2016). Several evidences have demonstrated that chlorogenic acids possess antioxidant, anti-inflammatory and anticarcinogenic activities; numerous epidemiologic studies have focused on these properties (Baeza et al., 2014; Liang & Kitts, 2015; Rocha, Monteiro, & Teodoro, 2012; Sato et al., 2011). It has been proposed that they can have positive effects on type-2 diabetes, obesity, Alzheimer's disease, stroke, endothelial function and blood pressure (Tajik, Tajik, Mack, & Enck, 2017). An important aspect to focus on, is that coffee beverages are the richest source of CGAs in human diet and the daily intakes of modest and heavy coffee drinkers ranged from 0.1 to 2 g (De Rosso et al., 2018). Numerous factors, such as preparation methods, roasting degree and type of coffee blend, can influence the levels of chlorogenic acids in cup (Fujioka & Shibamoto, 2008; Gloess et al., 2013; Tfouni et al., 2014). Most of published information about quantitative analysis of CGAs in dietary sources were based on HPLC-UV-VIS system (Caprioli et al., 2013; Craig, Fields, Liang, Kitts, & Erickson, 2016) while HPLC-MS techniques have gained popularity recently (Farah, 2019).

### 1.9.3 Phytoestrogens: lignans and isoflavones

Phytoestrogens are a group of compounds, functionally characterized by their estrogenic activities in mammals, which are structurally similar to mammalian estrogen 17β-estradiol ($E_2$). They commonly

derive from plants; ubiquitous molecules are isoflavones, lignans and coumestans. Also other classes of molecules can be classified as phytoestrogens, e.g., anthraquinones, chalcones, saponins, etc, but those are found in few species (Ososki & Kennelly, 2003). The present work is focused on two widespread classes: isoflavones and lignans. The most studied and best-known phytoestrogens are isoflavones, also considered a subclass of flavonoids. In the 1940s some Australian sheep, after a diet predominately based on subterranean clover (*Trifolium subterraneum* L.), have suffered from a reproductive disorder, called "clover disease". This triggered to start investigations on isoflavones and their estrogenic activities. These molecules occur in the Fabaceae (Leguminosae) family, the main sources being soybeans and soy products, kidney beans, red clover, kudzu root, mung bean sprouts, navy beans, lupines and fava beans. The main isoflavones naturally occurring that have shown estrogenic activities are: glycosides genistin and daidzin, aglycones genistein and daidzein and the 4-methyl ethers forms, formononetin and biochanin A. Soybean is the most studied matrix because it contains the highest levels of isoflavone (Mortensen et al., 2009); it has been reported that these molecules occur in four different forms: aglycons (daidzein, genistein and glycitein), glucosides (daidzin, genistin and glycitin), acetylglucosides and malonylglucosides. The methylated derivatives, biochanin A and formononetin, are present in other sources such as in alfalfa, red clover and beans (Ososki & Kennelly, 2003; Zaheer & Humayoun Akhtar, 2017). Lignans, another important class of phytoestrogens, are characterized by dimerization of two phenylpropanoid units, β,β'-linked or, as the IUPAC suggests, 8,8'-linked (Moss, 2000). Several compounds with different chemical structures take part of lignan family and they are generally classified under different subclasses, i.e., furofuran, furan, dibenzylbutan, dibenzylbutyrolactol, dibenzylbutyrolactones, aryltetralin, arylnaphtalene and dibenzocyclooctadienes, according to the way which the oxygen is incorporated into the skeleton and to the cyclisation patterns (Cunha, Andrade e Silva, Sola Veneziani, Ambrósio, & Kenupp Bastos, 2012; Floss, 1997; Knaggs, 2003). Lignans were firstly identified in plants where they aid in the formation of lignin used to construct the plant cell wall (Ososki & Kennelly, 2003), and the highest concentration has been found in the knots of Norway spruce trees (*Picea abies* (L.) H. Karst.) with

7-hydroxymatairesinol (HMR) as predominant compound. Other molecules have been isolated from other trees too, e.g. pinoresinol (PINO), a furofuran lignan, from the oleoresin of black pine (*Pinus nigra* J. F. Arnold) and lariciresinol (LARI), a member of furan subgroup, from European larch (*Larix decidua* Mill.) (Holmbom et al., 2003); but the main edible sources are flaxseeds and sesame seeds. Secoisolariciresinol (SECO) is the most abundant lignan content in flaxseeds but PINO, LARI and matairesinol (MAT) are present in substantial amount too. Other sources of lignans are nuts and oilseeds, cereals and breads, legumes, fruits, vegetables, soy products, meat products, and alcoholic and non-alcoholic beverages (Landete, 2012; Mazur et al., 1998; Milder, Arts, Putte, Venema, & Hollman, 2005; Thompson, Boucher, Liu, Cotterchio, & Kreiger, 2006). They can be present in free-form, glyosidic form or more complex forms, such as SECO in flaxseed, which occurs in diglucoside-hydroxymethyl glutaryl ester-linked oligomers (Ford, Huang, Wang, Davin, & Lewis, 2001; Smeds et al., 2007). Isoflavones and lignans are considered phytoestrogens because are able to act as agonists/antagonists of estrogen receptors (ER). As estrogen agonists, phytoestrogens mimic endogenous estrogens and cause estrogenic effects. As estrogen antagonists, they may block or alter ER and prevent estrogenic activity, causing anti-estrogenic effects. For this double action, phytoestrogens are sometimes classified as selective estrogen receptor modulators (SERMs) (Ososki & Kennelly, 2003) and several studies have investigated the use of phytoestrogens for possible treatment of breast cancer, endometrial cancer, prostate cancer and menopausal symptoms (Adlercreutz, 2002; Krebs, Ensrud, MacDonald, & Wilt, 2004; Landete, 2012; Ososki & Kennelly, 2003). It has been reported that for having these characteristics, lignans should be converted into mammalian lignans also called enterolignans, i.e., enterolactone (ENL), enterodiol (END) and enterofuran (ENF). Since various intestinal bacteria are involved in these bioactivations, enterolignan bioavailability and their biological effects are associated with the presence of bacteria capable of performing these chemical transformations. These two classes of phytoestrogens possess also other biological activities, such as antioxidant, antimicrobial, anti-inflammatory and antitumor (Heinonen et al., 2001; Landete, 2012). For example, genistein is the most potent antioxidant among the soy

isoflavones, followed by daidzein, and seems to increase the production of superoxide dismutase (SOD), which removes the free radicals (Ososki & Kennelly, 2003).

#### 1.9.3.1 Analytical methods for phytoestrogen analysis in coffee

The analytical methods for lignans and isoflavones quantification vary according to studied matrix and monitored compounds, and instruments often used are HPLC coupled with ultraviolet (UV) detector, electrochemical or mass spectrometric (MS) detection and gas chromatography (GC) coupled with MS. Various sample preparation techniques and extraction processes have been reported, such as acid and base hydrolysis, enzymatic digestion, organic solvent extraction, and solid-phase extraction (SPE), aimed to release these molecule from the complex matrix structure and to clean-up sample. (Slanina & Glatz, 2004; Wang, Prasain, & Barnes, 2002). The analysis of phytoestrogens in coffee are investigated in few works and specifically, some authors have studied their content especially in ground coffee and in not many coffee brews. Mazur et al., (1998) pioneered phytoestrogen quantitation in ground coffee, using a very long extraction and derivatization process coupled with isotope dilution-gas chromatography-mass spectrometry (ID-GC-MS); they found SECO in quantities ranging from 3.93 to 7.16 mg $kg^{-1}$, daidzein (n.d. to 0.66 mg $kg^{-1}$), genistein (n.d. to 0.29 mg $kg^{-1}$) and formononetin (n.d. to 0.78 mg $kg^{-1}$). Many studies have followed since then. Horn-Ross et al., (2000) reported, in an unspecified coffee sample, a daidzein concentration of 0.503 mg $kg^{-1}$ and only traces of SECO and genistein (≤0.25 mg $kg^{-1}$, their detection limits), while they did not detect any MAT, formononetin or biochanin A. Later, Milder et al., (2005) analysed four lignans in various foods, including coffee beverage, by using enzymatic hydrolysis followed by double ethyl ether extraction coupled with HPLC- MS/MS. They found a total concentration of lignans from 187 to 313 µg $L^{-1}$, the most abundant ones being SECO (92.0- 161.0 µg $L^{-1}$) and LARI (90.0- 131.0 µg $L^{-1}$). In a study of six coffee brews, Thompson et al., (2006) detected daidzein (1 µg $L^{-1}$), formononetin (2 µg $L^{-1}$), genistein (1 µg $L^{-1}$), SECO (34-47 µg $L^{-1}$), LARI (9-11 µg $L^{-1}$) and MAT (1-2 µg $L^{-1}$). Kuhnle et al., (2008) analysed coffee beverages reporting a SECO concentration of 100-160 µg $L^{-1}$ whereas genistein, biochanin A, formononetin and matairesinol were found at

concentrations below 10 µg $L^{-1}$. They also analysed two instant ground coffees and found SECO (6.10-8.62 mg $kg^{-1}$) and genistein (0.04-5.94 mg $kg^{-1}$) as the most abundant phytoestrogens. Alves, Almeida, Casal, & Oliveira, (2010) reported, in ground coffee, daidzein, genistein and formononetin concentrations of 8.7, 3.0 and 30.7 mg $kg^{-1}$, respectively. Sapozhnikova, (2014) developed a simple method for polyphenol quantitation in various beverages; in coffee brews they found the isoflavones daidzein (3.2-5.2 mg $L^{-1}$), genistein (0.9-1.4 mg $L^{-1}$) and formononetin (3.0-6.0 µg $L^{-1}$). Caprioli et al., (2016) used a SPE clean-up procedure and a HPLC-MS/MS to quantify isoflavones in espresso and ground coffees. They detected biochanin A (espresso: 0.59 to 3.26 µg $L^{-1}$; ground: 0.71 to 3.95 µg $kg^{-1}$) and formononetin (espresso: 0.36 to 0.41 µg $L^{-1}$; ground: 0.52 to 4.27 µg $kg^{-1}$). Therefore, most of the authors have analysed the levels of different phytoestrogens in several foodstuffs provoking a lack of a specific quantitative method for lignans analysis in ground coffee. Moreover, to the best of our knowledge, none investigated the concentration of lignans in espresso coffee and their content, together with those of isoflavones, in green beans. Indeed, one of our goals was to develop specific analytical methods for: a) lignan quantification in espresso and R&G coffee; b) lignan and isoflavone quantitation in green coffee. For further detail on project aims please refer to chapter 2 (2. Aims of the project).

### 1.10 Aroma compounds in coffee

Coffee beverages and mainly espresso coffee present an intense and characteristic flavour. Flavour is defined as a complex sensation described as a combination of aroma, taste, mouthfeel and texture. The aroma, or odor, is arguably the most important component of coffee flavour (Sunarharum et al., 2014). Therefore, several studies have analysed and investigated the aroma fraction of coffee beans and coffee beverages (Bicchi, Panero, Pellegrino, & Vanni, 1997; Blank, Sen, & Grosch, 1991; Caprioli et al., 2012; Michael Czerny & Grosch, 2000; Risticevic et al., 2008). Although more than 1,000 volatile compounds, occurring from ppt to ppm levels, have been identified in coffee, only 20-30 specific molecules are important for coffee aroma and could be responsible for the overall coffee odor (Blank et al., 1991; M Czerny, Mayer, & Grosch, 1999; Michael Czerny & Grosch, 2000;

Semmelroch & Grosch, 1996). Several factors related to species and plant cultivation, harvesting and processing methods, roasting, grinding and preparation techniques, play an important role on coffee quality and its aroma (Blank et al., 1991; Gonzalez-Rios et al., 2007a; Sanz, Maeztu, Jose Zapelena, Bello, & Cid, 2002; Sunarharum et al., 2014). The roasting process is likely the most import step for flavour formation, since transforms the green pea-like and bell pepper-like smell of green coffee into unique and pleasant coffee aroma of roasted beans (M Czerny et al., 1999; Michael Czerny & Grosch, 2000). In fact, several chemical mechanisms, such as Maillard reaction, which occurs between sugars and amino acids, Strecker degradation, degradation of sugars, minor lipid degradation, interaction between intermediate decomposition products, etc, lead an increment of volatile compounds numbers (Buffo & Cardelli-Freire, 2004; Caprioli et al., 2015; Sunarharum et al., 2014). The aroma fraction contains different molecules, which are part of various chemical classes. From a quantitative point of view furans and pyrazines are the most important volatile classes, while qualitatively sulfur-containing compounds and pyrazines are the most relevant (Sunarharum et al., 2014). Other important groups of volatiles are furanones, phenolic compounds, pyrroles, pyridines, alcohols, aldehyde, ketones and esters. Furans are ubiquitous molecules present in thermally processed foods and their attention has rapidly boosted since 1995, when they have been classified as "possibly carcinogenic to humans" (group 2B) from the International Agency for Research on Cancer (IARC) (Rahn & Yeretzian, 2019; W. IARC (Ed.), 1995). These compounds possess sensory thresholds that are relatively high compared to other groups of coffee volatiles, but they are present in high concentrations and therefore are relevant for coffee aroma quality (Sunarharum et al., 2014). Pyrazines are well-known molecules present in various foodstuff including coffee. Some alkyl pyrazines, such as 3-isopropyl-2-methoxypyrazine, 3-isobutyl-2-methoxypyrazine, 2-ethyl-3,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, described as nutty, earthy, roasty, green aromas, are reported to be potent odorants of coffee aroma (Blank et al., 1991; W. Grosch, 1998). 2-Furfurylthiol is likely the most-known sulfur-containing compounds present in coffee and possesses a roasted, pungent and coffee-like aroma. Moreover, it has one of the highest Odor Activity Values (OAV) and

a low level of odor threshold. Therefore, it is considered a key aroma compound of coffee brew (W. Grosch, 1998; Semmelroch & Grosch, 1996; Sunarharum et al., 2014). Important for coffee aroma are also some phenolic compounds such as guaiacol, 4-vinylguaiacol, 4-ethylguaiacol and vanillin. These phenols arise from thermal degradation of chlorogenic acids and these volatiles could have a role on flavour differentiation between arabica and robusta since these two species contain significative differences on CGA contents (Sunarharum et al., 2014). Several approaches have been employed for the analysis of coffee volatiles. One of the most frequently adopted is the headspace solid phase microextraction (HS-SPME) (Bressanello et al., 2017; Caprioli et al., 2012; Mondello et al., 2005; Risticevic et al., 2008), a fast preparation technique which consents to extract volatile analytes prior to analyse them, for instance by GC-MS. The choice of a SPME fiber is dependent on the specific physico-chemical characteristics of the target solutes to be extracted (Mondello et al., 2005), and for coffee it is commonly used the Divinyl-benzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Bressanello et al., 2017; Caprioli et al., 2012; Risticevic et al., 2008). Another approach is to characterize the aroma of coffee by the Aroma Extract Dilution Analysis (AEDA) and, as analytical instrument, to exploit a gas chromatographer-olfactometer (GC-O). This technique, coupled with an olfactometric instrument, which commonly possesses another detector (e.g., flame ionization detector (FID), MS), permits to assign an odor description for each studied molecule and moreover, to measure the potency of odorants by calculating the Flavour Dilution (FD) factor (Werner Grosch, 1994). These analysis are part of a more complex concept called sensomic approach that led to improve knowledge about key aroma compounds in coffee (Blank et al., 1991; M Czerny et al., 1999).

### 1.11 Coffee residues and coffee silverkin

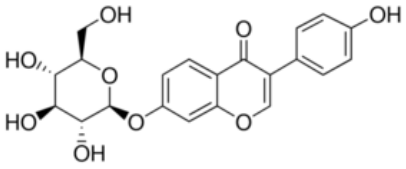
Coffee is one of the most consumed beverages in the word; in addition, it is an important agricultural product in the international trade. Therefore, to satisfy the enormous consumer demand, coffee industry must process a huge quantity of beans addressed to coffee beverage production. In this manner, coffee companies generate an elevated amount of liquid and solid residues, since the 90% in

weight of coffee cherries (mostly pulp) is discarded during processing as agricultural waste or by-product (Iriondo-DeHond et al., 2019). For this reason, in the latest years, several authors have proposed original mechanisms to reuse the coffee by-products in order to manage and reduce its disposal (Hijosa-Valsero, Garita-Cambronero, Paniagua-García, & Díez-Antolínez, 2018; Machado, Mussatto, Teixeira, Vilanova, & Oliveira, 2018; Martinez-Saez et al., 2014; Mussatto, Machado, Martins, & Teixeira, 2011; Narita & Inouye, 2014; Scully, Jaiswal, & Abu-Ghannam, 2016). Among these by-products, coffee silverskin (CS) is the major residue generating from the roasting process. It is a thin tegument that directly covers coffee seeds; during roasting, coffee beans expand and this thin layer is detached (Bessada, Alves, & Oliveira, 2018). Although CS accounts for only a minimal fraction of the whole coffee cherry (1-2%), it contains high level of dietary fiber, bioactive compounds and possesses antioxidant activities (Janissen & Huynh, 2018; Mussatto et al., 2011). That is why in recent years, some authors proposed the use of CS as raw material for the recovery of functional compounds of potential interest. Indeed, CS is a rich source of soluble and insoluble dietary fibers (3.7 and 64%, respectively), which can be used for food enrichment (Iriondo-DeHond et al., 2019). Moreover, recent studies have evidenced that CS is a valuable source of bioactive compounds such as melanoidins, caffeine and polyphenols, which allow potentials applications of CS extracts as functional ingredient in cosmetic and nutraceutical formulations (Bertolino et al., 2019; Bessada et al., 2018). Other author have suggested to apply this coffee residue as feedstock in biofuel production (Hijosa-Valsero et al., 2018), as adsorbent material to remove potential toxic metals in water (Malara et al., 2018), as a source of cellulose for paper production (Mussatto et al., 2011) and as an ingredient to be exploit in food industry. Indeed, Martinez-Saez et al., (2014) have proposed the use of CS for a novel beverage production aimed to body weight control. Recapitulating, several studies have reported the nutritional composition of coffee silverskin and the content of bioactive compounds such as caffeine, chlorogenic acids, melanoidins and other polyphenols (Janissen & Huynh, 2018; Narita & Inouye, 2014; Toschi, Cardenia, Bonaga, Mandrioli, & Rodriguez-Estrada, 2014). Moreover, different authors have proposed innovative CS reuses and applications, but to the best our knowledge,

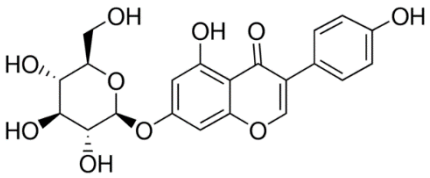
the aroma fraction of this coffee by-product has not been studied yet. Therefore, there are a dearth of investigation focused on CS volatile compounds which could be fascinating for food and food flavour industry. Furthermore, the characterization of odor-active compounds assumes an important role in development of novel foods. Hence, one of our objectives was to characterize the odor-active compounds in CS. For further detail on project aims please refer to chapter 2 (2. Aims of the project).

# 2. Aims of the project

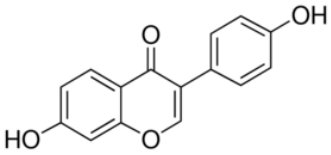
The first part of the project was performed in collaboration with illycaffè S.p.A. (Trieste, Italy) and the objective was to develop analytical methods for quantification of lignans and isoflavones in espresso coffee, R&G coffee and green coffee, aiming at characterizing the contents of these molecules in the whole coffee chain. The analytes were three lignans in glycosidic form (lariciresinol, matairesinol and secoisolariciresinol) and six isoflavones, two of them in glycosidic form (daidzin and genistin) and four aglycons (daidzein, formononetin, genistein and biochanin A). The chemical structures of monitored molecules are shown in **Figure 1**. For this purpose, analytical methods for quantitative analysis of lignans in espresso and R&G coffee and for isoflavones and lignans in green coffee were developed and optimized by using HPLC-MS/MS, operating in "multiple reaction monitoring" (MRM) mode. A previous study (Caprioli et al., 2016), from our research group, quantified isoflavones in R&G and espresso coffee; therefore, we omitted to investigate them in those matrices. The method optimizations concerned also the extraction efficiency evaluation of various procedures. Acid and base hydrolysis, enzymatic hydrolysis, organic solvent extraction, and a combination of these have been studied; the best performing procedures, in term of recovery and quantitative data, were chosen, validated and finally applied to different samples, from various geographical origins.



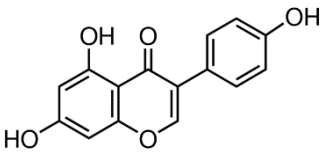
Daidzin



Genistin



Daidzein



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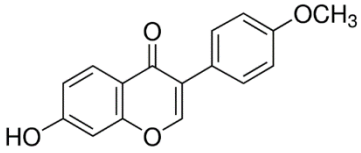
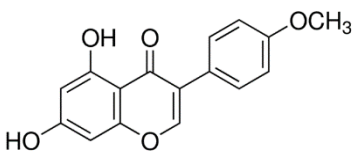
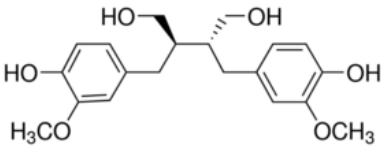


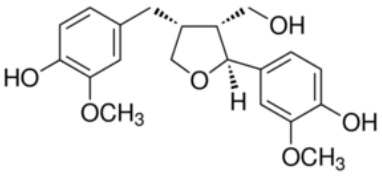
Formononetin



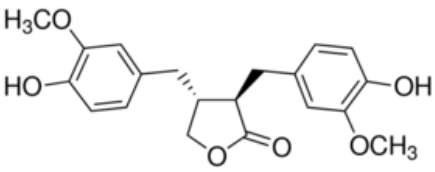
Biochanin A



Secoisolariciresinol



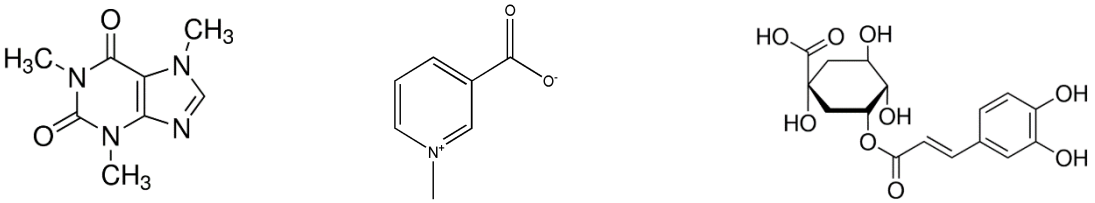
Lariciresinol



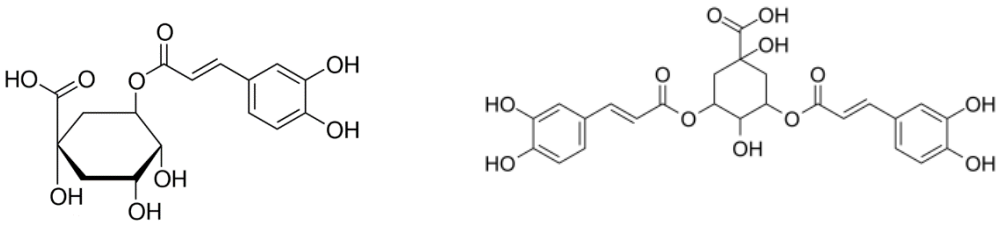
Matairesinol

**Figure 1**. Chemical structures of monitored phytoestrogens in coffee.

The second part of the project was performed together with Simonelli Group S.p.A, one of the founders of "International Hub for Coffee Research and Innovation" (Coffee HUB). The Coffee HUB is an international no-profit research network (organization) who cooperates to study some of the most important topics influencing the quality of coffee. It represents an outcome of the willingness of the industry and the university community to work together and to push forward the bounds of coffee knowledge (International Hub for Coffee Research and Innovation, 2017). This second part of research concerned the optimization of espresso coffee extraction by modifying some machine variables with the scope to produce a good quality espresso coffee, lowering the amount of coffee powder used for obtaining the espresso. The variables under study were the particle size distribution (PSD) of R&G coffee, the design of the filter basket and the height of perforated disc. For this purpose, specific particle size distribution (200-1000 μm) of R&G coffee in three different designed filter baskets (A, B and C) were used to prepare espresso coffee employing standard and lower amount of powder for a double EC extraction (14 and 12 g, respectively). Moreover, various heights of perforated disc (4-7 mm) were assembled into the machine and espresso coffee were extracted with 14 g and 12 g. The EC quality was investigated from chemical point of view by studying and comparing the content of some well-known compounds in coffee, brewed with different conditions. In detail, chemical studies concerned the total solids (TS) analysis and the quantification of caffeine, chlorogenic acids and trigonelline (chemical structures shown in **Figure 2**). In addition, some analysis on volatile fraction were performed, as well.



Caffeine Trigonelline 3-*O*-Caffeoylquinic acid



5-*O*-Caffeoylquinic acid 3,5-*O*-Dicaffeoylquinic acid

**Figure 2**. Chemical structures of target molecules quantified in ECs.

The third part of the project has been carried out at “Leibniz-Institute for Food Systems Biology” at “Technical University of Munich” under the supervision of Dr. Martin Steinhaus. The project focused on promoting and adding value to coffee silverskin (CS), a coffee by-product, in the perspective of its application in the food industry, for instance as an ingredient for novel food production. The work aimed to characterize the odor-active compounds of this coffee by-product and compare with those present in coffee beans. The characterization of aroma fraction was carried out by sniffing the silverskin extract through gas chromatography-olfactometry/flame ionization detector (GC-O/FID) and comparing the odors and odorants with those found in coffee beans. The identification was carried out by comparing with reference compounds the retention index, calculating on two capillary columns, and the odor quality. Finally, for unequivocal identification, samples were submitted to fractionation and then each fraction was injected into comprehensive two-dimensional gas chromatography-mass spectrometry (GCxGC-MS). Moreover, the Aroma Extract Dilution Analysis (AEDA) was performed to assess the potency of each odors/odorants and have an idea which of them were the more important for silverskin aroma.

# 3. Experimental

## 3.1 Lignan and isoflavone in coffee

### 3.1.1 Materials and Standards

Isoflavones, namely, daidzin (analytical standard, $C_{21}H_{20}O_9$, molecular weight 416.38, CAS No 552-66-9), genistin (analytical standard, ≥98%, $C_{21}H_{20}O_{10}$, molecular weight 432.38, CAS No 529-59-9), daidzein (analytical standard, $C_{15}H_{10}O_4$, molecular weight 254.24, CAS No 486-66-8), genistein (analytical standard, $C_{15}H_{10}O_5$, molecular weight 270.24, CAS No 446-72-0), formononetin (analytical standard, $C_{16}H_{12}O_4$, molecular weight 268.26, CAS No 485-72-3) and biochanin A (analytical standard, $C_{16}H_{12}O_5$, molecular weight 284.26, CAS No 491-80-5) and lignans, namely, lariciresinol (LARI, ≥95.0%, $C_{20}H_{24}O_6$, molecular weight 360.40, CAS No 27003-73-2), matairesinol (MAT, ≥95.0%, $C_{20}H_{22}O_6$, molecular weight 358.39, CAS No 580-72-3) and secoisolariciresinol (SECO, ≥95.0%, $C_{20}H_{26}O_6$, molecular weight 362.42, CAS No 29388-59-8) were purchased from Sigma Aldrich (St. Louis, MO, USA). Individual stock solutions of each isoflavone and lignan, at a concentration of 1000 mg $L^{-1}$, were prepared by dissolving pure standard compounds in HPLC grade methanol and storing them in glass-stoppered bottles at 4°C. Afterwards, standard working solutions at various concentrations were prepared daily by appropriate dilution of the stock solution with methanol. HPLC-grade acetonitrile and methanol were supplied by Sigma-Aldrich (Milano, Italy). HPLC-grade formic acid (99%) was obtained from Merck (Darmstadt, Germany). Analytical-grade hydrochloric acid (37%) was obtained from Carlo Erba Reagents (Milan, Italy). Ascorbic acid 99% was purchased from Sigma Aldrich (Steinheim, Germany). All other chemicals were analytical grade. taka-diastase from *Aspergillus oryzae* (~ 100 U/mg), clara-diastase (≥ 35 U/mg), Papain from papaya latex (1.5-10 U/mg), and Protease from *Rhizopus* sp. (≥0.2 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water (> 18 MΩ cm resistivity) was further purified using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All solvents and solutions were filtered through a 0.2 μm polyamide filter from Sartorius Stedim (Goettingen, Germany). Before HPLC analysis, all samples were filtered with Phenex™ RC 4 mm 0.2 μm syringeless filter, Phenomenex (Castelmaggiore, BO, Italy).

### 3.1.2 ESPRESSO AND R&G COFFEE samples and preparations

The espresso coffee samples (total volume 25 ± 1 mL) were prepared using an Iperespresso X7.1 coffee machine (illycaffè spa, Trieste, Italy). The water used for the extraction was a commercial mineral water (Sant'Anna di Vinadio, Cuneo, Italy, total hardness 3.2 French degrees, f). Nine different espresso samples, investigated for lignan content, were supplied by illycaffè spa (Trieste, Italy) in their commercially available capsule forms (Monoarabica™ and Idillyum Iperespresso Capsules). Four of them were 100% *Coffea arabica* L. samples having 4 different geographical origins (Brazil, Colombia, Ethiopia, and India) and available in 2 different production batches (lots 1 and 2) and one from El Salvador (100% *C. arabica* L. var. Bourbon low caffeine (BLC)). The same samples were also analysed as R&G coffee by opening and mixed 10 capsules of each type. Another sixteen 100% *C. arabica* L. samples were provided by illycaffè spa (Trieste, Italy). Six of them were in their commercially available R&G coffee form for use in a mocha pot (Monoarabica™), and came from Brazil, Colombia, Costa Rica, Ethiopia, Guatemala and India. The last ten samples were packaged in compostable foil bags (Goglio spa, Milano, Italy) under inert atmosphere and were available as roasted beans. These coffee samples came from Burundi, El Salvador, Ethiopia, Guatemala, Kenya, Rep. Dominican, Rwanda, Timor-Leste, Uganda and Yemen. Finally, Simonelli Group S.p.A. (Belforte del Chienti, Italy) provided us five roasted bean samples: Le Piantagioni (100% *C. arabica* L.), Le Piantagioni (a blend composed of 85% *C. arabica* L. and 15% *C. canephora* Pierre ex A. Froehner), MonCafè, 100% *C. arabica* L. from Ethiopia and 100% *C. canephora* Pierre ex A. Froehner from Bali. At the end a total of thirty R&G coffee samples were investigated for lignan content. All roasted bean samples were ground in a “Mythos 1” grinder from Simonelli Group S.p.A. (Belforte del Chienti, Italy).

### 3.1.3 GREEN COFFEE samples

A total of 25 green coffee samples were examined on lignan and isoflavone content. Illycaffè SpA (Trieste, Italy) supplied twenty-four different wet-processed *Coffea arabica* L. green bean samples having different geographic origins, i.e., Rwanda (three commercial lots: samples 1, 2 and 3), Costa Rica (three commercial lots: samples 1, 2 and 3), Brazil (four commercial lots: samples 1, 2, 3 and

4), Ethiopia (four commercial lots: samples 1, 2, 3 and 4), India (four commercial lots: samples 1, 2, 3 and 4), Colombia (three commercial lots: samples 1, 2 and 3) and Nicaragua (three commercial lots: samples 1, 2 and 3), Simonelli Group S.p.A. (Belforte del Chienti, Italy) provided one 100% *C. canephora* Pierre ex A. Froehner (robusta) green coffee sample from Bali (Indonesia).

### 3.1.4 Lignan extraction process: ESPRESSO COFFEE

For espresso coffee (EC) a multitude of extraction methods, which can be classified under: "dilute and shoot" (Method 1-EC), acid hydrolysis (Method 2-EC) and enzymatic digestion (Method 3-EC), have been evaluated and compared among them (as shown in **Table 1**). Method 1-EC (not shown in **Table 1**) was characterized by two "dilute and shoot" procedures at 2 diluting levels. In the first, EC samples were diluted 10 times with 0.1% formic acid in water, whereas the second was obtained diluting EC sample 5 times with 0.1% formic acid in water. Afterwards, samples were centrifuged at 13300 rpm for 10 minutes and filtered with a 0.2 μm syringeless filter before HPLC-MS/MS injection. In Method 2-EC was composed of 11 acid hydrolysis performed varying the acid normality, the ratio of sample to acid, the time, and the temperature of hydrolysis. For instance, Method 2.1-EC of acid hydrolysis (**Table 1**) was performed by adding 1 mL of 2 N HCl to 2 mL of EC, after ascorbic acid addition as antioxidant, and keeping the sample at 80 °C for 1 hour in a water bath, under magnetic stirring. After cooling, the pH was raised to 5 and, before HPLC-MS/MS analysis, sample was centrifuged at 13300 rpm for 10 minutes and filtered with a 0.2 μm syringeless filter. Methods 3-EC were characterized by various enzymatic digestions which employed four enzymes (taka-diastase, clara-diastase, papain from papaya latex, and protease from *Rhizopus* sp.). Taka-diastase is an α-amylase enzyme derived from *Aspergillus oryzae* and clara-diastase is a mixture of enzymes that contains α-amylase, cellulase, invertase, peptidase, phosphatase and sulphatase. The enzyme solutions or dispersions were prepared dissolving exact amount of enzyme powder in water or, for the protease, in 0.001 N of HCl, according to Sigma-Aldrich data sheet, the pH optimum for that enzyme was 3. The enzymatic hydrolysis was carried out adding 0.5 mL of enzyme solution to 2 mL of EC, after ascorbic acid addition as antioxidant and keeping the sample at 25 °C for 3 hours in a

water bath, under magnetic stirring (**Table 1, Methods from 3.1-EC to 3.8-EC**). Afterwards, sample was centrifuged at 13300 rpm for 10 minutes and filtered with a 0.2 μm syringeless filter and then injected into HPLC-MS/MS. Regarding enzymatic extraction with protease, before centrifugation, the sample pH was raised to 5. To optimize the enzymatic hydrolysis procedures, two concentration levels for each enzyme have been tested varying also the temperature and the time of digestion. In this manner, a great amount of different enzymatic hydrolysis procedures, reported in **Table 1**, have been evaluated.

**Table 1**. Acid and enzymatic hydrolysis procedures for the extraction of studied lignans from EC.

| Acid hydrolysis with HCl [a] | Concentration (N) | Ratio of sample to acid (S/A) | Time (h) | Temperature (°C) |
|---|---|---|---|---|
| Method 2.1-EC | 2 | 2 | 1 | 80 |
| Method 2.2-EC | 5 | 2 | 1 | 80 |
| Method 2.3-EC | 1 | 2 | 1 | 80 |
| Method 2.4-EC | 2 | 0.5 | 1 | 80 |
| Method 2.5-EC | 2 | 1 | 1 | 80 |
| Method 2.6-EC | 2 | 2 | 2 | 80 |
| Method 2.7-EC | 2 | 2 | 1 | 50 |
| Method 2.8-EC | 2 | 2 | 1 | 25 |
| Method 2.9-EC | 5 | 0.5 | 1 | 80 |
| Method 2.10-EC | 5 | 2 | 1 | 50 |
| Method 2.11-EC | 5 | 2 | 1 | 25 |
| **Enzymatic digestion** | **Enzyme type** | **Concentration % (w/v)** | **Time (h)** | **Temperature (°C)** |
| Method 3.1-EC | taka-diastase | 1 | 3 | 25 |
| Method 3.2-EC | clara-diastase | 5 | 3 | 25 |
| Method 3.3-EC | papain | 2.5 | 3 | 25 |
| Method 3.4-EC | protease [a] | 1.25 | 3 | 25 |
| Method 3.5-EC | taka-diastase | 2 | 3 | 25 |
| Method 3.6-EC | clara-diastase | 10 | 3 | 25 |
| Method 3.7-EC | papain | 5 | 3 | 25 |
| Method 3.8-EC | protease [a] | 2.5 | 3 | 25 |
| Method 3.9-EC | taka-diastase | 2 | 3 | 37 |
| Method 3.10-EC | clara-diastase | 10 | 3 | 37 |
| Method 3.11-EC | papain | 5 | 3 | 37 |
| Method 3.12-EC | protease [a] | 2.5 | 3 | 37 |
| Method 3.13-EC | taka-diastase | 2 | 6 | 37 |
| Method 3.14-EC | clara-diastase | 10 | 6 | 37 |
| Method 3.15-EC | papain | 5 | 6 | 37 |
| Method 3.16-EC | protease [a] | 2.5 | 6 | 37 |

[a] Before centrifugation pH samples have been raised to 5 with KOH.

### 3.1.5 Lignan extraction process: R&G COFFEE

Different types of extraction methods were tested: acid extractions (strong and mild acid extraction), methanolic extractions (100% methanol and acid methanolic extraction) and enzymatic digestions (aqueous enzymatic mixture and water/methanol enzymatic mixture), as following described.

#### 3.1.5.1 Acid extraction

The strong acid extraction (Method 1-R&G) was carried out by adding 5 ml of 2 M HCl together with 1% (w/v) of ascorbic acid as antioxidant, to 0.5 g of ground coffee. Afterwards, the sample was kept at controlled temperature (80 °C) for 1 h in a water bath with thermostat control under magnetic stirring. After cooling at room temperature, the pH of the sample was raised to 5 with 10 M KOH and, prior to HPLC injection, the sample was centrifuged at 5000 rpm for 10 min and the supernatant was filtered with a 0.2 μm syringeless filter. The mild acid extraction (Method 2-R&G) was performed with the same steps as the strong acid extraction, but 5 ml of an acetic acid/sodium acetate buffer solution was added to the ground coffee, rather than the 5 ml of 2 M HCl used in Method 1-R&G, in order to obtain a pH of 5.

#### 3.1.5.2 Methanolic extraction

Methanolic extraction (Method 3-R&G) was carried out by adding 5 ml of methanol to 0.5 g of ground coffee and adding 1% (w/v) of ascorbic acid as antioxidant. Afterwards, the sample was sonicated at room temperature for 2.5 h. We also tested a fourth method, using an acid methanolic solution composed of 2 M HCl and MeOH (50:50) as extraction solvent. This acid methanolic extraction (Method 4-R&G) was performed adding 5 ml of that solution to coffee and sonicating the mixture at room temperature for 2.5 h. For both processes, the sample was prepared for HPLC-MS/MS analysis by centrifugation at 5000 rpm for 10 min; the supernatant was then collected and filtered with 0.2 mm syringeless filter.

#### 3.1.5.3 Enzymatic digestions

Two different types of enzyme, taka-diastase (Method 5-R&G) and clara-diastase (Method 6-R&G), were evaluated. For both enzymes, the digestion process was performed by adding 2 ml of enzymatic

mixture at 2% (w/v) and 20 mg of ascorbic acid as antioxidant to 0.2 g of ground coffee. The digestion was obtained by keeping the sample at controlled temperature (37 °C) for 3 h in a water bath under magnetic stirring. As in the previously described methods, the sample was centrifuged at 5000 rpm for 10 minutes and the supernatant was collected and filtered before analysis. In Method 7-R&G, we sought to enhance the extraction of lignans by using both enzyme and methanol. Specifically, we carried out the same enzymatic digestions but using 2 ml of a taka-diastase suspension prepared by dissolving an exact amount of enzymatic powder in a methanol and water solution (50:50) to obtain a concentration of 2% (w/v). A summary of extraction processes is reported on **Table 2**.

**Table 2**. Different tested methods for lignan extraction from R&G coffee.

| **Extraction processes** | **Acid hydrolysis** | | **Methanolic extraction** | | **Enzymatic digestion** | | |
|---|---|---|---|---|---|---|---|
| | **Method 1-R&G** | **Method 2-R&G** | **Method 3-R&G** | **Method 4-R&G** | **Method 5-R&G** | **Method 6-R&G** | **Method 7-R&G** |
| **Solvent** | HCl in $H_2O$ | $CH_3COOH$/ $CH_3COONa$ | MeOH | HCl in $H_2O$/MeOH | taka-diastase in $H_2O$ | clara-diastase in $H_2O$ | taka-diastase in $H_2O$/MeOH |
| **Concentration** | 2[a] | 5[b] | / | 1[a] | 2[c] | 2[c] | 2[c] |
| **Temperature of extraction (°C)** | 80 | 80 | 25 | 25 | 37 | 37 | 37 |
| **Time of extraction (min)** | 60 | 60 | 150 | 150 | 180 | 180 | 180 |

[a] Concentration expressed as (M); [b] value of pH; [c] concentration expressed as (% w/v).

### 3.1.6 Sample preparation and extraction procedures: lignans and isoflavones in GREEN COFFEE

Coffee samples were ground in an Ariete Blendy 570 grinder (Florence, Italy). Homogeneous portions of 100% *C. canephora* sample were used for development and optimization of the method. We evaluated different extraction procedures, namely, acid and base hydrolysis, enzymatic digestions, organic solvent extraction and a combination of these, as following described, and chose the best one for lignan and isoflavone quantification in different green coffee samples.

#### 3.1.6.1 Acid and base hydrolysis

Three different extraction processes, namely acid hydrolysis, base hydrolysis and base hydrolysis in MeOH, were chosen for their likely capacity to break glycosidic and ester bonds (Mortensen et al., 2009; Smeds et al., 2007). Acid hydrolysis (Method 1-GC) was performed by adding 6 mL of HCl 0.1 M and 60 mg of ascorbic acid (as antioxidant) to 0.5 g of green coffee powder. The hydrolysis was performed while keeping the sample at a controlled temperature of 80 °C for 30 min in a water bath under magnetic stirring. The sample was cooled at room temperature, its pH was adjusted to 5-6 by adding KOH 4 M, and then it was stored overnight at -18 °C to precipitate soluble polysaccharide and protein fraction. Later, the sample was thawed at room temperature and centrifuged at 5000 rpm for 10 min. Finally, before HPLC-MS/MS injection, an aliquot of supernatant was collected, centrifuged at 13000 rpm for 15 min and filtered with a 0.2 μm syringeless filter. Base hydrolysis (Method 2-GC) was carried out by adding 3 mL of KOH 0.1 M and 30 mg of ascorbic acid to 0.3 g of green coffee powder and keeping the sample at a controlled temperature of 80 °C for 30 min in a water bath under magnetic stirring. Afterwards, the sample was cooled at room temperature, and the pH was brought to 5-6 by adding HCl 2 M. After storage overnight at -18 °C, the sample was melted, then centrifuged at 5000 rpm for 10 min, and an aliquot of supernatant was collected and centrifuged at 13000 rpm for 15 min. Before analysis, the sample was filtered with a 0.2 μm syringeless filter. With the purpose of enhancing the extraction yield, we carried out the same process of Method 2-GC but used KOH 0.1 M dissolved in MeOH (Method 3-GC) instead of water.

### 3.1.6.2 Enzymatic digestions

Two different types of enzymes, taka-diastase and clara-diastase, were evaluated for their ability to extract the compounds of interest from the coffee matrix. Hydrolysis with taka-diastase (Method 4-GC) was performed by adding 4 mL of enzyme mixture at 2% (w/v) and 40 mg of ascorbic acid to 0.5 g of green coffee powder, followed by digestion in a water bath at a controlled temperature of 37 °C for 2 h, under magnetic stirring. After cooling at room temperature, the sample was stored overnight at -18 °C and, afterwards, was melted at room temperature and centrifuged at 5000 rpm for 10 min. Before HPLC analysis, an aliquot of the liquid part was centrifuged at 13000 rpm for 15 min and filtered with a 0.2 μm syringeless filter. Hydrolysis with clara-diastase (Method 5-GC) was carried out adding 2 mL of enzyme at 5% (w/v) together with 30 mg of ascorbic acid to 0.3 g of green coffee powder, after which the sample was kept in a water bath at a controlled temperature of 37 °C for 2 h, under magnetic stirring. After cooling at room temperature, 1 mL of MeOH was added and the sample was vortexed for 1 min. Afterwards, the sample was stored overnight at -18 °C and, after melting at room temperature, it was centrifuged at 5000 for 10 min. Before injection into the HPLC-MS/MS, an aliquot of supernatant was collected, centrifuged at 13000 rpm for 15 min and filtered with 0.2 μm syringeless filter.

### 3.1.6.3 Methanolic extraction

Methanolic process (Method 6-GC) was structured in the same manner as acid hydrolysis. Briefly, 4 mL of a methanol:water (85:15) solution together with 40 mg of ascorbic acid were added to 0.5 g of green coffee powder and the sample was kept in a water bath at a controlled temperature of 80 °C, for 30 min under magnetic stirring. After storing overnight at -18 °C, the sample was kept at room temperature to thaw it and then centrifuged at 5000 rpm for 10 min. Before quantification through HPLC-MS/MS, an aliquot of supernatant was collected, centrifuged at 13000 rpm for 15 min and filtered with a 0.2 μm syringeless filter.

### 3.1.6.4 Combination processes

Two other processes, Method 7-GC and Method 8-GC combined the two extractions previously tested. Method 7-GC was performed by adding 2 mL of 0.1 M KOH dissolved in MeOH and 30 mg of ascorbic acid to 0.3 g of green coffee powder and keeping the sample in a water bath at a controlled temperature of 80 °C for 30 min. After cooling at room temperature, the pH was adjusted to 6 by adding 2 M HCl and enzymatic digestion was carried out by adding 1 mL of clara-diastase 10% (w/v) and then keeping the sample at 37 °C for 2 h. Thus, the sample was centrifuged at 5000 rpm for 10 min and stored overnight at -18 °C. Finally, after melting, an aliquot of supernatant was collected, centrifuged at 13000 rpm for 15 min, filtered with a 0.2 μm syringeless filter and injected into the HPLC. Method 8-GC involved acid hydrolysis followed by enzymatic digestion. The acid extraction was carried out adding 2 mL of 0.1 M HCl together with 30 mg of ascorbic acid to 0.3 g of green coffee powder and keeping the sample at 80 °C for 30 min. After cooling, the pH was raised to 6 by adding 2 M KOH and after that, enzymatic digestion was performed, as described above for Method 7-GC. A summary of extraction processes is reported on **Table 3**.

**Table 3.** Different processes for lignan and isoflavone extraction from green coffee.

| **Extraction processes** | *Acid and base hydrolysis* | | | *Enzymatic digestions* | | *Methanolic extraction* | *Combination processes* | |
|---|---|---|---|---|---|---|---|---|
| | **Method 1-GC** | **Method 2-GC** | **Method 3-GC** | **Method 4-GC** | **Method 5-GC** | **Method 6-GC** | **Method 7-GC** | **Method 8-GC** |
| **Solvent** | HCl in $H_2O$ | KOH in $H_2O$ | KOH in MeOH | taka-diastase in $H_2O$ | clara-diastase in $H_2O$ | MeOH:$H_2O$ | a)KOH in MeOH b)clara-diastase in $H_2O$ | a)HCl in $H_2O$ b)clara-diastase in $H_2O$ |
| **Concentration** | 0.1[a] | 0.1[a] | 0.1[a] | 2[b] | 5[b] | 85[c] | a)0.1[a] b)10[b] | a)0.1[a] b) 10[b] |
| **Temperature of extraction (°C)** | 80 | 80 | 80 | 37 | 37 | 80 | a)80 b)37 | a)80 b)37 |
| **Time of extraction (min)** | 30 | 30 | 30 | 120 | 120 | 30 | a)30 b)120 | a)30 b)120 |

[a] Concentration expressed as (M); [b] concentration expressed as (% w/v); [c] concentration expressed as (% v/v).

### 3.1.7 HPLC-MS/MS parameters for lignan analysis in ESPRESSO AND R&G COFFEE

HPLC-MS/MS studies were performed using an Agilent 1290 Infinity Series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an ESI source operating in negative ionization mode. The separation of lignans was achieved on a Kinetex C18 analytical column (50 mm × 2.10 mm i.d., 2.6 μm) from Phenomenex (Castel Maggiore, Bologna, Italy). The mobile phase for HPLC-MS/MS analyses was composed by water (A) and HPLC-grade acetonitrile (B) both with 0.1% formic acid. The separation was obtained flowing at 0.4 mL/minute with this gradient elution: 0 minute (15% B), 5 minutes (40% B), 8 minutes (15% B) and then constant until the end of the run (10 minutes). Before use, all solvents and solutions were filtered through a 0.2 μm polyamide filter from Sartorius Stedim (Goettingen, Germany). The injection volume was 2 μL. The temperature of the column was 30 °C, and the temperature of the drying gas in the ionization source was 350 °C. The gas flow was 11 L/minute, the nebulizer pressure was 50 psi, and the capillary voltage was 4000 V. Detection was performed by electrospray ionization (ESI)-MS in the MRM mode. The MRM peak areas were integrated for quantification. To enhance the sensitivity, the acquisition time was divided into 2 periods. The most abundant product ions were used for quantitation, and the rest of the product ions were used for qualification. The selected ion transitions and the settings of the mass analyser are reported in **Table 4**.

**Table 4**. HPLC-MS/MS acquisition parameters (MRM mode) used for the analysis of the target lignans.

| Compounds | Time window (min) | Precursor ion (*m/z*) | Product ion (*m/z*) | Fragmentor (V) | Collision energy (V) | Retention time (min) | Polarity |
|---|---|---|---|---|---|---|---|
| **SECO** | 1.5-3.0 | 361 | 165[a]<br>121 | 135 | 21<br>37 | 2.23 | Negative |
| **LARI** | 1.5-3.0 | 359 | 329[b]<br>160 | 89 | 5<br>37 | 2.45 | Negative |
| **MAT** | 3.0-end | 357 | 83[c]<br>122 | 120 | 21<br>29 | 3.80 | Negative |

[a, b, c] These product ions were used for the quantification; the rest of the product ions were used for confirmatory analysis.

### 3.1.8 HPLC-MS/MS parameters for lignan and isoflavone analysis in GREEN COFFEE

HPLC-MS/MS studies were performed using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an electrospray ionization (ESI) source operating in negative and positive ionization mode. The separation of target compounds was achieved on a Kinetex C18 analytical column (50 mm x 2.10 mm i.d., 2.6 μm) from Phenomenex (Castel Maggiore, Bologna, Italy). The mobile phase for HPLC-MS/MS analyses was a mixture of 85% water (A) and 15% HPLC-grade acetonitrile (B), both with 0.1% formic acid. The separation was obtained by flowing at 0.4 mL/min with this gradient elution: 0 min (15% B), 5 min (40% B), 8 min (15% B) and then constant until the end of the run (10 min). All solvents and solutions were filtered through a 0.2 μm polyamide filter from Sartorius Stedim (Goettingen, Germany). The injection volume was 2 μL. The temperature of the column was 30 °C and the temperature of the drying gas in the ionization source was 350 °C. The gas flow was 11 L/min, the nebulizer pressure was 50 psi and the capillary voltage was 4000 V. Detection was performed in the "multiple reaction monitoring" (MRM) mode. The MRM peak areas were integrated for quantification. To enhance the sensitivity, the acquisition time was divided into three periods. The most abundant product ion was used for quantitation, and the rest of the product ions were used for qualification. The selected ion transitions and the mass spectrometer parameters are reported in **Table 5**.

### 3.1.9 Principal Component Analysis (PCA)

Chemical differences among the various green coffee samples, in terms of lignan and isoflavone contents, were analysed by PCA using the statistical software STATISTICA v.7.1 (Stat Soft Italia S.r.l., Vigonza, Italy). For the purpose, a covariance matrix composed of 25 coffee samples and 8 variables (200 data) was built and eigenvalues were calculated.

**Table 5**. HPLC-MS/MS acquisition parameters (MRM mode) for determination of lignans and isoflavones.

| Compounds | Time window | Precursor ion (*m/z*) | Product ion (*m/z*) | Fragmentor (V) | Collision energy (V) | Retention time (min) | Polarity |
|---|---|---|---|---|---|---|---|
| **Daidzin** | 0-3.2 | 417 | 255[a]<br>199 | 102 | 13<br>45 | 1.03 | Positive |
| **Genistin** | 0-3.2 | 433 | 271[a]<br>91 | 100 | 13<br>77 | 1.63 | Positive |
| **Secoisolariciresinol** | 0-3.2 | 361 | 165[a]<br>121 | 135 | 21<br>37 | 2.23 | Negative |
| **Lariciresinol** | 0-3.2 | 359 | 329[a]<br>160 | 89 | 5<br>37 | 2.45 | Negative |
| **Daidzein** | 0-3.2 | 253 | 208[a]<br>132 | 155 | 29<br>41 | 2.70 | Negative |
| **Matairesinol** | 3.2-4.2 | 357 | 83[a]<br>122 | 120 | 21<br>29 | 3.68 | Negative |
| **Genistein** | 3.2-4.2 | 269 | 159[a]<br>133 | 144 | 25<br>31 | 3.80 | Negative |
| **Formononetin** | 4.2-end | 267 | 252[a]<br>233 | 112 | 10<br>26 | 4.57 | Negative |
| **Biochanin A** | 4.2-end | 283 | 268[a]<br>239 | 135 | 17<br>33 | 6.05 | Negative |

[a] These product ions were used for quantitative analysis; the rest for confirmatory analysis.

## 3.2 Optimization of espresso coffee extraction

### 3.2.1 Materials and Standards

Standards of caffeine, trigonelline, 5-*O*-caffeoylquinic acid (5-CQA), 3-*O*-caffeoylquinic acid (3-CQA) and 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA) were purchased from Sigma-Aldrich (Milano, Italy). Divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS) Stable Flex fiber of 50/30 μm was acquired (Supelco, USA) and used for the HS-SPME. HPLC-grade methanol was supplied by Sigma-Aldrich (Milano, Italy) and HPLC-grade formic acid (99%) was supplied by Merck (Darmstadt, Germany). Individual stock solutions of caffeine, trigonelline, 5-CQA, 3-CQA and 3,5-diCQA were prepared by dissolving 10 mg of each compound in 10 ml of methanol (HPLC-grade, 99.9%; Sigma-Aldrich, Milano, Italy) and stored in glass-stoppered bottles at 4°C. Standard working solutions, at various concentrations, were daily prepared by appropriate dilution of aliquots of the stock solutions in methanol. Deionized water (> 8 MΩ cm resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA). All solvents and solutions were filtered through a 0.45 μm polyamide filter from Sartorius Stedim (Goettingen, Germany) before use. Before HPLC analysis, all samples were filtered with Phenex™ RC 4 mm 0.45 μm syringeless filter, Phenomenex (Castelmaggiore, BO, Italy).

### 3.2.2 Coffee samples and coffee bean preparation

Coffee beans, i.e., 100% *Coffea arabica* L. (arabica) from "Le Piantagioni del Caffè srl" (Livorno, Italy) roasting company, supplied by Simonelli Group S.p.A., have been used for the research project. The coffee, of certified geographical origin from America, was suggested by certified roasters for EC preparation. From now on, the coffee cultivar is simply referred to as arabica. The arabica beans were kept under sealed packages at room temperature and were opened just before grinding. Coffee beans were milled between fine and medium sizes using a Mythos 1 grinder from Simonelli Group S.p.A. (Belforte del Chienti, Italy) and were separated by AS 200 Control vibrational sieve machine from Retch (Germany), using various sieve plates. The grinded coffee was separated into sieve plates with

200-300 μm, 300-400 μm, 400-500 μm and 500-1000 μm. A Crystal Series analytical scale from Gibertini, (Italy) was used to weigh 12 and 14 g of separated ground coffee.

### 3.2.3 Particle size analysis

Coffee beans, after passing comminution process, were analysed by Mastersizer 3000 Aero Series dry dispersion unit (Malvern PANalytical Ltd., UK), which uses a laser diffraction to measure the size of particles (from 0.01 to 3500 μm). The instrument operates with continuous air flow, generated by industrial compressor at 6.5 bar, that penetrates into Aero dry dispersion unit, which transfers the particles at 2-3 bar to laser diffraction. In this way, the particles move in laminar flow and the vacuum extraction unit (KARCHER Professional NT 45/1 Tact, Germany) removes samples from Aero dry. The grinded and separated coffee powder with various particle sizes were collected: a portion for Mastersizer 3000 and the rest for extraction of espresso coffee. The size of particles for each sample were examined in fivefold and the mean value was used for comparison.

### 3.2.4 Espresso coffee preparation

Espresso coffee was extracted using a VA388 Black Eagle espresso coffee machine from Victoria Arduino (Simonelli Group S.p.A., Italy). Preliminarily, for each filter basket (A, B and C), the grinding machine has been tuned and calibrated to obtain optimal EC (for two espresso: 14±0.01 g in filter basket, 50 ± 2 ml in cup and 25 ± 1 s of extraction) without separating ground coffee by sieves. After calibration, grinded coffee was separated by a vibrational sieve. Separated microns (200-300 μm, 300-400 μm, 400-500 μm and 500-1000 μm) and weights (12 and 14 g) of ground coffee were transferred in three different filter baskets (A: standard, around 300 μm sized; B: 180 μm sized filter basket; C: net designed on the boundary of filter basket) to prepare different espresso coffee samples which were analysed for content of TS, bioactive compounds and aroma compounds. To prepare the EC samples, the machine was set at the following conditions: 25 s, 93 °C, 9 bars and 5 mm of perforated disc.

Later on, the influence of different perforated disc heights was evaluated using standard filter basket (A). After calibration, the EC extraction was performed using 12 and 14 g of ground coffee by

modifying the height of the perforated disc (4, 5, 6 and 7 mm). The perforated disc was assembled under the shower to adjust the distance between the coffee cake and the shower. The extraction of ECs for each perforated disc (4-7 mm with 12 and 14g) was implemented in two ways: first, the extraction time was kept constant and second, the extracted volume was maintained constant. These parameters were automatically adjusted by the program of the espresso machine (25 ± 1 seconds and 50 ± 2 ml for two espressos). The utilized water was the same production batch of a minimally mineralized water (Blues, Acqua Minerale Naturale, minimamenta mineralizzata, Italy). This water is commercially available and its mineral contents was: total solids at 180 °C (22.0 mg/L); $HCO_3^-$ (9.5 mg/L); $Ca^{2+}$ (2.8 mg/L); $Mg^{2+}$ (0.45 mg/L); $SiO_2$ (7.3 mg/L); $NO_3^-$ (1.0 mg/L); $Na^+$ (1;8 mg/L); $SO_4^{2-}$ (3.6 mg/L); $Cl^-$ (0.21 mg/L); $K^+$ (0.20 mg/L); $F^-$ (< 0.10 mg/L). All extracted EC samples were performed in triplicate and immediately collected from the portafilter of the espresso machine in a ceramic espresso cup, and the weight of the extracted EC samples was measured by Hario and Acaia balance.

### 3.2.5 Total solids (TS)

TS were measured by following a developed procedure (Caprioli et al., 2012; Parenti et al., 2014), with some modification. Briefly, 1 ml of espresso coffee was collected and oven-dried until constant weight was reached (12 h, 100 ± 2 °C). TS was defined as ratio between dry coffee residue and the volume of EC (w/v) expressed in mg $mL^{-1}$.

### 3.2.6 Analysis of caffeine, chlorogenic acids and trigonelline

The analysis of caffeine, trigonelline and chlorogenic acids were performed following previous developed methods (Caprioli et al., 2014, 2013). 1 mL of espresso coffee was diluted 50 times in mobile phase and an aliquot of supernatant was collected and centrifuged at 13300 rpm for 10 min. Before HPLC-variable wavelength detector (VWD) injection, the sample was filtered using a 0.45 μm syringeless filter. For caffeine and trigonelline analysis the analytical column was a Gemini C18 110 Å (250 x 3 mm I.D., 5 μm, Phenomenex, Cheshire, U.K.). The mobile phase was composed of water with 0.3% of formic acid (A) and methanol (B). The flow rate was 0.4 mL $min^{-1}$ with this

gradient elution: 0 min, 25% B; 0–10 min, 60% B; 10–15 min, 60% B; 15–20 min, 25% B; and B was kept constant until the end of the run (25 min). The injection volume was 10 µL and HPLC-VWD experiments were carried out using a Hewlett Packard (Palo Alto, CA, USA) HP-1090 Series II, made of an autosampler and a binary solvent pump, equipped with a variable wavelength detector (VWD). HPLC-VWD analyses were performed at two different wavelengths in the same run: 265 nm for trigonelline and 270 nm for caffeine. The quantification of chlorogenic acids, such as 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA) and 3,5-*O*-dicaffeoylquinic acid (3,5-diCQA), was carried out through the same instrument but using as analytical column a Polar-RP 80 Å (150 x 4.6 mm I.D., 4 µm) from Phenomenex (Chesire, U.K.). The mobile phase was composed of water (A) and methanol (B), both containing 0.1% of formic acid and the flow rate was 1 mL $min^{-1}$. The solvent composition varied from 0-5.5 min: 25% B (v/v); 5.5-8 min: 50% B (v/v); 8-13.5 min: 50% B (v/v); 13.5-18 min: 25% B (v/v). The injection volume was 5 µL. HPLC-VWD analysis were performed monitoring 325 nm for all chlorogenic acids.

**3.2.7 Analysis of volatile compounds**

Just after brewing, 2 ml of EC was placed in 20 mL screw top vials for the analysis of volatile compounds using headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GS-MS). The instrument was equipped with PAL3 auto sampler system. DVB/CAR/PDMS fiber (Sigma Aldrich, Milan, Italy; 100 µm thickness) was used according to previous works (Bressanello et al., 2017; Caprioli et al., 2012; Risticevic et al., 2008). The vial was tightly screwed on magnetic cap with PTFE-silicon septum and the system was set for automatic functioning mode. The sample was placed into a stirrer, incubated at 60° C, and stirred at 250 rpm for 20 mins. Then, HS-SPME automatically was inserted in the sample and remained 20 mins for the extraction. After adsorption, HS-SPME automatically injected the analytes into the gas-chromatographic system. A desorption time of 5 min was sufficient to desorb analytes from the fiber. Cleaning was automatically performed with PAL system by inserting the fiber in the conditioning port at 230° C for 20 min after each process. A gas chromatograph/mass selective detector (GC/MSD

– Agilent, Santa Clara, CA, USA, Agilent 7890B GC Hardware with Agilent 5977 Series MSD and Mass Hunter GC/MSD Data Acquisition) was used. The column used for separation was DB-WAX (0.25 mm x 60 m x 0.25 µm - Agilent 122-7062, CA, USA). The workstation in the GC-MS system was an Agilent Chem. The flow rate (He) was 1.2 mL $min^{-1}$ under splitless mode. The temperature of the injector was 260 °C. The temperature for the column was programmed: from 35 °C (4 min) to 120 °C (2.5 °C per min), and from 120 °C to 250 °C (15 °C per min); then, 240 °C for 3.33 min remained plateau and the total run time was 49.3 min. Data were acquired through the electron impact (EI) mode and full-scan acquisition mode by monitoring from 25 to 500 m/z. Each sample was injected three times and values were expressed as the means of three replicates.

### 3.2.8 Statistical analysis

Data on selected volatile compounds were examined by principal component analysis (PCA) using Statistica v.7.1 (Stat Soft Italia, Vigonza, Italy). PCA was applied in order to visualize information at various particle sizes that were used for EC extraction in three filter baskets.

## 3.3 Studies on coffee silverskin

### 3.3.1 Materials and Standards

Reference compounds were purchased from Sigma Aldrich (Taufkirchen, Germany), Alfa Aesar (Karlsruhe, Germany), Symrise (Holzminden, Germany) and Merck (Darmstadt, Germany). Dichloromethane, diethyl ether, and pentane were freshly distilled before use. Silica gel 60 (0.040−0.063 mm) was purchased from VWR (Darmstadt, Germany) and purified as detailed in a previous work (Steinhaus, 2015). Mercurated agarose gel was prepared from Affi-Gel 10 (Bio-Rad, Munich, Germany) ("Bio-Rad Laboratories, Munich, Germany," n.d.). All other chemicals were analytical grade.

### 3.3.2 Coffee samples and volatile isolation

Coffee silverskin (CS) and coffee bean (CB) samples, namely 100% C*offea arabica* L. var. Catuai Rosso coming from Naranjo, Santa Cruz region, (Guatemala) were provided by Perfero Caffè (Altidona, Italy) roasting company. The coffee berries were submitted to natural method and dried for 24 days in African bed. About 200 g of coffee silverskin were collected from 20 kg of green coffee after the roasting process. Samples were kept in vacuum sealed bags at -20 °C.

Just before the extraction process, CS was immersed in liquid nitrogen and milled by GM 200 Retsch GrindoMix (time: 10 s; speed: 4000 rpm; in both rotation direction). CB was processed into a powder through 6875 Freezer/Mill High Capacity Cryogenic Grinder (SPEX SamplePrep, Stanmore, UK) using following program: pre-cool, 2 min; run time, 1 min; cool time, 1 min; cycle, 3; rate, 14 cps. The volatile compounds in 20 g of CS and CB powder were extracted with 250 ml of dichloromethane under stirring at room temperature for 2 h. After filtration with filter paper, the volatile compounds were removed from the extract by Solvent Assisted Flavour Evaporation (SAFE) at 40 °C (Engel, Bahr, & Schieberle, 1999). The SAFE distillate was dried by adding anhydrous sodium sulfate and concentrated to 1 mL by using a Vigreux column (50 x 1 cm) and then a Bemelmans microdistillation device (Neiens & Steinhaus, 2019). The concentrated volatile extracts were kept at -20 °C and the odor evaluation of a small amount of CS and CB extracts using fragrance test strips demonstrated the aroma equivalence to starting materials.

### 3.3.3 GC-O/FID

A Trace GC Ultra gas chromatograph (Thermo Scientific, Dreieich, Germany) was equipped with a cold-on-column injector, a flame ionization detector (FID) and a tailor-made sniffing port (Steinhaus, Sinuco, Polster, Osorio, & Schieberle, 2008). Two types of fused silica columns were used for volatile separation: a) DB-FFAP (30 m x 0.32 mm i.d., 0.25 µm film thickness); b) DB-5 (30 m x 0.32 mm i.d., 0.25 µm film thickness) (both Phenomenex, Aschaffenburg, Germany). The carrier gas was helium (He) at 60 KPa (DB-FFAP) and 65 KPa (DB-5) and the initial temperature of the oven was 40 °C ( 2 min) and ramped at 6 °C/min to 230 °C (DB-FFAP) and to 240 °C (DB-5). The temperature was then maintained constant for 5 min (both columns). The end of the analytical column was connected to a deactivated Y-shaped glass splitter which divided the column effluent in two equal parts that were directed via deactivated fused silica capillaries (50 cm x 0.25 mm i.d.) to the FID (250 °C) and the sniffing port (230 °C), respectively. The injection volume was 1 µL. A trained person, during the GC-O analysis, placed the nose in the region above the top of the sniffing port and evaluated the odor effluent. The positions and the descriptions of the odors were marked on the FID chromatogram registered by a recorder. On both columns, a linear retention index (RI) of each odor was calculated from their retention times and the retention times of adjacent n-alkanes by linear interpolation.

### 3.3.4 AEDA

The concentrated volatile extracts of CS and CB were injected into GC-O/FID. The GC-O/FID analysis were carried out by three different trained and experienced sniffers (two males, one female; age 26−40) using DB-FFAP column as well as DB-5 column. The training consisted in weekly sensory evaluation sessions of reference odorants dissolved in water and the evaluation of reference mixtures by GC-O analysis. Each sniffer repeated the analysis until data was reproducible. Aroma Extract Dilution Analysis (AEDA) was performed by stepwise diluting, with dichloromethane (1:2, 1:4, 1:8, 1:16, 1:32, etc), the coffee concentrated volatile extracts. Each diluted sample was then injected to GC-O/FID using DB-FFAP column. A flavour dilution (FD) factor was assigned to each

odor-active compound, representing the dilution factor of the highest diluted sample in which the odorant was detected during GC-O/FID analysis by any of the three sniffers.

### 3.3.5 Fractionation of Coffee Silverskin and Coffee Bean volatiles

The fractionation of volatile extracts was performed to simplify the CS and CB SAFE distillate and, consequently, to have less coelution during GC separation, aimed to facilitate the MS identification. Seven different fractions, i.e., acid volatile fraction, 5 neutral and basic volatile fractions and thiol volatile fraction, were prepared according to odor-active compounds commonly reported in coffee (Czerny et al., 1999; Czerny & Grosch, 2000; Sunarharum et al., 2014). A SAFE distillate was fractionated with acid-base extraction into a fraction of acidic volatiles and neutral and basic volatiles. In detail, SAFE distillates of CS and CB were extracted with aqueous sodium carbonate solution (0.5 mol $L^{-1}$) in three portions (300 mL total). The organic phase (dichloromethane), containing the basic and neutral volatiles, were dried with anhydrous sodium sulfate and concentrated to 0.5 mL by using a Vigreux column and then a Bemelmans microdistillation device (NBF). The aqueous phase, containing the acidic volatiles, was washed with dichloromethane (50 mL) and then acidified with hydrochloric acid (32%) to pH 2. Subsequently, volatiles were extracted in three portions with dichloromethane (300 mL total) and the remaining water was removed by drying over anhydrous sodium sulfate. Finally, organic phase was concentrated to 0.5 mL (AF). The neutral and basic volatile fraction (NBF) was separated onto a slurry of purified silica gel (9 g) in pentane using a water-cooled (12 °C) glass column (1 cm i.d.). The elution was carried out with five different mixtures of pentane:diethyl ether: A, 100:0; B, 90:10; C, 70:30; D, 50:50; E, 0:100 (v:v; 50 mL each). The eluate was collected in five portions of 50 mL and eluate portions were concentrated to 0.5 mL (NBFA-E). Another SAFE distillate was used to prepare thiol volatile fraction. The thiol volatile fraction was carried out following a published procedure (Steinhaus, 2015). Briefly, the concentrated volatile extracts of CS and CB were injected onto mercurated agarose gel (1 g) in a glass column (0.5 cm i.d.). Then, the column was rinsed with dichloromethane (50 mL) and the thiol volatiles were eluted

with dithiothreitol (10 mmol/L) in dichloromethane (50 mL). The excess of dithiothreitol was removed by SAFE distillation, and the distillate was concentrated to 0.5 mL (TF).

### 3.3.6 GCxGC-TOFMS

The system consisted of a 6890 Plus gas chromatograph (Agilent) and a Pegasus III TOFMS (Leco, Mönchengladbach, Germany). The GC was equipped with a KAS4 injector (Gerstel, Mühlheim/Ruhr, Germany). The injector was connected to a fused silica column, DB-FFAP, 30 m × 0.25 mm i.d., 0.25 µm film (Agilent). The end of this column was connected to a second fused silica column, DB-5, 2 m × 0.15 mm i.d., 0.30 µm film (Agilent). The front part of this column was passed through a liquid nitrogen-cooled dual-stage quad-jet thermal modulator (Leco), the major part was installed in a secondary oven mounted inside the primary GC oven, and the column end was connected via a heated (250 °C) transfer line to the MS inlet. Helium at 2 mL/min constant flow served as the carrier gas. The temperature of the first oven was 40 °C for 2 min, it ramped up at 6°/min to 230 °C, and held for 5 min at 230 °C. The modulation time was 4 s. The temperature of the secondary oven was 70 °C for 2 min, it ramped up at 6°/min to 250 °C, and held for 5 min at 250 °C. The mass spectrometer was operated in the EI mode at 70 eV, with a scan range of m/z 35−350, and a scan rate of 100 spectra/s. Data evaluation was performed by means of GC Image (GC Image, Lincoln, NE, USA).

# 4. Results and Discussion

## 4.1 Lignan and isoflavone in coffee

### 4.1.1 Optimization of HPLC-MS/MS analytical method for lignan quantification in ESPRESSO AND R&G COFFEE

The HPLC-tandem mass spectrometry instrument composed of triple quadrupole analyser has been chosen for its capacity to separate, identify, and quantify biological compounds in sensitive and specific manner. MS/MS acquisition parameters in MRM mode of our newly developed analytical method are shown in **Table 4**. Each lignan showed different transitions with different intensities, and the most abundant product ion was used for the quantification analysis while the others to confirm the identification. Different conditions of the ion source have been tested, in particular at the gas temperature of 300 °C, LARI precursor ion was $[M+HCOO]^-$ ion with m/z 405. The product ion spectrum of the $[M+HCOO]^-$ ion produced prominent $[(M+HCOO)-HCOOH-CH_2O]^-$ ions by a loss of HCOOH and $CH_2O$ (giving the *m*/*z* 329). When the temperature of the gas was raised to 350 °C, precursor ions for all 3 lignans were deprotonated molecules $[M-H]^-$ in negative polarity, as shown in **Table 4**. The product ion spectrum of the *m*/*z* 359 (precursor ion chosen for LARI) produced prominent $[M-H-30]^-$ ions, probably because of formaldehyde lose. This behaviour is common for butanediol lignans, like SECO, but lignans contained hydroxymethyl groups can have similar loss as well. For SECO, the fragmentation of its precursor ion (*m*/*z* 361) produced mainly *m*/*z* 165 species, probably because of a cleavage in β-position (Eklund, Backman, Kronberg, Smeds, & Sjöholm, 2008). Moreover, at 350 °C, as temperature of the drying gas in the ionization source, we found an increase of product ion abundances for all 3 compounds; therefore, these conditions were chosen for the lignan monitoring. Regarding the optimization of lignan separation, two were the best chromatograms, as shown in **Figure 3**, obtained after a series of attempts using different conditions. The first (**Figure 3a**) has been achieved flowing at 0.4 mL/minute, with this gradient elution: 0 minute (30% B) in isocratic conditions until 4.5 minutes, 4.5-6 min (80% B), 6-6.5 min (80% B), 6.5-8.5 min (30% B) and constant until the end of the run (10 min). The mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The studied compounds were eluted within 6

minutes. The second chromatogram (**Figure 3b**) has been achieved with the optimized and chosen chromatography conditions reported previously (chapter 3.1.7 HPLC-MS/MS parameters for lignan analysis in espresso and R&G coffee). The mobile phase in this case was composed by 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Under these conditions, narrower peaks and highest resolution has been obtained as well. Moreover, lignans were eluted within 4 minutes. Therefore, these parameters (i.e., with acetonitrile as mobile phase (B)), have been chosen for our studies.

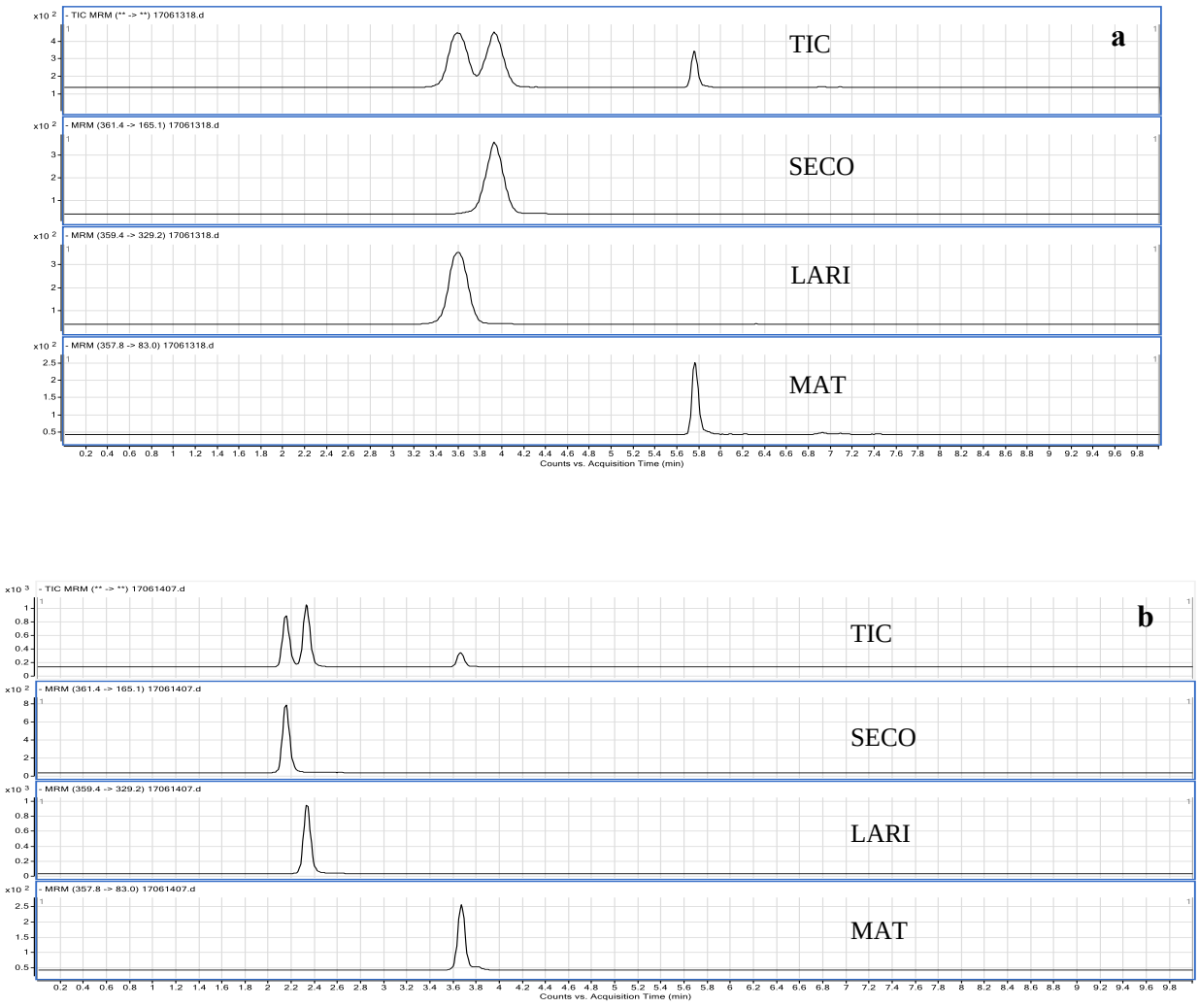



**Figure 3a** and **b.** HPLC-MS/MS chromatograms of a standard mixture of investigated lignans by using two different elution conditions: (**a**) mobile phase: water and methanol both with 0.1% formic acid and (**b**) water and acetonitrile both with 0.1% formic acid. Total ion chromatograms (TIC) and MS/MS product ion of the quantitative transitions are reported for all compounds.

### 4.1.2 Validation of HPLC-MS/MS analytical method for lignan quantification in ESPRESSO AND R&G COFFEE

Various parameters such as limits of detection (LOD), limits of quantitation (LOQ), linearity, repeatability, specificity, and matrix effects have been chosen and investigated for the validation of developed analytical methods for lignans quantification in espresso and R&G coffee (**Table 6**).

**Table 6**. Validation data of HPLC-MS/MS analytical method for lignan analysis: concentration range of calibration curve, regression equation, coefficient of determination ($R^2$), limit of detection (LOD), limit of quantification (LOQ), inter-day and intra-day repeatability.

| Compounds | SECO | MAT | LARI |
|---|---|---|---|
| **Concentration range (μg $L^{-1}$)** | 5-10000 | 10-10000 | 5-10000 |
| **Regression equation** | y= 663.66x + 101 | y= 179.97x + 32.68 | y= 814.92x + 146.54 |
| **$R^2$** | 0.9968 | 0.9964 | 0.9944 |
| **LOD (μg $L^{-1}$)** [a] | 2 | 3 | 2 |
| **LOQ (μg $L^{-1}$)** [b] | 5 | 10 | 5 |
| **Intra-day repeatability (RSD %)** | 1.75 | 7.34 | 4.18 |
| **Inter-day repeatability (RSD %)** | 5.72 | 12.12 | 8.38 |

[a] LOD (limit of detection) = ratio of signal to noise (S/N) = 3. [b] LOQ (limit of quantification) = ratio of signal to noise (S/N) = 10.

LOD and LOQ were calculated as 3:1 and 10:1 signal-to-noise ratio obtained by injecting gradually lower concentration of standard mixtures containing three lignans. The LOD and LOQ for SECO and LARI were 2 and 5 μg $L^{-1}$ respectively, whereas those for MAT were 3 and 10 μg $L^{-1}$. These values are lower than (Horn-Ross et al., 2000; Kuhnle et al., 2008; Mazur et al., 1996) or similar to (Milder et al., 2004; Sapozhnikova, 2014) those reported in literature. Linearity was calculated injecting seven different dilution levels, from 5 to 10000 μg $L^{-1}$, of standard mixtures of three lignans, in triplicate.

Calibration curves were based on peak areas of standard mixtures at seven different diluting levels. Determination coefficients, $R^2$, for SECO, LARI, and MAT were 0.9968, 0.9944, and 0.9964 respectively, which implies good linearity. Intraday repeatability or run-to-run precision and interday repeatability or day-to-day precision were obtained injecting 5 replicates for each concentration over the course of 5 days. The RSDs ranged from 1.75 to 7.34% for intraday repeatability and from 5.72 to 12.12% for interday repeatability. Using tandem mass spectrometry, high specificity has been achieved. The specificity of method was evaluated both measuring retention time stability and utilizing multiple pairs of precursor/product ions. Retention time stability, for each compound, was examined 5 times over a period of 5 days (n = 25). The retention times were stable, with RSD% values ≤0.48%.

For espresso and for R&G coffee, we also evaluated matrix effects, which are defined by the IUPAC as "The combined effect of all components of the sample other than the analyte on the measurement of the quantity. If a specific component can be identified as causing an effect then this is referred to as interference" ("Matrix Effect," 2008). In mass spectrometry with an ESI source, interferences are compounds present in the matrix that can influence the efficiency of analyte droplet formation and affect the amount of the analyte ions formed in the gas phase that reaches the detector (Gosetti, Mazzucco, Zampieri, & Gennaro, 2010). Therefore, the analysis results may differ depending on the solvent or matrix containing the analytes. For this reason, signal suppression/enhancement per cent (SSE %) should be studied. We did so by dividing (the slope of the matrix-matched calibration curve) by (the slope of the calibration curve) and multiplying by 100 (Sulyok, Berthiller, Krska, & Schuhmacher, 2006). The matrix-matched calibration curve was prepared by adding certain amounts of standards to extracted samples and subtracting, for each concentration, the amount of lignans measured in the blank extract. An SSE (%) of 100 indicates that there is no matrix effect, while values <100 indicate signal suppression, and values >100 indicate signal enhancement. Our results on espresso coffee showed signal suppression for SECO and LARI with values of 26.2% and 31.5% whereas for MAT a signal suppression slightly lower, 44.2%. As shown in **Table 7**, also ground

coffee samples showed high values of matrix effects for SECO (SSE= 21.71%) and LARI (SSE= 38.28%) but a lower value for MAT (SSE= 76.14%). This could be due to the presence of some interferences, like ionic species (inorganic electrolytes or salts) and polar compounds (phenols), that could be extracted from the water used for enzymatic digestion, since higher levels of ion suppression were found for more hydrophilic analytes (SECO and LARI). In fact, it has been reported that signal suppression also depends on the hydrophobicity of the analyte and its affinity for the stationary phase. When using reverse-phase stationary packings, the effect is generally lower for the more hydrophobic compounds like MAT (Gosetti et al., 2010). Matrix effects have been considered for the quantification of lignans in 9 ECs and in 30 different ground coffee samples.

**Table 7.** Evaluation of matrix effect: comparison between slopes of calibration curves and signal suppression/enhancement SSE (%) for the target lignans in ground coffee hydrolysed with taka-diastase 2% (w/v) (Method 5-R&G).

| **Compounds** | **Slope** | | **$R^2$** | | |
|---|---|---|---|---|---|
| | *Matrix* | *Solvent* | *Matrix* | *Solvent* | *SSE (%)* |
| **SECO** | 276.81 | 1271.10 | 0.9907 | 0.9981 | 21.71 |
| **LARI** | 589.48 | 1539.70 | 0.9990 | 0.9994 | 38.28 |
| **MAT** | 134.32 | 176.41 | 0.9986 | 0.9996 | 76.14 |

### 4.1.3 Optimization and validation of HPLC-MS/MS analytical method for lignan and isoflavone analysis in GREEN COFFEE

A new analytical method for simultaneous quantitation of three lignans (secoisolariciresinol, lariciresinol and matairesinol) and six isoflavones (daidzin, daidzein, genistin, genistein, formononetin and biochanin A) was developed by using HPLC-MS/MS triple quadrupole. For isoflavones in glycosidic form, the precursor ions were protonated molecules $[M+H]^+$ in positive polarity, whereas for the aglyconic form and lignans the precursor ions were deprotonated molecules $[M-H]^-$ in negative polarity (Caprioli et al., 2016). The chromatographic separation was characterized by good resolution and short run time, with the target phytoestrogens eluted within 6 min (**Table 5**). As an example, **Figure 4** shows the HPLC-MS/MS chromatogram of a standard mixture of the nine isoflavones and lignans plotted as overlapped multiple reaction monitoring (MRM) transition of each compound. The validation parameters studied were linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, specificity and matrix effect (**Table 8**). Linearity was tested by injecting seven different concentrations of the three lignans and six isoflavones, in triplicate, then plotting and calculating calibration curves with the respective determination coefficients ($R^2$). All target molecules showed good linearity, since the $R^2$ equalled or exceeded 0.9952. LOD and LOQ were calculated by injecting gradually lower concentrations of standard compounds and measuring the signal-to-noise ratio (SNR), using MassHunter Software from Agilent Technology (Santa Clara, CA). The standard concentration with SNR=3 was assigned to LOD, while that with SNR=10 to LOQ. The method revealed good sensitivity for both isoflavones and lignans, since the LODs ranged from 0.1 to 5 $\mu g\ L^{-1}$ and from 1.5 to 15 $\mu g\ L^{-1}$, respectively, levels lower than those reported by (Horn-Ross et al., 2000; Kuhnle et al., 2008) but similar to the results of (Alves et al., 2010; Caprioli et al., 2016). Method repeatability was tested by injecting 5 replicates for each standard concentration over the course of five days. The intra-day repeatability or run- to- run precision and inter-day repeatability or day- to- day precision were expressed by Relative Standard Deviation (RSD) percent. The RSD ranged from 0.40 to 4.17% for run-to-run precision and from 4.64 to 10.37% for day-to-day precision. Repeatability was also evaluated by measuring the RSDs of recovery replicates studied for each

method. High specificity was obtained using HPLC-MS/MS working in MRM mode. The method specificity was evaluated by measuring retention time stability and setting multiple pairs of precursor/product ions. Retention time stability for each molecule was studied 5 times over a period of 5 days (n=25) and expressed by RSD. Hence, RSDs were in all cases lower than or equal to 1.93%. We also evaluated the matrix effect by studying signal suppression/enhancement (SSE). SSE (%) was calculated by dividing the slope of the standard addition curve by the slope of the calibration curve and multiplying by 100 (Sulyok et al., 2006). An SSE (%) of 100 means no matrix effect, while values <100 mean signal suppression and values >100 indicate signal enhancement. Slight signal enhancement was found for formononetin (SSE=111.6%), while other compounds showed marked signal suppression (from 22.7 to 75.9%). Those values could be due to the presence of interferences that can reduce the ionization process by affecting the analyte droplet formation or evaporation as well as the amount of the analyte ions formed in the gas phase that reaches the detector (Gosetti et al., 2010). Matrix effects were considered for the quantification of lignans and isoflavones in 25 green coffee samples.

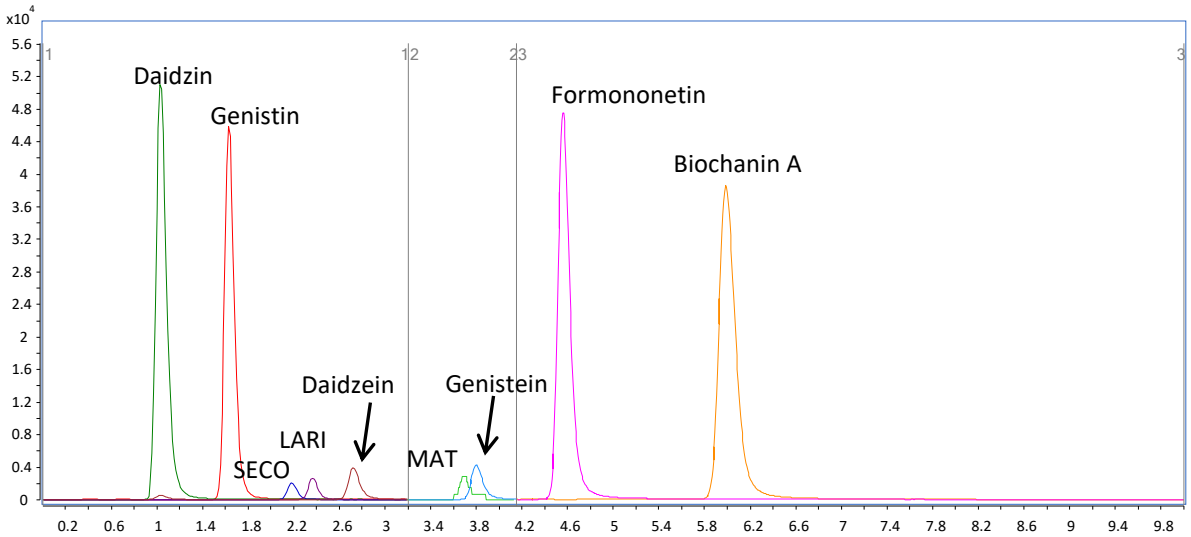



**Figure 4.** HPLC-MS/MS chromatogram of a standard mixture of nine isoflavones and lignans plotted as overlapped multiple reaction monitoring (MRM) transition of each compound.

**Table 8**. Validation data of the HPLC-MS/MS method for analysis of lignans and isoflavones.

| Compounds | Concentration range (µg $L^{-1}$) | Regression equation | $R^2$ | LOD (µg $L^{-1}$) [a] | LOQ (µg $L^{-1}$) [b] | Repeatability Intra-day (RSD %) | Repeatability Inter-day (RSD %) | SSE% [c] |
|---|---|---|---|---|---|---|---|---|
| Biochanin A | 1-10000 | y = 39043x + 5091.10 | 0.9970 | 0.3 | 1 | 1.01 | 6.81 | 74.8 |
| Daidzein | 1-10000 | y = 2794x + 626.71 | 0.9952 | 5 | 15 | 0.40 | 8.03 | 22.7 |
| Daidzin | 1-10000 | y = 33416x + 1541.60 | 0.9993 | 0.2 | 0.6 | 4.17 | 8.98 | 36.5 |
| Formononetin | 1-10000 | y = 36112x + 6052.20 | 0.9956 | 0.1 | 0.5 | 1.87 | 6.44 | 111.6 |
| Genistein | 1-10000 | y = 3052.2x + 527.99 | 0.9970 | 1.5 | 5 | 0.87 | 7.44 | 58.2 |
| Genistin | 1-10000 | y = 27136x – 454.38 | 0.9985 | 0.5 | 1.5 | 3.73 | 9.75 | 57.4 |
| Lariciresinol | 1-10000 | y = 1537x – 49.64 | 0.9983 | 1.5 | 5 | 1.54 | 9.68 | 28.7 |
| Matairesinol | 1-10000 | y = 228.06x – 2.38 | 0.9982 | 15 | 50 | 3.56 | 4.64 | 75.9 |
| Secoisolariciresinol | 1-10000 | y = 1177.10x – 39.24 | 0.9983 | 5 | 15 | 2.99 | 10.37 | 48.4 |

[a] LOD (limit of detection) = ratio of signal to noise (S/N) = 3. [b] LOQ (limit of quantification) = ratio of signal to noise (S/N) = 10. [c] SSE % (Signal suppression/enhancement percent) = slope of standard addition curve -to-slope of solvent calibration curve ratio × 100.

### 4.1.4 Extraction optimization for lignan quantification in ESPRESSO COFFEE

Three different methods, Method 1-EC, i.e. dilute and shoot, Method 2-EC, i.e. acid hydrolysis, and method 3-EC, i.e. enzymatic hydrolysis, have been tested to identify the best process for the extraction of lignans from EC matrix. Firstly, dilute and shoot procedures have been carried out diluting the EC sample in the aqueous portion (A) of mobile phase and injecting into HPLC-MS/MS. With these 2 procedures, we obtained the worst results. In fact, recoveries were very low (range 5%-10%) whereas relative standard deviations (RSD) were between 12.12% and 30.74%. These facts could be explained through matrix effect that generated ionic signal suppression. To enhance the extraction procedure, we decided to use acid hydrolysis to release bond lignans from coffee sample. A high number of acid hydrolysis procedures have been carried out (**Table 1**) and compared among them. The best recoveries of acid hydrolysis procedures are shown in **Table 9**.

**Table 9.** The best Recovery % (R %) values of lignans, in EC, for acid hydrolysis procedures at one spiking level (0.5 mg $L^{-1}$).

| | **Compounds** [a,b] | | |
|---|---|---|---|
| | **SECO** | **LARI** | **MAT** |
| **Method 2.1-EC** | 101 | 0 | 97 |
| **Method 2.2-EC** | 84 | 0 | 100 |
| **Method 2.3-EC** | 106 | 0 | 117 |
| **Method 2.4-EC** | 99 | 0 | 93 |
| **Method 2.6-EC** | 103 | 0 | 100 |
| **Method 2.7-EC** | 106 | 0 | 103 |
| **Method 2.8-EC** | 96 | 0 | 93 |

[a] Each sample was analyzed in triplicate (n=3); [b] RSD % (relative standard deviation percent) for each method was lower than 8%.

Recoveries were achieved spiking the matrix at 500 μg $L^{-1}$ with three target lignans. From the **Table 9**, one thing is particularly remarkable: in all acid procedures, the recovery for LARI was always at 0%. This fact was probably because of LARI sensitivity in acid conditions. Specifically, LARI could rearrange to cyclolariciresinol (cLARI) at strong acidic conditions (Willför, Smeds, & Holmbom, 2006). However, SECO and MAT showed good recoveries in the range of 84% to 106% and in the range of 93% to 117% respectively. Hence, varying some conditions of acid hydrolysis like hydrochloric acid normality, ratio of sample to acid and time, and temperature of extraction, recovery amounts remained at similar levels. With the main purpose of increasing the recovery of LARI, we decided to evaluate the enzymatic hydrolysis. We carried out different enzymatic procedures, reported in **Table 1**, using 4 enzymes, i.e. taka-diastase, clara-diastase, papain, and protease at two concentrations, testing also two temperatures and two times of hydrolysis. Some enzymatic hydrolysis procedures with the best recovery levels are reported in **Table 10**.

**Table 10.** The best Recovery % (R %) values of lignans for enzymatic hydrolysis procedures at one spiking level (0.5 mg $L^{-1}$).

| | **Compounds** [a,b] | | |
|---|---|---|---|
| | **SECO** | **LARI** | **MAT** |
| **Method 3.5-EC** | 70 | 65 | 53 |
| **Method 3.6-EC** | 82 | 78 | 91 |
| **Method 3.7-EC** | 81 | 76 | 70 |
| **Method 3.8-EC** | 96 | 81 | 71 |
| **Method 3.9-EC** | 98 | 79 | 82 |
| **Method 3.10-EC** | 97 | 98 | 93 |
| **Method 3.11-EC** | 91 | 76 | 75 |
| **Method 3.12-EC** | 91 | 71 | 76 |

[a] Each sample was analyzed in triplicate (n=3); [b] RSD % (relative standard deviation percent) for each method was lower than 7%.

It is evident that recoveries of LARI, in the rage of 65% to 98%, were much over the zero in all these processes. Moreover, recoveries of SECO and MAT resulted to be in the range of 70% to 98% and 53% to 93% respectively. Thus, enzymatic hydrolysis gave a significant improvement with respect to the previous method, especially for LARI. Changing the temperature of hydrolysis from 25 °C to 37 °C, recovery levels were increased for each monitored enzyme type. The sole exception was for procedure with Protease because its values decreased with temperature increasing. In fact, at 25 °C, the recovery levels by using taka-diastase, clara-diastase, papain, and protease procedures were in the ranges of 53 to 70%, 78 to 91%, 70 to 81%, and 71 to 96% respectively, while at 37 °C were in the ranges of 79 to 98%, 93 to 98%, 75 to 91%, and 71 to 91% respectively. Instead, comparing different enzymes, the best procedures at 25 °C were obtained with clara-diastase (Method 3.6-EC) and protease (Method 3.8-EC) with the ranges of 78 to 91% and of 71 to 96% respectively, whereas at 37 °C with clara-diastase (Method 3.10-EC) with a recovery from 93 to 98%. The latter procedure, Method 3.10-EC, was the best method for the extraction of lignans from EC samples, with R% almost equal to 100% for the 3 lignans. To conclude, various methods of dilution, acidic hydrolysis, and enzymatic digestion have been evaluated. The best recovery values (SECO: 97%, LARI: 98%, and MAT: 93%) were obtained with the clara-diastase at 10% (w/v), keeping the sample at 37 °C for 3 hours (Method 3.10-EC). For these reasons, this method has been chosen and then applied to quantify lignans in 9 different EC samples.

### 4.1.5 Extraction optimization for lignan quantification in R&G COFFEE

Since the extraction method significantly influences the determination of lignan content in coffee, it is essential to choose the optimum method. We evaluated acid extractions, methanolic extractions and enzymatic digestions in terms of recovery. Recovery % was performed by spiking the matrix, with three target lignans, at 500 μg $kg^{-1}$. To develop the extraction method, the same production batch of MocCafè coffee was used. First, we tried a strong acid extraction procedure to release lignans from ground coffee matrix. We supposed that these molecules could be present in coffee as oligomers, as is the case with flaxseeds, in which SECO occurs as diglucoside-hydroxymethyl glutaryl ester linked

oligomers, whose bonds can be broken under strong acid conditions (Ford et al., 2001; Smeds et al., 2007).

**Table 11**. Lignan recoveries % (R %) obtained using different extraction methods. Recovery was achieved by spiking the R&G coffee with three monitored compounds at 0.5 mg $kg^{-1}$.

| EXTRACTION PROCESSES[a, b] | SECO | LARI | MAT |
|---|---|---|---|
| **Method 1-R&G** | 48 | 0 | 54 |
| **Method 2-R&G** | 73 | 51 | 39 |
| **Method 3-R&G** | 96 | 92 | 79 |
| **Method 4-R&G** | 79 | 0 | 65 |
| **Method 5-R&G** | 98 | 73 | 73 |
| **Method 6-R&G** | 111 | 122 | 80 |
| **Method 7-R&G** | 82 | 66 | 75 |

[a] All samples were analysed in triplicate (n=3), [b] RSD (Relative Standard Deviation) % for each sample was lower than 12.68 %.

As shown in **Table 11**, the recovery levels of SECO and MAT for strong acid extraction were 48% and 54% respectively, while for LARI, it was 0% (Method 1-R&G). Therefore, this process was not chosen for our studies because it gave low recovery values for SECO and MAT, and also because under strong acid conditions LARI can be converted to cyclolariciresinol (cLARI) (Eklund, Sillanpää, & Sjöholm, 2002; Smeds et al., 2007; Willför et al., 2006). Given that LARI can be stable in mild acid conditions (Smeds et al., 2007), we tried a mild acid extraction (Method 2-R&G), which yielded SECO, LARI and MAT recoveries of 73%, 51% and 39%, respectively, which, except for the SECO, were not satisfactory results. The last attempt, in acid conditions, was carried out using a solution of 50% of MeOH and 50% of HCl 2M and sonicating the sample for 2.5 h (Method 4-R&G). The recoveries of SECO (79%) and MAT (65%) were the highest obtained thus far, but once again LARI recovery was 0%. Hence, acid processes were abandoned and we moved on to test methanolic

extractions, and then enzymatic digestions with two different enzymes, clara-diastase and taka-diastase. Comparing the methanolic and enzymatic results, it emerged that the best recovery values were obtained with methanolic extraction (Method 3-R&G), which yielded SECO 96%, LARI 92% and MAT 79%, while high recovery percentages were also found with extraction by enzymatic digestion when we used clara-diastase (Method 6-R&G), which yielded SECO 111%, LARI 122% and MAT 80%, and taka-diastase (Method 5-R&G), which yielded SECO 98%, LARI 73% and MAT 73%. The combined enzyme and methanol method (taka-diastase in water/methanol, Method 7-R&G) yielded lower levels: SECO 82%, LARI 66% and MAT 75%. In summary, the best recovery results were obtained with Methods 3, 5 and 6-R&G. Thus, to choose the most performing process, the total lignan concentrations, found it carrying out all extraction methods, have been evaluated, as well. As it is shown in **Figure 5**, the highest concentrations of lignans were obtained with enzymatic digestion using taka-diastase 2% (w/v) (Method 5-R&G).

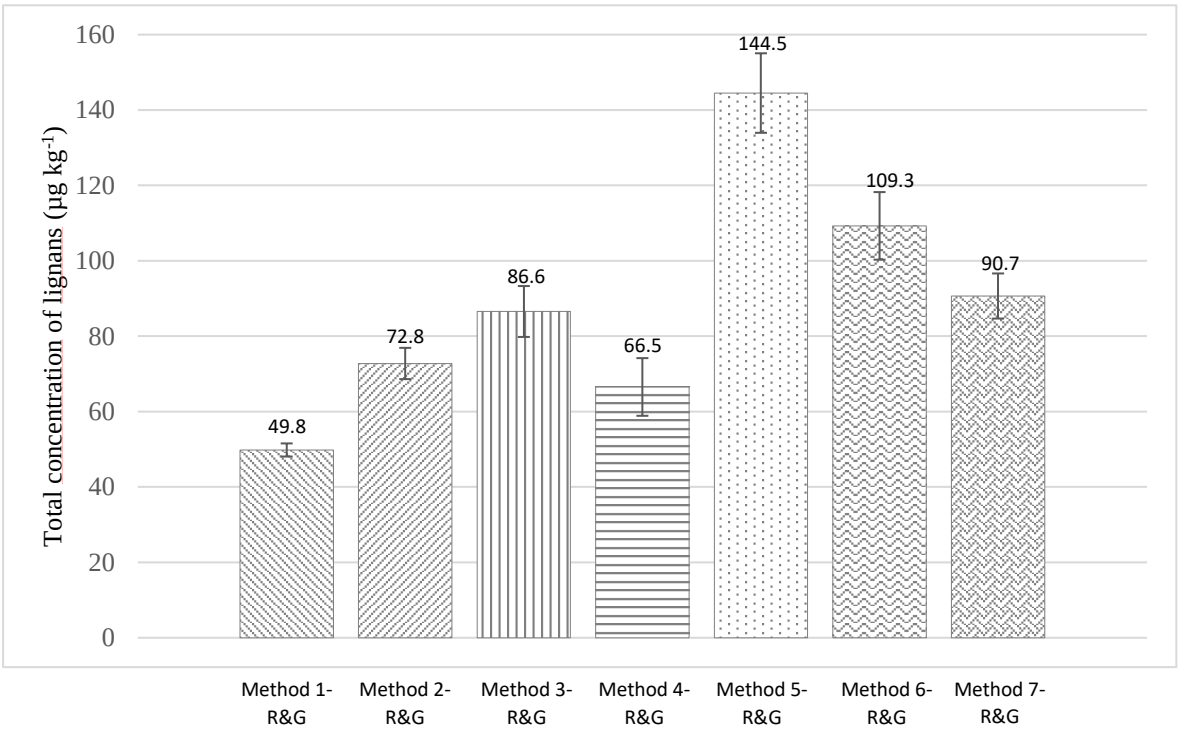



**Figure 5.** Concentration of the total lignans, expressed in µg $kg^{-1}$, found by carrying out different extraction methods. Each sample was analyzed in triplicate. RSD % for each sample was lower than 11.5%.

Considering that coffee beans contain low level of starch, the α-amylase (taka-diastase) could break glycosidic bonds of other polysaccharides too, e.g. arabinogalactans, and make more available monitored compounds (Redgwell, Trovato, Curti, & Fischer, 2002). Therefore, evaluating the recovery levels of the single lignans and total concentration of all three lignans, Method 5-R&G was chosen and then applied to quantify lignans in 30 different ground coffee samples.

### 4.1.6 Extraction optimization for lignan and isoflavone quantification in GREEN COFFEE

The extraction procedure is considered one of the most important stages of sample preparation in food analysis and should be developed and optimized for each analyte in each food matrix (Mortensen et al., 2009). Therefore, we evaluated the different extraction methods - acid (Method 1-GC) and base hydrolysis (Methods 2 and 3-GC), enzymatic digestions (Methods 4 and 5-GC), methanolic extraction (Method 6-GC) and combination processes (Methods 7 and 8-GC) - for their ability to release bound and free compounds from the coffee matrix. For each method, recovery (R%) levels were calculated by spiking the matrix with following levels: 0.5 mg $kg^{-1}$ for lignans and 0.05 mg $kg^{-1}$ for isoflavones. Considering that isoflavones in soy products have been found to occur in an unconjugated form (or aglycone) or in a conjugated form (glycoside, acetyl-glucoside and malonyl-glucoside) (Ren, Kuhn, Wegner, & Chen, 2001), and that lignans in flaxseed occur mainly in oligomer forms as glutaryl ester-linked oligomers (Ford et al., 2001), we assumed that these compounds might be present in green coffee beans under these forms as well, and reasoned that acid or base hydrolysis might be useful for breaking glycosidic and ester bonds. Thus, we evaluated the extraction efficiency in acid and base conditions. **Table 12** reports recoveries for each extraction procedure. Method 1-GC (acid hydrolysis) showed good recoveries for secoisolariciresinol (94%), matairesinol (86%), daidzin (77%) and genistin (72%), but lower levels for biochanin A (28%), daidzein (53%), genistein (54%) and formononetin (41%) and a total lack of lariciresinol, probably because under acid conditions lariciresinol rearranges to (+)-cyclolariciresinol (cLARI) (Smeds et al., 2007).

**Table 12.** Comparison of the efficiency of several extraction processes, evaluating recovery % (R %) values. Recoveries were achieved by spiking the matrix with the 9 target compounds at 0.5 mg $kg^{-1}$ for lignans and 0.05 mg $kg^{-1}$ for isoflavones.

| Compounds | Extraction Processes [a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Method 1-GC** | **Method 2-GC** | **Method 3-GC** | **Method 4-GC** | **Method 5-GC** | **Method 6-GC** | **Method 7-GC** | **Method 8-GC** |
| Lariciresinol | 0 | 39 | 75 | 38 | 90 | 93 | 94 | 5 |
| Matairesinol | 86 | 20 | 68 | 24 | 87 | 83 | 81 | 15 |
| Secoisolariciresinol | 94 | 57 | 104 | 63 | 97 | 90 | 74 | 48 |
| Daidzin | 77 | 69 | 68 | 1 | 8 | 89 | 83 | 0 |
| Genistin | 72 | 58 | 61 | 2 | 12 | 90 | 79 | 0 |
| Biochanin A | 28 | 0 | 78 | 5 | 1 | 94 | 75 | 0 |
| Daidzein | 53 | 0 | 73 | 33 | 15 | 94 | 89 | 0 |
| Genistein | 54 | 0 | 79 | 32 | 2 | 95 | 80 | 0 |
| Formononetin | 41 | 1 | 82 | 12 | 11 | 94 | 84 | 0 |

[a] All samples were analysed in triplicate (n=3); RSD (Relative Standard Deviation) % for each sample was less than or equal to 14.3 %.

Method 2-GC (hydrolysis in water) revealed low recovery levels for all compounds, in contrast to base hydrolysis in MeOH (Method 3-GC). The latter process showed good recoveries for lariciresinol (75%), secoisolariciresinol (104%), biochanin A (78%), genistein (79%) and formononetin (82%) and slightly lower ones for matairesinol (68%), daidzin (68%), genistin (61%) and daidzein (73%). Further on, we evaluated the efficacy of enzymatic digestions, in particular the activity of two different enzymes: taka-diastase (Method 4-GC) and clara-diastase (Method 5-GC). Neither enzyme was appropriate for extraction of isoflavones, since recovery levels using taka-diastase ranged from 1 to 33%, and those using clara-diastase from 1 to 15%. Considering the complex conjugated structure with which isoflavones occur in some foodstuffs, it may be that the enzymes we tested are unable to hydrolize the compounds. On the other hand, for lignan extraction, the process performed with clara-diastase revealed excellent recoveries of lariciresinol (90%), matairesinol (87%) and secoisolariciresinol (97%). Similar results were obtained in espresso coffee.

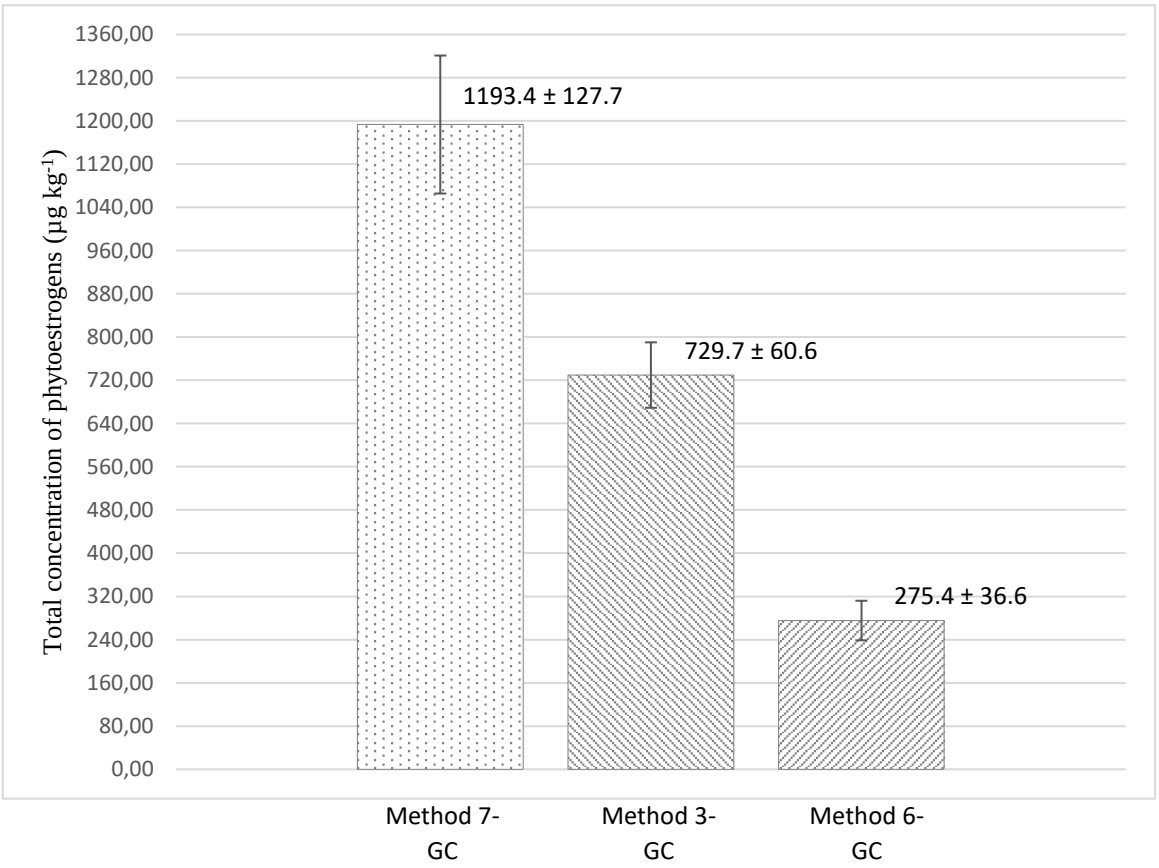



**Figure 6.** Extraction efficiency of the best three methods, Method 7-GC, Method 3-GC and Method 6-GC.

The third step was to study the extraction efficiency of a methanol:water (85:15) solution (Method 6-GC). Method 6-GC showed the highest recovery levels for isoflavones (from 89 to 95%,) and for lignans (from 83 to 93%) of all 6 methods tested up to that point, but lower concentrations of total isoflavones and lignans than those obtained with Method 3-GC. For this reason, in order to obtain good recovery levels and higher amounts of the target compounds, we decided to perform other two procedures, Methods 7 and 8-GC, that combined two different extractions, and found that Method 7-GC had the highest amounts of the compounds under study (1193.4 μg $kg^{-1}$), as shown in the **Figure 6**. Moreover Method 7-GC was chosen, validated, and then applied to 25 different green coffee samples.

### 4.1.7 Analysis of ESPRESSO COFFEE samples

The Method 3.10-EC, once validated, was applied to 9 different EC samples from 5 different countries (Brazil lots 1 and 2, Colombia lots 1 and 2, Ethiopia lots 1 and 2, India lots 1 and 2, and El Salvador (BLC)) to quantify the monitored lignans whose concentrations, expressed in μg $L^{-1}$, are shown in **Table 13**.

**Table 13.** Content of lignans in different EC samples from various countries, expressed in μg $L^{-1}$.

| EC sample[a] | SECO | MAT | LARI | Total |
|---|---|---|---|---|
| Brazil lot 1 | 33.4 | n.d.[b] | 10.5 | 43.9 |
| Colombia lot 1 | 44.2 | n.d. | 15.8 | 60.0 |
| Ethiopia lot 1 | 48.9 | n.d. | 27.8 | 76.7 |
| India lot 1 | 52.0 | n.d. | 8.3 | 60.3 |
| Brazil lot 2 | 27.9 | n.d. | 10.5 | 38.4 |
| Colombia lot 2 | 51.2 | n.d. | 18.8 | 70.0 |
| Ethiopia lot 2 | 52.0 | n.d. | 27.1 | 79.1 |
| India lot 2 | 41.9 | n.d. | 8.3 | 50.2 |
| BLC | 27.9 | n.d. | 5.3 | 33.2 |

[a] Each sample was analysed in triplicate. %RSDs in all cases were ≤ 12.8 %. [b] n.d., not detectable.

The quantification was carried out comparing areas of the samples with those of standard mixtures. SECO and LARI were found in all EC samples from 27.9 to 52.0 μg $L^{-1}$ and from 5.3 to 27.8 μg $L^{-1}$ respectively, contrary to MAT that it was not possible to detect it in each type of coffee. The highest level of SECO was found in the EC from Ethiopia lot 2 and India lot 1 at the concentration level of 52.0 μg $L^{-1}$, while the lowest was in the samples from Brazil lot 2 and El Salvador (BLC) at the concentration level of 27.9 μg $L^{-1}$. The latter showed also the lowest levels of LARI, 5.3 μg $L^{-1}$, whereas the highest concentration of LARI, 27.8 μg $L^{-1}$, was found in the sample from Ethiopia lot 1. The ECs from Ethiopia both lots 1 and 2 showed the highest levels of total lignans, 76.7 and 79.1 μg $L^{-1}$ respectively. Our results on lignan concentrations in ECs are in agreement with those of Thompson et al., (2006), who reported a concentration of SECO and LARI from 34 to 47 μg $L^{-1}$ and from 9 to 11 μg $L^{-1}$ in coffee brews. On the other hand, Milder et al., (2005), who used an enzymatic hydrolysis followed by double ethyl ether extraction coupled with HPLC-MS/MS to quantify monitored compounds in a filter coffee, reported a lignan concentrations different from our quantitative data, finding SECO levels from 92 to 161 μg $L^{-1}$, LARI levels from 90 to 131 μg $L^{-1}$, and MAT levels from 0 to 7 μg $L^{-1}$. Our data also differ from those of Kuhnle et al., (2008), who used LC-MS/MS to analyse only two lignans in coffee infusions finding the concentrations of SECO from 100 to 160 μg $L^{-1}$ and the concentrations of MAT <10 μg $L^{-1}$.

### 4.1.8 Analysis of R&G COFFEE samples

Method 5-R&G was applied to 30 different ground coffee samples from different countries, and available in three forms: capsule, powder and roasted bean. The 30 ground coffee samples were obtained by opening the capsules, using the powder as it is, and grinding the beans. Each sample was analysed in triplicate and the quantifications were calculated comparing areas of the samples with those of standard mixtures. As shown in **Table 14**, two lignans, SECO and LARI, were found in all ground coffee samples from 84.4 to 257.8 μg $kg^{-1}$ and from 26.1 to 91.5 μg $kg^{-1}$, respectively. In contrast, in all the samples, MAT was under its limit of detection.

**Table 14**. Concentration of lignans, determined in various coffee powder samples from different countries, expressed in μg $kg^{-1}$.

| Coffee powder samples [a] | SECO | MAT | LARI | TOTAL |
|---|---|---|---|---|
| **Capsule form [c]** | | | | |
| Brazil lot 1 | 143.7 | n.d.[b] | 26.1 | 169.8 |
| Colombia lot 1 | 157.4 | n.d. | 86.2 | 243.6 |
| Ethiopia lot 1 | 227.9 | n.d. | 83.0 | 310.9 |
| India lot 1 | 166.5 | n.d. | 44.4 | 210.9 |
| Brazil lot 2 | 125.5 | n.d. | 26.1 | 151.6 |
| Colombia lot 2 | 198.5 | n.d. | 60.1 | 258.6 |
| Ethiopia lot 2 | 234.9 | n.d. | 91.5 | 326.4 |
| India lot 2 | 175.6 | n.d. | 34.0 | 209.6 |
| BLC | 107.2 | n.d. | 26.1 | 133.3 |
| **Powder form [c]** | | | | |
| Brazil | 89.0 | n.d. | 28.7 | 117.7 |
| Colombia | 152.8 | n.d. | 28.7 | 181.5 |
| Costa Rica | 100.4 | n.d. | 39.2 | 139.6 |
| Ethiopia | 166.5 | n.d. | 78.4 | 244.9 |
| Guatemala | 187.0 | n.d. | 34.0 | 221.0 |
| India | 107.2 | n.d. | 47.0 | 154.2 |
| **Roasted bean form [c]** | | | | |
| Burundi | 107.2 | n.d. | 60.1 | 167.3 |
| El Salvador | 116.3 | n.d. | 28.7 | 145.0 |
| Ethiopia | 180.2 | n.d. | 86.2 | 266.4 |
| Guatemala | 134.6 | n.d. | 44.4 | 179.0 |
| Kenya | 120.9 | n.d. | 34.0 | 154.9 |
| Rep. Dominican | 116.3 | n.d. | 39.2 | 155.5 |
| Rwanda | 98.1 | n.d. | 54.9 | 153.0 |
| Timor-Leste | 257.8 | n.d. | 83.6 | 341.4 |
| Uganda | 157.4 | n.d. | 65.3 | 222.7 |
| Yemen | 134.6 | n.d. | 60.1 | 194.7 |
| Le Piantagioni (100% Arabica) | 143.7 | n.d. | 28.7 | 172.4 |
| Le Piantagioni (85% Arabica and 15% Canephora) | 98.1 | n.d. | 36.6 | 134.7 |
| MonCafè | 84.4 | n.d. | 60.1 | 144.5 |
| 100% Arabica from Ethiopia | 189.3 | n.d. | 70.5 | 259.8 |
| 100% Canephora from Bali | 216.7 | n.d. | 44.4 | 261.1 |

[a] Each sample was analysed in triplicate. RSDs % in all cases were ≤ 13.8 %; [b] n.d., not detectable. [c] All samples without specifications were 100% arabica except MonCafè whose composition was unknown.

The highest concentration of SECO was found in the sample from Timor-Leste, with 257.8 μg $kg^{-1}$, while the lowest was in MonCafè sample, with a concentration of 84.4 μg $kg^{-1}$. The highest level of LARI was found in the powder from Ethiopia lot 2 capsules, with a concentration of 91.5 μg $kg^{-1}$ while the lowest concentrations (26.1 μg $kg^{-1}$) were found in three capsule samples, Brazil lots 1 and 2, and BLC. The highest concentration of total lignans was found in the Timor-Leste sample, 341.4 μg $kg^{-1}$ (roasted bean form), followed by the Ethiopia lot 2 capsule sample, 326.4 μg $kg^{-1}$. Ethiopian samples showed the highest levels of lignans in samples available in capsule and powder form and the second levels in roasted bean form. The highest concentrations of lignans in Ethiopian samples were noticed also in espresso coffee. Among the difference samples available in capsule, also Colombia lots 1 and 2 contained good amount of total lignans (243.6 and 258.6 μg $kg^{-1}$, respectively). On the other side, the lowest concentration of total lignans was found in Brazil sample, 117.7 μg $kg^{-1}$ (powder form), followed by BLC sample (capsule form) with 133.3 μg $kg^{-1}$ and Le Piantagioni (85% arabica and 15% robusta), roasted bean form, with a total lignans concentration of 134.7 μg $kg^{-1}$. Our data agree with those reported by others (Horn-Ross et al., 2000), who, using HPLC-MS/MS to analyse some phytoestrogens in various foodstuffs, found SECO in ground coffee at concentrations <250 μg $kg^{-1}$. On the other hand, our results are in contrast with those reported by Mazur et al., (1998) and Kuhnle et al., (2008). The first study, which was characterized by a very long extraction and derivatization process, reported SECO concentrations from 3.93 to 7.16 mg $kg^{-1}$ and did not detect MAT, in six samples of ground coffees. HPLC-MS/MS instrument was used in the second study and they found concentrations of SECO and MAT from 6.1 to 8.62 mg $kg^{-1}$ and from 320 to 580 μg $kg^{-1}$, respectively, in two different instant coffee powders.

### 4.1.9 Lignan extraction yield

The lignan extraction yield was evaluated by comparing the amount of total lignan present in espresso coffees with that present in ground coffee. As shown in **Table 15**, lignans seem to be completely or almost completely extracted during the coffee percolation. Coffee percolation is a very complex process during which the water and ground coffee interact and many phenomena take place. First, the

hydrophilic components dissolve in the water phase, and second, the less water-soluble molecules or physically entrapped ones are forced out by physical mechanisms. Third, the heat leads to thermal degradation, making select components more soluble and therefore more available for extraction, and fourth, mobile water will physically lift and migrate coffee fines (the smallest particles in coffee) and emulsify coffee oil into the suspension (Chen Zhang et al., 2012). Lignans are probably extracted during the first three steps because in the first, their presence in a glycosidic form can cause an increase in hydrophilicity, and in the second and third phases, the heat and physical mechanisms probably degrade and extract bound lignans from the coffee matrix. The extraction yield average was 95.2%. Considering the lignan chemical structures and their chemical and physical properties, it is likely that most of the lignans are extracted during the passage of hot water under high pressure.

**Table 15.** Lignan extraction yield (%) achieved using nine different coffee samples.

| Sample | Total lignans in ECs (μg $L^{-1}$) | Total lignans in ground coffees (μg $kg^{-1}$) | Amount (μg) of total lignans in a cup (25ml) | Amount (μg) of total lignans in a capsule (6.7 g) | Extraction yield (%) |
|---|---|---|---|---|---|
| **Brazil lot 1** | 43.90 | 169.80 | 1.10 | 1.14 | 96.5 |
| **Colombia lot 1** | 60.00 | 243.60 | 1.50 | 1.63 | 92.0 |
| **Ethiopia lot 1** | 76.70 | 310.90 | 1.92 | 2.08 | 92.3 |
| **India lot 1** | 60.30 | 210.90 | 1.51 | 1.41 | 107.1 |
| **Brazil lot 2** | 38.40 | 151.60 | 0.96 | 1.01 | 95.0 |
| **Colombia lot 2** | 70.00 | 258.60 | 1.75 | 1.73 | 101.1 |
| **Ethiopia lot 2** | 79.10 | 326.40 | 1.98 | 2.19 | 90.4 |
| **India lot 2** | 50.20 | 209.60 | 1.25 | 1.40 | 89.3 |
| **BLC** | 33.20 | 133.30 | 0.83 | 0.89 | 93.2 |
| **Average** | 54.39 | 212.97 | 1.36 | 1.43 | 95.2 |

### 4.1.10 Analysis of GREEN COFFEE samples

The new analytical method for nine phytoestrogen quantification was applied to 24 different 100% *C. arabica* green coffees from different geographical origins (Rwanda, Costa Rica, Brazil, Ethiopia, India, Colombia and Nicaragua). **Table 16** displays the concentration, expressed in μg $kg^{-1}$, of the target phytoestrogens in the coffee samples, including also robusta sample used for the extraction optimization. Quantitative analysis was performed by comparing sample areas with those of a standard mixture. The highest phytoestrogen concentration was found in the Ethiopian samples: sample 1 (8137.1 μg $kg^{-1}$), followed by samples 4, 3 and 2 (7117.1 μg $kg^{-1}$, 5128.2 μg $kg^{-1}$ and 3703.8 μg $kg^{-1}$, respectively). SECO (172.6-5714.1 μg $kg^{-1}$, with an average of 1335.1 μg $kg^{-1}$) and LARI (113.9-2417.7 μg $kg^{-1}$, with an average of 615.1 μg $kg^{-1}$) were the most abundant compounds under study in green coffee. These two lignans occur in various foodstuffs such as flaxseed, soy products, cereals, legumes, roasted coffee beans and coffee beverages (Kuhnle et al., 2008; Milder et al., 2005; Thompson et al., 2006). Our results about the lignan content in green coffee are similar to those reported by Mazur et al., (1998) who found a SECO concentration from 3.93 to 7.16 mg $kg^{-1}$ in roasted coffee. Lower levels of lignans in coffee beverages were reported by Thompson et al., (2006) and were noticed from our studies in espresso coffee. Thompson et al., (2006) used a SPE coupled with GC-MS analysis and identified the following contents: SECO, from 34 to 47 μg $L^{-1}$; LARI, from 9 to 11 μg $L^{-1}$; MAT, from 1 to 2 μg $L^{-1}$. We reported in EC concentrations for SECO and LARI from 27.9 to 52.0 μg $L^{-1}$ and from 5.3 to 27.8 μg $L^{-1}$, respectively. Regarding isoflavones, our data revealed genistin as the most abundant isoflavone in 17 out of 25 green coffee samples (12.6-204.8 μg $kg^{-1}$). In samples where genistin occurred in high concentrations, except for Brazil 4 and India 4, also its aglycon genistein was found (21.8-40.7 μg $kg^{-1}$). This was also the case for daidzin (12.6-95.2 μg $kg^{-1}$) and its free form daidzein (3.4-25.3 μg $kg^{-1}$), with the exception that its aglycone was present in almost all green coffees, even in samples where the glycosidic form was not found. We can assume that these two molecules occur with different structures and/or bond types in green coffee, that can be the cause of different partial degradations of the glycosidic bonds. The low quantity of genistein

and daidzein found in green coffee in this study together with possible constituents/ion suppressors presents in the matrix, make challenging the detection of isoflavones in coffee. These aspects might be the cause that lead our research group to not quantify genistein and daidzein in roasted and ground coffee in a previous work (Caprioli et al., 2016). On the other side, formononetin (4.3-46.7 μg $kg^{-1}$) and biochanin A (4.7-42.1 μg $kg^{-1}$) were detected in fewer than half of the samples. These two molecules were detected in similar amounts by Caprioli et al., (2016) in R&G coffee (formononetin, 0.36-4.27 μg $kg^{-1}$ and biochanin A, 0.71-3.95 μg $kg^{-1}$) and by Thompson et al., (2006) in coffee brews (formononetin, 2 μg $L^{-1}$). In contrast, our data on the isoflavone quantitation differ significantly from those on coffee powder using acid hydrolysis reported by Alves et al., (2010) who found daidzein in the range from 6.2 to 10.8 mg $kg^{-1}$, genistein from 0.5 to 7.1 mg $kg^{-1}$ and formononetin from 12.0 to 58.0 mg $kg^{-1}$.

**Table 16**. Concentration, expressed in µg $kg^{-1}$, of isoflavones and lignans determined in various green coffee samples.

| Sample[a] | Genistin | Daidzin | SECO | LARI | Daidzein | MAT | Genistein | Biochanin A | Formononetin | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| **Rwanda 1** | 52.0 | 37.7 | 1537.9 | 734.2 | 13.2 | n.d.[b] | 29.0 | 21.2 | 20.0 | 2445.2 |
| **Rwanda 2** | 20.6 | n.d. | 620.6 | 386.1 | 5.4 | n.d. | n.d. | n.d. | n.d. | 1032.7 |
| **Rwanda 3** | n.d. | n.d. | 672.6 | 351.3 | 6.4 | n.d. | n.d. | 42.1 | 7.3 | 1079.7 |
| **Costa Rica 1** | 49.5 | 25.6 | 666.2 | 351.3 | 11.3 | n.d. | 26.6 | 13.6 | 15.0 | 1159.1 |
| **Costa Rica 2** | n.d. | n.d. | 1569.7 | 648.7 | 3.4 | n.d. | n.d. | n.d. | n.d. | 2221.8 |
| **Costa Rica 3** | 21.5 | 16.8 | 848.1 | 288.0 | 5.9 | n.d. | n.d. | n.d. | n.d. | 1180.3 |
| **Brazil 1** | n.d. | n.d. | 215.1 | 139.2 | 4.9 | n.d. | n.d. | n.d. | 16.9 | 376.1 |
| **Brazil 2** | n.d. | n.d. | 259.8 | 132.9 | 4.9 | n.d. | n.d. | n.d. | 19.4 | 417.0 |
| **Brazil 3** | 27.0 | n.d. | 336.8 | 151.9 | 3.4 | n.d. | n.d. | n.d. | n.d. | 519.1 |
| **Brazil 4** | 81.9 | 40.1 | 567.4 | 319.6 | 6.9 | n.d. | n.d. | n.d. | n.d. | 1015.9 |
| **Ethiopia 1** | n.d. | n.d. | 5714.1 | 2417.7 | n.d. | n.d. | n.d. | 5.3 | n.d. | 8137.1 |
| **Ethiopia 2** | 45.9 | 38.2 | 2364.6 | 1255.1 | n.d. | n.d. | n.d. | n.d. | n.d. | 3703.8 |
| **Ethiopia 3** | 22.5 | n.d. | 3694.3 | 1411.4 | n.d. | n.d. | n.d. | n.d. | n.d. | 5128.2 |
| **Ethiopia 4** | 41.0 | n.d. | 5317.4 | 1708.9 | 6.4 | n.d. | 25.8 | 7.8 | 9.8 | 7117.1 |
| **India 1** | 98.6 | 29.2 | 1764.1 | 509.5 | 13.2 | n.d. | 22.6 | 8.3 | 9.8 | 2455.3 |
| **India 2** | 33.2 | n.d. | 608.9 | 310.1 | n.d. | n.d. | n.d. | 4.7 | 4.5 | 961.4 |
| **India 3** | 39.2 | n.d. | 577.8 | 468.4 | 5.4 | n.d. | n.d. | 6.7 | 4.3 | 1101.8 |

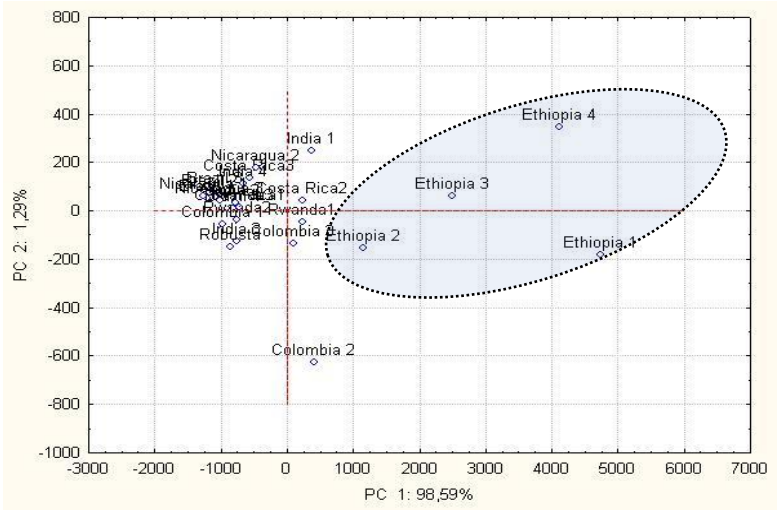
| Sample | Genistin | Daidzin | SECO | LARI | Daidzein | MAT | Genistein | Biochanin A | Formononetin | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| **India 4** | 204.8 | 95.2 | 746.7 | 281.7 | n.d. | n.d. | n.d. | n.d. | n.d. | 1328.4 |
| **Colombia 1** | n.d. | n.d. | 408.6 | 329.1 | n.d. | n.d. | n.d. | n.d. | n.d. | 737.7 |
| **Colombia 2** | n.d. | n.d. | 1503.8 | 1338.6 | 7.4 | n.d. | n.d. | n.d. | n.d. | 2849.8 |
| **Colombia 3** | 89.7 | 30.2 | 1378.8 | 775.3 | 11.3 | n.d. | 24.2 | 6.4 | 7.5 | 2323.4 |
| **Nicaragua 1** | 12.6 | 12.6 | 172.6 | 113.9 | 8.3 | n.d. | n.d. | n.d. | n.d. | 320.0 |
| **Nicaragua 2** | 39.0 | n.d. | 954.0 | 278.5 | 9.3 | n.d. | 21.8 | 7.7 | 8.7 | 1319.0 |
| **Nicaragua 3** | n.d. | n.d. | 397.8 | 218.4 | 4.9 | n.d. | n.d. | n.d. | n.d. | 621.1 |
| **robusta** | 71.1 | 50.8 | 480.0 | 458.9 | 25.3 | n.d. | 40.7 | 19.9 | 46.7 | 1193.4 |

[a] Each sample was analysed in triplicate. RSDs % in all cases were ≤ 13.8 %; [b] n.d., not detectable.

### 4.1.11 PCA

PCA scores and loading plots of the various green coffee samples analysed are reported in **Figure 7a** and **b**. The variability of data was mostly explained by the first principal component (98.59%), which was mainly influenced by the content of the two lignans SECO (values of eigenvectors: 1485; 62) and LARI (values of eigenvectors: 546; -170). Most of the samples were close to each other in the score plot (central part of the plot), as they were composed of similar amounts of phytoestrogens. However, the Ethiopian coffee samples differed significantly from the others, with higher contents of SECO and LARI, indicating that they are a good source of lignans.

**a**




**b**

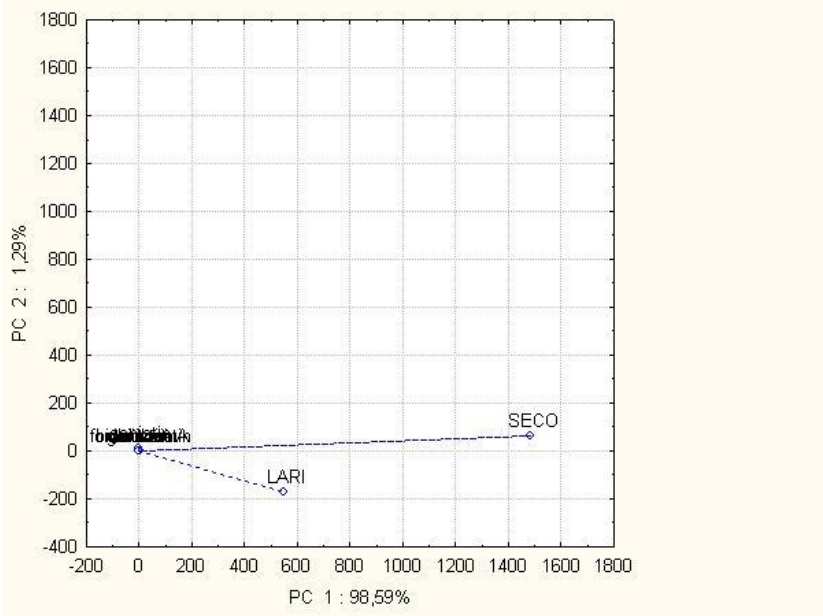
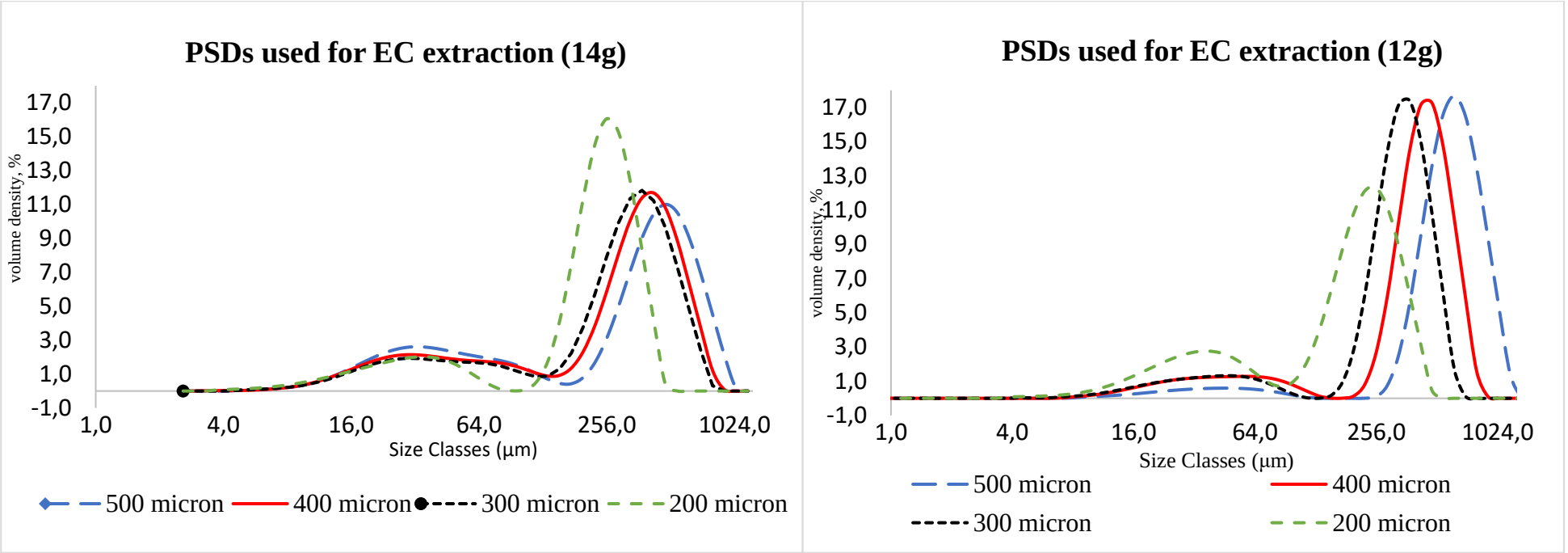



**Figure 7a** and **b.** PCA scores (**a**) and loading plots (**b**) of the green coffee samples, displaying chemical variance for lignans and isoflavones.

## 4.2 Optimization of espresso coffee extraction

### 4.2.1 Particle size analysis

The analysis of particle size distribution was performed by using Mastersizer 3000 instrument. The process of analysis was rapid due to the laser diffraction. Measurements were repeated fivefold for single sample and the mean value was used to create the Gaussian graph. The graphs (**Figure 8a** and **b**) deliver information of particles through percentage of volume density. The percentage of volume density comes through scattered light, because large particles scatter light at small angles with the laser beam, whereas small particles scatter light at large angles. This angular scattering intensity data are used to calculate the size of particles. Data analysis is based on the Mie theory (Do, Hargreaves, Wolf, Hort, & Mitchell, 2007). **Figure 8a** and **b** highlights those sizes of particles separated through vibrational sieve, which are distinguishable. These results show differences of particles (from 200 µm up to 1000 µm) used to extract EC with 14 g and 12 g, using arabica coffee and standard filter basket (A). The various percentages of volume density in both figures in similar dimensional ranges are superior and inferior, due to the prior extraction of EC during calibration, where mixed particles are used for brewing at 25 s with 25 mL of EC, using 14 g in one case and 12 g in the other. Therefore, different PSDs were employed for brewing coffee with 14 g and 12 g. Afterwards, the grinding condition was kept constant and the grinded coffee was filtered through vibrational sieve; the separated particles of different dimensions were utilized for extraction of EC. The average size of particles between 200-300 µm was 256 µm, 300-400 µm was 325 µm, 400-500 µm was 440 µm and 500-1000 µm was 580 µm.




**a** **b**

**Figure 8a** and **b**. Particle size distribution measured through Mastersizer 3000. PSDs of arabica ground coffee used for extraction of EC with 14 g (**a**) and 12 g (**b**).

### 4.2.2 Total solid (TS): influence of particle sizes and filter baskets

Total solids or also called dry matter measures the strength or concentration of coffee brew, which is the first indicator of coffee extraction efficiency. TS can be measured by drying a portion of espresso coffee and weighting the remaining solids. The remaining dry matter, in correlation with the volume of coffee, measures the strength of the beverage (Folmer, 2017). Three filter baskets, A (standard), B (180 μm sized) and C (net on boundary part) and four size range of particles (200-300 μm, 300-400 μm, 400-500 μm and 500-1000 μm) using 12 and 14 g of ground coffee were evaluated to study their influence on coffee extraction. **Table 17** shows the total solids, expressed in mg $mL^{-1}$, for 30 different EC samples, prepared varying the variables under study. Before separating different particle sizes, mixed particles of ground coffee were put in each filter basket directly from the grinding machine, as a calibration of the EC extraction. The resulting EC samples were used as a reference for the rest of samples, extracted with different sizes of particles. Starting from the standard condition (filter basket A, 14 g and mixed particles) and run down on the **Table 17** it is possible to notice that the highest content of total solids (53.7 mg $mL^{-1}$) was found using particles at 200-300 μm and a growing trend from 500-1000 to 200-300 μm (from 29.15 to 53.70 mg $mL^{-1}$) was obtained. These results are in agreement with those reported by Andueza et al., (2003) and Severini et al., (2015), i.e., higher TS levels were found when finer particles were used. Moreover, looking at averages of cup volumes, it is evident that using course particles long espressos with low levels of TS were obtained (volume: 42.5 mL, TS: 29.15 mg $mL^{-1}$ for 500-1000 μm; volume: 43.5 mL, TS: 35.15 mg $mL^{-1}$ for 400-500 μm; volume: 44 mL, TS: 32.10 mg $mL^{-1}$ for 300-400 μm). This suggests that extracting coffee with coarse particles could decrease extraction efficiency, yielding under-extracted coffee because the volume specific surface would be too small to retain enough water and allow a good coffee compounds solubilization and emulsification (Andueza et al., 2003). On the other hand, when fine particles were used to extract coffee, we obtained a comparable level of volume (28.5 mL) with the reference one but higher levels of TS, 53.70 mg $mL^{-1}$.

**Table 17.** Total solids (TS), expressed in mg $mL^{-1}$, in arabica EC samples, by using 14 and 12 g of ground coffee with various particle size distributions in three different filter baskets.

| **14 g of ground coffee** | | | | | | |
|---|---|---|---|---|---|---|
| | ***Filter A*** | | ***Filter B*** | | ***Filter C*** | |
| ***Particle sizes (μm)*** | **Volume per cup (ml)**[a] | **TS**[b] | **Volume per cup (ml)** | **TS** | **Volume per cup (ml)** | **TS** |
| **Mixed particles** | 26 | 44.60 | 26 | 52.20 | 24 | 35.96 |
| **500-1000** | 42.5 | 29.15 | 17.5 | 69.80 | 37 | 29.20 |
| **400-500** | 43.5 | 35.15 | 20.5 | 66.10 | 45.5 | 25.10 |
| **300-400** | 44 | 32.10 | 44 | 40.10 | 47 | 23.70 |
| **200-300** | 28.5 | 53.70 | 45 | 35.16 | 38 | 34.15 |
| **12 g of ground coffee** | | | | | | |
| | ***Filter A*** | | ***Filter B*** | | ***Filter C*** | |
| ***Particle sizes (μm)*** | **Volume per cup (ml)** | **TS** | **Volume per cup (ml)** | **TS** | **Volume per cup (ml)** | **TS** |
| **Mixed particles** | 26 | 46.50 | 26 | 44.30 | 25 | 43.00 |
| **500-1000** | 54 | 15.50 | 46 | 13.00 | 54 | 24.35 |
| **400-500** | 52 | 16.80 | 50 | 17.85 | 55 | 20.75 |
| **300-400** | 50 | 22.75 | 52 | 23.35 | 56 | 22.10 |
| **200-300** | 46 | 29.05 | 49 | 29.85 | 57 | 24.35 |

[a] RSD for EC volumes were 1.2-4.8 % for filter basket A, 2.2-5.7 % for B and 1.3-8.2% for C. [b] RSD for TS were 2.3-7.7 % for filter basket A, 3.6-9.2 % for B and 4.5-10.8 % for C.

Decreasing the amount of ground coffee from 14 to 12 g, determined similar level for reference samples (from 44.60 to 46.50 mg $mL^{-1}$, respectively) and a decrease of total solids in the various size ranges. Moreover, same as for 14 g, a growing trend from 500-1000 to 200-300 μm was present (from 15.5 to 29.05 mg $mL^{-1}$, volume: from 54 to 46 mL). Investigating the two prototypes of filter basket, the highest TS content, for mixed particles using 14 g, was obtained in the B prototype (52.20 mg $mL^{-1}$). With filter basket B, using 14 g of ground coffee, the highest results also for different size ranges (500-1000 μm: 69.80 mg $mL^{-1}$, 400-500 μm: 66.10 mg $mL^{-1}$, 300-400 μm: 40.10 mg $mL^{-1}$ and 200-300 μm: 35.16 mg $mL^{-1}$) were obtained. In this case, in contrast with filter A, a decreasing trend was evident. By decreasing the amount from 14 to 12 g lower levels were found, in particular in

reference samples (14-12 g: 52.20-44.30 mg $mL^{-1}$) and in 200-300 μm (14-12 g: 35.16-29.85 mg $mL^{-1}$, volume: 45-49 mL). Studying the prototype C, which possessed the net on the boundary surface, we can observe a lower level of total solids, especially in the reference and in 200-300 μm samples. Decreasing the amount of R&G coffee the samples showed good results for reference EC (from 35.96 to 43.00 mg $mL^{-1}$) but changing the sizes no large differences were notated (500-1000 μm: 24.35 mg $mL^{-1}$, volume: 54 mL; 400-500 μm: 20.75 mg $mL^{-1}$, volume: 55 mL; 300-400 μm: 22.10 mg $mL^{-1}$, volume: 56 mL; 200-300 μm: 24.35 mg $mL^{-1}$, volume: 57 mL).

### 4.2.3 Total solid (TS): influence of perforated discs

**Table 18** shows the total solids measured in different EC samples extracted with filter basket A using 12 and 14 g of arabica ground coffee, modifying the height of perforated disc (4, 5, 6, 7 mm). After calibration, the particle sizes were kept constant and the experiment was applied in two way: at first, maintaining time constant (25 s) and then, volume constant (50 ml). Eventually, a total of 16 samples were analysed. The highest levels of total solids were found with perforated disc 5 mm, time constant (97 mg $mL^{-1}$) and perforated disc 4 mm, volume constant (86 mg $mL^{-1}$), when 14 g were used. On the other hand, using 12 g high amount of TS was found in 6 mm for time constant (86.3 mg $mL^{-1}$) and 7 mm for volume constant (83.8 mg $mL^{-1}$). Considering both constants for each perforated disc the highest content of total solids were in perforated disc 4 mm (time constant: 84 mg $mL^{-1}$; volume constant: 86 mg $mL^{-1}$) and 5 mm (time constant: 97 mg $mL^{-1}$; volume constant: 83.5 mg $mL^{-1}$), when 14 g where used. Decreasing the amount of ground coffee from 14 to 12 g the best results were observed with perforated disc 5 mm (time constant: 84.5 mg $mL^{-1}$; volume constant: 77 mg $mL^{-1}$) and 6 mm (time constant: 86.3 mg $mL^{-1}$; volume constant: 74.5 mg $mL^{-1}$). Comparing perforated discs 6 and 7 mm when 14 and then 12 g were used, higher TS were found extracting coffee with less amount of coffee powder. Concluding, the best results for 14 g were obtained using 4 and 5 mm perforated disc, while for 12 g using 5 and 6 mm. Moreover, our results could suggest that decreasing the amount of coffee allow to obtain similar amount of TS when increasing the heights of perforated disc.

**Table 18.** Total solids (mg $mL^{-1}$) in EC samples prepared by brewing 14 and 12 g of R&G coffee and assembling different height of perforated discs. The extractions were performed setting time constant (25 s) and then volume constant (50 mL for two cups).

| **Filter A** | | **Total solids (TS) (mg $mL^{-1}$)** | |
|---|---|---|---|
| **Mass of ground coffee[a]** | **Perforated disc (mm)** | **Time constant (25 s)** | **Volume constant (50 mL)** |
| 14 g | 4 | 84.0 | 86.3 |
| 14 g | 5 | 97.0 | 83.5 |
| 14 g | 6 | 79.0 | 74.0 |
| 14 g | 7 | 88.0 | 78.5 |
| **Mass of ground coffee** | **Perforated disc (mm)** | **Time constant (25 s)** | **Volume constant (50 mL)** |
| 12 g | 4 | 74.0 | 73.8 |
| 12 g | 5 | 84.5 | 77.0 |
| 12 g | 6 | 86.3 | 74.5 |
| 12 g | 7 | 73.5 | 83.8 |

[a] RSD were 2.3-6.8 % for ECs extracted with 14 g and 1.8-7.2 % for ECs extracted with 12 g.

### 4.2.4 Validation of HPLC-VWD methods

The validation parameters studied were linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability and specificity (Caprioli et al., 2014, 2013). Linearity was tested by injecting five different concentrations (from 10 to 250 mg $L^{-1}$) of the three chlorogenic acids, caffeine and trigonelline, in triplicate, then plotting and calculating calibration curves with the respective determination coefficients ($R^2$). All target molecules showed good linearity, since the $R^2$ equalled or exceeded 0.9972. The LODs and LOQs of target compounds (estimated in matrix and expressed in mg $L^{-1}$) were calculated through signal to noise ratio (SNR) of 3:1 and 10:1. For chlorogenic acids LODs and LOQs were in the range of 0.06-0.08 mg $L^{-1}$ and 0.18-0.24 mg $L^{-1}$. Method repeatability was tested by injecting 5 replicates for each standard concentration over the course of five days. The intra-day repeatability or run-to-run precision and inter-day repeatability or day-to-day precision were expressed by Relative Standard Deviation (RSD) percent. The RSD ranged from 0.85 to 2.86% for run-to-run precision and from 1.25 to 3.79% for day-to-day precision. The method specificity was evaluated by measuring retention time stability for each molecule. The retention time stability was studied 5 times over a period of 5 days (n=25) and expressed by RSD. RSDs were in all cases lower than or equal to 1.68%.

### 4.2.5 Bioactive compounds in ECs: influence of particle sizes and filter baskets

The effect of particle sizes on EC extraction using 14 and 12 g in different filter baskets has been studied. EC samples (30 samples of arabica) were analysed for detecting caffeine, trigonelline and chlorogenic acids (3-CQA, 5-CQA and 3,5-diCQA). Results, expressed in mg $L^{-1}$, are shown in **Table 19** for caffeine and trigonelline and **Table 20** for chlorogenic acids. Starting from standard condition, such as filter basket A and 14 g, we found that the content of caffeine was higher in 200-300 μm (3005.68 mg $L^{-1}$) than mixed particles (2597.01 mg $L^{-1}$) and other sizes (**Table 19**). Similar behaviour was noticed for trigonelline (1557.51 mg $L^{-1}$ in 200-300 μm particle sizes and 1408.68 mg $L^{-1}$ in mixed particles). The inverse increment of caffeine and trigonelline levels, with respect to particle sizes, where found also in other papers (Andueza et al., 2003; Severini et al., 2018).

**Table 19**. Influence of particle sizes and filter baskets on EC extraction, in term of caffeine and trigonelline content (mg $L^{-1}$), using 14 and 12 g of arabica ground coffee.

| **14 g of ground coffee**[a] | | | | | | |
|---|---|---|---|---|---|---|
| ***Particle sizes (µm)*** | ***Filter A*** | | ***Filter B*** | | ***Filter C*** | |
| | **Caffeine** | **Trigonelline** | **Caffeine** | **Trigonelline** | **Caffeine** | **Trigonelline** |
| **Mixed particles** | 2597.01 | 1408.68 | 2811.88 | 1522.45 | 2034.14 | 1085.93 |
| **500-1000** | 1722.34 | 938.02 | 3536.42 | 2048.31 | 1740.54 | 942.77 |
| **400-500** | 2028.19 | 1080.76 | 3540.03 | 1907.57 | 1681.56 | 875.16 |
| **300-400** | 1881.10 | 971.36 | 1949.21 | 951.37 | 1473.81 | 796.97 |
| **200-300** | 3005.68 | 1557.51 | 2113.71 | 1013.76 | 2013.63 | 1076.15 |
| **12 g of ground coffee**[a] | | | | | | |
| ***Particle sizes (µm)*** | ***Filter A*** | | ***Filter B*** | | ***Filter C*** | |
| | **Caffeine** | **Trigonelline** | **Caffeine** | **Trigonelline** | **Caffeine** | **Trigonelline** |
| **Mixed particles** | 2894.63 | 1586.49 | 2668.92 | 1480.31 | 2420.59 | 1420.74 |
| **500-1000** | 695.87 | 421.36 | 717.87 | 430.54 | 594.32 | 397.80 |
| **400-500** | 954.59 | 550.42 | 1032.88 | 583.03 | 1182.29 | 709.79 |
| **300-400** | 955.28 | 541.38 | 1239.36 | 707.78 | 1146.04 | 667.80 |
| **200-300** | 1391.95 | 683.90 | 1490.18 | 795.91 | 1310.12 | 731.37 |

[a] RSD were 0.1-12.5 % for caffeine, 0.2-14.1 % for trigonelline using 14 g and 2.3-13.3 % and 1.8-12.6 % using 12 g.

Decreasing the amount of ground coffee from 14 to 12 g, results showed an increase in caffeine and trigonelline levels in mixed particles (from 2597.01 to 2894.63 mg $L^{-1}$and from 1408.68 to 1586.49 mg $L^{-1}$, respectively) and lower content for other sizes. Comparing the reference ECs extracted with different filter baskets, using 14 g, the highest levels of caffeine and trigonelline were found in B (2811.88 and 1522.45 mg $L^{-1}$). When filter basket B was employed, the highest levels of caffeine and trigonelline, in all different particle sizes, were found (from 500-1000 µm to 200-300 µm: 3536.42-

2113.71 mg $L^{-1}$ for caffeine and 2048.31-1013.76 mg $L^{-1}$ for trigonelline). When filter basket C was assembled into the filter holder, the lowest content of caffeine and trigonelline, in reference samples, were obtained. Moreover, we noticed a decreasing of detected compounds in all particle sizes comparing with those found in the other two filter baskets; quite good results were obtained, anyway, with 200-300 µm.

**Table 20** shows the concentration (mg $L^{-1}$) of chlorogenic acids (3-CQA, 5-CQA and 3,5-diCQA) in EC samples extracted with three filter baskets, using 12 and 14 g of different particle sizes. According to previous research studies (Labbe, Sudre, Dugas, & Folmer, 2016; Severini et al., 2015) particle size influences the amount of the extracted bioactive components. Therefore, also chlorogenic acids level can be affected from the particle sizes; our results showed that in standard condition (A, 14 g), modifying the particle sizes, the highest concentration of total CQAs was found in 200-300 µm (1507.23 mg $L^{-1}$). Hence, also for chlorogenic acids a greater extraction efficiency was obtained with fine particles, as already reported in literature (Andueza et al., 2003). Moreover, this concentration was higher than that in reference sample (1250.63 mg $L^{-1}$). Using 12 g the highest concentration of total CQAs was found in mixed particles (1415.42 mg $L^{-1}$). In reference samples the highest content of total CQAs were obtained in filber basket B (1351.76 mg $L^{-1}$). As already noticed for caffeine and trigonelline, the ECs prepared with prototype B showed also the highest concentration of total CQAs in ranges 500-1000 and 400-500 µm (1829.23 and 1750.94 mg $L^{-1}$). Decreasing the amount of R&G coffee (filter basket B) we found lower concentration of total CQAs than in 14 g. Studying also the extraction efficiency of filter basket C, the lowest levels were recovered in reference samples, with respect to those obtained with other filter baskets. The highest levels, with filter basket C, of total CQAs were found in mixed particles (12g) (1256.36 mg $L^{-1}$) and in 200-300 µm (14 g) (1039.36 mg $L^{-1}$).

**Table 20**. Influence of particle sizes and filter baskets on EC extraction, in term of chlorogenic acid content (mg $L^{-1}$), using 14 and 12 g of arabica coffee.

| ***Particle sizes (µm)*** | **14 g of ground coffee**[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***Filter A*** | | | | ***Filter B*** | | | | ***Filter C*** | | | |
| | **5-CQA** | **3-CQA** | **3,5-diCQA** | **Total CQA** | **5-CQA** | **3-CQA** | **3,5-diCQA** | **Total CQA** | **5-CQA** | **3-CQA** | **3,5-diCQA** | **Total CQA** |
| **Mixed particles** | 756.80 | 427.98 | 65.85 | 1250.63 | 825.03 | 465.01 | 61.72 | 1351.76 | 596.03 | 340.45 | 43.53 | 980.01 |
| **500-1000** | 499.17 | 287.98 | 41.18 | 828.33 | 1119.64 | 627.63 | 81.96 | 1829.23 | 499.55 | 287.38 | 35.08 | 822.01 |
| **400-500** | 591.25 | 343.74 | 49.59 | 984.58 | 1065.83 | 609.37 | 75.74 | 1750.94 | 479.52 | 276.60 | 34.60 | 790.72 |
| **300-400** | 553.76 | 318.45 | 42.89 | 915.1 | 536.29 | 317.99 | 43.53 | 897.81 | 436.64 | 256.88 | 30.47 | 723.99 |
| **200-300** | 922.54 | 511.85 | 72.84 | 1507.23 | 602.73 | 348.76 | 49.63 | 1001.12 | 631.26 | 360.93 | 47.17 | 1039.36 |
| ***Particle sizes (µm)*** | **12 g of ground coffee**[a] | | | | | | | | | | | |
| | ***Filter A*** | | | | ***Filter B*** | | | | ***Filter C*** | | | |
| | **5-CQA** | **3-CQA** | **3,5-diCQA** | **Total CQA** | **5-CQA** | **3-CQA** | **3,5-diCQA** | **Total CQA** | **5-CQA** | **3-CQA** | **3,5-diCQA** | **Total CQA** |
| **Mixed particles** | 863.35 | 479.19 | 72.88 | 1415.42 | 791.20 | 440.68 | 66.93 | 1298.81 | 767.69 | 426.80 | 61.87 | 1256.36 |
| **500-1000** | 198.83 | 116.53 | 13.91 | 329.27 | 195.45 | 115.26 | 14.36 | 325.07 | 177.67 | 106.08 | 12.13 | 295.88 |
| **400-500** | 279.64 | 161.31 | 21.39 | 462.34 | 303.27 | 171.84 | 24.96 | 500.07 | 359.37 | 207.74 | 28.20 | 595.31 |
| **300-400** | 282.76 | 163.72 | 22.21 | 468.69 | 371.78 | 216.57 | 31.70 | 620.05 | 350.49 | 200.91 | 27.27 | 578.67 |
| **200-300** | 409.84 | 224.63 | 39.06 | 673.53 | 467.53 | 259.93 | 42.49 | 769.95 | 413.99 | 233.79 | 34.15 | 681.93 |

[a] RSD were 2.2-11.6 % for 5-CQA, 3.4-9.2 % for 3-CQA and 5.7-12.1 % for 2,5-diCQA using 14 g and 2.8-8.3 %, 1.6-9.1 % and 3.7-11.7 % using 12 g.

### 4.2.6 Bioactive compounds in ECs: influence of perforated discs

A total of 16 EC samples were tested to evaluate the influence of different heights of perforated disc on coffee extraction. After calibration, the EC were prepared by maintaining constant the particle sizes but modifying the perforated disc and working in two way: fixing constant time of extraction (25 s) and fixing constant the final volume in the cup (50 mL for two espressos). Perforated disc is a metal disc that is assembled under the shower in order to adjust the distance between the coffee cake and the group shower. **Table 21** shows the content, expressed in mg $L^{-1}$, of caffeine, trigonelline and chlorogenic acids, in ECs prepared using 14 and 12 g of arabica ground coffee. With constant time the highest levels of caffeine, trigonelline and total chlorogenic acids were found using 5 mm perforated disc (5723.00, 3485.78, 2717.37 mg $L^{-1}$, respectively). The same results were obtained when the volume was kept constant (caffeine: 5500.00 mg $L^{-1}$, trigonelline: 3194.09 mg $L^{-1}$ and total CQAs: 2492.57 mg $L^{-1}$). The highest concentrations of bioactive compounds were found in 6 mm perforated disc using 12 g of ground coffee and maintaining constant time. Specifically, caffeine was 5619.39 mg $L^{-1}$, trigonelline 3227.27 mg $L^{-1}$ and total CQAs 2553.76 mg $L^{-1}$. With constant volume, the highest levels were obtained in 6 and 4 mm. Considering both constants, the best results, in term of quantitative data, were found in 5 mm perforated disc using 14 g and in 6 mm perforated disc using 12 g. These results confirm what we already found for total solid analysis: using lower amount of ground coffee to obtain similar extraction efficiency, in term of TS and bioactive compounds, when the height of perforated disc is increased.

**Table 21**. Content (mg $L^{-1}$) of caffeine, trigonelline and chlorogenic acids (3-CQA, 5-CQA and 3,5-diCQA) by brewing 14 and 12 g of R&G coffee by changing the heights of perforated disc in two condition: constant time (25 s) and constant volume (50 mL for two cups).

| ***14 g of R&G coffee*** | ***Constant time*** | | | | | |
|---|---|---|---|---|---|---|
| **Perforated disc (mm)** | **Trigonelline**[a] | **Caffeine** | **3-CQA** | **5-CQA** | **3,5-diCQA** | **Total CQA** |
| **4** | 3270.53 | 5410.67 | 870.04 | 1481.90 | 104.35 | 2456.29 |
| **5** | 3485.78 | 5723.00 | 921.73 | 1665.62 | 130.02 | 2717.37 |
| **6** | 2901.46 | 5013.90 | 770.35 | 1355.20 | 94.49 | 2220.04 |
| **7** | 3168.25 | 5715.86 | 825.75 | 1482.89 | 125.74 | 2434.38 |
| ***14 g of R&G coffee*** | ***Constant volume*** | | | | | |
| **Perforated disc (mm)** | **Trigonelline** | **Caffeine** | **3-CQA** | **5-CQA** | **3,5-diCQA** | **Total CQA** |
| **4** | 3195.26 | 5150.15 | 843.16 | 1459.85 | 99.52 | 2402.53 |
| **5** | 3194.09 | 5500.00 | 847.82 | 1517.15 | 127.60 | 2492.57 |
| **6** | 2679.81 | 4689.42 | 715.23 | 1261.86 | 88.17 | 2065.26 |
| **7** | 2673.72 | 4990.81 | 716.19 | 1281.75 | 112.17 | 2110.11 |
| ***12 g of R&G coffee*** | ***Constant time*** | | | | | |
| **Perforated disc (mm)** | **Trigonelline** | **Caffeine** | **3-CQA** | **5-CQA** | **3,5-diCQA** | **Total CQA** |
| **4** | 2832.99 | 5022.70 | 774.05 | 1373.80 | 101.75 | 2249.60 |
| **5** | 2985.79 | 5074.84 | 788.17 | 1397.53 | 109.19 | 2294.89 |
| **6** | 3227.27 | 5619.39 | 881.14 | 1548.36 | 124.26 | 2553.76 |
| **7** | 2569.56 | 4567.07 | 692.88 | 1244.54 | 112.17 | 2049.59 |
| ***12 g of R&G coffee*** | ***Constant volume*** | | | | | |
| **Perforated disc (mm)** | **Trigonelline** | **Caffeine** | **3-CQA** | **5-CQA** | **3,5-diCQA** | **Total CQA** |
| **4** | 2910.99 | 4964.10 | 804.63 | 1420.47 | 121.09 | 2346.19 |
| **5** | 2583.93 | 4448.27 | 687.39 | 1233.12 | 98.21 | 2018.72 |
| **6** | 2906.54 | 5163.92 | 807.92 | 1415.06 | 118.49 | 2341.47 |
| **7** | 2750.00 | 5048.21 | 725.10 | 1308.43 | 121.65 | 2155.18 |

[a] RSD were 0.2-13.7 % for trigonelline, 0.5-11.3 % for caffeine, 0.3-11.1 % for 3-CQA, 0.7-10.5 % for 5-CQA and 1.0-10.9 % for 3,5-diCQA.

### 4.2.7 Volatile compounds analysis

The volatile fraction of ECs was analysed trough HS-SPME-GC-MS. Just after extraction, ECs were collected into screw top vial and injected using PAL autosampler. Volatile compounds were identified by comparing the mass spectra of the analytes with those of NIST 17 Mass Spectral Library with a similarity above 85% and then, the identified compounds' areas were integrated. Relative peak area percentage of individual components was expressed as percent peak areas relative to total peak areas (RPA%) (Thammarat, Kulsing, Wongravee, Leepipatpiboon, & Nhujak, 2018; Cong Zhang, Qi, Shao, Zhou, & Fu, 2007). **Table 22** provides information about odour description and RPA% of identified volatiles which are divided into chemical classes (Caporaso, Whitworth, Cui, & Fisk, 2018; Thammarat et al., 2018). Between 3 and 40 key odorants are typically composing a specific odour code of a food, which could feature in fact more than 10000 volatiles (Dunkel et al., 2014). Furfural, furfuryl acetate, methylpyrazine, 2,5-dimethylpyrazine, 2-ethyl-6-methylpyrazine, 3-ethyl-2,5-dimethylpyrazine, pyridine and 1-hydroxy-2-propanone were found with the highest means of RPA in all samples. Signals at retention time 22.315 and 33.144 min are identified as member of Pyridine group; mean RPA of single volatiles found in this group corresponds to 7.55% of pyridine and 0.46% of 3-ethylpyridine. A key odorant of this group presents bitter, astringent, roasted and burnt aroma notes. In fact, coffee roasting defects can be associated to the presence of compounds of the pyridine family (Yang et al., 2016). The members of pyrazine group are found in extracted EC at the following mean RPA: 5.62% methylpyrazine, 4.69% 2,5-dimethylpyrazine, 3.61% ethyl pyrazine, 4.41% 2-ethyl-6-methylpyrazine, 3.95% 2-ethyl-5-methylpyrazine, 3.71% 2-ethyl-3-methylpyrazine, 0.41% 2-(n-propyl)-pyrazine, 4.13% 3-Ethyl-2,5-dimethylpyrazine, 1.49% 2-Methyl-5-propylpyrazine and 0.46% 3,5-Diethyl-2-methylpyrazine; the retention times of these compounds were within 26.816 - 37.555 min. They generate nutty, roasted, popcorn, earthy, grassy and hazelnut-like aroma notes. The components of furan group in EC were obtained at different mean RPA%: 0.78% 2-(methoxymethyl)-furan, 0.98% dihydro-2-methyl-3 (2H)-furanone, 0.99% 2-furanmethanol, 0.96% 5-ethylmethyltetrahydro-2-furanmethanol, 3.55% 1-(2-furanyl)-ethanone, 1.44% 2-N-Butylfuran,

4.56% furfuryl acetate, 3.84% 5-methylfurfural, 1.13% 2-furanmethanol propanoate, 2.77% 2-propionylfuran, 2.05% furan, 2,2'-methylenebis; these compounds were detected in the range 25.165 - 45.252 min. The general key odorants of furan groups are caramel, ethereal, rum, cocoa note, and nutty. The fluctuation in detecting the furan groups shows that EC aroma is quite dense and complex. Notably, the furan group components are higher in Arabica than in Robusta (Cordeiro, Valente, Santos, & Rodrigues, 2018). A derivative group of furans is furfural, found with 5.66% in EC aromas, and its detected retention time is 37.261 min. The generated aroma notes are almond, sweet, toasted odour and burnt. Chemicals of phenol group were 1.60% for 4-ethyl-2-methoxyphenol and 1.91% for 2-methoxyphenol, detected at retention times 45.587 and 47.139 min. Key-odorants that come with this group are mostly associated with spicy, clove-like and smoky description. RPA values for ketone and aldehyde groups were: 4.36% for 1-hydroxy-2-propanone, 0.92% for 3-hydroxybutanone and 1.89% for benzaldehyde. Normally, the aroma of these groups is close to buttery, alcoholic-fruity, almond, and fruity (Niu et al., 2011). Terpene alcohol groups, as linalool with 0.48%, mostly present sweet, fruity and citrus notes. Components of pyrrole groups, as 2-formyl-1-pyrrole (0.68%), 2-acetylpyrrole (1.04%) and 1-furfurylpyrrole (2.05%) in EC aroma, release nutty, musty, hay-like, mushroom-like, and herbaceous notes (Yang et al., 2016). They are detected from 42.028 to 46.652 min. Volatile compounds found in different EC samples were compared by extraction conditions and PCA was applied to evaluate the relationship among studied variables.

**Table 22.** Tentative volatile compounds of EC samples by using 14 and 12 g of ground coffee in various filter baskets at 200-300 µm and 400-500 µm obtained by HS-SPME-GC-MS, mean relative peak area (RPA) percentage, retention time (Rt) of volatiles and odour description (OD).

| Tentative compounds | A 500-400 µm, 14g | A 500-400 µm, 12g | A 300-200 µm, 14g | A 300-200 µm, 12g | B 500-400 µm, 14g | B 500-400 µm, 12g | B 300-200 µm, 14g | B 300-200 µm, 12g | C 500-400 µm, 14g | C 500-400 µm, 12g | C 300-200 µm, 14g | C 300-200 µm, 12g | Mean RPA, % | Rt (m) | OD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Furans* | | | | | | | | | | | | | | | |
| 2-(methoxymethyl)-furan | 0.84 | 1.25 | 0.84 | 1.01 | 0.68 | 0.55 | 0.61 | 0.5 | 0.54 | 1.20 | 0.57 | 0.79 | 0.78 | 25.165 | Coffee |
| Dihydro-2-methyl-3 (2H)-furanone | 0.49 | 1.14 | 1.40 | 1.16 | 1.08 | 1.13 | 1.06 | 0.80 | 0.84 | 0.94 | 0.86 | 0.82 | 0.98 | 26.591 | Bready |
| 2-furanmethanol | 0.45 | 0.96 | 1.05 | 1.01 | 1.06 | 1.32 | 1.12 | 1.01 | 0.96 | 1.00 | 0.98 | 0.98 | 0.99 | 36.408 | Chocolatey |
| Furfural | 5.97 | 5.45 | 5.83 | 5.53 | 5.18 | 3.03 | 4.85 | 5.76 | 5.95 | 6.96 | 6.22 | 7.23 | 5.66 | 37.261 | Bready |
| 5 ethylmethyltetrahydro-2 furanmethanol | 0.92 | 0.99 | 1.08 | 1.07 | 1.02 | 1.03 | 0.92 | 0.94 | 0.91 | 0.90 | 0.91 | 0.77 | 0.96 | 37.868 | - |
| 1-(2-furanyl)- ethanone | 3.53 | 3.36 | 3.53 | 3.43 | 3.42 | 3.75 | 3.30 | 3.68 | 3.79 | 3.65 | 3.51 | 3.60 | 3.55 | 39.158 | Balsamic |
| 2-N-Butylfuran | 1.53 | 1.50 | 2.10 | 1.92 | 1.42 | 1.39 | 1.26 | 1.21 | 1.09 | 1.38 | 1.19 | 1.25 | 1.44 | 39.663 | - |
| Furfuryl acetate | 4.42 | 4.39 | 4.54 | 4.39 | 4.46 | 4.91 | 4.10 | 4.67 | 4.79 | 4.87 | 4.41 | 4.75 | 4.56 | 40.224 | Fruity |
| 5-methylfurfural | 3.73 | 3.87 | 4.12 | 5.04 | 3.64 | 3.62 | 3.03 | 3.95 | 4.09 | 4.09 | 4.67 | 2.25 | 3.84 | 41.197 | Caramelly |
| 2-Propionylfuran | 3.06 | 3.17 | 3.56 | 3.36 | 2.85 | 2.20 | 1.93 | 2.69 | 2.66 | 2.75 | 2.44 | 2.54 | 2.77 | 41.715 | - |
| 2-Furanmethanol propanoate | 1.30 | 1.70 | 1.93 | 1.68 | 1.22 | 1.01 | 0.45 | 0.72 | 1.16 | 1.13 | 0.66 | 0.56 | 1.13 | 41.981 | Fruity |

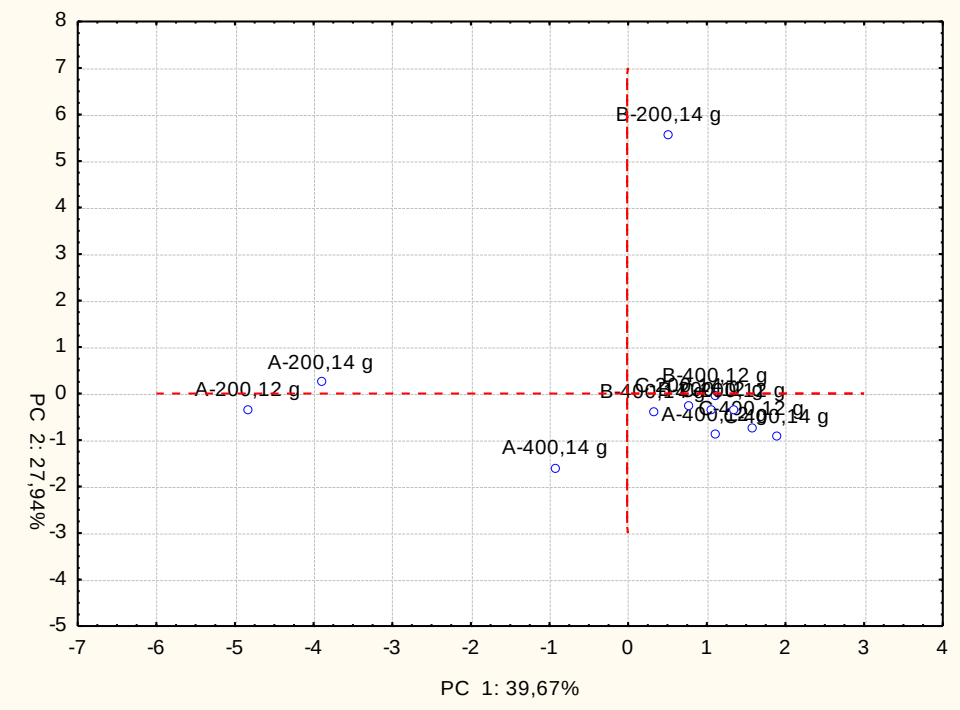
| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Furan, 2,2'-methylenebis | 1.95 | 1.98 | 2.47 | 1.92 | 2.02 | 2.21 | 1.78 | 2.06 | 1.97 | 2.24 | 1.95 | 2.03 | 2.05 | 45.252 | Roasted |
| Total percentage (furans) | 28.19 | 29.76 | 32.45 | 31.52 | 28.05 | 26.15 | 24.41 | 27.99 | 28.75 | 31.11 | 28.37 | 27.57 | 28.69 | | |
| *Pyrazines* | | | | | | | | | | | | | | | |
| Methylpyrazine | 6.77 | 5.54 | 6.38 | 6.57 | 5.03 | 5.69 | 4.86 | 5.27 | 5.62 | 5.20 | 5.28 | 5.26 | 5.62 | 26.816 | Nutty |
| 2,5-Dimethylpyrazine | 4.85 | 4.51 | 4.86 | 4.57 | 5.74 | 4.94 | 4.43 | 4.51 | 4.56 | 4.65 | 4.37 | 4.33 | 4.69 | 29.954 | Chocolatey |
| Ethyl pyrazine | 3.67 | 3.46 | 3.53 | 3.67 | 3.49 | 3.83 | 3.44 | 3.37 | 3.69 | 3.59 | 3.15 | 4.37 | 3.61 | 30.577 | Nutty |
| 2,3-Dimethylpyrazine | 0.97 | 0.92 | 1.04 | 0.98 | 0.90 | 0.98 | 0.91 | 0.83 | 0.88 | 0.89 | 0.83 | 0.81 | 0.91 | 31.249 | Nutty |
| 2-Ethyl-6-methylpyrazine | 4.44 | 4.41 | 4.40 | 4.37 | 4.37 | 4.80 | 4.13 | 4.33 | 4.51 | 4.55 | 4.34 | 4.24 | 4.41 | 33.379 | Potato |
| 2-Ethyl-5-methylpyrazine | 3.80 | 4.06 | 4.18 | 4.08 | 4.06 | 4.10 | 3.64 | 3.62 | 4.08 | 3.95 | 3.66 | 4.14 | 3.95 | 33.702 | - |
| 2-Ethyl-3-methylpyrazine | 3.78 | 3.72 | 4.02 | 3.77 | 3.70 | 3.92 | 3.51 | 3.63 | 3.70 | 3.70 | 3.64 | 3.43 | 3.71 | 34.392 | Nutty |
| 2-(n-propyl)-pyrazine | 0.40 | 0.45 | 0.49 | 0.45 | 0.41 | 0.46 | 0.41 | 0.34 | 0.40 | 0.39 | 0.37 | 0.37 | 0.41 | 35.082 | - |
| 3-Ethyl-2,5-dimethylpyrazine | 4.59 | 4.12 | 4.18 | 4.13 | 4.05 | 3.88 | 3.90 | 4.10 | 4.20 | 4.28 | 4.12 | 4.06 | 4.13 | 36.527 | Roasty |
| 2-Methyl-5-propylpyrazine | 1.59 | 1.59 | 1.66 | 1.69 | 1.33 | 1.54 | 1.38 | 1.30 | 1.09 | 1.62 | 1.58 | 1.46 | 1.49 | 37.408 | - |
| 3,5-Diethyl-2-methylpyrazine | 0.48 | 0.51 | 0.50 | 0.48 | 0.50 | 0.51 | 0.48 | 0.44 | 0.41 | 0.37 | 0.48 | 0.37 | 0.46 | 37.555 | Nutty |
| Total percentage (pyrazine) | 35.34 | 33.29 | 35.24 | 34.76 | 33.58 | 34.65 | 31.09 | 31.74 | 33.14 | 33.19 | 31.82 | 32.84 | 33.39 | | |
| *Pyrroles* | | | | | | | | | | | | | | | |
| 2-Formyl-1-pyrrole | 0.30 | 0.54 | 0.73 | 0.64 | 0.60 | 0.85 | 1.37 | 0.62 | 0.74 | 0.59 | 0.61 | 0.55 | 0.68 | 42.028 | Roasted |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2-Acetylpyrrole | 1.48 | 1.21 | 1.55 | 1.06 | 0.91 | 0.87 | 0.78 | 0.98 | 0.93 | 0.96 | 0.84 | 0.96 | 1.04 | 46.652 | Musty |
| 1-Furfurylpyrrole | 1.95 | 1.98 | 2.47 | 1.92 | 2.02 | 2.21 | 1.78 | 2.06 | 1.97 | 2.24 | 1.95 | 2.03 | 2.05 | 45.252 | Vegetable |
| Total percentage (pyrroles) | 3.73 | 3.73 | 4.75 | 3.62 | 3.53 | 3.93 | 3.93 | 3.66 | 3.64 | 3.79 | 3.4 | 3.54 | 3.77 | | |
| *Pyridines* | | | | | | | | | | | | | | | |
| Pyridine | 7.98 | 7.24 | 9.27 | 8.83 | 7.54 | 7.50 | 7.18 | 7.84 | 6.52 | 5.89 | 6.36 | 8.39 | 7.55 | 22.315 | Fishy |
| 3-Ethylpyridine | 0.49 | 0.40 | 0.49 | 0.52 | 0.42 | 0.55 | 0.41 | 0.42 | 0.42 | 0.50 | 0.40 | 0.45 | 0.46 | 33.144 | - |
| Total percentage (pyridine) | 8.47 | 7.64 | 9.76 | 9.35 | 7.96 | 8.05 | 7.59 | 8.26 | 6.94 | 6.39 | 6.76 | 8.84 | 8.00 | | |
| *Phenolic compounds* | | | | | | | | | | | | | | | |
| 4-Ethyl-2-methoxyphenol | 0.90 | 1.89 | 1.37 | 1.40 | 1.38 | 1.34 | 1.28 | 1.78 | 1.98 | 1.96 | 1.93 | 1.97 | 1.60 | 45.587 | Spicy |
| 2-Methoxyphenol | 0.85 | 1.42 | 1.75 | 1.35 | 1.31 | 1.42 | 7.02 | 1.60 | 1.36 | 1.59 | 1.45 | 1.74 | 1.91 | 47.139 | Phenolic |
| Total percentage (phenolic compounds) | 1.75 | 3.31 | 3.12 | 2.75 | 2.69 | 2.76 | 8.3 | 3.38 | 3.34 | 3.55 | 3.38 | 3.71 | 3.50 | | |
| *Esters* | | | | | | | | | | | | | | | |
| Isobutyl acetate | 0.43 | 0.65 | 0.15 | 1.19 | 0.90 | 0.35 | 0.15 | 0.30 | 0.22 | 0.16 | 0.17 | 0.14 | 0.40 | 37.038 | Fruity |
| Total percentage | 0.43 | 0.65 | 0.15 | 1.19 | 0.90 | 0.35 | 0.15 | 0.30 | 0.22 | 0.16 | 0.17 | 0.14 | 0.40 | | |
| *Ketones* | | | | | | | | | | | | | | | |
| 1-Hydroxy-2-propanone | 4.67 | 5.89 | 0.92 | 0.63 | 4.64 | 5.54 | 3.69 | 5.38 | 6.08 | 5.25 | 4.19 | 5.42 | 4.36 | 35.491 | Caramelly |
| 3-Hydroxybutanone | 1.01 | 1.04 | 1.00 | 1.14 | 0.98 | 0.97 | 0.93 | 1.27 | 0.83 | 0.87 | 0.88 | 0.91 | 0.99 | 40.072 | Buttery |

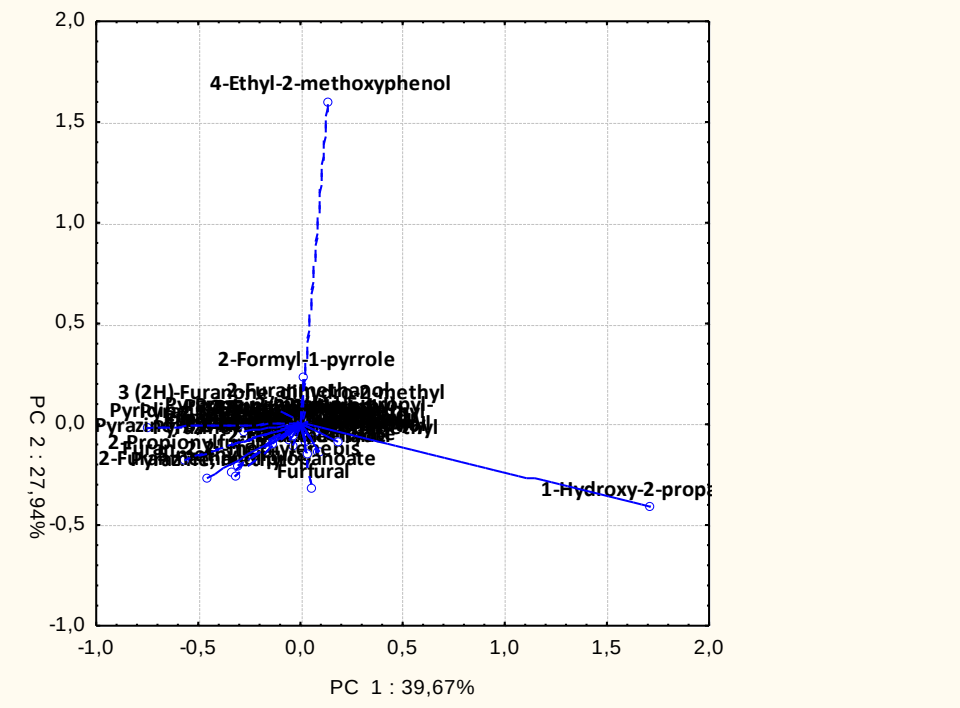
| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total percentage | 5.68 | 6.93 | 1.92 | 1.77 | 5.62 | 6.51 | 4.62 | 6.65 | 6.91 | 6.12 | 5.07 | 6.33 | 5.34 | | |
| *Aldehydes* | | | | | | | | | | | | | | | |
| Benzaldehyde | 1.98 | 1.93 | 1.69 | 1.78 | 1.82 | 1.89 | 1.48 | 1.82 | 1.91 | 2.25 | 1.97 | 2.19 | 1.89 | 39.779 | Almond |
| Total percentage | 1.98 | 1.93 | 1.69 | 1.78 | 1.82 | 1.89 | 1.48 | 1.82 | 1.91 | 2.25 | 1.97 | 2.19 | 1.89 | | |
| *Terpenes* | | | | | | | | | | | | | | | |
| Linalool | 0.31 | 0.46 | 0.43 | 0.42 | 0.49 | 0.56 | 0.48 | 0.52 | 0.53 | 0.50 | 0.57 | 0.44 | 0.48 | 40.541 | Fruity |
| Total percentage | 0.31 | 0.46 | 0.43 | 0.42 | 0.49 | 0.56 | 0.48 | 0.52 | 0.53 | 0.50 | 0.57 | 0.44 | 0.48 | | |
| **Total percentage of all compounds** | **85.88** | **87.70** | **89.51** | **87.16** | **84.64** | **84.85** | **82.05** | **84.32** | **85.38** | **87.06** | **81.51** | **85.60** | **85.47** | | |

### 4.2.8 Principal component analysis (PCA)

The principal component analysis was applied to evaluate the relationship among the different particle sizes (200 μm and 400 μm) and three different filter baskets (A, B and C), by reducing the amount of ground coffee in the filter baskets from 14 to 12 g (**Figure 9a**). Statistical data analysis indicates that filter baskets play a key role in brewing coffee. Three filter baskets, named A, B and C, proved that lowering the amount of ground coffee at the same particle size had little impact on the release of volatile compounds. With filter basket A, left side of **Figure 9a**, 14 grams of coffee are capable of generating nearly the same percentage of volatile compounds produced by 12 grams at the same size of particles. Indeed, the two filter baskets B and C show no significant percentage variance of volatile compounds with different particle sizes at 12 and 14 g of ground coffee. **Figure 9b** illustrates the percentage variance of volatiles in PCA. The variables most contributing to data variability were 1-hydroxy-2-propanone (caramelly) on the first principal component and 4-ethyl-2-methoxyphenol (spicy) on the second principal component. They explained 67.61% of variance. Most of samples bearing baskets B and C were positively correlated with toasty caramel aroma, whereas only one sample with 200 μm mesh size and equipped with basket B was characterized by spicy aroma. Finally, the samples on left side of **Figure 9b** were those from two samples with 200 μm mesh size and equipped with basket A; they were correlated with several compounds such as pyrazine, furan, pyridine, carboxylic acid, aldehyde, alcohol and pyrrole groups.




**a**



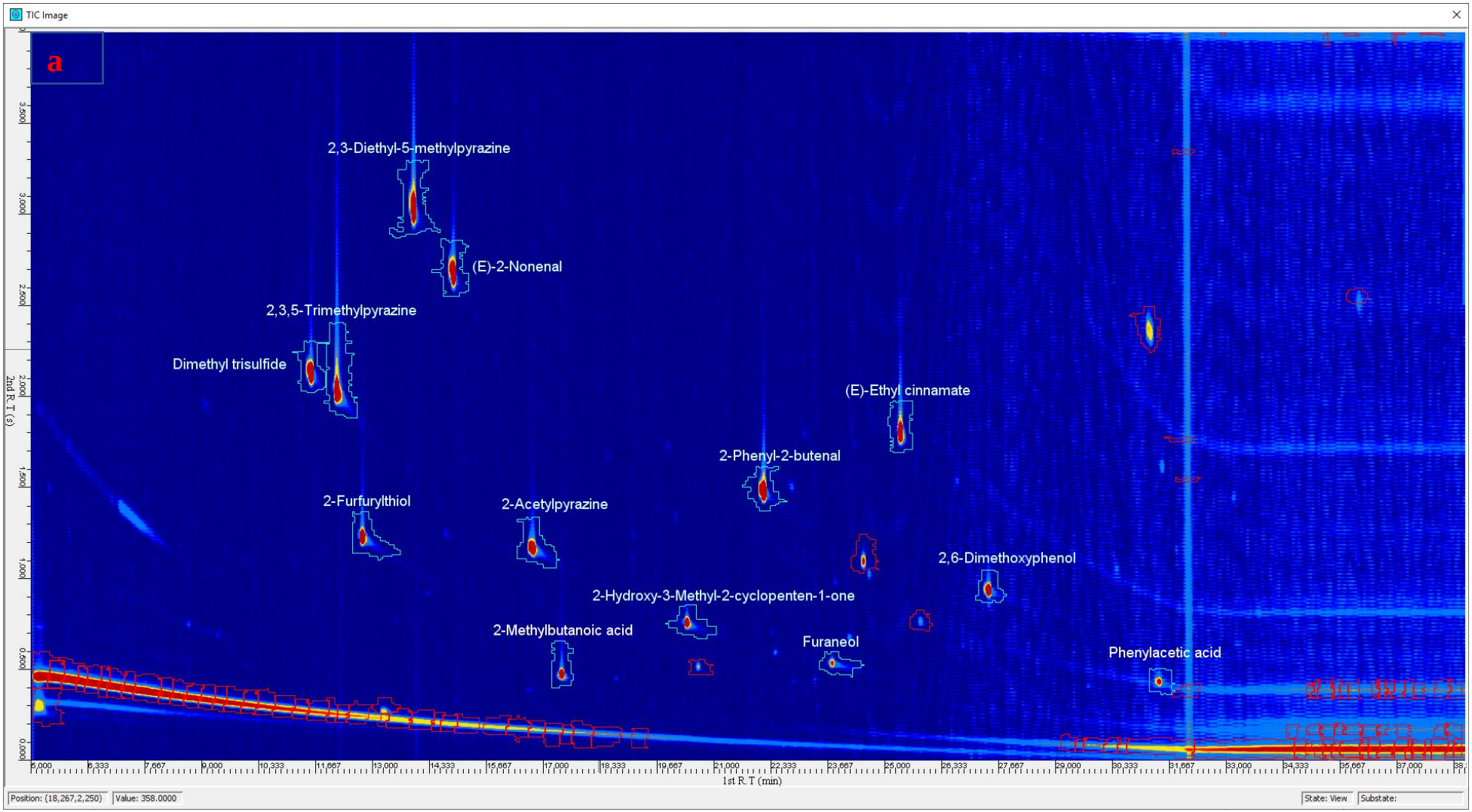

**b**

**Figure 9a** and **b.** Principal component analysis (PCA) of different filter baskets with 12 and 14 g of ground coffee used for extraction of EC.

## 4.3 Studies on Coffee Silverskin

### 4.3.1 Volatile identification

The first identification step of odor-active compounds in coffee silverskin and coffee beans were performed by comparing the retention indices and the odor descriptions of the odorants present in different chromatogram regions, recorded during the AEDA, to outcomes obtained in previous works of coffee odorants (Sanz, Czerny, Cid, & Schieberle, 2002; Scheidig, Czerny, & Schieberle, 2007) and to date on roughly 1600 food odorants, compiled in an in-house database provided by "Leibniz-Institute for Food Systems Biology". In case of matching, authentic reference compounds were injected into GC-O/FID to confirm the proposed structure. The second step of identification concerned the GC-O comparative analysis of the concentrated volatile extracts and reference compounds using a second column with different polarity (DB-5). Finally, to confirm the proposed structure, samples were analysed by GCxGC-ToF. Before injection, volatile extracts of CS and CB were separated in different fractions. Acid-base extraction permitted the separation of acid volatile fraction (AF) from basic and neutral volatile fraction (BNF). The volatiles in the latter fraction were further separated in five fractions (BNFA-E) through silica gel liquid chromatography. In a separate experiment, the thiols in CS and CB were selectively separated by covalent trapping using mercurated agarose gel (TF). Each fraction was, then, analysed by gas chromatography-olfactometry to detect the odorants previously identified and by comprehensive two-dimensional gas chromatography-mass spectrometry, together with reference compounds, to confirm the odorant structures, using both capillary columns such as DB-FFAP and DB-5. As an example, **Figure 10a**, **b** reports the TIC of 2D-GC-MS plots of a reference compounds mixture of thirteen reference compounds (**a**) and a sample of coffee silverskin AF (**b)**.


TIC Image
a
2,3-Diethyl-5-methylpyrazine
(E)-2-Nonenal
2,3,5-Trimethylpyrazine
Dimethyl trisulfide
(E)-Ethyl cinnamate
2-Phenyl-2-butenal
2-Furfurylthiol
2-Acetylpyrazine
2,6-Dimethoxyphenol
2-Hydroxy-3-Methyl-2-cyclopenten-1-one
2-Methylbutanoic acid
Furaneol
Phenylacetic acid
2nd R. T (s)
1st R. T (min)
Position: (18,267,2,250)
Value: 358.0000
State: View
Substate:

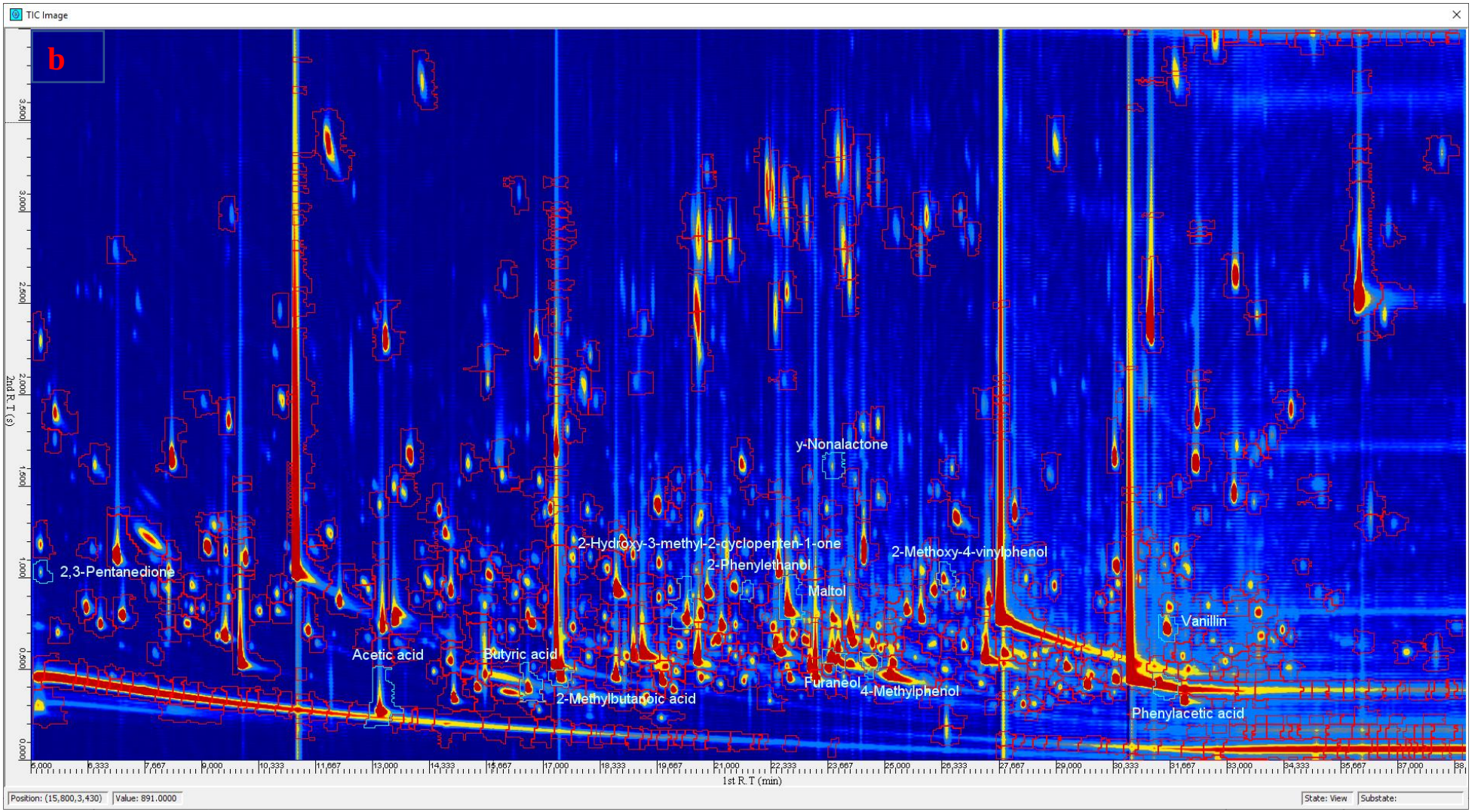



**Figure 10a, b.** The two plots report the 2D-GC-MS total ion current chromatogram (TIC) of a mixture of thirteen reference compounds (**a**) and a sample of silverskin acid volatile fraction (**b**). The regions highlight in sky blue represented the identified compounds.

### 4.3.2 Odor-active compounds in Coffee Silverskin

The characterization of odor-active compounds in coffee silverskin was based on the concordance of retention indices measured in two columns, odor descriptions and mass spectrums with respective data obtained from the analysis of authentic reference compounds. **Table 23** shows all odors smelt using GC-O system and the assigned odor structures (odorants) with their retention indices calculated in DB-FFAP and DB-5 column, the odor descriptions and the FD factors. A case of unseparated odorants was for 3-methylbutanoic acid and 2-methybutanoic acid, which were characterized by cheesy aroma. These two compounds were not separated in DB-FFAP column as well as in DB-5; the MS studies demonstrated the present of both isomers in silverskin aroma fraction. In the other hand, we can only assert the presence of 2-methylbutanal or 3-methylbutanal or both isomers. A total of 38 odorants have been identified in coffee silverksin. The odorants with the highest FD factors were furaneol with 8192, 2-methoxy-4-vinylphenol (4-vinylguaiacol), 4096, and 2-methoxyphenol (guaiacol), 1024. The first, belonging to furanone class, possessed caramel-like notes, while the others of phenol group were described as clove-like and phenolic aroma. These volatiles were reported in several studies as important odor-active compounds, which contribute to coffee flavour (Bicchi et al., 1997; Blank et al., 1991; Czerny et al., 1999; Semmelroch & Grosch, 1996; Sunarharum et al., 2014). High FD factors, from 512 to 128, were also found for 4-methyloctanoic acid, 512, trans-4,5-epoxy-(E)-2-decenal, 256, vanillin, 256, methional, 128, 2-isobutyl-3-methoxypyrazine, 128, 2-/3-methylbutanoic acid, 128, and phenylacetic acid, 128. 4-Methyloctanoic acid is a 4-alkyl-branched-chain fatty acids (vBCFAs), which are responsible for the goaty-sheepy flavour of sheep and goat milk (Teng, Reis, Ma, & Day, 2018) and for the first time, we identified this fatty acid in coffee and coffee products. Trans-4,5-epoxy-(E)-2-decenal is an important volatile compounds associated to the odor metallic and blood-like; some behavioural studies reported that mammalians predators are as attracted from this single volatile compound as they are from the odor of real blood (Lahger & Laska, 2018; Nilsson et al., 2014). It has been identified in a coffee surrogate, namely chicory coffee (Wu & Cadwallader, 2019) but, to the best of our knowledge, never in coffee and coffee beverages. Vanillin,

methional, 2-isobutyl-3-methoxypyrazine, 2-/3-methylbutanoic acid and phenylacetic acid are common odorants reported in coffee beans and brews (Blank et al., 1991; Czerny et al., 1999; Miyazato, Nakamura, Hashimoto, & Hayashi, 2013b). With FD factors from 64 to 16, fifteen volatiles were identified in coffee silverskin: 2-furfurylthiol, 2,3-diethyl-5-methylpyrazine, (E)-2-nonenal, (E,E)-2,4-decadienal, maltol, y-nonalactone, 2-isopropyl-3-methoxypyrazine, y-decalactone, 3-methylindole, 2,3-butanedione, 2-acetyl-1-pyrroline, dimethyl trisulfide, acetic acid, 2-acetylthiazole and 4-methylphenol. 2-Furfurylthiol and 2-isopropyl-3-methoxypyrazine, are important volatiles of coffee aroma which possessed, the first, coffee-like, roasty and pungent odor and, the second, green-like, earthy aroma. (Blank et al., 1991; Buffo & Cardelli-Freire, 2004; Sanz, Czerny, et al., 2002). Moreover, for 2-furfurylthiol was reported a high Odor Activity Value (OAV) in arabica and robusta coffee. (Buffo & Cardelli-Freire, 2004; Semmelroch & Grosch, 1996). The other odor-active compounds, with FD factor 64-16, have been already described in roasted beans and coffee beverages, except for y-nonalactone. This lactone has never been detected in those matrices but some studies reported its content in green beans (Gonzalez-Rios et al., 2007b; Holscher & Steinhart, 1995). Volatiles identified in coffee silverskin with low FD factors (8-1) were 3-methyl-2-buten-1-thiol, 2,3,5-trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, 2-acetylpyrazine, 2-acetyl-2-thiazoline, 2-hydroxy-3-methyl-2-cyclopenten-1-one, 2-phenyl-2-butenal, indole, 2-/3-methylbutanal, butyric acid, 2-phenylethanol, 2,3-pentanedione and linalool. All of these volatiles have been identified in previous works on coffee (Gonzalez-Rios et al., 2007a; Lee et al., 2017; Ryan et al., 2004).

**Table 23.** Odors and Odorants described and identified in Coffee Silverskin with their RIs and FD factors measured in DB-FFAP.

| No. | Odorant | Odor | RI[b] | | FD factor |
|---|---|---|---|---|---|
| | | | FFAP | DB-5 | |
| 1 | 2-/3-methylbutanal[a] | malty | 953 | 659 | 4 |
| 2 | 2,3-Butanedione[a] | butter | 980 | 606 | 16 |
| 3 | 2,3-Pentanedione | butter | 1055 | 700 | 2 |
| 4 | 3-Methyl-2-buten-1-thiol[a] | garlic-like, thiol | 1097 | 819 | 8 |
| 5 | unknown | mushroom-like | 1293 | 983 | 16 |
| 6 | 2-Acetyl-1-pyrroline[a] | roasty | 1330 | 922 | 16 |
| 7 | Dimethyl trisulfide | spicy, cabbage, sulfurous | 1364 | 967 | 16 |
| 8 | 2,3,5-Trimethylpyrazine | fatty, roasty, earthy | 1398 | 1007 | 8 |
| 9 | 2-Isopropyl-3-methoxypyrazine[a] | green, earthy | 1420 | 1093 | 32 |
| 10 | 2-Furfurylthiol[a] | pungent, coffee-like | 1427 | 907 | 64 |
| 11 | 3-Ethyl-2,5-dimethylpyrazine | roasty, popcorn, earthy | 1439 | 1084 | 8 |
| 12 | Acetic acid | pungent, vinegar | 1443 | 624 | 16 |
| 13 | Methional[a] | cooked potato | 1452 | 904 | 128 |
| 14 | 2,3-Diethyl-5-methylpyrazine | roasty, fatty | 1480 | 1157 | 64 |
| 15 | unknown | meaty, fatty | 1495 | 1145 | 16 |
| 16 | 2-Isobutyl-3-methoxypyrazine[a] | green pea-like, green bell pepper | 1512 | 1177 | 128 |
| 17 | (E)-2-Nonenal | greasy, green, roasty | 1524 | 1157 | 64 |
| 18 | Linalool | sweet, fruity, citrus | 1536 | 1102 | 1 |
| 19 | unknown | fatty, roasty | 1581 | - | 32 |
| 20 | Butyric acid | sweaty, cheese | 1618 | 819 | 4 |
| 21 | 2-Acetylpyrazine | roasty | 1625 | 1020 | 8 |
| 22 | 2-Acetylthiazole | roasty | 1638 | 1020 | 16 |
| 23 | 2-/3-Methylbutanoic acid | cheese-like | 1658 | 860 | 128 |
| 24 | unknown | meaty, greasy | 1693 | 1212 | 32 |
| 25 | unknown | sweet | 1703 | - | 2 |
| 26 | unknown | silverskin-like | 1718 | - | 512 |
| 27 | unknown | spicy | 1724 | - | 32 |
| 28 | unknown | spicy, sweaty | 1734 | - | 2 |
| 29 | 2-Acetyl-2-thiazoline | roasty | 1750 | 1100 | 8 |
| 30 | (E,E)-2,4-Decadienal | meaty, gravy-like | 1800 | 1315 | 64 |
| 31 | 2-Hydroxy-3-methyl-2-cyclopenten-1-one | spicy, burnt paper, smoky | 1827 | 1029 | 8 |
| 32 | unknown | pungent, spicy, clove-like | 1832 | - | 128 |

| | | | | | |
|---|---|---|---|---|---|
| 33 | 2-Methoxyphenol | clove-like, phenolic | 1854 | 1087 | 1024 |
| 34 | unknown | clove-like, sweet, spicy | 1876 | - | 16 |
| 35 | unknown | citrus, metallic, sweet | 1895 | - | 2 |
| 36 | 2-Phenylethanol | sweet, honey | 1906 | 1111 | 4 |
| 37 | 2-Phenyl-2-butenal | green, phenolic | 1926 | 1278 | 8 |
| 38 | unknown | smoky | 1957 | 1123 | 64 |
| 39 | Maltol | caramel-like | 1969 | 1115 | 64 |
| 40 | unknown | sweet | 1986 | - | 1 |
| 41 | trans-4,5-Epoxy-(E)-2-decenal[a] | metallic | 1997 | 1377 | 256 |
| 42 | y-Nonalactone | fruity, coconut | 2026 | 1364 | 64 |
| 43 | Furaneol | caramel-like | 2032 | 1064 | 8192 |
| 44 | unknown | burnt paper, seasoning, | 2059 | - | 128 |
| 45 | 4-Methylphenol | fecal, horse stable-like, pee-like | 2079 | 1080 | 16 |
| 46 | 4-Methyloctanoic acid[a] | goaty, sheepy | 2091 | - | 512 |
| 47 | unknown | fenolic, seasoning | 2128 | 1469 | 8 |
| 48 | unknown | sweet | 2134 | - | 4 |
| 49 | y-Decalactone[a] | peach-like, lemon-like | 2147 | 1471 | 32 |
| 50 | unknown | rubber-like | 2178 | - | 32 |
| 51 | 2-Methoxy-4-vinylphenol | clove-like | 2197 | 1315 | 4096 |
| 52 | unknown | burnt paper, rubber-like, | 2228 | - | 16 |
| 53 | unknown | mouldy | 2247 | - | 16 |
| 54 | unknown | seasoning, phenolic | 2269 | 1357 | 128 |
| 55 | unknown | phenolic, burnt, spicy | 2297 | - | 8 |
| 56 | unknown | mouldy | 2331 | - | 8 |
| 57 | unknown | seasoning, phenolic | 2345 | - | 4 |
| 58 | Indole | fecal | 2448 | 1298 | 8 |
| 59 | 3-Methylindole | fecal | 2493 | 1387 | 32 |
| 60 | unknown | rubber-like, burnt | 2517 | - | 8 |
| 61 | Phenylacetic acid | honey, | 2555 | 1259 | 128 |
| 62 | Vanillin | vanilla, chocolate-like | 2572 | 1402 | 256 |
| 63 | unknown | saliva-like | 2619 | - | 64 |

[a] GCxGC-MS analysis did not result in a clear mass spectrum, but comparison of retention index and odor quality with respective data of an authentic reference compound allowed for unequivocal structure assignment. [b] Retention index (RI), calculated from the retention time of the compound and the retention times of adjacent n-alkanes by linear interpolation.

### 4.3.3 Odor-active compounds in Coffee Beans and comparison with CS

The GC-O/FID analysis of concentrated volatile extract of coffee beans resulted in a large number of odors (about 150, 1≤FD≤16384) and almost 2.5 times more than those of coffee silverskin (63 odors, 1≤FD≤8192). For the aroma comparison it was necessary to keep, during the extraction process, the same amount of two matrices and the same solvent/sample ratio. Therefore, we analysed through GC-O/FID the stepwise diluted (1:2, 1:4, 1:8, 1:16) volatile extracts of coffee beans. In sixteen times diluted sample (1:16) we found a reasonable number of odors for GC-O analysis and we chose this one as starting point of our studies. **Table 24** shows the odors and the identified compounds (odorants) in coffee silverskin and beans with their retention indices, odor description and FD factor. We can notice that in coffee beans almost all odorants occurred with higher FD factor than the respective compound in coffee silverskin. Some volatiles were identified only in coffee beans, such as 2-ethyl-5-methylpyrazine, 6-acetyl-2,3,4,5-tetrahydropyridine, 5-methyl-2-methoxyphenol and 3-ethylphenol. To the best of our knowledge, for the first time, we found in coffee beans 5-methyl-2-methoxyphenol, a phenol derivate, and 6-acetyl-2,3,4,5-tetrahydropyridine, a pyridine derivate. It has been reported that phenol compounds can be formed during roasting process from quinic and caffeic acid and maybe also 5-methyl-2-methoxyphenol could be formed from this coffee molecules. (Dorfner, Ferge, Kettrup, Zimmermann, & Yeretzian, 2003; Moon & Shibamoto, 2010). 6-acetyl-2,3,4,5-tetrahydropyridine is commonly present in the aroma fraction of baked products, e.g., bread, pretzels, etc and possesses a roasty and popcorn odor (De Kimpe & Stevens, 1993; Schoenauer & Schieberle, 2019). 3-ethylphenol was described, after evaluation on the sniffing port, as phenolic, clove-like and has been already found in coffee (Miyazato, Nakamura, Hashimoto, & Hayashi, 2013a; Ryan et al., 2004). In the other hand, two odorants were identified only in coffee silverskin, such as 2-phenylethanol and y-decalactone. The ethanolic compounds was described as sweet and honey-like while the lactone possessed peach-like and lemon-like notes. The most powerful odor, in term of FD factor, found in coffee beans were: 2-methoxyphenol, 16384, furaneol, 16384, and 2-methoxy-4-vinylphenol, 8192. These molecules were the most intense in CS as well. Other thirteen identified

compounds occurred with high values of FD factors (from 1024 to 4096): 3-methyl-2-buten-1-thiol, 3-ethyl-2,5-dimethylpyrazine, butyric acid, 2-/3-methylbutanoic acid, (E,E)-2,4-decadienal, y-nonalactone, 2-isopropyl-3-methoxypyrazine, 2-furfurylthiol, 2,3-diethyl-5-methylpyrazine, 6-acetyl-2,3,4,5-tetrahydropyridine, 4-methyloctanoic acid, methional and 2-isobutyl-3-methoxypyrazine. Among these, 3-methyl-2-buten-1-thiol, 3-ethyl-2,5-dimethylpyrazine, 6-acetyl-2,3,4,5-tetrahydropyridine and butyric acid determined more intense roasty and popcorn aroma and also notes of thiol-like, and cheese-like in coffee beans since the ratio of CB and CS FD factors were 128, 128, >2048, and 256 respectively, for above-mentioned molecules. Other identified odorants with high FD factors (from 256 to 512) were 2,3-pentanedione, acetic acid, (E)-2-nonenal, 2-acetylthiazole, 2-acetyl-2-thiazoline, 5-methyl-2-methoxyphenol, 4-methylphenol, 3-methylindole, 2,3-butanedione, 2-hydroxy-3-methyl-2-cyclopenten-1-one, maltol, trans-4,5-epoxy-(E)-2-decenal, 3-ethylphenol, phenylacetic acid and vanillin. The FD factor ratio of CB and CS demonstrated that butter-like (2,3-pentanedione, ratio: 128), spicy and smoky (2-hydroxy-3-methyl-2-cyclopenten-1-one, ratio: 64), phenolic and clove-like (5-methyl-2-methoxyphenol, ratio >256) and phenolic and leather-like (3-ethylphenol >512) were more intense in coffee beans than silverskin. In contrast some odorants, including two important actors of coffee flavour, were found with similar FD factor in both matrices: furaneol, 2-methoxy-4-vinylphenol, vanillin, trans-4,5-epoxy-(E)-2-decenal, 2-Acetyl-1-pyrroline and 2-acetylpyrazine. Our study revealed an important aroma fraction in CS and therefore, this co-product can be considered as a potential source of interesting and pleasant aroma compounds and should be exploited in the food sector.

**Table 24.** Odor-active compounds identified in CS and CB: FD factor comparison of odors, as perceived in the sniffing port, and identified odorants.

| No. | Odorant | Odor | RI[b] | | FD[c] | |
|---|---|---|---|---|---|---|
| | | | FFAP | DB-5 | CS | CB |
| 1 | 2-/3-methylbutanal[a] | malty | 953 | 659 | 4 | 64 |
| 2 | 2,3-Butanedione[a] | butter-like | 980 | 606 | 16 | 512 |
| 3 | 2,3-Pentanedione | butter-like | 1055 | 700 | 2 | 256 |
| 4 | 3-Methyl-2-buten-1-thiol[a] | garlic-like, thiol | 1097 | 819 | 8 | 1024 |
| 5 | unknown | roasty, fatty | 1298 | - | <1 | 256 |
| 6 | 2-Acetyl-1-pyrroline[a] | roasty | 1330 | 922 | 16 | 32 |
| 7 | Dimethyl trisulfide | spicy, cabbage, sulfurous | 1364 | 967 | 16 | 128 |
| 8 | 2-Ethyl-5-Methylpyrazine | roasty, nutty | 1380 | 989 | <1 | 128 |
| 9 | 2,3,5-Trimethylpyrazine | fatty, roasty, earthy | 1398 | 1007 | 8 | 128 |
| 10 | unknown | roasty, fatty | 1402 | - | <1 | 256 |
| 11 | 2-Isopropyl-3-methoxypyrazine[a] | green, earthy | 1420 | 1093 | 32 | 2048 |
| 12 | 2-Furfurylthiol[a] | pungent, coffee-like | 1427 | 907 | 64 | 2048 |
| 13 | 3-Ethyl-2,5-dimethylpyrazine | roasty, popcorn, earthy | 1439 | 1084 | 8 | 1024 |
| 14 | Acetic acid | pungent, vinegar | 1443 | 624 | 16 | 256 |
| 15 | Methional[a] | cooked potato | 1452 | 904 | 128 | 4096 |
| 16 | 2,3-Diethyl-5-methylpyrazine | roasty, fatty | 1480 | 1157 | 64 | 2048 |
| 17 | 2-Isobutyl-3-methoxypyrazine[a] | green pea-like, green bell pepper | 1512 | 1177 | 128 | 4096 |
| 18 | (E)-2-Nonenal | greasy, green, roasty | 1524 | 1157 | 64 | 256 |
| 19 | Linalool | sweet, fruity, citrus | 1536 | 1102 | 1 | 64 |
| 20 | 6-Acetyl-2,3,4,5-tetrahydropyridine[a] | roasty, popcorn | 1556 | 1054 | <1 | 2048 |
| 21 | unknown | roasty, green | 1572 | - | <1 | 2048 |
| 22 | unknown | fatty, roasty | 1581 | - | 32 | <1 |
| 23 | Butyric acid | sweaty, cheese | 1618 | 819 | 4 | 1024 |
| 24 | 2-Acetylpyrazine | roasty | 1625 | 1020 | 8 | 16 |
| 25 | 2-Acetylthiazole | roasty | 1638 | 1020 | 16 | 256 |
| 26 | 2-/3-Methylbutanoic acid | cheese-like | 1658 | 860 | 128 | 1024 |
| 27 | unknown | meaty, greasy | 1693 | 1212 | 32 | 32 |
| 28 | unknown | silverskin-like | 1718 | - | 512 | 512 |
| 29 | unknown | spicy | 1724 | - | 32 | 256 |
| 30 | 2-Acetyl-2-thiazoline | fatty, roasty | 1750 | 1100 | 8 | 256 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 31 | unknown | caramel-like | 1787 | - | <1 | 128 |
| 32 | (E,E)-2,4-Decadienal | meaty, gravy-like | 1800 | 1315 | 64 | 1024 |
| 33 | 2-Hydroxy-3-methyl-2-cyclopenten-1-one | spicy, burnt paper, smoky | 1827 | 1029 | 8 | 512 |
| 34 | unknown | pungent, spicy, clove-like | 1832 | - | 128 | 256 |
| 35 | 2-Methoxyphenol | clove-like, phenolic | 1854 | 1087 | 1024 | 16384 |
| 36 | 2-Phenylethanol | sweet, honey | 1906 | 1111 | 4 | <1 |
| 37 | 2-Phenyl-2-butenal | green, phenolic | 1926 | 1278 | 8 | 64 |
| 38 | 5-Methyl-2-Methoxyphenol[a] | phenolic, clove-like | 1937 | 1185 | <1 | 256 |
| 39 | unknown | smoky | 1957 | 1123 | 64 | <1 |
| 40 | Maltol | caramel-like | 1969 | 1115 | 64 | 512 |
| 41 | trans-4,5-Epoxy-(E)-2-decenal[a] | metallic | 1997 | 1377 | 256 | 512 |
| 42 | y-Nonalactone | fruity, coconut | 2026 | 1364 | 64 | 1024 |
| 43 | Furaneol | caramel-like | 2032 | 1064 | 8192 | 16384 |
| 44 | unknown | burnt paper, seasoning, | 2059 | - | 128 | 256 |
| 45 | 4-Methylphenol | fecal, horse stable-like, pee-like | 2079 | 1080 | 16 | 256 |
| 46 | 4-Methyloctanoic acid[a] | goaty, sheepy | 2091 | - | 512 | 2048 |
| 47 | y-Decalactone[a] | peach-like, lemon-like | 2147 | 1471 | 32 | <1 |
| 48 | unknown | rubber-like | 2178 | - | 32 | <1 |
| 49 | 3-Ethylphenol | phenolic, leather-like | 2181 | 1169 | <1 | 512 |
| 50 | 2-Methoxy-4-vinylphenol | clove-like | 2197 | 1315 | 4096 | 8192 |
| 51 | unknown | seasoning, phenolic | 2269 | 1357 | 128 | <1 |
| 52 | Indole | fecal | 2448 | 1298 | 8 | 64 |
| 53 | 3-Methylindole | fecal | 2493 | 1387 | 32 | 256 |
| 54 | Phenylacetic acid | honey, | 2555 | 1259 | 128 | 512 |
| 55 | Vanillin | vanilla, chocolate-like | 2572 | 1402 | 256 | 512 |
| 56 | unknown | saliva-like | 2619 | - | 64 | 128 |

[a] GCxGC-MS analysis did not result in a clear mass spectrum, but comparison of retention index and odor quality with respective data of an authentic reference compound allowed for unequivocal structure assignment. [b] Retention index (RI), calculated from the retention time of the compound and the retention times of adjacent n-alkanes by linear interpolation. [c] Only more powerful odors (unknown chemical structure) with, for CS, FD>16 and for CB, FD>128 are reported.

# 5. Conclusions

## 5.1 Lignan and isoflavone in coffee

The project on quantitative analysis of two classes of phytoestrogens in coffee, resulted in the development of analytical methods for lignan analysis in espresso and in R&G coffee and another method for lignan and isoflavone analysis in green coffee. To extract target compounds from different coffee matrix we evaluated various extraction processes studying recovery and quantitative data. The most performing were chosen and applied to coffee samples. Specifically, for espresso and R&G coffee two enzymatic digestions were the most efficient: clara-diastase (Method 3.10-EC) and taka-diastase (Method 5-R&G), respectively, both coupled to HPLC-MS/MS. These two methods avoided long clean-up and enrichment sample procedures and were characterized by short chromatographic run time (the separation of monitored compounds was obtained within 4 minutes). We found that SECO was the lignans present at highest concentration in espresso (27.9-52.0 μg $L^{-1}$) and in R&G coffee (84.4-257.8 μg $kg^{-1}$) followed by LARI (espresso: 5.3-27.8 μg $L^{-1}$ and R&G: 26.1-91.5 μg $kg^{-1}$). In contrast, MAT was not detected in all coffee samples. Moreover, the extraction yield of lignans revealed that they are almost completely extracted during coffee percolation with an average of 95.2%. Hence, regular consumption of EC can contribute to the dietary intake of lignans that, recently calculated in 5 European countries, ranges from 1 to 2 mg/day (Tetens et al., 2013). Regarding green coffee matrix, for the first time an analytical method for the simultaneous quantitation of three lignans (secoisolariciresinol, matairesinol and lariciresinol) and six isoflavones (genistin, daidzin, daidzein, genistein, biochanin A and formononetin) has been developed. The best performing process was Method 7-GC, a double extraction composed of base hydrolysis in MeOH and enzymatic digestion with clara-diastase, since it showed satisfactory recovery levels, ranging from 74 to 94%, and the highest total concentration of all studied compounds (1193.4 μg $kg^{-1}$). This method was validated and then applied to 25 different green coffee samples, found that lignans (286.5-8131.8 μg $kg^{-1}$) were more abundant than isoflavones (3.4-300.0 μg $kg^{-1}$). This work provided new insight into two important bioactive compounds (i.e. lignans and isoflavones) of green coffee, one of the most

important agricultural products in the international trade. The future perspective will be to study these substances during the fruit ripening.

## 5.2 Optimization of espresso coffee extraction

The project on optimization of espresso coffee extraction aimed to enhance the extraction efficiency in order to prepare the same quality EC using lower amount of R&G coffee. We studied different variables, i.e., particle sizes, filter baskets and perforated discs, by brewing coffee with standard and reduced amount. From our results it emerged that extracting coffee with different particle sizes dramatically influenced total solids and bioactive compounds. Specifically, when standard filter was set in the machine, we found higher levels of TS and target molecules for reference samples (ECs extracted with mixed particles), and for 200-300 μm mesh size. Decreasing the amount of R&G coffee, similar TS levels and contents of bioactive compounds to standard condition were obtained for reference compounds. The filter basket played a key role on coffee extraction, and our results demonstrated that the best filter baskets, in term of quantitative data, were A and B. The prototype C was the worst, probably for its own design characterized by the holes on the boundary surface and not on the bottom. Data on aroma compounds showed that lowering the amount of ground coffee at the same particle size had little impact on the release of volatile compounds, especially in standard filter basket. Another important variable to be optimized during the EC extraction was the distance between the coffee cake and the shower. Installing different perforated disc with their specific heights is possible to adjust that distance. The study on perforated discs demonstrated that using 14 g the best, in term of quantitative data, was 5 mm while higher height (6 mm) was necessary for 12 g to obtain similar content of bioactive compounds and TS. These results may confirm the empirical knowledge of using 5 mm perforated disc with 14 g of ground coffee in standard filter basket. Moreover, our data suggest that using lower amount of ground coffee permits to obtain the same extraction yield increasing the height of perforated disc. The implementation of different filters for smaller particle sizes, and of different heights of perforates discs for reducing amount of ground coffee, are both easy adjustments to apply in coffee houses (bars, coffee shops etc.). Simple and feasible as it is, this optimization of the coffee brewing process could lead in fact, in the long run, to a more sustainable

consumption of EC, by reducing the amount of the raw material and, in the end, producing lower amount of spent coffee while maintaining the same quality of beverage.

## 5.3 Studies on Coffee Silverskin

For the first time, we studied the aroma fraction of coffee silverskin by using GC-O/FID and 2D-GC-MS. The identification of odorants was performed by comparative analysis with reference compounds by evaluating three characteristics of each odor: linear retention index, odor description and mass spectrum. The potency of aroma was studied by AEDA, which established a ranking list based on odor persistence after a series of dilution. From our investigations, four volatile compounds were identified for the first time in coffee. In detail, 4-methyloctanoic acid and trans-4,5-epoxy-(E)-2-decenal were characterized in coffee beans and silverskin, while 6-acetyl-2,3,4,5-tetrahydropyridine and 5-methyl-2-methoxyphenol only in coffee beans. The highest FD factors in coffee silverskin were obtained for furaneol, 2-methoxy-4-vinylphenol and 2-methoxyphenol and, moreover, other typical coffee aroma volatiles were found, e.g., 2-furfurylthiol, 2,3-butanedione, vanillin, 2-isobutyl-3-methoxypyrazine, etc (Amanpour & Selli, 2016; W. Grosch, 1998). After aroma comparison of the two matrices, in coffee beans we found about 2.5 times more odorants than in silverskin and almost all of those were more intense, in term of FD factor. On the other hand, some aromas, such as furaneol, 2-methoxy-4-vinylphenol, vanillin, trans-4,5-epoxy-(E)-2-decenal, 2-acetyl-1-pyrroline and 2-acetylpyrazine, occurred with similar FD factors in CS and CB. Our studies demonstrated that coffee silverskin contains an interesting odor-active compound fraction with high similarity to coffee beans. Although beans are characterized by more complex and intense aroma, coffee silverskin remains an important co-product to be exploited in food industry, for instance in novel food production. Moreover, this study increases knowledge on coffee silverskin and can contribute to develop an its original application in food sector and, in the optical of more sustainable environmental, to decrease the cost of its disposal of.

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