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Bioprospection of Bioactive Compounds from Coffee Waste Biomass as a Useful Strategy for its Valorization

MASTER DISSERTATION

Carolina Fátima Pereira Andrade

MASTER IN APPLIED BIOCHEMISTRY

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ABSTRACT

Coffee is one of the most popular beverages in the world and its consumption generates copious amounts of waste. The most relevant by-product of the coffee industry are the spent coffee grounds, with 6 million tons being produced worldwide per year. Although generally treated as waste, spent coffee grounds are a rich source of several bioactive compounds, with applications in diverse industrial fields. The present work aimed the analysis of spent coffee grounds from different geographical origins (Guatemala, Colombia, Brazil, Timor, and Ethiopia) for the identification of bioactive compounds with industrial interest. For this purpose, the volatile fingerprint was established by headspace solid-phase microextraction coupled with gas chromatography mass spectrometry (HS-SPME/GC-MS). Additionally, the bioactive and antioxidant potential was assessed through a μ-QuEChERS methodology coupled to spectrophotometric techniques (total phenolic content (TPC), and DPPH and ABTS scavenging assays). Finally, the identification of the bioactive compounds responsible for the antioxidant activity attributed to the spent coffee grounds was attempted, using miniaturized solid-phase extraction (μ-SPEed) coupled to ultrahigh performance liquid chromatography with photodiode array detection (UHPLC-PDA). The volatile fingerprint analysis enabled the identification of a total of 111 volatile organic compounds (VOCs) in the spent coffee grounds belonging to different chemical families. Furanic compounds, nitrogen compounds, esters, carbonyl compounds, volatile phenols, and terpenoids comprised most of the volatile fingerprint, with the remaining chemical families only constituting less than 2%. Some of the major VOCs identified have been reported in literature to have great potential as raw material for applications in food and/or in non-food products. The highest value for the TPC was obtained for the sample from Brazil (53.68 ± 3.120 mg GAE/100 g DW), while Colombia presented the lowest value (41.56 ± mg GAE/100 g DW). As for the DPPH assay, the highest scavenging activity was presented by Brazil (78.11 ± 7.333 mg TE/100 g DW) and the lowest for Guatemala (50.64 ± 5.257 mg TE/100 g DW). Finally, the UHPLC-PDA analysis permitted the identification and quantification of seven bioactive compounds in the spent coffee grounds, which were 3- and 5-cafeoylquinic, 4,5-, 1,5-, 3,4-dicaffeoylquinic and caffeic acids, and caffeine. 5-cafeoylquinic acid and caffeine were the most abundant compounds found in all of the samples analyzed and highest concentrations were registered for the Guatemala sample (385.3 ± 24.38 e 391.9 ± 27.31 mg/100 g, respectively). These bioactive compounds are responsible for several biological effects in the human organism, with high potential applications in cosmetic, pharmacological, and food industries, as well as in plague control. The results obtained in this work show that spent coffee grounds are a rich source of several bioactive compounds, supporting its bioprospection based on circular economy concept closing the loop of coffee value chain, toward the valorization of coffee by-products.

Keywords: Spent coffee grounds; Bioactive compounds; Waste valorization; HS-SPME; GC-MS; μ-QuEChERS; antioxidant potential; μ-SPEed; UHPLC-PDA.

RESUMO

O café é uma das bebidas mais populares no mundo e o seu consumo gera enormes quantidades de resíduos. O resíduo mais significativo produzido pela indústria do café é a borra, com 6 milhões de toneladas a serem produzidas anualmente a nível mundial. Apesar de ser tratada como resíduo, a borra de café é uma fonte rica de compostos bioativos, com aplicações em diversas áreas industriais. O presente trabalho teve como objetivo analisar borra de café de diferentes origens geográficas (Guatemala, Colômbia, Brasil, Timor e Etiópia), para a identificação de compostos bioactivos com interesse industrial. Para este fim, estabeleceu-se o perfil volátil por microextração em fase sólida em espaço de cabeça acoplada a cromatografia em fase gasosa e espectrometria de massa (HS-SPME/GC-MS). Adicionalmente, o potencial bioativo e antioxidante foi determinado por uma metodologia de µ-QuEChERS acoplada a técnicas espetrofotométricas (conteúdo total de compostos fenólicos (TPC), e capacidade antioxidante). Finalmente, a identificação dos compostos bioativos foi realizada por extração em fase sólida miniaturizada (µ-SPEed) acoplada a cromatografia em fase líquida de alta eficiência (UHPLC-PDA). O estabelecimento do perfil volátil permitiu a identificação de 111 compostos orgânicos voláteis (VOCs) nas borras analisadas, pertencentes a diferentes famílias químicas. Compostos furânicos, compostos nitrogenados, ésteres, compostos carbonílicos, fenóis voláteis, e terpenoides compuseram a maioria do perfil volátil, com as restantes famílias químicas apenas representando menos de 2%. Alguns dos VOCs maioritários foram descritos na literatura com grande potencial como matéria-prima para aplicações em produtos alimentares e não alimentares. O teor mais elevado obtido para o TPC foi observado na amostra do Brasil (53.68 ± 3.120 mg GAE/100 g DW) e menor para o da Colômbia (41.56 ± mg GAE/100 g DW). A atividade antioxidante mais elevada foi observada para a amostra do Brasil (78.11 ± 7.333 mg TE/100 g DW) e a menor para a da Guatemala (50.64 ± 5.257 mg TE/100 g DW). Por fim, a análise por UHPLC-PDA permitiu a identificação e a quantificação de sete compostos bioativos, que foram os ácidos cafeico, 3- e 5-cafeoilquínicos e 4,5-, 1,5-, 3,4-dicafeoilquínicos, e a cafeína. O ácido 5-cafeoilquínico e a cafeína foram os compostos mais abundantes encontrados nas amostras, e as concentrações mais elevadas foram registadas para a amostra de Guatemala (385.3 ± 24.38 e 391.9 ± 27.31 mg/100 g, respetivamente). Estes compostos bioativos são responsáveis por diversos efeitos biológicos no organismo humano, e apresentam potencial em muitas aplicações nas indústrias cosméticas, farmacêuticas e alimentar, bem como no controlo de pragas. Os resultados obtidos neste trabalho mostram que a borra de café é uma fonte de compostos bioativos, apoiando a sua bioprospecção baseada no conceito da economia circular, fechando o ciclo de valorização do café.

Palavras-chave: Borra de café; Compostos bioativos; Valorização de resíduos; HS-SPME; GC-MS; µ-QuEChERS; UV-Vis; µ-SPEed; UHPLC-PDA.

ABBREVIATURES

%RSD	Percentage of relative standard deviation
%Rec	Percentage of recovery
µ-SPEed	Miniaturized solid-phase extraction
µ-QuEChERS	Miniaturized quick, easy, cheap, effective, rugged and safe
1,5-diCQA	1,5-Dicaffeoylquinic acid
3-CQA	3-Caffeoylquinic acid
3-FQA	3-Feruloylquinic acid
3-pCoQA	3-*p*-Coumaroylquinic acid
3,4-diCQA	3,4-Dicaffeoylquinic acid
3,5-diCQA	3,5-Dicaffeoylquinic acid
4-CQA	4-Caffeoylquinic acid
4-FQA	4-Feruloylquinic acid
4-pCoQA	4-*p*-Coumaroylquinic acid
4,5-diCQA	4,5-Dicaffeoylquinic acid
5-CQA	5-Caffeoylquinic acid
5-FQA	5-Feruloylquinic acid
5-pCoQA	5-*p*-Coumaroylquinic acid
ABTS	2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACN	Acetonitrile
a_w	Water activity
C_{18}	Octadecylsilane
CFQA	Caffeoylferuloylquinic acid
CGA	Chlorogenic acid
cLC-DAD	Capillary liquid chromatography coupled with diode array detection
CQA	Caffeoylquinic acid
diCQA	Dicaffeoylquinic acid
DI-SPME	Direct immersion solid-phase microextraction
DPPH	2,2-diphenyl-1- picrylhydrazyl
dSPE	Dispersive solid-phase extraction
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
DW	Dry weight
ECD	Electron capture detector
EtAc	Ethyl acetate
FA	Formic acid
FID	Flame ionization detector

FQA	Feruloylquinic acid
FRAP	Ferric reducing antioxidant power
GA	Gallic acid
GAE	Gallic acid equivalents
GC	Gas chromatography
GCB	Graphitized carbon black
GLC	Gas-liquid chromatography
GSC	Gas-solid chromatography
HCA	Hierarchical cluster analysis
HS-SPME	Headspace solid-phase microextraction
HPLC-DAD	High performance liquid chromatography coupled with diode array detection
IARC	International Agency for Research on Cancer
ICO	International Coffee Organization
IR	Infrared spectroscopy
IS	Internal Standard
LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave-assisted extraction
MeOH	Methanol
MEPS	Microextraction in packed sorbent
MRP	Maillard reaction products
MS	Mass spectrometry
MV	Missing values
OTs	Odor thresholds
PBS	Phosphate-buffered saline
PCA	Principal component analysis
pCoQA	*p*-Coumaroylquinic acid
PLS-DA	Partial least squares-discriminant analysis
PS/DVB-RP	Porous polystyrene-divinylbenzene reversed phase
PSA	Primary secondary amine
PTFE	Polytetrafluoroethylene
QSM	Quaternary solvent manager
SD	Standard deviation
SM	Sample manager
SPE	Solid-phase extraction

SPME	Solid-phase microextraction
TCD	Thermal conductivity detector
TE	Trolox equivalents
TPC	Total phenolic content
UAE	Ultrasound-assisted extraction
UPLC-UV	Ultrahigh performance liquid chromatography coupled with ultraviolet detection
UV-Vis	Ultraviolet-visible
VOCs	Volatile organic compounds

Table of Contents

List of Figures

List of Tables

Chapter I

1. Overview

1.1. Coffee's origin and commercial importance

Coffee is one of the most popular beverages in the world and is obtained from the beans of plants belonging to the *Coffea* genus. Historically, the stimulating effect of coffee was discovered in the Ethiopian province of Kaffa when Kaldi, an Arab goatherd, noticed that his goats behaved strangely after eating the red berries of *Coffea* plants. He tried tasting them, but the bitter taste and large seeds made him spit them out. However, he could already feel his heart rate quickening as his energy level surged. This led to different experiments with the mysterious berries, including making a brew with crushed berries. The interesting effects of this drink spread, and by the fifteenth century, wild coffee plants were being cultivated in the Arabian Peninsula. Around the seventeenth century, coffee drinks were served in famous coffee houses in several European countries, and their popularity only increased after that [1].

Nowadays, coffee has become firmly entrenched in people's daily routines. With studies proving that its consumption is linked to decreased risks of heart disease and some cancers [2], global coffee consumption is estimated to be about 2.25 billion cups per day [3]. Moreover, advances in science and technology facilitated the identification of the bioactive compounds responsible for the stimulating and health-promoting effect of coffee.

Economically, coffee is the second most traded commodity globally after crude oil [4] and acts as a major source of income for dozens of developing countries, with over 100 million people relying on it, worldwide. In 2020, the International Coffee Organization (ICO) estimated that coffee production surpassed 169 million 60 kg bags, with its top producing countries being Brazil (4140 tons), Vietnam (1740 tons), Colombia (858 tons), Indonesia (726 tons), and Ethiopia (442.5 tons). According to the coffee imports data published by the ICO, the United States of America (USA) is the biggest coffee importer in the world. In the period from December 2020 - November 2021 the USA imported 1754553 tons of coffee, followed by Germany (1324670 tons), Italy (646504 tons), France (479763 tons), and Japan (450934 tons). Although the first processing steps occur in the producing countries (e.g., de-pulping, drying, and mucilage removal), green coffee beans are exported to other countries, and re-exported to consuming countries, with or without undergoing processing (e.g., roasting, grinding, instant coffee production). For instance, in the same period mentioned above for the importations, Germany and Italy each re-exported over 50% and Belgium over 85% of the amount of coffee they imported [5].

The *Coffea* genus covers about 500 species of which only a few have commercial interest [6]. The two main coffee varieties cultivated for commercial purposes are *Coffea arabica* and *C. canephora* variety Robusta, which are responsible for approximately 70 and 30% of the global production, respectively. Other minor cultivated species include *C. liberica* and *C. excelsa*, which are mainly restricted to West Africa and Asia, and only account for a very slight percentage of the global production [7,8].

Coffee is a climate-sensitive perennial crop and is likely susceptible to changes in climate [9], which limits its production. The optimal growth conditions for coffee are in tropical climates, with its biggest producers being countries situated in the tropical bean belt, which is the horizontal strip that stretches across Central and South America, Africa, and Asia (Figure 1). From an agricultural perspective, both main varieties, Arabica and Robusta, enjoy a tropical climate, rich soils, and plenty of rainfall. However, Robusta is easier to cultivate. Growing in flat lands, it allows a more mechanized production and originates bigger yields of product. Moreover, Robusta beans contain higher concentrations of caffeine and antioxidants compared to Arabica, making them more resistant to disease, and producing a strong, bitter beverage with an intense flavor. Conversely, Arabica plants require distinct rainy and dry seasons, a year-round temperature range of 15 and 24 °C, and are generally cultivated on mountain slopes, making their production more laborious and costlier. This allows Arabica coffee to have superior organoleptic properties than Robusta and is consequently considered to be of higher quality, with its beverage being described as mild, fruity, floral, sweet, and acid, with a pronounced flavor profile [1,8,10].

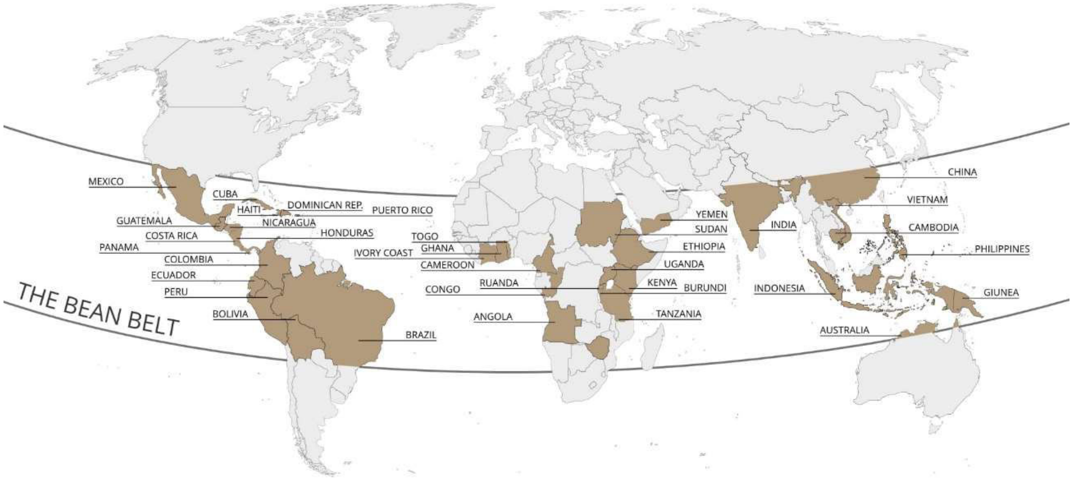


Figure 1: “The Bean Belt”: Major coffee producing countries highlighted in brown [11].

1.2. Coffee’s chemical composition

The organoleptic properties of coffee are key aspects in the determination of its quality, and they include aspects related to taste, sight, smell, and touch. When it was first discovered, coffee was recognized for its stimulating properties but was highly unpleasant to drink due to its bitter taste. Evolution in coffee production and preparation techniques revealed that certain practices have a positive effect on coffee's quality. For this reason, coffee can be seen both as a craft or a science, because the quality of the final product is a result of art and knowledge, and it depends on several factors, such as terroir, climate, variety, and postharvest methods [11]. However, all these factors only serve as modulators for the real aspect that influences the organoleptic properties of coffee – its chemical composition.

Although generally, flavor properties are related to the volatile organic compounds (VOCs) present in the matrix, some non-volatile compounds influence its taste. For instance, in roasted coffee, the non-volatile compounds, which include alkaloids (e.g., caffeine, trigonelline), proteins, polysaccharides, melanoidins, carboxylic acids, chlorogenic acids, lipids, and minerals contribute to the basic taste sensations of sourness, bitterness, astringency and sweetness [12]. Furthermore, these non-volatile compounds often act as precursors of VOCs [13].

Additionally, several positive biological effects have been attributed to coffee. Studies have proven that coffee constituents present antioxidant [14–16], anti-inflammatory [17,18], antibacterial [19,20], anti-hyperlipidemic [21], antidiabetic [22,23], anti-hypertensive [24,25], anti-cariogenic [26], anticancer [27,28], and neuroprotective [29,30] properties.

The chemical composition of coffee has been widely studied, and a plethora of compounds have been analyzed and identified, as well as their biological effect on the human organism, leading to the beverage being named a functional food. Although the final product is very different from the original coffee beans harvested in terms of chemical composition, the original matrix contains the precursors that will originate the final compounds. For this reason, the phytochemistry of green coffee, which corresponds to the coffee beans before roasting, has been investigated, and over 1000 phytochemicals identified [6,31,32]. The tissue of green coffee beans contains primarily water, carbohydrates and fiber, proteins and free amino acids, lipids, minerals, organic acids, chlorogenic acids, trigonelline, and caffeine. Also, smaller concentrations of some diterpenes (e.g., cafestol, kahweol), alkaloids (e.g., theobromine, theophylline), and minerals (mainly potassium and phosphorus) have been found in green coffee [33,34].

1.2.1. Changes in the chemical composition of coffee during harvest and postharvest processing

1.2.1.1. The coffee fruits

Even after the *Coffea* plants are fully grown and bear fruits, coffee undergoes several processes that have an impact on its chemical composition. Coffee cherries are composed of a series of layers, as can be seen in Figure 2. The husk and pulp (exocarp and mesocarp, respectively) are the outer layers of the cherry and are rich in carbohydrates, minerals, and proteins, as well as in some organic compounds such as tannins, chlorogenic acid, and caffeine [35]. The husk color can range from yellow to red when the fruit matures, depending on the genotype of the species, and is green, when the fruit is premature. Next is a pectinaceous layer, also known as mucilage, that contains protein, fat, lipids, minerals, tannins, polyphenols, and caffeine [36], and envelops the endocarp or parchment. The parchment is a strong, fibrous, yellowish layer, mainly composed of lignocellulose, that covers both coffee beans and separates them from each other [37]. The silverskin or integument is the layer that surrounds the bean and is essentially composed of polysaccharides (e.g., cellulose, hemicelluloses), monosaccharides, proteins, and phenolic compounds [35,38]. Finally, in the heart of the cherry, there are two elliptical or egg-shaped seeds containing endosperm and embryos [36,38].

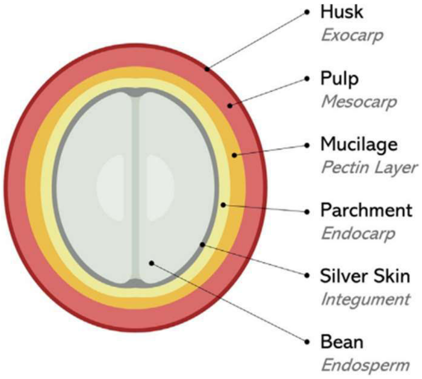


Figure 2: Anatomy of the coffee fruit [36].

1.2.1.2. Coffee harvesting

During harvest, various harvesting procedures can be applied, from mechanical procedures to manual picking of the cherries. The fruit's ripeness can be classified as green (immature), cherry (ripe), and raisin (overripe), with cherry fruit being the stage that gives the coffee bean better quality because it has lower astringency and higher volatile composition (e.g., aldehydes, ketones, and alcohols) [39]. When manual picking is used, only the ripe, non-defective cherries are harvested, allowing the production of a more uniform, high-quality coffee. In this sense, mechanical procedures are disadvantageous since all cherries are harvested independently of their stage of ripeness, and an additional step of sorting the fruits is generally necessary. Either way, to reduce economic loss, the lower quality beans (unripe and/or defective) are all mixed and sold. The harvesting practices applied usually depend on the size of the plantation and access to technology, with manual picking being used in smaller plantations, such as in family coffee growing [40].

1.2.1.3. Postharvest processing

The first step of postharvest processing intends to remove the pulp of the fruit, to obtain the green coffee beans, and must be initiated immediately to avoid fruit spoilage. This can be done through three different methods – dry, wet, and semi-dry – each leading to a distinct resulting product. The easiest and oldest method is dry processing. In this practice, the cherries are dried under the sun or by mechanical drying until the moisture level is about 10-11%, and then mechanically peeled [40]. In this process, since the moisture of harvested cherries is about 70%, mechanical drying is usually not applied because it presents an unnecessary cost [41]. During sun-drying, natural microbial fermentation occurs, secreting enzymes that break down the pulp and mucilage. Fermentation of the pectinaceous sugars originates from ethanol and acetic, lactic, butyric, and other higher carboxylic acids. The dry method produces the called unwashed or natural coffee and is commonly used in countries with less rainfall and long periods of sunshine. The formation of butyric and propionic acids, that diffuse into the beans, lower the quality. Therefore, the dry method is generally used for the production of cheaper brands, such as Robusta and low-quality Arabica coffees [42,43].

Wet processing is normally applied in the production of higher-quality coffee (usually called washed-coffee) [40]. In this method, an initial step of washing and separation of the fruits is done, in which the better-quality cherries are separated from others by density difference. For this, all fruits are dumped into water low-density fruits are separated from denser ones. Ripe cherries present a higher density than low-quality fruit (also called floating coffee). Floating coffee are fruits that may exhibit seed malformation, insects or microorganisms attack, or advanced ripening stage [36,44]. After this separation, the cherries that sunk are passed through sieves to remove liquid effluent and are submitted to de-pulping, in which their husk and pulp are removed by mechanical pulpers, which leaves the mucilage, parchment, and silver skin firmly attached to the beans. The removal of mucilage is done by biological degradation, with fermentation occurring spontaneously after the cherries are exposed to the environment and contaminated with microorganisms or by the addition of microorganisms and enzymes [38,45]. The fermentation is carried out in tanks at ambient temperature for 12-36 hours, depending on local temperature, with the cherries immersed in water (wet fermentation) or not (dry fermentation). Fermentation ends when all the mucilage is degraded, and it can be recognized when the beans are no longer slippery [46]. The beans are then dried by direct sun exposure in yards or by using mechanical air dryers. The use of mechanical dryers in wet-processed beans is advantageous since it accelerates the process and helps to avoid microbial contamination that would reduce coffee quality [39]. When the water content is reduced to 10-12%, the dried parchment that is still attached to the beans is removed by a peeler [36].

Finally, the third method is called semi-dry processing (also known as semi-wet, semi-washed, or pulped natural process), which is a hybrid technique of wet and dry processing. In this process, the cherries go through the same process as in wet processing, excluding the fermentation step. The use of this method is said to have begun in Brazil in the early 1990s and demonstrated to produce excellent coffee when well processed [46].

Independently of the method by which the cherries are processed changes in the chemical composition of beans occur, and each different process will lead to the formation of different compounds. Subsequently, distinct flavor profiles will be obtained. Whereas coffees obtained through the wet process are characterized by their full aroma and pleasant acidity, dry-processed coffees typically exhibit a so-called full body, with a medicinal flavor [41].

1.2.1.4. Roasting

When the beans are submitted to roasting, some of the existing compounds are destroyed and others are formed, due to the reactions, principally Strecker degradation, pyrolysis, caramelization, and Maillard reactions, induced by the high temperatures. The roasting conditions can be tuned to initiate the array of complex reactions, that will lead to the desired aromatic and physical characteristics. Usually, the maximum temperatures that modern roasters reach range between 180 and 240 °C [34,47]. During roasting, the color of coffee beans changes drastically, from the light brown raw coffee to the dark brown (sometimes almost black) roasted beans. The main reason for this change is due to the formation of high-molecular-weight compounds called melanoidins that are common in heat-processed food products and have characteristic dark brown color [33]. Consequently, color is a direct indicator of the degree of roasting. Darker beans have been roasted for a longer time and/or at higher temperatures, while lighter-colored beans are derived from shorter and lower-temperature roasts.

Few modifications happen during the initial phase of the roasting procedure. Generally, the only significant occurrence in this endothermic phase is the evaporation of free water. This is followed by a dry heating stage, with multiple chemical reactions taking place, including dehydration, hydrolysis, enolization, cyclization, cleavage, fragmentation, recombination of fragments, pyrolysis, and polymerization. When the temperature reaches 130 °C, with the beginning of sucrose caramelization, coffee beans start to brown and swell. At temperatures above 170 °C begins the exothermic phase, accompanied by the intensification of the brown color and a considerable increase in the volume of the beans. The key reactions responsible for aroma formation are triggered at approximately 190 °C, namely the Maillard and Strecker reactions. The beans have then to be rapidly cooled, to interrupt the reactions at the desired point [11,33]. Concerning aromatic characteristics, a light roast produces sweet, cocoa, and nutty aromas, while dark roasting is responsible for burnt/acrid, acid/sooty, pungent, coffee, and roasted characteristics [48]. In Figure 3 the principal reactions that lead to the formation of flavor compounds during the roasting of coffee beans are shown.

Besides originating important flavor compounds, roasting can also lead to the formation of compounds that affect negatively human health. These include furan (discussed in section 1.2.2.1.) and acrylamide, two Maillard reaction products (MRPs) that raise concerns regarding public health. Acrylamide is formed during food processing temperatures above 120 °C, from the reaction of the amino acid asparagine with reducing sugars, and is associated with several toxic properties, including genotoxicity, carcinogenicity, and neurotoxicity [49,50]. Various methodologies to mitigate the formation of these health-demoting compounds have been investigated, such as optimization of the roasting conditions and the enzymatic treatment of green coffee beans to reduce the content of asparagine [50,51].

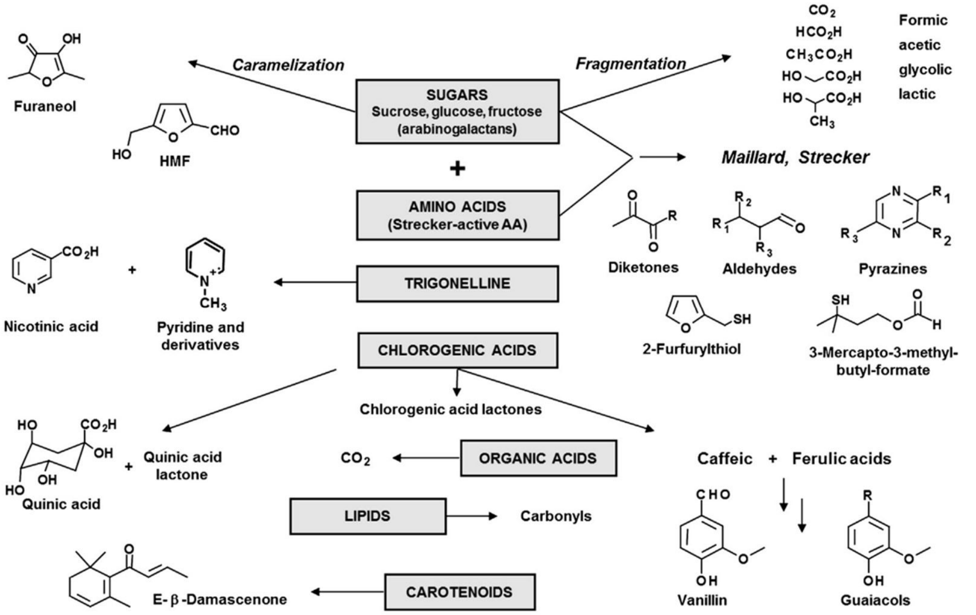


Figure 3: Principal reactions involved in the formation of flavor compounds during roasting of coffee beans [52].

1.2.1.5. Storage

The green coffee beans are stored on the farms before being sold to importer countries. The duration of the storage depends on the prices and demand for coffee, as farmers wait for the best moment to sell it. During this storage period, the beans are kept in bags made of natural fibers (fique or jute) or in polyethylene bags. Farm storerooms lack control of temperature, humidity, and light which can lead to the depletion of coffee's quality [53].

Due to the high temperatures to which coffee beans are submitted during the roasting process and to their lower water activity (a_w), no enzymatic and microbial spoilage occurs in roasted coffee. This makes roasted coffee a shelf-stable product. Despite this, physical and chemical changes take place during storage. These changes can be classified as either coffee aging or coffee stalling. Aging is a short length of time, just after roasting, in which coffee is allowed to rest under proper technological conditions, to improve its sensorial properties [54]. On the other hand, stalling usually happens after grinding and is characterized by the reduction of the fresh, mild roast notes and the appearance of strong spicy notes in coffee's flavor. The main responsible for coffee stalling is the very potent sulfur compounds that deteriorate in contact with air. Stalling reactions can be decelerated by packaging under inert gas and freezing, or by keeping coffee stored in a cool, dry, and dark place [55].

1.2.2. Volatile fingerprint in roasted coffee

Even though all processing steps have a great influence on the physical and chemical properties of coffee, roasting is the one responsible for the most drastic alterations, especially in the volatile fraction [56,57]. While only about 200 VOCs have been found in green coffee, over 1000 have been identified in roasted coffee [39]. Many different chemical classes comprise the volatile fraction of roasted coffee, including hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids, esters, pyrazines, pyrroles, pyridines, other bases (e.g., quinoxalines, indoles), sulfur compounds, furans, furanones, phenols, oxazoles, among others [47]. This happens because the exposure to high temperatures during the roasting procedure triggers a chain of complex mechanisms. The main reactions involved are Maillard reactions, Strecker degradations, sulfur and hydroxy-amino acids' breakdown, proline and hydroxyproline's breakdown, degradation of trigonelline, quinic acid moiety, and pigments, and minor lipid degradation. Additionally, several interactions between intermediate degradation products occur, originating from other compounds [12]. The VOCs present in coffee have been widely studied [52,58–62] since the flavor is greatly influenced by coffee's volatile component. Nevertheless, a small number of these VOCs are key coffee aroma compounds, since many have relatively high odor thresholds (OTs). According to López-Galilea et al., only four classes of compounds appear to have a distinctive impact on the aroma of coffee: pyrazines, furans, aldehydes, and ketones [63]. An important class of VOCs that provide roasted coffee with its distinctive aroma are heterocyclic compounds, including pyrroles, furans, pyrazines, thiazoles, oxazoles, thiophenes, and imidazoles. These are mainly formed during roasting and are rarely found in green coffee beans. However, the precursors to most odorous compounds are non-volatiles present in green coffee beans, such as carbohydrates (produce furans, aldehydes, ketones, and phenols), lipids (produce small amounts of aldehydes and ketones), proteins, peptide, and free amino acids (originate ketones, pyrroles, and pyrazines), chlorogenic acids (produce volatile phenols), and trigonelline (produces pyrroles, pyridines, and pyrazines) [33,64]. Additionally, some VOCs are present in green coffee, mainly those of nonthermal origin (hexanal, isoamyl alcohol, 1-hexanol, 1-pentanol, 2-heptanol, 1-octen-3-ol, benzyl alcohol, and benzaldehyde) [65]. Conversely, thiophenes, oxazoles, and thiazoles are only formed during roasting and are not found in green coffee [33].

As previously mentioned, processing highly affects coffee's chemical composition, and the formation of VOCs is especially influenced by the degree of roasting. Additionally, it also depends on the stability of their precursors and location within the seed. Major VOCs recognized in coffee that may be affect by roasting conditions include pyridine, 2-methylpyrazine, furfural, furfuryl formate, 2-furanmethanol acetate, 5-methyl-furancarboxaldehyde, 1-(2-furanylmethyl)-1H-pyrrol, 1-(1H-pyrrol-2-yl)-ethanone, 2-methoxyphenol, 4-ethyl-2-metoxyphenol, 2(5)-ethyl-4-hydroxy-5(2)-methyl-3(2H)-furanone, guaiacol, 4-ethylguaiacol, 2-furfurylthiol, 3-methyl-2-buten-1-thiol and methanethiol [33,62,66]. Furanic and nitrogenated compounds (e.g., pyrroles, pyrazines, and pyridines) are the two most abundant chemical families of VOCs found in roasted coffee and will be further described below.

1.2.2.1. Furanic compounds

Furan and furan derivates are heterocyclic compounds, produced in coffee during the roasting of the beans. As long known normal components of coffee flavor volatiles, furans are major contributors to coffee's volatile fingerprint, imparting flavor characteristics described as burnt, roasted, malty, sweet, and caramel base notes to coffee [67,68]. Although most furans present relatively higher sensory thresholds when compared with other coffee compounds, they are still considered important in coffee flavor, due to the high concentrations they appear [47]. The mechanisms through which furanic compounds are formed still need further clarification. However, ascorbic acid, carbohydrates, amino acids, MRPs, polyunsaturated fatty acids, and carotenoids have been pointed to as precursors of furan [69]. Cheong et al. [70] investigated the volatile composition of four types of Arabica beans and were able to identify and quantify 12 furans in the roasted coffees. This study showed that furanic compounds comprised, on average, approximately 30% of the total compounds quantified, with furfuryl alcohol being the most abundant compound found, taking up almost 19% of the total volatile composition. Furans are commonly found in heat-treated foods, and concerns over their possible negative effects on health have strengthened the need for their quantification in such products. Hence, furan is considered to be "possibly carcinogenic to humans" (Group 2B) based on animal trials (IARC, International Agency for Research on Cancer, 1995) [71] and its concentration in coffee is, consequently, highly monitored [49]. Moreover, minimization of the occurrence of furan and furan derivates in coffee has been studied, through the optimization of the roasting conditions of commercial coffee [51].

1.2.2.2. Nitrogen compounds

Nitrogenated compounds are also a class of heterocyclic compounds found in roasted coffee, which include pyrroles, pyrazines, and pyridines. Their routes of formation are complex and not completely elucidated, but proteins and amino acids can be precursors to these compounds, as well as the alkaloid trigonelline [33]. Pyrroles were described as furan degradation products or amino acid derivates and exhibit a peculiarly sweet and slightly flared smell in coffee [72]. Pyrazines are a class of compounds with key importance to the flavor of roasted coffee. Although they are the second most abundant class after furanic compounds, they also present a low OTs. Generally, the aroma of pyrazines has been described as nutty, earthy, roasty, and green [58,72]. Volatile pyridine derivates are also formed when coffee beans are submitted to high temperatures and result from the degradation of trigonelline. Literature shows that, from all the VOCs that have been detected in coffee, approximately 20 compounds contain a pyridine ring, including methyl, ethyl, acetyl, and vinyl derivates [73,74]. Generally, pyridines impart an unpleasant and bitter plant-like odor to coffee, and their content tends to increase at higher roasting degrees [75]. Pyridines have been a target of investigation for their potential carcinogenicity since excess amounts of these VOCs can be harmful to the human organism. However, this effect has only been verified in animal tests, and no study has proved the carcinogenic effect of pyridine in the human organism [74].

1.2.3. Non-volatile compounds in coffee

Carbohydrates account for the most components, making up about 60% of the total weight of raw coffee beans, and slightly less in roasted coffee. After carbohydrates, lipids and proteins also comprise a significant part of the chemical composition, followed by some secondary metabolites [76]. Several bioactive compounds among the non-volatiles in coffee, including methylxanthines (e.g., caffeine, theobromine, theophylline), diterpene alcohols (e.g., cafestol, kahweol), phenolic compounds (e.g., chlorogenic acids, catechins, anthocyanins, hydroxycinnamic acids, tocopherols), and melanoidins [77].

1.2.3.1. Caffeine

Caffeine is a purine alkaloid (a nitrogenous secondary metabolite that contains a purine ring in its structure), also known as 1,3,7-trimethylxanthine [73], and is the most favorable used psychostimulant worldwide [78]. The chemical structure of caffeine is represented in Figure 4. It is one of the most widely consumed active food ingredients and can be found in varying concentrations in the beans, leaves, and fruits of more than 60 plants, including kola nut (*Cola acuminate*), cacao bean (*Theobroma cacao*), yerba mate (*Ilex paraguariensis*), guarana berries (*Paullinia cupana*), coffee beans (*Coffea* spp.), and tea leaves (*Camelia siniensis*) [79]. Coffee is the primary dietary source of caffeine since it contains the highest caffeine content [80]. In green coffee, the caffeine content depends on many factors, with variety being one of the most notable ones. Robusta coffee usually presents approximately twice the concentration of caffeine found in Arabica coffee [33]. Caffeine is highly heat stable and does not suffer degradation during roasting. However, caffeine content tends to be lower in dark roast coffee due to sublimation loss at high roast temperatures [81,82]. This was confirmed by the results of Awwad et al. [83], that studied the effect of roasting degree on caffeine and observed that caffeine content increased from green to medium roasted coffee (roasted at 175-185°C) and decreased when dark roasting was applied (roasted at 205-215°C). In roasted coffee beverages alkaloids are some of the compounds that impart a bitter taste. Caffeine alone is responsible for about 10% of the perceived bitterness [33].

Among the plethora of bioactive compounds found in coffee, caffeine is one of the most abundant. The effect of caffeine on the human organism has been extensively investigated [84–89]. Some of the effects on the human organism attributed to caffeine include its role in the prevention of neurodegenerative diseases (e.g., Alzheimer's and Parkinson's diseases) [86] and cardiovascular diseases [89], in the treatment of neonatal apnea [87], and as an analgesic adjuvant [88].

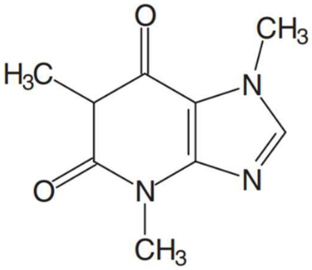


Figure 4: Schematic representation of the structure of caffeine.

1.2.3.2. Trigonelline

Trigonelline is another alkaloid, which structure is represented in Figure 5(b), that is found in green coffee beans and has nicotinate as a precursor. It is produced by the methylation of nicotinic acid (also known as pyridine-3-carboxylic acid, Figure 5(b)), using methionine, a sulfur-containing amino acid. Its content in green coffee ranges between 0.6 and 1%. However, a great part degrades during roasting breaking down into nicotinic acid and pyridines. Trigonelline is known to have a bitter taste, and because it is 100% soluble in water, it is a substantial element that contributes to coffee bitterness [74,90]. The content of trigonelline differs among coffee varieties. For instance, in *C. canephora* its content is about two-thirds of the amount found in *C. arabica* [33]. This vitamin B3 derivate is a highly bioactive compound with several bioactive effects related to its ingestion: hypoglycemic, hypolipidemic, antimigraine, antibacterial, antiviral, antioxidant, antitumor, antithrombotic, and neuroprotective properties [91].

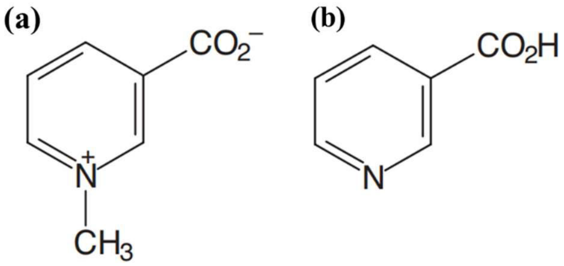
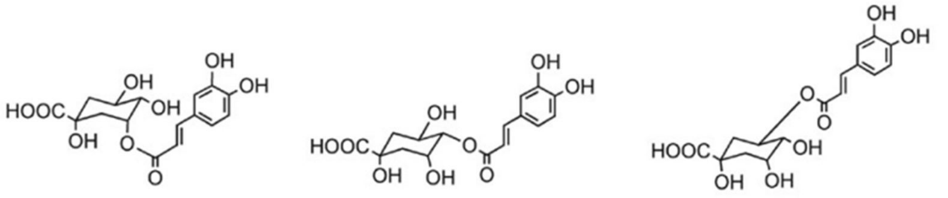


Figure 5: Schematic representation of the structures of (a) trigonelline and (b) nicotinic acid.

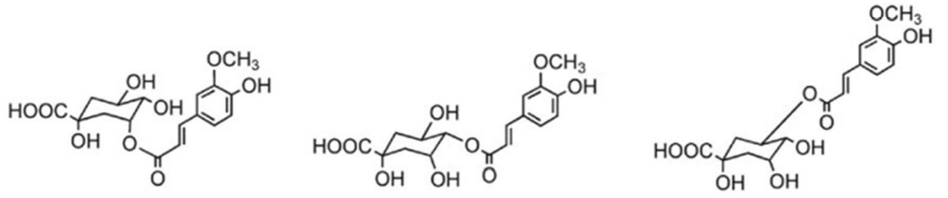
1.2.3.3. Chlorogenic acids

Chlorogenic acids (CGAs) are the main group of phenolic compounds in coffee. These compounds are derived primarily from the esterification of *trans*-cinnamic acids (e.g., caffeic, ferulic, and *p*-coumaric acids) with quinic acid, and are subdivided according to nature and number of cinnamic substituents, as well as the esterification position in the cyclohexane ring of the quinic acid [92]. Caffeoylquinic acids (CQAs), with its 3 isomers 3-CQA, 4-CQA, and 5-CQA, dicaffeoylquinic acids (diCQAs), with its 3 isomers 3,4-diCQA, 3,5-diCQA and 4,5-diCQA, feruloylquinic acids (FQAs), with its 3 isomers 3-FQA, 4-FQA and 5-FQA, p-coumaroylquinic acids (pCoQAs), with its 3 isomers 3-pCoQA, 4-pCoQA and 5-pCoQA, and mixed diesters of caffeoylferuloylquinic acids (CFQAs) [6]. The structures some of the most common CGAs in coffee are represented in Figure 6. Although CGA is highly distributed among plants, including apple, pear, prune, cabbage, kale, burdock, yacon, and sweet potato, green coffee beans are one of the main edible sources [93]. In green coffee beans, about 80% of the total CGA content corresponds to caffeoylquinic acids. 5-CQA was the first of these compounds identified and is the most abundant in green coffee, corresponding to almost 60% of the total CGA content. For this reason, 5-CQA is the most studied isomer and is usually called chlorogenic acid [33].

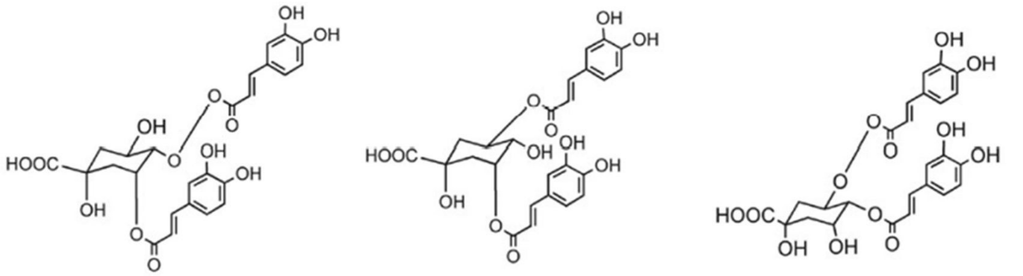
Although green coffee beans are a rich source of CGA, coffee is mostly consumed after roasting. During this procedure, because CGA are thermolabile, they suffer chemical changes being isomerized, epimerized, and lactonized, including a considerable amount that is lost by degradation [94]. Consequently, most studies investigating the effect of roasting degree on CGA content report that its concentration decreases as the roasting degree gets higher [83,95–97]. Some of the products of CGA degradation (hydroxycinnamic acids) can be incorporated into MRP melanoidins during roasting [94]. Also, CGA hydrolysis products such as quinic and ferulic acids, which further degrades forming important phenolic odorants (e.g., guaiacol, 4-vinylguaiacol) [98]. The effects of CGA on human health have been widely investigated, given the popular biological activity attributed to coffee consumption. However, most biological properties reported for these compounds are related to CQA, particularly 5-CQA. Nevertheless, CGA have been showed to present antioxidant [99], anti-inflammatory [100], anticancer [101], hepatoprotective [102], antidiabetic [103], cardioprotective and antihypertensive [104], antiobesity [105], neuroprotective [106], antimicrobial [107], and potential prebiotic [108] activity.



3-*O*-Caffeoylquinic acid 4-*O*-Caffeoylquinic acid 5-*O*-Caffeoylquinic acid



3-*O*-Feruloylquinic acid 4-*O*-Feruloylquinic acid 5-*O*-Feruloylquinic acid



3,4-*O*-Dicaffeoylquinic acid 3,5-*O*-Dicaffeoylquinic acid 4,5-*O*-Dicaffeoylquinic acid

Figure 6: Principal chlorogenic acids found in coffee [109].

1.2.3.4. Cafestol and kahweol

Cafestol and its dehydro derivate kahweol are two coffee-specific diterpenes found in considerable amounts in coffee beans and coffee brews [110,111]. The occurrence of these two pentacyclic diterpene alcohols has only been verified in plants of the *Coffea* genus. While cafestol is found in both *C. arabica* and *C. canephora*, kahweol is specific to the arabica variety [112].

Cafestol and kahweol's individual isolation is extremely difficult due to the similarities in their structures (Figure 7), making even chromatographic separation challenging [113,114]. For this reason, their properties are usually studied as a mixture of both. Hence, kahweol is highly unstable when purified [113]. Despite this, successful chromatographic separation and quantification of cafestol and kahweol without derivatization has been attained according to the literature [112,115]. These compounds comprise about 20% of the lipid fraction in coffee [116]. Both compounds present low stability to heat, acids, and light. Consequently, during roasting, they are degraded to dehydrokahweol and dehydrocafestol. The effects on diterpenes depend on the intensity of the process [117]. These compounds have several pharmacological properties, including anti-inflammatory [118], anti-cancer [119], anti-diabetic [120], hepatoprotective [121], and anti-osteoclastogenesis [122] activities.

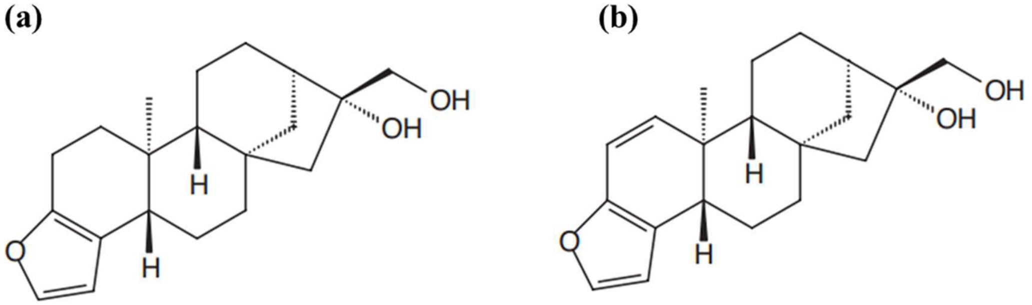


Figure 7: Chemical structure of the two main diterpenes found in coffee: (a) kahweol and (b) cafestol [33].

1.2.3.5. Melanoidins

Melanoidins are brown heterogeneous polymeric compounds that have high molecular weight and contain nitrogen-bearing components [123]. These MRP account for 23-25% of the dry matter in roasted coffee. The structure of melanoidins is complex and variable, incorporating components such as chlorogenic acids, galactomannans, and arabinogalactan-proteins [33,81]. They can act as roasting monitoring agents since they are responsible for the change in the bean color [11,124]. Some studies indicate that melanoidins present bioactivity and are partially responsible for the antioxidant [125], antimicrobial [126], prebiotic [127], antihypertensive [128], and metal-chelating [33] properties of coffee, among others.

1.3. Coffee by-products and their valorization

Along the several steps that coffee undergoes from harvesting until it meets the final consumer, numerous residues are generated. The amount of the coffee cherries that end up in the final beverage is only roughly 10% [129]. The scheme in Figure 8 describes synthetically the steps that coffee goes through, either during dry, semi-dry, or wet processing, and the residues that are discarded along the way. These residues include wastewater from the first washing step, husk, pulp, mucilage, fermented broth (wet processing), parchment, silver skin, and spent coffee grounds [36]. The chemical composition of the residues depends on the characteristics of the fruit used and the level of processing applied [130].

1.3.1. Spent coffee grounds

Spent coffee grounds represent the final by-product of the coffee industry and are the biggest contributors to coffee's biowaste. This coffee residue mainly results from the soluble coffee industry, where roasted and ground coffee beans are heat or steam treated to produce coffee extract for consumption, but also from domestic commerce. Approximately 6 million tons of spent coffee grounds are produced worldwide per year [130,131]. Despite this, spent coffee grounds still lack adequate management, with most of them having landfills as the final destination [132]. However, this practice is environmentally hazardous, since spent coffee grounds are disposed of without prior treatment leading to the emission of dioxide carbon (CO_2) and other greenhouse gas, and the release of chemicals such as caffeine, polyphenols, and tannins into the environment [4,133]. Furthermore, spent coffee grounds' degradation involves great oxygen consumption, given its high carbon content [134] and there is an eminent risk of spontaneous combustion [131]. Another practice used in Europe for the management of this type of waste is incineration, which is also an environmental hazard [135]. For this reason, the demand for proper management of spent coffee grounds is increasing, especially with climate change being an escalating threat in the past decades, leading to various catastrophic events. The valorization of materials previously treated as waste is one of the measures encouraged by international organizations to minimize climate change's effects [136]. Hence, investigation has focused on finding an adequate valorization approach for spent coffee grounds [137–141].

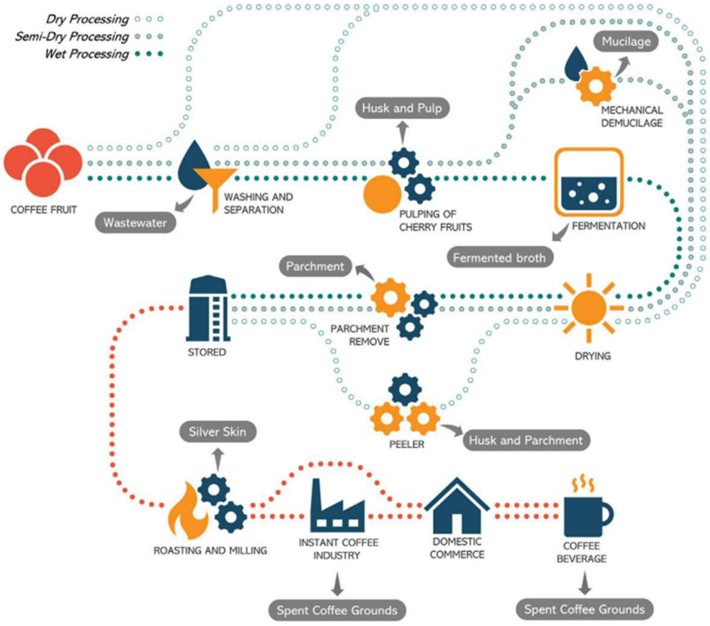


Figure 8: Schematic representation of the different types of postharvesting processing with the main residues generated [39].

Some recent approaches to use it for animal feeding showed that spent coffee grounds are not adequate for this purpose, since they displayed nutritional limitations, such as very low digestible energy and negative metabolizable energy contents. Also, the presence of toxic compounds (e.g., caffeine, tannins, and other polyphenols) represents a potential problem when spent coffee grounds are used to feed animals for human consumption [135].

Table 1: Chemical composition of spent coffee grounds [35,142–144].

Component	Value	Reference
Cellulose (% w/w dry matter)	20.6	[143]
Hemicellulose (% w/w dry matter)	25.57	
Lignin (% w/w dry matter)	12.28	
Lipids (wt%)	6	[35]
Fatty acids (% of coffee oil)		[143]
Palmitic acid	37.78	
Stearic acid	8.58	
Oleic acid	10.07	
Linoleic acid	34.06	
Linolenic acid	0.83	
Arachidonic acid	3.49	
Tocopherols (mg/100g)	14.23	
α-Tocopherols	4.64	
β-Tocopherols	9.48	
γ-Tocopherols	0.05	
δ-Tocopherols	0.07	
Total fiber (wt%)	60.5	[35]
Proteins(wt%)	13.6	[144]
Caffeine (wt%)	0.4	[35]
Tannins (wt%)	0.02	
Chlorogenic acid (wt%)	11.5	
Minerals (mg/100g)		[142]
Potassium	258.2	
Magnesium	49.6	
Calcium	37.2	
Manganese	1.8	
Copper	1.2	
Sodium	1.1	
Iron	0.9	
Zinc	0.1	

The potential of spent coffee grounds for bioenergy production has likewise been investigated. Because the content of triglycerides and long fatty acids on spent coffee grounds is high, it is a promising substrate to produce biodiesel [145,146]. Spent coffee grounds can also be used for the production of bioethanol, categorized as second-generation feedstocks, which are lignocellulosic biomass [147,148]. The production of biogas from spent coffee grounds happens when anaerobic digestion or composting is applied [149,150]. Although the richness in chemical compounds in spent coffee grounds can represent great toxicity to the environment, if well managed it can be a precious source of compounds for several applications. The recovery of added value compounds from spent coffee grounds has been a topic of recent interest [142,151–153]. Table 1 shows the chemical composition of spent coffee grounds. Apart from the component that can be used to produce bioenergy, there are nutrients and bioactive compounds, some of them in significant concentrations [35,142–144]. These can be recovered and used for applications in several fields (e.g., cosmetics, pharmaceutics, and food additives) [154].

Although a significant number of research works have recently focused on investigating spent coffee grounds' valorization, we still lack an integrated approach. In this sense, the "cascade pyramidal biorefinery hierarchy" framework is the promoted strategy, allowing the complete valorization of organic waste materials, including spent coffee grounds. In this framework, the recovery of valuable compounds is prioritized [136].

1.4. Analytical approaches for the identification and quantification of bioactive compounds

1.4.1. State-of-the-art

As mentioned in the previous section, the recovery of bioactive compounds from spent coffee grounds and their application in the industry is prioritized. Therefore, it is of great importance to assess the type of compounds that are present in this coffee residue and their concentration. For that, several extraction and analytical approaches have been studied [155–167].

The extraction of phenolic compounds from spent coffee grounds has been reported in several works. Extraction techniques such as conventional solid-liquid extraction [156–158,161], microwave-assisted extraction (MAE) [159,162,163], and ultrasound-assisted extraction (UAE) [155,165,167,168], among others, have been employed for this purpose. Ramón-Gonçalves et al. [160] conducted an investigation focused on the extraction of bioactive compounds from spent coffee grounds via subcritical treatment and were able to identify and quantify the phenolic compounds chlorogenic, *p*-coumaric, and ferulic acids, rutin, naringin, resveratrol, and kaempferol, as well as caffeine, through capillary liquid chromatography coupled with diode array detection (cLC-DAD). Also, Pettinato et al. [164] used a high pressure and temperature extraction methodology to identify and quantify chlorogenic acid and caffeine in spent coffee grounds, through high-performance liquid chromatography coupled with diode array detection (HPLC-DAD). Another research work reported the application of UAE coupled with UPLC-UV, which allowed the identification and quantification of quinic, gallic, caffeic, coumaric, and ferulic acids in spent coffee grounds. Various works also focused on the determination of phenolic content and antioxidant potential via spectrophotometric assays, including Folin-Ciocalteu, Ferric reducing antioxidant power (FRAP), 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical assays [157–159,161–165,167,168].

Despite this, emerging green extraction techniques, such as headspace solid-phase microextraction (HS-SPME), miniaturized solid-phase extraction (μ-SPEed), and miniaturized quick, easy, cheap, effective, rugged and safe (μ-QuEChERS) have gained significant attention in recent years, since they are environmentally friendly [169].

1.4.2. Extraction techniques

1.4.2.1. Headspace solid-phase microextraction

Developed in the early 1990s by Arthur and Pawliszyn [170], solid-phase microextraction (SPME) is a solvent-free, rapid, simple, and flexible technique, that allows extraction, enrichment, and injection in a single step [171]. As shown in Figure 9, the SPME device is a syringe-like assembly, in which a cylindrical-shaped fused silica fiber coated with the stationary phase (sorbent) can be protruded outside or drawn in when needed [172,173]. The extraction of analytes through this technique involves the partitioning of the analytes from the sample into the sorbent coating of the SPME fiber. Thus, since the adsorption of the analytes depends on the intermolecular interactions or affinity between the sorbent and the analytes, the type of sorbent used in the extraction is variable depending on the nature of the target analytes [171,174]. Moreover, SPME permits the pre-concentration and sampling of analytes from different kinds of samples (e.g., biological, environmental, and food samples) and enables the detection of VOCs and non-volatile compounds [169].

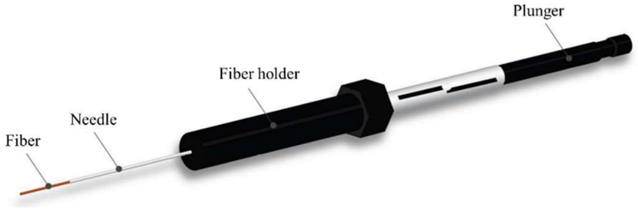


Figure 9: Solid-phase microextraction syringe-like device. Adapted from Gonçalves et al. [173].

There are three common modes in which this technique can be performed: headspace SPME (HS-SPME); direct-immersion SPME (DI-SPME); and membrane extraction. HS-SPME consists of the adsorption of the compounds present in the gas phase in equilibrium with the samples. For this reason, this modality is the most adequate for the extraction of volatile compounds, and it also avoids interference caused by the high molecular weight substances present in the sample matrix. To aid the volatilization of the analytes, the extraction conditions are optimized, the temperature being the key parameter used to influence the kinetics of the process. However, this process is also assisted by the addition of salts, which increases the ionic strength of the solution and reduces the solubility of the analytes (salting-out) [169,174]. A major advantage of this extraction technique is that it is compatible with chromatographic analysis methodologies, such as gas and liquid chromatography [171].

1.4.2.2. *Miniaturized solid-phase extraction*

Solid-phase extraction (SPE) is an extraction process that consists of passing a liquid sample through an immobilized phase followed by the extraction of the retained analytes with a suitable organic solvent [175]. Novel adaptations of this methodology have been developed envisioning miniaturization, automation, high-throughput performance, on-line coupling with the analytical instrument, and low-cost operation of sample preparation.

Microextraction by packed sorbent (MEPS) is a miniaturized form of SPE that offers several advantages to the conventional procedure. It was first introduced by Abdel-Rehim [176] and was adopted as a simple, fast, and on-line sample preparation technique. The miniaturization also results in reduced volumes of sample and solvent, making it more eco-friendly. In MEPS, a syringe-like device is used with the sorbent packed in a small container between the barrel and the needle [177]. MEPS cartridges with a variety of different sorbents are commercialized and can be used in automated and semi-automated methodologies, wherein the cartridge is connected to an automatic syringe, aspiring, and dispensing the programmed volumes of sample and solvent. Typically, MEPS comprises sorbent conditioning, sample loading, washing (optional), and analyte elution. In this technique, a two-directional flow is applied (up and down), providing duplication of each step (increases sample-sorbent), and allowing pre-concentration and enhanced analyte elution [178]. A drawback of this method is that its application is limited when it comes to highly viscous or concentrated samples that can block the flow through the device. For this reason, sample dilution previous to the extraction is sometimes necessary [179].

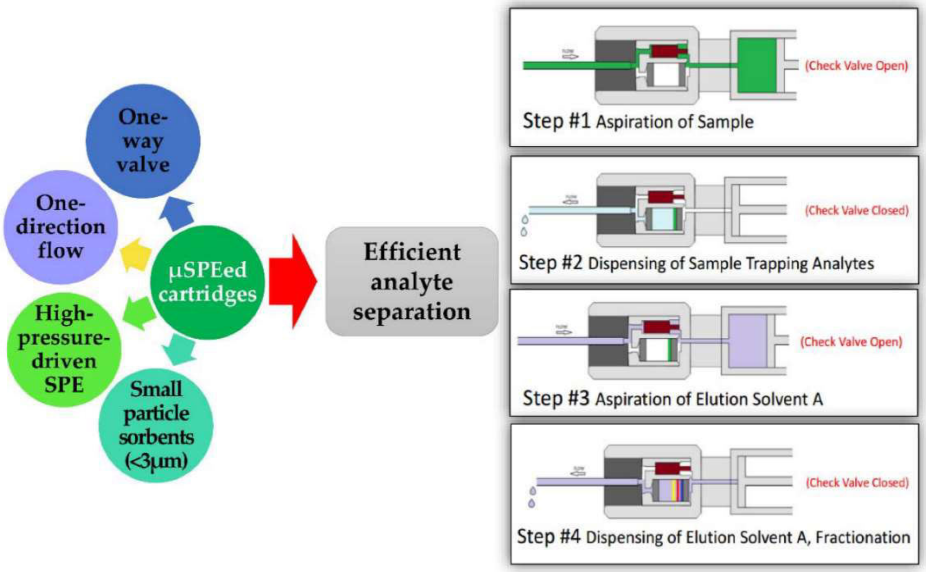


Figure 10: Schematic representation of µ-SPEed®. Adapted from Câmara et al. [169].

More recently, a modified version of MEPS has been proposed, µ-SPEed®, which differs from the former for having a unidirectional valve, in addition to the high pressure conferred by the small diameter of the sorbent particles. In this case, cartridges have two compartments: one where the liquid is aspired, and another that contains the sorbent bed and in which the liquid is dispensed, as can be seen in the scheme presented in Figure 10. This assures that the analytes retained in the solvent bed are not disturbed by solvent aspiration, leading to more efficient extraction of the target analytes [169].

1.4.2.3. Miniaturized quick, easy, cheap, effective, rugged and safe

QuEChERS is an innovative extraction method first presented by Anastassiades et al. [180] for the quantification of multi-residue pesticides in fruits and vegetables. The development of the QuEChERS methodology was supported by the need of researchers to find a simple, effective, and inexpensive way to extract and clean residual analytes from different chemical classes from a wide variety of sample matrices. In recent years, this methodology has been applied for the analysis of polyphenols and other bioactive compounds in foods [181–186]. Additionally, a miniaturized version of this technique, μ-QuEChERS, minimizes the amounts of sample and organic solvents used, making it more environmentally friendly [187].

The procedure involves only two main steps, minimizing the occurrence of errors: the first step of extraction and partitioning of the analytes, and a clean-up step. The first stage consists of sample extraction with an organic solvent, with the addition of partitioning salts (e.g., anhydrous magnesium sulfate ($MgSO_4$), sodium chloride (NaCl)), assisting in the removal of water. Several organic solvents can be applied for the extraction, but acetonitrile (ACN) was proved to efficiently isolate nonpolar and relatively polar analytes as well as minimize the amount of lipophilic material. ACN is water-soluble, allowing a good penetration in the water phase of samples, helping the extraction. However, the organic phase ends up containing water residues, which are removed by the addition of $MgSO_4$ (drying agent). Moreover, this hydration reaction is exothermic, generating heat at around 40 °C, which aids the extraction of nonpolar compounds without the use of nonpolar solvents. The extraction and partitioning are usually aided by homogenization of the mixture in the vortex and ultrasonication [188].

The second and final stage comprises the clean-up step, denominated dispersive SPE (dSPE). In this step, the use of SPE aims the removal of undesirable matrix components coextracted (e.g., organic acids, fatty acids, pigments, and sugars) and traces of water. The advantage of using dSPE instead of the traditional SPE is that it is carried out in the bulk solution, without requiring tedious and long procedures. The sorbents used in the clean-up stage are generally primary secondary amine (PSA), octadecylsilane (C_{18}), graphitized carbon black (GCB), and $MgSO_4$. The sorbents employed in this phase can be selected according to the complexity of the sample matrix and the type of interferences to be removed [188,189].

1.4.3. Chromatographic separation techniques

Chromatography is a biophysical technique that enables the separation, identification, and purification of the components in a mixture for qualitative and quantitative analysis. Chromatographic separation is based on the distribution of the components in a mixture between two phases: a porous bed, bulk liquid, layer, or film phase that is generally immobile, called the stationary phase, and fluid or gaseous phase, also called the mobile phase, that percolates through or over the stationary phase. The separation is based on the interactions between both stationary and mobile phases and the molecules in the sample, and it depends on the characteristics of the components, such as adsorption, partition, affinity, and differences in molecular weight. Based on this principle, some components stay more retained in the stationary phase, taking longer to exit the system, while others pass rapidly with the mobile phase [190,191].

To achieve adequate separation of the components belonging to a variety of matrices, various chromatography methods have been developed, including column chromatography, thin-layer chromatography, paper chromatography, paper chromatography, gas chromatography (GC), ion exchange chromatography, gel permeation chromatography, HPLC, and affinity chromatography [191].

Chromatographic procedures are widely used in research as well as routine medical, industrial, food, and environmental analysis. Particularly, GC and HPLC approaches have been extensively employed for the determination of bioactive compounds in food samples [192–198].

1.4.3.1. Gas chromatography

The GC system uses a gaseous mobile phase (carrier gas) and is used for the separation of VOCs. The carrier gas must have low viscosity, high purity, favorable solute diffusivity, and be chemically inert and non-inflammable. Nitrogen and helium are two of the most common gases employed in GC analysis, each of them having advantages and disadvantages: nitrogen is low-priced but has reduced sensitivity, while helium has excellent thermal conductivity but is expensive. The selection of the carrier gas is made according to the type of detector used [190,199].

There are two types of GC, based on the nature of the stationary phase: gas-solid chromatography (GSC), which uses a solid as the stationary phase, and gas-liquid chromatography (GLC), where the stationary phase is a liquid immobilized in a solid surface. GLC is the most common type of GC used due to its numerous advantages, including high-resolution capacity, low sample volume, speed, and sensitivity of detection, and it allows qualitative and quantitative analysis. However, GLC columns have a shorter life when compared to GSC columns, since the immobilized liquid slowly runs out from the solid surface [199].

Several detection methods can be coupled to GC, with some of the most common being flame ionization detector (FID), thermal conductivity detector (TCD), electron capture detector (ECD), mass spectrometry (MS), and infrared spectroscopy (IR) [190].

1.4.3.2. Ultrahigh performance liquid chromatography

HPLC is a form of liquid chromatography developed in the 1970s based on the principle of column chromatography. This methodology is usually employed in the characterization of more polar and less volatile or nonvolatile analytes and can be used either for analytical or preparative purposes. The HPLC system works by pumping the mobile phase through a packed column under high pressure (approximately 3000 psi). The sample is injected into the column and the components pass through the column at a different rate according to their affinity to the stationary phase [200]. Ultrahigh performance liquid chromatography (UHLPC) is an upgraded version of the HPLC system that can operate at up to 17500 psi and offers better resolution sensitivity, and speed of analysis [201].

To obtain an output of the separation results, UHPLC needs to be coupled with a detector, that provides the results in the form of a chromatogram. The most common detectors hyphenated to technique are absorbance detection techniques, particularly photodiode array (PDA) and ultraviolet (UV), and MS [200].

1.5. Aims and scope

Considering the information mentioned in the previous sections, the present work aims to:

Characterize five spent coffee grounds samples, from the Arabica variety, from different geographical origins (Guatemala, Colombia, Brazil, Timor, and Ethiopia) in terms of their chemical composition.

Establish the volatile fingerprint of the spent coffee grounds through HS-SPME/GC-MS, to assess the compounds with potential industrial applications.

Determine the bioactive and antioxidant potential of the spent coffee grounds using µ-QuEChERS coupled to spectrophotometric techniques (TPC, DPPH and ABTS scavenging assays).

Identify and quantify the bioactive compounds present in the spent coffee grounds via a µ-SPEed/UHPLC-PDA methodology.

Chapter II

2. Materials and Methods

2.1. Reagents and standards

The SPME fiber holder for manual sampling together with 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber, with 1 cm length, was purchased from Supelco (Bellefonte, PA, USA). Fiber conditioning was performed daily according to the manufacturer's recommendations to avoid carryover between sets of analyses. Helium (GC carrier gas) of purity 5.0 was supplied by Air Liquide (Portugal). The 2 mL DisQuE^{TM} dSPE tubes containing the sorbents (150 mg of $MgSO_4$ and 25 mg of PSA) used in the µ-QuEChERS clean-up step were obtained from Waters (Milford, MA, USA). Ultrapure water (H_2O)(18 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Burlington, MA, USA). 4-Methyl-2-pentanol (internal standard (IS), $C_6H_{14}O$, 99.0%) was acquired from Acros Organics (Geel, Belgium) and the Folin-Ciocalteu solution, 2,2-diphenyl-1-picrylhydrazyl, (DPPH, $C_{18}H_{12}N_5O_6$), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, $C_{14}H_{18}O_4$, 98.0%) and gallic acid (GA, $C_7H_6O_5$, 98.0%), caffeic acid ($C_9H_8O_4$, 99.0%) were supplied by Fluka (Munich, Germany). Potassium persulfate ($K_2S_2O_8$, 99.0%) and sodium nitrite ($NaNO_2$) were purchased from Merck® (Buchs, Switzerland), whilst sodium chloride (NaCl, 99.5%), disodium phosphate dihydrate ($Na_2HPO_4.2H_2O$, 99.0%), potassium dihydrogen phosphate (KH_2PO_4, 99.0%), trisodium citrate dihydrate ($C_6H_9Na_3O_9$, 99.0%) and formic acid (FA, CH_2O_2, 98.0%) were acquired from Panreac Applichem (Barcelona, Spain). Aluminum chloride ($AlCl_3$), potassium chloride (KCl, 99.5%), and ethyl acetate (EtAc, $C_4H_8O_2$, 99.7%) were supplied by Riedel-de Haën® (Seelze, Germany). 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS, $C_{18}H_{18}N_4O_6S_4$, 98.0%), sodium hydrogencitrate sesquihydrate ($C_6H_9NaO_8$, 99.0%) and 5-CQA ($C_{16}H1_8O_9$, ≥95.0%) were obtained from Sigma-Aldrich (Buchs, Switzerland) and sodium hydroxide (NaOH, 98.0%) was purchased from Eka Chemicals AB (Amsterdam, The Netherlands). Sodium carbonate (Na_2CO_3, 99.7%) was supplied by Labsolve® (Lisboa, Portugal). HPLC grade ACN (CH_3CN) and methanol (MeOH, CH_3OH) were acquired from Fisher Scientific (Loughborough, UK). 3-CQA, 1,5-diCQA, 3,4-diCQA, and 4,5-diCQA (>98.0%), were purchased from Biopurify Phytochemicals LTD (Chengdu, China).

2.2. Establishment of volatile fingerprint

2.2.1. Sample preparation

The roasted coffee samples were purchased from the Awaked company (Caldas da Rainha, Portugal). The samples acquired were composed of 100% Arabica coffee and were single-origin coffees Guatemala, Colombia, Brazil, Timor, and Ethiopia. The five roasted coffee samples were grounded until a thin powder was obtained. After that, 6 g of each sample were used to make 20 mL of expresso, in a coffee machine, and the spent coffee grounds were collected and stored in a flask for posterior analysis.

2.2.2. Headspace solid-phase microextraction procedure

The VOCs were extracted from the samples via HS-SPME. Three g of spent coffee ground sample, 1 g of NaCl, 3 mL of distilled water, and 5 µL of 4-methyl-2-pentanol (IS, 0.401 g/L) and a stirring bar were added to the extraction vial (20 mL). The vial was then hermetically closed and submitted to the extraction temperature (50 ºC) for 50 min, at a 450 rpm stirring rate, following the insertion of the DVB/CAR/PDMS fiber. All analyses were carried out in triplicate.

2.2.3. Gas chromatography-mass spectrometry conditions

After the extraction was completed, the fiber holder was removed from the vial and inserted into the injector port of an Agilent Technologies 6890N Network gas chromatograph system (Palo Alto, California, USA), where the thermal desorption of the analytes was conducted at 250 ºC during 6 min. The gas chromatographer was equipped with a 60 m × 0.25 mm I.D. × 0.25 µm film thickness HP-5 (SGE, Dortmund, Germany) fused silica capillary column and interfaced with an Agilent 5975 quadrupole inert mass selective detector. The oven temperature program was set as follows: initially set at 40 ºC, increased until 220 ºC at a rate of 2.5 ºC/min, and maintained for 20 min at 220 ºC. The total run time was 83 min. The column flow was constant at 1.0 mL/min using helium of purity 5.0. The injection port was operated in the splitless mode and held at 250 ºC. For the 5975 MS system, the temperature of the quadrupole detector was 220 ºC. Data acquisition was performed in the scan mode (30–300 m/z) with electron ionization at an energy of 70 eV. VOC identification was accomplished through manual interpretation, comparing the spectra with the data system library (NIST, 2005 software, Mass Spectral Search Program v.2.0d; NIST 2005, Washington, DC, USA) with a similarity threshold higher than 80%. The semi-quantification of VOCs was performed using 4-methyl-2-pentanol (IS) through the following equation: VOCs concentration = (VOC GC peak area/IS GC peak area) × IS concentration.

2.2.4. Statistical analysis

Statistical analysis was carried out using MetaboAnalyst 5.0, which comprises the data pre-processing to eliminate VOCs with missing values (MV) and normalization (data transformation using data scaling by mean-center and cubic root). The normalized data was processed using the one-way ANOVA followed by Tukey's test for post hoc multiple comparisons of means and multivariate statistical analysis, including principal component analysis (PCA), an unsupervised method that was used to visualize group tendencies of the data set, using the information contained in the VOCs fingerprint as several variables to obtain insights into the separations among sample sets according to geographical origin, and partial least squares-discriminant analysis (PLS-DA), a supervised method performed using the relative peak area of the VOCs, that allows the investigation of the differences in the VOCs levels of spent coffee grounds and the identification of the VOCs that can be used to differentiate the spent coffee grounds samples. Finally, Pearson's correlation was done to build the heat map of the spent coffee grounds using the VOCs identified to recognize clustering patterns.

2.3. Determination of bioactive compounds

2.3.1. Evaluation of the bioactive and antioxidant potential of spent coffee grounds

2.3.1.1. μ-QuEChERS extraction

The μ-QuEChERS extraction was performed according to the procedure optimized by Casado et al. [181]. All samples were lyophilized before the extraction. Briefly, 0.5 g of the lyophilized spent coffee grounds were added to 0.4 g of the μ-QuEChERS mixture (buffered salts) in the following proportion 4:1:1:0.5 ($MgSO_4$, NaCl, $C_6H_9Na_3O_9$, $C_6H_9NaO_8$, respectively). Then, 2 mL of a solution of ACN:EtAc (1:1, v/v), containing 0.1% of FA were added and the flask was vortexed for 10 s. The mixture was then submitted to ultrasonic agitation for 5 min, in an ultrasonic bath, and centrifuged for 5 min at 5000 rpm. After this, the supernatant (~1400 μL) was transferred to a 2 mL DisQue^{TM} dSPE clean-up tube containing 150 mg of $MgSO_4$ and 25 mg of PSA. The tube was vortexed for 30 s and centrifuged for 5 min at 4000 rpm. The extract was, finally, filtered through 0.22 μm PTFE syringe filters (BGB Analytik, VA, USA) into a vial, and stored at -20 °C until the analysis.

2.3.1.2. Total phenolic content

The total phenolic content (TPC) of the spent coffee grounds was determined using the Folin-Ciocalteu procedure, as described by Figueira et al. [202], with a few modifications. Briefly, the extracts obtained by the µ-QuEChERS procedure were diluted in water up to 3 mL final volume. Then, 300 µL of Folin-Ciocalteu solution was added to the reaction tube, followed by 1200 µL of 20% (w/v) Na_2CO_3 solution and 1500 µL of H_2O. The mixture was homogenized and incubated for 30 min in the dark and at room temperature (25 ± 1 ºC). After the incubation time, the absorbance was measured using a UV-Vis spectrophotometer (Lambda 25, Perkin Elmer, Waltham, MA, USA) at λ = 765 nm. The results were expressed in mg of GA equivalents (GAE)/100g DW (dry weight) after the absorbance registered was interpolated in the calibration curve ($y = 0.0517x + 0.0082$, where y is the absorbance and x the concentration, $R^2 = 0.9916$) prepared with standard solutions with different concentrations of GA (from 0.5 to 15 µg/mL). The TPC was assessed in triplicate for each sample.

2.3.1.3. 2,2-Diphenyl-1-picrylhydrazyl scavenging assay

The DPPH assay was performed according to Woraratphoka et al. (2007) [203], to measure the free radical scavenging properties of the spent coffee grounds. A DPPH stock solution was prepared by dissolving 24 mg of DPPH in 100 mL of MeOH. Before the reaction, the stock solution was diluted to obtain a working solution with absorbance ~0.9, and the absorbance value was registered. Then, the µ-QuEChERS extracts were diluted to a final volume of 100 µL and added to a reaction tube containing 3.9 mL of the DPPH working. The mixture was homogenized and incubated for 45 min in the dark and at room temperature. The absorbance was measured at λ = 515 nm using a UV-Vis spectrophotometer. The free radical scavenging capacity (AAR(DPPH)) against DPPH was calculated using the following formula (calibration curve): $\ln(\%\Delta A515) = 0.7631 \times \ln(AAR(DPPH)) - 0.0765$ (R2 = 0.9852), where $\%\Delta A515 = [(A515(0) - A515(45))/ A515(0)] \times 100$, A515(0) is the absorbance value measured at the beginning of the reaction and A515(45) is the absorbance value measured after 45 min of reaction. The calibration curve was obtained by performing the reaction procedure using standard solutions with different concentrations of Trolox (from 5 to 400 µg/mL), and the results were expressed in mg Trolox equivalents (TE)/100g of DW. The antioxidant capacity against DPPH was assessed in triplicate for each sample.

2.3.1.4. 2,29-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid assay

The ABTS assay was adapted from the procedure reported by Paixão et al. [204] to determine the antioxidant capacity of spent coffee grounds against the stable $ABTS^{\cdot +}$ radical cation. Briefly, a stock solution of ABTS (20 mM) was prepared in 50 mL of phosphate-buffered saline (PBS, pH 7.4) and 200 µL of 70 mM potassium persulfate solution was added. The solution was stored in the dark at room temperature (25 ± 1 °C) for 16 h. The ABTS solution was diluted with PBS until an absorbance value of ~0.9 was obtained, and the absorbance of the working solution was registered. Then, 12 µL of the µ-QuEChERS extracts were added to 3 mL of the diluted ABTS solution. The mixture was then homogenized and incubated for 20 min in the dark and at room temperature. After the incubation, the absorbance was measured at 734 nm using a UV-Vis spectrophotometer. The free radical scavenging capacity against ABTS (AAR(ABTS)) was calculated using the following formula (calibration curve): $I = 0.0448 \times AAR(ABTS) + 0.7094$ ($R^2 = 0.9912$), where $I = [(AB - AA)/ AB] \times 100$, with I being the percentage of inhibition of ABTS*, AB the absorbance of a blank sample (t = 0 min) and AA the absorbance after 20 min of adding the extracts. The calibration curve was obtained by performing the reaction procedure using standard solutions with different concentrations of Trolox (from 10 to 600 µg/mL), and the results were expressed in mg Trolox equivalents (TE)/100g of DW. The antioxidant capacity against ABTS was assessed in triplicate for each sample.

2.3.2. Analysis of bioactive compounds by UHPLC-PDA

2.3.2.1. Sample preparation

All spent coffee grounds samples were lyophilized before the extraction of phenolic compounds. The samples were then subjected to maceration to obtain a liquid extract, in which 100 mL of distilled water were added to 1.5 g of each sample and left under magnetic stirring for 1 h in a bath at 50 °C. After maceration, the sample was centrifuged, and the supernatants were transferred to a flask and stored in the cold until the extraction was conducted.

2.3.2.2. Extraction of phenolic compounds via µ-SPEed

The extraction of the phenolic compounds from the spent coffee grounds was obtained using µ-SPEed®. The extraction conditions applied were adapted from the procedure optimized by Casado et al. [193]. Before the extraction, sample pH was adjusted to pH 2, and all samples were filtered through 0.22 µm PTFE syringe filters (BGB Analytik, VA, USA). A programmable digital syringe driver, digiVOL®, equipped with a 250 µL syringe (ePrep®, Oakleigh, Australia), was used for the µ-SPEed® experiments. The sorbent used was a porous polystyrene-divinylbenzene reversed phase (PS/DVB-RP). For each extraction, the sorbent was activated with 100 µL of MeOH and conditioned with 100 µL of acidified H_2O (0.1% FA). After this, the sample was passed through the sorbent 10 times. No washing step was applied, and the analytes were directly eluted with 50 µL of MeOH:H_2O (95:5, v/v) containing 0.1% FA, into the vial for chromatographic analysis. After each extraction, the sorbent was reconditioned by passing

2×100 μL of MeOH and 100 μL of acidified H_2O (0.1% FA), to prevent the carry-over of analytes to the next extraction. The extraction was performed in duplicate for each sample.

2.3.2.3. UHPLC-PDA analysis

The chromatographic analysis of the spent coffee grounds was performed using a UHPLC system (Waters Ultra-High Performance Liquid Chromatography Acquity H-Class system) (Milford, MA, USA) equipped with a quaternary solvent manager (QSM), an Acquity sample manager (SM), a column heater, a degassing system, and a photodiode array (2996 PDA) detector. The column used for the separation of the analytes was an Acquity HSS T3 analytical column ($2.1\ \text{mm} \times 50\ \text{mm}, 1.7\ \mu\text{m particle size}$) packed with a trifunctional C18 alkyl phase (Waters, Milford, MA, USA). The chromatographic separation of the target analytes was achieved with the column at 40 °C, using a mobile phase composed of acidified H_2O (0.1% FA) (solvent A) and ACN (solvent B). The gradient conditions applied are presented in Table 2. Each analysis was followed by a 2 min re-equilibration time before the next injection. The total analysis time was 10 min. The injection volume was 2 μL and the sample manager compartment was kept at 20 °C. The PDA data was registered at 273 and 326 nm, according to the maximum wavelength of the analyzed compounds. The Empower software 2.0 (Waters, Milford, MA, USA) was used to drive the whole UHPLC configuration and for data collection. The identification of the target analytes was achieved by comparing the retention times and UV spectrum with those obtained for pure standards using the same instrumental conditions. Each extract was analyzed in triplicate.

Table 2: Gradient conditions applied for the UHPLC-PDA analysis of bioactive compounds in spent coffee grounds.

Time (min)	Flow (mL/min)	%A	%B
0	0.250	85.0	15.0
7	0.250	63.0	37.0
8	0.250	85.0	15.0

2.3.2.4. Validation of the μ-SPEed/UHPLC-PDA methodology

The analytical performance of the proposed methodology (μ-SPEed/UHPLC-PDA) was validated in terms of selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision (intra-day and inter-day, expressed as percentage of relative standard deviation, %RSD) and accuracy (expressed as percentage of recovery, %Rec.), to assure that the method is suitable for the determination of the target analytes in the spent coffee grounds samples.

2.3.2.4.1. Selectivity

The selectivity of an analytical method corresponds to the extent to which it can determine particular analytes in a complex mixture without interference from other components in the mixture [205]. The selectivity was assessed by analyzing the spent coffee grounds using the µ-SPEed/UHPLC-PDA method and comparing them with the standard solutions. The absence of interferences in the retention time and wavelength of the target analytes prove that the proposed methodology is selective.

2.3.2.4.2. Linearity

The linearity of a method is the capacity of an analytical method of producing results directly proportional to the concentration of an analyte within a range of concentration. It can be assessed by a measure of how well a calibration plot of response (usually chromatographic area of the peak) *vs.* concentration approximates a straight line [206]. The linearity of the µ-SPEed/UHPLC-PDA method was determined by preparing a calibration curve with seven points ($n = 7$) with concentration ranging from 0.2 to 75 µg/mL.

2.3.2.4.3. Limits of detection (LOD) and quantification (LOQ)

The LOD is the lowest concentration of an analyte from which it is possible to deduce its presence in the sample. Similarly, the LOQ is the smallest concentration of an analyte that can be determined in the sample [207]. The calculation of LOD and LOQ are similar, and one can be inferred from the other. The instrumental LOD can be determined by using a very low concentration of the target analytes and comparing its signal-to-noise (S/N) ratio. For LOD, the signal (chromatographic peak) should be at least three times superior to noise and should yield acceptable identity and purity. Regarding LOQ, the signal should be at least ten times the noise [207,208]. In the present work, the LOD and LOQ of the µ-SPEed/UHPLC-PDA method applied were determined considering the concentration that produced a signal-to-noise ratio equal or higher than 3 and 10, using the lowest standard concentration of the calibration curve.

2.3.2.4.4. Precision

Precision is a measure of the ability of the method to generate reproducible results. Precision can be considered into three level: repeatability, intermediate precision, and interlaboratory precision (also known as reproducibility), and its evaluation should be performed on homogenous authentic samples or artificially prepared samples [207,208]. In the present work, precision was assessed in terms of repeatability, by performing a series of repeated analyses within a short period of time (intra-day) and intermediate precision, by repeating the analyses in different, non-consecutive days (inter-day). One of the spent coffee grounds samples (Timor) was used for the evaluation of precision and was spiked in three different levels of concentration: low level (0.2 µg/mL), medium level (25 µg/mL), and high level (75 µg/mL). Intra-day precision determined by analyzing two replicates in triplicate (n = 6) for each spiking level, while inter-day precision was determined by analyzing one replicate in triplicate (n = 9) for each spiking level. The precision was expressed in terms of percentage of relative standard deviation (%RSD).

2.3.2.4.5. Accuracy

The accuracy consists in the closeness in agreement between the accepted true value or a reference value and the actual result obtained. Usually, accuracy is evaluated by determining the recovery of the analyte in spiked sample analyzed [208]. In this work, the accuracy was determined by spiking a spent coffee grounds sample (Timor) in three different levels of concentration: low level (0.2 µg/mL), medium level (25 µg/mL), and high level (75 µg/mL). The percentage of recovery (%Rec.) was calculated as showed in the following equation:

$$\%Rec = \frac{[Spiked\ sample] - [Sample]}{[Standard\ added]} \times 100$$

where [Spiked sample] corresponds to the concentration of the analyte in the fortified sample, [Sample] is the concentration of the analyte in the non-fortified sample, and [Standard added] is the theoretical concentration of the analyte added to the sample.

2.3.3. Statistical analysis

Statistical analysis was carried out using MetaboAnalyst 5.0, which comprises the data pre-processing to eliminate metabolites with missing values (MV) and normalization (data transformation using data scaling by mean-center and cubic root). The normalized data was processed using the one-way ANOVA followed by Tukey's test for post hoc multiple comparisons of means to identified significant differences between the spent coffee grounds from different geographical origins.

Chapter III

3. Results and discussion

3.1. Volatile fingerprint of spent coffee grounds

The volatile fingerprint of the spent coffee grounds Arabica samples, from Guatemala, Colombia, Brazil, Timor, and Ethiopia, was determined using HS-SPME/GC-MS methodology, and the VOCs detected and identified are listed in Table 3, along with their relative concentration expressed in µg/L ± standard deviation for the different samples. The GC-MS chromatograms of spent coffee grounds from different geographical origins were mapped in Figure 11.

A total of 111 VOCs was tentatively identified in the spent coffee grounds samples, 60 of which are common to all samples. These VOCs consisted of 29 nitrogen compounds, 20 carbonyl compounds, 16 furanic compounds, 11 terpenoids, 7 esters, 7 volatile phenols, 4 alcohols, 3 acids, 3 sulfur compounds, and 11 others. In semi-quantitative terms, furanic compounds (13150 ± 903.0 µg/L for the total relative concentration of volatile fingerprint), nitrogen compounds (11125 ± 733.3 µg/L), esters (6862 ± 426.5 µg/L), carbonyl compounds (2772 ± 122.3 µg/L), volatile phenols (1310 ± 85.6 µg/L) and terpenoids (1122 ± 89.6 µg/L) were the chemical families with the highest contribution to the volatile fingerprint of spent coffee grounds. The contribution of the remaining families to the total volatile fingerprint was lower than 2%. Figure 12 shows the distribution of the VOCs, according to the chemical family. Significant statistical differences were observed in terms of relative concentration among chemical families identified in spent coffee grounds from different coffee investigated samples. Furanic and nitrogen compounds found in coffee are mainly products of the Maillard reaction as well as thermal and Strecker degradations of their precursors (carbohydrates and proteins) [209], which occur during the roasting of the coffee beans. Furan compounds have been shown to have potential as alternative commodity chemicals to fossil-fuel-based platform chemicals, through the oxidation, dehydration, and hydrogenation process of functionalities attached to their furan ring [210], producing a variety of value-added chemicals [211]. Spent coffee grounds from Timor seem to be the richest in furanic compounds (21953 ± 1373 µg/L), followed by spent coffee grounds produced in Colombia (21091 ± 1298.9 µg/L), Brazil (9722 ± 840.5 µg/L), Guatemala (6531 ± 919.9 µg/L) and Ethiopia (6453 ± 517.1 µg/L). Furfural (2359 ± 224.9 µg/L of the total relative concentration of volatile fingerprint), 5-methylfurfural (4391 ± 415.6 µg/L), and 2-furanmethanol (3339 ± 311.3 µg/L) were the most abundant furanic compounds identified in all spent coffee grounds. Furfural can be transformed into useful fuels and chemicals used in oil refining, plastics production, and the pharmaceutical and agrochemical industries [212].

Table 3: Relative concentration (µg/L) of volatile organic compounds in the spent coffee grounds from different geographical origin using HS-SPME/GC–MS.

Peak nº	**RT (min)**	**Chemical Families**	**Relative concentration (µg/L) ± Standard deviation**				
			Guatemala	**Colombia**	**Brazil**	**Timor**	**Ethiopia**
Furanic compounds							
3	10.69	2-Methylfuran	32.35 ± 2.807	165.8 ± 18.27	157.6 ± 3.476	279.3 ± 35.10	81.75 ± 11.61
8	13.68	2,5-Dimethylfuran	32.45 ± 3.151	67.83 ± 10.29	45.82 ± 3.605	59.84 ± 7.554	30.22 ± 2.647
11	19.48	Vinylfuran	-	68.22 ± 10.09	62.12 ± 3.754	80.80 ± 14.44	25.15 ± 0.591
29	27.45	2-(2-Propenyl)furan	57.81 ± 9.025	174.4 ± 12.13	109.3 ± 1.837	128.0 ± 24.93	78.47 ± 14.94
31	28.67	2-Penthylfuran	153.7 ± 27.17	123.1 ± 6.383	81.27 ± 3.392	72.71 ± 11.59	50.92 ± 6.991
33	29.18	2-(Methoxymethyl)furan	40.75 ± 4.517	130.8 ± 4.067	64.74 ± 10.38	114.1 ± 11.85	55.75 ± 0.328
59	42.77	Furfural	1052 ± 185.5	3846 ± 303.1	1537 ± 195.4	4317 ± 344.7	1037 ± 95.63
65	45.12	2-Acetylfuran	611.6 ± 80.38	1319 ± 79.80	517.9 ± 38.71	1262 ± 100.6	360.5 ± 42.45
66	45.31	Benzofuran	-	-	118.5 ± 19.35	-	-
71	48.89	5-Methylfurfural	1876 ± 365.1	7150 ± 635.8	3071 ± 98.47	7625 ± 788.2	2235 ± 191.8
73	49.98	2,2'-Bifuran	68.59 ± 6.937	406.7 ± 38.41	233.6 ± 78.03	321.8 ± 6.142	167.6 ± 26.19
75	50.26	2-Methylbenzofuran	-	-	139.0 ± 19.08	101.6 ± 6.604	61.61 ± 6.493
77	50.47	2,2'-Methylenebisfuran	205.6 ± 15.90	1205 ± 40.91	777.4 ± 149.7	747.6 ± 56.68	442.8 ± 38.63
81	52.74	2-Furanmethanol	2131 ± 408.7	5270 ± 382.1	2096 ± 118.6	5916 ± 464.1	1280 ± 183.2
84	53.98	2-Furfuryl-5-methylfuran	153.0 ± 29.48	688.7 ± 60.06	380.1 ± 65.47	447.8 ± 23.57	294.8 ± 17.45
104	68.38	Difurfuryl ether	116.2 ± 13.61	471.7 ± 63.78	330.5 ± 50.35	479.4 ± 49.76	251.1 ± 32.65
Carbonyl compounds							
1	9.21	2-Methylpropanal	-	39.23 ± 5.756	20.99 ± 2.912	42.71 ± 3.692	15.65 ± 2.433
2	9.27	Acetone	36.77 ± 6.790	47.40 ± 3.951	25.49 ± 3.514	88.42 ± 12.57	18.87 ± 1.544
4	11.65	2-Butanone	18.21 ± 1.393	48.25 ± 6.338	23.08 ± 2.668	53.50 ± 4.322	18.04 ± 1.360
5	12.12	2-Methylbutanal	37.91 ± 6.963	185.7 ± 29.20	98.24 ± 8.734	280.1 ± 25.59	101.5 ± 11.28
6	12.27	3-Methylbutanal	19.09 ± 3.443	101.6 ± 13.12	48.51 ± 3.580	128.3 ± 0.579	47.23 ± 2.425
10	18.33	3-Hexanone	-	50.66 ± 7.312	26.30 ± 2.368	32.27 ± 0.898	16.48 ± 1.638
13	20.00	Hexanal	161.6 ± 21.45	76.59 ± 10.58	123.2 ± 4.822	84.80 ± 16.42	35.43 ± 1.386
15	20.85	2-Methyl-2-butenal	-	34.98 ± 2.586	-	49.81 ± 7.886	20.91 ± 2.539
49	37.46	2-Methylcyclopent-2-en-1-one	30.82 ± 4.513	24.43 ± 2.215	15.80 ± 1.861	51.17 ± 4.746	-
57	42.30	1-(Acetyloxy)-2-propanone	262.7 ± 50.82	772.4 ± 44.97	485.1 ± 14.64	742.4 ± 66.74	386.0 ± 36.64

64	44.76	2,3,4-Trimethylcyclopent-2-ene-1-one	47.01 ± 4.102	97.00 ± 14.88	69.10 ± 7.897	133.5 ± 23.56	-
68	46.10	1-(Acetyloxy)-2-butanone	69.79 ± 8.213	192.8 ± 11.79	98.62 ± 7.483	239.5 ± 38.96	78.67 ± 5.236
80	51.66	1-Methylpyrrole-2-carboxaldehyde	409.1 ± 60.99	1009 ± 96.13	408.2 ± 44.55	863.8 ± 125.8	337.4 ± 6.290
85	54.50	1-(5-Methyl-2-furanyl)-1-propanone	51.11 ± 8.913	193.3 ± 19.20	161.9 ± 15.62	245.7 ± 18.78	104.3 ± 3.237
88	55.44	3-Thiophenecarboxaldehyde	81.95 ± 6.049	194.1 ± 13.32	128.1 ± 4.330	230.0 ± 12.46	110.1 ± 3.956
89	55.77	4-(5-Methyl-2-furanyl)-2-butanone	55.04 ± 7.713	150.9 ± 9.911	117.7 ± 14.19	168.3 ± 13.30	99.93 ± 1.155
96	60.67	3,4-dimethylpyrrole-2-carboxaldehyde	79.86 ± 15.15	238.2 ± 23.10	163.8 ± 24.02	229.2 ± 28.86	133.4 ± 4.687
100	64.50	3-Ethyl-2-hydroxy-2-cyclopenten-1-one	48.21 ± 10.05	140.3 ± 27.11	-	142.8 ± 11.36	45.20 ± 3.894
101	65.20	4-(2-Furanyl)-3-buten-2-one	75.43 ± 12.29	222.4 ± 5.494	154.6 ± 18.53	197.4 ± 30.26	89.65 ± 16.57
109	73.79	1-Methylpyrrole-2-carboxaldehyde	73.36 ± 11.76	186.6 ± 24.95	137.8 ± 22.56	207.6 ± 25.33	98.89 ± 7.737
Nitrogen compounds							
19	23.48	1-Methylpyrrole	-	56.41 ± 9.041	43.53 ± 1.601	113.8 ± 17.90	-
25	25.89	Pyridine	231.5 ± 16.62	465.3 ± 17.32	549.1 ± 52.99	672.2 ± 104.2	180.8 ± 31.07
30	27.77	Pyrazine	19.67 ± 1.841	47.68 ± 5.511	35.46 ± 4.561	67.02 ± 8.104	-
38	30.99	Methylpyrazine	594.3 ± 66.29	1206 ± 62.03	621.6 ± 80.05	1252 ± 100.3	375.4 ± 51.81
44	34.31	2,5-Dimethylpyrazine	4870 ± 74.89	1228 ± 89.84	631.7 ± 77.06	1075 ± 106.1	446.4 ± 58.79
45	34.68	2,6-Dimethylpyrazine	534.2 ± 75.54	1157 ± 81.97	577.6 ± 86.41	989.7 ± 94.39	412.0 ± 58.61
46	35.09	Ethylpyrazine	324.31 ± 26.88	623.1 ± 82.54	405.2 ± 66.44	610.5 ± 55.32	279.3 ± 31.50
47	35.80	2,3-Dimethylpyrazine	100.6 ± 16.08	193.4 ± 6.569	135.2 ± 20.53	201.6 ± 12.12	76.31 ± 11.12
48	37.33	1-Pentylpyrrole	-	-	12.25 ± 0.496	-	-
50	38.03	2-Ethyl-6-methylpyrazine,	495.6 ± 84.41	1396 ± 104.9	860.8 ± 130.4	1216 ± 113.5	737.2 ± 83.66
51	38.42	2-Ethyl-5-methylpyrazine	381.5 ± 70.70	1056 ± 127.9	695.6 ± 68.88	906.9 ± 108.8	590.8 ± 53.03
52	39.13	Trimethylpyrazine	390.7 ± 54.93	1029 ± 123.9	644.4 ± 72.31	897.8 ± 95.26	497.1 ± 36.64
53	40.03	2-(n-Propyl)pyrazine	44.63 ± 7.261	80.94 ± 3.038	71.45 ± 10.83	105.7 ± 12.70	44.33 ± 3.517
54	40.84	2,6-Diethylpyrazine	91.39 ± 13.22	1329 ± 27.96	274.7 ± 25.70	280.7 ± 28.03	233.4 ± 21.70
55	41.37	3-Ethyl-2,5-dimethylpyrazine	413.5 ± 62.74	1329 ± 150.5	1086 ± 55.40	1079 ± 80.68	894.1 ± 66.24
56	41.96	2,3-Dimethylpirazine	-	88.19 ± 7.728	77.43 ± 2.614	90.05 ± 15.41	53.59 ± 3.506
58	42.56	2-Methyl-5-propylpyrazine	-	-	54.63 ± 0.121	86.75 ± 9.378	67.38 ± 4.048
61	44.02	2,3-Diethyl-5-methylpyrazine	51.43 ± 6.440	-	-	-	112.7 ± 9.891
62	44.09	3,5-Diethyl-2-methylpyrazine	77.78 ± 13.28	277.9 ± 35.88	571.2 ± 35.19	423.6 ± 31.38	290.9 ± 12.74
63	44.49	2-Ethenyl-6-methylpyrazine	-	156.5 ± 18.49	107.1 ± 5.82	170.3 ± 32.77	66.40 ± 1.476
67	45.82	2-Methoxy-3-(2-methylpropyl)pyrazine	-	-	258.5 ± 30.03	528.2 ± 48.95	-

74	50.07	Isopropenylpyrazine	40.46 ± 8.075	186.7 ± 20.50	79.01 ± 12.64	178.6 ± 20.04	83.51 ± 11.45
78	50.56	2-Acetylpyridine	-	-	-	134.0 ± 19.05	69.45 ± 8.223
82	53.22	2-Acetyl-1-methylpyrrole	253.6 ± 48.89	847.5 ± 99.88	534.5 ± 44.33	683.6 ± 21.41	337.4 ± 33.81
87	55.22	2-Acetyl-3-methylpyrazine	63.38 ± 8.495	237.2 ± 35.77	136.8 ± 9.104	252.8 ± 24.67	114.8 ± 9.090
90	56.25	3-Methylpyrrole	53.57 ± 7.528	204.2 ± 22.15	87.33 ± 12.65	213.9 ± 26.46	-
97	61.57	1-(2-Furanylmethyl)pyrrole	403.7 ± 77.24	1615 ± 89.00	1291 ± 108.4	1462 ± 92.84	977.4 ± 83.77
103	67.99	2-Acetylpyrrole	263.2 ± 52.03	803.2 ± 156.7	291.3 ± 36.40	837.9 ± 73.19	234.3 ± 37.07
107	70.52	Pyrrole-2-carboxaldehyde	304.7 ± 55.57	1081 ± 188.2	502.1 ± 76.74	1102 ± 44.02	483.3 ± 75.45
Esters							
17	21.41	Methyl-2-butanoate	-	-	-	-	15.76 ± 1.038
32	28.77	Undecyl benzoate	-	-	13.51 ± 1.205	-	-
36	30.00	3,3-Dimethylallyl acetate	-	37.87 ± 2.202	46.03 ± 9.062	47.30 ± 1.309	32.02 ± 0.934
69	46.38	2-Furfuryl acetate	3090 ± 497.2	7930 ± 144.8	6097 ± 737.2	8499 ± 633.6	4250 ± 270.1
72	49.57	2-Furfuryl propanoate	205.4 ± 29.76	638.7. ± 22.90	438.7 ± 33.69	504.0 ± 55.12	328.2 ± 39.06
86	54.54	Furfuryl 3-methylbutanoate	89.78 ± 15.27	379.5 ± 46.98	198.8 ± 39.16	354.2 ± 23.96	224.8 ± 6.479
93	59.60	Methyl-2-hydroxybenzoate	40.45 ± 6.367	251.1 ± 38.54	121.0 ± 17.56	344.2 ± 40.12	133.9 ± 8.486
Terpenoids							
18	22.06	β-Terpinene	28.99 ± 1.445	17.29 ± 2.617	-	-	-
21	23.74	3-Carene	23.20 ± 3.721	-	-	-	-
23	24.43	β-Myrcene	26.69 ± 3.975	58.45 ± 9.665	37.32 ± 2.044	39.95 ± 5.829	38.64 ± 1.532
24	25.19	α-Terpinene	26.13 ± 3.925	-	-	-	-
28	26.80	Limonene	194.1 ± 25.74	200.0 ± 16.20	103.3 ± 9.771	106.9 ± 16.01	164.8 ± 9.548
34	29.65	γ-Terpinene	87.64 ± 11.33	54.32 ± 6.037	-	-	-
35	29.83	β-cis-Ocimene	-	68.74 ± 6.549	34.20 ± 1.713	34.79 ± 5.172	30.30 ± 3.454
39	31.30	Cymene	107.0 ± 17.59	51.05 ± 7.566	-	-	-
76	50.35	Caryophyllene	172.7 ± 33.19	-	-	-	-
92	59.16	2-Menthene	40.14 ± 6.087	199.9 ± 21.73	78.83 ± 11.04	244.1 ± 15.15	62.24 ± 8.815
111	77.92	Carvacrol	96.52 ± 14.20	855.3 ± 124.3	653.5 ± 126.8	1393 ± 118.5	270.3 ± 32.90
Volatile phenols							
22	24.05	3-Methylphenol	-	156.2 ± 17.66	126.1 ± 10.80	113.0 ± 1.997	66.73 ± 9.176
98	63.00	Guaiacol	263.7 ± 41.87	852.5 ± 118.3	457.4 ± 26.62	903.4 ± 83.59	441.3 ± 36.06

99	63.93	*o*-Cresol	85.86 ± 15.10	222.8 ± 36.49	178.5 ± 9.106	231.5 ± 19.46	151.9 ± 17.41
105	69.00	*p*-Cresol	63.46 ± 5.820	141.5 ± 3.574	82.15 ± 3.970	167.8 ± 12.53	63.80 ± 9.344
106	69.26	Phenol	119.9 ± 19.63	287.0 ± 28.80	142.9 ± 8.188	345.9 ± 32.88	117.3 ± 20.18
108	72.78	*m*-Cresol	61.88 ± 5.393	164.9 ± 27.65	131.1 ± 20.10	281.9 ± 39.97	84.74 ± 12.16
110	76.46	*m*-tert-Butylphenol	43.97 ± 0.994	-	-	-	-
Sulphur compounds							
12	19.65	Dimethyldisulfide	-	33.51 ± 5.493	-	47.00 ± 6.383	15.04 ± 0.262
14	20.67	2-Methylthiophene	-	21.62 ± 0.893	21.65 ± 1.543	-	13.39 ± 0.668
102	66.71	2-Thiophenemethanol	72.11 ± 10.73	266.0 ± 46.03	-	-	-
Alcohols							
42	33.30	3-Methylbenzyl alcohol	-	-	15.21 ± 1.480	-	-
43	33.44	2-Heptanol	-	-	49.17 ± 8.216	48.71 ± 5.321	40.74 ± 4.082
60	43.31	2-Ethyl-1-hexanol	131.5 ± 24.21	129.0 ± 24.51	95.27 ± 4.130	-	-
70	46.98	1-Octanol	-	-	80.54 ± 12.42	-	-
Acids							
83	53.51	3-Methylbutanoic acid	418.9 ± 48.21	437.0 ± 77.70	-	297.7 ± 46.61	95.85 ± 16.71
94	59.90	(Pyrrol-3-yl) acetic acid	-	182.2 ± 15.59	93.53 ± 9.126	276.5 ± 45.85	115.9 ± 7.289
95	60.13	3-Methyl-2-butenoic acid	59.71 ± 5.449	105.8 ± 9.025	-	132.0 ± 11.38	73.68 ± 11.98
Others							
7	13.49	2,2,4,6,6-Pentamethylheptane	15.30 ± 1.552	-	-	-	-
9	15.30	Decane	31.96 ± 3.145	16.74 ± 1.903	8.31 ± 0.4633	-	-
16	21.14	2,3,6-Trimethyl-1,5-heptadiene	-	28.64 ± 3.653	21.54 ± 2.496	-	19.32 ± 2.044
20	23.52	*p*-Xylene	20.14 ± 2.173	-	-	-	-
26	26.14	Dodecane	53.13 ± 4.700	127.3 ± 14.52	64.57 ± 6.056	62.26 ± 12.11	-
27	26.33	Trimethyloxazole	25.72 ± 4.273	39.29 ± 2.386	43.27 ± 7.174	-	47.78 ± 5.174
37	30.61	Styrene	-	53.67 ± 0.409	33.55 ± 3.740	31.48 ± 1.251	32.17 ± 3.835
40	31.71	2,7-Dimethyloxepine	-	58.62 ± 7.032	31.20 ± 1.046	-	-
41	32.34	3-Methylanisole	-	252.9 ± 20.84	133.1 ± 3.533	111.7 ± 16.03	81.05 ± 5.853
79	51.04	1-Acetyl-2-methylcyclopentene	108.3 ± 18.89	327.0 ± 48.98	164.3 ± 21.58	265.4 ± 28.01	136.5 ± 12.05
91	57.86	Cyclopentene	45.19 ± 4.663	-	-	-	-

RT - Retention time.

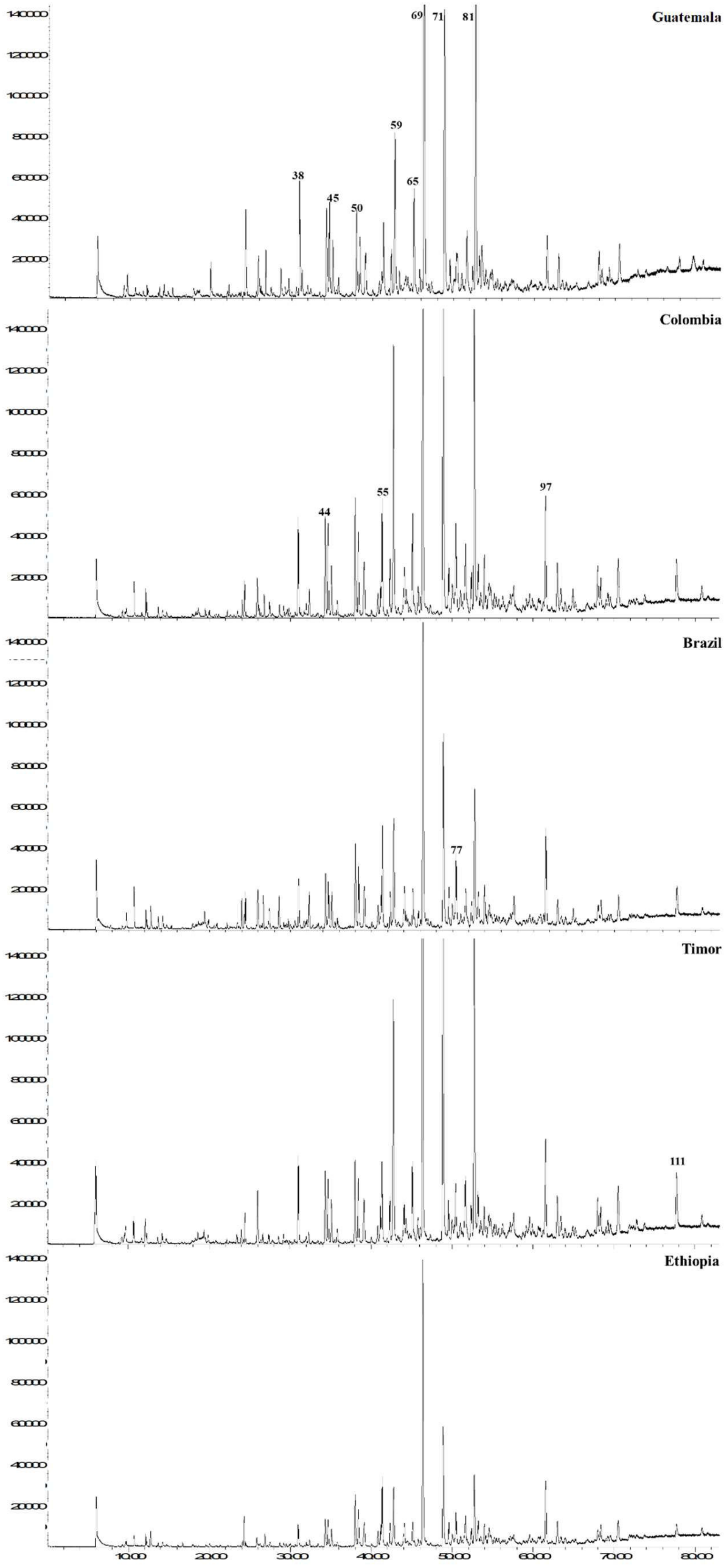


Figure 11: Chromatograms obtained through HS-SPME/GC-MS for spent coffee grounds from different geographical origin (attribution of the peak number is shown in Table 1).

This furanic compound was identified in all the spent coffee ground samples analyzed in high relative concentrations but was more abundant in spent coffee grounds from Colombia and Timor, Table 3. On the other hand, benzofuran was only detected in spent coffee grounds from Brazil, whereas vinyl furan was not detected only in spent coffee grounds from Guatemala.

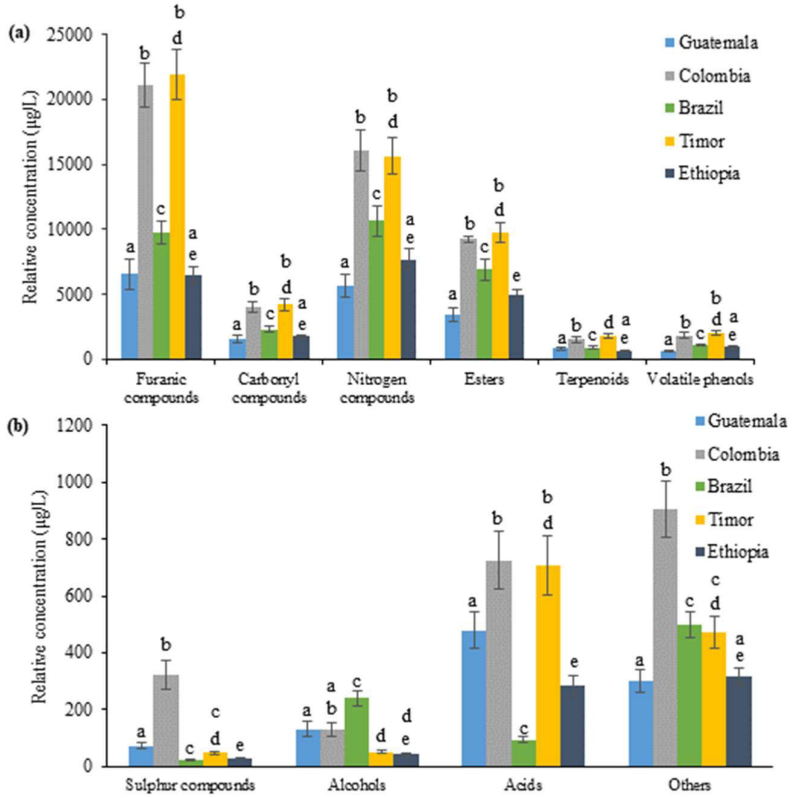


Figure 12: Relative concentration (µg/L) of the VOCs identified grouped by major (a) and minor (b) chemical families for the spent coffee grounds Different superscript letters in the same chemical family indicate significant differences ($p < 0.05$) among spent coffee grounds from different geographical origin.

Nitrogen compounds represent the second most abundant chemical family identified in the analyzed samples, of which the majority consisted of pyrazines and pyrroles. Pyrazine and its derivates have various applications as ingredients in pesticides, insecticides, dyes, pharmaceutical products, corrosion inhibitors, organic photovoltaics, and organic emitting diodes [213]. Moreover, alkylated pyrazines are used as flavoring agents in the food industry due to their strong aromatic properties, while methoxylated pyrazines in the perfume industry are used to improve the odor of cosmetics and toiletries [214]. On the other hand, pyrrole and its derivates have been proven to play a significant role in material science, as a component of optoelectronic equipment, and in pharmaceutical chemistry, since several pyrrole-based drugs have been discovered [215]. The nitrogen compounds' contribution to the total volatile fingerprint is similar between the spent coffee grounds from Ethiopia and Brazil, 33.27 and 32.72%, respectively. Nevertheless, this chemical family has a less contribution to the total volatile fingerprint in spent coffee grounds from Colombia (28.81%), Guatemala (28.74%), and Timor (27.58%). 2-Ethyl-6-methylpyrazine (941.2 ± 103.4 µg/L of the total relative concentration of volatile fingerprint), 3-ethyl-2.5-dimethylpyrazine (960.4 ± 83.11 µg/L), and 1-(2-furanylmethyl)pyrrole (1150 ± 90.24 µg/L) were the most abundant nitrogen compounds in spent coffee grounds independently of geographical origin.

Esters, synthesized through the oxidation of fatty acids, contribute to the fruity and floral flavor of spent coffee grounds, and depending on their specific properties have a wide range of applications in the cosmetic (e.g., emollients in creams, surfactants in shampoos, antioxidants in antiaging creams, fragrances in perfumes) [216] and food (e.g., flavor and aroma of the diversity of food-related products) industry [217]. Spent coffee grounds from Timor seem to be the richest in esters (9749 ± 599.0 µg/L), followed by spent coffee grounds produced in Colombia (9237 ± 195.5 µg/L), Brazil (6915 ± 652.3 µg/L), Guatemala (3426 ± 436.0 µg/L) and Ethiopia (4985 ± 249.8 µg/L). 2-Furfuryl acetate (5973 ± 456.6 µg/L of the total relative concentration of volatile fingerprint) was the predominant ester identified in all spent coffee grounds independently of geographical origin.

Carbonyl compounds (aldehydes and ketones) were a similar contribution to the total volatile fingerprint among the spent coffee grounds from Guatemala (7.97%), Ethiopia (7.64%), Timor (7.43%), Colombia (7.18%), and Brazil (7.10%). 1-Methylpyrrole-2-carboxaldehyde (605.4 ± 66.74 µg/L of the total relative concentration of volatile fingerprint) and 1-(acetyloxy)-2-propanone (529.7 ± 42.76 µg/L) were the most abundant carbonyl compounds identified in spent coffee grounds analyzed. 2-Methylpropanal and 3-hexanone were not detected in spent coffee grounds from Guatemala, 2-methylcyclopent-2-en-1-one and 2,3,4-trimethylcyclopent-2-en-1-one in spent coffee grounds from Ethiopia, and 3-ethyl-2-hydrodoxy-2-cyclopenten-1-one in spent coffee grounds from Brazil. Terpenoids are compounds produced by plants (phytochemicals) and are one of the largest families of natural compounds, owing to their chemical diversity. Therefore, they can have a wide range of industrial applications, such as flavors, fragrances, high-grade lubricants, biofuels, agricultural chemicals, and medicines [218]. The terpenoid's contribution to the total volatile fingerprint is similar among the spent coffee grounds from Brazil (2.83%), Colombia (2.70%), and Ethiopia (2.46%), being highest in spent coffee grounds from Guatemala (4.11%). Carvacrol (653.6 ± 83.32 µg/L of the total relative concentration of volatile fingerprint), limonene (153.8 ± 15.45 µg/L), and 2-menthene (125.0 ± 12.56 µg/L) were the most abundant terpenoids in spent coffee grounds independently of geographical origin. Carvacrol has been progressively employed in the food and pharmaceutical industries due to its strong antibacterial and anti-inflammatory properties [219]. Nevertheless, carvacrol is volatile, easily oxidized, and difficult to dissolve in water, and for this reason, several studies have been developed to improve its stability through encapsulation methods [211,219]. Limonene was used as a green and non-toxic solvent alternative to produce polystyrene fiber matrix by electrospinning [211] and applied in the agri-food industry as an antimicrobial, herbicidal, and antioxidant agent [220].

3.1.1. Potential applications of spent coffee grounds volatile compounds

In nature, VOCs, perform numerous functions for the communication between insects and/or plants for (insect) mating or even, because of their pleasant smell or taste, as fragrances and flavors. In fruits and vegetables [221], these secondary metabolites are generated from fatty acids or amino acid precursors during ripening, whiles in other foods several pathways can explain its formation such as the yeast metabolism in fermented beverages, the oxidation/reduction processes during storage/aging, the Maillard and Strecker reactions which occur as a result of some thermal procedures to which some foods are subjected, the coffee roasting and wine heating (*estufagem* in Madeira wine), for instance, with the formation of compounds that impact the aroma of different products such as furan, sulfur, and nitrogen-containing compounds [222].

Table 4: Potential properties of some important volatile organic metabolites (VOCs) identified in spent coffee grounds analyzed in this study.

VOCs	**Antidiabetic**	**Anti-inflammatory**	**Antimicrobial**	**Antioxidant**	**Antiproliferative**	**Antitumor**	**Cytotoxic**	**Flavors**	**References**
Furfural		x	x	x					
5-Methylfurfural			x			x		x	
2-Furanmethanol		x	x	x					
2-Ethyl-6-methyl-pyrazine								x	
3-Ethyl-2,5-dimethylpyrazine								x	
Pyrrole-2-carboxaldehyde			x						
Hexanal			x						[221,223–231]
β-Myrcene			x	x			x		
Limonene	x	x	x	x	x	x	x		
Carvacrol		x	x	x	x	x	x		
Menthene		x	x	x					
Dimethyl disulfide				x					
Guaiacol			x	x					

There is increasing demand for natural compounds which can act as food preservatives to inhibit the growth of microorganisms (Table 4), especially pathogenic microorganisms, and to control the natural spoilage process. In this context, the use of waste from the agrifood industry constitutes an important natural, cheap, and renewable source of compounds with a potential preservative effect, among which, in addition to phytochemicals, a wide variety of VOCs can be highlighted [226].

Some of the VOCs identified in the spent coffee grounds analyzed have been pointed out for presenting biological activity, including antidiabetic, anti-inflammatory, antimicrobial, antioxidant, antiproliferative, and antitumor activities, potentiating their applications in the food industry as preservatives, additives, flavoring agents, among others (Table 2) [221,223–231].

A semi-quantitative analysis of the volatile profile of spent coffee ground was performed by Page et al. [232]. The most abundant chemical families identified in the evaluated samples were furan compounds, pyrroles, phenolics, and carboxylic acids. Terpenes, alcohols, ketones, aldehydes, thiophenes, thiazoles, and benzoxazoles were also identified in minor quantities. In addition, Zambonin et al. [233] also identified pyrazines, furans, pyrroles, and phenolic compounds as the most abundant classes of compounds when studying coffee brews using HS-SPME/GC–MS.

Ten of these VOCs were indicated to have antimicrobial activity which were furfural [225], 5-methylfurfural [230], 2-furanmethanol [224], pyrrole-2-carboxaldehyde [231], hexanal, β-myrcene, limonene, guaiacol [221], carvacrol [223], and menthene [221]; eight compounds with antioxidant activity, including furfural, 2-furanmethanol [226], β-myrcene, limonene, guaiacol [221], carvacrol [223], menthene [229], and dimethyl disulfide [228]; five compounds with anti-inflammatory activity including furfural, 2-furanmethanol [226], limonene [221], carvacrol [223], and menthene [229]; three compounds with antitumor activity 5-methylfurfural

[230], limonene [221], and carvacrol [223]; three compounds that present cytotoxic activity, including β-myrcene, limonene [221], and carvacrol [223]; and one compound, limonene [221], with antidiabetic activity. Additionally, three of the identified VOCs were used as flavoring agents, including 5-methylfurfural [230], 2-ethyl-6-methylpyrazine, and 3-ethyl-2,5-dimethylpyrazine [227].

These VOCs properties enhance its potential use in different areas including the food, pharmaceutical, and cosmetics industry, valuing a natural, renewable, and low-cost raw material, from a circular economy perspective.

3.1.2. Statistical analysis

The one-way ANOVA with post-hoc Tukey test ($p < 0.05$) performed showed that the p values obtained proved that the identified 111 VOCs presented statistically significant differences for the analyzed spent coffee grounds from different geographical origins. Statistical analysis was applied to the spent coffee grounds through the principal component analysis (PCA) multivariate pattern recognition procedure, to determine the differences in the volatile fingerprint. Figure 13(a,b) present the PCA score plot and loading plot from the spent coffee grounds from different geographical origins, respectively. All spent coffee grounds were adequately separated, underlining that the total 111 VOCs identified in the five different samples allowed their discrimination.

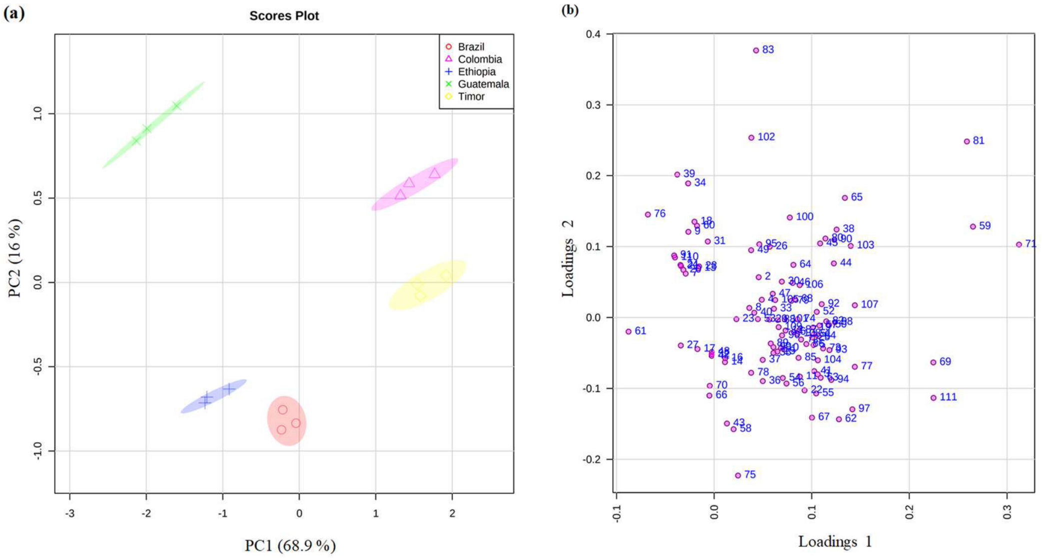


Figure 13: PCA of the volatile fingerprint of spent coffee grounds from different geographical origin. (a) PC1 × PC2 score scatter plot and (b) loading weight plot (attribution of the peak number is shown in Table 3).

The variances of PC1 and PC2 were 68.9 and 16%, respectively, representing 84.9% of the total variability of data. Colombia spent coffee grounds, projected in PC1 and PC2 positive, was characterized by 2-furanmethanol (81), 2-acetylfuran (65), and furfural (59), whereas the spent coffee grounds from Ethiopia and Brazil projected in PC1 and PC2 negative by trimethyloxazole (27), methyl-2-butanoate (17), 1-octanol (70), 2-methylbenzofuran (75), 2-methyl-5-propylpyrazine (58), and 2-heptanol (43). Guatemala spent coffee grounds projected in PC1 negative and PC2 positive, was characterized by caryophyllene (76), o-cymene (39), and γ-terpinene (34), while Timor spent coffee grounds placed in PC1 positive and PC2 negative by 2-furfuryl acetate (69), carvacrol (111), and 5-methylfurfural (71).

Moreover, partial least square-discriminant analysis (PLS-DA) was applied (Figure 14) and the 10 most significant VOCs (VIP score > 1.5) that allowed the discrimination of the spent coffee ground samples by geographical origin were 3-methylbutanoic acid (83), 3,5-diethyl-2-methylpyrazine (62), 2-furanmethanol (81), 2-acetylpyridine (78), 3-methyl-2-butenoic acid (95), 2,2'-methylenebisfuran (77), 3-ethyl-2-hydroxy-2-cyclopenten-1-one (100), 2,7-dimethyloxepine (40), 3-methylanisole (41), and benzofuran (66) (Figure 13(b)).

A random permutation test with 1000 permutations was performed with PLS-DA model (Figure 14) to evaluate the robustness of the model.

The heatmap created using Pearson's correlation for the VOCs with VIP scores > 1.5 is shown in Figure 14(c). The heatmap shows that most of these VOCs presented higher chromatographic area in Colombia spent coffee grounds, except for 2-acetylpyridine (78) and benzofuran (66), that presented higher values in Timor and Brazil spent coffee grounds, respectively. In addition, as seen in Figure 14(c), the volatile fingerprint of spent coffee grounds showed remarkable differences among the geographical regions. This variance could be an outcome of climate conditions factors (e.g., mean annual temperature), altitude and geographic location (e.g., longitude, latitude), which may result in an inhibition of the activity of certain odor-related enzymes [234,235].

In Figure 15(a) goodness of fit of 0.9944 ($R^2 = 99.44\%$) and a predicted ability of 0.9813 ($Q^2 = 98.13\%$) were obtained with three significant components, based on crossing-validation. The model is not overfitted, as can be proved by the difference between R^2 and Q^2, which was lower than 0.3 ($R^2 - Q^2 = 0.01$). This indicates that it presented an acceptable predictive ability for the discrimination of the spent coffee ground from different geographical origin.

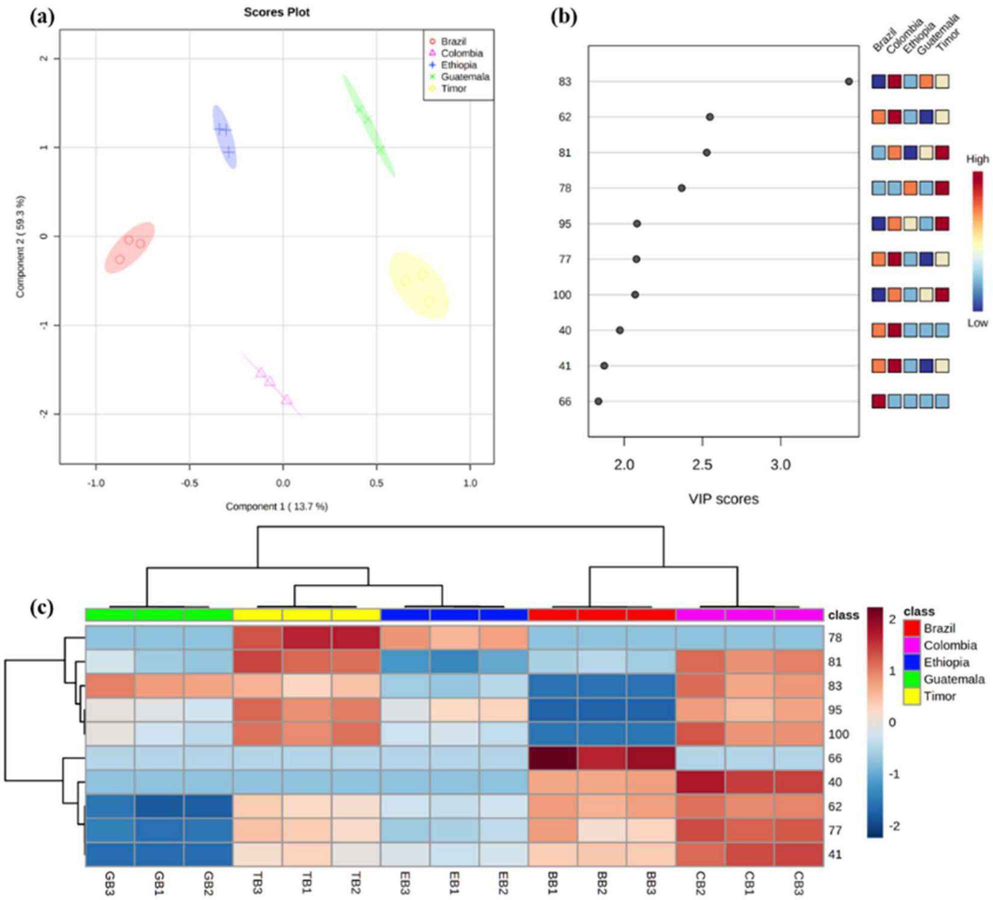


Figure 14: PLS-DA of the volatile fingerprint of spent coffee grounds from different geographical origins (a) score scatter plot, and (b) VIP scores and (c) hierarchical cluster analysis (HCA). (Attribution of the peak number is shown in Table 1).

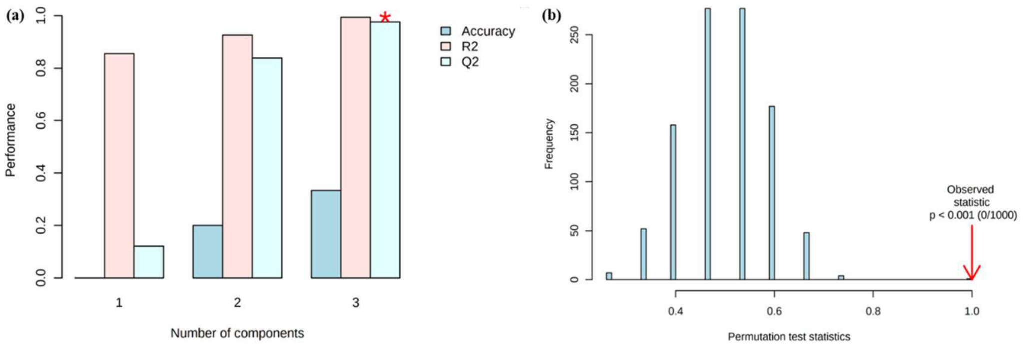


Figure 15: (a) 10-fold cross-validation performance and (b) model validation by permutation test based on 1000 permutations of VOCs obtained by GC-MS of spent coffee grounds samples (* means best Q^2 value).

3.2. Evaluation of the bioactive and antioxidant potential of spent coffee grounds

To evaluate the bioactive and antioxidant potential of the spent coffee grounds, a μ-QuEChERS extraction procedure was performed. The extracts were, then, analyzed through spectrophotometric assays (TPC, DPPH, and ABTS).

Phenolic compounds are recognized for having high antioxidant activity due to their redox properties, playing important roles in absorbing and neutralizing free radicals [236]. In the present work, the TPC was assessed using the Folin-Ciocalteu method, which is a colorimetric assay based on electron transfer reactions between the Folin-Ciocalteu reagent and phenolic compounds [237]. The TPC results for the spent coffee grounds samples from different geographical origins can be found in Figure 16. The values obtained for TPC ranged from 41.56 ± 2.085 to 53.68 ± 3.120 mg GAE/100 g DW, for Colombia and Brazil spent coffee grounds, respectively. No statistically significant differences ($p < 0.05$) were found between the samples from Guatemala, Brazil, Timor, and Ethiopia. However, the TPC obtained for Colombia was significantly different to all samples except for Ethiopia. Additionally, the TPC obtained for the spent coffee grounds analyzed was considerably lower than the results reported in literature. For instance, Mussatto et al. [238] was able to extract 18 mg GAE/g DW, while Panusa et al. [239] obtained extracts with TPC up to 36 mg GAE/g DW. The differences observed between the TPC values obtained for the spent coffee grounds samples could be related to the edaphoclimatic conditions (e.g., temperature, humidity, altitude, geographical location) that influence the quantities in which these secondary metabolites occur in the coffee plants [240,241].

The antioxidant potential of the spent coffee grounds was determined using the DPPH and ABTS assays. Because these two assays use model synthetic radicals which are not directly associated with food or biological systems, frequent objection is raised. However, they are commonly applied due to their simplicity, acceptable repeatability, and low cost. Moreover, literature demonstrates that DPPH and ABTS are the most preferably employed synthetic radicals in antioxidant assays [242]. The scavenging of the DPPH, which is a highly colored and stable free radical, is the basis of the popular DPPH antioxidant assay [243]. The values obtained for the DPPH scavenging assay, presented in Figure 17, differed from 50.64 ± 5.257 (Guatemala) to 78.11 ± 7.333 mg TE/100 g DW (Brazil). There were no statistically significant differences between the samples from Guatemala, Colombia, and Timor, while the value obtained for Ethiopia was only significantly different from Guatemala. Brazil spent coffee grounds presented antioxidant capacity significantly superior to all samples, except for Ethiopia. Additionally, the ABTS assay was performed to measure the antioxidant potential of the spent coffee grounds. The green-blue stable radical cationic chromophore $ABTS^{\bullet+}$ is generated by the reaction of the ABTS salt with a strong oxidizing agent (in this case potassium persulfate). The ABTS assay is based on the suppression of its characteristic long wave absorption spectrum, caused by the reduction of the ABTS radical by hydrogen-donating antioxidants [244]. The values obtained for the scavenging of the ABTS radical (Figure 18) ranged between 0.494 ± 0.039 (Guatemala) and 1.787 ± 0.210 (Ethiopia) mg TE/100 g DW. The statistical analysis showed that the ABTS scavenging capacity was significantly different between all the samples, except for Colombia and Brazil. The values observed for the ABTS assay were considerably lower than those obtained the DPPH assay for all samples. This could be related to the specific antioxidant capacity of the compounds present in the extracts against the free radicals used. Nenadis et al. [242] compared the order of antioxidant activity of specific phenolic compounds against the DPPH and ABTS radicals. The results showed that caffeic and chlorogenic acids, two phenolic compounds typically found in coffee, presented higher scavenging capacity against DPPH than ABTS. A correlation between the TPC values and the antioxidant capacity should be expected, since phenolic compounds are potent antioxidants, meaning higher TPC corresponds to superior antioxidant capacity. However, this was not observed in this works results. This could be explained by the presence of non-phenolic compounds in the extracts analyzed that possess antioxidant capacity (for instance, caffeine), and aid the reduction of the free radicals.

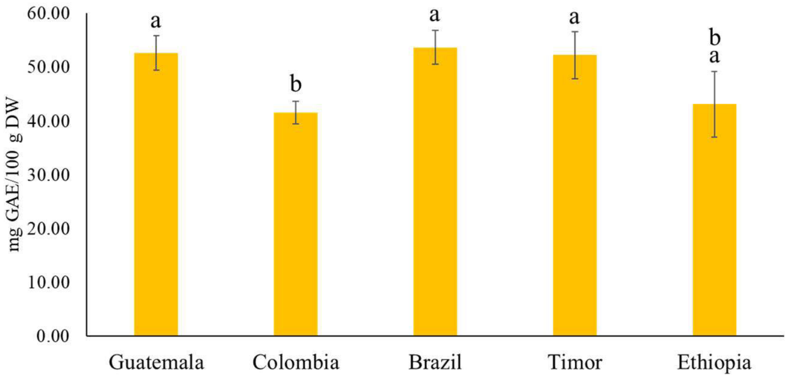


Figure 16: Total phenolic content of spent coffee grounds from different geographical origins, expressed in mg GAE/100 g DW. Different superscript letters indicate significant differences ($p < 0.05$) among spent coffee grounds from different geographical origins.

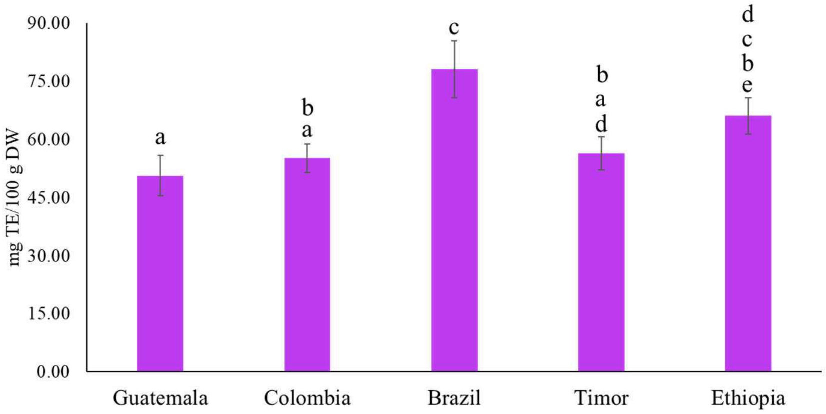


Figure 17: DPPH scavenging assay for spent coffee grounds from different geographical origins, expressed in mg of TE/100 g DW. Different superscript letters indicate significant differences ($p < 0.05$) among spent coffee grounds from different geographical origins.

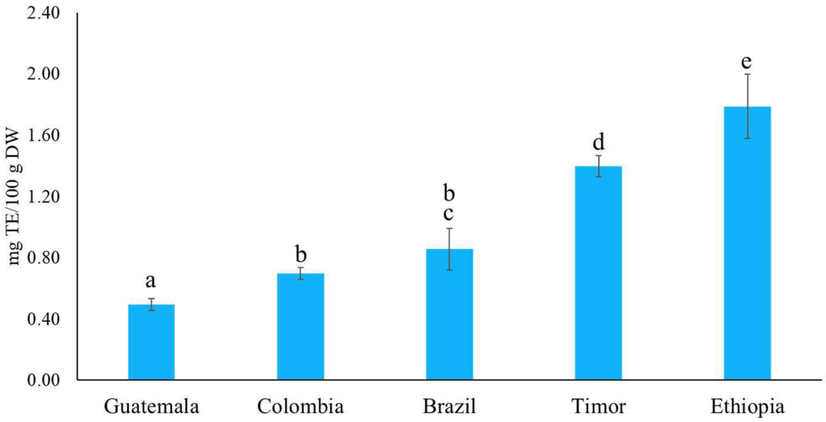


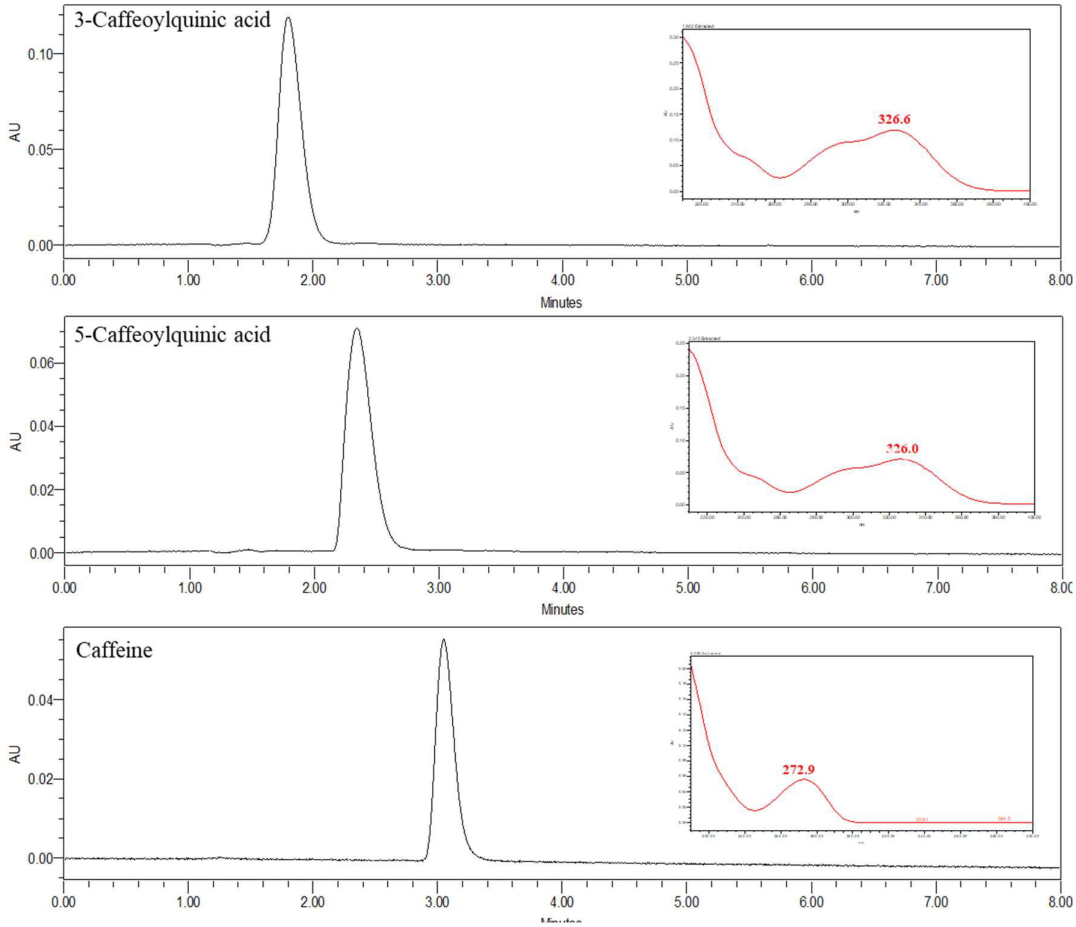
Figure 18: ABTS assay for spent coffee grounds from different geographical origins, expressed in mg of TE/100 g DW. Different superscript letters indicate significant differences ($p < 0.05$) among spent coffee grounds from different geographical origins.

3.3. Analysis of bioactive compounds by UHPLC-PDA

3.3.1. Validation of the μ-SPEed/UHPLC-PDA methodology

The performance of the μ-SPEed/UHPLC-PDA method applied was validated in terms of selectivity, linearity, LOD, LOQ, precision, and accuracy, to demonstrate that the method is adequate for the quantification of the proposed bioactive compounds in spent coffee grounds.

The selectivity of the method was assessed through the comparison of the chromatograms and PDA spectra obtained for the spent coffee grounds UHPLC-PDA analysis, with those of analytical standards. The standards used for the identification were 3-CQA, 5-CQA, caffeine, caffeic acid, 4,5-diCQA, 1,5-diCQA, and 3,4-diCQA. The chromatograms obtained the individual standards used in the identification of bioactive compounds are shown in Figure 19. There were not considerable interferences in the retention times at which the analytes of interest appear, proving that the method is selective and allowing the identification of these analytes in the different spent coffee grounds.



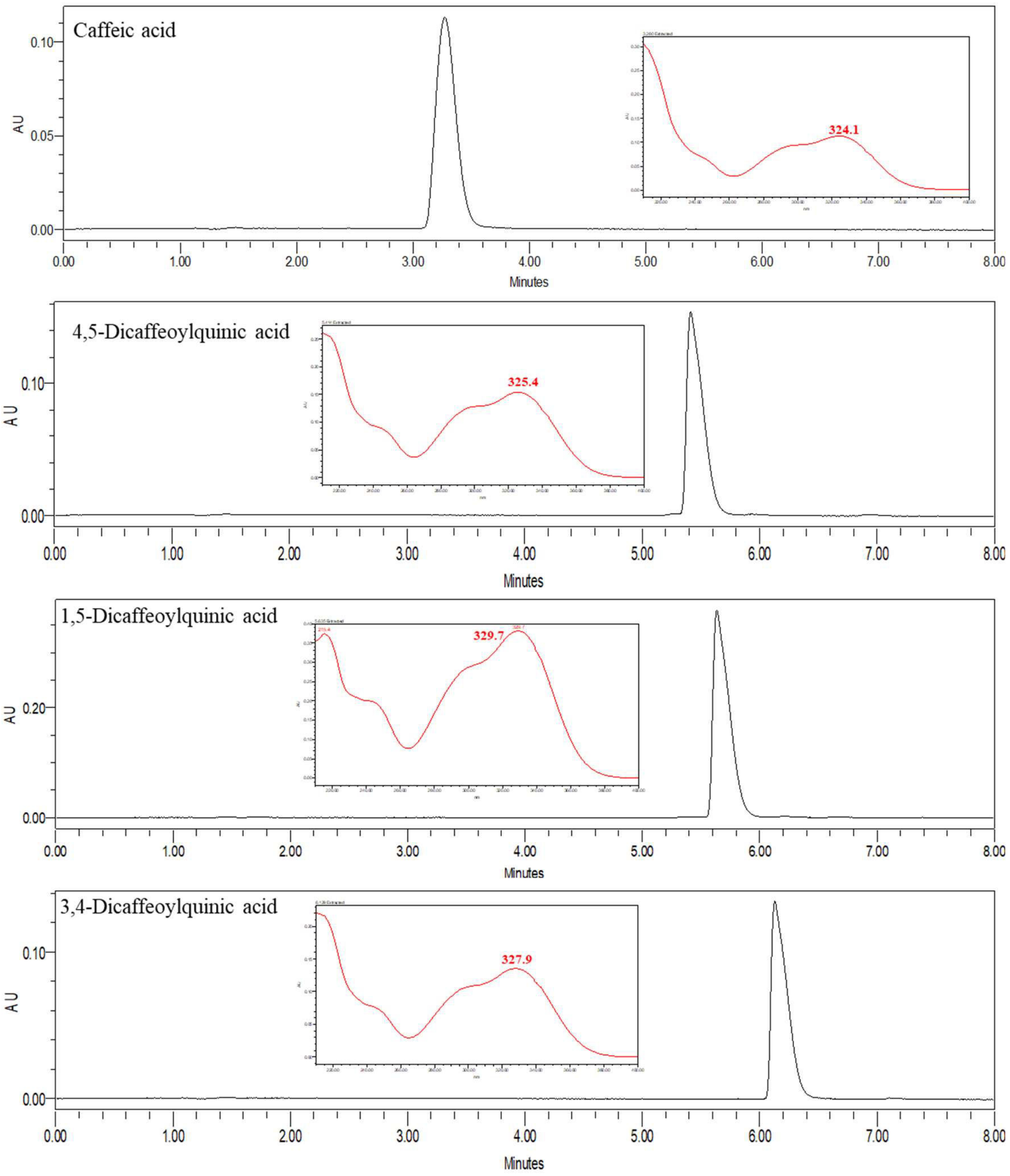


Figure 19: Chromatograms obtained for the individual standard solutions and PDA spectra acquired for each peak used for the identification of the bioactive compounds in spent coffee grounds (acquired at 273 nm for caffeine and at 326 nm for the remaining analytes).

Further, the capacity of the method to produce results directly proportional to the concentration of the analytes was evaluated. The calibration curves were obtained by performing the methodology with seven different concentrations of a mix solution of 5-CQA, caffeine and caffeic acid, ranging from 0.5 to 75 µg/mL. The analytes 3-CQA, 4,5-diCQA, 1,5-diCQA, and 3,4-diCQA were quantified in 5-CQA equivalents. The results of the linearity evaluation are presented in Table 5. Linearity was observed for all the compounds within the concentration range utilized. The coefficients of correlation and determination were superior to 0.998, proving a satisfactory adjustment of the obtained value to the calibration curve.

Table 5: Results obtained for the linearity evaluation and limits of detection and quantification through the µ-SPEed/UHPLC-PDA method.

RT (min)	Analyte	λ_{max} (nm)	Linear range (µg/mL)	Calibration Curve		LOD (µg/mL)	LOQ (µg/mL)
2.45	5-CQA	326	0.2 - 75	Equation R^2	y = 72508x + 57908 0.9984	0.010	0.032
2.85	Caffeine	273	0.2 - 75	Equation R^2	y = 67275x - 23743 0.9997	0.030	0.100
3.94	Caffeic acid	326	0.2 - 75	Equation R^2	y = 128542x + 57103 0.9992	0.031	0.103

RT – Retention time; 5-CQA – 5-Caffeoylquinic acid; LOD – Limit of detection; LOQ – Limit of quantification.

The LOD and LOQ values correspond to the lowest concentrations at which the analytes can be detected and quantified in the sample, respectively. In this work, the LODs and LOQs for the target analytes were determined using the lowest concentration of the calibration curve (0.2 µg/mL). The S/N ratio should be equal or higher than 3 and 10 for LOD and LOQ, respectively. The LOD and LOQ values calculated for each compound are shown in Table 5. The LODs observed were low, ranging between 0.010 µg/mL (5-CQA) and 0.031 µg/mL (caffeic acid). Regarding the LOQs, the values obtained ranged from 0.032 µg/mL (5-CQA) and 0.103 µg/mL (caffeic acid). Similar results were observed in previous works [245], indicating that the method utilized is acceptable for the quantification of small amounts of these bioactive compounds in spent coffee grounds.

The precision and accuracy of the µ-SPEed/UHPLC-PDA method used were also assessed by spiking one of the spent coffee grounds samples (Timor). The sample was spiked in three different concentrations of 0.2 µg/mL (low level), 25 µg/mL (medium level), and 75 µg/mL (high level). The results for the precision and accuracy of the method, are presented in Table 6. Precision was evaluated to determine if the ability of the method to generate reproducible results [207,208]. The parameters evaluated were the repeatability (intra-day), in which the analyses were performed in the same day repeatably, and intermediate precision (inter-day), in which the analyses were performed in different, non-consecutive days. The precision was expressed in percentage of relative standard deviation (RSD%). The values obtained for the repeatability and intermediate precision were all below 16% indicating good performance of the method regarding these parameters.

Finally, the accuracy of the method was assessed to determine the degree of agreement between the reference value (concentration of standard analyte spiked) and the actual value obtained after the analysis [208]. The accuracy results were expressed in percentage of recovery (Rec%). The closer to 100% the value obtained is the more accurate the method is. Usually, the acceptability limits for the accuracy of an analytical method are set as ±25% [246]. The accuracy results obtained for the analytes used in this work (Table 6) ranged from 94.5% to 119.5%. Since these values are within the acceptable limits defined, we can conclude that the µ-SPEed/UHPLC-PDA methodology used is capable of giving accurate results.

Overall, the validation results suggest that the µ-SPEed/UHPLC-PDA method is adequate for the extraction and quantification of these bioactive compounds in spent coffee grounds.

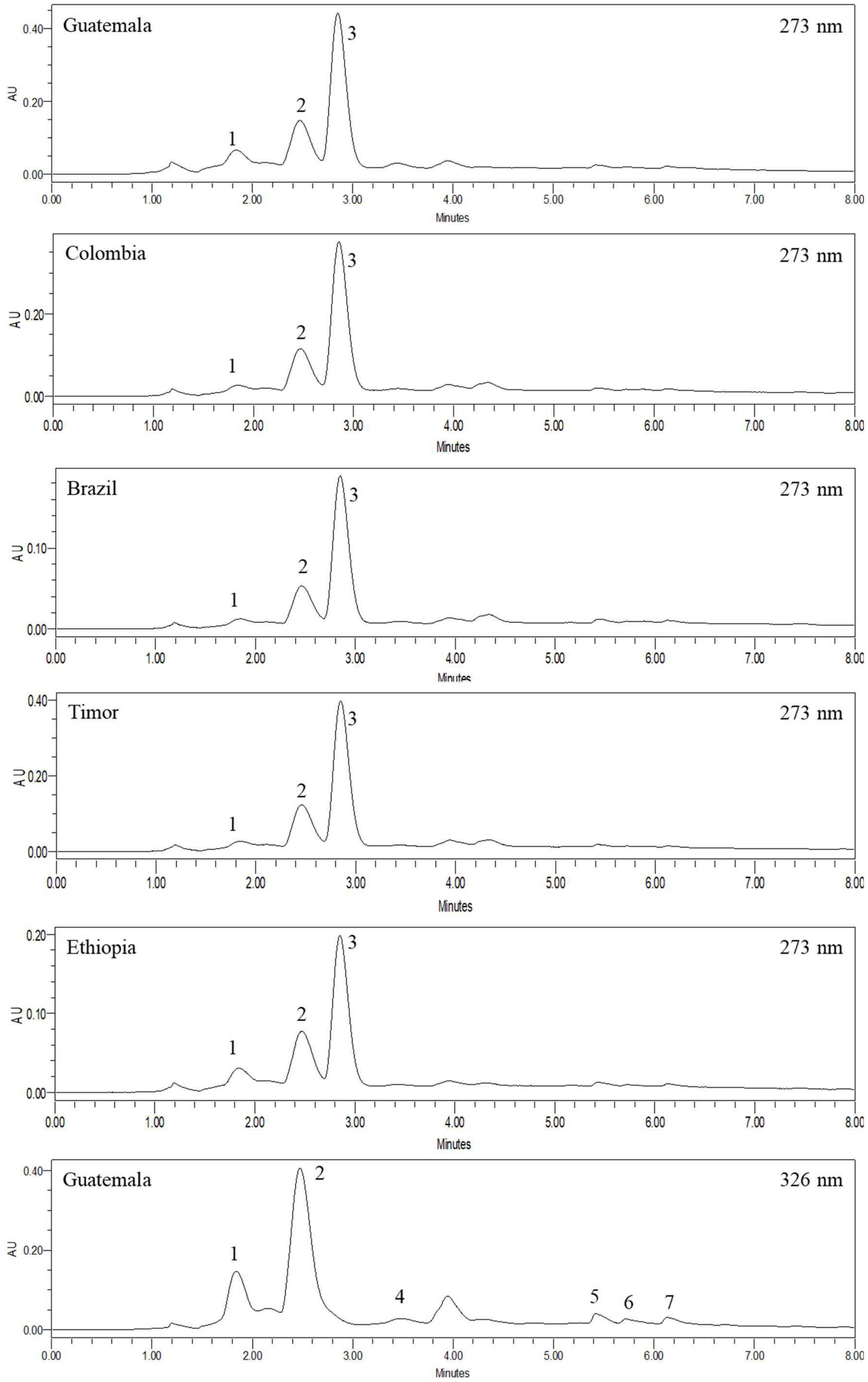
Table 6: Results obtained for the precision and accuracy of the µ-SPEed/UHPLC-PDA method.

Analyte	Spiking level (µg/mL)	Precision (RSD%)		Accuracy (Rec%)
		Intra-day	Inter-day	
5-CQA	0.2	1.47	15.7	98.5 ± 9.5
	25	6.21	8.47	111.6 ± 2.7
	75	6.06	6.19	108.2 ± 2.6
Caffeine	0.2	4.68	15.6	94.5 ± 4.9
	25	2.90	12.9	100.8 ± 4.9
	75	2.69	6.88	119.5 ± 1.5
Caffeic acid	0.2	4.23	11.3	112.3 ± 5.9
	25	1.78	10.5	110.1 ± 7.3
	75	1.52	7.91	110.1 ± 8.0

RSD% - percentage of relative standard deviation; Rec% - percentage of recovery; 5-CQA – 5-Caffeoylquinic acid.

3.3.2. Application of the µ-SPEed/UHPLC-PDA method in the spent coffee grounds samples

After the µ-SPEed/UHPLC-PDA method was proved to be suitable for the determination of the target analytes, it was performed in the spent coffee grounds samples from different geographical origins. The chromatograms obtained for each sample are presented in Figure 20, and the results for the concentration of each bioactive compounds found in the samples are shown in Table 7. The chromatographic areas for the bioactive compounds were collected according to the maximum wavelength of each compound's PDA spectra: at 273 nm for caffeine and at 326 nm for 3-CQA, 5-CQA, caffeic acid, 4,5-diCQA, 1,5-diCQA, and 3,4-diCQA. The results were expressed in mg of the target analyte per 100 g of DW (mg/100 g DW).



Guatemala
273 nm
0.40
0.20
0.00
AU
1
2
3
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Minutes
Colombia
273 nm
0.20
0.00
AU
1
2
3
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Minutes
Brazil
273 nm
0.10
0.00
AU
1
2
3
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Minutes
Timor
273 nm
0.40
0.20
0.00
AU
1
2
3
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Minutes
Ethiopia
273 nm
0.20
0.10
0.00
AU
1
2
3
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Minutes
Guatemala
326 nm
0.40
0.20
0.00
AU
1
2
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7
0.00
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2.00
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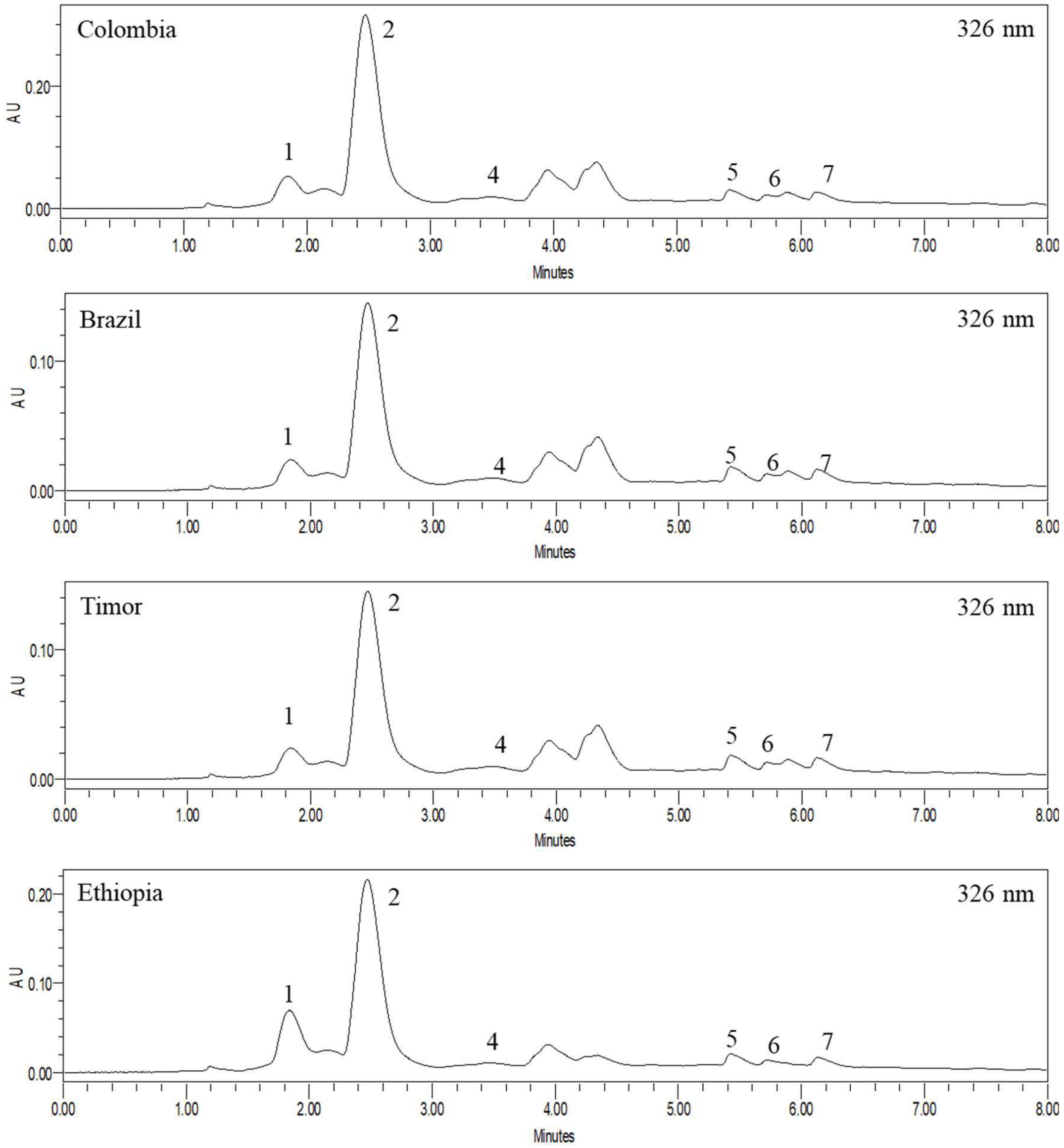

Figure 20: Chromatograms obtained for the spent coffee grounds samples from different geographical origins, extracted at wavelength 273 and 326 nm. Peak number correspondence: 1 – 3-CQA; 2 – 5-CQA; 3 – Caffeine; 4 – Caffeic acid; 5 – 4,5-diCQA; 6 – 1,5-diCQA; 7 – 3,4-diCQA.

As can be seen in the chromatograms displayed in Figure 20, the bioactive profile of the different spent coffee grounds analyzed are similar to one another, only differing in the concentrations in which they appear. Caffeine and 5-CQA were the two most abundant bioactive compounds found in all of samples. Particularly, caffeine was the most concentrated compound in the spent coffee grounds from Guatemala (391.9 ± 27.31 mg/100 g DW), Colombia (386.8 ± 25.61 mg/100 g DW), Brazil (194.1 ± 4.763 mg/100 g DW), and Timor (381.1 ± 9.330 mg/100 g DW), while the sample from Ethiopia was richer in 5-CQA (247.7 ± 11.83 mg/100 g DW). After caffeine and 5-CQA, 3-CQA was the third compound found in higher concentration in all the samples, ranging from 8.251 ± 1.539 mg/100 g DW (Brazil) to 104.1 ± 20.72 mg/100 g DW (Guatemala). The other compounds analyzed were present in lower concentrations in the spent coffee grounds. The amount of 4,5-diCQA ranged from 3.586 ± 0.278 to 10.98 ± 1.136 mg/100 g DW in Brazil and Guatemala spent coffee grounds, respectively, while 3,4-diCQA was found between 0.642 ± 0.058 mg/100 g DW (Brazil) and 7.005 ± 0.670 mg/100 g DW (Guatemala). Finally, caffeic acid and 1,5-diCQA were only quantified in the sample from Guatemala, as the other samples presented concentrations of these bioactive compounds below the LOD values. Guatemalan spent coffee grounds presented higher concentrations of all the compounds analyzed in this work, whilst spent coffee grounds from Brazil presented lower abundance. Among the CGAs identified in spent coffee grounds, only CQAs were found, and 5-CQA was most abundant compound quantified. This outcome was expected, as about 80% of the total CGA content corresponds to CQAs in green coffee, and 5-CQA corresponds to almost 60% of the total CGA content [33]. Generally, monocaffeoylquinic acids (e.g., 3-CQA and 5-CQA) occur in higher concentrations in coffee when compared to dicaffeoylquinic acids (e.g., 4,5-diCQA, 1,5-diCQA, and 3,4-diCQA), with the latter ones appearing in such lower concentra-tion that cannot be quantified or even detected [247]. Similar results were obtained in the present work, with some differences being observed between the values obtained from the different samples, probably due to the samples being from different geo-graphical origins.

Like the results obtained in this work, relatively high amounts of caffeine and CQAs have been observed in spent coffee grounds. However, literature reports the recovery of higher concentrations of these compounds from different types of spent coffee grounds. For instance, Panusa et al. [248] were able to recover as much as 6.1 mg of CQAs/g DW and 11.5 mg of caffeine/g DW from spent coffee grounds of a blend of 40% Arabica and 60% Robusta. But in the same work, they report the recuperation of about 2.3 mg of CQAs/g DW and 1 mg of caffeine/g DW from 100% Arabica spent coffee grounds, which in less than the obtained in the present work. Additionally, Shang et al. [139] analyzed several spent coffee samples from the Arabica variety and one Robusta spent coffee grounds and observed that the different Arabica samples presented caffeine concentrations ranging from 3.19 to 7.45 mg/g DW and 5-CQA concentrations between 51.72 and 213.98 mg/g DW. The differences observed in the concentrations at which these bioactive compounds are found in the spent coffee grounds samples are probably related to the edaphoclimatic factors that influence the concentrations at which these secondary metabolites are present [240,241]. Shang et al. [139] also observed that Robusta presented the highest content of caffeine. This is due to the Robusta variety being richer in secondary metabolites than Arabica coffee [1], in particular its caffeine content is usually twice the concentration of caffeine found in Arabica coffee [33].

Caffeic acid was only found in concentration superior to the LOD value in the spent coffee grounds from Guatemala, where its content was determined to be 3.655 ± 0.689 mg/100 g DW. This result is below the caffeic acid content reported in literature in spent coffee grounds (9.36 mg/100 g of oil extracted from spent coffee grounds) [249]. Caffeic acid is an hydroxycinnamic acid generally found in coffee esterified with quinic acid to form chlorogenic acids [250]. Although a part of chlorogenic acid is degraded during coffee roasting, originating caffeic acid, it can undergo further reactions to form aromatic compounds [92]. For these reasons, the content in caffeic acid can depend on several factors, including coffee variety and roasting conditions [251].

3.3.3. Possible applications for the bioactive compounds identified in the spent coffee grounds samples

As the results obtained in the present work indicate, spent coffee grounds can be a rich source of some bioactive compounds. Depending on the extraction method used in the preparation of the coffee beverage, great amounts of these compounds remain in the coffee grounds matrix and are usually thrown away as waste. In this section, some possible applications for the bioactive compounds identified in the spent coffee grounds analyzed (caffeine, chlorogenic acids and caffeic acid) will be discussed.

As can be seen in the results presented in Section 4.3.2., caffeine is one of the most abundant bioactive compounds in coffee, and great amounts of it remain in spent coffee grounds after the beverage's preparation. Caffeine is the most popularly used psychostimulant worldwide [78], and has been proved to have several biological effects in the human body, including its role in affecting the pathophysiology of neurodegenerative disorders (e.g., Alzheimer's and Parkinson's Diseases) and cardiovascular diseases, in the treatment of neonatal apnea, and as an analgesic adjuvant [86–89]. These properties are highly related to its rapid absorption by the gastrointestinal tract [252]. Because of the benefic effects attributed to caffeine, it can have an array of applications in the pharmaceutical, cosmetic, biocide and food industries. In pharmaceutics, caffeine is used as a respiratory stimulant, as an additive in drugs to improve the analgesic effect, and as a regulator of appetite [253]. In the cosmetic filed, the application of caffeine in topical cosmetic products proved to prevent excessive fat accumulations in skin, promote lymphatic drainage and protect skin from photodamage [254]. Caffeine has been highly reported to enhance the beneficial properties of dermatologic products due to its antioxidant and phosphodiesterase inhibitory effects, as well as being a cosmetic and nutraceutical ingredient [255]. Another interesting application of caffeine that has been reported is as a biocide, more specifically against decaying fungi and termites in wood protection [256], and it proved to be an eco-friendly alternative to the artificial biocidal agent. Also, Hollingsworth et al. [257] showed that caffeine can be a potential molluscicide to repel slugs and snails from food crops in agriculture.

CGAs are the main class of phenolic compounds in coffee, and identically to caffeine, copious sums of them remain in spent coffee grounds. The effects of CGAs in human health derive mainly from its antioxidant, anti-inflammatory, anticancer, hepatoprotective, among other properties. These characteristics are mainly attributed to 5-CQA, since it is the most abundant of its class present in coffee [99–102]. CGAs can also be applied in several field in the industry, such as in the pharmaceutical, cosmetic, and food industries. The topical application of CGAs was proved to have an inhibitory effect on tumor promotion in mouse skin [258] and to accelerate the process of excision wound healing in rats [259]. Moreover, Shao et al. [260] proposed the encapsulation of CGA in β-cyclodextrin and the potential application of the complexes in the food industry as enhanced antioxidants and co-pigments. Other authors reported the creation of a chitosan grafted with chlorogenic acid to be used as a potential preservative agent and edible coating material in peach fruit [261].

Caffeic acid is naturally occurring hydroxycinnamic acid produced as a secondary metabolite of the sikimate way. This phenolic compounds is widely recognized for its antioxidant and anticoagulant activity [262]. Although caffeic only appeared in concentrations above the LOD in the spent coffee grounds from Guatemala, it is a potent antioxidant, some applications have been studied. Hallan et al. [263] encapsulated caffeic acid within solid-lipid nanoparticles and ethosomes to improve the therapeutic potential.

Overall, the compounds identified and quantified in the spent coffee grounds analyzed present several bioactivities. However, the amounts quantified in the spent coffee grounds are well below the concentrations that can be found in ground coffee. The caffeine content in ground coffee can be as high as 6 g/100 g of DW (for the Arabica variety) [264], which is approximately twenty times the value obtained in this work. This makes spent coffee grounds a not so viable source of these compounds, because higher yields can be obtained from other raw materials (e.g., coffee beans and black tea leaves). Nevertheless, the recovery of value-added compounds from waste materials is highly prioritized, in light of the biorefinery framework. This causes spent coffee grounds to be an interesting source of bioactive compounds and an important contribution to circular economy.

Table 7: Results obtained for the identification and quantification of bioactive compounds in spent coffee grounds from different geographical origins through µ-SPEed/UHPLC-PDA.

RT (min)	Analyte	λ_{max} (nm)	Concentration (mg/100 g DW) ± standard deviation				
			Guatemala	Colombia	Brazil	Timor	Ethiopia
1.83	3-CQA*	326	104.1 ± 20.72^{a}	23.91 ± 3.990^{b}	8.251 ± 1.539^{c}	24.80 ± 2.232^{b}	53.80 ± 5.304^{e}
2.45	5-CQA	326	385.3 ± 24.38^{a}	348.9 ± 26.29^{a}	167.3 ± 10.45^{b}	330.6 ± 29.64^{a}	247.7 ± 11.83^{c}
2.85	Caffeine	273	391.9 ± 27.31^{a}	386.8 ± 25.61^{a}	194.1 ± 4.763^{b}	381.1 ± 9.330^{a}	198.6 ± 6.803^{c}
3.94	Caffeic acid	326	3.655 ± 0.6889	<LOQ	<LOQ	<LOQ	<LOQ
5.41	4,5-CQA*	326	10.98 ± 1.136^{a}	8.818 ± 1.352^{b,a}	3.586 ± 0.2775^{c}	7.457 ± 0.5027^{b}	4.538 ± 0.2237^{c}
5.71	1,5-CQA*	326	2.280 ± 0.4542	<LOQ	<LOQ	<LOQ	<LOQ
6.12	3,4-CQA*	326	7.005 ± 0.6701^{a}	4.806 ± 0.6892^{b}	0.6419 ± 0.0581^{c}	3.520 ± 0.3231^{d}	1.559 ± 0.1810^{e}

RT – retention time; <LOQ: below the limit of quantification; 3-CQA – 3-Caffeoylquinic acid; 5-CQA – 5-Caffeoylquinic acid; 4,5-diCQA – 4,5-Dicaffeoylquinic acid; 1,5-diCQA – 1,5-Dicaffeoylquinic acid; 3,4-diCQA – 3,4-Dicaffeoylquinic acid.

*5-CQA equivalents.

Different superscript letters indicate significant differences ($p < 0.05$) among spent coffee grounds from different geographical origins.

Chapter IV

4. Conclusions and future perspectives

Since ancient times, coffee has become a significant part of people's lives, as well as an important economical asset. Furthermore, from the numerous by-products generated by the coffee industry, spent coffee grounds are the biggest residue originated, with 6 million tons being produced worldwide per year. Even though it is usually treated as waste, this coffee by-product can the raw material for several applications, including for the recovery of bioactive compounds with industrial interest. However, similarly to other bio-residues, there is still need to create an integrate approach for the valorization of spent coffee grounds. Considering this, the present work aimed for the bioprospection of valuable compounds recovered from spent coffee grounds, using analytical techniques for the identification of and quantification of bioactive compounds. Since coffee's chemical composition changes depending on their origin, due to the differences in the edaphoclimatic conditions the *Coffea* plants are exposed to, we used five coffee samples from different geographical origins (Guatemala, Colombia, Brazil, Timor, and Ethiopia).

The HS-SPME/GC-MS methodology permitted the establishment of the volatile fingerprint of the spent coffee grounds, with a total of 111 VOCs being identified. The majority of the volatiles identified belonged to the following chemical families: furanic compounds, nitrogen compounds, esters, carbonyl compounds, volatile phenols, and terpenoids. The remaining compounds constituted only less than 2% of the volatile composition. Additionally, the statistical analysis performed revealed a clear discrimination of the spent coffee grounds from different geographical origins. Some of the major VOCs identified in the spent coffee grounds showed great potential to be used as raw material for applications in food (e.g., as an ingredient and/or value-added components) and/or in non-food products (e.g., pharmaceuticals, cosmetics, food packing), supporting its valorization based on circular economy concept closing the loop of coffee value chain, toward the valorization of coffee by-product.

Furthermore, the bioactive and antioxidant potential, determined by µ-QuEChERS/UV-Vis, showed that the maximum TPC was obtained for the sample from Brazil (53.68 ± 3.120 mg GAE/100 g DW), which still was a value lower than those reported in literature. The extracts presented higher scavenging capacity against the DPPH radical, in comparison the ABTS, with the highest value registered for DPPH being 78.11 ± 7.333 mg TE/100 g DW for the sample from Brazil. The values obtained could be related to the fact that caffeic and chlorogenic acids, two phenolic compounds typically found in coffee, presented higher scavenging capacity against DPPH than ABTS.

Finally, the µ-SPEed/UHPLC-PDA methodology led to the identification of seven bioactive compounds (3-CQA, 5-CQA, caffeine, caffeic acid, 4,5-diCQA, 1,5-diCQA, and 3,4-diCQA). 5-CQA and caffeine were the most abundant compounds found in all the samples, and their highest concentrations were found in the sample from Guatemala (385.3 ± 24.38 and 391.9 ± 27.31 mg/100 g DW, respectively). These bioactive compounds have been recognized in literature for their impressive biological effect, hence why they present several applications in the pharmaceutical, cosmetic, and food industries, among others.

The results obtained in this work provide an insight into the chemical composition of spent coffee grounds, allowing the identification and quantification of the bioactive compounds that remain in the matrix. Moreover, we can conclude that this coffee industry residue can be a rich source of numerous compounds with industrial interest. However, more research must be done regarding the optimization of the extraction techniques used to recover as much of these compounds as possible. Also, more studies need to be conducted to achieve an integrate cascade biorefinery approach to properly valorize spent coffee grounds.

4.1. Future works

Regarding the results obtained in this work, more research is required to achieve the proper valorization of spent coffee grounds. Regarding this, the next steps could be:

- Isolation of bioactive compounds from spent coffee grounds.
- Exploring the biological properties of bioactive compounds from spent coffee grounds, as a way to determine their potential applications.

Scientific output

The results presented in the present work were published in the following articles:

- Andrade, C.; Perestrelo, R.; Câmara, J.S. Valorization of spent coffee grounds as a natural source of bioactive compounds for several industrial applications—a volatilomic approach. *Foods* **2022**, *11*, doi:10.3390/foods11121731.

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Bioactive Compounds and Antioxidant Activity from Spent Coffee Grounds as a Powerful Approach for Its Valorization

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