
Development of a fractionation strategy for a spent coffee ground-based biorefinery

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Development of a fractionation strategy for a spent coffee ground-based biorefinery

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Abstract

Biorefineries based on food wastes such as spent coffee grounds (SCG) are of growing interest to the EU owing to the push towards greener production and waste valorization. SCG from café Richard were studied in detail in terms of composition (41.1 % hemicelluloses, 16.2 % lipids, 1.5 % cellulose, 1 % moisture, 1 % ash, 0.4 % proteins, and 21.1 % Klason insoluble residues). Extractible fractions of SCG were studied using conventional and assistive extractions (ultrasound-assisted and accelerated solvent extractions (ASE)) for different solvents and temperatures. In addition, lignin separation methods such as dioxane organosolv (DO) and soda (NT) treatments were performed to study the non-sugar fraction. Results showed that residues obtained from DO and NT did not behave like lignins and were characterized in detail. The study of extractives at different temperatures showed that hexane showed the highest yield at room temperature (16.6 %). Furthermore, using ASE, methanol extractives (60 °C) yielded the best antioxidant capacity ($EC_{50}=0.4$ mg/mmol DPPH), while methanol extractives (125 °C) had the highest yields (20.4 %). In addition, water extraction at 180 °C resulted in the extraction of hemicelluloses. Based on the results of this study, an SCG sequential fractionation strategy consisting of hexane-based fractionation of lipids, methanol-based fractionation of antioxidants and water-based hemicellulose fractionation are proposed.

Abstraktne

Toidujäätmetel, nagu näiteks kasutatud kohvipuru, põhinevad biorafineerimistehased pakuvad ELile huvi, kuna nad püüavad saavutada keskkonnahoidlikumat tootmist ja jäätmete väärimist. Richard'i kohvikust saadud SCG koostist uuriti ja saadi, et see sisaldas 41.1 % hemitselluloosi, 16.2 % lipiide, 1.5 % tselluloosi, 1 % niiskust, 1 % tuhka, 0.4 % valke ja 21.1 % Klasoni lahustumatud jääke. SCG ekstraktiivaineid uuriti kasutades tavalist, ultraheli- ja kiirendatud lahustiekstraktsiooni (ASE)), erinevate lahuste ja temperatuuride juures. Lisaks sellele eraldati ligniini suhkruta fraktsioonist kahel meetodil: kasutades ekstrahendina dioksaani (organosolv, DO) ja sooda meetodit (NT). Tulemused näitasid, et DO ja NT ekstraheerimisel saadud jäägid ei olnud ligniiniga sarnased ja neid iseloomustati üksikasjalikult. Erinevatel temperatuuridel ekstraheerimisel leiti, et heksaan andis toatemperatuuril kõige suurema saagise (16,6 %). Lisaks sellele andsid ASE kasutamisel metanoolsed ekstraktid (60 °C) parima antioksidatiivsuse ($EC_{50} = 0.4$ mg/mmol DPPH), samas kui metanoolsete ekstraktide (125 °C) saagis oli kõige suurem (20.4 %). Lisaks sellele andis veeekstraheerimine 180 °C juures tulemuseks hemitsellulooside ekstraheerimise. Käesoleva uuringu tulemuste põhjal tehakse ettepanek SCG järjestikuse fraktsioneerimise strateegia kohta, mis koosneb lipiidide fraktsioneerimisest heksaaniga, antioksidantide fraktsioneerimisest metanooliga ja hemitselluloosi fraktsioneerimisest veega.

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1. Introduction

The sustainable development goals put forward by the UN 2030 Agenda for Sustainable Development has necessitated several steps by the European Union (1). Among these, reducing consumption and ensuring sustainable communities are important goals. In this regard, the EU has invested heavily in reducing food wastes, and the valorization of waste streams. Furthermore, reducing the overall waste generation and shifting to a greener, more efficient approach to valorize food wastes are considered important steps towards ensuring sustainability. The valorization of waste streams will significantly reduce the waste dumped into landfills, thereby ensuring a positive impact on the environment.

According to the EU, approximately 89 million tons of food wastes are annually generated in the EU (2). These food wastes can be from food industries, commercial and personal sources. In general, these wastes undergo incineration, composting or deposition in landfills. In all these cases, the food waste undergoes an end-of-life strategy. According to the waste hierarchy proposed by the European commission joint research center, the disposal of food waste is considered the least preferable option (3). However, these wastes can be a potential source of sugars, lipids, proteins or a biologically active compound, making the valorization of these waste streams interesting, in the race to complete the sustainable development goals.

Therefore, several groups are currently working on the valorization of waste streams that are produced during the processing or consumption of a food product. In this regard, spent coffee grounds (SCG) are interesting to researchers owing to the high consumption of coffee and the present disposal strategy for the waste (landfill or composting). Reuse of SCG towards the production of energy or chemicals can significantly reduce the SCG waste that ends up in the environment. Furthermore, owing to EU being the largest consumer of coffee beans and the consistent consumption of coffee in commercial and personal spaces, the valorization of SCG is interesting to researchers. In addition, the presence of various primary and secondary metabolites that could replace the fossil-fuel based chemicals should be studied. Moreover, any lacking knowledge regarding the structural properties of SCG or the effect of the fractionation strategy must be studied in detail.

Therefore, this master's thesis aims to improve the knowledge related to the compositional and structural properties of SCG and propose a fractionation strategy based on the results of the study. First, an overview of SCG, its composition, the different approaches to valorize it, and proposals for SCG-based biorefineries are presented in the 'State of the art'. Then, the various experiments and the characterization techniques used during this study are explained in the 'Materials and methods' section. Furthermore, the results of these experiments, its implications and the overall SCG fractionation strategy proposed by the author are presented in the 'Results and discussion'. A perspective is then presented discussing the potential future works, and the limitations of the present work. Finally, the conclusions from this study are presented in the 'Conclusions'.

2. State of the art

2.1 Coffee origin and processing

According to the International Coffee Organization, approximately 10^7 tons of green coffee beans are produced annually, with the EU being the largest consumer in the world, accounting for 32.5 % of the global consumption. The main producer of coffee beans is Brazil, accounting for 39 % of the global production, followed by Vietnam (16.5 %) and Colombia (8.1 %) (4). These countries mainly produce two varieties, *Coffea arabica* and *Coffea canephora* also known as Robusta. According to Clifford et al., green coffee beans generally consist of carbohydrates (~60 %), amino acids and proteins (9-16 %), lipids (8-18 %), chlorogenic acids (6-10 %) and caffeine (0.9-2.5 %). The green beans also contain trigonelline, and diterpenes such as cafestol and kahweol (5). The structures of these molecules are given in Figure 1.

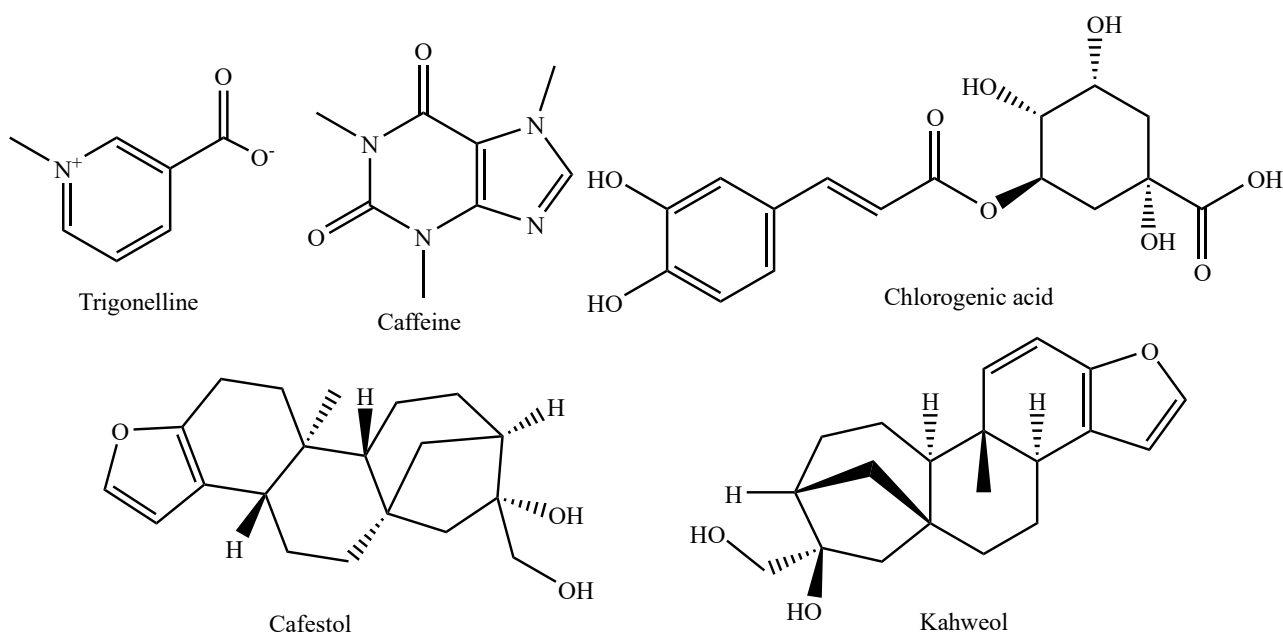


Figure 1. Chemical structures of some coffee compounds

The green beans are obtained from coffee cherries either with dry process, or wet process, depending on the country and capacity. In Brazil, the coffee cherries are commonly processed through the dry process (6). The dry process, which is generally attributed to low quality beans, involves drying the harvested coffee cherries under the sun and then dehulled using mechanical dehulsters. In the wet process, the cherries are first separated into high quality and lower quality through floatation, and then sent for pulping. The pulped cherries are fermented (6-80 h depending on temperature), to loosen the mucilaginous layer. The mucilage is removed, and the resulting beans, called parchment coffee, are dried under the sun and dehulled to produce the green coffee beans (7). Both the coffee pulp and coffee mucilage, which are residues of coffee beans production, are considered as a rich source of polysaccharides, protein, pectin and tannins (6). The cross section of a coffee cherry is given in Figure 2.

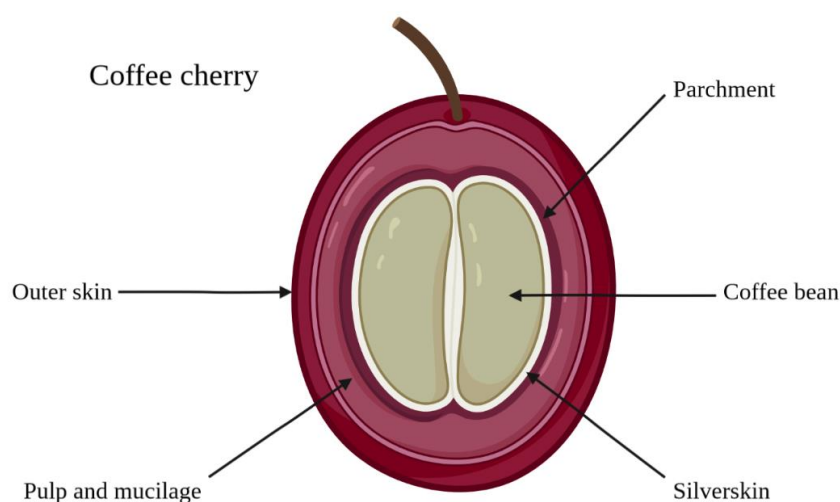


Figure 2. Cross section of a coffee cherry

The green coffee beans are usually shipped to roasting facilities where the beans undergo roasting, a series of thermochemical changes to give coffee its flavor, color and aroma, with the roasting parameters having a significant impact on these characteristics (8). The roasting conditions can vary between 180-250 °C and 2-25 mins, for varying degrees of roasting, affecting the flavor, aroma and color profiles with the change in process conditions (8,9). In brief, the roasting process involves the heating of green coffee beans for a specific duration, according to the roasters' preferences. In the beginning of the roasting, the loosely bound water is driven off, resulting in shrinkage, followed by an increase in the internal temperature of the beans. Exothermic pyrolysis occurs around 140-160 °C, with the pyrolysis peaking at 190-210 °C. During the pyrolysis, large quantities of CO₂ is produced inside the beans, increasing the internal pressure of the beans to approximately 5.5-8 atm and swelling, which in combination with the heat, acts as a pressure vessel, causing a series of reactions (Strecker degradation, Maillard's reaction, etc.) resulting in the formation of heterocyclic volatiles and the expansion of the beans, until it pops open (7). Interestingly, the popping sounds are used as a means of process control in traditional coffee roasting (10). The first pop, associated with the pyrolytic reactions is followed by a second pop as the roasting is continued (11). This is associated with dark roasting as the coffee begins to char and the lipids come to the bean surface. During roasting, owing to the high pressure and temperature conditions inside the beans, several reactions occur. The ferulic acid moieties are converted into guaiacol and its derivatives, and the quinic acid moieties are converted into pyrogallol and trihydroxybenzenes (12,13). Furthermore, melanoidins are formed during roasting. Melanoidins are complex Maillard reaction products formed by the reaction between sugars and proteins that gives thermally processed food its color, aroma and flavor (14). In addition, cinnamic and chlorogenic acids are said to have a role in the coffee melanoidin structure (5,12)

Fast roasting (higher temperature, shorter residence time) can reduce the degradation of chlorogenic acids, volatile losses and burnt taste, but lipid oxidation is greater, compared to traditional slow roasting (lower temperature, longer residence time) (8). Once the necessary level of roasting is obtained, the reaction is quenched quickly by the addition of water or cool air (11). Roasting results in the detachment of a thin outer membrane layer (endocarp) of the coffee bean, called coffee silverskin. This, being a major waste from the coffee roasting industry has been studied in detail, owing to its potential as a polyphenol source (15). The roasted coffee beans are either sold directly to preserve longer their aroma, or ground before sale for the ease of consumption. The residue of coffee grounds after brewing is called the spent coffee grounds (SCG).

In addition to the aforementioned commercial preparations of coffee, coffee is also sold as soluble coffee and decaffeinated coffee. To produce soluble coffee, roasted coffee grounds are percolated in water at different temperatures (ranging from 100-180 °C), which are optimized based on the required organoleptic properties (16). The extract is then dried either through lyophilization or spray drying to produce soluble coffee. However, during the production of 1 kg of soluble coffee, approximately 2 kg of wet SCG is expected (17). SCG produced after the brewing of coffee is estimated to have approximately 60 % water according to several studies (18,19).

In the coffee industry, the wastes are generated during the processing of coffee cherry into green coffee beans (cherry husks, cherry pulp, and parchment skins) and the consumption of green coffee beans (spent coffee ground, and silverskin)(17). The processing wastes are an issue in the country of harvest, which is mainly located outside the EU (Brazil, Vietnam, Colombia, etc.). However, SCG and coffee silverskin are a major source of waste in the EU owing to the scale of consumption of green coffee beans in the EU (10^7 tons per year) (4).

2.2 Chemical composition of spent coffee grounds

The composition of SCG has been studied by various authors. However, the reported compositions vary significantly from one study to another. This is commonly attributed to several factors such as coffee type, cultivation conditions, geographical location, degree of roasting, and brewing conditions (10). The coffee type (*Coffea arabica* or *Coffea canephora*) indeed affects the composition of the coffee beans and therefore the SCG obtained from it (5). In general, *Coffea arabica* contains more lipids than *Coffea canephora*. Bertrand et al. studied the effect of cultivation conditions such as altitude, temperature, precipitation, sunlight and relative humidity on the composition of the coffee beans and showed that the chlorogenic acid composition and lipid composition showed clear difference (20). Jenkins et al. studied the differences in the biodiesel profile obtained from SCG of coffee grounds from different geographical locations, composition and extraction process. They found that the lipid content and the fatty acid methyl esters (FAME) produced through transesterification varied with the studied factor, suggesting that the lipid composition is directly impacted by these factors (21).

According to Cordoba et al., the method for brewing the coffee can significantly affect the composition of the beverage, which in turn affects the composition of the SCG (22). They observed that the flavors observed in coffee and dissolved solids in the brew varies with the brewing method (boiling, vacuum decoction, filter, French press, or espresso). The various brewing methods are summarized in Figure 3. This in turn results in some variation in the composition of SCG obtained from these methods. The roasting process can impact the degree of degradation of sugars, lipids, proteins and chlorogenic acids into melanoidins. The quantity of melanoidins, sugars, proteins, lipids and chlorogenic acids in the SCG will depend on the brewing method (5,8).

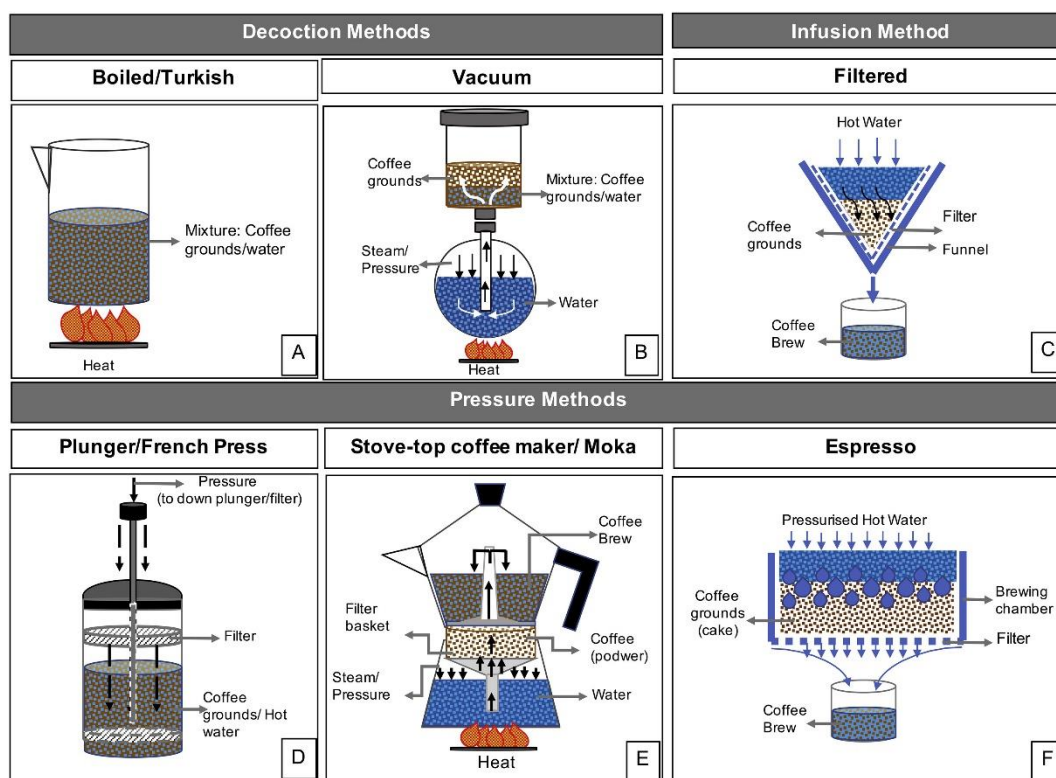


Figure 3. Summary of brewing methods

Table 1 summarizes the SCG composition presented in various reviews. In general, these references are used as a basis for the analysis of spent coffee ground composition. In addition to factors affecting the compositional

data available in the literature such as coffee type, cultivation conditions, degree of roasting and brewing method, the choice of characterization method also has a significant impact. In the case of the polysaccharide fractions such as cellulose and hemicellulose, different quantification methods are used, depending on the authors. The common sugar quantification method is based on the analytical procedure for the determination of structural carbohydrates and lignins in biomass by the national renewable energy laboratory (NREL) (23). This method consists of sequential hydrolysis of lignocellulosic biomass using 72 % H₂SO₄ (1 h, room temperature) and 5 % H₂SO₄ (2 h, 120 °C) solutions followed by the quantification of the hydrolysate using high pressure liquid chromatography with a refractive index detector. The solid residue and the hydrolysate can also be used for the quantification of the acid soluble and insoluble lignin (Klason lignin).

Table 1. SCG composition from literature reviews

#	Review	Cellulose (%)	Hemicellulose (%)	Lignins (%)	Lipids (%)	Proteins (%)	References
1	Bio-refinery approach for spent coffee grounds valorization	8.6-15.3	36.4	32.5-33.6	10-15	6.7-13.7	(24)
2	Valorization of spent coffee grounds: A review	10	30-40	19.8-29.8	7.9-26.4	10	(25)
3	Valorization of spent coffee grounds into biofuels and value-added products: Pathway towards integrated bio-refinery	8.6-24.3	NA	NA	7-14	8.9	(26)
4	A spent coffee grounds based biorefinery for the production of biofuels, biopolymers, antioxidants and biocomposites	13.0	42.0	25.0	2.0	18.0	(27)
5	Utilization of waste from coffee production	12.4	39.1	23.4	11-20	17.4	(28)
6	Interest of Coffee Melanoidins as Sustainable Healthier Food Ingredients	8.6	36.7	24.0	1.6-2.3	13-17	(14)
7	Conceptualization of a spent coffee grounds biorefinery: A review of existing valorisation approaches	7-13	32-42	0-26	2-24	10-18	(29)
8	Biotechnological conversion of spent coffee grounds into polyhydroxyalkanoates and carotenoids	8.6-13.3	30-40	25-33	10-20	6.7-13.6	(30)
9	Spent coffee grounds: A review on current research and future prospects	8.6	36.7	NA	10-15	13.6	(31)
10	A Review of Coffee By-Products Including Leaf, Flower, Cherry, Husk, Silver Skin, and Spent Grounds as Novel Foods within the European Union	12	39	NA	NA	NA	(32)
11	A review on valorization of spent coffee grounds (SCG) towards biopolymers and biocatalysts production	9	37	13	7.9-26.4	13	(33)

*NA: Not available

Giroto et al. quantified the composition of the SCG used in the study using an American oil chemists' society crude fiber analysis method (34). The analysis consists of a two-step acid & alkaline hydrolysis followed by the quantification of sugars and the lignin residue, similar to the NREL method. Vardon et al. quantified the neutral detergent fiber and acid detergent fiber using the Van Soest forage analysis (35). The neutral detergent fiber consists of lignins, cellulose and hemicelluloses and the acid detergent fiber consist of cellulose and lignins. Furthermore, the analytical method for the quantification of sugars vary with different research groups, with additional steps used, as in the case of Fang et al. (36). In this method, prior to the two-step hydrolysis using 72% H₂SO₄ and 5 % H₂SO₄ solutions, the biomass is treated with trifluoroacetic acid (TFA). The quantification of sugars was performed in a high-performance anion exchange chromatography system. In general, the total extractives are gravimetrically calculated after Soxhlet washing, according to the NREL lab analytical

procedure titled ‘determination of extractives in biomass’, which consist of Soxhlet washing using water, ethanol or both (37). Interestingly, the additional step of TFA hydrolysis in Fang et al. allows for the selective hydrolysis of hemicellulose, thus differentiating the glucose in the hemicelluloses and cellulose, which is not possible with the NREL procedure (23,36).

The recovery of lipids from SCG is usually quantified using gravimetric measurements of lipid yield. This consists of an extraction step, which can be conventional extraction at room temperature for a long duration (21), Soxhlet extraction for different durations (38) or ultrasound-assisted extraction using solvents such as hexane, ethanol, heptane and ethyl acetate. Although gravimetric quantification of lipids is common, some authors perform transesterification reaction and quantify the fatty acid methyl esters obtained (21,35,39).

In addition to these reviews, several studies have proceeded to present SCG compositions. Table 2 summarizes the various compositional data found in journal articles. As evident in Table 2, the composition of each fraction varies significantly, across the different studies. The only consistent information is that hemicelluloses account for the largest fraction of the SCG dry mass. Accordingly, the main hemicelluloses in SCG are reported to be galactomannans and arabinogalactan (40).

Table 2. Composition of SCG from journal articles

#	Journal article	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Lipids (%)	Proteins (%)	References
1	Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin	12.4%	39.1%	23.9%	2.3%	17%	(41)
2	Sequential Production of Lignin, Fatty Acid Methyl Esters and Biogas from Spent Coffee Grounds via an Integrated Physicochemical and Biological Process	9.0%	38.3%	28.0%	9.3-16.2%	13.6%	(19)
3	Spent Coffee Grounds Alkaline Pre-treatment as Biorefinery Option to Enhance their Anaerobic Digestion Yield	24.3%	24.8%	13.5%	NA	NA	(34)
4	Hydrothermal liquefaction of spent coffee grounds followed by biocatalytic upgradation to produce biofuel: a circular economy approach	19.3%	35.0%	10.5%	12.9%	12.71%	(42)
5	The cascade biorefinery approach for the valorization of the spent coffee grounds	30.6%	25.6%	12.3%	NA	NA	(43)
6	Spent coffee grounds for biodiesel production and other applications	13.8%	NA	33.6%	6.0%	13.7	(44)
7	Biodiesel, biogas and fermentable sugars production from Spent coffee Grounds: A cascade biorefinery approach	20.6%	46.5%	12.3%	10.0%	NA	(45)

Ballesteros et al. studied the functional, structural and chemical properties of SCG and coffee silverskin from an industrial source that commercially produces coffee mixtures and found that SCG consists mainly of hemicelluloses (39 %), lignins (24 %), proteins (17 %) and cellulose (12 %) with a small fraction of lipids (2 %) (41). For this, the authors performed a two-stage acid hydrolysis of SCG and quantified the sugars in the hydrolysate. The insoluble residue from the acid hydrolysis is considered as Klason lignin and attributed to the lignin content. They studied various properties of the SCG and found that SCG has interesting adsorption properties based on water and oil holding capacity studies.

Lee et al. studied the sequential extraction of lignins and fatty acid methyl esters from SCG consisting of hemicelluloses (38.3 %), lignins (28.0 %), lipids (14.9 %), and cellulose (9 %) as the major constituents (19). Similar to Ballesteros et al., the authors measured the hemicelluloses, cellulose and lignins content using the laboratory analytical procedure for the determination of structural carbohydrates and lignins in biomass by NREL (23). However, there was a significant difference in the lipid composition between the two studies.

Giroto et al. studied the potential of an alkaline pretreatment for enhancing the anaerobic digestion of SCG reported an SCG composition of 24.3 % cellulose, 24.8 % hemicelluloses and 13.5 % lignins (34). In all cases, the lignin quantification of the samples is obtained using the Klason method, where the acid insoluble residue

from the sequential acid hydrolysis of the biomass are gravimetrically calculated. In the case of the other articles mentioned in Table 2, the specific method used for the characterization has not been made clear. In many cases, the compositional data from these papers are referenced for the calculating the yield or proposing new experiments. However, the heterogeneity of the compositional data makes any proposal for SCG-based experiments complicated. Therefore, characterization of the SCG feedstock used in a study is a necessity. Furthermore, to the extent of the author's knowledge, no fundamental analysis of the lignins from SCG has been performed in the literature. As mentioned earlier, Klason method is generally used for quantifying the 'lignin' fraction of SCG. Therefore, an understanding of SCG lignins are lacking in the literature.

2.3 Valorization of spent coffee grounds

There have been many works studying the valorization of SCG. This can be owing to its physical, chemical and functional properties. The valorization strategies can be classified into high value applications and low value applications. This classification of high value or low value application is proposed based on the bio-based value pyramid proposed by several authors, given in Figure 4 (46,47). For the sake of simplicity, 'pharmaceutical & fine chemicals', 'food & feed', and 'bioplastics & polymers' are considered high value applications where as 'bulk chemicals & materials' and 'energy, heat & fuels' are considered as low value applications in this study.

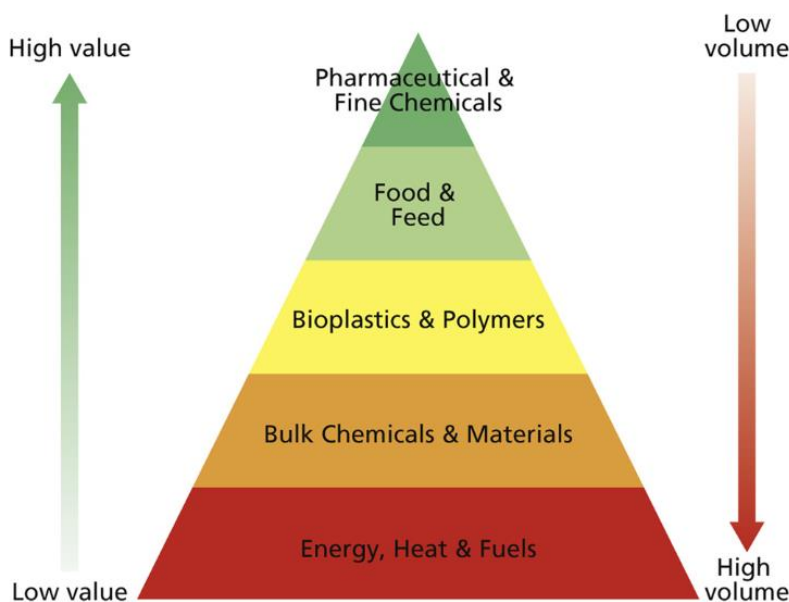


Figure 4. Bio-based value pyramid

2.3.1 Low value applications

Several studies have looked at low value valorization strategies for SCG. Owing to its high heating value (HHV) ranging between 19 MJ/kg and 26.9 MJ/kg, direct incineration of SCG have been studied for energy production (44). Colantoni et al. studied the use of SCG as a solid fuel in a thermal energy system, studying the heating value of pellets made of raw SCG and different ratios of SCG-saw dust mixtures (48). The authors observed an HHV of 22.24 MJ/kg for SCG, showing greater heating value than saw dust. However, higher emission of NO_x, and CO emissions were observed, which can be attributed to the nitrogen content of SCG and incomplete combustion, respectively. Allesina et al. studied the feasibility of using the SCG produced at a coffee roasting plant as the heat source for operation (49). The authors considered the recovery of the SCG from the local cafes and showed that the circular approach of collecting SCG and using as the heat source, coupled with saw dust can be economically feasible, obtaining the return of initial investment in 4 years. However, this study does not consider the environmental impact of this proposal. In addition, a startup in the United Kingdom has started commercial production of coffee pellets for solid fuel applications (50). The combustion of food waste residue for energy production is considered more sustainable than using wood

sources as there is no competition with food and is the reutilization of a waste, which would otherwise end up in a landfill (51).

Apart from the use as a solid fuel, the lipids from SCG could be converted through transesterification into biodiesel. This consists of the extraction of the coffee lipids and fatty acids using methods such as conventional and Soxhlet extraction, using solvents such as hexane, diethyl ether, chloroform, pentane, toluene, and heptane (27,29,52). In addition, several teams have studied the potential of using supercritical fluids (CO₂) for the extraction of lipids from lignocellulosic matrices such as SCG and other biomasses (27). The conventional solvent extraction methods for lipid extraction can be improved upon using assistive technologies such as ultrasonication or microwave technology (27). The extracted triglycerides are then converted into fatty acid methyl esters (FAME) through transesterification using chemical and biological (enzymes) catalysts (21,35,44,53). Chemical catalysts used for the conversion of lipids into FAME include homogenous and heterogeneous catalysts which can be of acidic or basic nature (35,53). Acid catalysts such as H₂SO₄ and HCl or basic catalysts such as NaOH and KOH can be used to produce FAME in an excess of methanol (6:1), to maximize the conversion (44).

The production of biodiesel from SCG lipids have been studied in detail. Caetano et al. produced biodiesel from SCG lipids, which were extracted by different solvents/mixtures, and produced biodiesel (in general, FAMEs) using base catalysis (NaOH), acid catalysis and two-stage transesterification (first stage: acid catalysis; second step: base catalysis) (44). The resulting biodiesel was not up to the European standard EN 14214. Similar biodiesel quality was observed by Vardon et al., where the authors synthesized biodiesel from SCG lipids, which were extracted through hexane Soxhlet extraction, using a two-stage transesterification system (35). The resultant biodiesel was not suitable according to EN 14214. However, blends containing this biodiesel were of acceptable standard for EN 14214. The production of biodiesel using SCG lipids are not limited to the studies mentioned above. Rather, the studies test different extraction solvents, catalysts (acids, bases, and lipases), process conditions, and assisted extraction strategies towards the production of biodiesel (21,35,44,45,53,54).

In addition to biodiesel, several studies have proposed thermochemical conversion of SCG into bio-oil, biochar and biocrude (26,55,56). Muller et al. studied the hydrothermal liquefaction (HTL) of SCG towards biofuel applications (42). This consisted of HTL at temperatures between 270 °C and 300 °C for 10 mins in a batch reactor pressurized to 1 MPa. The results showed a yield of 19 % of bio-oil. The bio-oil is collected and further upgraded using lipases. The HTL process also produced biochar, which can be used as solid fuel owing to its high heating value. The authors estimated the main fatty acids present in the bio-oil to be similar to fatty acids present in SCG lipids, although higher quantities of lipids were observed, compared to the starting material. Vardon et al. proposed a slow pyrolysis approach to obtain biochar and bio-oil from SCG heating the biomass up to 450 °C for 2 h under inert conditions (35). The authors observed that the bio-oil from raw SCG and defatted SCG had high heating value similar to other renewable fuels such as ethanol, with low oxygen content and moderate nitrogen content. Several authors have also proposed the valorization of SCG polysaccharides towards the production of bioethanol (57).

In addition to fuel-based applications, several groups have studied the feasibility of using SCG directly for its physicochemical properties. SCG is commonly used as a soil amendment in houses owing to the common beliefs. Cervera-Mata showed that SCG has soil amendment properties, improving the availability of several essential minerals in the soil, reduces the soil pH and improves the essential minerals in lettuce (58). Another study reported an improvement in the carotenoid production in lettuce when the cultivating soil is amended with SCG (59). Sadarik et al. mixed SCG with ferrofluid to produce a magnetic SCG which was used to adsorb water soluble dyes (60). The SCG-based adsorbent showed a maximum adsorption capacity of approximately 73.4 mg/g adsorbent for acridine orange dye. Pagalan Jr et al. produced activated carbon from SCG for producing an adsorbent and tested the adsorption capacity against aniline yellow dye, with the adsorbent achieving 89 % removal of the dye with an adsorption capacity of 2.6 mg/g adsorbent (61). In addition, Franca et al. studied the adsorption capacity of SCG using methylene blue, reporting the maximum adsorption capacity of 18.7 mg/g adsorbent, which is comparable to some untreated agricultural wastes (62). However, the same study reported coffee husks to be better suited for adsorption applications owing to the higher adsorption capacity (90 mg/g adsorbent).

The low value applications of SCG presented here are not an exhaustive list, with many more proposed applications. This state of the art aims to introduce the general possibilities of using SCG in low value applications.

2.3.2 High value applications

The valorization of SCG has been studied towards most of the high value applications listed in Figure 4 (63–67). Accordingly, the valorization of SCG towards food and feed are considered high value applications. Martinez-Saez et al. studied the use of SCG as an ingredient in biscuits owing to its high dietary fiber content (47.3 %) (68). The authors reported that SCG could be a source of antioxidant insoluble fibers, when incorporated into biscuits (at 4 % loading), no significant effect was observed in the sensory perception of these biscuits, when compared to commercial biscuits. The same group confirmed the bio-accessibility of the antioxidants present in SCG fibers during gastrointestinal digestion (69). In addition, SCG has also been proposed as a functional ingredient in cattle feed (70). San Martin et al. performed this study and reported that 5 % SCG in the feedstock did not affect the production of milk or its protein and lipid content in any significant way (70). This approach could reduce the quantity of SCG that ends up in the landfill.

The production of biopolymers, bio-composites and biopolymer monomers from SCG has also been studied through several studies. Obruca et al. studied the feasibility of converting the SCG lipids and polysaccharides into polyhydroxyalkanoates (PHAs) and carotenoids, respectively (30). Specifically, the authors synthesized polyhydroxybutyrates (PHBs) from SCG lipids, with a high productivity coefficient of 0.88 g PHB/ g SCG lipid. Furthermore, they hydrolyzed the lipid-free fraction and tested the PHA production of the hydrolysate, confirming the feasibility of this approach. In addition to the PHA production, the SCG hydrolysate was tested as a substrate for carotenogenic yeasts to produce carotenoids, which can have pharmaceutical, food and cosmetic applications (30). Soares et al. synthesized polyols from SCG through acid liquefaction, which could be the precursor to produce polymers including polyurethane foam (71).

Furthermore, SCG has been proposed as a source of biologically active compounds such as phytosterols, chlorogenic acids, caffeine, and peptides. To this end, several studies have proposed strategies towards valorization. In general, rather than purification of individual fractions, the works focus on the extraction of solvent-soluble fraction and test the antioxidant capacity and the total phenolic content (TPC) of the extractives. The antioxidant capacity of extractives is measured using assays such as DPPH assay, FRAP assay and ABTS assay. The TPC is measured using Folin-Ciocalteu assay. Mussatto et al. studied the extraction of antioxidants from SCG using different compositions of methanol-water mixtures, extraction times and biomass-solvent ratios (72). The authors reported the presence of flavonoids, chlorogenic acids and other phenolic compounds, with the maximum phenolic content to be approximately 18 mg gallic acid equivalent (GAE) per g of SCG. In addition, as part of the same research group, Ballesteros et al. studied the encapsulation of antioxidant extractives from SCG using different methods to preserve the antioxidant activity for food additive applications and showed the potential of using SCG-based extractive capsules with maltodextrin as the wall material. The encapsulation using freeze drying preserved the antioxidant activity by 73-86 % of the original content.

Similarly, Panusa et al. studied the extraction of antioxidants from SCG using water and water-ethanol mixtures at 60 °C, characterizing the TPC, flavonoid content and antioxidant activity (73). The results showed a maximum phenolic content of 28 mg GAE/g SCG when using a 6:4 ethanol-water mixture. Although the antioxidant capacity of the extractives was correlated to TPC, the flavonoid content did not show such a correlation with the antioxidant capacity. Furthermore, the authors reported that the source of SCG impacted the antioxidant activity, TPC and the flavonoid content of the extractives.

Gigliobianco et al. studied the extractives from SCG using water at different loading and temperatures (74). The authors performed a central composite design study to optimize the yield of caffeine, trigonelline and nicotinic acid, after which, the antioxidant activity and TPC of the best extract was studied (74). The authors reported that the optimal extraction conditions using water at 80 °C for 30 mins. The results showed a TPC of approximately 61 mg GAE/g SCG and an antioxidant activity of 324 µmol Trolox equivalents (TEAC) per g SCG using DPPH assay. Furthermore, these results were used to propose cosmetic preparations using SCG.

Ribeiro et al. also studied the potential of using the extractives from SCG for cosmetic applications through hydrogel preparation (75). The authors performed subcritical water extraction to produce extractives at different temperature conditions and studied the potential antiaging and skin lightening effects of the hydrogels. The authors compared the antioxidant capacity, TPC, and sugar content of extractives at 140 °C and 220 °C. At higher temperature, the sugar content of the extractive was higher, as compared to the lower temperature. TPC and antioxidant capacity showed the inverse behaviour. The authors were also able to show that the extract possessed antiaging and skin-lightening properties using enzyme inhibition tests.

The valorization of SCG extractives for several high value applications have been studied. However, because polysaccharides account for a large fraction of the SCG composition, several authors have tried to valorize it.

Kim et al. proposed a catalyst-free biphasic reaction system to convert the polysaccharides from SCG into levulinic acid and formic acid (76). The authors proposed a subcritical water system coupled with an organic phase (dichloroethane) to convert the polysaccharides into levulinic acid and formic acid with a yield of 47 % and 29 % total convertible monosaccharides, respectively. Similar biphasic systems could be used to produce 5-hydroxymethylfurfural owing to the large fraction of hexose monosaccharides obtainable from the SCG polysaccharides (77).

2.4 SCG-based biorefineries

As mentioned in the previous section, several studies have been reported on different valorization strategies for SCG. However, the techno-economic feasibility and life cycle assessment of any strategy must be considered for real world applications. Schmidt Rivera et al. performed a life cycle analysis (LCA) for six different valorization strategies for SCG (51). The authors considered incineration, anaerobic digestion, composting, direct application of SCG, biodiesel production and landfilling as the six possible routes to valorization. They performed an LCA according to the ISO 14040/44 guidelines and found that among the studied routes for valorization, incineration was the most environmentally sustainable. Furthermore, the authors found that biodiesel production was the least favorable for the environment. This is attributed to the use of fossil-based methanol and the better performance of incineration in net energy production. However, the authors did not study the life cycle impact of producing higher value-added products such as biopolymers, biochemicals or food products (51).

A SCG-based biorefinery could be very impactful owing to the valorization of the different possible product streams from SCG. A biorefinery aims to sustainably process biomass into marketable products and energy (78). In addition, this system could reduce the overall waste that is directly produced while using coffee products. This has been the basis for several proposed SCG-based biorefineries in the literature. In general, the SCG-based biorefineries consist of a fractionation strategy and a valorization strategy. The fractionation strategy consists of unit processes dedicated to separating SCG into valuable fractions, which could be enriched through extraction or thermochemical treatments. Once the SCG is fractionated into several fractions, the valorization of individual fractions is considered. In this regard, several authors have proposed SCG-based biorefinery systems.

Battista et al. proposed a cascade biorefinery approach to produce biofuels by first extracting the coffee lipids, followed by the hydrolysis of the lipid-free SCG and the fermentation of the sugars using *S. cerevisiae* to produce bioethanol and the anaerobic digestion of the solid residue for biogas production (43). The extracted SCG lipids consisted of tocopherols (mg/100 g SCG), cafestol (383 mg/100 g SCG) and kahweol (194 mg/100 g SCG). The same group proposed a cascade consisting of biodiesel from SCG lipids, followed by the production of fermentable sugars and biogas (45). The authors reported the transesterification of SCG lipids and a glycerol/sulphuric acid pretreatment of the lipid-free SCG followed by enzymatic hydrolysis to produce free sugars. The solid residue was proposed for anaerobic digestion, like the former study (45).

Massaya et al. proposed a SCG-based biorefinery using a water-based system to perform subcritical water extraction (SWE) followed by hydrothermal carbonization (79). In this system, SWE was performed (127 °C to 200 °C) to produce extracts consisting of polysaccharides, chlorogenic acids, and polyphenols. The remaining solid residues underwent hydrothermal carbonization in order to produce hydrochar. Pereira et al. proposed a fractionation strategy using methyl isobutyl ketone (MIBK)-ethanol-water, MIBK-acetone-water and MIBK-water organosolv treatments (140 °C, 1 h) with H₂SO₄ as catalyst to fractionate SCG into cellulose rich, hemicellulose rich and lignin rich fractions (66). The authors proposed the production of 5-hydroxymethylfurfural (%-HMF) from the cellulose rich fraction and the fermentation of the hemicellulose rich fraction.

Because any SCG-based biorefinery will require a fractionation strategy to maximize profits and minimize the environmental impact, a complete understanding of the feedstock, SCG and the individual unit processes must be present. In this regard, the quantification and a structural understanding of SCG is a must, including the lignins, which at present lack the understanding, owing to a lack of structural study. Furthermore, owing to the heterogeneity of SCG compositions, the different considerations are necessary when developing a SCG-based biorefinery. Therefore, in this study, these problems will be addressed. The characterization of SCG, effect of different extraction strategies, treatments to separate lignins, are performed to develop a fractionation strategy.

3. Materials and methods

3.1 Preliminary methods

3.1.1 Collection and preparation

Spent coffee grounds (SCG) was collected from Café Richards. The coffee was originally from Mexico. It consists of *Coffea arabica* (70 %) and *Coffea canephora* (30 %), also known as Robusta. The spent coffee grounds collected were lyophilized before being stored in dry containers at room temperature.

3.1.2 Soxhlet washing

Previously dried thimbles (in a 105 °C oven for 2 h and then cooled to room temperature under vacuum) and SCG were weighed and placed in the Soxhlet. The heating was adjusted to ensure reflux of solvent. Two combinations were performed in the Soxhlet washing, (1) water followed by ethanol, and (2) hexane. The extractions were performed exhaustively, to ensure the complete removal of extractives. The thimbles were collected and dried under vacuum before weighing to estimate the insoluble residues.

3.1.3 Detergent washing

Approximately 50 g of SCG was weighed and transferred into a flask with 7.2 g of sodium dodecyl sulfate (SDS) and 500 mL of water. The sample was continuously stirred for 24 h. The mixture was allowed to settle and approximately 50 mL of the supernatant was collected for analysis. The detergent washed residue was transferred into 50 mL eppendorf tubes and centrifuged (4800 rpm for 10 mins at 5 °C), and further washed with water five times. The residue was lyophilized and weighed.

3.2 Extractions

3.2.1 Conventional solvent extraction

Approximately 400 mg of SCG was weighed and transferred to a 50 mL Eppendorf tube. Solvent, depending on the experiment, was added at a 10 g SCG/ L of solvent ratio. The mixture was shaken at room temperature for 30 mins using an orbital shaker at 450 rpm. The mixture was then centrifuged at 5 °C and 4800 rpm for 10 mins. The clear supernatant was transferred into a round bottom flask and concentrated under vacuum. The solid residue was further extracted twice following the same steps. The supernatant was transferred into the same round bottom flask after each extraction. Once the solvent was removed using a rotary evaporator, the extractive fraction and the solid residue were dried under vacuum at 40 °C overnight and weighed.

In cases of floating solids in the supernatant after extraction and centrifugation, the supernatant was passed through a 0.22 µm nylon syringe filter before transferring into the round bottom flasks. The temperature of centrifugation was changed to 20 °C only in the case of dioxane systems, owing to the freezing point of dioxane (11.8 °C). Each solvent experiment was performed in duplicates.

The extraction yield was estimated using Equation 1.

Equation 1

$$\text{Extraction yield (\%)} = \frac{(\text{mass of extractives}) * 100}{\text{SCG mass} * (\text{organic content \%})}$$

Here, the organic content of SCG is considered as the fraction of SCG excluding the moisture and ash content.

3.2.2 Ultrasound assisted extraction

SCG was weighed (approximately 200 mg) in a glass tube and loaded with the solvent at a rate of 10 g SCG/L of solvent. The samples were placed in a low frequency ultrasound bath (VWR ultrasonic cleaner) at

the maximum intensity for 30 mins. The temperature of the bath was not controlled. The samples were then centrifuged at 5 °C and 4800 rpm for 10 mins to ensure that the solids are settled. Supernatants were transferred into flasks and rotary evaporated. The solid residue was further extracted twice using the same steps, to ensure maximum extraction. The supernatant was transferred into the same round bottom flask after each extraction, rotary evaporated, dried under vacuum at 40 °C overnight and weighed.

The experiment was started at room temperature and monitored after each extraction. In the case of floating particles after centrifugation, the supernatant was passed through a syringe filter. The extraction yield was calculated using Equation 1. Each solvent experiment was performed in duplicates.

3.2.3 Accelerated solvent extraction

Accelerated solvent extraction (ASE) was performed using a Dionex ASE 350 extractor (Thermo Fischer Scientific Inc.). The samples were weighed and loaded into a cell (34 mL) containing an ASE cellulose filter, and the solvent choice was made (hexane, water, methanol or methanol-water mixture). The parameters of the extraction such as the number of cycles, rinse volume, static time, and temperature were adjusted according to the experiment.

After the extraction, the extractives were collected in bottles, and transferred to weighed flasks for rotary evaporation. Once the solvent was removed, the samples were kept in a vacuum oven at 40 °C overnight. The solid residues were transferred into Eppendorf tubes and dried. The samples were weighed to measure the yield and stored in glass tubes for further analysis.

3.3 Treatments

3.3.1 Dioxane-HCl treatment

The dioxane-HCl solution was prepared using 900 mL of dioxane and 100 mL of 0.2 M HCl. The SCG residue (raw SCG, SCG-EF and SCG-HW) is weighed and transferred into a 500 mL flask equipped with a condenser and 150 mL of dioxane-HCl solution was added. The mixture was refluxed for 30 mins under magnetic stirring and N₂ flow.

The solution was cooled to room temperature and filtered under vacuum through a Whatman filter No. 3. The solid residue was washed with 150 mL dioxane-water solution (9:1 v/v), twice. The pH of the filtrate was adjusted to 3-4 using saturated NaHCO₃ solution. The filtrate was concentrated under vacuum at 40 °C, then precipitated in 600 mL of cold water. The precipitates were allowed to settle, the supernatant was largely siphoned off, and the residue was centrifuged at 4800 rpm at 5 °C for 10 mins. The residue was collected, lyophilized and weighed before further characterization.

3.3.2 Room temperature NaOH treatment

Approximately 5 g of residue (raw SCG or SCG-HW) was weighed and transferred into a flask containing 500 mL of 1 M NaOH solution and magnetically stirred for 24 h. Once the reaction finished, solids were separated using a No. 3 glass filter under vacuum. The filtrate was transferred into another beaker, and the pH of the filtrate was adjusted to 2-3 using 96 % H₂SO₄. The acidified filtrate was cooled in an ice bath and allowed to precipitate, then half of the supernatant was siphoned off and collected. The rest of the supernatant and precipitate were separated via centrifugation at 4800 rpm, 5 °C, and 10 mins. The precipitates were collected and washed with deionized water prior to lyophilization for 48 h.

3.3.3 High temperature NaOH treatment

The feedstock (SCG or hexane Soxhlet washed SCG) was loaded with 1 M NaOH at a 10 g biomass per L solvent into a flask fitted with a condenser. The reaction was magnetically stirred for 3 h at 95 °C. The mixture was cooled down to room temperature and filtered through a cellulose filter (616 grade). The pH of the filtrate was adjusted to 2-3 using 96% H₂SO₄ solution in a new beaker and cooled in an ice bath for precipitation. The precipitates were allowed to settle, half the supernatant siphoned off and collected. The precipitates were separated using centrifugation at 4800 rpm, 5 °C, and 10 mins, collected, washed with deionized water, and lyophilized for 48 h.

3.4 Characterization

3.4.1 Moisture and ash content

Approximately 500 mg of SCG were weighed and deposited in weighed metal plates (replicates) and placed in a 105 °C oven for 18 h. The samples were cooled to room temperature under vacuum and then weighed again. These samples were then placed in a muffle furnace, with the following temperature profile-ramp function from 20 °C to 575 °C in 2 h, and the temperature maintained at 575 °C for 5 h. The samples were cooled to room temperature and then weighed, to calculate the ash content.

3.4.2 Protein assay

Protein extraction method was based on proteomic analysis performed by Natarajan et al. (). The protein quantification was performed using the BCA Protein Assay from ThermoFischer™. Protein extraction was initially performed on 100 mg of sample, using 1.5 mL of extraction reagent, which consists of 5 M urea, 2 M thiourea, 65 mM dithiothreitol (DTT) and 4 w/v % of 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) detergent. The samples were mixed for 5 mins at room temperature and then centrifuged at 15000 g for 15 mins. The supernatant was collected in a 2 mL Eppendorf tube, centrifuged again at 20800 g for 15 mins. Approximately 800 µL of the supernatant was transferred into another Eppendorf tube and 200 µL of trichloroacetic acid (concentration 500 mg/mL) was added to it. This was kept in ice for 1 h, then centrifuged at 20800 g for 15 mins and the supernatant was removed. The pellet (or lower fraction if not visible) was washed with cold acetone and centrifuged twice. The washed precipitated proteins were dried and dissolved in 50 µL of phosphate-buffered saline (PBS) buffer. The samples were diluted in PBS buffer (10x, 100x, and 1000x). Bovine serum albumin (BSA) standards were prepared at different concentrations along with a blank using PBS buffer for dilutions.

The working reagent was prepared by mixing Micro BCA reagents MA, MB, and MC, respectively, in a 25:24:1 ratio. Approximately 150 µL of samples, standards or blank were transferred into a microplate and 150 µL of the working reagent was added to it. The microplate was closed and kept at 37 °C for 1 h, after which, the absorbance was measured at 562 nm using a Tecan microplate reader.

3.4.3 Elementary analysis

Elementary analysis was performed in duplicates using a FLASH 2000 organic elementary analyzer-isotope ratio mass spectrometer (EA-IRMS) system from ThermoFischer Scientific™. Samples were weighed (1-2 mg) in tin capsules with an automated scale to tabulate the mass. The capsules were closed and stored in a well plate for analysis. The samples were analyzed using the Dumas method with atropine as the reference molecule to estimate the total nitrogen content. The crude protein content is estimated using ISO 16634-2:2016, using a conversion factor of 6.25 (80).

3.4.4 Klason analysis

Klason analysis was performed according to Dence et al. (1992) (81). In brief, approximately 300 mg of samples were transferred into beakers and 3 mL of 72 % H₂SO₄ were added. These mixtures were homogenized during the span of 2 h and kept at room temperature. This was followed by the addition of 43 mL of deionized water to each sample to reduce the concentrations to 5 % H₂SO₄ solution, then refluxed in a pre-heated sand bath for 3 h.

The samples were cooled to room temperature and filtered through the previously weighed crucibles with nylon filters and the filtrates were transferred for the characterization of acid soluble residue. The insoluble residue was washed with water and the crucible was dried at 105 °C overnight. The dried crucible was cooled to room temperature, weighed, and then placed in a muffle furnace at 550 °C for 5 h. The crucibles were cooled to room temperature and weighed again, to measure the ash content.

Klason content is calculated using Equations 2-3.

Equation 2

$$\text{Klason content with ash (\%)} = \frac{(M_{\text{Dry}} - M_{\text{Tare}})}{M_{\text{Initial}}} * 100$$

Equation 3

$$\text{Ash content (\%)} = \frac{(M_{\text{Ash}} - M_{\text{Tare}})}{M_{\text{Initial}}} * 100$$

$$\text{Klason content (\%)} = \text{Klason content with ash} - \text{Ash content}$$

Where M_{Initial} , M_{Tare} , M_{Dry} , and M_{Ash} are the masses of initial sample, crucible with filter, after drying the residue at 105 °C, and after burning the sample for ash content, respectively.

3.4.5 NMR spectroscopy

All NMR spectra were recorded on Bruker Ascend 400 MHz spectrometer.

3.4.5.1 31P NMR spectroscopy

Approximately 15 mg of sample and 15 mg of triphenylphosphine (TPP), the internal standard, were added to a glass tube and dissolved in 400 μL of a solution consisting of anhydrous pyridine and deuterated chloroform (1.6:1, v/v). Then, 50 μL of the phosphitylating reagent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, (TMDP) was added to the mixture. A total of 128 scans were acquired with a delay time of 6 s between successive pulses. The spectra were processed using Topspin 3.1. All chemical shifts are reported relative to the water-TMDP signal (132.2 ppm).

3.4.5.2 2D NMR spectroscopy

The samples were weighed (5-15 mg) and dissolved in 500 μL of deuterated dimethyl sulfoxide (DMSO- d_6). Several experiments were run on each sample: HSQC, COSY, HMBC. Different pulse programs and acquisition parameters (NS, O1 and WS) were chosen depending on the samples.

3.4.6 Thioacidolysis and subsequent gas chromatography-mass spectrometry

Approximately 10 mg of sample were taken in a glass tube and 100 μL of internal standards (C_{18} , C_{21} , and C_{22}) of known concentration (2 mg/mL). Thioacidolysis reagent consists of 10 mL of ethanethiol and 2.5 mL of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ and made up to 100 mL using dioxane. Approximately 5 mL of the thioacidolysis reagent was added to each tube, closed and placed in an oil bath (100 °C) for 4 h. The mixture was cooled to room temperature, quenched with 5 mL of 0.2 M NaHCO_3 solution and then acidified using 0.5 mL of 6 M HCl. The phenolic compounds were extracted using 5 mL of dichloromethane. The organic phases were dried over anhydrous Na_2SO_4 and then rotary evaporated to 2 mL.

Approximately 5 μL of the concentrated samples were added to 100 μL of N, O-Bis (trimethylsilyl)trifluoroacetamide (BSTFA), the silylating reagent and 10 μL of pyridine in vials with inserts. Silylation occurred at room temperature for 2 h, after which, the samples were injected into a GC-MS Varian 4000 instrument (Varian, Les Ulis, France) with an autosampler, splitless injector (280 °C) and an ion trap mass spectrometer (electronic impact mode: source at 220 °C, interface at 280 °C, and scanning range- 50-650 m/z) (82). A VF-1 ms 15 m \times 0.25 mm polydimethylsiloxane capillary column operating in the temperature program mode (45 °C to 180 °C at 30 °C/min and 180 °C to 260 °C at 3 °C/min) was used with helium (1.5 mL/min) as the carrier gas. Ion chromatograms reconstructed at m/z 239, 269, and 299 are used to determine H, G, and S lignin monomers. The monomer concentrations are calculated in $\mu\text{mol/g}$.

3.4.7 Sugar quantification

Approximately 10 mg of the sample were weighed and transferred into Pyrex glass tubes with PTFE screw caps. The samples were hydrolyzed using 500 μ L of 2.5 M trifluoroacetic acid (TFA) for 2 h at 120 $^{\circ}$ C and then cooled to RT. These hydrolyzed samples were centrifuged (3700 rpm, 10 mins, 4 $^{\circ}$ C) and the supernatants were recovered. The solid residue in the glass tube were used for glucose quantification. Approximately 10 μ L of the supernatants were diluted 1000 times and the remaining supernatants were stored at -20 $^{\circ}$ C for further use.

The hydrolysis residues in the glass tubes were washed first with 3 mL water, 1.5 mL 96% ethanol twice, and finally, with 1.5 mL of acetone. After each of these washes, the samples were centrifuged (3700 rpm, 10 mins, 4 $^{\circ}$ C), ensuring the maximum removal of supernatant and minimum loss of the solid residue. The solid residues were then dried overnight and further hydrolyzed using 72 % H_2SO_4 for 1 h at RT. Each tube was diluted with 1.5 mL of water and further hydrolyzed in a water bath at 100 $^{\circ}$ C for 2 h. The tubes were cooled to room temperature, centrifuged (3700 rpm, 10 mins, 4 $^{\circ}$ C) and the supernatant was transferred into Eppendorf tubes. For the quantification of hemicelluloses, the supernatants were diluted 1000 times and stored (-20 $^{\circ}$ C). The TFA hydrolyzed sample fractions were filtered through a 22 μ m syringe filter and quantified using a Dionex DX-600 high performance anion exchange chromatography instrument equipped with a CarboPac PA1 analytical anion exchange column (4 mm \times 250 mm; ThermoFischer Scientific), PA1 guard column (4 mm \times 50 mm; ThermoFischer Scientific) and an amperometric detector. The samples underwent a 15 min equilibration, after which, they were eluted using an isocratic gradient of 50 mM NaOH from 0 min to 40 min. Arabinose, fructose, galactose, glucose, mannose and xylose were used as standards, undergoing the same characterization steps as the hydrolyzed samples (36).

For the quantification of cellulose, instead of 1000 times, the supernatants (after H_2SO_4 hydrolysis) were diluted 10 times. Approximately 10 μ L of these samples were added to vials with inserts and heated (70 $^{\circ}$ C for 2 h) to remove water traces. Then, 100 μ L of N, O-Bis (trimethylsilyl)trifluoroacetamide (BSTFA), the silylating reagent and 10 μ L of pyridine were added in the vials. Silylation occurred at 70 $^{\circ}$ C for 4 hours, after which, the samples were injected into a GC-MS Varian 4000 instrument.

3.4.8 Size exclusion chromatography

Size exclusion chromatography (SEC) was conducted in a styrene-divinylbenzene PL-gel Mixed E column (Agilent technologies, 3 μ m, 2 \times 300 mm, 7.5 mm inner diameter) with a photodiode array detector (Dionex UltiMate 3000 UV/Vis detector) set at 280 nm and 320 nm (UV) and tetrahydrofuran (1 mL.min⁻¹) as eluent. The samples were dissolved in BHT-stabilized THF containing 1 % toluene at a concentration of 1 mg/mL. The mixture was passed through a 0.22 μ m nylon syringe filter into SEC vials and run through the chromatographic system.

3.4.9 Liquid chromatography-mass spectrometry (LC-MS)

A qualitative LC-MS analysis was performed by dissolving the dried samples in either methanol, water, acetonitrile or water (10%) in acetonitrile at a concentration of 1 mg sample/mL, depending on the solubility. The dissolved fraction of the sample was passed through a 0.22 μ m nylon syringe filter and placed in LC vials. Liquid samples were directly filtered through 0.22 μ m nylon syringe filters. The samples were passed through an UltiMate 3000 Thermo UHPLC system (ThermoFischer) with a Nucleoshell RP 18 plus reversed-phase column (2 mm \times 100 mm, 2 μ m, Macherey-Nagel) coupled to a quadrupole time of flight mass spectrometer (Q-ToF Impact II Bruker Daltonics) for metabolomic analysis. Two mobile phases were used for chromatographic separation: (1) 0.1 % formic acid in water; and (2) 0.1 % formic acid in acetonitrile.

3.4.10 Fatty acid quantification

The samples for fatty acid quantification were weighed (5-10 mg for lipid-rich samples, 20-25 mg for solid residues), and transferred into borosilicate glass tubes. Approximately 2 mL of transmethylating reagent (5 % H_2SO_4 in methanol containing 0.5 mg/mL methyl margarate) were added to the glass tubes containing the sample and were heated to 85 $^{\circ}$ C for 2 h. The samples were then cooled down to room temperature and the FAMES were extracted using 2 mL of hexane and 1 mL of deionized water. The tubes were shaken and then

centrifuged (4800 rpm, 5 °C, and 10 mins). Approximately 1 mL of the hexane fraction (upper layer) was collected from the tubes and transferred to GC vials for analysis. Standard calibration curves were prepared using various concentrations of FAME mixture consisting of C_{16:0}, C_{18:2}, C_{18:0} and C_{18:1} methyl esters that underwent the same steps as the samples.

3.4.11 DPPH assay

The antioxidant capacities of samples were studied using the DPPH assay. A DPPH solution of known concentration (1.48 mM) was prepared in the solvent (methanol or methanol/water (1:1 v/v)). Six dilutions of the samples were prepared from a stock of approximately 10 mg/mL (1×, 2×, 4×, 8×, 16×, and 32×). The absorbances were measured using a Tecan microplate reader, set to 517 nm in the absorbance mode. Approximately 190 µL of the DPPH solution was added to each microplate well followed by the addition of 10 µL of sample. A zero concentration well filled with 200 µL of DPPH solution and a blank with the pure solvent were also placed. The arrangement of the wells was defined in the Spectrostar NanoMars software and the measurements were made every 30 mins until 21 h. The EC₅₀ value was calculated by plotting the concentration to the radical scavenging activity (RSA), given by Equation 4.

Equation 4

$$RSA = \frac{(A_0 - A_i)}{A_0} * 100$$

where A₀ is the absorbance of the control and A_i is the absorbance of the sample at different dilutions, once the absorbance remains constant. A trendline with a good fit ($r^2 > 0.95$) was used to calculate the EC₅₀ value, which is the concentration corresponding to RSA of 50 %.

4. Results and discussion

An overview of the experiments and the various fractions are presented in Figure 5.

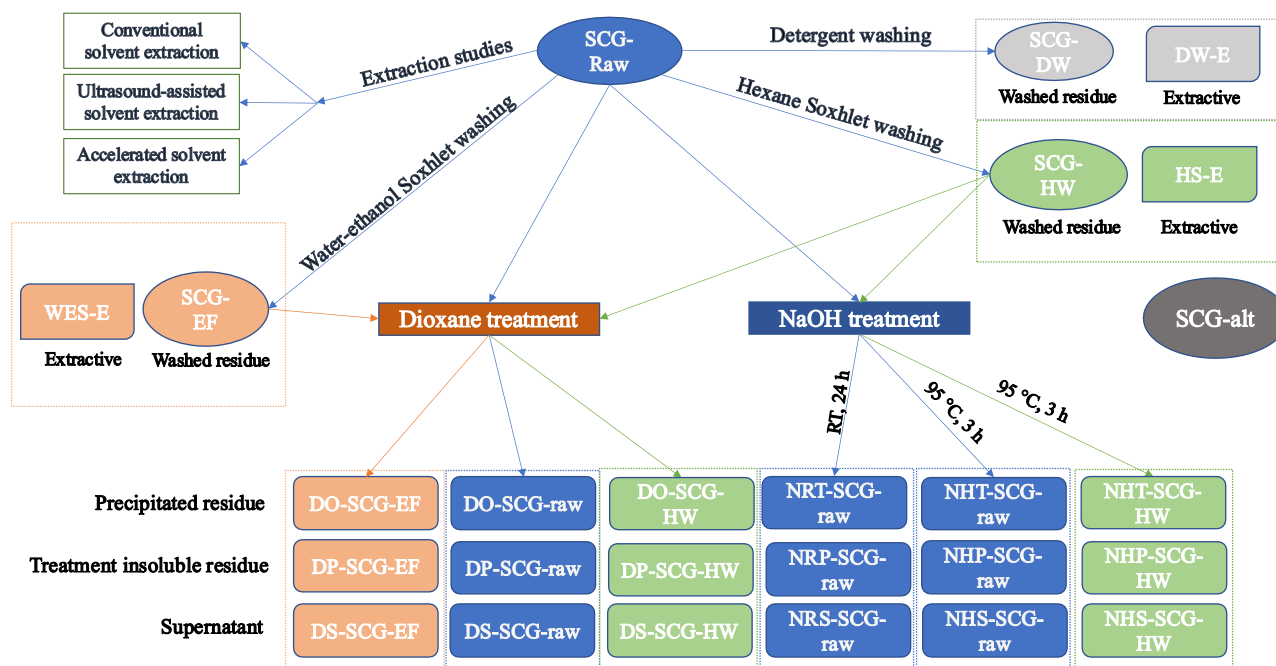


Figure 5. Experimental overview

4.1 SCG washing

The SCG obtained from Café Richards (SCG-raw) was lyophilized upon collection and stored in dry containers for studies. The moisture and ash content of the SCG-raw was performed in quadruplicate. The moisture and ash content of the lyophilized raw SCG after storage in ambient conditions were 1.1 ± 0.1 % and 1.2 ± 0.4 %, respectively. In this study, several methods were used to remove the extractives from the SCG for further analyses. In the first method, SCG-raw underwent a two stage Soxhlet washing with (1) water and then (2) ethanol. To ensure exhaustive extraction, the Soxhlet washing was continued until the solvent appeared colorless inside the Soxhlet tube. This required approximately 10 and 3 days for the water and ethanol extraction steps, respectively. The results of the water-ethanol Soxhlet extraction showed that the raw SCG had an extractive content of 31.3%. For the sake of simplicity, water-ethanol Soxhlet washed SCG will be called extractive-free SCG (SCG-EF), from here on. The fraction extracted during the ethanol wash (WES-E) was rotary evaporated and stored for analysis. SCG-EF had a moisture content of 5.4 ± 1.0 %. Similarly, SCG-raw underwent Soxhlet washing with hexane, exhaustively, to remove the lipid fraction. To maximize the lipid extraction, the Soxhlet washing lasted 8 days. This washed SCG will be called hexane-washed SCG (SCG-HW). The extractives obtained from this Soxhlet washing accounted for 16.2 % of the raw SCG mass. These two washed fractions and SCG-raw were studied to obtain their compositional data and their potential for valorization. In addition, another fraction of SCG-raw was washed with sodium dodecyl sulfate (SDS), to remove extractives, which is referred to as the detergent-washed SCG (SCG-DW). The effect of solvents and extraction methods on the extractive composition were studied using the SCG-raw. The SCG-EF and SCG-HW were used for further experimentation to verify the properties of the different treated fractions. In addition to SCG-raw, an alternative SCG (SCG-alt) was collected from the team APSYNTH cafeteria at INRAE Versailles. This was a mixture of several brands of SCGs, and therefore was used just for comparison in several fractional analyses.

4.1.1 Protein content

According to the literature, the protein content of spent coffee grounds can be estimated using three approaches. The first approach is a crude estimation of proteins based on the nitrogen content of the sample determined by elemental analysis (83), which calculates the protein content by multiplying a constant value (6.25) to the nitrogen composition of the sample. The second approach involves the selective extraction of proteins and colorimetric quantification (84). However, this approach involves relative quantification (to BSA standards) and, can be hindered by the extraction strategy and artifacts affecting the absorption. In the third approach, the proteins undergo acid hydrolysis and are derivatized to heptafluorobutyrate before the quantification of the amino acids using standards with a gas chromatography with flame ionization detection system (40). Recently, Massaya et al. proposed a modified nitrogen-to-protein conversion factor specific to the SCG they studied, based on the amino acid composition of the SCG, reporting a value of 7.9, rather than 6.25 and the total protein content of 21.79 % (79). The authors performed the hydrolysis of the proteins and quantified the amino acids using an LC/MS system. However, the authors point out there are possibilities of error arising from the various steps involved including sample preparation, chromatographic separation, and protein hydrolysis.

In this study, a colorimetric quantification and crude protein estimations were performed. The analyses were performed on SCG-raw, SCG-EF, and SCG-HW. The results of the analyses are given in Table 3. In the case of crude protein estimation, the conversion factors were chosen as 6.25 and 7.9, based on ISO 16634-2:2016, and Massaya et al., respectively (26,41,79). However, it must be mentioned that the choice of conversion factor as 6.25 assumes that SCG can be considered in the category of cereals, pulses and milled cereal products (80). A modified conversion factor can be developed, as in the case of Massaya et al. (79), based on the amino acid composition and nitrogen content of SCG or roasted coffee beans similar to the work from Mossé (1990), but this is beyond the scope of this study (85).

Table 3. Protein content determined using elemental analysis (conversion factor of 6.25 and 7.9) and colorimetric method

Sample	Crude protein content (%)	Protein content based on modified factor (7.9) (%)	Colorimetric protein content (%)
SCG-raw	13.9	17.5	0.4
SCG-EF	15.1	19.1	0.2
SCG-HW	16.7	21.1	0.4

where SCG-raw is the raw spent coffee grounds; SCG-EF is extractive-free SCG; SCG-HW is hexane Soxhlet washed SCG.

As shown in Table 3, the protein content is different, based on the quantification method. The crude protein estimation based on the nitrogen content can be an overestimation in the case of SCG owing to the various nitrogen containing compounds such as caffeine and trigonelline (84). Furthermore, the roasting of coffee beans prior to brewing creates Maillard reaction products (MRPs) from proteins and sugars called melanoidins (86), which can be attributed to the protein content of SCG, when using a crude protein analysis such as the Dumas method. Therefore, the colorimetric protein quantification should be considered as the more accurate method, although the bottleneck here is the extraction step. This could lead to an underestimation of the protein content.

The results from this study show that the protein content based on the nitrogen content slightly varies with different samples. In the case of SCG-raw, the crude protein content was estimated to be 13.9 % or 17.5 %, depending on the conversion factor. The colorimetric assay estimated a protein content of approximately 0.4 %. However, in the case of SCG-EF, the protein content should reduce, owing to the consecutive washing with water and ethanol, which should in theory remove most of the soluble proteins. However, the crude estimation based on the nitrogen content suggests that the protein content is higher than in the case of SCG-raw. The colorimetric assay however, estimates that the protein content did, in fact, reduce after the water-ethanol Soxhlet washing. Although an increase in the protein content was expected in the case of SCG-HW owing to the removal of lipids, the results do not represent this. The colorimetric protein content of SCG-HW is similar to that of SCG-raw.

Passos & Coimbra studied microwave assisted extraction of polysaccharides from SCG using water and quantified the protein content using GC-FID after the hydrolysis of proteins into amino acids and derivatizing them (40). They reported a protein content of approximately 4.9 %, based on the amino acid content. Valdés et al. studied the protein content of defatted SCG using the Bradford assay, which is a colorimetric quantification method, using BSA standard curves (84). They showed that the protein concentration varied with the extraction reagent that was used, ranging from 0.3 mg of protein/100 mg of SCG to 2.89 mg of protein/100 mg of SCG (84). Furthermore, they concluded that the choice of protein extraction method and the brewing method for the coffee affects the estimated protein content of the SCG. This could explain the low protein content of the SCG-raw. In addition, melanoidin is expected to be a large fraction of the SCG, and the Maillard reaction product would account for a large fraction of nitrogen content in SCG, different studies in the literature assumes this as proteins, which creates a problem for proposing a fractionation strategy (87). Furthermore, the results show that Soxhlet washing with water and ethanol extracts approximately 1/3rd of the proteins initially present in the SCG. Therefore, a water-ethanol based extraction strategy could be pursued for removing the proteins from the SCG, mainly for cleaning rather than valorization.

4.1.2 Sugar content

The polysaccharide composition of the SCG-raw, SCG-EF and SCG-HW were analyzed by sequentially hydrolyzing the biomass and quantifying the concentration of fructose, glucose, arabinose, galactose, xylose, and mannose using a calibration curve based on standard solutions. The hemicellulose and cellulose compositions of these samples are given in Table 4.

Table 4. Sugar composition of SCG samples determined by ionic LC after TFA hydrolysis followed by H₂SO₄ hydrolysis

Sugar content in sample (% monosaccharide per g sample)								
Sample	Hemicellulose					Fructose	Cellulose	Total sugar content
	Mannose	Galactose	Arabinose	Glucose	Xylose		Glucose	
SCG-raw	24.3 ± 3.4	12.9 ± 1.7	2.5 ± 0.4	1.1 ± 0.2	0.3 ± 0	0	1.5 ± 0.2	42.6 ± 5.5
SCG-EF	36.9 ± 3.5	16.6 ± 1.3	2.8 ± 0.3	1.7 ± 0.2	0.3 ± 0.1	0	3.3 ± 1.5	61.5 ± 6.0
SCG-HW	34.8 ± 5.5	18.1 ± 3.2	3.6 ± 0.7	1.6 ± 0.4	0.3 ± 0.1	0	1.9 ± 0.4	60.3 ± 9.8

where SCG-EF and SCG-HW are extractive-free SCG and hexane-washed SCG, respectively.

The results of the sugar composition suggest that mannose and galactose are the major hexoses found in the hydrolysate from SCG. The sugars mainly consist of mannose, being nearly twice that of galactose, with a small fraction of arabinose and glucose also present. In addition, no fructose was observed in the studied SCG samples. These compositions suggest that SCG-raw consists mainly of hemicelluloses, accounting for approximately 96.5 % of the total sugars and cellulose, being the remaining 3.5 %. Although the composition of hemicelluloses found in SCG-raw is in accordance with the results observed in the literature (30-42 %), the cellulose content of the SCG-raw is 1.5 %, which is relatively low, according to the literature (25,31). The cellulose content found in the literature is in the range of 7-24 % (31,41). In addition, the results show the presence of some glucose in the hemicelluloses (1.1 %). This is a new observation as the studies found in literature do not perform separate hydrolysis of hemicelluloses and cellulose. They assume that the glucose observed is part of the cellulose fraction.

The relatively low cellulose content could be a result of the chosen method for analysis, which consists of three stages, hydrolysis using TFA, followed by hydrolysis using 72 % H₂SO₄ and 5 % H₂SO₄ solutions followed by quantification. It is also possible that the dilutions used in the quantification step were too high for the anion exchange chromatography (AEC) system. This is slightly different from the common approach used in the literature, which consists of a two-step hydrolysis that cannot differentiate the glucose from the hemicelluloses

and cellulose as it only identifies the structural monosaccharides. Therefore, to quantify the cellulose content, a GC/MS based approach was used. The results of the sugar analysis suggest that the hemicelluloses in SCG-raw are mainly galactomannan and arabinogalactans, which is observed in other studies (40,88).

However, when a mass balance is performed on the sugar content of SCG-raw and SCG-HW, considering the extraction yield for HS-E (16.2 %), the expected sugar content of SCG-raw would be 50.3 %. When performing the sample balance for SCG-raw and SCG-EF, the expected sugar content of SCG-raw would be 42 %. In addition, the GC/MS metabolic analysis of WES-E shows significant presence of mannose, galactose, and arabinose, which suggests that the long duration of the Soxhlet washing (10 days) may have resulted in the partial hydrolysis of the hemicelluloses since the free sugars are expected to be removed during the water Soxhlet wash, which suggests that some of the extractive yield of WES-E will be owing to hydrolyzed hemicelluloses. Considering these two observations, it is likely that the sugar content of SCG-raw is underestimated, owing to hinderances caused by the extractible fractions such as lipids, proteins, alkaloids and chlorogenic acids. Therefore, the sugar content of SCG-raw is likely closer to 50 % than the estimated 42.6 %. Therefore, in the future, the Soxhlet washing step should be shorter to ensure that the structural sugars are not hydrolyzed. Further analysis should be performed on these fractions. Moreover, owing to the large contribution of sugars to the overall composition of SCGs, the valorization of SCG-raw must consider strategies towards using these polysaccharides.

4.1.3 Klason content (KL)

The acid insoluble residues of SCG-raw, SCG-EF, and SCG-HW were quantified as the Klason content (KL). In general, Klason analysis is performed to quantify the total lignins present in a biomass. Therefore, the KL of the various feedstocks and washed fractions were studied and are presented in Table 5.

Table 5. Klason content of feedstocks (ash-free)

Sample	Klason content (%)
SCG-raw	32.3 ± 0.5
SCG-EF	21.1 ± 0.3
SCG-HW	22 ± 0.3
SCG-DW	26.5 ± 1.6
SCG-alt	36.6 ± 0.1

In general, the KL of a biomass is studied after the removal of the extractives including lipids, proteins and polyphenols. However, for comparative purpose, KL was estimated for SCG-raw, SCG-EF, SCG-HW, SCG-DW and SCG-alt. The results show that SCG-alt had slightly higher KL than SCG-raw, suggesting that the total acid insoluble content could vary with the source of SCG. Furthermore, the KL of different washed fractions suggest that the washing does not completely remove the extractives compared to ethanol-water Soxhlet washing, which is considered as the exhaustive washing step. Because KL content is generally used to quantify the lignins in a biomass, it is assumed that SCG-raw consists of approximately 21.1 % of lignins, based on the KL of SCG-EF. This is the common approach to quantify KL in the literature, i.e., after the removal of the extractives. Furthermore, the hexane Soxhlet washing results in the lowering of KL from the 32.3 %, observed in the case of SCG-raw, to 22 %. This suggests that the presence of the extractives impacts the KL of SCG-raw, but no direct correlation can be made because the gravimetric yield of HS-E (16.2 %) does not directly account for the difference in KL (32.3 % – 22 % = 10.3 %). However, lipids, proteins and other extractible molecules are known to contribute to KL of feedstocks (81).

4.1.4 Lipid content

The lipid content of the feedstock was estimated using two approaches, as used in the literature (39,44,45). In the first approach, the gravimetric lipid content of SCG-raw was estimated to be 16.2 % based on hexane Soxhlet washing. The extraction yield of individual thimbles in the Soxhlet varied between 15.8 %

and 16.3 %, suggesting that either the heterogeneity of the SCG-raw causes variance in lipid content, or the vertical position of the thimble inside the Soxhlet affects the complete extraction.

In addition to the gravimetric estimation of lipids, the fatty acid content of the SCG-raw, SCG-EF, extractives from water-ethanol Soxhlet washing, SCG-HW, and the extractives from the hexane Soxhlet washing were quantified using GC/MS after the transmethylation (5 % H₂SO₄ in methanol, 2 h, 85 °C) of the samples with methyl margarate as the internal standard. A trial run showed the presence of 4 significant peaks in the chromatogram, which were identified as methyl esters of palmitic acid (C_{16:0}), linoleic acid (C_{18:2}), stearic acid (C_{18:0}) and oleic acid (C_{18:1}). Therefore, only these FAMES were quantified in this study. The fatty acid content of the different fractions is summarized in Table 6.

Table 6. Fatty acid quantification by transmethylation followed by GC-MS analysis

Sample	mg C _{16:0} / g DW	mg C _{18:0} / g DW	mg C _{18:1} / g DW	mg C _{18:2} / g DW	Total fatty acids mg/ g DW
<i>Solid residues</i>					
SCG-raw	53.8	9.9	11.6	74.6	149.8
SCG-EF	7.3	1.3	1.3	8.6	18.6
SCG-HW	13.3	1.9	1.9	14.7	31.8
SCG-DW	29.1	3.7	3.5	29.6	65.9
SCG alt	58.4	10.1	10.2	63.4	142.1
<i>Extractives</i>					
HS-E	229.0	42.4	39.5	233.7	544.6
WES-E	226.4	37.7	43.2	253.3	560.5

The fatty acid quantification of different solid residues shows similar fatty acid content for the two sources of SCG. This was also visible in the gravimetric measurement of the hexane extractives, with SCG-raw having a gravimetric yield of 16.2 %, compared to SCG-alt (14.7 %). However, the fatty acid composition differed between the two sources. A higher concentration of linoleic and oleic acids was observed in SCG-raw, compared to SCG-alt. In the case of SCG-alt, the fatty acids consisted of similar concentrations of palmitic and linoleic acids, and similar concentrations of stearic and oleic acids. In both cases the major fatty acids present in coffee lipids are linoleic and palmitic acids, with minor quantities of stearic and oleic acids. A representative chromatogram of the FAMES obtained through the transmethylation of SCG-raw without standard is shown in Figure 6 with their corresponding fatty acids labelled.

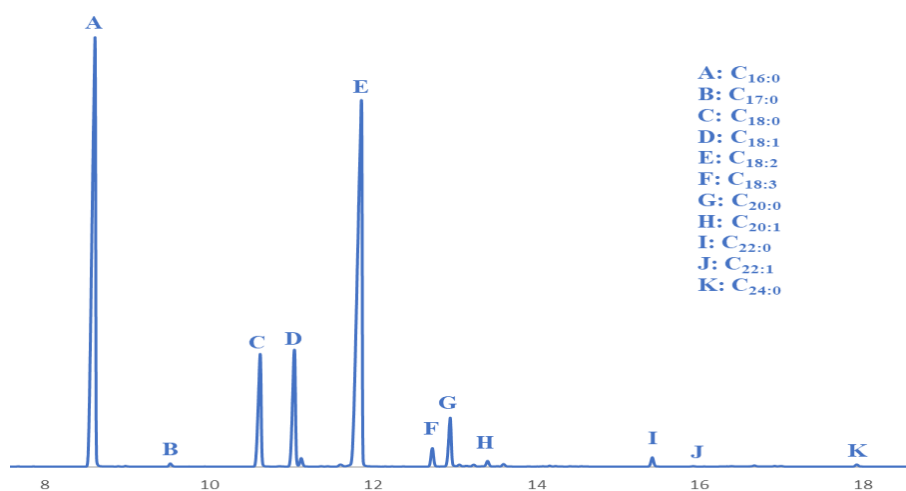


Figure 6. Representative GC-MS chromatogram of transmethylated SCG-raw

Although the four FAMES dominate the composition, methyl esters of arachidic acid (C_{20:0}), linolenic acid (C_{18:3}), erucic acid (C_{22:1}), gondoic acid (C_{21:1}), margaric acid (C_{17:0}), and lignoceric acid (C_{24:0}), were observed in the samples. The choice of internal standard for fatty acid quantification was based on previous trials to ensure a distinct quantifiable peak in the chromatogram. However, because of the trace of C_{17:0} observed in Figure 6, the fatty acid quantification using methyl margarate as the internal standard will be slightly underestimated. Furthermore, according to the literature, SCG may contains unsaponifiable contents such as diterpenes (kahweol, cafestol, etc..) which can account for some of the undetermined fractions (21,89). Battista et al. reported a 0.2 % and 0.4 % of cafestol and kahweol in the SCG lipids extracted using hexane (43), whereas Jenkin et al. reported the unsaponifiable fractions can account for 0-40 % of the gravimetric lipid content (21). However, the heterogeneity in SCG owing to the different stages of processing can impact the composition of these terpenes in SCG-raw. Owing to technical constraints, only a qualitative analysis of the lipid fractions was performed using the GC/MS for analyzing the metabolites. Interestingly, tocopherol, stigmasterol and sitosterol were detected in the HS-E and WES-E.

Furthermore, Campos-Vega et al. consolidated the fatty acid composition of SCG available in the literature (31). The composition of SCGs from this study in comparison with other studies are presented in Table 7. In the cases where traces of fatty acids were observed, no quantification was performed.

Table 7. Fatty acid composition of SCG from literature compared to this study

Reference	% C _{12:0}	% C _{14:0}	% C _{16:0}	% C _{18:0}	% C _{18:1}	% C _{18:2}	% C _{18:3}	% C _{20:0}	% C _{22:0}
<i>Literature</i>									
Jenkin et al. (21)	nd	nd	35.4	6.7	6.7	49.9	tr	1.2	nd
Jenkin et al. (21)	nd	nd	40.4	13.5	24.0	22	tr	0	nd
Ahangari and Sargolzaei (90)	3.6	2.0	43.6	6.6	8.2	21.4	1.3	2.4	nd
Couto et al. (39)	3.6	2	43.7	6.5	8.2	32.5	1.3	2.4	nd
Vardon et al. (35)	nd	nd	33.9	7.3	8.3	45.0	1.5	2.5	0.6
<i>This study</i>									
SCG-raw	nd	nd	35.9	6.6	7.7	49.9	tr	tr	tr
SCG alt	nd	nd	41.1	7.0	7.2	44.7	nd	nd	nd

*nd: not detected; tr- traces.

The fatty acid composition obtained in this study is similar to the ones observed in the literature with a composition dominated by linoleic acid moieties. Therefore, the SCG extracted lipids could find applications in food and cosmetic industries, due to the presence of long fatty acids (> C₁₆) and valuable Omega 3, rather than for biodiesel applications, as commonly proposed in the literature, where shorter chains are preferred (35).

4.1.5 Metabolites analyses

The metabolites in the SCG-raw, SCG-EF, SCG-HW, WES-E and HS-E were analyzed qualitatively using LC/MS and metabolomic GC/MS systems and presented in hierarchical clusters (Annex). In the case of LC/MS, features are made using the retention time and mass spectra data. The results of GC/MS metabolomic characterization were tabulated based on spectral matches with different databases. These qualitative approaches were used to identify the molecules of interest in the different fractions and compare the effect of the washing strategies. Using the LC/MS system, cafestol, kahweol, caffeine, dehydrocafestol and chlorogenic acids were detected in WES-E, suggesting that ethanol extraction can separate these compounds from the solid residue. In addition, tocopherol, stigmasterol and sitosterol were also observed in WES-E and HS-E fractions. These metabolites find various applications owing to their biological activities. Cafestol and kahweol have anticarcinogenic properties and antioxidant properties (91). Caffeine is an alkaloid with known physiological activity and finds application in food industry for energy drinks and other products (9). Furthermore, phytoosterols such as stigmasterol and sitosterol have been known to shown antioxidant, anticarcinogenic, and anti-inflammatory properties (63).

4.1.6 Structural properties

The structural properties of SCGs and the washed fractions were studied using various methods including NMR spectroscopy (1D and 2D), size exclusion chromatography, and thioacidolysis (a specific degradative lignin analysis). Because of the heterogenous nature of SCG, the combinations of these approaches are necessary to understand the different fractions. The thioacidolysis results are presented in Table 8.

Table 8. Amount of guaiacyl (G) and syringyl (S) monomers released and thioacidolysis yield of the SCG samples

Sample	G units ($\mu\text{mol/g}$)	S units ($\mu\text{mol/g}$)	Total (G+S) ($\mu\text{mol/g}$)	S/G
SCG-raw	1.0 ± 0.3	0.3 ± 0.1	1.3 ± 0.4	0.3 ± 0
SCG-EF	1.4 ± 0.2	0.3 ± 0.1	1.7 ± 0.2	0.2 ± 0
SCG-HW	1.3 ± 0	0.4 ± 0.1	1.7 ± 0.1	0.3 ± 0
SCG-alt	0.5 ± 0.1	0.1 ± 0	0.6 ± 0.1	0.3 ± 0
SCG-DW	0.7 ± 0	0.1 ± 0	0.8 ± 0	0.2 ± 0

Lapierre et al. studied the yield of H, G and S units from the thioacidolysis of suberized potato periderms and lignified cell walls (poplar wood and wheat straw) to compare the phenolic moieties in suberin and lignins (92). The reported total monomeric units of poplar wood and wheat straw were approximately $540 \mu\text{mol/g}$ and $177 \mu\text{mol/g}$, respectively. The potato suberin fraction had a total of $17 \mu\text{mol/g}$ of monomeric units. However, the results of the thioacidolysis show much lesser quantities of G and S units. Interestingly, the detergent washing reduced the total monomers compared to SCG-raw. However, this was not investigated further owing to time constraints. Furthermore, thioacidolysis results of SCG-alt show that the low monomeric yield is not unique to SCG-raw. The ^{31}P NMR was performed on SCG-raw, SCG-EF and SCG-HW. The results are presented in Table 9.

Table 9. ^{31}P NMR spectroscopic analysis of the SCG samples after derivatisation in pyridine/ CDCl_3

Sample	mmol of OH/g sample studied			Solubility
	Aliphatic OH	Phenolic OH	Carboxylic acid OH	
SCG-raw	0.1	0.0	0.7	Poor
SCG-EF	0.0	0.0	4.5	Poor
SCG-HW	0.0	0.0	0.0	Poor

The poor solubility of these residues in the pyridine/ CDCl_3 solution is inconsistent with lignins from other sources. The quantification is impossible but the ^{31}P NMR results show that the phenolic OH groups associated with uncondensed lignins are very limited in the raw feedstock and the washed fractions. Therefore, further separation of these monomers and the acid insoluble can be interesting towards understanding the structure of these fractions.

4.2 Extraction

In this study, the extractability of the biologically active compounds in SCG-raw was studied using three different methods. (i) conventional extraction at room temperature; (ii) ultrasound assisted extraction; and (iii) accelerated solvent extraction.

4.2.1 Conventional solvent extraction (CSE)

The experiment consisted of a three-stage extraction with the solvent fraction filtered after each stage. Solvents tested in this study include hexane, acetone, ethanol, ethyl acetate, tetrahydrofuran (THF), acetonitrile, dichloromethane (DCM), methanol, dioxane, water. Various solvents and solvent mixtures were studied for their extractability, including methanol (70 % v/v) in water, methanol-DCM (1:2) and dioxane (90% v/v) in water. To ensure repeatability, each experiment was conducted in duplicates, except for the methanol (70 % v/v) in water, due to a broken Eppendorf tube. The extractive yield of different solvents and mixtures are presented in Figure 7.

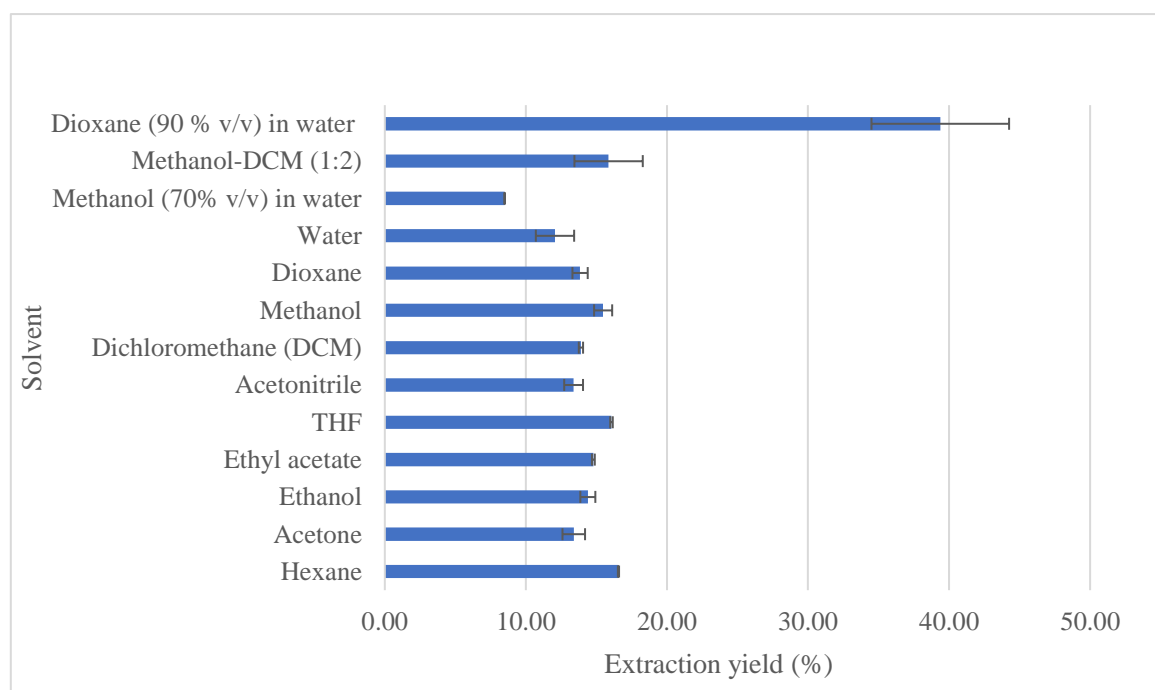


Figure 7. Conventional solvent extraction (CSE) yields of SCG-raw with various solvents at RT

Interestingly, the highest yield was observed in the case of dioxane in water (39.4 ± 4.9 %). However, the metabolic GC/MS analysis of this sample suggests that dioxane underwent degradation in water, which could contribute to the obtained yield. In addition, the mass balance performed using the mass of the extractives, residue and the feed, shows an excess mass, suggesting the presence of dioxane or its degradation products in the extractives, residue or both.

The next highest yields were observed for hexane (16.6 ± 0.1 %), followed by THF (16.0 ± 0.1 %), methanol-DCM (15.9 ± 2.4 %) and methanol (15.5 ± 0.6 %), respectively. Owing to the broad range of solvents tested, the preliminary analyses of the extractives were performed using SEC (Figure 8) to compare the extractive size profile and the gravimetric yield. The peak at a retention time of 20.2 minutes is the standard toluene peak. In most cases, the extractives were not completely soluble in THF. Overall, these solvents showed very similar SEC profiles. The SEC profile of THF extractives was unique owing to contamination (the peak at 17.8 min). The use of THF stabilized with butylated hydroxytoluene (BHT) as the solvent for extraction affects the antioxidant activity calculation for the SCG extractives owing to the antioxidant capacity of BHT, as evident in the work of Leow et al., where the authors observed high antioxidant activity and BHT content in the SCG extractives using THF as the solvent (93). However, the authors misidentify the source of BHT as the SCG

rather than the use of THF as the solvent. Because the antioxidant capacity of different extractives was planned to be tested, this solvent was abandoned.

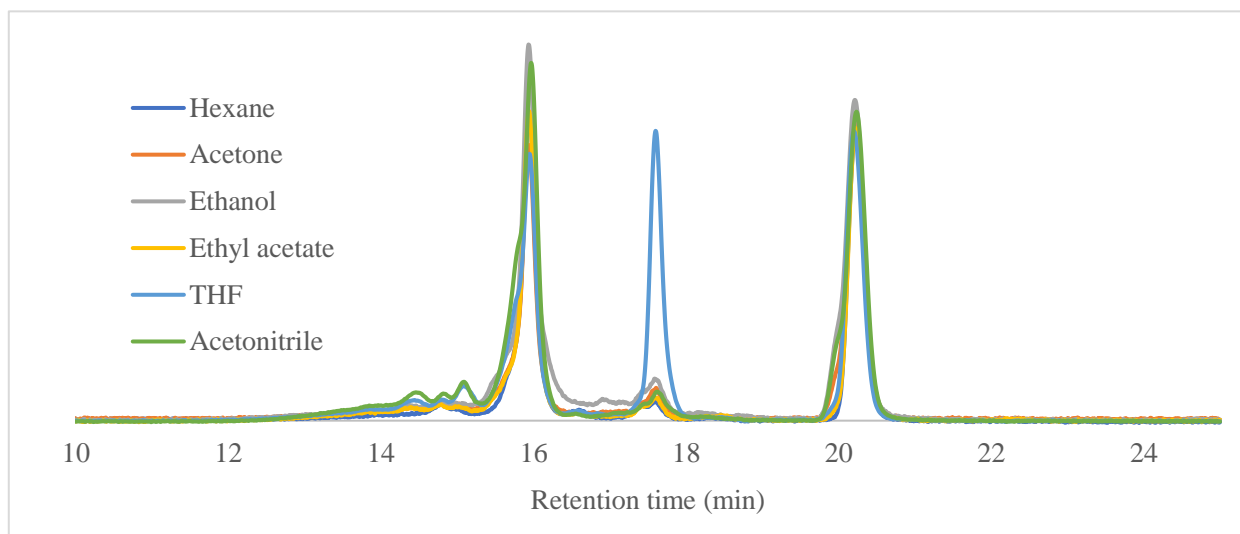


Figure 8. SEC chromatograms of CSE; analysis in THF with UV detection at 280 nm, normalized on toluene peak

In the case of hexane, the extraction yield is higher than that reported from the hexane Soxhlet washing, which could be due to a lack of dynamic mixing or incomplete extraction of lipids at the higher levels of the Soxhlet tube. In addition, during CSE, some SCG particles can be transferred into the extractive fraction owing to density differences. This is avoided by filtration of the solvent before transferring into flasks, but some solids could still pass owing to the size. Another reason for the difference in the extraction yield between HS-E and CSE of hexane could be the heterogeneity of SCG-raw, which can have slight differences.

The solvents for further experiments were chosen based on the extractive yield, SEC and NMR profiles and the greenness of the solvent. The greenness of the solvent was compared using the solvent-selection guide proposed by McElroy et al. (94). Among the studied solvents, DCM, methanol-DCM, hexane and THF were chosen owing to their capacity to extract lipids. However, these are not considered as green solvents, owing to their toxicity, environmental impact, safety concerns and the suitability of antioxidant study in the case of THF. Therefore, for further studies, only hexane was chosen owing to the highest yield (16.6 ± 0.0 %) and the fact that it is acceptable by the European union guidelines as a food grade solvent (95). Although ethyl acetate is considered greener and is accepted as a food grade solvent, the gravimetric yield of the extract was lower (14.8 ± 0.1 %). In addition to the lipids, antioxidant chemicals have been reported in SCG (41,72). Therefore, solvents such as methanol, ethanol, water, acetone and acetonitrile were compared. However, owing to the similarity of the $^1\text{H-NMR}$ spectra of acetone and hexane extractives (Figure 9), and the lower extract yield obtained with acetonitrile, only methanol was chosen among these solvents for further studies.

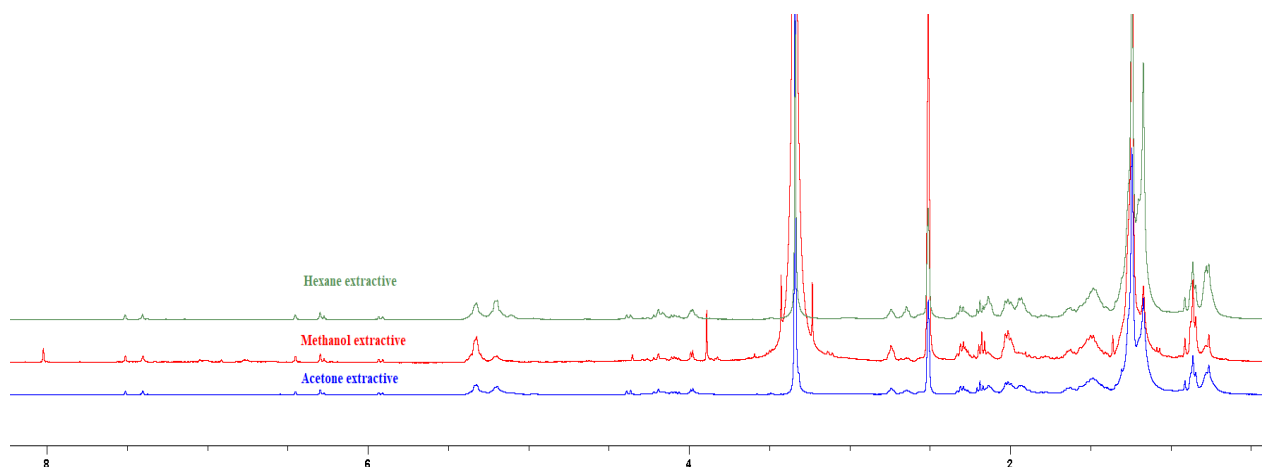


Figure 9. $^1\text{H-NMR}$ spectra of hexane, methanol and acetone CSE extractives; spectra recorded in DMSO-d_6

In the previous experiments, the three stages of extraction used the same solvent. However, in a biorefinery approach, the fractionation of the extractives using different solvents must be considered to maximize the recovery of extractives. Therefore, the effect of a three stage-three solvent extraction strategy was also studied gravimetrically. In this regard, three setups were tested, (i) hexane-ethyl acetate-methanol, (ii) water-hexane-methanol and (iii) hexane-methanol-water. The results of these extractions are presented in Table 10.

Table 10. Three stage extraction yields of SCG-raw

Three stage solvents	Gravimetric yield (%)			
	Stage 1	Stage 2	Stage 3	Total
Hexane-Ethyl acetate-Methanol	11.5 ± 0.5	0.7 ± 0.1	2.8 ± 0.3	15.0 ± 0.1
Water-Hexane-Methanol	5.6 ± 0.3	12.7 ± 0.7	2.9 ± 0.7	21.2 ± 0.4
Hexane-Methanol-Water	11.5 ± 1.2	4.1 ± 4.4	6 ± 0.2	21.7 ± 3.0

Interestingly, total yield of the latter two setups remained the same (21.2 ± 0.4 and 21.7 ± 3.0 %, respectively), although the extraction by hexane (12.7 ± 0.7 % and 11.5 ± 1.2 %, respectively) and water (5.6 ± 0.3 % and 6 ± 0.2 %, respectively) remaining similar. However, the choice of ethyl acetate is avoided as it is evident that additional extraction did not result in a significant recovery (0.7 ± 0.1 %). In the first two setups, methanol consistently extracted 2.8 % of extractives after hexane, which when comparing the $^1\text{H-NMR}$ spectra above (Figure 9), could be attributed to molecules other than lipids. In addition, because water extractives remain 5-6 %, irrespective of the stage, it is interesting to study this fraction in further detail. Therefore, for further studies, hexane, methanol, water and ethanol are used as extraction solvents. In addition, methanol-water mixture was also studied even though this was the lowest yielding extraction solvent.

4.2.2 Ultrasound assisted extraction (UAE)

The effect of low frequency ultrasound on the extractability of bioactive compounds in SCG was studied using selective solvents, consisting of hexane, acetone, methanol, methanol, and ethanol. Similar to conventional solvent extraction, the UAE experiments were performed in three stages, with the fresh solvents introduced in each stage. The results of the ultrasound assisted extraction are given in Table 11.

Table 11. Results of the ultrasound assisted extraction (UAE) of SCG-raw

Solvent	Average UAE yield (%)	Average CSE yield (%)
Hexane	14.1 ± 0.5	16.6 ± 0.1
Acetone	14.5 ± 0.1	13.4 ± 0.8
Methanol	17.0 ± 2	15.5 ± 0.6
Ethanol	11.1 ± 0.6	14.4 ± 0.5

Interestingly, the ultrasound assisted extraction slightly improved the extraction yield in the case of acetone and methanol whereas a lower yield was observed for ethanol and hexane, compared to the conventional extraction. Because the same duration of extraction was performed for UAE and conventional extraction, the lower extraction yield is surprising and is difficult to explain.

4.2.3 Accelerated solvent extraction (ASE)

The extractability of different fractions were also studied using Dionex ASE 350 accelerated solvent extractor. In brief, an accelerated solvent extractor uses temperature and pressure to perform extractions in a short time (seconds to minutes). The extractor first heats up the cell containing the biomass to the preset temperature and sustains the temperature for the duration of the extraction. The solvent is then pumped into the cell and the cell is pressurized by the closing of the static valve. The extractor ensures that the internal pressure does not increase beyond 200 psi of the set point and adds fresh solvent to adjust the pressure. This is continued until the predefined time (static time), after which, a predefined volume of fresh solvent (rinse volume) is pumped into the cell to remove the extractives, through a filter into a collection tube. This constitutes an extraction cycle, the number of which, can be modified as needed. A unique set of the parameters make up a defined method. Furthermore, an array of extractions can be performed with just one method in series, or an array of different methods can be studied in series using a defined sequence. This makes the use of ASE, very easy for the extraction of analytes from a biological matrix.

In this study, four different solvents and a mixture, including hexane, methanol, water, ethanol and a 70 % v/v methanol-water mixture were used to understand the effect of different parameters on the extractability of extractives from SCG-raw using ASE. First, an optimization of ASE specific parameters was performed keeping hexane as the solvent. Because the number of cycles, static time, rinse volume, biomass loading, and the temperature could be modified, the effect of these parameters were studied to propose an optimized strategy. Each experiment was performed in duplicates to ensure reproducibility.

4.2.3.1 Optimization of ASE specific parameters

First, the effect of the number of cycles on the extractive yield was performed using a fixed mass accurately weighted of approximately 400 mg of SCG-raw in a 34 mL cell. This ratio was used to keep the experiments more comparable to the results of the conventional hexane extraction. The number of cycles were adjusted to 3 and 1. Then, the effect of the biomass loading was tested, keeping the parameters including the number of cycles constant. Here, three different loadings were studied, with loadings of 4 and 8 times the original loading, to see if the extractive yield is significantly affected by the biomass loading. In addition to the parameters mentioned, the Dionex ASE 350 extractor suggests the use of a dispersing agent such as sand in the case that the sample can form aggregates. Therefore, sand was used as a dispersing agent in the next experiment, with approximately 5 g of sand used in each replicate. Then the effect of rinse volumes on extraction yield were tested. The results of these optimization trials are summarized in Table 12.

Table 12. ASE parameter optimization on SCG-raw extracted by hexane

#	Mass of SCG-raw (g)	Cycles	Static time (min)	Rinse volume (% v)	Sand	Extraction yield (%)
1	0.4	1	5	50%	No	12.2 ± 0.1
2	0.4	3	5	50%	No	12.7 ± 0.3
3	1.6	1	5	50%	No	12.2 ± 0.1
4	3.2	1	5	50%	No	12.1 ± 0.1
5	1.6	3	30	50 %	No	11.9 ± 0.2
6	1.6	1	5	20%	No	11.6 ± 0.2
7	1.6	1	5	150%	No	11.8 ± 0.1
8	1.6	1	5	50%	Yes	11.8 ± 0.1

The cell volume, solvent and temperature were unchanged, using, 34 mL, hexane and room temperature, respectively.

As shown in Table 12, the number of cycles nor the biomass loading had a significant impact on the extraction yield (entries 1-4). Although a slight increase in the extraction yield was observed when the number of cycles is increased from 1 to 3 (from 12.2 % to 12.7 %), this increase is too small for practical applications. Therefore, in the experiments related to temperature and solvents, as a compromise, the number of cycles was set as 2. Furthermore, the static time studied (5 and 30 mins) did not have a significant impact on the extraction yield.

In addition, extraction yield remained constant with the three different biomass loadings tested (12.1 %), which was then set as 1.6 g for future experiments.

Furthermore, when the rinse volume was studied, the optimal yield was observed at a rinse volume of 50 % (entry 3), as opposed to 20 % or 150 % (entries 6 and 7). Similarly, the addition of dispersant (sand) resulted in a lower extraction yield (11.8 %), compared to the control (12.2 %). Therefore, no sand is used in future experiments. These results suggest that the extraction yield was not impacted by the various ASE parameters for hexane. Although similar study should be performed for individual solvents, this work is kept for future works.

Based on the optimization studies, the solvent and temperature studies were performed to study the extractives from SCG-raw. In this regard, the choice of solvent was studied using hexane, methanol, water, methanol-water (7:3 v/v) mixture and ethanol. The effect of temperature on the extraction yield was also studied for the chosen solvent systems.

4.2.3.2 Effect of solvent and temperature on ASE

The effect of the solvent and temperature on the extraction of SCG extractives using ASE was tested using hexane, methanol, water, methanol-water (7:3 v/v) mixture and ethanol at temperatures between room temperature (20 °C) and 125 °C. The temperatures of 20 °C, 60 °C, 100 °C and 125 °C were tested for all solvents except ethanol, with only the highest and lowest temperatures tested. The ASE extraction yields of these solvents with respect to temperature are presented in Figure 10.

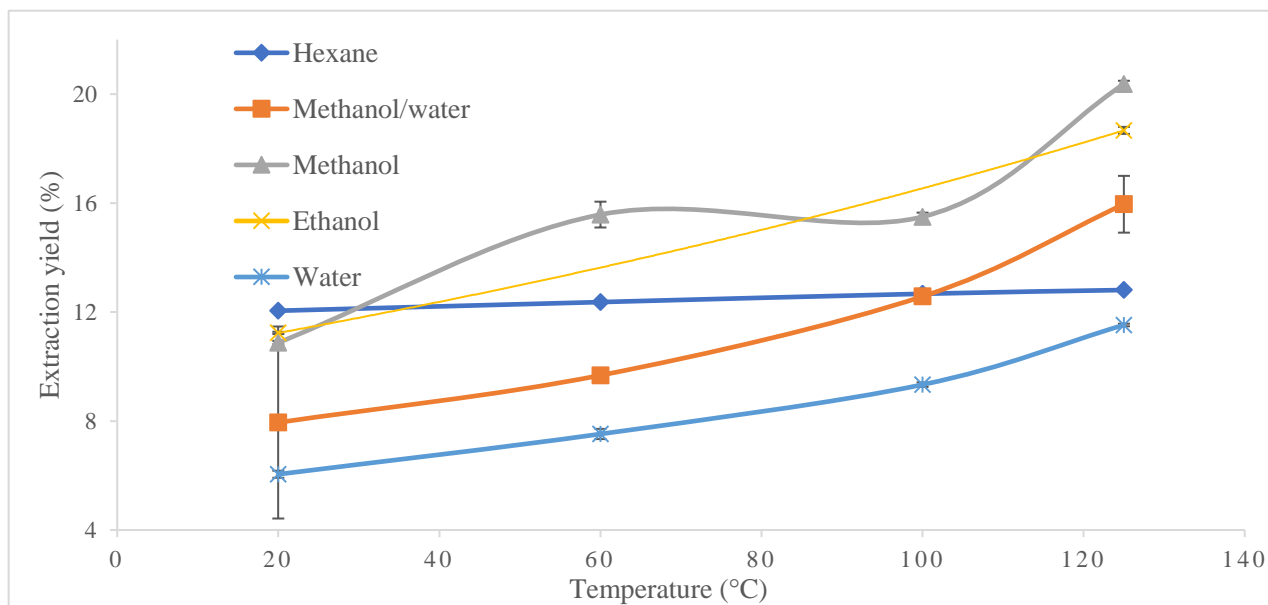


Figure 10. Effect of solvent and temperature on extraction yield of SCG-raw by ASE

As shown in Figure 10, the extraction yield shows increase with temperature in all cases except hexane. The extraction yield for hexane at 20 °C was approximately 12 ± 0.1 %, which only increases to 12.8 ± 0.1 % at 125 °C. Efthymiopoulos et al. reported lipid extraction efficiency of approximately 60 % (relative to HS-E) at 60 °C, which increased to 83 % at 125 °C. However, when similar units are used in this study, the extraction efficiency would be 74.5 % and 79.5 % (relative to HS-E), respectively for ASE using hexane. Furthermore, they reported that the extraction decreased to 75 % (relative to HS-E), when the temperature was increased beyond 125 °C. The minimal increase in the extraction yield with increase in temperature for hexane can be attributed to the apolar nature of hexane.

The extraction yields nearly doubled with a rise of temperature from 20 °C to 125 °C, for polar solvents such as ethanol, water, methanol/water and methanol. However, the effect of temperature on the solvents were not identical. In the case of methanol, similar extraction yields were observed at 60 °C and 100 °C (approximately 15.5 %), but it increases to approximately 20 % when the temperature reaches 125 °C. Furthermore, the maximum yield was obtained at 125 °C in all cases. Owing to time constraints, the antioxidant activity of only few samples were studied. The results of the DPPH assay are given Table 13.

Table 13. Results of DPPH assay on different extractable fractions from SCG-raw

Solvent	Temperature (°C)	EC ₅₀ (mg/mmol DPPH)	Gravimetric yield (%)
Methanol*	RT	2.0 ± 0.6	10.9 ± 0.1
Methanol*	60	0.4 ± 0.1	15.6 ± 0.5
Methanol	125	4.6	20.4 ± 0.1
Methanol-water (7:3)	60	2.6 ± 0.6	9.7 ± 0.1
Methanol-water (7:3)	125	3	16.0 ± 1.0
Water	125	2.8 ± 1.2	11.5 ± 0.1

*DPPH methanol solution used for quantification; the other DPPH assays used a 50 % methanol-water solution.

The results show that the highest antioxidant activity was observed for the methanol extractives at 60 °C. This was followed by the methanol extractive at room temperature. Among the three extractions performed at 125 °C, both methanol-water mixture and water yielded similar antioxidant activities. However, the antioxidant activity of methanol decreased at 125 °C, compared to the lower temperatures. This could be owing to the thermal degradation of antioxidant molecules during the extraction or the relative dilution of antioxidants owing to improved gravimetric yield. The choice of solvent for the DPPH assay could also have impacted this result. The comparison of the SEC profiles for methanol extractions performed at room temperature and 125 °C is given in Figure 11. It should be noted that the peak at approximately 19 min is a contaminant and must not be considered for analysis. However, additional peaks are observed in the methanol extractive from room temperature, especially around 24 mins. These differences could be responsible of the difference in the antioxidant activity. However, further studies are necessary to identify the exact chemical differences.

However, the direct comparison of the EC₅₀ value from this study with the literature is complicated because of the units used. Owing to a lack of standard DPPH assay methodology, the EC₅₀ values can be observed in units such as GAE, v/v%, mM, Trolox equivalents, etc. Mishra et al. reported an EC₅₀ values of gallic acid and ferulic acid as 11.9 mg/mmol DPPH and 69.9 mg/mmol DPPH (96). Similarly, Brand-Williams reported the EC₅₀ values of 13.6 mg/mmol DPPH and 83 mg/mmol DPPH (97). Compared to these, the extractives studied show high antioxidant activity.

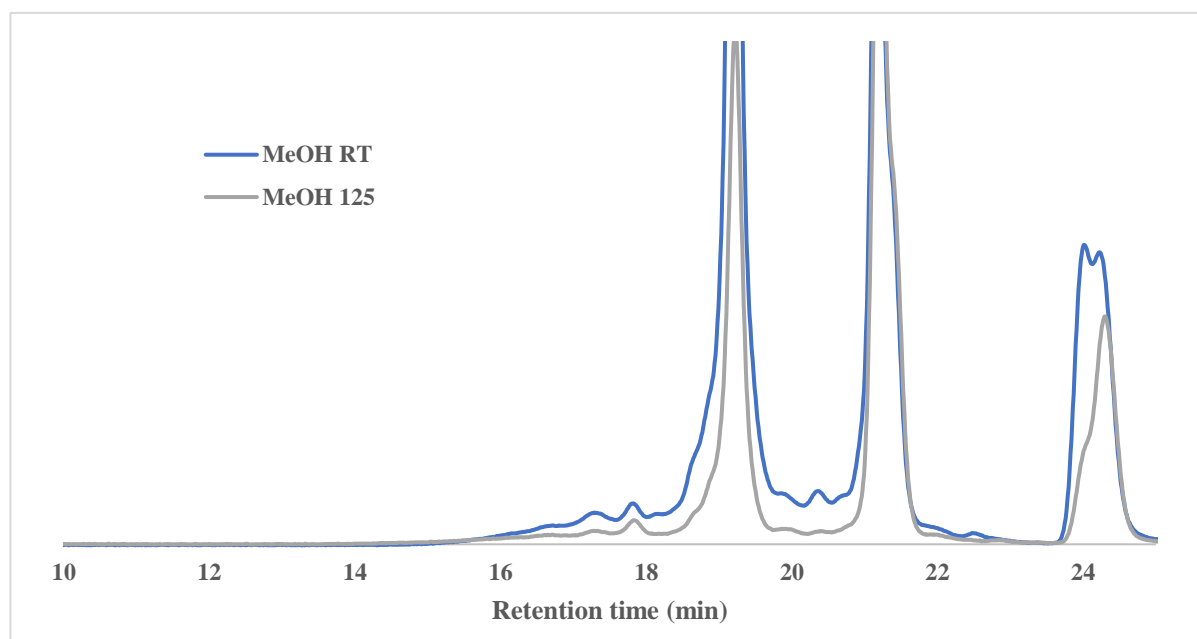


Figure 11. SEC profile of ASE methanol extractives at different temperatures, normalized on toluene peak; analysis in THF with UV detection at 280 nm

In addition to the experiments mentioned above, the effect of temperature and time on the extractive yield using water was tested to maximize the yield. Therefore, ASE was performed at 180 °C, with 3 cycles and 30 mins of static time for each cycle. Interestingly, in this case, the extraction yield obtained was $51.3 \pm 0.9 \%$, which is higher than the yield of WES-E, (31.3 %). In addition, the KL content of this fraction was approximately 12.34 %. This extractive also showed numerous spots in the sugar region in the 2D HSQC studies (Figure 12), which coupled with the high yield and the subcritical water system used suggests that hemicelluloses were hydrolyzed and extracted. The HSQC of extractive also showed aromatic signals in the range of 6.5-7.5 ppm/120-140 ppm.

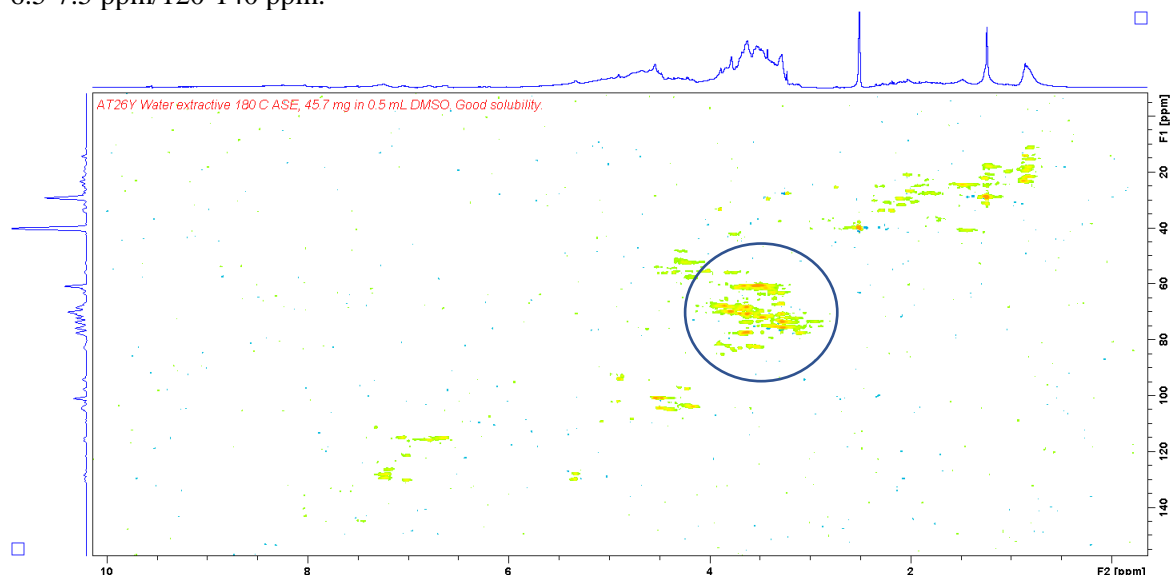


Figure 12. 2D-HSQC spectra of extractives from 180 °C water extraction; spectrum recorded in DMSO- d_6

Similar results were obtained using subcritical water systems such as Massaya et al., where the authors hydrolyzed the hemicellulose using subcritical water systems and performed hydrothermal carbonization of the remaining residues (79). Passos & Coimbra performed microwave-assisted subcritical water extraction at 200 °C for 2 min to extract the polysaccharides (40). These results also suggest that polysaccharides were extracted from SCG-raw during the extraction at 180 °C.

Further confirmation can be obtained by performing the sugar quantification of this extractive fraction, but it is left for future work owing to a shortage of time. The results suggest that subcritical water systems could be used for a hemicellulose-based approach to the valorization of SCG-raw.

4.2.4 Comparison of extraction methods

Among the studied methods for extraction, CSE resulted in highest yields when the extractions were performed using identical volumes of solvents. In this study, CSE and UAE were performed for identical duration and stages. However, the results were mixed, with the UAE yield improved for acetone and methanol but not for hexane or ethanol. In the future, the effect of a shorter extraction time and higher temperatures must be considered, as similar extraction yields might be observed in UAE at shorter times. When using polar solvents with ASE, such as water methanol and ethanol, an increase in temperature, and therefore in pressure, allowed a higher extraction yield.

In this study, owing to technical and time constraints, the quantification of the known metabolites or the individual characterization of antioxidant activity and total phenolic content (TPC) of each sample was not performed. Only a global analysis was performed to understand whether the temperature, and the choice of solvent impacted the yield of extractives and its antioxidant property. Although the features in the LC/MS spectra suggest that caffeine, cafestol, kahweol etc. were found during the different extractions, quantitative LC/MS must still be performed.

However, as shown through the three-stage extraction experiment, fractionation of extractives using different solvents in a sequence could be interesting towards the valorization of lipids, antioxidants, chlorogenic acids and caffeine. Furthermore, the choice of temperature and solvent must be optimized towards individual fractions.

4.3 Lignin separation treatments

According to the literature, SCG consists mainly of hemicelluloses, lignins, lipids, cellulose, proteins, and small amounts of polyphenols and alkaloids (31,44,98). Among these fractions, lipids, proteins, polyphenols and alkaloids can be extracted using various solvents. Moreover, because hemicelluloses and cellulose make up approximately 42.6 %, 61.5 % and 60.3 % of the SCG-raw, SCG-EF and SCG-HW, respectively, the valorization of these fractions is of significant interest. However, the valorization of the hemicelluloses and celluloses in lignocellulosic biomasses are limited by the recalcitrance of the lignins. Therefore, separating the lignins from the sugars is a necessary step in the development of a SCG fractionation strategy. In this regard, two treatments were studied for the fractionation of lignins from SCG, with the lignin content of SCG reported in the literature to be 2-32 % in SCG (66). The treatments used in this study are: (i) organosolv and (ii) soda treatments.

An organosolv treatment consists in the dissolution of lignins in organic solvents in the presence or absence of a catalyst, usually inorganic acids, to degrade the hemicelluloses and dissolve the fragmented lignins (99). The lignins are then recovered when the organosolv liquor is separated from the polysaccharide residue by filtration, concentrated, and precipitated in water. In the soda treatment, an alkaline solution of NaOH (pH 13-14) is used to disrupt the hemicellulose linkages, ether linkages and improve the solubility of lignins in water. The soda liquor is separated from the polysaccharides by filtration, and then the lignins are precipitated by lowering the pH (<10) (99). These treatments were used to study lignins owing to their reputation to extract lignins.

4.3.1 Dioxane-HCl treatment

In the first approach, an organosolv treatment using a dioxane-HCl mixture was performed on the SCG-raw. Use of the dioxane organosolv treatment (DO) for the fractionation of lignins has been reported by various research teams (100,101). Organosolv treatments and specifically DO treatment are considered as a good approach to preserve the native lignin structure during fractionation (101). The DO treatment was first performed on the SCG-raw and studied. During the precipitation of the DO lignin residue in water, the solids formed two fractions, a less dense fraction that stayed at the surface of the water, and a denser residue that fell to the bottom. Both fractions were collected to obtain a gravimetric yield of approximately 20.9 ± 2.3 %, with the experiment performed in replicates. The effect of biomass loading on the gravimetric yield of DO lignin residue was also tested, with the same volume of solvent used for twice the biomass loading. The results showed that the gravimetric yield remained similar (19.4 %), suggesting that a higher biomass loading does not impact the gravimetric yield.

DO treatments were also performed on SCG-HW and SCG-EF to study the feasibility of extracting lignins from SCG. For clarity, the precipitated DO residue from SCG-HW and SCG-EF are called DO-SCG-HW and DO-SCG-EF, respectively. The aqueous supernatant from the DO treatments were collected from the respective SCG residues and are called DS-SCG-raw, DS-SCG-EF and DS-SCG-HW. Interestingly, the proposed dioxane degradation products that were observed when CSE was performed using dioxane-water mixture as the solvents were not observed in these supernatants. This could be owing to the diluted nature of the supernatant and because of all residues were washed many times. In addition, the fraction of the SCG-raw, SCG-EF, and SCG-HW, insoluble during the DO treatment were collected during the filtration and are labelled as DP-SCG-raw, DP-SCG-EF, and DP-SCG-HW, respectively, as it is assumed that this fraction mainly consists of polysaccharides. The DO residue yields for SCG-raw, SCG-EF and SCG-HW were 20.4 ± 1.8 %, 6.0 ± 1.4 % and 10.1 %, respectively. The lipid composition, protein content, KL, and structural studies (NMR and thioacidolysis) were performed on these fractions. The results of the various analyses on these fractions are presented in the following subsections.

4.3.2 NaOH treatment

In the second approach, NaOH treatments were performed on owing to its capacity to solubilize lignins. The process usually results in the degradation of β -O-4 linkages and the improved solubility of the phenolic groups owing to the high pH. In addition, this approach has the added advantage of saponifying the lipids, thereby making the removal easier (19). In this study, two separate NaOH treatments (NT) were performed and compared to DO treatment results. NaOH treatment (1 M) at room temperature (NRT) for 24 h, and NaOH

treatment (1 M) at high temperature (95 °C) for 3 h (NHT). The high temperature and molarity ensure that saponification does not hinder the yield of the treatment. Interestingly, this treatment made the filtration of the precipitated residue quite complicated, owing to its fine size. The precipitated lignin residues from SCG-raw at room temperature and high temperature soda treatments are labelled as NRT-SCG-raw and NHT-SCG-raw. NHT was also performed on SCG-HW, which resulted in lignin residues labelled NHT-SCG-HW. The yields of NRT-SCG-raw, NHT-SCG-raw, and NHT-SCG-HW, were 13.2 ± 1.9 %, 13.8 % and 16.6 %, respectively.

4.4 Characterization of lignin separation treatments

Owing to the interconnectedness of the aim of the two lignin separation treatments, the results of the various characterizations are presented together for the sake of clarity and ease.

4.4.1 Protein fraction

Although the protein quantification was not performed for all the studied fraction, the LC/MS analysis of the aqueous supernatants (DS, NRS, and NHS) from the NT and DO treatments showed features associated with amino acids, which was also shown by GC-MS after silylation. Although the quantification was not performed due to technical constraints, the presence of amino acids in the aqueous fraction is expected in both treatments. Furthermore, SCG-raw, SCG-EF, and SCG-HW contained a small fraction of proteins (Table 3). The results of the colorimetric protein assay are reflected in the amino acid profile from the LC/MS data of DO-residues, with DO-SCG-raw and DO-SCG-HW clustered together, while DO-SCG-EF shows similarity except in terms of intensity. In the future, a well-defined approach must be developed to quantify the proteins since the methods used in this study and the literature has several disadvantages. However, this was beyond the scope of this work.

4.4.2 Thioacidolysis and ^{31}P NMR results

The results of the thioacidolysis for the DO and NT precipitated residues are given in Table 14.

Table 14. Yield of guaiacyl (G) and syringyl (S) monomers released during thioacidolysis of the precipitated residues

Sample	G units ($\mu\text{mol/g}$)	S units ($\mu\text{mol/g}$)	Total (G+S) ($\mu\text{mol/g}$)	S/G
DO-SCG-raw	0.5 ± 0	0.2 ± 0	0.7 ± 0	0.3 ± 0
DO-SCG-EF	3.5 ± 0.1	1.0 ± 0	4.5 ± 0.1	0.3 ± 0
DO-SCG-HW	1.4 ± 0.1	0.4 ± 0	1.7 ± 0.1	0.3 ± 0
NRT-SCG-raw	0.2 ± 0	0.1 ± 0	0.3 ± 0	0.3 ± 0
NHT-SCG-raw	0.1 ± 0	0.0 ± 0	0.1 ± 0	0.3 ± 0.1
NHT-SCG-HW	0.4 ± 0	0.1 ± 0	0.5 ± 0	0.3 ± 0

As observed in Table 14, the total G and S units in the residues are low, compared to lignin samples observed in Lapierre et al (92). DO-SCG-EF contained the highest quantity of G and S units among the studied residues. Compared to feedstocks, the total monomeric units reduce for DO-SCG-raw, and in NaOH treatments. The opposite was observed for DO-SCG-EF and DO-SCG-HF. However, the S/G ratio remains constant in all residues. The low G and S monomeric units of these samples are more comparable to the suberized tissue studied by Lapierre et al. than lignins. This could be owing to the condensed nature of the DO and NT residues.

The ^{31}P NMR studies also showed very low quantities of phenolic OH groups associated with lignins (Table 15).

Table 15. ^{31}P NMR spectroscopic analysis of the DO and NT residues after derivatisation in pyridine/ CDCl_3

Sample	mmol of OH/g sample tested			Solubility
	Aliphatic OH	Phenolic OH	Carboxylic acid OH	
DO-SCG-raw	1.2	0.9	1.1	Good
DO-SCG-EF	1.1	0.5	0.5	Good
DO-SCG-HW	2.2	1.4	0.4	Good
NHT-SCG-raw	1.5	1.3	0.7	Good
NHT-SCG-HW	0.6	0.4	2.1	Good

Although the solubility of the residues improved in pyridine/ CDCl_3 mixture, the complete solubilization was still not observed. This coupled with the low quantities of the observed OH groups suggest that the precipitated residues likely had a very condensed structure or that it was not lignins. The ^{31}P NMR results are quantified in the units of mmol OH groups per gram of initial sample tested. However, because the residues are not completely soluble, these values can only be used for comparison between the studied samples.

4.4.3 Klason content

The KL of the various residues are summarized in Table 16. The results are arranged based on the feedstock. In Table 16, the precipitated residues from dioxane treatment, NaOH treatment at room temperature and $95\text{ }^\circ\text{C}$ are given as DO, NRT, and NHT, and the insoluble residues collected in the filter are given by DP, NRP, and NHP, respectively.

Table 16. Ash-free Klason content of precipitated residues

Feedstock	Klason content (KL) %						
	DO	DP	NRT	NRP	NHT	NHP	NHT washed*
SCG-raw	88.1 ± 5.0	18.6 ± 0.5	43.4 ± 0.4	24.9 ± 1.5	36.3	18.6	80.3
SCG-EF	81.95 ± 2.0	21.9 ± 0.4	NA	NA	NA	NA	NA
SCG-HW	84.3 ± 1.3	21.1 ± 0.5	NA	NA	80.3	12.5 ± 0.5	NA

* washed with water exhaustively; NA: not available

During the visual inspection of NRT-SCG-raw and NHT-SCG-raw, large amounts of salts were observed. This was further confirmed by the ash content of NHT-SCG-raw, with inorganic compounds accounting for approximately 58.8 % of the sample. The KL of the NHT-SCG-raw before and after exhaustive washing with water showed a significant difference, suggesting that intensive washing is necessary for NaOH treatments. Therefore, during the production of NHT-SCG-HW, intensive washing was performed. Therefore, it is assumed that even though the KL of NRT-SCG-raw is 43.4 %, this is an underestimation as further washing and KL analysis was not performed on the washed fraction.

The DO residues showed relatively higher KL than NaOH treatment residues. However, among the insoluble fractions (DP, NRP and NHP), the lowest KL was observed in the case of NHP-SCG-HW, suggesting that most of the acid insoluble content was removed during NHT. However, DP residues have similar KL irrespective of the feedstock. In general, the results of the KL analysis show that the precipitated residues from both lignin separation treatments showed high value of KL. This is usually reported in the literature as the lignin content (19,54). The combination of KL and thioacidolysis results suggest that obtained residue could be condensed lignin or pseudo lignin, i.e., something that behaves like lignin (acid insoluble residue). This behavior makes the chemical valorization difficult owing to lack of reactive β -O-4 bonds. Therefore, the valorization of these fractions might require similar approaches to technical lignins such as the production of nanoparticles or nanocomposites (102).

4.4.4 Lipid fraction

The fatty acid composition of SCG-raw, SCG-EF, SCG-HW, and its DO precipitated residues are presented in Table 17. As shown in Table 17, the choice of feedstock had an impact on the gravimetric yield of precipitated residue after DO treatment. Interestingly, the DO residue yield increased with the estimated fatty acid content of the feedstock. Furthermore, the fatty acid content seems to contribute to the KL of these samples as shown in Table 16, although a relation does not seem proportional.

Table 17. Average fatty acid content of DO residues determined by transmethylation followed by GC-MS

Feedstock	Average fatty acids of sample (mg/g DW)	Average fatty acids of DO residue mg/g DW	Gravimetric yield of DO residues (%)
SCG-raw	149.8 \pm 10.3	539.6 \pm 31.2	20.4 \pm 1.8
SCG-EF	18.6 \pm 3.2	367.8 \pm 28.5	6.0 \pm 1.4
SCG-HW	31.8 \pm 2.8	359.3 \pm 3.9	10.1

The results of Table 17 show that a large fraction of these residues consists of fatty acids. Furthermore, the gravimetric mass balance comparing the total fatty acid content of the feedstock and the DO residues (considering the yields) shows that more fatty acids are present in the DO residues, compared to the original feedstock.

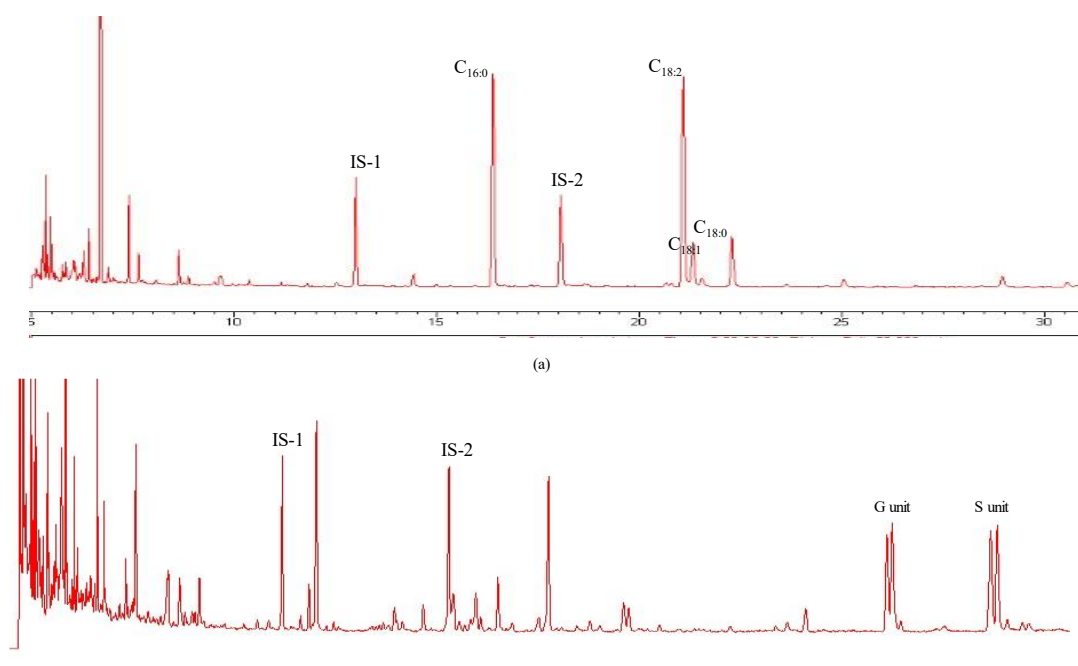


Figure 13. Thioacidolysis-GC-MS chromatogram of (a) DO-SCG-EF and (b) Alkaline lignin from Green-Value

In addition, the thioacidolysis chromatograms of DO residues suggest that some fatty acids were released during thioacidolysis. An example of the DO residue chromatogram after thioacidolysis and silylation is presented along with a lignin chromatogram in Figure 13.

Normally, the thioacidolysis chromatogram of a lignin residue does not consist of lipid peaks, as shown by the alkaline lignin from GreenValue (Figure 13 b). However, in the case of DO-SCG-EF, no visible characteristic isomeric peaks associated with G and S units are observed. The presence of these units was only confirmed with the quantification of ions specific to G and S units. This is interesting, especially in the case of DO-SCG-EF because SCG-EF had the lowest quantity of lipids among the feedstocks. In addition, DO-SCG-EF was exhaustively washed (3 cycles with 40 mL hexane on 150 mg sample), to remove any free lipids and then analyzed using 2D HSQC NMR (Figure 14). The washed DO-SCG-EF had a total fatty acid content of approximately 4.7 ± 0.2 mg/g sample (0.5 %). Some of the peaks can be attributed to lipids (red) even if the residue was extensively washed. The presence of spots in the region marked in green can be attributed whether to sugars or glycerol, but not really to the lignins substructures observed normally. Moreover, the high definition of aromatic spots (blue circle), along with the absence of dominating methoxy groups (green circle) doesn't confirm that this sample is made of lignin. The combination of these observations leads to the hypothesis that the aliphatic fatty acid chains are covalently bound to some aromatic and aliphatic moieties.

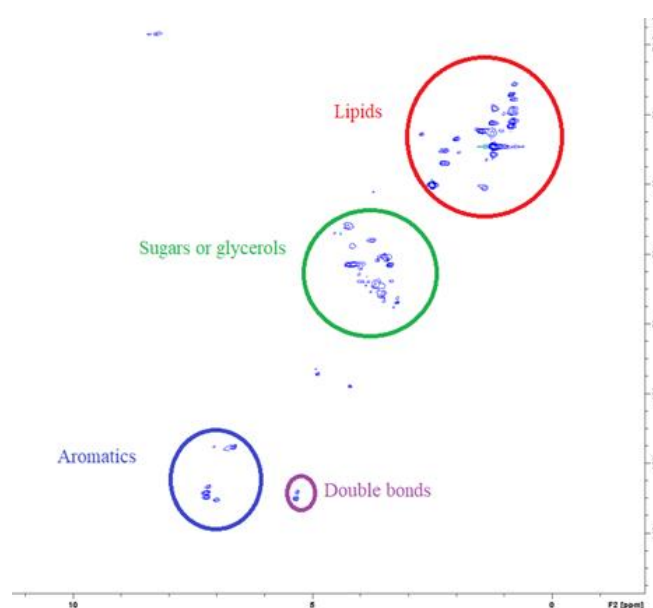


Figure 14. 2D HSQC spectra of hexane washed DO-SCG-EF; spectrum recorded in DMSO-d₆

Similarly, the average fatty acid content of NT residues and gravimetric yields are presented in Table 18. Average fatty acid content of NT residues determined by transmethylation followed by GC-MS. However, no direct inferences can be derived from these samples except to show its low lipid content. This is expected as NaOH treatment can result in the saponification of lipids to form fatty acids (in acidified medium).

Table 18. Average fatty acid content of NT residues determined by transmethylation followed by GC-MS

Sample	Average fatty acids of NT residue mg/g DW	Gravimetric yield of residues (%)
NRT-SCG-raw	88.1 ± 3.9	13.2 ± 1.9
NHT-SCG-raw	NA	13.8
NHT-SCG-HW	50.4 ± 0.6	16.6

NA: not available.

4.4.5 Sugar content

Owing to technical constraints, only the sugar composition of some treatment insoluble residues (DP and NRP) were quantified. The results are presented in Table 19. Furthermore, the aqueous supernatants of these treatments were analyzed using GC/MS metabolomic analyses after silylation.

Table 19. Polysaccharide composition of insoluble treatment residues determined by hydrolysis using (i) TFA (ii) 72 % H₂SO₄ and 5 % H₂SO₄ and quantification using ionic LC and silylation-GC-MS

Sample	Hemicellulose (wt. %)					Cellulose (wt. %)		
	Mannose	Galactose	Arabinose	Glucose	Xylose	Fructose	Glucose	Total
DP-SCG-raw	42.6 ± 2.3	5.7 ± 0.4	0 ± 0	2.4 ± 0.2	0.2 ± 0	0 ± 0	3.1 ± 1.0	53.9 ± 2.2
DP-SCG-EF	39.8 ± 8.8	10.2 ± 2.2	0.3 ± 0.2	3.3 ± 0.7	0.2 ± 0	0 ± 0	3.6 ± 2.0	57.5 ± 13.9
DP-SCG-HW	35.7 ± 5.9	10.5 ± 1.9	0.7 ± 0.3	2.4 ± 0.4	0.2 ± 0	0 ± 0	2.6 ± 0.5	52.0 ± 9.0
NRP-SCG-raw	18.3 ± 2.0	8.1 ± 0.6	1.3 ± 0	1.4 ± 0.4	0.2 ± 0	0 ± 0	1.7 ± 0.8	30.9 ± 3.5

When compared to the feedstocks, the DP-SCG-raw shows higher sugar content. The sugar content of SCG-raw was estimated to be 42.6 ± 5.5 % and increased to 53.9 ± 2.2 %. However, the DO treatment did not show the same response in the cases of SCG-EF and SCG-HW, as the sugar content showed a decrease. This could also indirectly suggest the underestimation of the SCG-raw sugar content. In addition, NRP-SCG-raw shows the lowest sugar content among the samples. Furthermore, compared to the feedstocks, the composition of various monosaccharides varies in these insoluble residues. This suggests that the lignin separation treatment hydrolyzes the various hemicelluloses present in the feedstocks. Compared to the feedstock (Table 4), the galactose and arabinose underwent significant hydrolysis. In the case of DP-SCG-raw (42.6 %), the mannose content was nearly double that of its feedstock, SCG-raw (24.3 %). In addition, the metabolomic GC/MS (Annex 1) confirms the hydrolysis of hemicelluloses with high concentrations of galactose, mannose and arabinose in DS-SCG-raw, DS-SCG-HF and NHT-SCG-raw, which were the aqueous supernatant from the corresponding treatments that were analyzed.

4.4.6 SEC results

The size distribution of the different residues and their solubilities in THF are presented in Figure 15. All the solid residues showed poor solubility in THF. However, the size distribution of the partially dissolved fractions is presented. However, the peak at 17.8 min should be disregarded as it was confirmed as a contaminant using a blank. The overall profiles of NRT and NHT residues show poor solubility and very few peaks, compared to DO treatment.

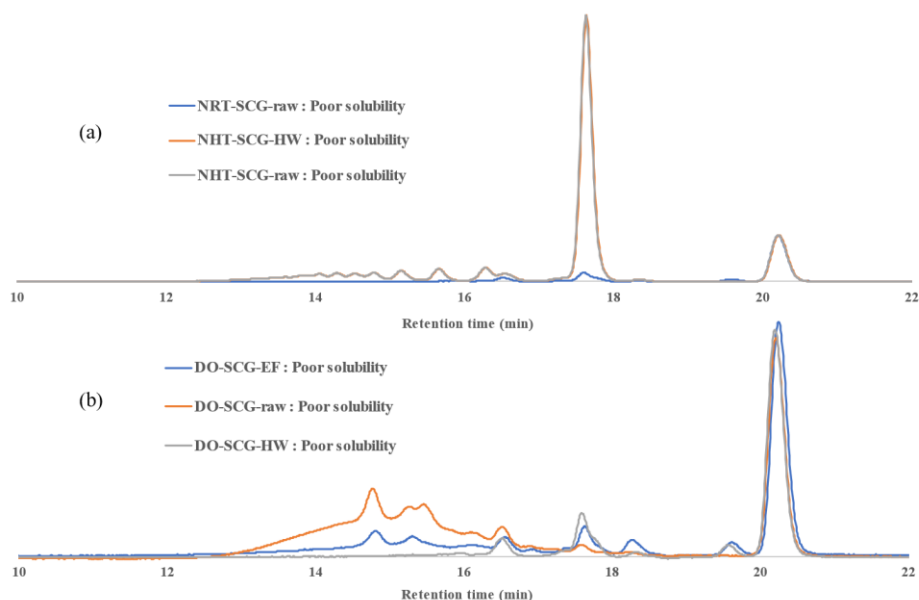


Figure 15. SEC chromatogram of residues; analysis in THF with UV detection at 280 nm, normalized on toluene peak

The overall profile of DO-SCG-raw and DO-SCG-EF were very similar. Compared to other DO residues, the profile between 13 min and 16 min was distinct for DO-SCG-HW. However, the poor solubility of all samples studied suggests that the residues are very condensed although the size distribution varied with the choice of treatment. Indeed, the profiles seem more polymeric after dioxane/HCl treatment than after NaOH treatment. In cases of dioxane/HCl treatment, the molar mass distribution seems to depend on substrate and going this order: SCG-raw > SCG-EF > SCG-HW.

4.4.7 Overall conclusions of the lignin separation treatments

Because hemicelluloses account for a large fraction of SCG, and the aqueous supernatants contain dioxane or salts, in the cases of dioxane and NaOH treatments, respectively, the valorization of these hemicelluloses are not practical. In addition, the insoluble residues would require further hydrolysis, as shown by the compositional data, which suggests that approximately 50 % of the insoluble residues are still polysaccharides. Therefore, these lignin separation treatments may not be ideal for the fractionation of SCG. Moreover, the precipitated residues obtained through lignin separation methods seem condensed (low quantities of G and S units, and phenolic OH) and chemically bound to fatty acid chains. Therefore, these residues could be considered as condensed lignins either as pseudo-lignins. The combination of the bound fatty acids and the low quantity of G and S units of the various residues suggests similarities towards suberized tissues. However, no fatty acids characteristic of suberin (dicarboxylic or hydroxyl fatty acids) were observed during the fatty acid quantifications performed in this study (103). Therefore, only hypotheses can be made towards what the precipitated residues are. These residues could be the result of the complex pyrolytic reactions that happen during the roasting process. However, this would require the comparison of the residues obtained before and after roasting. In this case, the removal of the layer, commonly called coffee silverskin, from the unroasted coffee beans would also be necessary as the lignin content associated with this fraction differs from that of coffee beans (41).

4.5 Fractionation strategy for a SCG-based biorefinery

Based on the results of this study, several fractionation strategies can be proposed. The main conclusions drawn from the lignin separation treatments is that such approaches are not suitable for an SCG-based biorefinery. Furthermore, owing to the condensed nature of the lignin-like residue, technical lignin valorization strategies such as the production of lignin nanoparticles or colloidal lignin particles might be more suited for this residue than uses as an intermediate or primary chemical feedstock. Therefore, a hemicellulose-based approach coupled with lipid valorization might be better suited for SCG-raw. In this regard, the results of ASE experiments suggest an intriguing strategy towards an SCG-based biorefinery.

4.5.1 SCG-based biorefinery using ASE systems

An interesting strategy to consider would be an ASE based biorefinery system consisting of sequential solvent extraction followed by subcritical water treatment. This approach simply put would consist of the sequential extractions using hexane, methanol and water, respectively. This was based on the three stage extraction studies that were performed as part of this study, coupled with the ASE results. A representation of this process is given in Figure 16. Because temperature did not seem to impact the overall lipid yield using hexane during ASE extraction, the room temperature extraction of SCG would be considered the best for the recovery of the lipid fraction. SCG lipids have long fatty acid chains ($>C_{16}$) and large fraction of unsaturated fatty acids (~50 %), which can find applications in food and cosmetic formulations (73). Furthermore, based on the measured antioxidant activity of the methanol extractives from ASE studies, the maximum antioxidant activity was observed at 60 °C. Therefore, the second stage methanol extraction must be performed at 60 °C to maximize the antioxidant activity. Even though it is possible that some molecules capable of antioxidant activity can be extracted using hexane, this was not confirmed in this study owing to time constraints. Therefore, this must be considered in the future works. Furthermore, another consideration for future studies is the replacement of methanol with ethanol since the NMR profiles for these two samples are similar and ethanol is considered greener according to McElroy et al (94). However, the antioxidant capacity of ethanol extractives was not studied in this study owing to time constraints.

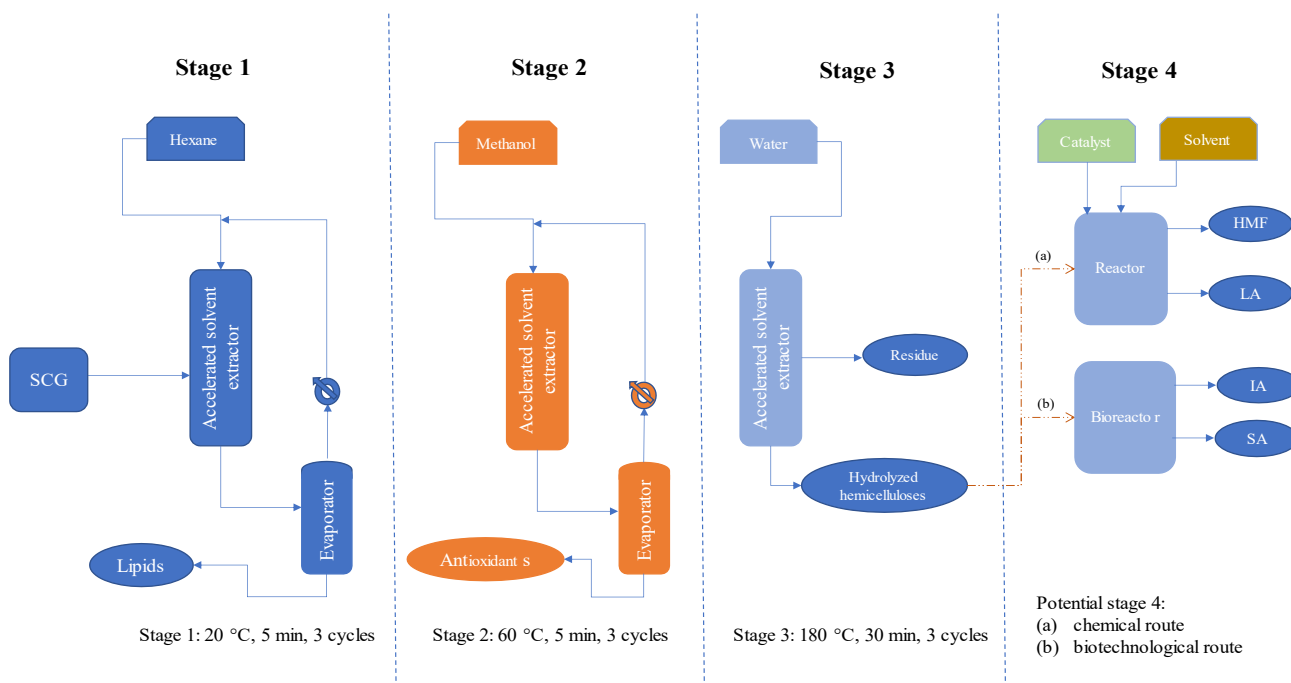


Figure 16. ASE-based SCG fractionation strategy. HMF: hydroxymethylfurfural, LA: Levulinic acid, IA: Itaconic acid, and SA: Succinic acid

In the next stage, subcritical water treatment is performed to hydrolyze the hemicelluloses. Although the optimization of this treatment is left for future work, results from the literature predicts good hydrolyzability (40,79). Furthermore, the removal of extractives in the prior steps should improve the hydrolysis of hemicel-

luloses. In addition, the sugar content of SCG-raw suggests that the main monosaccharides in this polysaccharide fraction would be mannose and galactose, which are both hexoses that can be converted into platform chemicals such as levulinic acid (LA) or 5-hydroxymethylfurfural (5-HMF) through chemical processes (55). Several authors have reported the use of biphasic systems and other catalytic systems towards the production of 5-HMF and LA from sugar hydrolysates at high temperatures (greater than 150 °C) (104,105). As an alternative, owing to the prior removal of extractives, this hydrolysate could also be used to produce platform chemicals such as itaconic acid (IA) or succinic acid (SA). However, these processes would require further studies (106–108). The feasibility of these steps must be further studied for an SCG-based biorefinery. Moreover, the solid residue from the subcritical water treatment will likely be useful for confirming the structural abnormalities of the lignin separation residues as the polysaccharides and lipids could be further removed during this fractionation strategy. However, owing to technical constraints, these steps were not pursued.

5. Conclusions

In this study, a fractionation strategy for the valorization of spent coffee grounds was proposed based on the study of extraction methods (CSE, UAE, and ASE) and lignin separation treatments such as dioxane organosolv (DO) and NaOH treatments (NT). The composition of the raw SCG, SCG-raw (41.1 % hemicelluloses, 16.2 % lipids, 1.5 % cellulose, 1 % moisture, 1 % ash, 0.4 % proteins, and 21.1 % Klason insoluble residues), water-ethanol Soxhlet washed residue, SCG-EF (58.2 % hemicelluloses, 3.3 % cellulose, 21.1 % Klason insoluble residues, 0.2 % proteins, 1.9 % lipids, 5.4 % moisture and 1 % ash), and hexane-Soxhlet washed residue SCG-HW (58.4 % hemicelluloses, 1.9 % cellulose, 22 % Klason insoluble residue, 1 % ash, 0.4 % proteins, and 3.2 % lipids) were quantified. The solubility of the extractable fraction of SCG-raw was studied using an array of solvents in CSE. The highest extraction yield was observed in the case of hexane (16.6 %). UAE was then compared with CSE, showing higher yields for methanol and acetone, while lesser yields for hexane and ethanol. Then, the effect of temperature (20 °C to 125 °C) on the extractable fraction was studied using ASE, with the parameters optimized keeping hexane as solvent. Hexane did not show a significant difference in the gravimetric yield with temperature (12 % to 12.8 %). The gravimetric yield of polar solvents such as methanol, methanol-water (7:3), water and ethanol doubled, compared to 20 °C, with the rise of temperature to 125 °C. The antioxidant capacity of the extractives was selectively tested, with methanol extractives (60 °C) showing the lowest EC₅₀ value (0.45 mg/mmol DPPH). In addition, an ASE-based subcritical water extraction (180 °C) resulted in the hydrolysis and extraction of hemicelluloses, which can be an approach towards valorization.

Furthermore, the non-polysaccharide fraction was also studied using known lignin separation methods such as DO and NT to fill the knowledge lacking in the literature. The residue, characterized through various analyses, do not behave like lignins, and therefore could be condensed lignins or pseudo lignins (acid insoluble residue which are not lignin). For instance, the precipitated residues showed very low G and S monomer concentrations compared to lignins from other sources. The results also suggest aromatic moieties covalently bound to fatty acids. The results of these experiments were used to propose an ASE-based SCG fractionation strategy, consisting of sequential extraction of lipids using hexane, antioxidants using methanol and hemicelluloses using subcritical water. The potential valorization strategy for individual fractions are also discussed.

6. Perspectives

Although the proposed strategy is based on the results of various experiments, this does not necessitate that it will be economically or environmentally acceptable. Therefore, a technoeconomic analysis and a life cycle assessment must be carried out on the proposed system. The boundaries for this analysis could be based on whether the SCG-based biorefinery is part of the coffee industry or if it is a separate enterprise. The proposed strategies should be considered more as a starting point for further studies than as the final proposals. Owing to a lack of time, the modelling and simulation of such SCG-based biorefinery was not considered. Furthermore, the heterogeneity of SCG owing to the type of brewing, degree of roasting, and green coffee bean preparation makes its valorization quite a challenge. The pyrolytic processes involved results in degradation products such as melanoidins and peptides (109). These products affect the compositional measurements and will vary with coffee processing and consuming parameters (87,88). Therefore, the impact of roasting and brewing on the fractionation strategy must be studied to possibly modulate the properties of different fractions. Although this would require the study of several parameters, it could significantly enhance the technoeconomic feasibility of an SCG-based biorefinery.

The initial plan of this work included the valorization of lignins obtained from SCG based on the literature review. However, the residues obtained through lignin separation strategies do not behave like lignins found in other food residues such as wheat straw (92). To completely understand the structure of these residues, further 2D NMR and ³¹P NMR studies using standard model compounds (sugars, phenols bound to fatty acids, and glycerol to name a few) should be performed.

Owing to a shortage of time and other technical constraints, several characterizations were not performed. Specifically, the quantification of chlorogenic acids, caffeine and other polyphenols in both the feedstocks, extractives and residues must be performed to propose an optimized fractionation strategy. In addition, the analysis of the total phenolic content and other antioxidant assays must also be performed to complement the results of the DPPH assay.

Although two assistive extraction methods (ASE & UAE) were studied for different solvents, the use of microwave assisted extraction (MAE) was not tested owing to a shortage of time. However, this would be a good approach for polar solvents such as methanol, water, and ethanol for enhanced extraction (110). Several studies have shown that MAE can significantly improve the extraction yield when used over very short time scale (min). Therefore, a comparison of MAE, UAE, and ASE to conventional extraction would be interesting in the view of a SCG-based biorefinery. The choice of solvents is another aspect that could be improved upon, for example, the replacement of hexane, with a greener alternative such as 2-methyltetrahydrofuran or supercritical fluids must be considered (39,111). Moreover, the potential applications of SCG lipids for food and cosmetic applications should be studied.

In addition, the hydrolysis and valorization of hemicelluloses must be further studied to propose more strategies towards an SCG-based biorefinery. The optimization of the subcritical water treatment towards the hydrolysis of hemicelluloses must also be studied. However, there is still more to be known, and should be studied by maximizing the separation of hemicelluloses and lipids, and further experimentation.

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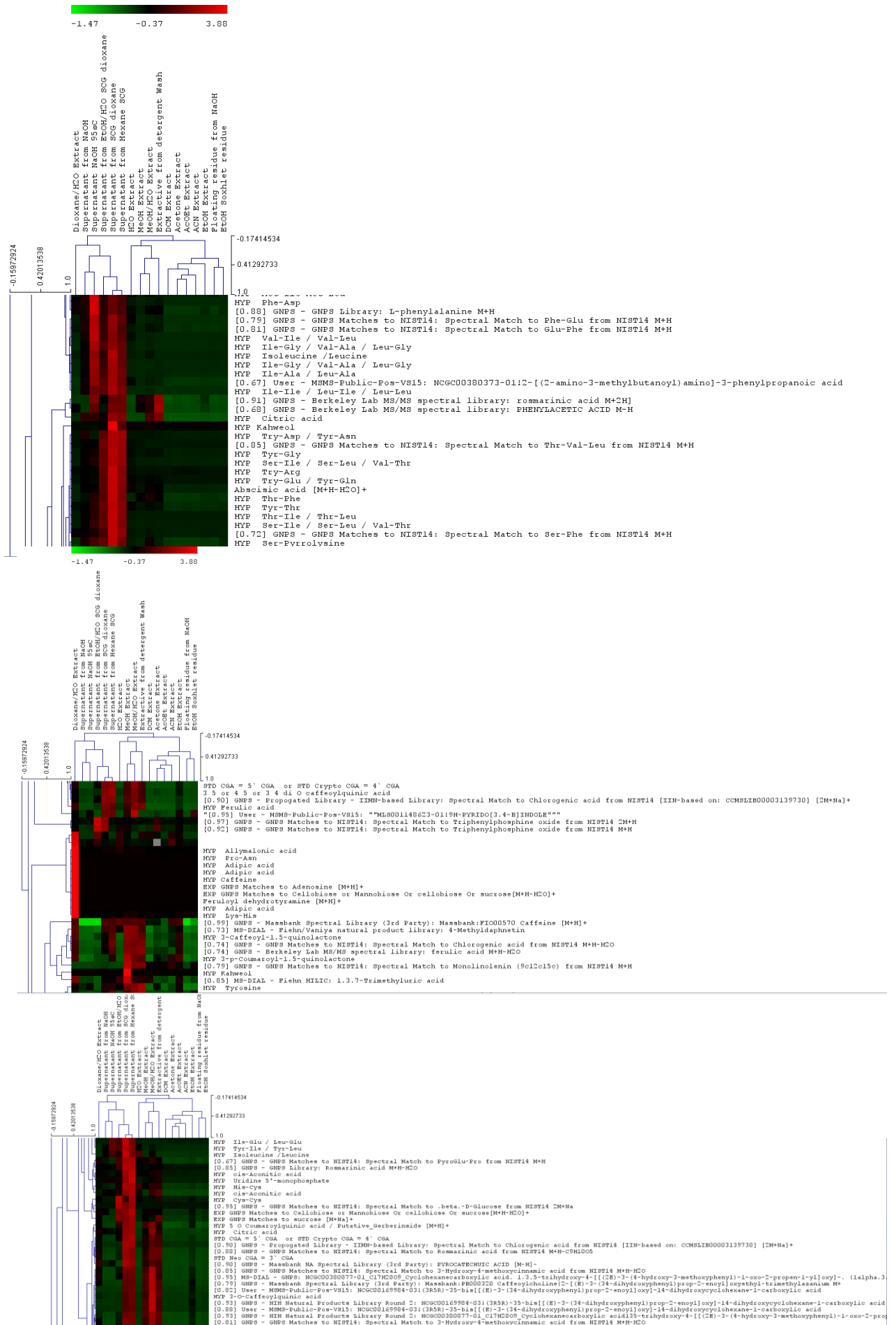
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Annex

LC-MS features



GC metabolomic analysis clusters

