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Multistep Extraction Transformation of Spent Coffee Grounds to the Cellulose-Based Enzyme Immobilization Carrier

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Abstract: The present study investigated the possibility of spent coffee ground (SCG) transformation to a cellulose-based enzyme immobilization carrier using a multistep extraction procedure. In the first step, SCGs were extracted with *n*-hexane by Soxhlet extraction in order to obtain SCG oil, while the remaining solid residue was subjected to continuous solvent flow sequential subcritical extraction with 96% and 50% ethanol and water. Afterwards, the obtained solid residue was subjected to alkaline liquefaction with 8% NaOH in order to remove lignin and produce cellulose-enriched SCGs as a potential enzyme immobilization carrier. Multistep extraction transformation of SCGs was monitored by chemical analysis of extracts and obtained solid residues. Soxhlet extraction of 100 g of SCGs yielded 10.58 g of SCG oil rich in linoleic and palmitic acid, while continuous solvent flow sequential subcritical extraction of 100 g of defatted SCGs yielded a total of 1.63 g of proteins, 5.58 g of sugars, 204 mg of caffeine, 76 mg of chlorogenic acid, and 11.97 mg of 5-(hydroxymethyl)furfural. Alkaline liquefaction of 100 g of sequentially extracted defatted SCGs by 8% NaOH yielded 7.45 g of proteins, 8.63 g of total polyphenols, 50.73 g of sugars, and 20.83 g of cellulose-enriched SCGs. Based on the characteristics of cellulose-enriched SCGs including a volume-weighted mean particle size of 277 µm, relative narrow particle size distribution with a span value of 1.484, water holding capacity of 7.55 mL/g, and a lack of carrier leakage, it could be safely concluded that produced cellulose-enriched SCGs fulfills criteria to be used as potential enzyme immobilization carrier. Overall, it seems that the proposed multistep extraction transformation of SCGs has great potential to be used for the production of several high-value added products.

Keywords: spent coffee grounds; Soxhlet extraction; continuous solvent flow sequential subcritical extraction; alkaline liquefaction; cellulose-based enzyme immobilization carrier

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1. Introduction

There is nothing better than to start the morning waking up with a joyful cup of refreshing and stimulating coffee, at least for coffee drinkers. However, the preparation of this beverage in the form of espresso and/or Turkish coffee, whether it is consumed at home, or in a local coffee shop or restaurant, generates a great amount of solid residue known as spent coffee grounds (SCGs) obtained after the treatment of coffee powder with hot water or steam [1–6]. This is even more evident if one considers that for the preparation of 1 kg of soluble coffee 2 kg of wet SCGs are produced, and the fact that 650 kg of spent coffee grounds are generated from one ton of green coffee beans turned into a coffee beverage [4,7]. In addition, it should be pointed out that consumption of various types

of instant coffee produced by coffee industries, results in an additional amount of SCG generated in the coffee industry [1,4].

The latest available data on worldwide green coffee production reported by the Food Agricultural Organization (FAO) [8] and the International Coffee Organization (ICO) [9] shows that 10,712,040 tons of green coffee was produced in 2022, with Europe the leading consumer. Based on the report on annual green coffee production and fact that 650 kg of spent coffee grounds are generated from one ton of green coffee beans [5] the amount of generated SCGs in 2022 could be estimated to be 6,962,826 tons. Unfortunately, even today, the majority of generated SCGs end up on landfills being highly polluting due to significant amounts of organic substances demanding a great quantity of oxygen for decomposition [2], as well as due to the fact its degradation products may produce an odor and harborage for insects [7].

However, if one considers the chemical composition of SCGs, as reported by Ballesteros and coworkers [1], it is more than evident that SCGs presents a resourceful material for the production of a multitude of valuable products. This was recognized by the scientific community, where numerous scientific groups investigated the possibility of SCG transformation to value added products. This included the possibility of the production of polyphenols and antioxidants for the functionalization of food products and development of new cosmetic and pharmaceutical products [2,4,10–20], biodiesel production [21], as well as development of the concept of biorefinery with the production of solid fuel, biodiesel, biogas, bioethanol, and biopolymers [22–30]. While the aforementioned clearly evidenced the versatility of SCGs for the production of numerous products, according to our knowledge, there are no data in the available literature on the possibility of SCG use for the production of cellulose-based carriers for enzyme immobilization.

In this respect, the present study investigated the possibility of the production of SCG-derived cellulose-enriched enzyme immobilization carrier using multistep extraction transformation, oriented toward valorization of each of extract obtained. The extraction started with well-known recovery of SCG oil from spent coffee grounds by *n*-hexane Soxhlet extraction [21], and continued by subcritical extraction of defatted SCG residue. Subcritical extraction has been selected over conventional extraction techniques as an innovative, selective, and sustainable alternative for the isolation of valuable compounds from spent coffee grounds. It uses environmentally friendly solvents, moderate temperatures and pressures, preserving the integrity and functionality of delicate compounds such as polyphenols, proteins and other bioactive ingredients present in SCGs [6,13,19,31–35]. While the possibility of the use of subcritical extraction in SCG transformation is well established, it should be pointed out that SCG treatment by subcritical extraction so far, has been performed only by single solvent (water or ethanol) [6,15,19,32,34,36]. The novelty of the current research presented is the application of continuous solvent flow sequential subcritical extraction of defatted SCGs by consecutive use of 96% and 50% ethanol and water as extraction solvents. The production of an SCG-derived cellulose-enriched enzyme immobilization carrier continued through a well-known procedure for lignin removal, alkaline hydrolysis [25,29,37], where sequentially extracted defatted SCGs were examined for the possibility of complete lignin removal by the use of 2, 4, 6, and 8% NaOH, and the suitability of the obtained solid residue as potential enzyme immobilization carrier monitored by leakage. Finally, the obtained SCG-derived cellulose-based immobilization carrier was confirmed to be suitable for enzyme immobilization by analysis of particle size distribution.

Based on the obtained results, it can be safely concluded that the currently proposed multistep extraction transformation of SCGs has great potential to be used for the production of several high-value added products, including an SCG-derived enzyme immobilization carrier. In addition, it seems to us that proposed SCG multistep extraction transformation presents an additional step toward sustainable SCG transformation approaching the "zero waste" model.

2. Materials and Methods

2.1. Material and Chemicals

Spent coffee grounds (SCGs) from the production of espresso coffee generously supplied by a local coffee shop located in the city of Osijek, Croatia, were dried in a thin layer of 1 cm thickness on aluminum plates during 24 h at 60 °C. Afterwards, the dried SCGs were kept in hermetically sealed plastic containers until further use.

Defatting dried SCGs was performed by *n*-hexane purchased from Acros Organics (Geel, Belgium), while continuous solvent flow sequential subcritical extraction by 96 and 50% ethanol obtained from Chem-Lab NV (Zedelgem, Belgium). Alkaline liquefaction of solid SCG residue was performed with aqueous solutions of sodium hydroxide (NaOH) prepared from NaOH pellets purchased from Grammol (Zagreb, Croatia). Supelco F.A.M.E. Mix (C4–C24) purchased from Sigma-Aldrich (Darmstadt, Germany) was used as a reference material for the determination of the SCG oil fatty acid profile. Ferulic, caffeic, and chlorogenic acid purchased from Sigma-Aldrich (Darmstadt, Germany) and caffeine from Acros Organics (Geel, Belgium) were used as authentic standards of phenolic compounds used for the construction of calibration curves (HPLC) and sample spiking. Cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate, ethylenediaminetetraacetic disodium salt, sodium borate, sodium phosphate dibasic, triethylene glycol, sodium sulfite, and heat-stable bacterial α-amylase (17,400 Liquefon Units/mL) purchased from ANKOM Technology (Macedon, NY, USA), and sulfuric acid 96% and acetone from Fisher scientific (Loughborough, UK) were used to determine the fiber content on an ANKOM^{2000} fiber analyzer (ANKOM Technology, Macedon, NY, USA). All other chemicals used in this research were of pro-analysis purity.

2.2. Determination of Chemical Composition of Spent Coffee Grounds and Spent Coffee Ground Derived Cellulose-Based Carrier

Spent coffee grounds and SCG-derived cellulose-based enzyme immobilization carriers were analyzed for dry matter, protein, fat, crude fiber, total polyphenol, and ash content. Dry matter content was determined by drying at 103 °C until constant mass, protein content by Kjeldahl method [38], while ash content by incineration at 550 °C for 4 h. Fat content was determined by Soxhlet extraction of 3 g of sample with 90 mL of *n*-hexane using a SoxROC SX-360 extractor (Opsis Liquidline, Furulund, Sweden). Crude fibers were analyzed by the ISO 6865:2000 method [39], while cellulose, hemicellulose, and lignin by the Van Soest et al. [40] method, described in Appendix A, using an ANKOM^{2000} fiber analyzer. The total polyphenol content in 80% methanolic extracts was determined using the Folin-Ciocalteu method as described by Matić et al. [41], with methanolic extracts prepared by ultrasound-assisted extraction in 80% methanol.

2.3. Multistep Extraction of Spent Coffee Grounds

Multistep extraction of dried spent coffee grounds included: (a) Soxhlet extraction with *n*-hexane in order to obtain SCG oil; (b) continuous solvent flow sequential subcritical extraction of defatted spent coffee grounds (DSCGs) with 96 and 50% ethanol and water for the extraction of phenolics, free sugars, and proteins; and (c) alkaline liquefaction of sequentially extracted defatted spent coffee grounds (SEDSCGs) for lignin removal (Figure 1). All this was performed in order to obtain a potential SCG-derived cellulose-based enzyme immobilization carrier.

SCG oil extraction was performed in a SoxROC SX-360 extractor (Opsis Liquidline, Furulund, Sweden) using *n*-hexane as the extraction solvent. The extraction conditions were as follows: SCG mass to *n*-hexane volume of 10 g/90 mL per thimble, extraction temperature of 155 °C (aluminum cups), and total extraction time of 80 min (30 min boiling, 40 min reducing/rinsing, 5 min solvent recovery, 5 min drying). Following the extraction, the obtained SCG oil was dried for 1 h at 103 °C and used to determine the fatty acid profile, while solid residue dried at 60 °C for 12 h was used for continuous solvent flow sequential subcritical extraction.

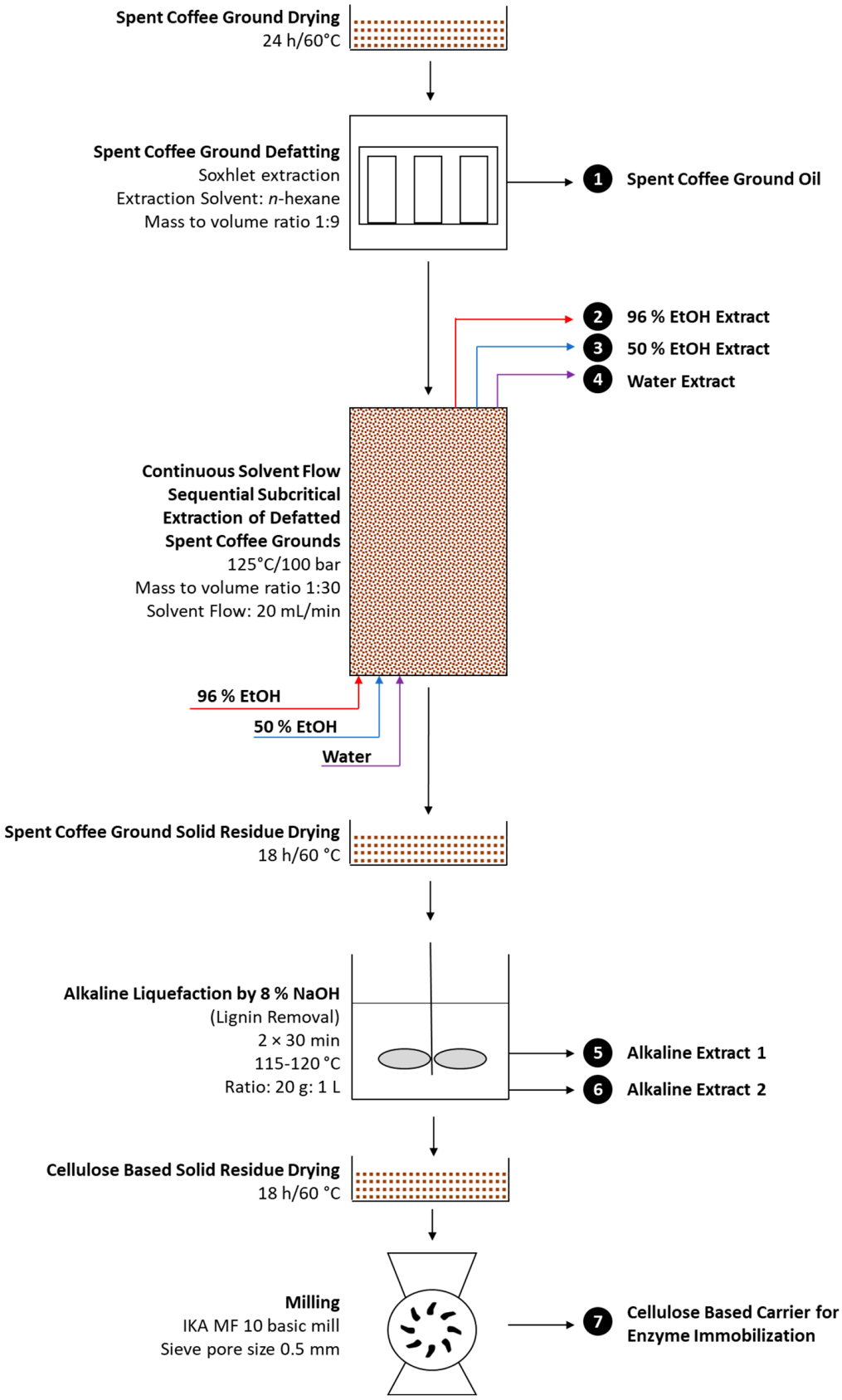

Figure 1. Schematic presentation of the performed transformation of spent coffee grounds to cellulose-based enzyme immobilization carrier and valuable compounds present in the obtained extracts.

Continuous solvent flow sequential subcritical extraction of DSCGs was performed in a designed subcritical system for continuous extraction in laboratory conditions shown in Figure 2. The system contained the following components: compressor, valves, extraction solvent tank, extractor, heating oven, pump, manometer, thermoregulated heating plate, cooling coil (bath), temperature sensor and temperature regulated controller, where the extractor and tubes were made of stainless steel (AISI 304). Sequential extraction included three different solvents of increasing polarity, 96% ethanol, 50% ethanol and water, where the sample mass and solvent volume ratio was 100 g: 3000 mL, solvent flow was 20 mL/min, pressure 100 bars, and extraction temperature 125 °C. In brief, continuous solvent flow sequential subcritical extraction of DSCGs started by filling the extractor with 100 g of DSCGs, followed by mounting the filter near the top of the extractor with Profissimo bags (DM-drogerie markt d.o.o., Croatia) in order to prevent leakage of DSCGs into the pipe. The extractor was then hermetically sealed, and heated solvent (~125 °C) was introduced from the bottom of extractor under an initial pressure of 50 bars. Upon reaching the desired pressure of 100 bars and a temperature of 125 °C in the extractor, continuous solvent flow extraction started with a solvent flow of 20 mL/min regulated by needle valve and the work of the pump. Once complete, the volume of the desired solvent (3000 mL) was used, new solvent of increased polarity was introduced into the extraction solvent tank and extraction continued. The obtained extracts of DSCGs (96% ethanolic, 50% ethanolic, and water extracts) were collected and analyzed for their chemical composition, while the remaining solid residue (SEDSCGs; sequentially extracted defatted spent coffee grounds) was dried at 60 °C for 18 h and used for analysis and/or alkaline liquefaction.

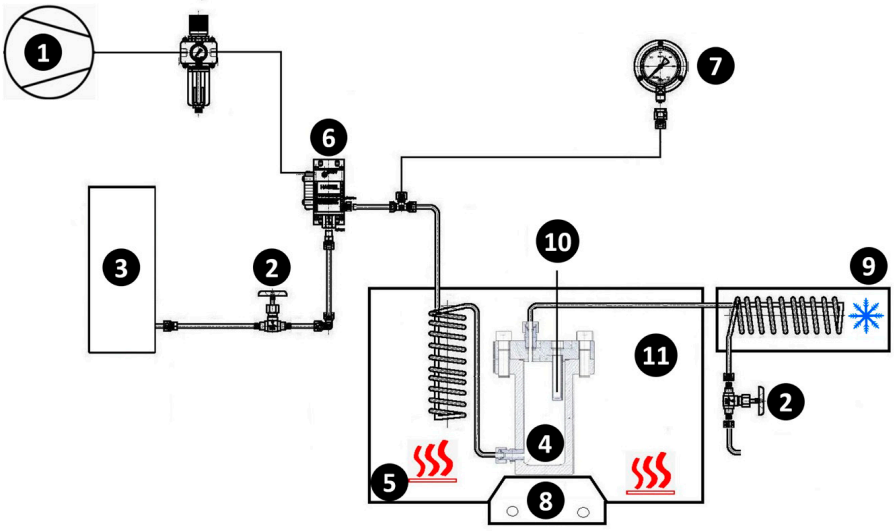

Figure 2. Schematic presentation of constructed continuous solvent flow subcritical extraction system. Legend: 1—compressor; 2—valves; 3—extraction solvent tank; 4—extractor; 5—heating oven; 6—pump; 7—manometer; 8—thermoregulated heating plate (with optional stirring); 9—cooling coil (bath); 10—temperature sensor; 11—temperature regulated controller.

Alkaline liquefaction of SEDSCGs (i.e., removal of lignin) was performed by a combination of methods from Pujol et al. [42], Girotto et al. [25], and Menezes et al. [43]. Briefly, alkaline liquefaction of SEDSCGs with NaOH aqueous solutions of 2, 4, 6, 8% w/v, and 1:50 solid–liquid ratio (g/mL) was performed 2 × 30 min in a stirred glass reactor with reflux at 115–130 °C. The obtained alkaline extracts were separated by vacuum filtration through filter paper Whatman 113 and examined for their chemical composition, while cellulose-based SCG residue (ALSEDSCGs; alkaline liquefied sequentially extracted defatted spent coffee grounds) was washed with distilled water until the pH of the filtrate reached pH = 7, followed by acetone washing, and subsequent drying at 60 °C for 18 h.

2.4. Chemical Analysis of Obtained Extracts

Extracts obtained after multistep extraction of spent coffee grounds (Figure 1) were analyzed for the chemical composition of targeted compounds, as follows: (a) SCG-derived oil was examined for its fatty acid profile; (b) 96% and 50% ethanolic and water extracts of DSCGs of continuous solvent flow sequential subcritical extraction for the protein, total polyphenol, sugar, and 5-(hydroxymethyl)furfural (HMF) content; while alkaline liquefaction extracts of SEDSCGs for the protein, total polyphenol, and sugar content. In addition, 96% and 50% ethanolic, and water extracts of DSCGs were analyzed by HPLC for the amount of dominant polyphenols, as well as for dry matter content (i.e., amount of extractives).

The fatty acid profile of SCG-derived oil was performed according to Ostojčić et al. [44]. Analysis of FAME (fatty acid methyl esters) was conducted on a Shimadzu GC-2010 Plus gas chromatography system equipped with a flame-ionization detector fitted with an SH-FameWax capillary GC column (30 m, 0.32 mm i.d., and 0.25 µm thick stationary phase). The carrier gas was nitrogen with flow at a constant linear velocity of 1.25 mL/min. The split ratio was 100.0, split/spitless, injector temperature was set at 240 °C, and the injection volume was 2 µL. The temperature program was set as follows: 120 °C hold for 5 min, temperature increase 5 °C/min up to 220 °C and hold 220 °C for 20 min. Total analysis time was 45 min and FID temperature was set to 250 °C. Identification of the FAME content was conducted with comparison of retention times with those of the certified reference standard (Supelco F.A.M.E. Mix. C4–C24) analyzed under identical conditions.

Protein content in extracts obtained after continuous solvent flow sequential subcritical extraction, as well as alkaline liquefaction was determined by the Bradford method [45], sugar content by the phenol-sulfuric acid method according to Nielsen [46], while total polyphenol content by the Folin–Ciocalteu method according to Matić et al. [41].

HMF content in extracts obtained after continuous solvent flow sequential subcritical extraction was determined by the spectrophotometric method according to White [47].

The amounts of dominant polyphenols in extracts obtained after continuous solvent flow sequential subcritical extraction was determined by HPLC analysis. Samples were analyzed using a HPLC system (1260 Infinity II, with a quaternary pump, a PDA detector, and a vial sampler) (Agilent technology, Santa Clara, CA, USA). Compounds were separated by injecting 10 µL of samples into a Poroshell 120 EC C-18 column (4.6 × 100 mm, 2.7 µm) protected with a Poroshell 120 EC-C18 4.6 mm guard-column. The mobile phases used to separate compounds were 0.1% H_3PO_4 (mobile phase A), and 100% methanol (mobile phase B) with gradient conditions as follows: 0 min 5% B, 5 min 25% B, 14 min 34% B, 25 min 37% B, 30 min 40% B, 34 min 49% B, 35 min 50% B, 58 min 51% B, 60 min 55% B, 62 min 80% B, 65 min 80% B, 67 min 5% B, and 72 min 5% B. The flow was set to 0.5 mL/min. Compounds were identified by spiking samples with authentic standards, and by comparing UV–vis spectra of authentic standards and peaks in samples (200 to 600 nm). Calibration curves obtained by measuring different concentrations of standards were linear (r^2 = 0.9971–0.9996). Caffeine was quantified at 280 nm and phenolic acids at 320 nm. The total phenolic acids were obtained by adding the areas of all peaks at 320 nm belonging to phenolic acids, and quantifying them with the chlorogenic acid calibration curve.

2.5. Characterization of Spent Coffee Ground Derived Cellulose-Based Enzyme Immobilization Carrier

The obtained spent coffee grounds-derived cellulose-based immobilization carriers (ALDESCGs; alkaline liquefied sequentially extracted defatted spent coffee grounds) were characterized by basic chemical composition as described in Section 2.2, as well as, by FTIR-ATR analysis. Moreover, the produced SCG-derived cellulose-based enzyme immobilization carrier was analyzed for its suitability to be used as enzyme immobilization carrier by determining particle size distribution, water and oil holding capacity, as well as leakage.

FTIR-ATR spectra of solid residues obtained during the production of SCG-derived cellulose-based enzyme immobilization carriers from spent coffee grounds (SCGs, DSCGs, SEDSCGs, ALDESCGs) was performed on a Cary 630 FTIR ATR spectrometer (Agilent, Santa Clara, CA, USA) in the range of 650–4000 cm^{-1}.

Particle size distribution of the spent coffee grounds and the cellulose-based SCG-derived enzyme immobilization carrier was investigated by the laser light scattering method using a Mastersizer Scirocco 2000 analyzer (Malvern Instruments, Malvern, UK), where obtained results presented averaged values from three independent measurements.

The water holding capacity (WHC) and oil holding capacity (OHC) of ALDESCGs were determined by the method from Ballesteros et al. [1] with minor modifications. Briefly, 0.5 g of the sample was mixed with 5 mL of water or olive oil, vortexed for 1 min, and centrifuged at 2795× *g* for 30 min using a Centric 150 centrifuge (Tehtnica, Podplat, Slovenia). Afterwards, the volume of supernatant was measured, and WHC expressed as mL of water held per grams of sample, while OHC as mL of oil held per grams of sample.

The leakage of ALDESCGs was determined according to Corici et al. [48] but with modification. In brief, 0.5 g of ALDESCGs were mixed with 50 mL of distilled water and boiled in an Erlenmeyer flask with refluxing for 1 h. Afterwards, the obtained water extracts were separated by vacuum filtration through Whatman 1 filter paper and analyzed for soluble protein, total polyphenol, and sugar content as previously described.

3. Results and Discussion

The present study investigated the possibility of the complete transformation of spent coffee grounds to high-value products using multistep extraction transformation, with a cellulose-based SCG-derived enzyme immobilization carrier as the primary transformation product, and potentially emerging products revealed by compound-targeted analysis of each of the extracts obtained during SCG multistep extraction.

This approach was selected for the fact that one of the five major challenges of sustainable waste management in the agri-food industry using the "zero-waste" model of the circular economy is development of innovative waste transformation techniques used for the production of chemicals, fine chemicals, bioactive compounds, enzymes, and functional materials [10,49]. Such products have at least twice the added value of products obtained from currently dominant outdated waste management strategies, including the production of animal feed, fertilizers, treated waste from the processes of composting, anaerobic digestion, and incineration [10].

The current research presented here clearly offers the development of novel innovative techniques of SCG transformation by multistep extraction for the production of several high-value products from SCG waste, oriented toward the "zero-waste" model.

Nevertheless, the first step in the monitoring of case-oriented SCG multistep transformation to value-added products was the analysis of the chemical composition of the starting material, SCGs collected from a local coffee shop located in the vicinity of the city of Osijek, Croatia.

3.1. Chemical Composition of Spent Coffee Grounds

The chemical analysis of SCGs shown in Table 1 revealed that spent coffee grounds present a rich source of total fibers, with a dominance of cellulose (~24%) and hemicellulose (~23%), and a somewhat lower amount of lignin (~12%), justifying SCG potential to be used for the production of the cellulose-based enzyme immobilization carrier. Moreover, SCGs were found to contain a significant amount of proteins (~17%), and *n*-hexane extractable fats (~10.6%), while total polyphenols were found to be present in amounts of ~2.9%, and ash in the amount of ~1.9% per SCG dry weight basis. The obtained data on the chemical composition of SCGs were well in accordance with reports by Ballesteros et al. [1], Pujol et al. [42], Cruz et al. [20], Scully et al. [16], Campos-Vega et al. [24], and Murthy and Naidu [28].

Table 1. Chemical composition of spent coffee grounds (SCGs) and the cellulose-based SCG-derived enzyme immobilization carrier [1].

Parameter	Spent Coffee Grounds	Cellulose-Based SCG-Derived Enzyme Immobilization Carrier
Dry matter [g/100 g]	39.88 ± 0.75	92.25 ± 0.18
Proteins [g/100 g $_{d.w.b.}$] [2]	16.68 ± 0.06	1.12 ± 0.01
Fats [g/100 g $_{d.w.b.}$]	10.58 ± 1.63	n.d. [3]
Crude fiber [g/100 g $_{d.w.b.}$] [4]	29.86 ± 3.13	54.65 ± 1.31
Crude fiber (NDF) [g/100 g $_{d.w.b.}$] [5]	58.73 ± 1.31	81.87 ± 0.04
Cellulose (ADF-ADL) [g/100 g $_{d.w.b.}$]	23.59 ± 0.15	59.36 ± 1.74
Hemicellulose (NDF-ADF) [g/100 g $_{d.w.b.}$]	23.35 ± 1.51	21.30 ± 1.67
Lignin (ADL) [g/100 g $_{d.w.b.}$]	11.79 ± 0.35	1.21± 0.04
Total polyphenols [g/100 g $_{d.w.b.}$]	2.88 ± 0.40	n.d.
Ash [g/100 g $_{d.w.b.}$]	1.98 ± 0.13	1.21 ± 0.05

[1] Results are presented as average values ± standard deviation of at least three independent determinations. [2] d.w.b.—dry weight basis. [3] n.d.—not detected. [4] Crude fiber determined by ISO 6865:2000 method. [5] Crude fiber determined by Van Soest et al. method using an ANKOM^{2000} fiber analyzer (ANKOM Technology, Macedon, NY, USA).

3.2. Multistep Extraction of Spent Coffee Grounds Oriented toward Production of Cellulose-Based Enzyme Immobilization Carrier

Multistep extraction of SCGs followed the rule of increasing extraction solvent polarity and consisted of Soxhlet extraction with *n*-hexane in order to obtain SCG oil, followed by continuous solvent flow sequential subcritical extraction by consecutive use of 96% and 50% ethanol and water for the extraction of proteins, soluble carbohydrates, and total polyphenols, and finally alkaline liquefaction with 8% NaOH in order to remove lignin and produce cellulose-enriched SCGs as a potential enzyme immobilization carrier (Figure 1).

Soxhlet extraction with *n*-hexane was the first step of multistep SCG extraction where SCG oil of 10.58 ± 1.63 g per 100 g of dry SCGs was obtained. SCG oil was found to be rich in linoleic (46.28%) and palmitic acid (33.59%) (Table 2), which was congruent with reports by McNutt and Hee [5], Cruz et al. [20], Obruca et al. [50], Couto et al. [51], Mota et al. [52], and Vu et al. [14]. The most dominant role of SCG oil reported in the available literature [5,21,23] is its suitability for biodiesel synthesis. Therefore, it can be safely concluded that SCG oil produced by *n*-hexane extraction of SCGs, as the first step of multistep SCG extraction performed within this research, clearly presents a valuable raw material as a "cheap source" of waste oil intended to be used for biodiesel production.

Table 2. Fatty acid profile of spent coffee ground (SCG) oil.

Fatty Acid	Amount in SCG Oil [%]
Palmitic (C16:0)	33.59
Stearic (C18:0)	7.02
Oleic (C18:1)	9.56
Linoleic (C18:2)	46.28
α-Linolenic (C18:3)	0.92
Arachidic (C20:0)	2.63

The second step of the multistep SCG extraction transformation (Figure 1) was continuous solvent flow sequential subcritical extraction of defatted SCGs with 96% and 50% ethanol and water, where extraction of soluble proteins, carbohydrates, and polyphenols was expected. Continuous solvent flow sequential subcritical extraction of defatted SCGs was performed in the constructed subcritical system for continuous extraction in laboratory conditions (Figure 2) capable of extracting 100 g of defatted SCGs present in the extractor. Extraction conditions were as follows: defatted SCG mass vs. solvent volume ratio of 100 g per 3000 mL, solvent flow of 20 mL/min, pressure of 100 bars, and temperature of 125 °C.

Subcritical extraction of defatted SCGs was chosen as second extraction step, due to its superiority in the extraction of a wide range of organic compounds over standard laboratory extraction methods. By the use of subcritical conditions (temperature above boiling point of solvent at standard pressure; high pressure application), the extraction solvent remains in its liquid state but with changed properties including decreased dielectric constant, polarity, viscosity, and surface tension. This enables enhanced solvent penetration inside the extracting material, an improved diffusion rate, and accelerated extraction of targeted compounds [31,53–55]. It is well known that increasing the temperature of subcritical extraction increases the entropy of system enabling better penetration of the solvent into the porous structure of the extracting material and consequently increases the solubility of target compounds [31,54]. However, if the compounds of interest are prone to thermal degradation at high temperatures, a compromise between the selected temperature of subcritical extraction and the temperature of thermal degradation of the targeted compound must be made. This was the case in current research where one of the main aims was the production of an SCG-derived cellulose-based enzyme immobilization carrier. Thus, based on reports by Park et al. [32], Getachew et al. [56], Mayanga-Torres et al. [33], and Pereira et al. [30] on the thermal degradation of constitutive sugars at elevated temperatures during subcritical extraction, a temperature of 125 °C was selected as the most promising.

Figure 3 shows the distribution of soluble proteins, carbohydrates, total polyphenols, and dry matter content in the obtained extracts, as well as the amount of HMF formed. The application of continuous solvent flow sequential subcritical extraction of defatted SCGs by consecutive use of 96% and 50% ethanol and water as extraction solvents, ended with a total amount of 27.04 ± 1.19 g extracted matter per 100 g of dry defatted SCGs (Figure 3a), where the highest amount of SCG extracted matter of 9.37 ± 1.36 g/100 g was found in extracts obtained by 96% EtOH, followed by 50% EtOH (8.38 ± 0.80 g/100 g), and water (7.88 ± 0.33 g/100 g). The obtained data on the amount of SCG extracted matter were quite close to the value of 37.7 ± 2.6 g/100 g reported by Pedras and coworkers [6], where SCGs were extracted at subcritical conditions with water and a temperature of 150 °C. Chemical analysis of the extracts obtained (Figure 3b–e) revealed that each of the extracts contains proteins, sugars, polyphenols, as well as some amount of HMF formed during the applied subcritical extraction temperature of 125 °C. A total amount of 1.62 ± 0.13 g of proteins was extracted from 100 g of dry defatted SCGs, with the majority present in 50% EtOH extracts (1.03 ± 0.10 g/100 g) (Figure 3b). Much higher amount of proteins extracted from SCGs by subcritical water extraction were reported by Getachew and Chun [34] ranging from 2.05 to 4.74 g/100 g. However, it should be pointed out that the authors performed subcritical water extraction at temperatures of 180, 220, and 240 °C. Therefore, the lower amount of proteins extracted in the current research is not surprising, considering the fact that temperature is a key player in subcritical extraction. Total polyphenols extracted from dry defatted SCGs (Figure 3c) were the highest in 96% EtOH, followed by 50% EtOH and water, with a total amount of 8.70 ± 0.49 g/100 g. Getachew and Chun [34] reported somewhat lower amounts of total polyphenol extracted by subcritical water ranging between 3.3 and 5.5 g per 100 g of SCGs; Pedras and corworkers [6] in the range of 2.3–4.0 g/100 g; Xu et al. [13] up to 4.7 g/100 g; while Okur and corworkers [19] up to 9 g of total polyphenols per 100 g of SCGs.

Continuous solvent flow sequential subcritical extraction of defatted SCGs by consecutive use of 96% and 50% ethanol and water as extraction solvents, ended with a total of 5.58 ± 0.55 g of sugars per 100 g of dry defatted SCG, where the highest amount of sugars was extracted by water (3.10 ± 0.28 g/100 g) (Figure 3d). The results of total sugar content present in the extracts obtained were rather lower from those reported by Getachew and coworkers [56] of 18.80 ± 0.5%; Getachew and Chun [34], ranging from 8.88 to 37.91 g/100 g; and Mayanga-Torres et al. [33] where up to 17.23% of sugars were extracted from defatted SCGs. However, observed differences in total sugar content could be explained by the fact that a lower temperature (125 °C) of subcritical extraction was

used in the present research, preventing any possibility of SCG-constitutive oligo and polysaccharide degradation.

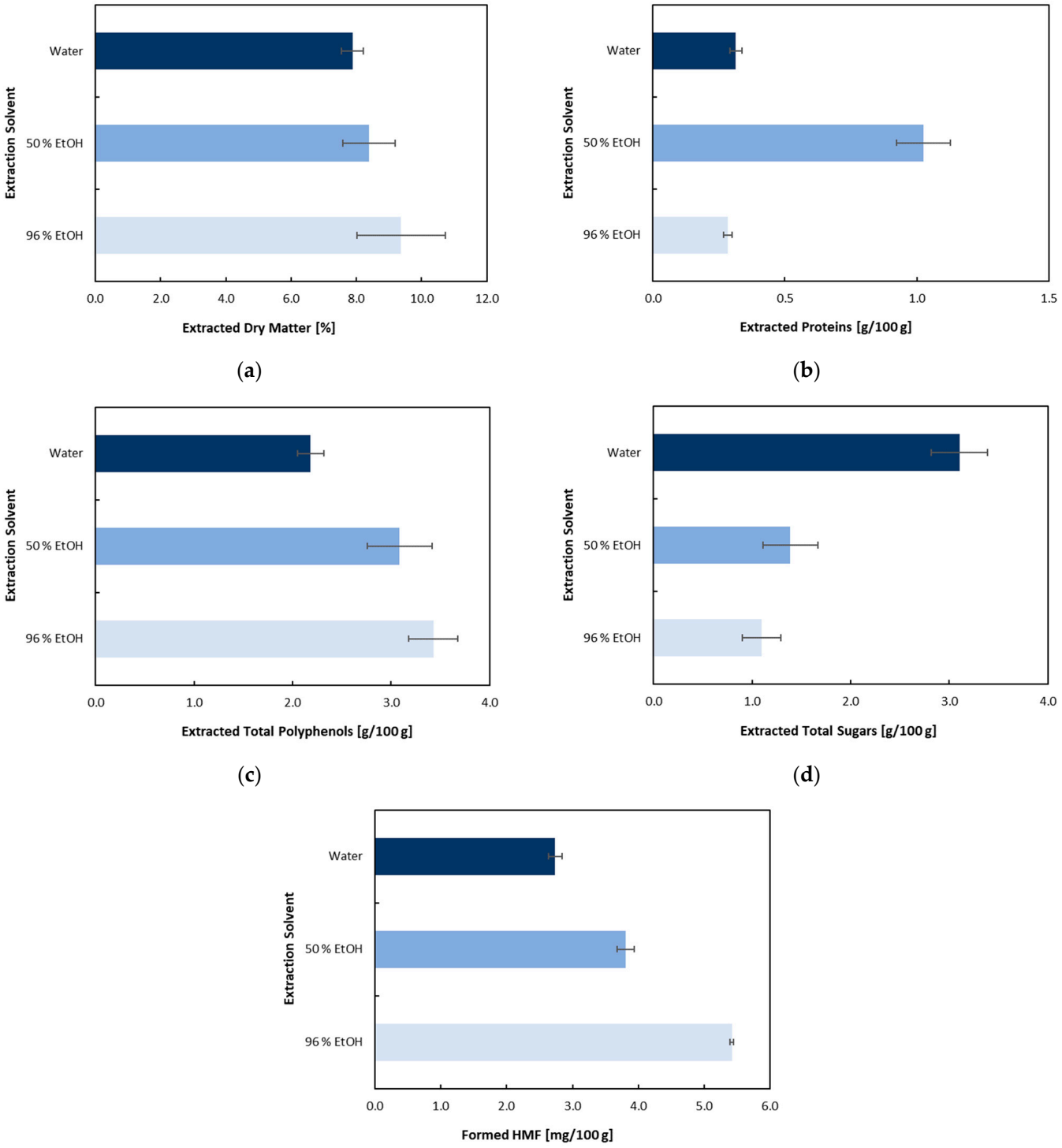

Figure 3. Composition of extracts obtained by continuous solvent flow sequential subcritical extraction of defatted spent coffee grounds by 96% and 50% ethanol (EtOH) and water: (**a**) dry matter content; (**b**) protein content; (**c**) total polyphenol content; (**d**) total sugar content; (**e**) HMF content. Results are presented as average values ± standard deviations of the independent extractions, where each determination was performed in triplicate.

Temperature dependent transformation of SCG reducing sugars to 5-(hydroxymethyl) furfural (HMF), with small amounts of HMF (0.34 mg/100 g) formed even at a subcritical extraction temperature of 120 °C using water as the extraction solvent, was reported by Park and coworkers [32]. Considering the fact that the authors [32] used HPLC for HMF detection, as well as only one extraction solvent (water), the higher amount of HMF determined in the current research (Figure 3e) is not surprising, due to the fact that three consecutive solvents were used, and HMF was determined by the spectrophotometric method. In brief, a total of 11.97 ± 0.75 mg of HMF was formed during continuous solvent flow sequential subcritical extraction of 100 g of defatted SCGs, with the highest amount of HMF detected in 96% EtOH extracts (5.42 ± 0.03 mg/100 g), followed by 50% EtOH (3.81 ± 0.13 mg/100 g) and water (2.74 ± 0.10 mg/100 g).

Besides the major class compound content determination (proteins, sugar, and total polyphenols, including the presence of HMF), extracts obtained by continuous solvent flow sequential subcritical extraction of defatted SCGs by consecutive use of 96% and 50% ethanol and water as extraction solvents, were examined on the amounts of major phenolics reported to be present in SCGs [11,17–19,24,36,57] including caffeine, chlorogenic, caffeic and ferulic acid via HPLC analysis. Figure 4 shows the total extracted amount and distribution of caffeine, chlorogenic, caffeic and ferulic acid in 96% and 50% EtOH and water extracts obtained after continuous solvent flow sequential subcritical extraction of defatted SCGs.

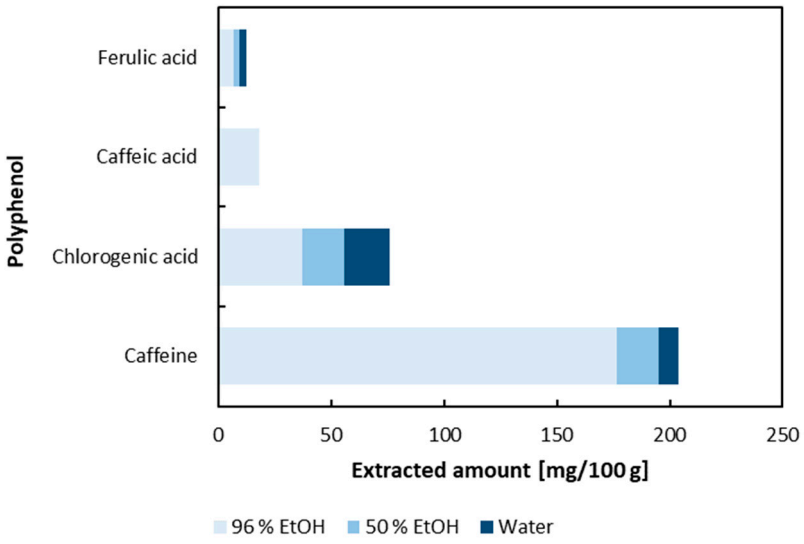

Figure 4. Major phenolics in extracts obtained by continuous solvent flow sequential subcritical extraction of defatted spent coffee grounds by 96% and 50% ethanol (EtOH) and water. Individual phenolics were determined by HPLC.

Caffeine was found to be the most abundant phenolic present in SCGs (204 mg/100 g), followed by chlorogenic acid (76 mg/100 g), and a much lower amount of caffeic (18 mg/100 g) and ferulic acid (13 mg/100 g), with the majority of examined phenolics dominantly present in the 96% EtOH extracts. Obtained data on the amount of caffeine were well in accordance with reports by Andrade et al. [11], Choi and Koh [57], Shang et al. [36], Ramón-Gonçalves et al. [17], and Campos-Vega et al. [24]; those on the amounts of chlorogenic and caffeic acid with reports by Andrade et al. [11], Okur et al. [19], and Ramón-Gonçalves et al. [17]; while those on ferulic acid content with reports by Ramón-Gonçalves et al. [17].

Based on the aforementioned, it seems that continuous solvent flow sequential subcritical extraction of defatted SCGs by consecutive use of 96% and 50% ethanol and water as the extraction solvents used at a temperature of subcritical extraction of 125 °C shows great potential to be used for SCG utilization, with the possibility of partial fractionation of targeted value-added SCG compounds. By such an approach, 96% EtOH extracts enriched

with total phenolics including caffeine and caffeic acid, as well as HMF were obtained, followed by 50% EtOH extracts rich in proteins and total phenolics, and finally water extracts enriched in total sugars (Figures 3 and 4). Nevertheless, the necessity of finding appropriate procedures for the separation and purification of SCG-targeted-compounds in the obtained extracts still remains to be elucidated, which is one of tasks of future perspectives. The application of continuous solvent flow sequential subcritical extraction of defatted SCGs by the consecutive use of 96% and 50% ethanol and water as the extraction solvents presented in the current research, according to our knowledge, is the first report of this kind available. Thus, based on the presented data, it could be safely concluded that this represents a new "window" for the future research of sustainable SCG transformation oriented toward the "zero-waste" model.

The production of a SCG-derived cellulose-based carrier for enzyme immobilization was one of the major tasks of the current research. According to our knowledge, there were no reports in the available literature on the possibility of the use of SCGs for the production of cellulose-based carriers for enzyme immobilization. However, there were several reports dealing with the possibility of sustainable SCG transformation, with cellulose-enriched SCG solid residues designated for the production of biogas and/or bioethanol and fuel pellets [25–27,29,37,58], and few reports on the possibility of production cellulose or hemicellulose from exhausted SCGs by direct [22,59] or indirect SCG transformation [60]. One of the key determinants of the majority the aforementioned publications was the use of alkaline treatment/liquefaction of obtained SCG residues for the purpose of constitutive lignin removal. In this respect, alkaline liquefaction of obtained sequentially extracted defatted spent coffee grounds (SEDSCGs) was selected as the last step of the multistep extraction transformation of SCG in the current research. Based on the reports by Passadis et al. [29], Girroto et al. [25], and Kim et al. [37], alkaline liquefaction of obtained SEDSCGs was performed by 2, 4, 6, and 8% (w/v) aqueous solution of NaOH, but at an elevated temperature in order to obtain an SCG-derived cellulose-enriched enzyme immobilization carrier devoid of lignin content. The process of lignin removal from SEDSCGs by alkaline liquefaction was monitored indirectly by FTIR-ATR analysis of obtained solid residues (Figure 5), where changes in the absorbance intensities at defined wavenumbers were found attributable to the increase in cellulose content [61–63]. In parallel with FTIR-ATR analysis, the produced ALDESCGs were examined for yield (Table 3) and presence of extractives ("leakage") in the ALDESCGs obtained by alkaline liquefaction by 2–8% NaOH (Table 4). The lack of leakage, i.e., chemical and thermal stability and insolubility of the enzyme carrier under reaction conditions is one of the desirable properties of an enzyme immobilization carrier [64], therefore it was necessary to pinpoint the percentage of NaOH at which the produced ALDESCGs, as a potential enzyme immobilization carrier, do not leak.

FTIR-ATR analysis (Figure 5) revealed that the application of alkaline liquefaction leads to an increase of SCG-constitutive polysaccharides including cellulose, as was observed by significant increase in the intensity of peaks centered at 1.010, 1.025, and 1.058 cm^{-1} and attributable to symmetric and/or asymmetric stretching vibration of C-O, C-H, C-O-C groups in cellulose and hemicellulose [61–63]. In addition, the removal of SCG oil from SCGs by Soxhlet extraction with *n*-hexane could be clearly noted by a decrease in the absorbance of intensity peaks centered at 2.920 and 2.850 cm^{-1} (Figure 5) attributable to symmetric and asymmetric stretching vibration of CH_2 of acyl chains in constitutive SCG lipids and phospholipids [62].

An increase of NaOH percentage in the solution for alkaline liquefaction led to a significant decrease in the mass yield of obtained ALSEDSCGs (Table 3), where the use of 8% NaOH aqueous solution for alkaline liquefaction of SEDSCGs resulted in a mass yield of only 20.83 $\pm$ 0.1%. However, the loss in the mass yield of obtained ALSEDSCGs (Table 3) was expected, due to the delignification process. Passadis et al. [29], Wongsiridetchai et al. [65], Girotto et al. [25], and Kim et al. [37] reported concentration and/or temperature dependent delignification of SCGs by an aqueous solution of NaOH, while Procentese et al. [66] of coffee silverskins. An increase in the temperature of alkaline liquefaction and

an increase in the percentage of NaOH used, were found to be key factors for successful lignin removal, but resulted in solid residue recovery between 40 and 50% [29,66]. Contrary to the reported, recovery (mass yield) of ALDESCGs obtained by alkaline liquefaction with 6 and 8% NaOH (Table 3) was much lower (33.98 ± 0.1 and 20.83 ± 0.1%, respectively) which could be explained by the fact that besides delignification, degradation of structural polysaccharides, especially hemicellulose occurs [29,37,66]. This was additionally proved by differences in the fiber content of SEDSCGs and ALSEDSCGs (Table 5), where alkaline liquefaction of SEDSCGs by 8% NaOH caused a ~25% decrease in hemicellulose and a ~91% decrease in lignin content, as well as by the great amount of total sugars (~50 g per 100 g of SEDSCGs) present in alkaline liquefaction extracts (Table 6). The observed decrease in lignin content of SEDSCGs treated with 8% NaOH (Table 5) was much higher in comparison with reports by Passadis et al. (63.0–79.2%), Girotto et al. [25] (24%), and Kim et al. [37] (60–80%), with the amount of lignin that remained in alkaline treated SCGs of 1.21 ± 0.04 g/100 g, which was 10-fold lower than those of 12.0 ± 1.1% reported by Girroto et al. [25]. On the contrary, the liquid phase of alkaline liquefaction extract (Table 6) contained a much higher amount of total sugars (~50 g per 100 g of SEDSCG) than those reported by Passadis et al. [29] (0.17–0.49 g/100 g) and Procentese et al. [66] (20 g/100 g). However, it should be pointed out that the authors measured glucose content by enzymatic test [29] or by HPLC [66] instead of total sugar content by the phenol-sulfuric acid method performed within this research, and used a lower NaOH concentration for alkaline liquefaction.

Table 3. Effect of alkaline liquefaction of sequentially extracted defatted spent coffee grounds (SEDSCGs) by 2, 4, 6, and 8% NaOH on the mass yield of obtained solid residue.

NaOH [%]	Mass Yield [%] [1]
2	83.25 ± 0.1
4	45.05 ± 0.3
6	33.98 ± 0.1
8	20.83 ± 0.1

[1] Results are presented as average values ± standard deviation of at least three independent determinations.

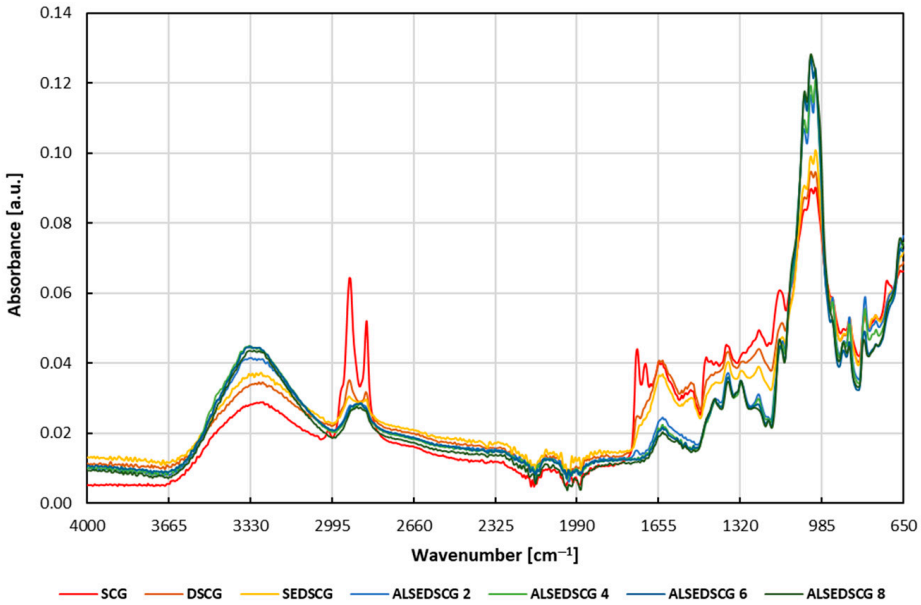

Figure 5. FTIR-ATR spectra of solid residues obtained during the production of cellulose-based enzyme immobilization carriers from spent coffee grounds. Legend: SCG—spent coffee grounds; DSCG—defatted spent coffee grounds; SEDSCG—sequentially extracted defatted spent coffee grounds; ALSEDSCG 2–8—alkaline liquefied sequentially extracted defatted spent coffee grounds (numbers 2–8 equals to the NaOH percentage used in alkaline liquefaction).

Table 4. Leakage of spent coffee ground-derived cellulose-based enzyme immobilization carrier in comparison to the percentage of sodium hydroxide used for alkaline liquefaction of sequentially extracted defatted spent coffee grounds (SEDSCGs) [1].

NaOH [%]	Polyphenols [%]	Proteins [%]	Sugars [%]
0 [2]	0.30 ± 0.04	0.24 ± 0.04	0.96 ± 0.21
2	0.68 ± 0.07	n.d.	0.37 ± 0.06
4	0.36 ± 0.09	n.d.	0.37 ± 0.08
6	0.32 ± 0.06	n.d.	0.21 ± 0.05
8	n.d. [3]	n.d.	n.d.

[1] Results are presented as average values ± standard deviations of three independent extractions, where each determination was performed in triplicate. [2] The number "0" resembles to the leakage of sequentially extracted defatted spent coffee ground (SEDSCG) samples. [3] n.d.—not detected.

Table 5. Crude fiber content changes during preparation of spent coffee ground-derived enzyme immobilization carrier [1].

Fiber	SCG [2]	DSCG [3]	SEDSCG [4]	ALSEDSCG [5]
Crude fiber (NDF) [g/100 g $_{d.w.b.}$]	58.73 ± 1.3	60.31 ± 1.27	79.10 ± 1.04	81.87 ± 0.04
Cellulose (ADF-ADL) [g/100 g $_{d.w.b.}$]	23.59 ± 0.15	24.77 ± 0.49	37.23 ± 0.56	59.36 ± 1.74
Hemicellulose (NDF-ADF) [g/100 g $_{d.w.b.}$]	23.35 ± 1.51	23.48 ± 0.65	28.39 ± 0.10	21.30 ± 1.67
Lignin (ADL) [g/100 g $_{d.w.b.}$]	11.79 ± 0.35	12.07 ± 0.14	13.48 ± 0.38	1.21 ± 0.04

[1] Results are presented as average values ± standard deviations of the independent determinations. [2] SCG—spent coffee grounds. [3] DSCG—defatted spent coffee grounds. [4] SEDSCG—sequentially extracted defatted spent coffee grounds. [5] ALSEDSCG—alkaline liquefied sequentially extracted defatted spent coffee grounds.

Table 6. Chemical composition of spent coffee ground alkaline liquefaction extracts [1].

Component	1st Alkaline Liquefaction	2nd Alkaline Liquefaction
Proteins [g/100 g]	7.33 ± 0.14	0.12 ± 0.04
Total Polyphenols [g/100 g]	8.26 ± 0.71	0.37 ± 0.06
Total sugars [g/100 g]	44.41 ± 3.14	6.32 ± 0.14

[1] Results are presented as average values ± standard deviation of at least three independent alkaline liquefactions.

As previously mentioned, chemical and thermal stability and insolubility of the enzyme carrier under the reaction conditions (i.e., lack of leakage) is one of the desirable properties of an enzyme immobilization carrier [55]. Therefore, it was necessary to pinpoint the percentage of NaOH at which the produced ALDESCGs, as a potential enzyme immobilization carrier, do not leak. Based on the results on ALDESCG leakage presented in Table 4, it could be safely concluded that alkaline liquefaction of SEDSCG by 8% NaOH enables the production of SCG residue resistant to hot water extraction. Thus, the chemical stability and insolubility under the reaction conditions of the potential SCG-derived enzyme carrier was achieved.

Since data on leakage of the produced ALDESCGs (Table 4), as well as cellulose enrichment (Figure 5), clearly pinpointed 8% NaOH as the most desirable for the production of an SCG-derived cellulose-based enzyme immobilization carrier by alkaline liquefaction of SEDSCGs, it was necessary to more closely examine the changes in cellulose, hemicellulose, and lignin content of SCG solid residues obtained during multistep extraction transformation. In this respect, SCGs, DSCGs, SEDSCGs, and finally ALDESCGs obtained by 8% NaOH alkaline liquefaction were examined for cellulose, hemicellulose, and lignin content (Table 5). Multistep extraction transformation of SCGs (Table 5) ended with a significant increase in cellulose (~250%) and a concomitant decrease in lignin content (~91%), while hemicellulose content of SCG-derived solid residue slightly decreased ~25% during the

alkaline liquefaction of SEDSCGs. Moreover, significant changes in SCG solid residue color from dark to light brown could be observed (Figure 6).

Figure 6. Color changes of spent coffee ground solid residues during the production of cellulose-based enzyme immobilization carriers. Legend: SCG—spent coffee grounds; DSCG—defatted spent coffee grounds; SEDSCG—sequentially extracted defatted spent coffee grounds; ALSEDSCG 2–8—alkaline liquefied sequentially extracted defatted spent coffee grounds (numbers 2–8 represent the NaOH percentage used in alkaline liquefaction).

Based on the current evidence (Tables 4 and 5, Figures 5 and 6), it is evident that the proposed procedure for multistep extraction transformation of SCG, including *n*-hexane defatting by Soxhlet extraction, continuous solvent flow sequential subcritical extraction of defatted SCGs by consecutive use of 96% and 50% ethanol and water as the extraction solvents, and finally alkaline liquefaction of SEDSCGs by 8% NaOH, results in cellulose-enriched SCG-derived residue with the potential to be used as an enzyme immobilization carrier.

In order to "close the circle", i.e., approach the "zero waste" model of sustainable SCG waste transformation, alkaline liquefaction extracts of SEDSCGs by 8% NaOH were examined for total sugar, protein, and polyphenol content (Table 6) in order to arrive at an idea of the possibility of their potential use. It was found that alkaline extracts are rich in total sugars, total polyphenols, and proteins, where 1st alkaline liquefaction extracts contained the majority of extracted compounds, and second alkaline liquefaction, the rest of the unextracted compounds (Table 6). Besides a high amount of total sugars released from 100 g of SEDSCGs during alkaline liquefaction by 8% NaOH of ~50 g/100 g, alkaline liquefaction extract contained approximately 8.6 g of total polyphenols and 7.5 g of proteins released from 100 g od SEDSCGs (Table 6). The obtained values (Table 6) were much higher than those reported by Passadis et al. [29] who reported lower amounts of total phenolics (0.135–0.421 g/100 g) and total nitrogen (1.47–2.59 g/100 g) present in alkaline liquefaction extracts. Such differences can be explained by the fact that the authors performed alkaline liquefaction of SCGs at a lower temperature (50 °C) and a lower concentration of NaOH. Nevertheless, more detailed analysis of the observed compounds present in the alkaline liquefaction extract should be performed in order to pinpoint their potential uses. One of the major problems of alkaline liquefaction by NaOH is the difficulty of recovering the NaOH used [37]. Therefore, it will be necessary to find adequate procedures for NaOH removal and subsequent more detailed analysis of the present compounds. One of the possibilities is neutralization of NaOH by sulfuric or *o*-phosphoric acid where sodium sulfate (Na_2SO_4) or sodium phosphate (Na_3PO_4) can be produced and precipitated from alkaline liquefaction extracts by lowering the temperature and/or by the use of precipitation by ethanol.

Table 7 shows the cumulative mass yield of SCG solid residues produced from dried spent coffee grounds by the use of SCG multistep extraction transformation, where 13.75 ± 0.04 g of ALDESCGs as a potential enzyme immobilization carrier could be produced from 100 g of dry SCGs. However, it was necessary to characterize the produced ALDESCGs in order to prove their suitability to be used as an enzyme immobilization carrier.

Table 7. Mass yield of cellulose-based enzyme immobilization carrier produced from spent coffee grounds.

Process	Product	Mass Yield [%] [1]
Initial drying at 60 °C	Spent Coffee Ground (SCG)	100.00
Soxhlet extraction	Defatted Spent Coffee Ground (DSCG)	89.42 ± 0.01
Continuous solvent flow sequential subcritical extraction	Sequentially Extracted Defatted Spent Coffee Grounds (SEDSCG)	66.01 ± 0.57
Alkaline liquefaction by 8% NaOH	Alkaline Liquefied Sequentially Extracted Defatted Spent Coffee Grounds (ALSEDSCG)	13.75 ± 0.04

[1] Results are presented as average values ± standard deviation of at least three independent production batches.

3.3. Characterization of the Produced SCG-Derived Cellulose-Based Enzyme Immobilization Carrier

The chemical composition of the produced ALDESCGs and the starting material (SCGs) is shown in Table 1. It can be seen that ALDESCGs, as a potential enzyme immobilization carrier, are dominantly a cellulose-based material (59.36 ± 1.74 g/100 g), containing 21.30 ± 1.67 g/100 g of hemicellulose, and a minor amount of lignin (1.21 ± 0.04 g/100 g) and proteins (1.12 ± 0.01 g/100 g). Among several cellulose-enriched wastes generated by the agri-food industry [10,67], rice husks have been proven to be a valuable carrier for enzyme immobilization [48,68]. However, in contrast to the ALDESCGs, prepared rice husks for enzyme immobilization have been reported to possess 46.5% of cellulose, 31.9% of lignin, and 22.1% of pentosans [48].

Corici and coworkers [48] stated that the majority of commercial beads generally employed for enzyme immobilization have a particle size between 150 and 300 µm, while Biró et al. [69] and Ferrario et al. [70] widen the desirable enzyme immobilization carrier particle size from 20 up to 500 µm. In this respect, it was necessary to determine the particle size distribution of the produced ALDESCGs. Figure 7 shows volume-weighted particle size distribution curves of spent coffee grounds (SCG) and ALDESCGs as a potential immobilization carrier. Both curves indicated relatively narrow particle size distribution confirmed with low span values of 1.896 and 1.484 for SCGs and ALDESCGs, respectively. However, an evident shift from bimodal particle size distribution in the SCG sample toward monomodal particle size distribution in the ALDESCG sample could be observed. The volume-weighted mean diameter of the SCG sample was 264.9 µm while those of ALDESCGs were found to be slightly higher, 277.2 µm. Regardless, the largest volume fraction of particles in both samples were in the range of mean particle size diameters from 200 to 400 µm, 39.12% for SCG and 39.22% for ALDESCG. Based on the desirable particle size [48,69,70], it could be safely concluded that the produced ALDESCGs by their particle size fulfilled the criteria to be used as a carrier for enzyme immobilization.

Zdarta and coworkers [64] stated that the chemical and thermal stability and insolubility of enzyme carriers under reaction conditions is one of the desirable properties of an enzyme immobilization carrier. The produced ALDESCGs fulfilled this criterion as can be seen in Table 4, where no leakage of proteins, polyphenols, and sugars could be observed at the highest NaOH percentage of 8% used for alkaline liquefaction.

Since data on the chemical composition, particle size distribution, and the lack of leakage indicated that the produced ALDESCGs might be used as a successful enzyme immobilization carrier, it was necessary to determine their water and oil holding capacity. This was carried out in order to determine the minimal volume of enzyme solution which should be added to the 1 g of enzyme immobilization carrier (ALDESCGs) prior to enzyme immobilization performed in the presence of a buffer and/or oil. Corrici et al. reported [48] much a better enzyme immobilization efficiency of rice husks when rape seed oil was used for enzyme immobilization by adsorption in comparison with a buffer.

The obtained data on water and oil holding capacity of ALDESCGs (Table 8) were different from those reported by Ballesteros and coworkers [1], where 5.73 ± 0.10 g of water were found to be adsorbed by 1 g of SCGs, and 5.20 ± 0.30 g of oil per 1 g of dry SCGs.

However, the increased water holding capacity of ALDESCGs of 7.55 ± 0.13 mL/g could be attributed to the higher amount of crude fibers present in ALDESCGs in comparison with SCGs (Table 1). Raghavendra et al. [71] reported that water holding capacity is higher in materials containing more elevated amounts of total dietary fibers.

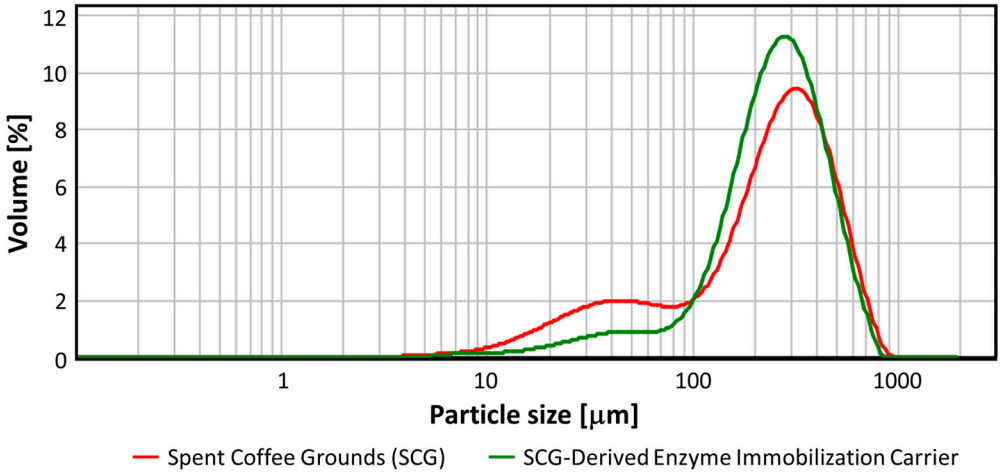

Figure 7. Particle size distribution of spent coffee grounds (SCGs) and SCG-derived cellulose carriers for enzyme immobilization.

Table 8. Water and oil holding capacity of the produced cellulose-based enzyme immobilization carrier [1].

Liquid Examined	Carrier Holding Capacity [mL/g]
Water	7.55 ± 0.13
Oil	2.93 ± 0.10

[1] Results are presented as average values ± standard deviation of four independent determinations.

The oil holding capacity of ALDESCGs (Table 8) was almost twice as low (2.93 ± 0.10 mL/g) as compared with capacity determined for SCGs of 5.20 ± 0.30 mL of oil per 1 g of dry SCG [1]. Femenia et al. [72] reported that materials richer in lignin show a higher oil holding capacity. Based on the fact that SCGs contain 11.79 ± 0.35 g/100 g of lignin, and ALDESCGs only 1.21 ± 0.04 g/100 g (Table 1), the much lower oil holding capacity value determined for ALDESCGs does not come as a surprise.

Overall, it can be safely concluded that ALDESCGs produced by multistep extraction transformation of SCGs, show great potential to be used as a successful SCG-derived cellulose-enriched carrier for enzyme immobilization.

4. Conclusions

In search for innovative transformation techniques oriented toward the "zero waste" model of sustainable waste management, herein we report the possibility of spent coffee ground (SCG) transformation by multistep extraction to several highly valuable products. Soxhlet extraction of SCGs with *n*-hexane was found to be suitable for the production SCG oil (10.58 ± 1.63 g/100 g SCG) intended for biodiesel production. Application of continuous solvent flow sequential subcritical extraction of defatted SCGs with 96% and 50% ethanol and water resulted in the production of extracts where caffeine (204 mg/100 g SCG), chlorogenic acid (76 mg/100 g SCG), and 5-(hydroxymethyl) furfural (11.97 mg/100 g SCG) were found as highly valuable extraction products, in addition tothe observed high amount of extracted proteins and soluble sugars. Further examination of the possibility of the use of proteins and soluble sugars present in the extracts obtained by solvent flow sequential subcritical extraction still remains to be elucidated in future research. The alkaline liquefaction of the remaining solid SCG residue by 8% NaOH was the last step oriented toward

the production of a cellulose-based enzyme immobilization carrier. The process of alkaline liquefaction resulted in cellulose-enriched SCGs and a multitude of compounds present in the alkaline liquefaction extracts (sugars, proteins, polyphenols) whose suitability for the production of valuable products still remains to be elucidated. Cellulose-enriched SCGs produced by alkaline liquefaction were found to be suitable for use as a potential enzyme immobilization carrier due to their defined properties including weighted mean particle size of 277 μm and lack of carrier leakage. On the other hand, the possibility of the use of alkaline liquefaction extracts for the production of value-added products is one of the tasks intended to be examined by future research. The possibility of the neutralization of NaOH present in alkaline liquefaction SCG extracts by sulfuric and *o*-phosphoric acid opens up a completely new area of research, where the obtained Na-salts might be precipitated due to their different solubility at different temperatures, as well as solubility in different organic solvents. Thus, it seems quite possible that alkaline liquefaction extracts of SCGs could find their future usage in the complete transformation of spent coffee grounds oriented toward the "zero-waste" approach. Nevertheless, based on the presented results, it seems that the currently proposed multistep extraction transformation of SCGs has great potential to be used for the production of several high-value added products.

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Appendix A. Determination of Crude Fiber, NDF, ADF and Lignin Contents by Van Soest Method Using ANKOM^{2000} Fiber Analyzer

The crude fiber, NDF, ADF, and lignin contents were determined gravimetrically after appropriate extraction in an ANKOM^{2000} fiber analyzer according to the ANKOM protocols. After milling, an amount of 0.45–0.50 g of sample was placed in F57 filter bags for NDF, ADF, and lignin determination, while 0.95–1.00 g was required for crude fiber extraction. Filter bags were placed into an ANKOM^{2000} fiber analyzer and the instructions on the ANKOM^{2000} display were followed depending on the required analysis (crude fiber, NDF, or ADF). Crude fiber extraction was carried out by automated addition of 0.255 N H_2SO_4 and 0.313 N NaOH as extraction solutions, with a rinsing step in between. The NDF extraction required the use of the neutral detergent solution that was automatically added and manual addition of 8.0 mL of α-amylase, whereas the ADF extraction required the use of the acid detergent solution only. After each extraction procedure, the filter bags were automatically rinsed with tap water. When the extraction and rinsing procedures were completed, the filter bags were placed in a 250 mL beaker and acetone was added to cover bags and to soak bags for 3–5 min. The filter bags were then removed from the acetone and placed on a wire screen to air-dry. Completely dried bags were placed in an oven at 102 ± 2 °C for 2–4 h and weighed after reaching room temperature. Crude fiber analysis required the additional step of a dry ashing procedure at 600 ± 15 °C to calculate the loss of weight of organic matter.

Lignin was determined after performing ADF determinations by placing the dried bags with samples into a 3 L beaker and completely covering them with 72% sulfuric acid.

The bags were agitated at the start and at 30 min intervals by gently pushing and lifting the 2 L beaker up and down approximately 30 times. After 3 h, the 72% sulfuric acid was poured off and rinsed with tap water to remove all acid until pH paper showed neutral color when touching the bags. The bags were then placed in an oven at 102 ± 2 °C for 2–4 h and weighed after reaching room temperature.

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