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ROYAL SOCIETY OF CHEMISTRY

Sustainability spotlight

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In the context of circular economy, the agri-food chain represents the sector in which there is the highest production of wasted biomasses, with a direct strong potential to be further investigated. This, joint with the growing interest in the nutraceutical industry that is continuously raising, pushes research to seek techniques to extract bioactive compounds starting from these wastes. Our research merges the valorization of a biomass residual, coffee silverskin, responsible for the production of 2400 tons of waste per year only in Italy, with the development of a green and sustainable technique, supercritical CO_2, to target its polar fraction to be exploited in nutraceutical field. This approach is perfectly aligned with the following UN sustainable development goals: Responsible consumption and production (SDG 12), industry, innovation, and infrastructure (SDG 9) and climate action (SDG 13).

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ARTICLE

Circular Valorizaron of Coffee Silverskin through Supercritical CO_2 for Functional Extracts Production

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The development of sustainable procedures for the valorization of industrial biomass wastes represents a major challenge for the scientific community. Among residual biomasses, coffee silverskin could represent an attractive source of bioactive compounds. In spite of promising applications, silverskin is still underutilized and nowadays still discarded by the roasters as solid waste in landfills. This work presents the application of a green unconventional extraction strategy, supercritical CO_2, with a particular focus on the extractability of polar compounds from green coffee beans and their related wasted biomass, coffee silverskin, by means of the addition of ethanol as co-solvent, allowing to shift the window of extractables towards more polar molecules. Results point out the ability of the supercritical fluid to efficiently target chlorogenic acids, minimizing the excessive use of organic solvents and avoiding the presence of inorganic or organic acids. The use of a co-solvent, at the expense of reducing the selectivity towards target species, was demonstrated to efficiently co-extract other classes of compounds directly connected with the relevance of silverskin's phytochemical profile, dealing mainly with its antioxidant and prebiotic properties, pointing to interesting applications in functional food products.

Introduction

Biomasses valorization constitutes a key aspect of circular economy and an opportunity to unlock the full potential of bioeconomy and enhance the possible synergies between different sectors. Within circular models, agriculture, and more in general the agri-food chain, represents the sector in which there is the highest production of wasted biomasses, with a direct strong potential to be further investigated [1]. This, joint with the growing interest in the nutraceutical industry that is continuously raising, pushes academic research to seek new bioactive molecules and techniques to extract and isolate them starting from these wastes. Among food-chain related wastes, plant residuals could represent an attractive source of more sustainable bioactive compounds towards nutraceutical and/or functional foods sector [2].

The exploitation of byproducts towards generation of added-value compounds should anyway meet and merge the need to develop alternative and innovative extraction techniques, that are able to guarantee the sustainability of the entire process, from biomass selection to the technological methods, to final added value generation. In this context, unconventional extraction techniques are promising approaches able to enhance compounds recovery in a greener way compared to conventional solvent-based technologies. Among them, subcritical water extraction and supercritical carbon dioxide are raising more and more interest. These extraction techniques are efficient, economical and promising routes for bioactives recovery from different matrices. When the target compounds could undergo thermal degradation, supercritical CO_2 (sc-CO_2) might be a preferable choice, in order to maintain all the antioxidant profile in the extracts[3,4]. sc-CO_2 technology, employing CO_2 as an extraction fluid is indeed characterized by low environmental impacts: the use of organic solvents can be avoided or minimized ensuring safer and selective processes directly on the biomass without any pretreatment, with the possibility to recycle the employed CO_2 in the industrial plants [5]. Following these global trends, where the interest in "green" products and technologies is growing, this work presents as core strategy, a supercritical CO_2 (sc-CO_2) extraction methodology with a particular focus on the extractability of polar compounds. Since sc-CO_2 alone displays a solvating power similar to *n*-hexane, most of the literature is focused on the primary and direct use of the supercritical fluid, targeting lipidic fraction and, more in general, non polar compounds [6]. On the other hand, the addition of a co-solvent in relatively low ratio enables to modify the supercritical fluid polarity, allowing to shift the window of extractables towards more polar molecules [7]. This approach is already discussed in the literature, remaining however still underdeveloped, probably due to the loss of a certain selectivity attributable to the presence of a co-solvent [8,9]. Nevertheless, if the loss of selectivity is evaluated together with an investigation of the co-extracted compounds and their potential added-value, a sustainable platform might be designed, in a more complete biorefinery concept, where the effect of co-extraction has an interesting economical outcome from the process perspective. Other molecules, apart from the targeted ones could be beneficial towards specific applications in particular in the nutraceutical field, where they could have a great interest as prebiotics or as building blocks for functional materials [10–12].

In this wide context, this work presents for the first time the valorization of the polar fraction from coffee silverskin by means of supercritical CO_2, in the presence of a co-solvent. Silverskin, the thin layer directly in contact with the coffee bean removed during the

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roasting process, representing about 4.2 % (w/w) of coffee beans and accounting for a production of 2400 tons of waste produced per year only in Italy, was selected as interesting case study together with its related original biomass, green coffee beans, as term of comparison [13]. In spite of promising applications, silverskin is still underutilized and nowadays still discarded by the roasters as solid waste in landfills. To date, exploitation of silverskin results mainly focused on its lipidic content, explored also by means of environmental-friendly techniques such as sc-CO_2, anyhow ending up losing the high value of its polar content [14,15]. This latter connects with the relevance of silverskin's phytochemical profile, dealing mainly with its antioxidant and prebiotic properties, mostly related to the presence of phenolics and, in particular, chlorogenic acids [16–18]. To the best of authors' knowledge, no investigations are present in the literature related to phenolics extraction from coffee silverskin by means of a green extraction strategy like supercritical CO_2. In addition, the aim of the present work is to study the effect of the co-solvent addition to the supercritical fluid and investigate the presence and nature of the co-extracted compounds.

Materials and Methods

Reagents and starting biomasses

All chemicals were used without further treatment. Acetonitrile and water, when used for liquid chromatography, were purchased from Sigma-Aldrich (Italy) as ultra-performance liquid chromatography-grade. Formic acid, trifluoracetic acid, ethanol, *n*-hexane, caffeine (powder, ReagentPlus®) and 5-caffeoylquinic acid (≥ 95%) standards were purchased from Sigma-Aldrich (Italy). A carbon dioxide tube (CO_2 purity 4.5) was purchased from Sapio (Italy). p-coumaric, caffeic and ferulic acid standards were purchased from Sigma-Aldrich (SE).
Green coffee beans (GCB) of Robusta species from Vietnam and micronized coffee silverskin (CS) were kindly donated by an Italian roasting company (Ideal Caffè snc, Verderio) and then pulverized by a knife blender at its maximum velocity for 3 min.

Delipidization of GCB and CS

Lipids extraction yield was calculated as: $\frac{mass_{extract}}{mass_{starting\ biomass}} \cdot \mathbf{100}$.

Three replicates were performed for each experiment.

Supercritical CO_2 extraction

Supercritical CO_2 extractions (sc-CO_2) were performed using a pilot unit SFT110XW System (Supercritical Fluid Technologies, Inc., USA). It consists of an SFT-Nex10 SCF CO_2 Pump (Constant Pressure-Piston Pump) with a Peltier Cooler, a 100 cm^3 stainless steel extractor vessel inserted in an oven and a collection vial.
30.15 g of CS or 47.30 g of GCB were loaded in the vessel during their respective delipidization experiments.
Following the results from our previous work on coffee silverskin [19], the operative pressure was set as 300 bar in all experiments, while the temperature of the vessel and the temperature of the restrictor block were maintained at 60 °C and 75 °C, respectively, throughout the extraction period. For each extraction experiment many cycles, each one comprising 30 min of maceration time in static conditions and 10 min of dynamic conditions, were performed until no evident extracted mass was further gained. In dynamic conditions, the valves were opened and the extract was collected in a vial, keeping a CO_2 gas constant flow rate of 8 SCFH (standard cubic feet per hour). In the case of lab-scale supercritical fluid extractor, the duration of static and dynamic cycles (and hence the final number of cycles) was optimized on the basis of the need of specific soaking time in static conditions before opening the valves to run the dynamic process to collect the extract. The extracts were then stored at -20°C for subsequent analysis, whilst the defatted biomasses were collected from the vessel and subjected to further experiments.

Solvent-based extraction

As reference conventional method, *n*-hexane was employed as the extraction solvent. 10 g of the starting biomasses (GCB or CS) were suspended in 100 mL of *n*-hexane and: i) magnetically stirred for 6 h at room temperature, or ii) extracted by means of Soxhlet apparatus. The residual solid was dried under static air at 40°C. The solvent was evaporated from the filtered solution by rotary evaporator (Buchi) and then under vacuum pump.

Chlorogenic acids extraction

Supercritical CO_2 extraction + co-solvent

The same equipment employed for biomass supercritical delipidization was then connected to a co-solvent pump (515 HPLC pump Waters). 29.5 g of defatted CS or 46.6 g of defatted GCB were loaded during their respective extraction experiments. Before setting the supercritical conditions inside the vessel, 10% v/w (with respect to the loaded biomass) of a mixture water:ethanol (1:1) was injected inside the vessel, as a result of preliminary trials aimed to assess the best co-solvent mixture. The operative supercritical pressure was set at 300 bar, while the temperature of the vessel and the temperature of the restrictor block were maintained at 60 °C and 75 °C, respectively, throughout the extraction period. A static maceration 30 min period was maintained at the beginning, followed by a dynamic period of 1 hour maintaining an ethanol flow rate of 0.5 mL min^{-1} (corresponding to 0.25% v/v of the CO_2 amount) with a CO_2 gas flow rate constant at 8 SCFH (standard cubic feet per hour). After this first cycle, many cycles, comprising 15 min of maceration time in static conditions and 1 hour of dynamic conditions with co-solvent addition, were performed. The extract solutions were collected and ethanol was evaporated by rotary evaporator and then under vacuum pump.

Solvent-based extraction

A conventional solvent-based extraction method was employed as a reference in order to quantify the total chlorogenic acids and caffeine content of the biomasses and identify the co-extracted species. Some reference literature works were considered to select the most appropriate and effective strategy [20–22]. In details, 1 gram of biomass powder of CS or GCB was mixed with 10 mL of an acidic hydroalcoholic solution of water:ethanol=7:3 (+1% formic acid). The suspension was magnetically stirred at room temperature for 4 hours, centrifuged at 6000 rpm for 5 min and then filtered on a Buchner. The solvent was evaporated by rotary evaporator (Buchi) and then under vacuum pump.

Characterization of extracts

Chlorogenic acids and caffeine profile

The extracts composition in terms of chlorogenic acids and caffeine was analyzed with Waters ACQUITY UPLC (Ultra-high Performance Liquid Chromatography) system (Waters corp., United States) equipped with a quaternary solvent manager system, autosampler, thermostated column compartment and a dual-wavelength UV–Vis detector. Water solutions of samples were filtered (0.2 µm nylon filters) before injection (5 µL) in the system. The analytical separation was performed using an ACQUITY UPLC® BEH C18 column (1.7 µm, 2.1×50 mm). The mobile phase was composed of water containing 0.1% of formic acid (v/v) (A) and acetonitrile containing 0.1% of formic acid (v/v) (B). The flow rate was set at 0.25 mL min^{-1} and the linear gradient elution was: 0 min, 95% A; 3 min, 93% A; 7 min, 90%

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A; 15 min, 80% A; 18 min, 95% with a re-equilibration time of 3 min before the next injection. The column temperature was maintained at 34 °C and the wavelengths were set at 275 nm and 324 nm, corresponding to the maximum absorption of caffeine and chlorogenic acids, respectively. Data were processed with Empower 3 workstations.

Before samples injections, six dilutions of 5-caffeoylquinic acid (5-CQA) aqueous solution (515 μg mL^{-1}) and six dilutions of caffeine aqueous solution (220 μg mL^{-1}) were prepared in the range 5.15–515 μg mL^{-1} for 5-CQA and in the range 2.2–220 μg mL^{-1} for caffeine. Standard solutions were filtered (0.2 μm polypropylene filters) and injected three times in UPLC system. 5-CQA and caffeine were eluted as sharp peaks. In the operative concentration range the trend was linear, with no saturation effects that could bend the linearity.

The area under each peak was quantified by instrumental software and plotted versus the concentration. The best fit of experimental data in the plot "Peak area at 324 nm *vs*. [standard concentration]" was then used for 5-CQA and caffeine quantification in each extract. The best fit of experimental data in the plot of "$A_{324\ nm}$ vs. [5-CQA]" was a straight-line represented by the following mathematical equation: $y = 6.1 \cdot 10^7 x - 3 \cdot 10^5$, resulting in a R^2 equal to 0.996. The slope *m* and intercept *q* of the regression line with their respective standard deviations were ($6.1 \cdot 10^7 \pm 2 \cdot 10^6$) and ($-3 \cdot 10^5 \pm 4 \cdot 10^5$), respectively. The calculated equation of the regression line was then employed to determine the 5-CQA concentration in each extract.

The best fit of experimental data in the plot of "$A_{324\ nm}$ vs. [caffeine]" was a straight-line, represented by the following mathematical equation: $y = 4.91 \cdot 10^7 x + 7 \cdot 10^4$, resulting in a R^2 equal to 0.999. The slope *m* and intercept *q* of the regression line with their respective standard deviations were ($4.91 \cdot 10^7 \pm 8 \cdot 10^5$) and ($7 \cdot 10^4 \pm 9 \cdot 10^4$), respectively. The calculated equation of the regression line was then employed to determine the caffeine concentration in each extract.

When necessary, chromatographic separation was followed by a mass spectrometry (LCQ Fleet Thermofisher) analysis. A negative electrospray mode was used for the ionization of molecules with a capillary voltage of -42 V, at a capillary temperature of 275 °C. The heater temperature was set at 150 °C, the gas flow rate was 20 (arb) and the spray voltage was 5kV. The monitored mass range was from m/z 50 to 1000. Before sample injection, mass spectrometry parameters were optimized using a commercial standard of 5-caffeoyquinic acid.

Monosaccharides profile

The carbohydrates profile of the soluble extracts after supercritical CO_2 extraction (samples obtained during the 6th cycles in sc-CO_2) and solvent extraction were analyzed by direct solubilization in water or hydrolysed by trifluoracetic acid (TFA), followed by analysis of the released monosaccharides by high-performance anion-exchange chromatography-pulsed amperometric detection analysis (HPAEC-PAD). In summary, samples were weighed at 1 mg in glass tubes (triplicate) and 1 mL of MiliQ water or 2M TFA was added. In the case of TFA, the hydrolysis was performed at 120 °C for 3 h. Hydrolyzed samples were then dried under a stream of air and then re-suspended in water. Both sets of samples were analyzed using on the ICS6000 fitted with a Dionex CarboPac PA1 IC column (Thermo Scientific, Stockholm, Sweden), as previously described [23]. Analysis was performed in triplicate.

Hydroxycinnamic acids profile

Phenolic acids were released by saponification followed by analysis using high performance liquid chromatography (HPLC) as described in the literature [24,25]. 10 mg of each sample (solvent extracts and samples obtained during the 6th cycles in sc-CO_2) were saponified with 500 μL of 2 M NaOH at either 30°C (for soluble extracts) or 80 °C (for insoluble ones) for 17 h, with stirring in duplicate. The saponified samples were then acidified to pH 3.0 using 12 M HCl, partitioned with ethyl acetate and dried under nitrogen. The dried phenolic acid fractions were resuspended in methanol: 2% (v/v) acetic acid (1:1, v/v), filtered through Chromacol 0.2 μm filters (Scantec Nordic, Sweden) and analyzed on the HPLC using the ZORBAX StableBond C18 column (Agilent Technologies, USA) fitted with photodiode array detection at 200-400 nm (Waters 2695, MA, USA). Samples that were not saponified were diluted with methanol 2% (v/v) acetic acid (1:1, v/v), filtered through Chromacol 0.2 μm filters (Scantec Nordic, Sweden) and subjected to the HPLC for analysis. The phenolic acids were separated in accordance with a previous method using p-coumaric, caffeic and ferulic acid as standards [26].

Conjugates profile present in samples

The presence of single and conjugate phenolics of the extract (solvent extracts and samples obtained during the 6th cycles in sc-CO_2) was checked using Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC-ESI-MS), a Synapt G2 mass spectrometer (Waters Corporation, Milford, MA, USA). Saponified and non-saponified samples were diluted in acetonitrile 50% (v/v) with 0.1% (v/v) formic acid to 0.1 mg/mL and filtered through Chromacol 0.2 μm filters (Scantec Nordic, Sweden). Samples were then briefly passed through an Eclipse Plus C18 column 1.8 μm (2.1 x 150 mm) (Agilent Technologies, Santa Clara, CA) for automation and analyzed using positive-ion mode in the ESI-MS. The capillary and cone voltage were set to 3 kV and 25 kV, respectively. The phenolic dimers and conjugate of carbohydrate-phenolics were detected as $[M + H]^+$ or $[M + Na]^+$adducts.

Size exclusion chromatography

The molar mass distribution of the extracts (solvent extracts and samples obtained during the 6th cycles in sc-CO_2) was determined using the SECurity 1260 size exclusion chromatography (Polymer Standard Services, Mainz, Germany) coupled to a refractive index, as formerly described [27]. Samples were solubilized in water containing 100 mM $NaNO_3$ and 5 mM NaN_3.

Results and discussion

Biomasses milling

One of the most critical operating parameters affecting extraction yield is the particle size. The solid matrix must be blended to increase the mass transfer area. Enhancing the exposed surface area to the solvent, makes the extractions more effective in terms of yield, facilitating the mass transfers. Solutes can be in fact located on the surface of the cells of the vegetable matrix or in intracellular spaces. As the particle size of the sample decreases, the extracted solute would travel shorter diffusional paths along the solid substrate and mass transfer barriers, such as cell walls, would be destroyed. However, it was also reported by Coats *et al.* that some adverse hydrodynamic effects may occur when excessively small particles are treated in a packed bed [28,29]. Particle size is hence considered a critical parameter, influencing the yield of extraction processes [30].

Coffee silverskin (CS) was provided as already micronized in the form of fine powder. Optical microscopy assessed an average particle size around 100 μm. Green coffee beans (GCB) were subjected to blending before extractions. Due to the high content of lipids, the GCB fatty biomass could not be transformed into a fine powder as silverskin, not even by embrittlement by means of liquid nitrogen before blending. In this case, the minimum achievable particle size was in the range 500-1000 μm, as detected by optical microscope. GCB particle size was in any case in the range of recommendations found in the literature [30]. Pictures of the pristine and blended biomasses are reported in the Supplementary Information in Figure S1.

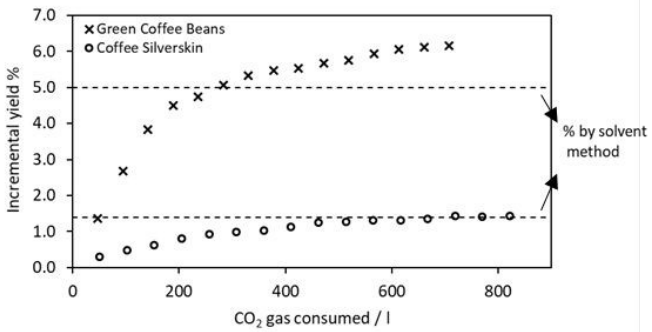

Fig. 1 - Extraction kinetics (incremental yield *vs.* CO_2 utilization) during supercritical delipidization of GCB and CS. Dashed lines correspond to extraction yields obtained by solvent method.

Delipidization extraction yields and kinetics

The lipid content of CS was already reported in a recent publication [19]. For the purpose of this work, a preliminary delipidization of both biomasses was performed in order to ease the successive extraction of the target polar compounds. The presence of excessive lipids would hinder the accessibility of the supercritical solvent (and the co-solvent) towards the generation of the necessary interface between the fluid and the solutes. The presence of lipids in the biomasses could be responsible of a certain hindrance in the targeted polar compounds extraction, rendering the biomass less prone to be penetrated by the extraction fluid. Moreover, lipids could contaminate the extracts later obtained in sc-CO_2 in the presence of the co-solvent.

The lipid content was determined by conventional solvent-based procedures by triplicate trials, corresponding to 5.0±0.5 % and 1.4±0.1 % (w/w on dry biomass) for GCB and CS samples, respectively.

When the extraction was performed in supercritical CO_2, the optimization of process parameters followed the results from the previous work by Nasti *et al.*, set at 60°C and 300 bar. Cycle by cycle, with an alternation of static and dynamic periods, an incremental mass of extract was obtained from both GCB and CS, and the extraction process was maintained active until no evident mass gain was observed, thus corresponding to the maximum extractable yield. These values corresponded to 6.2±0.1% and 1.4±0.2 % for GCB and CS, respectively. It was expectable that these yield values are comparable to the corresponding numbers obtained by conventional extraction. A slight difference was observed for GCB. The lipids yield obtained by means of sc-CO_2 was 20% higher than the conventional extract. This can be attributed to the impossibility to finely micronize GCB, hence making less effective the biomass-hexane solid-liquid interface contact, due to a lower exposed surface area. The penetration inside porosity of the supercritical fluid, partially behaving like a gas in terms of permeability, was on the other side more effective, reaching pores inaccessible to hexane, thus enhancing the mass transfer and hence the yield [31].

sc-CO_2 delipidization extraction kinetics are displayed in Figure 1. Both extractions follow the model explained by da Silva *et al.*, primarily based on two mass transfer mechanisms: solubility and diffusion [32]. The model assumes two district modes of extraction: initial constant rate extraction that is controlled by solubility, and falling rate extraction that is controlled by diffusivity. An initial period of extraction (the steepest, represented by the first four points in each dataset) respond to the depletion of lipids found on the particle surface, while the final period represents the extraction from inside the porosity, where the mass transfer is more hindered and the extraction rate slows down until it zeroes [32,33]. For the purpose of this work, during the delipidization process, extraction was interrupted after 15/16 cycles when a plateau was reached, meaning that no more lipids could be obtained in the process.

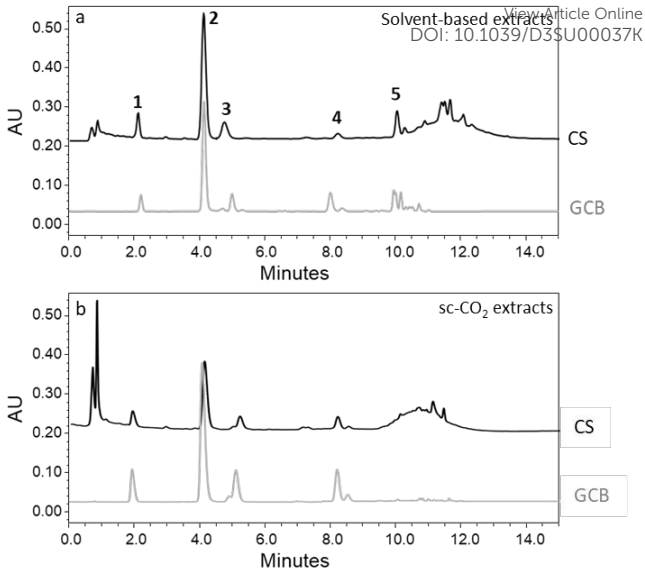

Fig. 2 - UPLC-UV chromatographic profile monitored at 324 nm of: a) conventional solvent-based extracts, b) sc-CO2 extracts. Peaks assignments: 1) 3-CQA, 2) 5-CQA, 3) 4-CQA, 4) FQA, 5) di-CQA.

All the extracts obtained in the delipidization process were subjected to UPLC analysis, and none of them displayed evidence of chlorogenic acid presence. The details on lipidic extracts composition has been reported in a recent paper by Nasti *et al* [19].

Chlorogenic acids extraction

Chlorogenic acids, due to their well-known antioxidant and interesting biological activities, were considered the main target compounds adopting supercritical fluid in the presence of a co-solvent [20,34]. For comparison purposes, a solvent-based extraction was carried out employing a literature method with the aim of effectively extracting all the chlorogenic acids content from the delipidized biomasses. In the conventional extraction, 1:10 solid-to-liquid ratio was employed since it represents a good compromise between not exceeding in the amount of solvent and, at the same time, guaranteeing an exhaustive extraction of targeted compounds. The first three sc-CO_2 extracts obtained in the presence of co-solvent from GCB delipidized biomass, and the first five extracts obtained in the presence of co-solvent from delipidized CS biomass, were all similar to samples obtained during delipidization and almost completely insoluble in water. After the mentioned cycles, successive extracts displayed an almost complete water-solubility.

Figure 2 displays the chromatograms recorded at 324 nm (maximum absorbance for chlorogenic acids) for the solvent-based extracts and a selection of representative samples from sc-CO_2 extraction cycles. The UPLC data show an almost complete overlap of the chromatograms at 324 nm, corresponding to the maximum absorption of chlorogenic acids, relative to all four samples, independently from their extraction method. This leads to a conclusion that sc-CO_2 in the presence of the co-solvent was effective in the extraction of chlorogenic acids to the same extent of solvent-based method, from a qualitative point of view. Some slight differences are detected in the last part of the chromatogram, after 10 min as retention time, where some small peaks are present in all chromatograms, differing from sample to sample. Due to the low area of the corresponding peaks, these differences were not further investigated, but tentative attributions to uniquitary flavonoids such as quercetin and kaempferol could be carried out on the bases of a work by Nzekoue *et al.*[35].

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Table 1 - Quantification of 5-CQA in each sample.

Sample name	Starting biomass	Extraction method	% 5-CGA (on dry biomass)	% 5-CGA (in extract)
GCB-solv	GCB	Solvent	4.4%	11.7%
CS-solv	CS	Solvent	0.29%	2.1%
GCB-scCO$_2$	GCB	sc-CO$_2$ + co-solvent	3.5%	16.8%*
CS-scCO$_2$	CS	sc-CO$_2$ + co-solvent	0.37%	33.9%*

* the concentration of 5-CQA changes in each extract from the different successive sc-CO$_2$ cycles. In the table are reported the highest values, obtained during the 11th sc-CO$_2$ cycle for GCB and during the 6th sc-CO$_2$ cycle for CS.

The UPLC-UV chromatogram shows a main peak characterized by a retention time of 4.1 min (peak 2 in Figure 2) attributable to 5-CQA, the most abundant chlorogenic acid in coffee, as confirmed by co-injection of the commercial standard and further mass spectrometry results.

The assignments of the other peaks in the chromatograms were confirmed by mass spectrometry. Peak 1, 2 and 3, attributed to 3-caffeoyquinic acid (3-CQA), 5-caffeoyquinic acid (5-CQA) and 4-caffeoyquinic acid (4-CQA), respectively, are all characterized by the presence of the molecular ion signal at m/z = 353.5 corresponding to $[M - H]^-$. Isomers were recognized based on literature data [36]. Peak 4 was attributed to feruloylquinic acid (FQA), characterized by the presence of the molecular ion signal at m/z = 367.7 corresponding to $[M - H]^-$. Peak 5 was assigned to dicaffeoylquinic acids (di-CQA), due to the presence of the molecular ion signal at m/z = 515.4 corresponding to $[M - H]^-$, with no further investigation on the identity of the specific isomers.

Extraction results are coherent with the literature, where a previous work on coffee silverskin extract obtained by acidified water (1% aqueous formic acid) at 70 °C for 1 h, reported the presence of 3-CQA, 4-CQA, 5-CQA and feruloylquinic acids [37].

Supercritical CO_2 was already employed in the literature targeting phenolics from green coffee beans. De Azevedo employed ethanol and isopropyl alcohol as co-solvents in temperature ranges similar to the present work, concluding that chlorogenic acids were only extracted from coffee beans when isopropyl alcohol was used. However, they evaluated chlorogenic acids extraction efficiency as poor, explained by competition of these molecules with more polar components present in coffee beans (ex. sugars) and by the inability of the solvents to break down the caffeine-chlorogenic acid interactions [38]. In the case of the present work, the presence of an enhanced possibility of hydrogen bonding due to the presence of ethanol/water co-solvent mixture, could result more competitive and hence able to brake the hydrogen bonds between caffeine and chlorogenic acid.

Some other coffee by-products were also investigated targeting the chlorogenic acid by sc-CO_2, such as spent coffee grounds and coffee husks for valorization purposes. 15% of ethanol as co-solvent was the best choice, providing the higher recovery of chlorogenic acids in supercritical conditions [39].

Concerning silverskin, to the best of authors' knowledge, no investigations are present in the literature related to chlorogenic acids extraction from this biomass by means of supercritical CO_2.

After identification of the main phenolics, the most abundant chlorogenic acid in samples, 5-CQA, was quantified.

As reported in Table 1, 5-CQA was quantified as 4.4% of green coffee beans and 0.29% of silverskin, when the extraction was run by means of conventional solvent-based strategy. Results for green coffee beans are consistent with the literature, depending on the plant species, harvest time and geographical origin of the plant. 2.7-6.5% of 5-CQA was reported by Ky *et al.* employing GCB of species *C. liberica* var. dewevrei from Ivory Coast [40]. Jeszka-Skowron *et al.* found chlorogenic acids content varying from 3.41 % per dry mass in Arabica type from Laos or Rwanda to 8.16 % in Robusta coffee from Indonesia [41].

The content of chlorogenic acid quantified in coffee silverskin is also consistent with the literature. In particular, 5-CQA was extracted at 0.1–0.2% (w/w dry matter) from CS in the temperature range of 25–180 °C by Narita *et al.* [42]. Bresciani *et al*. reported similar content of chlorogenic acid, with an abundance of *circa* 0.2% of 5-CQA in the dry biomass [43]. Subcritical water was also employed by Ginting *et al.* targeting chlorogenic acids, reaching a percentage of 0.27% (w/w dry CS) at 147.8 °C [44].

The solvent-based extraction was supposed to be exhaustive in chlorogenic acids complete extraction from both the delipidized biomasses. After assessing, by consistency with the literature, the effectiveness of extraction of chlorogenic acids from GCB and CS with solvents, data were assumed to represent the reference when studying the recovery of the same phenolics by means of supercritical fluids.

5-CQA was then quantified in all extracts deriving from supercritical extraction and results were compared to solvent-based extraction. When the water solubility of sc-CO_2 samples was low (first extracts obtained during the initial cycles in sc-CO_2), they were first dissolved in dichloromethane and then counter-extracted in water. Only the water phase was injected in UPLC.

In terms of 5-CQA recovery, results are displayed in Figure 3.

Figures 3a and 3b show the % of cumulative yield of 5-CQA (on dry biomass) extracted over time, for each cycle of sc-CO_2 in the presence of the co-solvent. The dashed lines represent the % of 5-CQA (on dry biomass) extracted by means of solvent method. The extractions of 5-CQA from both GCB and CS follow similar kinetics, with an initial absence of chlorogenic acids in the samples, then following again the model by da Silva *et al*. with a steep extraction rate followed by a lower extraction rate, where the mass transfer occurs mainly by diffusion inside the solid particles [32]. The initial period where no 5-CQA is detected in the samples can be explained by a too low wetting of the biomass by the co-solvent, unable to drag out (with the aid of the gas flow) the affine compounds in terms of polarity, such as chlorogenic acids.

For GCB (Figure 3a), the first three cycles were ineffective in 5-CQA extraction, whilst from the 4th cycle onward, 5-CQA was positively found in the extracts. After the 16th cycle, 3.5% of 5-CQA (on dry biomass) was reached. This value is slightly lower, but comparable, to the % of 5-CQA obtained by solvent-based extraction, equal to 4.4%. The extraction was not continued longer, due to amount of co-solvent already used. Further use of organic solvent, higher than 10 times the mass of the solid to be extracted, would not be reasonable to be employed when applying a "green technique" such as supercritical fluid extraction. Regarding chlorogenic acid recovery from CS extraction, as shown in Figure 3b, the first five cycles provided extracts in which 5-CQA was absent. Its extraction began with the 6th cycle. From the 11th cycle onward, the amount of extracted 5-CQA was even higher than what obtained by means of solvent strategy, reaching a maximum extraction yield of 0.37% (the content of 5-CQA in CS when the extraction was run in conventional

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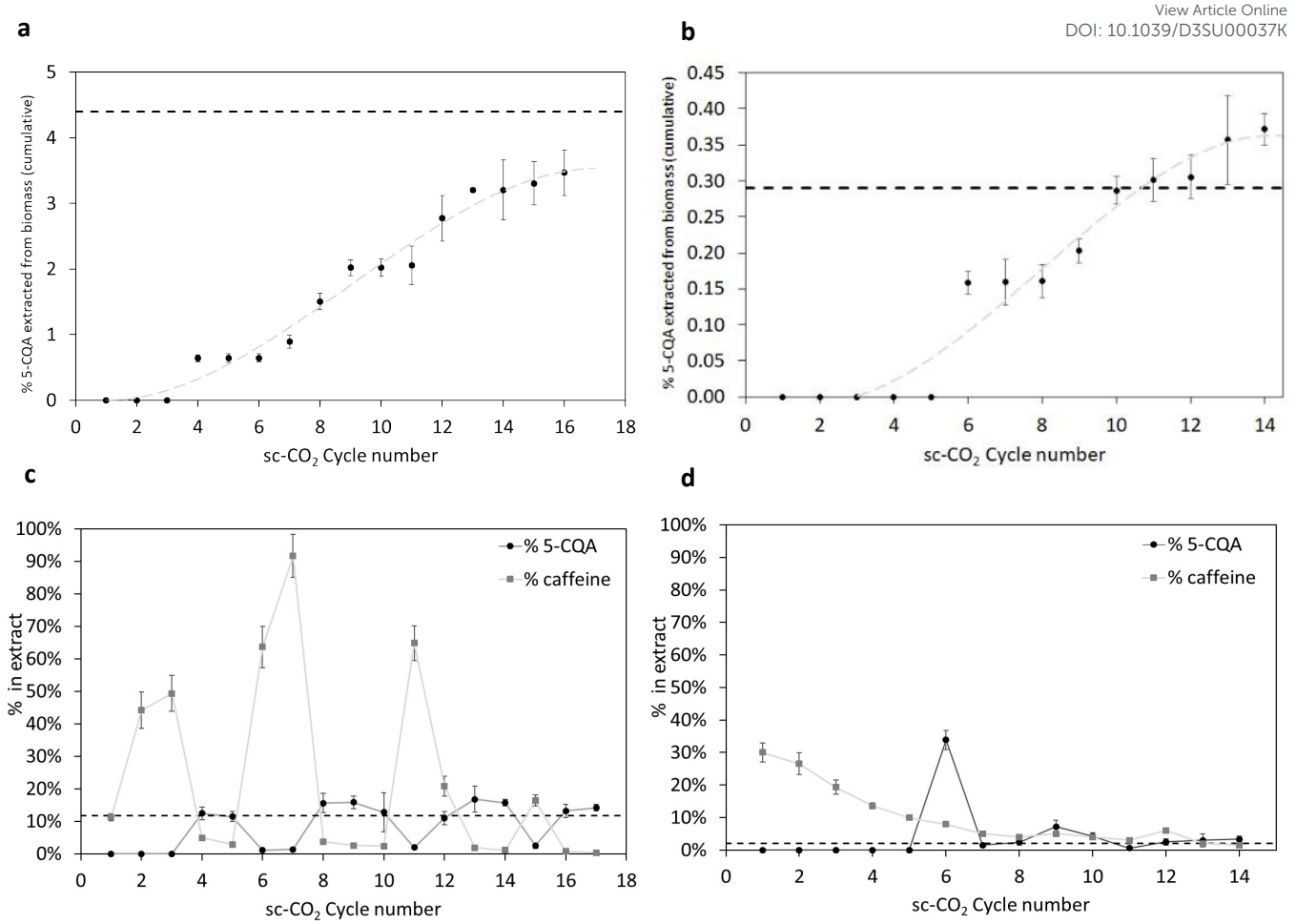

Fig. 3 - Extraction kinetics of 5-CQA (incremental yield on dry biomass vs. progressive extraction cycle) from a) delipidized GCB and b) delipidized CS. % of 5-CQA and % of caffeine in each extract from c) delipidized GCB and d) delipidized CS. Dashed lines represent the % of 5-CQA obtained by solvent-method.

conditions was 0.29%). The micronization of the sample, together with the enhanced penetrability of the fluid mixture in supercritical conditions, might have facilitated the mass transfer process of chlorogenic acids. For the same reason above (not to exceed in the logical amount of co-solvent employed), the extraction was stopped on the 14th cycle.

Besides the 5-CQA recovery, it was also interesting to quantify, cycle by cycle during sc-CO_2 extraction process, the 5-CQA content of each extract, as a necessary dataset to study how the selectivity towards chlorogenic acids varies over the time of sc-CO_2 extraction. Explicative data are displayed in Table 1 in the last column, where it appears evident that supercritical extracts are enriched in chlorogenic acids more than the solvent-based samples, confirming the increased selectivity of the sc-CO_2 technique. GCB solvent-based extract contains only 11.7% of 5-CQA, while one of the extracts obtained in sc-CO_2 reaches 16.8%. Results are even more rewarding when considering coffee silverskin: solvent based extract contains only 2.1% of 5-CQA, while a sc-CO_2 extract reaches 33.9%.

Apart from these data, relative to the most rewarding sc-CO_2 extracts (in terms of 5-CQA content), the % of 5-CQA in each extract changes during the extraction kinetics in supercritical conditions. Figure 3c and 3d are explicative. The dashed lines represent the 5-CQA enrichment in the conventional extracts, while each point of the plot represents the sc-CO_2 samples enrichment in terms of 5-CQA content. For GCB, the trend is oscillating. This might be explained by the non-micronized biomass particles, hindering the mass transfer

processes and hence releasing phenolics in a non-linear way, being trapped inside different channels' sizes and morphologies. Anyhow, most of the extracted samples display an enhanced 5-CQA content compared to the solvent-based extract, with most of the points laying above the dashed line. Interestingly, the caffeine content follows a complementary behavior: samples characterized by a high content of caffeine are composed by low content of 5-CQA and vice versa, as clearly visible in Figures 3c and 3d.

Caffeine is characterized by solubilities in organic solvents that are completely different from the solubilities of chlorogenic acids, almost insoluble in most of organic solvents [45,46].

CQA present in the dry biomass was extracted during the 6th cycle, likely facilitated by the micronized particles that favor the contact between the fluid and the biomass, and hence the mass transfers. This result points out the possibility to interrupt the extraction at this cycle, collecting an extract that is selectively enriched in 5-CQA. On the other side, caffeine follows a decreasing trend, being mostly extracted at the beginning cycles, then decreasing over time and over extraction cycles.

Overall, aiming at extracting polar compounds like phenolics in sc-CO_2, the presence of a co-solvent (such as ethanol) as polarity modifier is demonstrated to be indispensable, justified by the

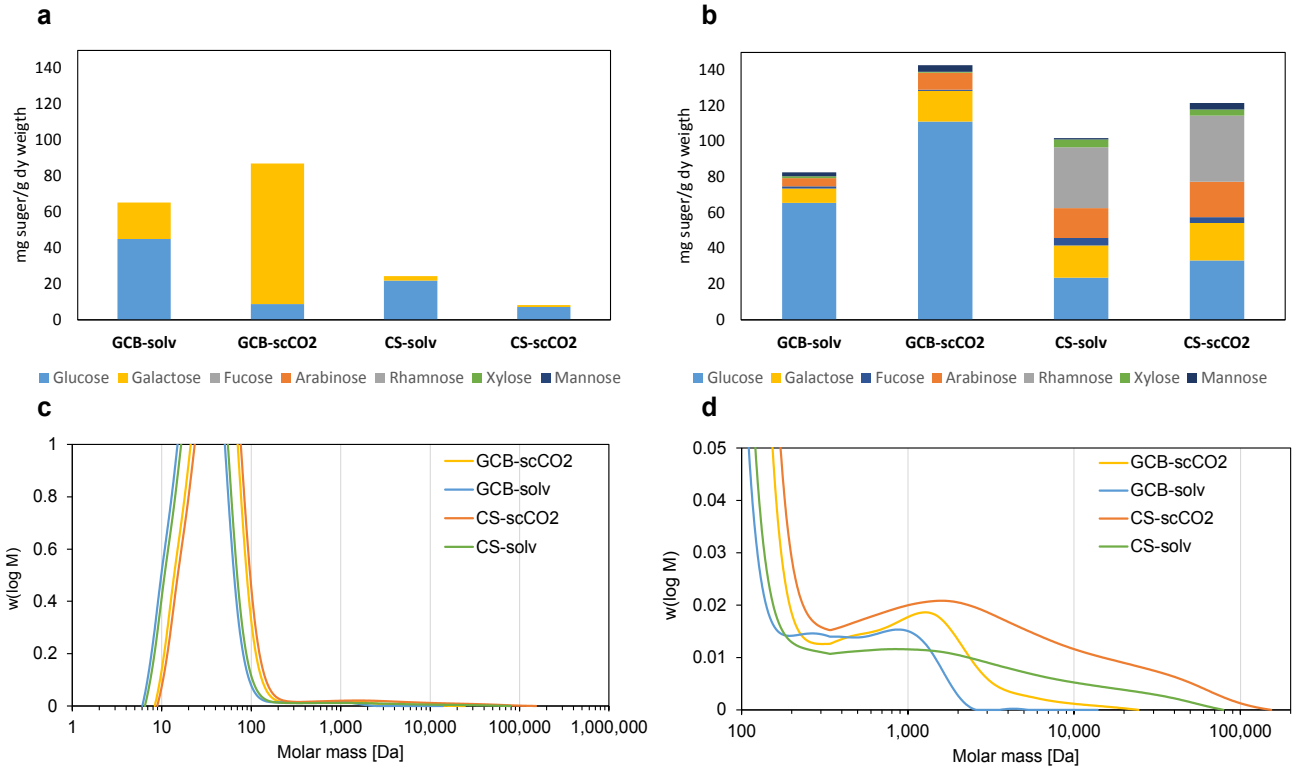

Figure 4 – Monosaccharide profile and molecular weight distribution (Da) of the extracts from conventional solvent extraction and supercritical CO_2 + EtOH. **a)** Carbohydrate composition after direct solubilization; **b)** Carbohydrate composition after TFA hydrolysis; **c)** Complete molecular weight distribution; **d)** Oligomeric and polymeric molecular weight distribution of the extracts after direct solubilization (zooming of c).

When the extraction was performed starting from GCB, caffeine was immediately extracted during the first extraction cycles (caffeine has a higher solubility in pure sc-CO_2 than chlorogenic acids), that were those ones characterized by a lower wetting of the matrix by the co-solvent. Over time (and over cycles) increasing the wetting of the matrix corresponded to an easier extractability of polar compounds, and in fact chlorogenic acids were extracted to a higher extent than caffeine, flushed out from the vessel together with the co-solvent. At the end of this process, the almost complete co-solvent removal corresponded to a decrease in the wetting of the matrix, thus making caffeine more easily extractable in the following cycles. This behavior can explain the alternation in the presence of more caffeine (or more chlorogenic acids) in the extracts, hence related to a higher and lower presence of co-solvent in the vessel. In the case of CS, due to the lower percentages of caffeine and chlorogenic acids present in the biomasses, this phenomenon might have been hidden.

CS extracts behave in a completely diff erent way. The first extract containing 5-CQA (6th cycle) is also the highest one in terms of 5-CQA content (33.9%), exceeding more than 15 times the content of the solvent-based extract (2.1%). About 50% by weight of the total 5-

increase in the solubility of polar compounds in the mixture ethanol/sc-CO_2, compared to the solubility in pure sc-CO_2 [41,47].

Carbohydrates, phenolics and conjugates profile

In consequence of the previous discussion, not only the solubility of target compound increases with the use of co-solvent, but also the diversity of other compounds solubilized by the same solvent, which increases the yield as mentioned above, at the expense of reducing the process selectivity.

In a biorefinery concept, the effect of co-extraction has anyway an interesting economical outcome from the process perspective. The use of conventional solvent extraction methods could solubilize other molecules apart from the targeted phenolic due to similar physicochemical properties or by molecular interactions of the phenolic with other macromolecules in the biomass as carbohydrates or lignin. The effect of sc-CO_2 extraction has been proven for the disruption of the plant cell wall as a pretreatment but also for the solubilization of carbohydrates [48–50]. Based on that, considering the potential co-extraction of different biomass fractions from the same treatment, the characterization of co-extracted

species was performed on both conventional extracts and on the 6th cycles of sc-CO_2 extracts, to understand the profile of the obtained samples. In the case of conventional solvent extraction, the method is based on the capacity of the specific solvent to solubilize polar compounds from a matrix. However, the sc-CO_2 also promotes a physical extraction process by the pressurized conditions on the flow rate in the reactor implying that other polar compounds from the biomass can be co-extracted. Since carbohydrates are the main components of GCB and CS biomasses, they have been analyzed to assess and quantify their co-extraction together with the polar phenolics. For this determination, the initial extracts were characterized by high-performance anion-exchange chromatography-pulsed amperometric detection analysis (HPAEC-PAD) in order to study the presence of free sugars. In addition, acidic hydrolysis of the extracts was performed with trifluoroacetic acid (TFA) aiming to check the presence of complex carbohydrates molecules in the extracts deriving from the biomasses used in the study. The comparison allows understanding the complexity of the carbohydrates in monomeric, oligomeric and polymeric forms co-extracted during the process. In the case of sc-CO_2 conditions, the carbohydrate co-extraction is more important in the case of GCB, as displayed in Figure 4a. After TFA hydrolysis it can be observed a clear increase in the complexity of monosaccharide profiles of the different samples, as displayed in Figure 4b. For instance, the presence of other sugars as fucose, arabinose, rhamnose, xylose and mannose was detected by HPAEC-PAD. The sugars confirm the most probable presence of galactoglucomannans, glucans, arabinogalactans and, in the case of CS, also pectins deducted by the presence of rhamnose. This is in agreement with previous studies on CS for the extraction of pectins [51,52]. The occurrence of those sugars is also followed by an increase of the solubilized glucose by the hydrolytic effect of the polymeric carbohydrate species. However, the galactose content seems to be affected by the TFA hydrolysis, more clearly in the GCB samples, in the case of GCB-sc-CO_2, the galactose after TFA hydrolysis is 20% of the initial free amount Figure 4a. The partial degradation of free sugars has been presented in previous work and discussed in the susceptibility of degradation in the case of arabinose and galactose more than in the case of free glucose [53,54]. From the SEC analysis, it was highlighted that most of the extracts were lower molecular size until 200 Da, clearly represented in Figure 4c, having most of the signal in the small range of the molecular size. The greater increase of monosaccharides after TFA hydrolysis from the CS samples is directly correlated with the presence of longer polymeric fractions, close to 100000 Da presented in the zoom in the Figure 4d. The molecular distribution of the CS extract showed the presence of longer polymeric fraction that were co-extracted by both conventional solvent and sc-CO_2. This also correlates with the lower amounts of free sugars shown in Figure 4a. In the case of GCB, the molecular distribution of the extracts is in the range of oligomers and short polymers, populations around 2000 Da for sc-CO_2 extract and 10000 Da for conventional solvent extraction. The use of LC-ESI-MS before and after saponification of samples allowed the identification of certain alternative phenolic molecules in monomeric forms such as ferulic acid at m/z=195 $[M + H]^+$ and m/z=177, corresponding to its dehydrated adduct $[M + H - H_2O]^+$, caffeic acid at m/z=181 $[M + H]^+$ and m/z=163 $[M + H - H_2O]^+$, as well as certain dimeric forms such as di-ferulic acid at m/z=369 $[M + H - H_2O]^+$ and m/z=351 $[M + H - 2H_2O]^+$ as reported in Table S1. These fractions, commonly present in other lignocellulosic materials as cereal brans [55], were also identified by comparing with available standards (reported in Figure S2). Moreover, it was possible to identify the presence of carbohydrates-phenolic conjugates at m/z=509 (feruloyl hexo-pentose), m/z=345 (caffeoyl hexose) and m/z=329 (feruloyl pentose), distinctly present in the CS samples, structures presented previously in other types of biomasses as tomatoes or cereals [56,57]. In the case of GCB, the possible presence of conjugates is only reported after sc-CO_2 treatment at m/z=499 (caffeoyl hexo-pentose) and m/z=367 (caffeoyl hexose), demonstrating the increased potential of co-extraction of this technique (Figure S3). The different elution times could correspond with different linkages in the sugar-phenolic conjugates. These results correlate with the previously mentioned co-extraction of phenolic with carbohydrates reported in Figure 4. In the case of sc-CO_2 is the first time, in our knowledge, that these conjugates are recognized after extraction. Other studies using subcritical water extraction have proven the protection of the link between carbohydrates and phenolic moieties present in the natural cell wall extracts [27]. These conjugates could have a great interest as prebiotics or as building blocks for functional materials since the phenolics are able to provide their characteristic antioxidant properties to the carbohydrates fraction [58,59].

Conclusions

Results point out the ability of the supercritical fluid to efficiently target polar compounds, such as chlorogenic acids in the presence of other beneficial co-extracted compounds, minimizing the excessive use of organic solvents and avoiding the presence of inorganic or organic acids conventionally employed when solvent-based methods are used. To the best of authors' knowledge, no other works are present in the literature targeting chlorogenic acids from coffee silverskin employing sc-CO_2 strategy. Under the view of the application, the feasibility of the technique of supercritical CO_2 in the presence of a co-solvent should meet a solvent usage threshold in order to be environmentally and economically convenient in comparison to conventional solvent-based methods. For example, lesser use of organic co-solvent is advisable and should be the object of future studies and technological improvemments, in order to further reduce the use of ethanol/water and to decrease the time and energetic requirements for solvents removal.

The scalability of sc-CO_2 technology is not well-documented in the literature, and future studies are needed to better understand the practical application of this technology and their related implications when co-solvents are employed.

The advantage of sc-CO_2 connects also with the possibility to recover extracts in different batches over time, since it requires alternation of static and dynamic periods. Extracts separation over time allows a selection and isolation of the most promising, as the ones characterized by high polyphenols content and negligible amount of caffeine. This profile is desirable since caffeine itself has some biological properties that are not always compatible with the nutraceutical purposes of extracts containing chlorogenic acids.

Overall, results further confirm that CS can be considered as a good raw material for the production of chlorogenic acids-enriched extracts, with the advantage of being cheaper and more sustainable than green coffee beans. A very interesting application could be related to prebiotics in food products such as yogurt and derivatives, since coffee chlorogenic acids have been already demonstrated to increase the growth of total bacteria to a similar magnitude to fructo-oligosaccharides, which are the positive prebiotic control [60]. This assertion could also be supported by the fact that a considerable proportion of ingested chlorogenic acids reaches the large intestine, giving them the possibility of exerting beneficial effects in the large gut [61]. Upcoming studies are desirable to assess the nutraceutical and antioxidant profiles of these extracts in order to confirm the potential commercial intererest in this ambitious and virtuous "waste-to-products" valorization chain.

Author Contributions

Data curation: S.M., A.M., R.N., A.Y.Q.; Funding acquisition: L.V., S.M., A.Y.Q.; Investigation: A.M., R.N., S.M., A.Y.Q.; Project administration: L.V., S.M., A.Y.Q.; Supervision: L.V., S.M., A.Y.Q.; Conceptualization: L.V., S.M., R.N., A.Y.Q.; Validation: A.M., R.N., S.M., A.Y.Q.; Visualization: A.M., R.N., S.M., A.Y.Q.; Writing – original draft: S.M., A.Y.Q.; Writing – review & editing: A.M., R.N., L.V., S.M., A.Y.Q.

Conflicts of interest

There are no conflicts to declare.

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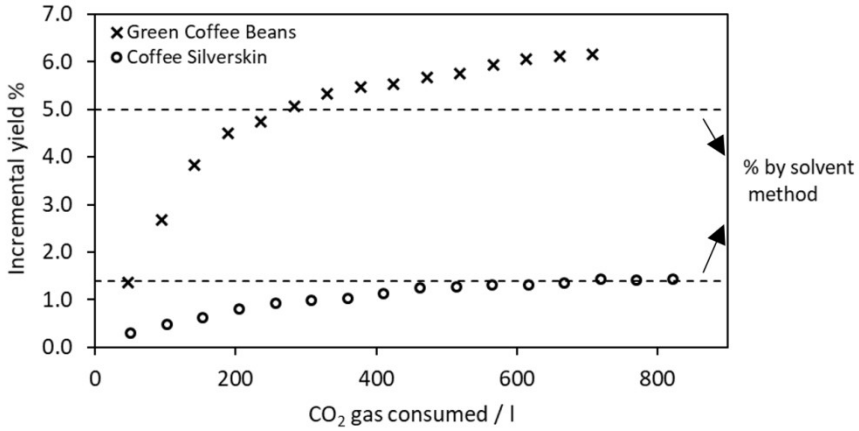

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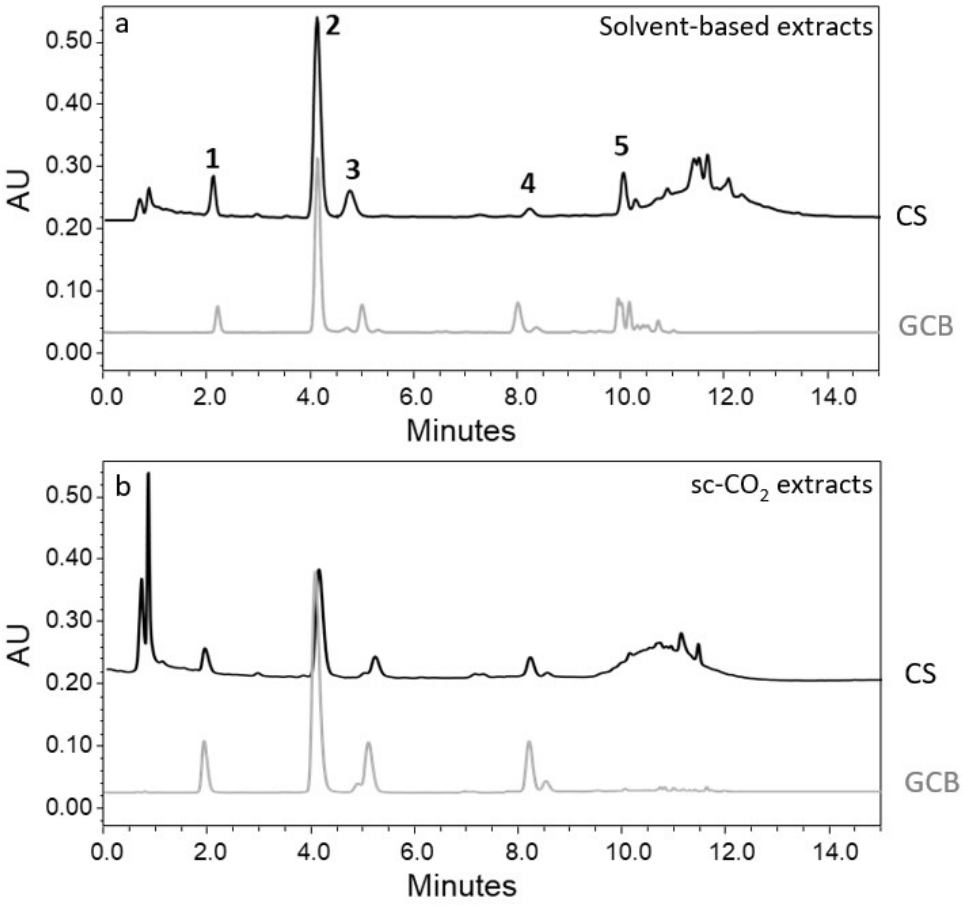

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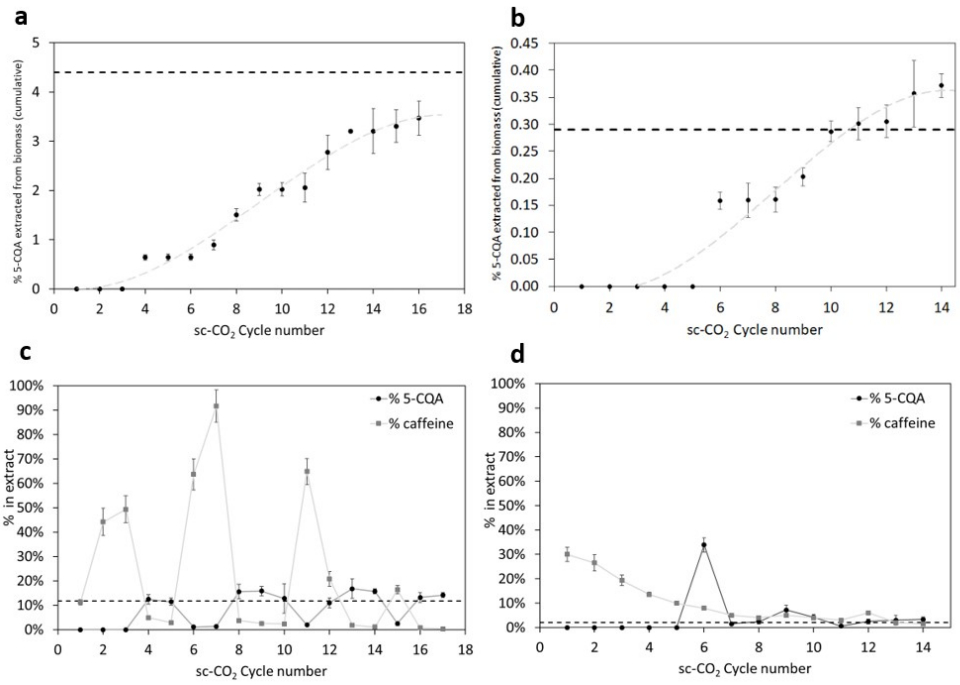

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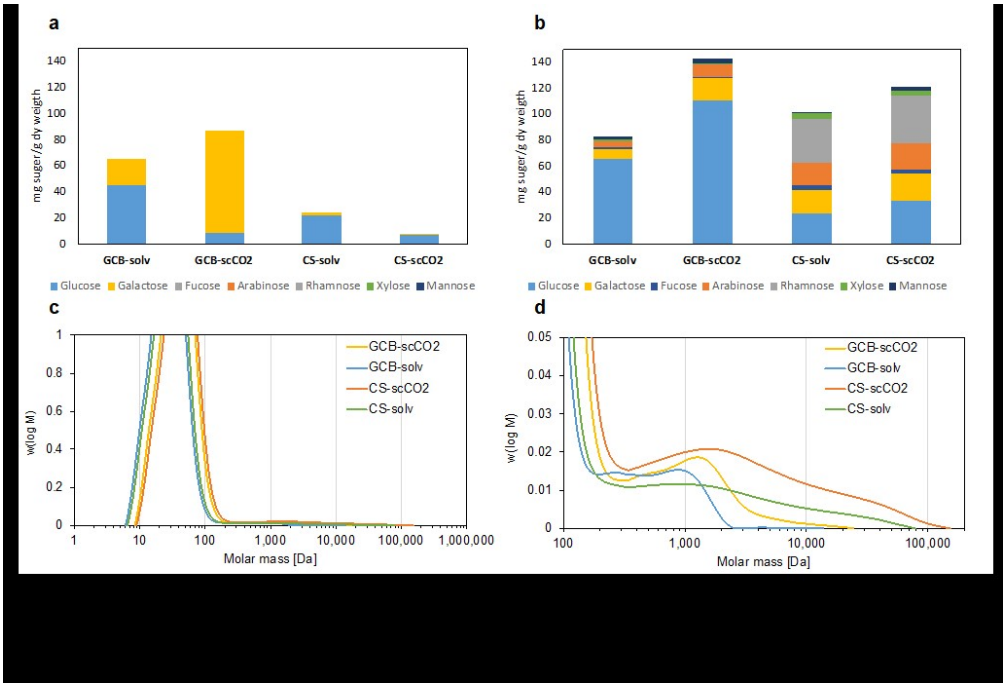

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