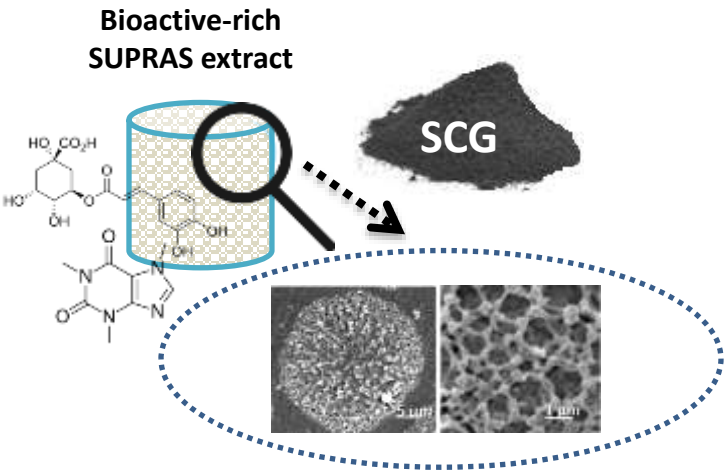
Graphical abstract



Highlights

- SUPRAS are applied for first time for valorization of food waste (spent coffee grounds)
- Bioactive compounds were extracted in an good rate for industrial applications
- SUPRAS extracts showed high polyphenolic content and antioxidant activity
- SUPRAS extracts exhibited antimicrobial activity (gram-negative bacteria)

Valorization of spent coffee grounds by supramolecular solvent extraction

Laura Sofía Torres-Valenzuela [a,b], Ana Ballesteros [a*], Alejandra Sanin [b], Soledad Rubio[a]

[a] Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Universidad de Córdoba, Cordoba 14071, Spain

[b] Faculty of Engineering, Universidad La Gran Colombia, Armenia 630004 Colombia

[*]Corresponding author, email: ana.ballesteros@uco.es

Abstract

In this study, we assess the potential of supramolecular solvents (SUPRAS) for valorization of spent coffee grounds (SCG). SUPRAS, made up of self-assembled amphiphilic aggregates dispersed in an aqueous or hydro-organic medium, are excellent extractants that provide multiple binding interactions (hydrogen bonds, dispersion, dipole-dipole, etc.) and microenvironments of different polarity due to their special internal architecture. In this work, SUPRAS made up of different amphiphiles (decanoic acid and hexanol) and hydro-organic media (water-ethanol and water-tetrahydrofuran) were investigated for extraction of bioactives from SCG. Extraction was optimized from the yield obtained for caffeine and 5-chlorogenic acid, that were considered as model compounds. Under optimal extraction conditions, the profile of bioactive compounds in the extracts was screened by liquid chromatography tandem mass spectrometry and the total phenolic content was estimated. The antioxidants and antimicrobial properties of the extracts were also evaluated. Bioactive compounds were extracted from wet SCG up to 3.32 $mg.g^{-1}$and 4.3 $mg.g^{-1}$ SCG of caffeine and chlorogenic acid, respectively. Extracts showed antioxidant capacity by different assays (DPPH, TEAC, FRAP) in accordance with their high total phenolic content (60.1 mg CGA per mg of extracted dry SCG). SUPRAS offered advantages in terms of rapidity (extraction for 1 min) and simplicity (the process involved stirring and centrifugation at room temperature), thus avoiding costly processes based on high pressure and temperature. Furthermore, SUPRAS extracts exhibited certain degree of antimicrobial effects against, *S. aureus* and *B. cereu*s and a high effect against *S. enterica* and *P. putida.*

Keywords: supramolecular solvents; spent coffee grounds; bioactive compounds; valorization

1. Introduction

The agricultural world production is continuously increasing as a result of the rising global demand for food and generates billion tons of by-products each year [1]. There is a growing interest in the recovery of bioactive compounds from agro-waste for application in functional foods and nutraceutical formulations [2]. The coffee industry alone generates about 2 billion tons of agro-waste, which represent a great pollution hazard [3]. Coffee pulp, husks, silverskin, peel and spend coffee grounds are common coffee by-products [4] and have been reported of interest as substrates for mushroom cultivation [5], immobilization of enzymes [6], production of bioethanol [7] composting [8], and extraction of bioactive compounds [9–11].

Spent coffee grounds (SCG), a high humidity residue (up to ~80%) obtained in coffee beverage preparation and instant coffee manufacturing, is the most abundant coffee by-product (45-50%) [12,13]. SCG is produced at a rate of 6 million tons a year [12]. Valorization of coffee by-products through the recovery of bioactives, particularly alkaloids and polyphenols, has increasingly become of interest for food, pharmaceutical and cosmetic industries [13–16]. The major alkaloid in coffee by-products is caffeine, which shows anti-inflammatory and immunosuppressant effects [16]. Regarding polyphenols, they include a broad range of compounds including tannins, flavanols, flavones, anthocyanins, proanthocyanidins, and phenolic, hydroxybenzoic and hydroxycinnamic acids [17]. Polyphenols have demonstrated antioxidant, anti-bacterial, anti-inflammatory and anti-carcinogenic activities [13–15].

Extraction of bioactives from SCG has been investigated using different solvents and techniques, including conventional solid-liquid extraction (SLE) [18,19], supercritical fluid extraction (SFE), with and without co-solvent [20], Soxhlet extraction [20], and ultrasound (USAE) [20–22] or microwave (MAE) [21,23] assisted extraction. Extraction efficiencies, usually given as total phenolic compounds (TPC) and expressed as gallic acid (GAE) or chlorogenic acid (CAE) equivalents [24], are highly dependent on the type of solvent, the solvent/solid ratio, the number of extraction steps and the extraction time and temperature, among others factors [15]. Extractions have been carried out using polar (e.g. methanol and ethanol) and medium or non-polar (e.g. dichloromethane, ethyl acetate, hexane) solvents [18–20,23], supercritical fluids [20],

subcritical water [21], and deep eutectic solvents [22]. Common conditions for conventional SLE include solvent/solid ratios of around 30-40 mL/g SCG, extraction temperatures in the range 50-65 ºC and extraction times for 1-2 h, which give extraction efficiencies for TPCs of about 16-18 mg GAE/g SCG [18–20]. Extraction efficiencies for TPCs in SFE increase in the presence of ethanol as co-solvent (e.g. around 42 mg CAE per gram of extract using 8% ethanol, which is equivalent to ~4 mg CAE/g SCG taking into account yields of about 10%) [20]. Extraction of phenolic compounds have been also reported for energy-assisted techniques, namely Soxhlet extraction [20], USAE [20] or MAE [23]. Thus, TPCs were in the range 119-167 mg CAE/g extract (18-22 mg CAE/g SCG; extraction yields 12-15%) with Soxhlet extraction using solvents of different polarity, solvent /solid ratios of 30, and 6 h of extraction at the boiling temperature of the solvent [20]. Likewise, the application of USAE for 2 h, at room temperature and solvent/solid ratios of 30, permitted to achieve extraction efficiencies for TPCs in the range of 221-588 mg CAE/g extract (21.9-71.7 mg CAE/g SCG; extraction yields 10-12%) [20]. Application of MAE was also assessed; it provided up to 399 mg GAE/g extract (21.5 mg/g SCG; extraction yield 5.4) with 40 s of irradiation and a solvent/solid ratio of 9 [23]. All these figures indicate that SCG is a valuable source for bioactives and that further research should be intended to reduce extraction efforts in order to make their valorization simpler and more cost-effective.

In this paper, we propose for the first time the use of supramolecular solvents (SUPRASs) for the extraction of bioactives from SCG. SUPRASs are nanostructured liquids spontaneously produced in colloidal suspensions of amphiphiles through a bottom-up approach based on sequential self-assembly phenomena [25,26]. The synthesis is made by a simple two-step process. First, amphiphiles spontaneously assemble into three-dimensional individual aggregates (mainly micelles and/or vesicles). The second stage generates a new highly packed phase by the assembly of the aggregates into a nano or microestructured liquid (SUPRAS phase). This second phase is triggered by an external stimuli such pH or temperature changes, addition of salt or addition of a poor solvent for the amphiphile, which diminishes the repulsion among the aggregates and promotes their assembly [25]. The SUPRAS phase remains in equilibrium with the bulk solution, which contains the amphiphile at the critical aggregation concentration. SUPRAS can be collected and stored if required (keeping its structure and properties) for

application to solid samples or applied together with the equilibrium solution, which acts as a wetting and dispersion phase for the matrix [27].

The capability of SUPRASs for developing efficient processes for extraction of bioactives is based on the presence of different polarity microenvironments into their ordered structures, the high concentration of amphiphiles make up them (up to 1 mg/µL), and the possibility of producing tailored SUPRASs by selection of the amphiphile or the environment for self-assembly [28]. Thus, SUPRASs are able to efficiently extract compounds spanning a wide polarity range using low solvent/solid ratios [27]. On the other hand, SUPRASs with restricted access properties (SUPRAS-RAM) have been reported that permit the extraction of low molecular weight compounds while excluding macromolecules [29]. These properties have allowed the development of innovative strategies for sample preparation in the determination of organic contaminants and metals in food, the environment and biological fluids [25,26]. More recently, SUPRASs have also proved promising for the extraction of bioactives from microalgae [30] and the removal of contaminants in wastewater [31].

The suitability of SUPRASs for the extraction of bioactives from SCG obtained by the drip filter method was here explored. For this purpose, two types of SUPRASs, synthesized from decanoic acid [32] and hexanol [33] in hydro-organic media (water and ethanol or tetrahydrofuran) were investigated. Extraction efficiencies were evaluated by monitoring caffeine and chlorogenic acid, two major representatives of alkaloids and polyphenols, respectively. Under the optimized conditions, the SUPRAS extracts were further analysed to identify the main bioactives, to estimate their total phenolic content and evaluate their antioxidant and antimicrobial properties. Below, the more relevant results are presented and discussed.

1. Materials and methods

1.1 Chemicals

Caffeine (1,3,7-trimethylxantine, HPLC grade), 5-chlorogenic acid (5-O-Caffeoylquinic acid, 5-CGA, 98%), (±)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (98,1%, Trolox), 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2 diphenyl-1-picrylhydrazyl (DPPH), decanoic acid (98%), ethanol (HPLC grade), methanol (99,9%), 2,3,5-triphenyltetrazolium chloride (TTC), glacial acetic acid and tetrahydrofuran (HPLC grade) were purchased from Sigma–Aldrich Co. (St. Louis, USA). 1-hexanol (98%), hydrochloric acid (37%), and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were supplied by Merck (Darmstadt, Germany). Potassium persulfate was purchased from Panreac (Barcelona, Spain), ferric chloride from Carlo Erba (Val-de-Reuil, France) and potassium acetate (99,4%) from JT Baker (Madrid, Spain). All chemicals were analytical reagent-grade and were used as supplied. Pure water was prepared using a Milli-Q, Ultrapure water purification system equipped with a 0.22-µm filter (MA, USA).

Three reagents were prepared for evaluation of the antioxidant capacity of SUPRAS extracts containing coffee bioactives. The DPPH reagent was freshly prepared by dissolving 1 mg of DPPH in 50 mL of methanol and diluted with methanol to give an absorbance of 1.057±0.005 at 529 nm. It was kept in the dark at room temperature when not used. The $ABTS^{+}$ radical reagent was freshly prepared by dissolving 97 mg of ABTS and 16.5 mg of potassium persulfate in 25 mL of distilled water and keeping the solution for 16 hours under dark. Then, it was diluted with ethanol to yield an absorbance of 0.635±0.005 at 732 nm. The reagent FRAP (ferric reducing antioxidant power) was prepared by the mixing of three solutions in a thermostatic bath at 35 ºC; 250 mL of acetic acid/acetate buffer (40 mM, pH 3.6), 2.5 mL of an aqueous solution of ferric chloride (20 mM) and 2.5 mL of TPTZ (10 mM) in 40 mM HCl. The absorbance of the reagent solution was 0.107±0.005 at 595 nm.

1.2 Apparatus

A high-performance liquid chromatograph (HPLC) coupled to a UV Detector (Shimadzu, Japan) was employed for the quantification of caffeine and 5-CGA. The stationary phase was an Ultra C_8 column (5 µm particle size, 150 mm length, 4.6 mm i.d.) from Restek (France). All data were acquired and processed using the LabSolutions Software (Shimadzu, Japan). For the target screening of bioactive compounds in SUPRAS extracts under optimal conditions (section 2.5) we performed LC-MS/MS analysis. The equipment consisted in an Agilent Technologies 1200 LC system with a column ACE 3 C18-PFP column (3 mm i.d., 150 mm length, 3.0 µm particle size) preceded by a precolumn Phenomenex KJ 0-4282 Security Guard Cartridge Kit, Ea. The detector was an Agilent Technologies 6420 Triple Quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in negative and positive modes. Raw data were controlled and processed using Agilent MassHunter Software® (version B.07.00). Other instrumentation used for sample preparation were a vortex-shaker REAX Top (Heidolph, Schwabach, Germany) and a 12 x 1.5 – 2 mL angle rotor Minicen centrifuge from Ortoalresa (Madrid, Spain). Optimization of the extraction of coffee byproducts was carried out in 2 mL-microtubes Safe-Lock from Eppendorf Iberica (Madrid, Spain). A vortex shaker from Vorterex (Heathrow Scientific, Vernon Hills, IL, USA) with an attachment for 4 tubes, and a high-speed brushless centrifuge BX 24 (Unico, USA) were used for sample preparation. Antimicrobial activity was evaluated in a laminar flow cabinet Physis (AirFlux, Malasya).

1.3 Spent coffee grounds

Spent coffee grounds (SCG) were obtained from a drip filter brewing method consisting in flowing water at 92–96 °C through a ground coffee bed so that the extract drips from the brewing chamber into the pot. The coffee used in all the experiments was the variety Castillo produced in Circasia (Colombia). The water content in the SCG was 74.0±0.8%. SCG samples were not dried and immediately processed or stored at -18 ºC.

2.4. SUPRAS production

SUPRASs of different composition were produced by adding ultrapure water to a colloidal suspension of decanoic acid or hexanol in THF or ethanol (total volume of the mixture: 2 mL). Under addition of water, the decanoic acid or hexanol aggregates in the colloidal suspension gave spontaneously oily droplets that associated as clusters and finally separated from the bulk solution as a new liquid phase named SUPRAS. The whole solution, containing both the SUPRAS (at the top) and the hydro-organic equilibrium solution, was added to the SCG. Figure 1 shows a schematic of the general procedure followed for SUPRAS production.

2.5.SUPRAS-based extraction of bioactives from SCG

The following variables were considered for the optimization of SUPRAS-based extraction of bioactives from SCG: (a) type of organic solvent used to produce the colloidal suspension (ethanol or THF); (b) type of amphiphile (decanoic acid or hexanol) making up the SUPRAS; (c) amphiphile concentration in the SUPRAS synthetic solution (8, 16 and 24 % v/v), and (d) organic solvent concentration in the SUPRAS synthetic solution (20, 30 and 40 % v/v). The extraction of bioactives was performed by adding 0.35 g of wet SCG to the SUPRAS synthetic solution (see section 2.4) in polypropylene centrifuge microtubes. The composition of the SUPRAS synthetic solution was varied as follows: hexanol or decanoic acid (66-574 μL), ethanol or THF (176-1024 μL) and distilled water (656-1560 μL). The sample size was kept constant at 0.35 g to ensure good sample dispersibility at the SUPRAS volume/sample size ratio that was set for the laboratory scale. The mixtures were vortex-shaken for 1 min at 3,000 rpm for the extraction of bioactives and then centrifuged for 20 minutes at 4,519 g to accelerate the separation of SUPRAS from the bulk equilibrium phase (in the middle) and precipitate (at the bottom). The volume of SUPRAS was measured using a digital caliper [33]. The volume of SUPRAS produced varies under different synthetic conditions (usually increasing with the concentration of both the amphiphile and the organic solvent) and consequently this affects concentration factors (ratio of SUPRAS volume/sample size). SUPRAS volumes varied in the range 61 – 1476 μL under the tested conditions. Experiments were done in triplicate. Figure 1 shows a schematic picture of the SUPRAS extraction procedure.

The final optimal SUPRAS synthesis conditions were 24% v/v hexanol and 30% v/v ethanol. The average SUPRAS volume was 980±10 μL (2.8 mL SUPRAS/g wet SCG). These conditions were finally tested for identification of bioactives, estimation of the total phenolic content and antioxidant and antimicrobial activity

2.6.Analysis of caffeine and chlorogenic acid by HPLC-UV

Caffeine and 5-CGA acid contents in the SUPRAS extracts were determined by HPLC-UV. The detector wavelength was set at 254 nm. The mobile phase consisted of 69.9% v/v of water, 30% v/v of methanol and 0.1% v/v of acetic in isocratic mode. The flow rate was set at 0.6 mL min^{-1} and the sample injection volume was 20 μL. Quantitative analysis was conducted by external calibration using standard solutions of caffeine and 5-CGA prepared in ultrapure water in the concentration range of 5 – 100 μg L^{-1}.

2.7.Profile of bioactive compounds in SUPRAS extracts by HPLC-MS/MS and estimation of total phenolic content

The presence of the main bioactives compounds present in SUPRAS extracts under optimal conditions (section 2.5.) was confirmed by target screening with LC-MS/MS experiments. The mobile phase was made up of Milli-Q water with 0.1% acetic acid (A) and MeOH:Acetonitrile 50:50 v/v (B) at a flow rate of 0.3 mL·min^{-1}. The injection volume was 5 μL. The gradient was as follow: initial 5% B hold for 0.1 min, linear gradient to 30% B in 25 min and to 40% B in the next 10 min. Finally, B was increased to 100% at 35.1 min and maintained for 10 min to remove possible hydrophobic compounds form the column. The column was re-conditioned for 10 min before injection. The MRM transitions for target masses of the bioactives identified in SUPRAS extracts are given in Table 1. The MS parameters were: fragmentor 100 V, collision energy 15 eV, cell accelerator voltage 4 V, dwell 20 ms. Source parameters were: gas temperature, 350°C; gas flow, 12 L·min^{-1}; nebulizer gas pressure, 30 psi; capillary voltage, -4000 V. Total phenolic content was estimated from the sum of chromatographic peaks of the identified phenolic compounds with external calibration against 5-CGA, due to the lack of authentic standards for all of them.

2.8.Antioxidant activity assays

The antioxidant activity of the SUPRAS extracts obtained under the optimal conditions specified in section 2.5 was evaluated by the DPPH, TEAC [34] and FRAP [35] methods. Control assays with Trolox were run in parallel for TEAC. The decrease of the absorbance of the reagent solutions, measured as inhibition, was calculated from the following equation [34]:

$$\%inhibition = \frac{Abs_0 - Abs_{30}}{Abs_0} * 100$$

where Abs_0 is the absorbance of DPPH, $ABTS^+$ or FRAP reagent solution at time zero and Abs_{30} is the absorbance of the reagent in the presence of the bioactive coffee compounds at 30 minutes of reaction (as mentioned below).

2.8.1. DPPH radical scavenging assay

Aliquots of 100 µL of SUPRAS (previously diluted in 1:10 with methanol) or methanol as blank were mixed with 2 mL of DPPH solution. The mixture was vortexed for a minute and placed in the dark for 30 min. Finally, the absorbance of the mixture was measured at 529 nm. The final concentration of extract tested was ~4.1 mg SUPRAS extract /mL.

2.8.2. Trolox equivalent antioxidant capacity (TEAC) assay

The assays were made by mixing 50 µL of methanol as blank or SUPRAS extracts (previously diluted in 1:10 with methanol) and 1450 µL of the free radical $ABTS^+$ stock solution prepared as indicated in section 2.1. The mixture was vortexed for a minute and placed in the dark for 30 min. The absorbance was measured at 732 nm and the percentage of inhibition was referred to TEAC. The final concentration of extract tested was 2.7 mg SUPRAS extract/mL.

2.8.3. Ferric reducing antioxidant potential (FRAP) assay

Aliquots of 30 µL of SUPRAS extracts (previously diluted in 1:10 with methanol) or methanol as blank, 90 µL of water and 900 µL of the FRAP reagent were mixed and incubated during 30

minutes at 37 ºC. The absorbance was measured at 595 nm. The final concentration of extract tested was 2.5 mg SUPRAS extract/mL.

2.9. Antimicrobial susceptibility testing method

The colorimetric broth microdilution method with 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (TTC) [36,37] was used to determine the lowest concentration of the assayed antimicrobial agent (minimal inhibitory concentration, MIC). Suspensions of *S. enterica* (ATCC 0363), *S. aureus* (ATCC 0496), *P. putida* (ATCC 49128) and *B. cereus* (ATCC 14579) were growth at 37°C in Tryptic Soy broth (TSB) until a concentration of 10^6 colonies forming units (cfu)/mL was reached. Initially, 100 µL of TSB with 1% of TTC (indicator of metabolic activity) were added in each well of a sterile 96-well microplate followed by 100 µL of SUPRAS extracts (undiluted and diluted at 1:10 and 1:100 with distilled water). Finally, 100 µL of the previously standardized microorganisms were inoculated. Final extract concentrations were 287, 28.7 and 2.87 mg SUPRAS extract/mL. After incubating for 24 hours, a color change in the wells was observed and those showing microbial growth were pink-colored.

3. Results and discussion

3.1.SUPRAS-based extraction of bioactives from SCG

The ability of SUPRASs to develop efficient and cost-effective processes for extraction of bioactives from SCG was evaluated by monitoring the extraction yield for caffeine and chlorogenic acid (5-CGA), which were selected as model compounds for alkaloids and polyphenols, respectively. These compounds can establish donor and/or acceptor hydrogen bonds, and polar and dispersion interactions, so the components making up the SUPRAS were selected to maximize these types of interactions.

Two amphiphiles (decanoic acid and hexanol) and two hydro-organic media (THF:water and ethanol:water) were chosen for SUPRAS production. Both, carboxylic acids [32] and alkanols [29] have been reported to give SUPRASs made up of inverted hexagonal aggregates where the

polar groups surround aqueous cavities and the hydrocarbon chains disperse in the organic solvent (see schematic in Figure 1). The amphiphile functional groups (-OH, -COOH) provide hydrogen bonds and polar interactions, while the alkyl chains give dispersion interactions, so both alkaloids and polyphenols can be solubilized in the hexagonal nanostructures of the SUPRAS by mixed mode mechanisms, which should enhance extraction. On the other hand, ethanol and THF, used to produce the colloidal suspension of the amphiphile, were selected on the basis of their different polarity, which should also influence the extraction of the target compounds.

Optimization of the SUPRAS-based extraction was carried out according to the procedure specified in section 2.5. The SCG obtained by the drip filter method were subjected to extraction as collected (viz. without drying the by-product) in order to reduce costs and speed up the valorization process. Although bioactives in the SCG were solubilized in the SUPRAS, the equilibrium solution generated in SUPRAS formation (see Figure 1) was also used in the extraction process with the aim of facilitating both the dispersion of the SCG and the SUPRAS extract overflows.

Figures 2 and 3 show the average extraction recoveries obtained for caffeine and 5-CGA, respectively, when the SCG were subjected to extraction with each of the SUPRAS investigated. Results are expressed as mg of bioactive per g of dry SCG in order to facilitate comparison with previous reported procedures. Each SUPRAS was produced at different proportions (expressed as volume percentages) of the ternary mixture (viz. amphiphile:organic solvent:water), which permitted to vary both SUPRAS composition and volume [29,32]. Thus, increased volume of SUPRAS was obtained by increasing the concentration of the amphiphile at constant organic solvent/water volume ratios in the synthesis. On the other hand, increased volume of SUPRAS was obtained by increasing the organic solvent/water volume ratios in the synthesis at constant amphiphile concentration.

According to the results (Fig. 2 and 3), hexanol was better extractant for both caffeine and 5-CGA than decanoic acid. The stronger hydrogen bonding ability of hexanol over decanoic acid (which is related to its shorter alkyl chain length) could explain this behavior. In general,

recoveries for both bioactives increased or kept constant as a function of amphiphile concentration, at least in the range 8-24%, due to the increase of available binding interactions. Regarding the organic solvent, maximal extraction yields were usually obtained for 40% of THF and 30% of ethanol, being the recovery slightly greater for ethanol. Since, in addition, this solvent is more biocompatible and authorized for use in author industry, ethanol was selected for the production of the colloidal suspension of hexanol. Hexanol is also an authorized food additive by FDA and EU (flavouring substance).

The maximum extraction rates of caffeine and of CGA (expressed both in dry weight) were 3.32 ± 0.07 mg g^{-1} and 4.3 ± 0.1 mg g^{-1}, respectively, by extraction of the SCG with a SUPRAS obtained from 24% v/v hexanol, 30% v/v ethanol and 46% v/v water. These extracts were selected as optimal for further characterization of functional properties.

The contents of caffeine and 5-CGA in SCG have been reported to be highly dependent on the extraction process and the SCG source [13, 38,39]. Caffeine and 5-CGA contents were previously reported in the ranges 3.59-8.09 $mg.g^{-1}$ and 1.18-3.59 $mg.g^{-1}$, respectively, in freeze-dried SCG from *Robusta* and *Arabica* varieties. The extraction procedure involved the drying of the SCG, the defatting with petroleum ether (1:11, w/v) for 3 h at 60 °C in a Soxhlet extraction system, the extraction of the SCG residue with water at 90 ºC for 6 min (16 mL/g SCG) and the freeze-drying of the extract [38], which is not cost-effective for SCG valorization. The concentration for both caffeine and 5-CGA obtained by the SUPRAS-based extraction were similar to those previously reported for the drip filter method [38] taking into account that actual concentrations will be influenced by coffee variety and roasting degree [39].

Since the optimization was done on the basis of caffeine and 5-CGA only, optimal SUPRAS extracts were further analysed by LC-MS/MS to confirm the presence of common bioactives expected in SCG (alkaloids, phenolic compounds and niacin) [40]. Abundant MS peaks corresponding to *n*-O-dicaffeoyl quinic acids, *n*-O-feruloylquinic acids, *n*-O-caffeoylquinic acids, *n*-O-feruloylquinic lactones, *n*-O-coumaroylquinic acids, *n*-O-caffeoylshikimic acid, *n*-O-caffeoylquinic lactones, caffeine, niacin, trigonelline and N-methylpyridinium were obtained

(Table 1). Fig. 4 A and B shows the MRM chromatograms recorded in negative and positive acquisition modes (only the most abundant isomer of the main classes are labelled).

3.2.Functional and microbiological properties of SUPRAS extracts

3.2.1. Total phenolic content (TPC)

Phenolic compounds are the main contributors to the strong antioxidant activity of coffee brews and processing by-products [39]. The use of HPLC measurements instead of the standard Folin-Ciocalteu assay has been recommended by different authors to avoid overestimation due to the presence of reducing sugars, proteins and ascorbic acids, among others [41,42]. As mentioned before, different isomeric peaks of the major groups of polyphenolic compounds present in SCG were identified, namely *n*-O-dicaffeoyl quinic acids (n=4), *n*-O-feruloylquinic acids (n=4), *n*-O-caffeoylquinic acids (n=4, being 5-CGA the most abundant), *n*-O-feruloylquinic lactones (n=5), *n*-O-coumaroylquinic acids (n=2), *n*-O-caffeoylshikimic acid (n=2) and *n*-O-caffeoylquinic lactones (n=2) . Their concentration were estimated by external calibration against 5-CGA due to the lack of authentic standards for all of them.

The TPC obtained with the SUPRAS extraction at optimal conditions (see section 3.1) was 14,4 ± 0.5 mg CGA/ g wet SCG (equivalent to 60.1 mg CGA per mg of extracted dry SCG). This value was near the TPC reported for USAE (71.7 mg CAE/g SCG, extraction for 2 h at room temperature and ethanol/solid ratio of 30) [20], that is, for the best of our knowledge, the highest reported for SCG. The high extraction efficiency of SUPRAS for phenolic compounds, the wide variety of phenolics extracted (see Table 1 and Figure 4), and the fact that samples were immediately processed, without further treatment, could account for the high TPC value found in our experiments. Values for SUPRAS were higher than those reported for conventional SLE (16-18 mg GAE/g) [18–20], SFE (~4 mg CAE/g) [20] or Soxhlet (18-22 mg CAE/g). However, it is known that TPC values depend on variables such as the roasting process [43], the preparation method (grinding degree or particle size, coffee:water ratio, water temperature, extraction time, etc.) and the technique followed for TPC estimation too, so that these factors can also influence results and differences between reported levels. Some advantages of SUPRAS were the low

solvent/solid ratio (11.7 mL/g dry SCG) and the fact that the extraction was done at room temperature during 1 min.

3.2.2. Antioxidant activity

SUPRAS extracts, rich in TPC, were further tested for antioxidant activity using three assays (see section 2.8). The maximum value for the antioxidant capacity (100%) means that the respective reagent was reduced by the effect of the antioxidants present in the SUPRAS extract. The antioxidant capacity with DPPH (4.1 mg SUPRAS extract/mL) and FRAP (2.5 mg SUPRAS extract/mL) was 21 ± 3% and 68 ± 4%, respectively. Values of DPPH antioxidant capacity have been reported in the range 14.4-93.5% in extracts from dry SCG, with those techniques enhancing TPC extraction too, such as Soxhlet, USAE and MAE (EC_{50} concentration values in the range 0.2-1 mg extract/mL). [20,23] The same Soxhlet and USAE extracts gave values in the range 160-381 µM TEAC/g extract. [20], while for SUPRAS a value of 405 ± 6 µM TEAC/g extract was measured. These results are in line with their high TPC content.

3.2.3. Antimicrobial activity

Previous studies have reported that phenolic substances, alkaloids and melanoidins present in the coffee have antibacterial activity [13]. However, even though the antimicrobial activity of coffee by-products can be attributed to any of their compounds, some studies suggest that bacteria are highly sensitive to phenolic acids [44], while other authors report that caffeine is the cause for the inhibition of growth in gram-negative bacteria, and that chlorogenic acid is less efficient against *S. enterica* [45].

The antimicrobial activity of SUPRAS was tested against *P. putida, S. enterica, S. areus and B. cereus.* Both *P. putida* and *S. enterica* have the ability to form biofilms [46], which is a strategy developed by bacteria to protect themselves from harmful substances such as antibiotics. For this reason, multiple studies are conducted to control these microorganisms in food. *S. aureus*, can produce different infections such pneumonia [47]. Respect to *B. cereus,* it has been reported to produce five enterotoxins and one emetic toxin and their spores are resistant to many processes

as low and high temperatures, desiccation, disinfectant agents, ionization, radiation and ultraviolet light [48]. *A priori,* the complex mixture of compounds that could be present in SUPRAS extracts from SCG, could be used for enhancing functional properties such as antimicrobial activity to be used in the food industry as a preservative, extending the shelf life of food, or even in the pharmaceutical and cosmetic sector.

The minimum inhibitory capacity (MIC), considered as the lowest concentration of SUPRAS extract that inhibited the growth of the microorganism tested (two gram-positive and two-gram negative bacteria), were calculated. SUPRAS extracts showed antimicrobial activities toward the growth of the target bacteria at varying degrees of concentrations. Thus, gram-positive bacteria (*B. cereus, S aureus)* were found more resistant, with a MIC value of 287 mg SUPRAS extract/mL. On the contrary, gram-negative bacteria (*S. enterica, P. putida)* were very sensitive with a MIC value of 2.87 mg SUPRAS extract/mL.

The literature reporting the antibacterial capacity of coffee waste is very scarce. Values ranged between 5 and 60 mg extract/mL for SCG extracted with subcritical water [21]. In this study, gram-positive (*B. cereus*, *S. aureus*) and gram-negative (*E. Coli, S. typhi*) bacteria were tested with methods involving different modifiers and pretreatments. MIC values were in the ranges 20-40 mg extract/mL for *B. cereus*, 5 mg extract/mL for *S. aureus*, 10-20 mg extract /mL for *E. Coli and* 20-60 mg extract/mL for *S. typhi.*

4. Conclusions

This study shows the first insights on the potential of SUPRAS, nanostructured solvents made up of assembled amphiphile aggregates, for valorization of coffee waste. Results proved that these solvents offer good extraction capacity of high-added value compounds from coffee by-products with interest for the food, pharmaceutical and cosmetic industry. Furthermore, extracts showed antioxidant capacity and antimicrobial effects to gram-negative bacteria. SUPRAS extraction offer rapid, simple and low cost methods and could be directly applied to the extraction of bioactives from wet by-products. Given the high number of biocompatible amphiphiles commercially available, the use of SUPRAS for agrifood by-product valorization is promising.

Acknowledgments

Authors gratefully acknowledge financial support from Spanish MINECO (Project CTQ2017-83823-R). A. Ballesteros-Gómez acknowledges the funding from Spanish Ministry of Science, Innovation and Universities for a Ramón y Cajal contract (RYC-2015-18482). L.S. Torres-Valenzuela thanks AUIP for her doctoral fellowship.

References

[1] Lai WT, Khong NMH, Lim SS, Hee YY, Sim BI, Lau KL, Lai OM. A review: Modified agricultural by-products for the development and fortification of food products and nutraceuticals, Trends Food Sci. Technol. 2017; 59:148–160. doi:10.1016/j.tifs.2016.11.014.

[2] Bandara N, Chalamaiah M. Bioactives From Agricultural Processing By-products, in: Reference Module in Food Science, Elsevier, 2018. doi:10.1016/B978-0-08-100596-5.22408-6.

[3] Dadi D, Mengistie E, Terefe G, Getahun T, Haddis A, Birke W, Beyene A, Luis P, Van der Bruggen B. Assessment of the effluent quality of wet coffee processing wastewater and its influence on downstream water quality. Ecohydrol. Hydrobiol. 2018; 18:201–211. doi:10.1016/j.ecohyd.2017.10.007.

[4] Janissen B, Huynh T. Chemical composition and value-adding applications of coffee industry by-products: A review, Resources, Conservation and Recycling. 2018; 128:110–117. doi:10.1016/j.resconrec.2017.10.001.

[5] Murthy PS, Manonmani HK. Bioconversion of Coffee Industry Wastes with White Rot Fungus Pleurotus florida, Res. J. Environ. Sci. 2008; 2:145–150. doi:10.3923/rjes.2008.145.150.

[6] Buntić AV, Pavlović MD, Antonović DG, Šiler-Marinković SS, Dimitrijević-Branković SI. Utilization of spent coffee grounds for isolation and stabilization of Paenibacillus chitinolyticus CKS1 cellulase by immobilization. Heliyon. 2016; 2 e00146. doi:10.1016/j.heliyon.2016.e00146.

[7] Woldesenbet AG, Woldeyes B, Chandravanshi BS. Bio-ethanol production from wet coffee processing waste in Ethiopia, Springerplus. 2016; 5: 1903. doi:10.1186/s40064-016-3600-8.

[8] Hachicha R, Rekik O, Hachicha S, Ferchichi M, Woodward S, Moncef N, Cegarra J, Mechichi T. Co-composting of spent coffee ground with olive mill wastewater sludge and

poultry manure and effect of Trametes versicolor inoculation on the compost maturity, Chemosphere. 2012; 88:677–682. doi:10.1016/j.chemosphere.2012.03.053.

[9] Jiménez-Zamora A, Pastoriza S, Rufián-Henares JA. Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties, LWT - Food Sci. Technol. 2015; 61:12–18. doi:10.1016/j.lwt.2014.11.031.

[10] Iriondo-DeHond A, Aparicio García N, Fernandez-Gomez B, Guisantes-Batan E, Velázquez Escobar F, Blanch GP, San Andres MI, Sanchez-Fortun S, del Castillo MD. Validation of coffee by-products as novel food ingredients. Innovative Food Sci. Emerging Technol. 2019; 51: 194-204. doi:10.1016/j.ifset.2018.06.010.

[11] Esquivel P, Jiménez VM, Functional properties of coffee and coffee by-products. Food Res. Int. 2012; 46:488–495. doi:10.1016/j.foodres.2011.05.028.

[12] Mussatto SI, Machado EMS, Martins S, Teixeira JA. Production, Composition, and Application of Coffee and Its Industrial Residues, Food Bioprocess. Technol. 2011; 4: 661. doi:10.1007/s11947-011-0565-z.

[13] Campos-Vega R, Loarca-Piña G, Vergara-Castañeda HA, Oomah BD. Spent coffee grounds: A review on current research and future prospects. Trends Food Sci. Technol.2015; 45:24–36. doi:10.1016/j.tifs.2015.04.012.

[14] Kovalcik A, Obruca S, Marova I. Valorization of spent coffee grounds: A review. Food Bioprod. Process.2018; 110:104–119. doi:10.1016/j.fbp.2018.05.002.

[15] McNutt J, He QS. Spent coffee grounds: A review on current utilization, J. Ind. Eng. Chem. 2019; 71:78–88. doi:10.1016/j.jiec.2018.11.054.

[16] Hall S, Desbrow B, Anoopkumar-Dukie S, Davey AK, Arora D, McDermott C, Schubert MM, Perkins AV, Kiefel MJ, Grant GD. A review of the bioactivity of coffee, caffeine and key coffee constituents on inflammatory responses linked to depression, Food Res. Int. 2015; 76:626–636. doi:10.1016/j.foodres.2015.07.027.

[17] Pathak L, Agrawal Y, Dhir A. Natural polyphenols in the management of major depression, Expert Opin. Investig. Drugs. 2013; 2:863–880. doi:10.1517/13543784.2013.794783.

[18] Mussatto SI,. Ballesteros LF, Martins S, Teixeira JA. Extraction of antioxidant phenolic compounds from spent coffee grounds. Sep. Purif. Technol. 2011; 83:173–179. doi:10.1016/j.seppur.2011.09.036.

[19] Zuorro A, Lavecchia R. Spent coffee grounds as a valuable source of phenolic

compounds and bioenergy. J. Cleaner Prod 2012;. 34:49–56. doi:10.1016/j.jclepro.2011.12.003.
[20] Andrade KS, Gonçalvez RT, Maraschin M, Ribeiro-do-Valle RM, Martínez J, Ferreira SRS. Supercritical fluid extraction from spent coffee grounds and coffee husks: Antioxidant activity and effect of operational variables on extract composition. Talanta; 2012; 88:544–552. doi:10.1016/j.talanta.2011.11.031.
[21] Getachew AT, Chun BS. Influence of pretreatment and modifiers on subcritical water liquefaction of spent coffee grounds: A green waste valorization approach, J. Cleaner Prod. 2017; 142: 3719–3727. doi:10.1016/j.jclepro.2016.10.096.
[22] Yoo DE, Jeong KM, Han SY, Kim EM, Jin Y, Lee J. Deep eutectic solvent-based valorization of spent coffee grounds, Food Chem. 2018; 255:357–364. doi:10.1016/j.foodchem.2018.02.096.
[23] Pavlović MD, Buntić AV, Šiler-Marinković SS, Dimitrijević-Branković SI. Ethanol influenced fast microwave-assisted extraction for natural antioxidants obtaining from spent filter coffee, Sep. Purif. Technol. 2013; 118 :503–510. doi:10.1016/j.seppur.2013.07.035.
[24] Chun OK, Kim DO. Consideration on equivalent chemicals in total phenolic assay of chlorogenic acid-rich plums. Food Res. Int. 2004; 37:337–342. doi:10.1016/j.foodres.2004.02.001.
[25] Caballo C, Sicilia MD, Rubio S, Chapter 5 - Supramolecular Solvents for Green Chemistry, in: F. Pena-Pereira, M. Tobiszewski (Eds.), The Application of Green Solvents in Separation Processes, Elsevier, 2017: pp. 111–137. doi:10.1016/B978-0-12-805297-6.00005-X.
[26] Ballesteros-Gómez A, Sicilia MD, Rubio S. Supramolecular solvents in the extraction of organic compounds. A review, Anal. Chim. Acta. 2010; 677:108–130. doi:10.1016/j.aca.2010.07.027.
[27] Ballesteros-Gómez A, Lunar L, Sicilia MD, Rubio S. Hyphenating Supramolecular Solvents and Liquid Chromatography: Tips for Efficient Extraction and Reliable Determination of Organics, Chromatographia. 2019; 82:111–124. doi:10.1007/s10337-018-3614-1.
[28] Steed JW, Turner DR, Wallace K. Core Concepts in Supramolecular Chemistry and Nanochemistry, John Wiley & Sons, Ltd, Chippenham, Witshire, 2007.
[29] Ballesteros-Gómez A, Rubio S. Environment-responsive alkanol-based supramolecular solvents: characterization and potential as restricted access property and mixed-mode extractants. Anal. Chem. 2012; 84: 342–349. doi:10.1021/ac2026207.

[30] Salatti-Dorado JA, García-Gómez D, Rodriguez-Ruiz V, Gueguen V, Pavon-Djavid G, Rubio S. Multifunctional green supramolecular solvents for cost-effective production of highly stable astaxanthin-rich formulations from Haematococcus pluvialis. Food Chem. 2019; 279:294–302. doi:10.1016/j.foodchem.2018.11.132.

[31] Ballesteros-Gómez A, Caballero-Casero N, García-Fonseca S, Lunar L, Rubio S. Multifunctional vesicular coacervates as engineered supramolecular solvents for wastewater treatment. Chemosphere. 2019; 223:569–576. doi:10.1016/j.chemosphere.2019.02.089.

[32] Ruiz FJ, Rubio S, Pérez-Bendito D. Water-induced coacervation of alkyl carboxylic acid reverse micelles: phenomenon description and potential for the extraction of organic compounds, Anal. Chem. 2007; 79: 7473–7484. doi:10.1021/ac0708644.

[33] Salatti-Dorado JA, Caballero-Casero N, Sicilia MD, Lunar ML, Rubi S. The use of a restricted access volatile supramolecular solvent for the LC/MS-MS assay of bisphenol A in urine with a significant reduction of phospholipid-based matrix effects, Anal. Chim. Acta. 2017; 950:71–79. doi:10.1016/j.aca.2016.11.026.

[34] Régnier P, Bastias J, Rodriguez-Ruiz V, Caballero-Casero N, Caballo C, Sicilia D, Fuentes A, Maire M, Crepin M, Letourneur D, Gueguen V, Rubio S, Pavon-Djavid G. Astaxanthin from Haematococcus pluvialis Prevents Oxidative Stress on Human Endothelial Cells without Toxicity, Mar Drugs. 2015; 13:2857–2874. doi:10.3390/md13052857.

[35] Jones A, Pravadali-Cekic S, Dennis GR, Bashir R, Mahon PJ, Shalliker RA. Ferric reducing antioxidant potential (FRAP) of antioxidants using reaction flow chromatography. Anal. Chim. Acta. 2017; 967:93–101. doi:10.1016/j.aca.2017.02.032.

[36] D'Amato S, Mazzarrino G, Rossi C, Serio A, López CC, Celano GV, Paparella A. Thymus Vulgaris (Red Thyme) and Caryophyllus Aromaticus (Clove) Essential Oils to Control Spoilage Microorganisms in Pork Under Modified Atmosphere. Ital. J. Food Saf. 2016; 5:5785. doi:10.4081/ijfs.2016.5785.

[37] Pellegrini M, Ricci A, Serio A, Chaves-López C, Mazzarrino G, D'Amato S, Lo Sterzo C, Paparella A. Characterization of Essential Oils Obtained from Abruzzo Autochthonous Plants: Antioxidant and Antimicrobial Activities Assessment for Food Application, Foods. 2018; 7:19. doi:10.3390/foods7020019.

[38] Bravo J, Juániz I, Monente C, Caemmerer B, Kroh LW, De Peña MP, Cid C. Evaluation of spent coffee obtained from the most common coffeemakers as a source of hydrophilic

bioactive compounds, J. Agric. Food Chem. 2012; 60:12565–12573. doi:10.1021/jf3040594.
[39] Belščak-Cvitanović, A, Komes D. Extraction and formulation of bioactive compounds, in: Handbook of Coffee Processing By-Products, Elsevier, 2017: pp. 93–140. doi:10.1016/B978-0-12-811290-8.00004-9.
[40] Angelino D, Tassotti1 M, Brighenti F, Del Rio D, Mena P. Niacin, alkaloids and (poly)phenolic compounds in the most widespread Italian capsule-brewed coffees. Sci. Rep. 2018; 8:17874. doi:10.1038/s41598-018-36291-6
[41] Escarpa A, González MC. Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods Anal. Chim. Acta 2001; 427: 119–127. doi: 10.1016/S0003-2670(00)01188-0
[42] Tsao, R., Yang, R. Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography. J. Chromatogr. A, 2003; 1018:29–40. doi: 10.1016/j.chroma.2003.08.034
[43] Duarte SMS, de Abreu CMP, de Menezes HC, dos Santos MH, Gouvêa CMCP. Effect of processing and roasting on the antioxidant activity of coffee brews, Food Sci. Technol.. 2005; 25: 387–393. doi:10.1590/S0101-20612005000200035.
[44] Monente C, Bravo J, Vitas AI, Arbillaga L, De Peña MP, Cid C. Coffee and spent coffee extracts protect against cell mutagens and inhibit growth of food-borne pathogen microorganisms, J. Funct. Foods. 2015; 12: 365–374. doi:10.1016/j.jff.2014.12.006.
[45] Almeida AAP, Farah A, Silva DAM, Nunan EA, Glória MBA. Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria, J. Agric. Food Chem. 2006; 54:8738–8743. doi:10.1021/jf0617317.
[46] Liu H, Xiao Y, Nie H, Huang Q, Chen W. Influence of (p)ppGpp on biofilm regulation in Pseudomonas putida KT2440, Microbiol. Res. 2017; 204:1–8. doi:10.1016/j.micres.2017.07.003.
[47] Paling FP, Wolkewitz M, Bode LGM, Klein Klouwenberg PMC, Ong DSY, Depuydt P, de Bus L, Sifakis F, Bonten MJM, Kluytmans JAJW. Staphylococcus aureus colonization at ICU admission as a risk factor for developing S. aureus ICU pneumonia, Clin. Microbiol. Infect. 2017; 23: 49 doi:10.1016/j.cmi.2016.09.022.
[48] Sadek ZI, Abdel-Rahman MA, Azab MS, Darwesh OM, Hassan MS. Microbiological evaluation of infant foods quality and molecular detection of Bacillus cereus toxins relating genes, Toxicol. Rep. 2018; 5: 871-877. doi:10.1016/j.toxrep.2018.08.013.

Figure captions

Figure 1. Schematic picture of SUPRAS production and SUPRAS-based extraction of SCGs

Figure 2. Extraction rate of caffeine (average of three replicates, relative standard deviation, RSD: 5-10%) from SCG with SUPRAS synthesized under different percentages of organic solvent (% v/v ethanol or THF) and amphiphiles (% v/v hexanol or decanoic acid). The optimal conditions are shown in a different color.

Figure 3. Extraction rate of 5-CGA (average of three replicates, RSD: 5-10%) from SCG with SUPRAS synthesized under different percentages of organic solvent (% v/v ethanol or THF) and amphiphiles (% v/v hexanol or decanoic acid). The optimal conditions are shown in a different color.

Figure 4. LC-(ESI) MS-MS peaks corresponding to the extracted ion chromatograms of *n*-*O*-dicaffeoyl quinic acids, *n*-*O*-feruloylquinic acids (n-FQA), *n*-*O*-caffeoylquinic acids (n-CQA), *n*-*O*-feruloylquinic lactones (n-FQL), *n*-*O*-coumaroylquinic acids (n-CSA), *n*-*O*-caffeoylshikimic acid, *n*-*O*-caffeoylquinic lactones (n-CQL), caffeine (C), niacin (N), trigonelline (T) and N-methylpyridinium (Table 1). Fig. A and B shows the MRM chromatograms recorded in negative and positive acquisition modes, respectively. Only the most intense isomers of the most abundant classes are labelled.

Table 1. Polyphenolic compounds, alkaloids and niacin identified in SUPRAS extracts, two main fragments were monitored for each class according reference [40]

Compound class	Abreviation	Parent ion	Fragment 1	Fragment 2	Retention times	[a] Area (sum of peaks)	Polarity
n-O-Dicaffeoylquinic acids	n-DCQAs	515	179	135	31.1, 31.8, 32.4, 35.2	43804	-
n-O-Feruloylquinic acids	n-FQAs	367	193	191	17.3, 18.4,22.8,23.7	118109	-
n-O-Caffeoylquinic acids	n-CQAs	353	191	173	13.0, 16.1, 17.7, 18.5	220745	-
n-O-Feruloylquinic lactones	n-FQLs	349	175	193	26.8,28.9, 30.3, 30.9, 32.8	829621	-
n-O-Coumaroylquinic acids	n-CouQAs	337	191	173	21.8, 22.2	5273	-
n-O-caffeoylshikimic acid	n-CSAs	335	179	173	20.6, 23.8	152796	-
n-O-caffeoylquinic lactones	n-CQLs	335	135	161	25.3, 26.4	1157812	-
Trigonelline	T	138	92	94	2.2	52738	+
Niacin	N	124	106	80	3.2	4776	+
Caffeine	C	195	138		17.8	14961897	+

[a]The most abundant fragment was used for quantifying each peak

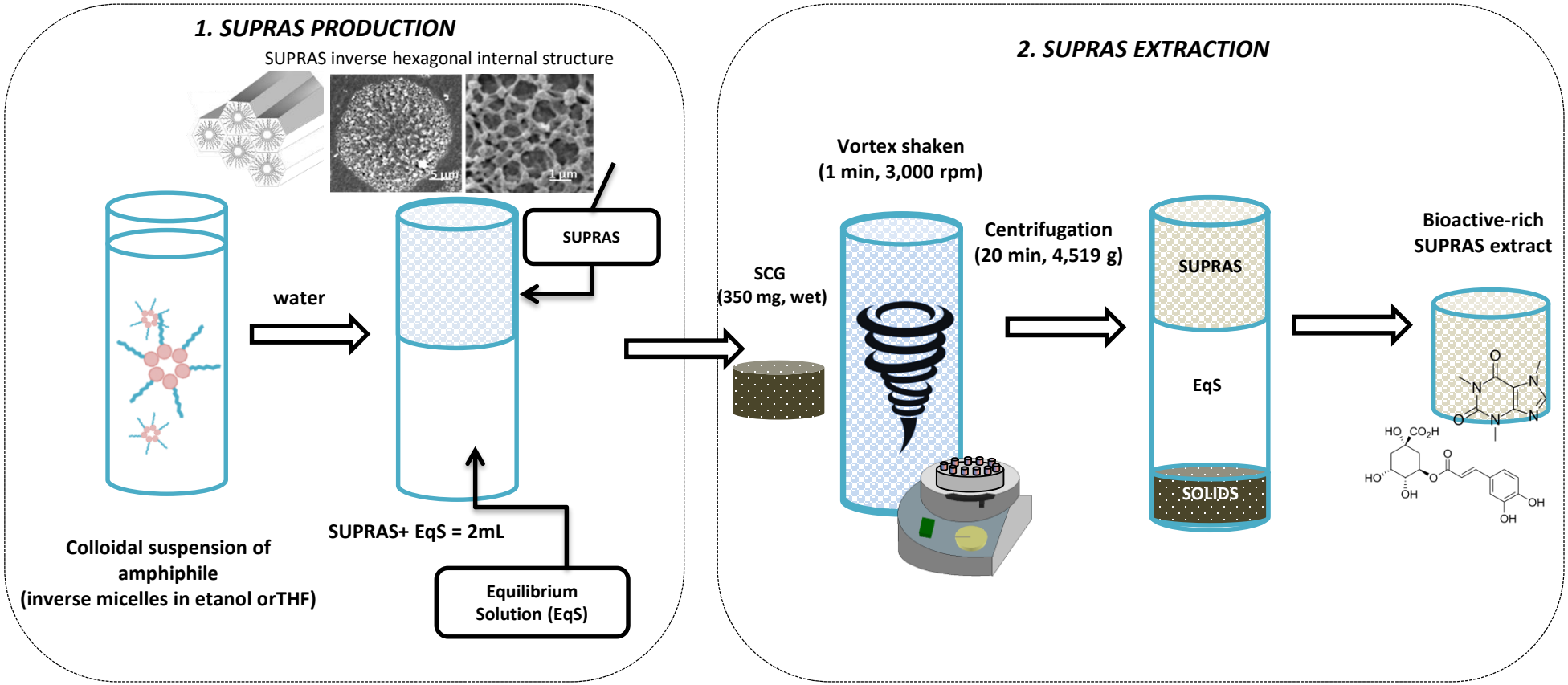
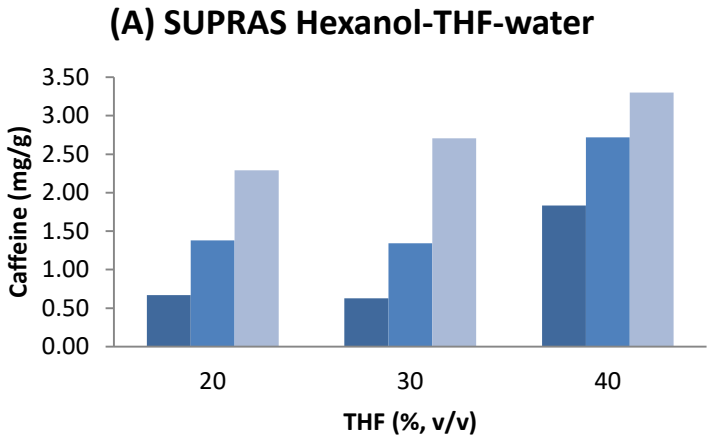
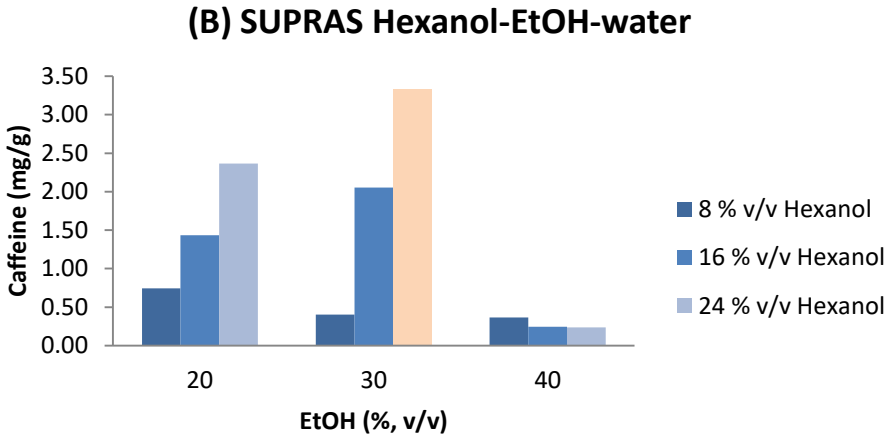
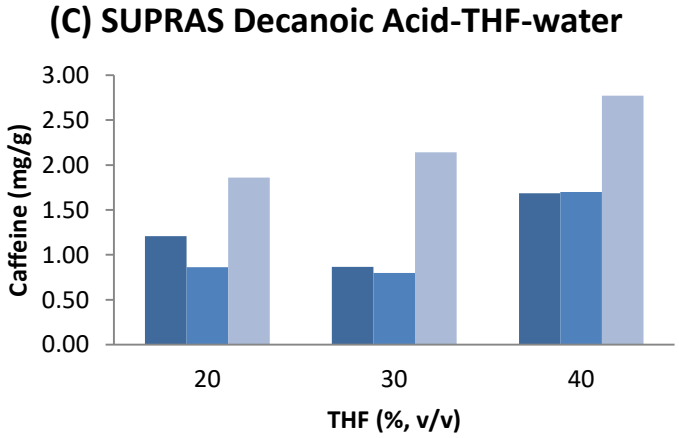

1. SUPRAS PRODUCTION
SUPRAS inverse hexagonal internal structure
5 µm
1 µm
water
SUPRAS
SUPRAS+ EqS = 2mL
Colloidal suspension of amphiphile (inverse micelles in etanol orTHF)
Equilibrium Solution (EqS)
2. SUPRAS EXTRACTION
SCG (350 mg, wet)
Vortex shaken (1 min, 3,000 rpm)
Centrifugation (20 min, 4,519 g)
SUPRAS
EqS
SOLIDS
Bioactive-rich SUPRAS extract

Fig.1


(A) SUPRAS Hexanol-THF-water
Caffeine (mg/g)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
30
40
THF (%, v/v)


(B) SUPRAS Hexanol-EtOH-water
Caffeine (mg/g)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
30
40
EtOH (%, v/v)
8 % v/v Hexanol
16 % v/v Hexanol
24 % v/v Hexanol


(C) SUPRAS Decanoic Acid-THF-water
Caffeine (mg/g)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
30
40
THF (%, v/v)

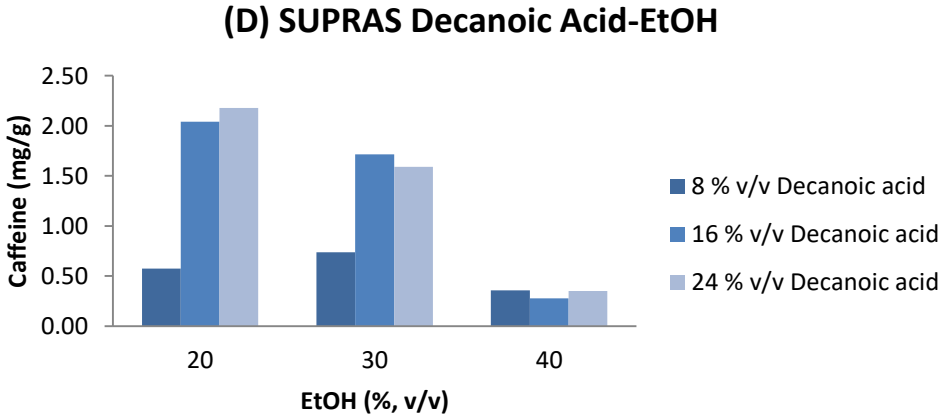
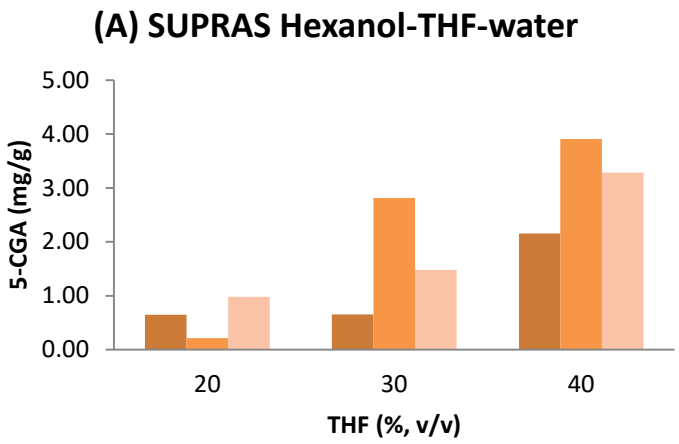
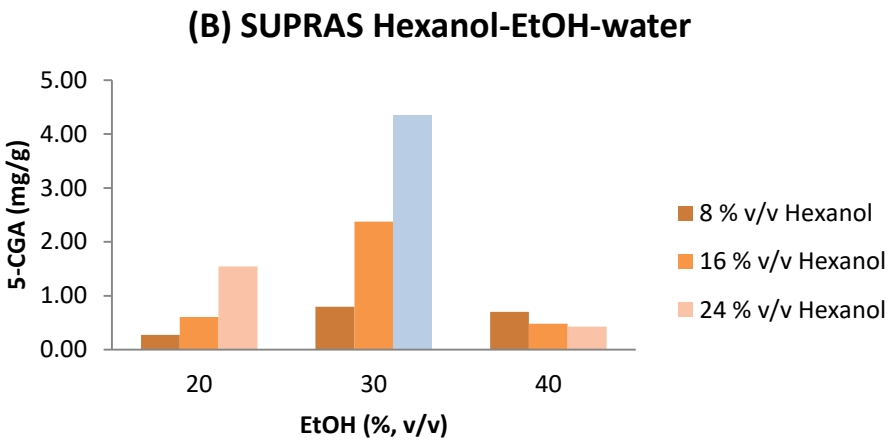
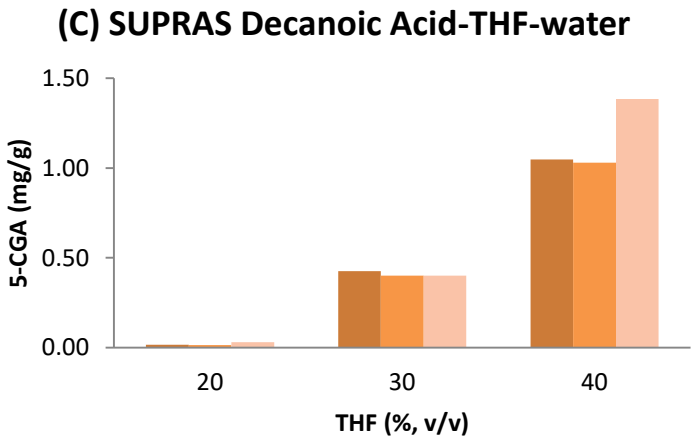

(D) SUPRAS Decanoic Acid-EtOH
Caffeine (mg/g)
2.50
2.00
1.50
1.00
0.50
0.00
20
30
40
EtOH (%, v/v)
8 % v/v Decanoic acid
16 % v/v Decanoic acid
24 % v/v Decanoic acid

Fig. 2


(A) SUPRAS Hexanol-THF-water
5-CGA (mg/g)
5.00
4.00
3.00
2.00
1.00
0.00
20
30
40
THF (%, v/v)


(B) SUPRAS Hexanol-EtOH-water
5-CGA (mg/g)
5.00
4.00
3.00
2.00
1.00
0.00
20
30
40
EtOH (%, v/v)
8 % v/v Hexanol
16 % v/v Hexanol
24 % v/v Hexanol


(C) SUPRAS Decanoic Acid-THF-water
5-CGA (mg/g)
1.50
1.00
0.50
0.00
20
30
40
THF (%, v/v)

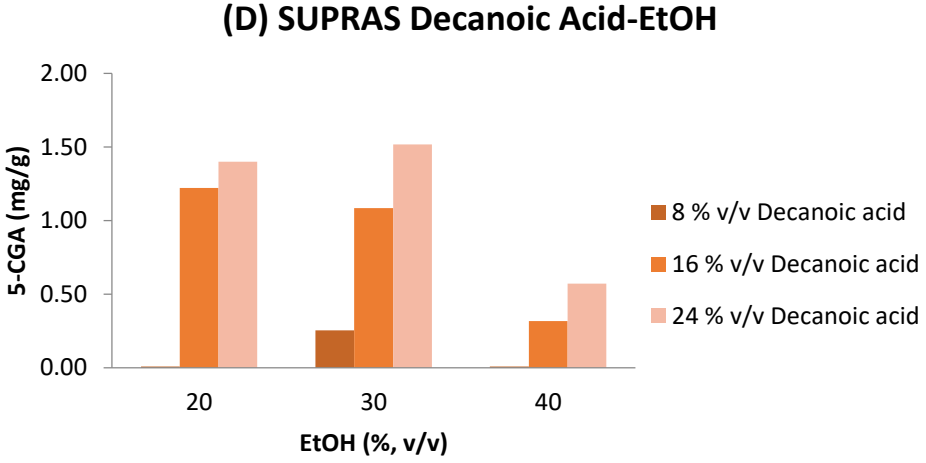
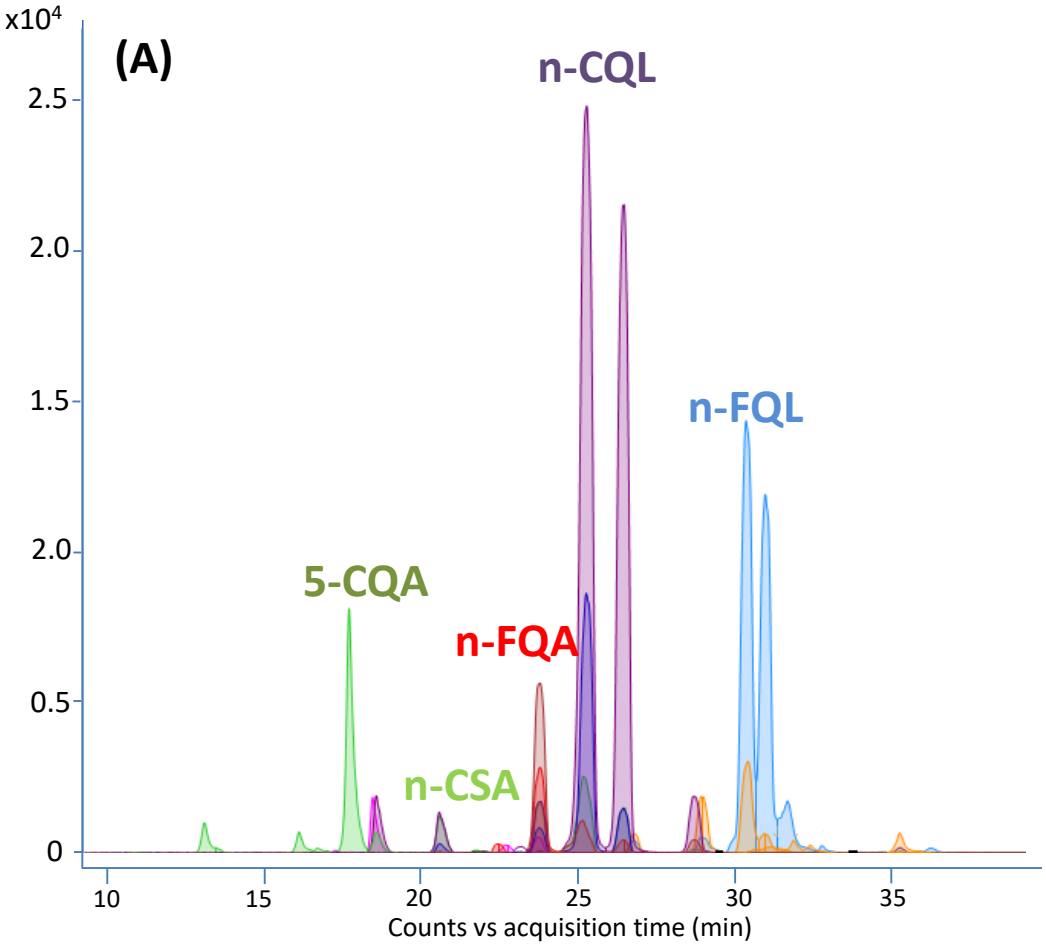

(D) SUPRAS Decanoic Acid-EtOH
5-CGA (mg/g)
2.00
1.50
1.00
0.50
0.00
20
30
40
EtOH (%, v/v)
8 % v/v Decanoic acid
16 % v/v Decanoic acid
24 % v/v Decanoic acid

Fig. 3


(A)
x10^4
2.5
2.0
1.5
2.0
0.5
0
n-CQL
n-FQL
5-CQA
n-FQA
n-CSA
10
15
20
25
30
35
Counts vs acquisition time (min)

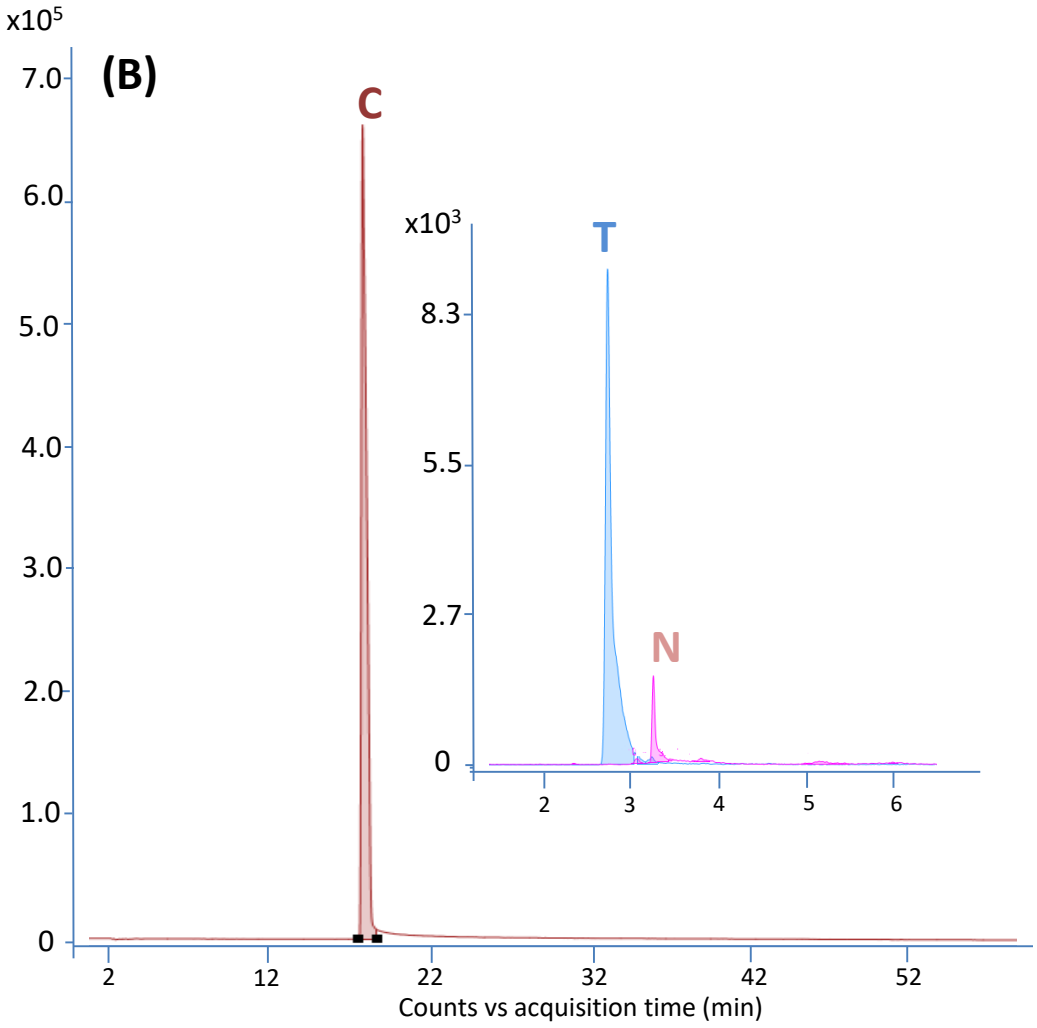

x10^5
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
(B)
C
x10^3
8.3
5.5
2.7
0
T
N
2
3
4
5
6
2
12
22
32
42
52
Counts vs acquisition time (min)

Fig.4