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A biorefinery approach for the valorization of spent coffee grounds to produce antioxidant compounds and biobutanol

Juan C. López-Linares [a,b], María Teresa García-Cubero [a,b], Mónica Coca [a,b], Susana Lucas [a,b,*]

[a] Institute of Sustainable Processes, Dr. Mergelina S/n, 47011, Valladolid, Spain
[b] Department of Chemical Engineering and Environmental Technology, School of Industrial Engineering, University of Valladolid, Dr. Mergelina, S/n, 47011, Valladolid, Spain

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ABSTRACT

Spent coffee grounds (SCG) has been proposed as a potential material within the biorefinery context. The optimization of the microwave assisted extraction of antioxidant compounds with natural deep eutectic solvents (NADES), a promising green solvent, was conducted in this study. Under the optimal extraction conditions (120 °C, 15 min, 20% water in NADES and 0.53 ChCl:Glyc molar ratio), the obtained extract contained: total phenolic content (TPC), 0.48 mg of gallic acid equivalents (GAE) g^{-1} SCG, coumaric acid being the most abundant; total flavonoid content (TFC), 0.44 mg of catechin equivalents (CE) g^{-1} SCG; antioxidant activities: DPPH, 0.55 mg trolox equivalents (TE) g^{-1} SCG; ABTS, 3.17 mg TE g^{-1} SCG; and FRAP, 1.52 mg TE g^{-1} SCG. Moreover, the residual NADES extracted SCG was microwave pretreated with dilute sulfuric acid, enzymatically hydrolyzed and fermented by *Clostridium beijerinckii*, generating 7.1 g L^{-1} butanol (81 kg butanol t^{-1} SCG and 126 kg acetone-butanol-ethanol (ABE) t^{-1} SCG). In this way, SCG displayed its potential as a source to be used in an integrated biorefinery approach.

1. Introduction

One of the most important and usual beverages in the world is coffee [1]. According to Buratti et al. [2], it is the second best-selling product worldwide. In this way, the coffee industry has greatly increased its production since 2010, going from 140 to 152 million/year according to data supplied by the International Coffee Organization [3]. On the other hand, it is worth mentioning that a great amount of residues are obtained in the process of coffee production (coffee processing and roasting, as well as drink generation), such as husk, pulp, mucilage, coffee silver skin and spent coffee grounds (SCG) [4]. Of these, the most abundant residue generated annually is SCG (6 million tonnes/year) in comparison with the generation of coffee pulp and husk (0.50 and 0.18 t t^{-1} fresh coffee) [3]. Although SCG has been used for bioethanol and biodiesel generation, and in wastewater treatment (for instance, in the reduction of cationic dyes due to its adsorption properties) [5], these applications are quite limited.

Nevertheless, an important content of bioactive compounds (e.g. polyphenols) can be found in SCG, such as chlorogenic, caffeic, caffeoylquinic, ellagic, *trans*-ferulic, feruloylquinic, gallic, p-hydroxybenzoic, p-coumaric, p-coumaroylquinic, protocatechuic and tannic acids, esters of caffeic and ferulic acids with quinic acid, flavonoids, catechin, epicatechin, rutin, trigonelline, caffeine and quercetin, among others [4,6]. These compounds present potent antioxidant, anticarcinogenic, anti-allergic, anti-inflammatory, antimicrobial and antitumor properties, as well as beneficial properties related to neuroprotection [7]. Due to all the above, they have potential applications in the pharmaceutical and health industries, as well as for cosmetics production, and the generation of functional foods and value added additives in food products [5,6]. Therefore, SCG could be considered as a potential and very interesting residue for the recovery of bioactive compounds [8].

Bioactive compounds contained in SCG have commonly been extracted using traditional extraction methods, such as autohydrolysis, boiling extraction, extraction with polar or intermediate polar solvents (ethanol or methanol), or soxhlet extraction with hexane [4,9,10]. On the contrary, due to the disadvantages of traditional extraction, such as the use of expensive, flammable, toxic and not environmentally-friendly solvents, natural deep eutectic solvents (NADES) are currently being reported as newfangled and hopeful green solvents [11–13]. NADES are

* Corresponding author. Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina s/n, 47011, Valladolid, Spain.
E-mail address: susana@iq.uva.es (S. Lucas).

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formed by natural metabolites from diverse cells and organisms [14]. They are a mixture of two or more pure compounds (hydrogen-bonding donor (HBD) and hydrogen-boning acceptor (HBA)) with a eutectic point temperature lower than those found for an ideal liquid mixture due to the strong hydrogen-bonding interaction between HBD and HBA, showing significant negative deviations from ideality ($\Delta T_2 > 0$) [15]. In particular, these intramolecular hydrogen bonds in NADES provide a high possibility of breaking the strong hydrogen bonds in biomass, hence the high biomass solubility and the favorable conversion rate [16,17]. Moreover, NADES are liquid at room temperature for a certain composition range, so they can be adequate for use in extraction processes [18]. In addition, they can easily be prepared using different combinations of compounds, depending on the extraction purpose, and both polar and apolar compounds can be successfully extracted [16]. Although NADES have the drawback of having a high viscosity in comparison with conventional solvents which might make extraction processes more difficult due to the low mass transfer, this viscosity can be reduced by the addition of such solvents as water and/or increasing the temperature, which can be explained by the increased molar volume of the solvent favored at higher temperatures [19,20]. On the other hand, NADES are not volatile, non-flammable, no toxic and extremely biodegradable solvents. Their price is relatively low and their preparation very simple. Then, due to the low toxicity, bioactive compounds extracted using NADES can be used in food and life sciences industries [21]. NADES extraction can also be carried out with microwave assistance, resulting in a higher homogeneity in the heating process, a shorter extraction time, higher efficiency and lower solvent usage [22,23].

As is well known, the integrated biorefinery concept endeavors to valorize lignocellulosic wastes and by-products (such as SCG) under the framework of the circular economy [24]. In this context, in order to guarantee the profitability of the microwave assisted NADES extraction process of bioactive compounds from SCG, these need to be co-produced in an SCG biorefinery along with other products. Therefore, the residual NADES extracted SCG needs to be valorized, as it has been reported that NADES extraction does not break the structural carbohydrates contained in the lignocellulosic residue [16]. Thus, for instance, it could be valorized through the production of such biofuels as biogas, bioethanol and biodiesel [25,26]. In addition, it could be used as substrate in an acetone-butanol-ethanol (ABE) fermentation to generate biobutanol, which is a 4-carbon alcohol that is regarded as a chemical building block as well as an advanced biofuel [27]. In this process, genus Clostridia such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccaroperbutylacetonicum* and *Clostridium saccharoacetobutylicum* have shown a better activity for the synthesis of biobutanol with higher yields [28]. Biobutanol offers many advantages that make it superior to bioethanol, such as a better energy density (energy content 30 vs 19 MJ L^{-1}) due to the doubling of the carbon atoms, a higher boiling point that causes biobutanol to take longer to burn in engines and a higher hygroscopicity [28–30]. What is more, biobutanol also has a low octane number that is comparable to gasoline, a low vapor pressure that eases transportation, low volatility, reduced sensitivity to temperature and it also shows less corrosion of the contact material [30–32]. Therefore, gasoline might be partially (it can be blended with gasoline up to 95%) or totally replaced by butanol, since existing engines do not need any modifications. In addition, butanol can be used as a chemical commodity in different industries, such as enamels, lacquers, antibiotics, pharmaceuticals, food and flavoring [32]. In this way, the potential of biobutanol generation from diverse agricultural residues (such as corn residue, sugarcane bagasse, rice waste, wheat residue, barley straw, cassava bagasse, bamboo, or wood waste) and agro-food waste (such as potato peelings, fresh cut fruits and vegetables, coffee silverskin, sugar beet pulp, grape marc and soft drinks, among others) [28,33] has been successfully studied. Giacobbe et al. [34] also studied the process of biobutanol production with good results (concentrations of 12.6 g L^{-1} ABE and 7.8 g L^{-1} butanol) starting from laccase pretreated brewer's spent grain followed by enzymatic saccharification, using *Clostridium acetobutilycum* in the ABE fermentation. Al-Shorgani et al. [32] also evaluated biobutanol production from such agro-industrial wastes, as rice bran, de-oiled rice bran, palm oil mill effluent and palm kernel cake, reporting high biobutanol and ABE concentrations (7.3 and 10.2 g L^{-1}).

On the other hand, SCG is rich in an oil phase containing different added-value molecules (tocopherols, cafestol, kahweol along with a great amount of fatty acids), which can be extracted and used as additives for food, cosmetic and pharmaceutical applications [25,26]. The three major fatty acids in coffee oil are linoleic, palmitic and oleic acids (over 82%), while stearic and arachidonic acids are present in lower amounts (8.5% and less than 1%, respectively). Linoleic and palmitic acids are known to be added-value compounds. Linolenic acid prevents skin cancer and diseases of the heart and blood vessels, while palmitic acid exerts multiple fundamental biological functions at cellular and tissue levels, representing a fundamental constituent for the synthesis of soap and cosmetics and for the production of high performance biodiesel. Regarding tocopherols (basic compounds of vitamin E), they are also considered antioxidants, being useful for the prevention of several diseases (from heart and nervous systems to infertility), as well as to improve the oxidation stability of biofuels. Considering cafestol and kahweol (two interesting coffee-specific diterpenes), they are important for health (being anticarcinogenic substances), as well as being used as additives in the production of many food products (such as candies, cakes and beverages) due to its peculiar taste and flavoring [25].

The present work is an attempt to study the use of SCG in an integrated biorefinery framework to achieve bioactive compounds with antioxidant activity as well as advanced biofuels. In this context, a microwave assisted NADES extraction process was proposed, the influence of four extraction parameters (temperature, time, percentage of water in NADES and ChCl:Glyc molar ratio) being evaluated using the response surface methodology. The evaluation criteria used was the recovery of highly bioactive extracts with antioxidant activity, thus simultaneously maximizing all the evaluated responses (total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (measured by DPPH, ABTS and FRAP)). On the other hand, the residual NADES extracted SCG was used for butanol generation by ABE fermentation using *Clostridium beijerinckii* DSM 6422, the butanol and ABE concentrations, yields and productivities achieved being determined. To the best of our knowledge, this is the first work on microwave assisted NADES extraction of bioactive compounds with antioxidant activity from SCG. On the other hand, a few references were found relating butanol production from coffee silverskin [35–37], but none used SCG as a source of butanol.

2. Materials and methods

2.1. Raw material

Spent coffee grounds (SCG), which were kindly donated by the coffee industry (Productos Solubles S.A., Palencia), were oven-dried at 50 °C, milled using a coffee grinder (Moulinex, A505, France) to get a particle size lower than 1 mm and homogenized, the moisture content being lower than 1%. The SCG were stored at 4 °C until use. The composition was (% w/w): cellulose, 16.3 ± 0.1; hemicellulose, 27.7 ± 0.7 (mannose, 31.3 ± 0.8); acid-insoluble lignin (AIL), 38.5 ± 0.7; acid-soluble lignin (ASL), 0.7 ± 0.1; extractives, 12.4 ± 0.4 (mannose in extractives, 0.2 ± 0.0); ash, 0.1 ± 0.0; acetyl groups, 0.4 ± 0.0; fat, 9.9 ± 0.16; and protein, 9.4 ± 0.7 [38].

2.2. Microwave assisted NADES extraction

The extraction of phenolic and antioxidants compounds from the SCG was carried out using a Multiwave PRO SOLV reactor 50 Hz with Rotor type 16HF100 (Anton Paar GmbH, Austria, Europe), with a solid-liquid ratio of 10% (for more details, see López-Linares et al. [39]). Once the temperature of each run had been reached, the extraction time was

started. At the end of the process, the reactor was cooled to 50 °C.

The solid and liquid fractions (extracted solid and liquid extract) were separated by vacuum filtration. The solid fraction was washed, dried at 40 °C and weighed, the solid recovery (g solid fraction/100 g SCG) being determined in this way. Moreover, the composition (structural carbohydrates, lignin and ash) of the optimal extracted solid was analyzed. On the other hand, the liquid extracts were measured to determine the content of phenolic compounds (as both total and individual phenols identified), the antioxidant capacity, monosaccharides and degradation products. In order to analyze the influence of microwave assisted NADES extraction in SCG, the recovery of carbohydrates in the liquid extracts was calculated as a percentage of the sugar content in the raw SCG.

2.3. Experimental design

In order to select the optimum conditions for the microwave assisted NADES extraction of phenolic and antioxidant compounds, a central composite experimental design was proposed ($\alpha = 1.414$). The NADES used was choline chloride: glycerol (ChCl:Glyc), which was chosen from previous results [16,40]. Four factors were selected: temperature (60–120 °C), time (5–15 min), percentage of water in NADES (20–70%) and the ChCl:Glyc molar ratio (0.5–1.5 M). In this way, a design with 30 experiments was carried out, which was composed of one point and five replicates at the center of the domain selected for each factor under study. The values of each factor, in both coded and uncoded terms, are shown in Table 1. The experimental data were analyzed using the commercial software Statgraphics Centurion XVIII.

On the other hand, microwave assisted extraction with a conventional organic solvent (ethanol-water 25:75, v/v) was also carried out to evaluate the extraction efficiency of the ChCl:Glyc NADES. The extraction was performed at 60 °C for 15 min, which were selected according to Ramón-Gonçalves et al. [7].

Table 1
Experimental design for microwave assisted NADES extraction of SCG.

Run	Temperature (°C)		Time (min)		Water in NADES (%)		ChCl:Glyc molar ratio	
	Coded	Real	Coded	Real	Coded	Real	Coded	Real
1	−1	60	−1	5	+1	70	+1	1.5
2	+1	120	−1	5	−1	20	−1	0.5
3	−1	60	+1	15	−1	20	−1	0.5
4	−1	60	+1	15	+1	70	+1	1.5
5	−1	60	+1	15	−1	20	+1	1.5
6	0	90	0	10	0	45	0	1
7	0	90	0	10	0	45	−1.41	0
8	+1	120	−1	5	+1	70	+1	1.5
9	+1	120	+1	15	−1	20	−1	0.5
10	−1	60	−1	5	−1	20	+1	1.5
11	0	90	0	10	0	45	0	1
12	+1	120	−1	5	+1	70	−1	0.5
13	−1	60	+1	15	+1	70	−1	0.5
14	+1	120	+1	15	−1	20	+1	1.5
15	0	90	0	10	0	45	0	1
16	0	90	0	10	0	45	0	1
17	0	90	0	10	0	45	0	1
18	−1	60	−1	5	−1	20	−1	0.5
19	0	90	0	10	0	45	0	1
20	+1	120	+1	15	+1	70	−1	0.5
21	+1.41	150	0	10	0	45	0	1
22	−1.41	30	0	10	0	45	0	1
23	+1	120	+1	15	+1	70	+1	1.5
24	0	90	−1.41	0	0	45	0	1
25	0	90	+1.41	20	0	45	0	1
26	0	90	0	10	0	45	+1.41	2
27	+1	120	−1	5	−1	20	+1	1.5
28	−1	60	−1	5	+1	70	−1	0.5
29	0	90	0	10	−1.41	0	0	1
30	0	90	0	10	+1.41	95	0	1

2.4. Butanol generation from extracted SCG

The extracted solid obtained under optimal extraction conditions was pretreated with sulfuric acid 1% (w/v) at 170 °C and 10% w/v solid-liquid ratio for 5 min, using the Multiwave PRO SOLV reactor mentioned in Section 2.2. The type and conditions of the pretreatment were chosen based on previous results (data not shown).

Next, the resulting slurry was enzymatically hydrolyzed, using Cellic CTec2 (a cellulolytic complex) and Pectinex Ultra SP-L (a commercial β-galactosidase), which were kindly provided by Novozymes A/S (Denmark). The enzymatic hydrolysis assays were performed at 40 °C, 150 rpm, 72 h and pH 4.8 in triplicate, using 1 L flasks. NaOH pellets were used to adjust the pH. 15 Filter Paper Units (FPU) g^{-1} solid was the enzyme load used for the Cellic CTec2, the double load being used for the Pectinex Ultra SP-L enzyme. The type and load of commercial β-galactosidase enzyme was chosen based on previous results (data not shown). In order to consider the monosaccharides content of the commercial enzymes, enzyme blanks were carried out. Samples were withdrawn at 24, 48 and 72 h, centrifuged and analyzed for monosaccharides and degradation products. The enzymatic hydrolysis yield was determined from the sugars released in the enzymatic hydrolysis with respect to the sugars contained in the acid pretreated SCG and raw SCG.

At the end of the enzymatic hydrolysis, the liquid hydrolysate was separated from the solid by vacuum filtration, detoxified with activated charcoal in a 5 and 10% (w/v) ratio in an orbital shaker (Comecta Optic Ivymen system) at 35 °C, 130 rpm and 1.5 h, and used in ABE fermentation (both detoxified and non-detoxified) by *C. beijerinckii* DSM 6422 at 35 °C, 135 rpm, 48 h and pH 5.5 (for more details, see López-Linares et al. [39]). All experiments were performed in triplicate.

2.5. Analytical methods

2.5.1. Determination of carbohydrate, lignin, ash, fat and protein content

The structural carbohydrate, lignin and ash content in extracted SCG were determined using the National Renewable Energy Laboratory (NREL) methodology [41,42].

High Performance Liquid Chromatography (HPLC) was the technique used to measure sugars (glucose, xylose and arabinose), degradation products (acetic and formic acids, furfural and HMF), ABE solvents (acetone, butanol and ethanol) and other organic acids (lactic and butyric acids), with an Aminex HPX-87H column (for more details, see López-Linares et al. [39]).

In order to determine the oligomeric sugar content in the liquid extracts, an acid hydrolysis process (120 °C, 3% w/v H_2SO_4, 30 min) was carried out, being calculated as the difference between the total free sugars before and after acid hydrolysis.

All analytical determinations were carried out in triplicate and the average results are shown.

2.5.2. Phenolic and flavonoid content determination

The Folin-Ciocalteu method [17] was used to analyze the total phenols content (TPC) using gallic acid as standard, the results being expressed as mg of gallic acid equivalents (GAE) g^{-1} of dry SCG. In addition, individual phenolic compounds (such as 4-hydroxybenzoic acid, vanillic acid, vanillin, syringic acid, syringaldehyde, coumaric acid, ferulic acid and caffeine) were also identified by HPLC using a Bondapak C18 column (Waters, Ireland), following the method described by Zarena and Sankar [43].

Total flavonoid content (TFC) was measured following the colorimetric method described by Zhishen et al. [44], using catechin as standard. The results were expressed as mg of catechin equivalents (CE) g^{-1} of dry SCG.

All analytical determinations were carried out in triplicate and the average results are shown.

2.5.3. Antioxidant capacity

The antioxidant capacity of the liquid extracts obtained was determined using three different methods: DPPH radical scavenging, ABTS cation radical scavenging and ferric reducing antioxidant power (FRAP). In the three methods, trolox (6-hydroxy-2,5,7,8-tetramethylchromen-2-carboxylic acid) was used as standard, expressing the results as mg of trolox equivalents (TE) g^{-1} of dry SCG. All analytical determinations were carried out in triplicate and the average results are shown.

DPPH tests were done following the method described by Brand-Williams et al. [45] with some modifications. 0.2 mL of liquid extract was added to 2 mL of $6{\cdot}10^{-5}$ M DPPH (2,2-diphenyl-1-picrylhydrazyl) methanol solution and mixed, the decrease in absorbance being measured at 517 nm after 15 min. Regarding the ABTS assay, it was performed according to the procedure described by Cano et al. [46], while the FRAP assay was carried out following the procedure described by Benzie et al. [47].

3. Results and discussion

3.1. Effect of the microwave assisted NADES extraction of antioxidants from SCG

In order to evaluate the conditions for the microwave assisted NADES extraction of phenolic and antioxidant compounds from SCG, a central composite experimental design was proposed, the influence of four factors (temperature, time, percentage of water in NADES and ChCl:Glyc molar ratio) being studied. Table 2 shows the experimental results achieved for the TPC, TFC, DPPH, ABTS and FRAP responses in the liquid extracts obtained after the microwave assisted NADES extraction for each experimental run.

3.1.1. Total phenolic and flavonoid content (TPC and TFC)

Phenolic compounds are considered to have high antioxidant activity, which is due to their capacity to donate hydrogen, leading to stable radical intermediates [48]. On the other hand, an interesting group of phenolic compounds is the flavonoids, which can be found in products of natural origin. They are considered very important, as they contain a great antioxidant capacity and are therefore potentially useful for maintaining human health. It is known, for instance, to prevent cardiovascular diseases, and to reduce the risk of Alzheimer and Parkinson [49].

As can be appreciated in Table 2, the TPC ranged between 0.05 and 0.06 mg GAE g^{-1} SCG (runs 12, 13, 20, 22, 23 and 28) and 1.15 mg GAE g^{-1} SCG (run 7), while the TFC ranged from 0.05 mg CE g^{-1} SCG (run 22) to 0.47 mg CE g^{-1} SCG (run 21). Around the central point (90 °C, 10 min, 45% water in NADES and 1 ChCl:Glyc molar ratio), an average TPC and TFC of 0.11 mg GAE g^{-1} SCG and 0.20 mg CE g^{-1} SCG, respectively, were measured.

Both TPC and TFC responses were adjusted by second-order polynomial equations (Eq. (1) and (2)):

$$TPC = 0.11 + 0.052\,T + 0.003\,t - 0.13\,W + 0.007\,M + 0.017\,Tt - 0.074\,TW - 0.012\,TM + 0.074\,W^2 \quad (R^2 = 0.9766;\ R^2adjust = 0.9649) \tag{1}$$

$$TFC = 0.19 + 0.11\,T + 0.012\,t - 0.029\,W + 0.068\,Tt - 0.034\,TW - 0.075\,tW + 0.018\,T^2 \quad (R^2 = 0.9886;\ R^2adjust = 0.9848) \tag{2}$$

where T is the temperature (°C), t is the time (min), W is the percentage of water in NADES (%) and M is the ChCl:Glyc molar ratio. In both re-

Table 2
Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity measured in the liquid extracts resulting from microwave assisted NADES extraction of SCG.

Run	TPC (mg GAE g^{-1} SCG)	TFC (mg CE g^{-1} SCG)	Antioxidant capacity (mg TE g^{-1} SCG)		
			DPPH	ABTS	FRAP
1	0.09 ± 0.01	0.11 ± 0.01	0.23 ± 0.01	1.09 ± 0.01	0.34 ± 0.01
2	0.42 ± 0.01	0.36 ± 0.02	0.57 ± 0.01	2.56 ± 0.02	1.27 ± 0.01
3	0.12 ± 0.03	0.12 ± 0.00	0.21 ± 0.02	1.13 ± 0.00	0.42 ± 0.01
4	0.09 ± 0.01	0.12 ± 0.00	0.16 ± 0.01	1.16 ± 0.02	0.38 ± 0.01
5	0.19 ± 0.02	0.10 ± 0.00	0.25 ± 0.02	1.02 ± 0.03	0.38 ± 0.01
6	0.13 ± 0.00	0.21 ± 0.01	0.23 ± 0.04	1.48 ± 0.00	0.50 ± 0.00
7	1.15 ± 0.04	0.16 ± 0.01	0.34 ± 0.00	1.24 ± 0.08	0.45 ± 0.01
8	0.14 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	1.64 ± 0.00	0.47 ± 0.00
9	0.46 ± 0.01	0.43 ± 0.01	0.56 ± 0.01	3.07 ± 0.06	1.54 ± 0.03
10	0.23 ± 0.01	0.09 ± 0.01	0.23 ± 0.02	0.93 ± 0.02	0.36 ± 0.01
11	0.12 ± 0.01	0.19 ± 0.01	0.26 ± 0.01	1.45 ± 0.03	0.46 ± 0.01
12	0.05 ± 0.01	0.25 ± 0.00	0.44 ± 0.01	2.07 ± 0.03	0.78 ± 0.02
13	0.06 ± 0.00	0.12 ± 0.01	0.22 ± 0.01	1.27 ± 0.01	0.42 ± 0.00
14	0.49 ± 0.09	0.41 ± 0.02	0.57 ± 0.01	3.12 ± 0.05	1.49 ± 0.01
15	0.13 ± 0.00	0.21 ± 0.01	0.37 ± 0.00	1.69 ± 0.08	0.59 ± 0.01
16	0.09 ± 0.02	0.18 ± 0.01	0.36 ± 0.01	1.66 ± 0.01	0.57 ± 0.01
17	0.10 ± 0.01	0.20 ± 0.01	0.34 ± 0.01	1.61 ± 0.04	0.55 ± 0.01
18	0.09 ± 0.01	0.08 ± 0.00	0.27 ± 0.01	1.14 ± 0.02	0.42 ± 0.01
19	0.10 ± 0.02	0.19 ± 0.01	0.28 ± 0.01	1.58 ± 0.00	0.52 ± 0.01
20	0.06 ± 0.01	0.27 ± 0.01	0.41 ± 0.02	2.05 ± 0.03	0.70 ± 0.01
21	0.20 ± 0.01	0.47 ± 0.00	0.55 ± 0.01	3.15 ± 0.01	1.58 ± 0.02
22	0.05 ± 0.00	0.05 ± 0.00	0.14 ± 0.01	0.78 ± 0.01	0.24 ± 0.00
23	0.06 ± 0.00	0.28 ± 0.01	0.34 ± 0.01	1.86 ± 0.05	0.64 ± 0.01
24	0.11 ± 0.01	0.17 ± 0.00	0.35 ± 0.02	1.59 ± 0.03	0.61 ± 0.02
25	0.11 ± 0.01	0.21 ± 0.01	0.47 ± 0.01	1.91 ± 0.03	0.73 ± 0.01
26	0.10 ± 0.00	0.20 ± 0.01	0.37 ± 0.02	1.81 ± 0.05	0.66 ± 0.01
27	0.33 ± 0.03	0.35 ± 0.00	0.56 ± 0.02	2.90 ± 0.02	1.41 ± 0.05
28	0.06 ± 0.01	0.11 ± 0.01	0.28 ± 0.01	1.33 ± 0.01	0.43 ± 0.01
29	0.27 ± 0.03	0.42 ± 0.05	0.31 ± 0.00	2.08 ± 0.01	0.80 ± 0.03
30	0.15 ± 0.01	0.12 ± 0.01	0.29 ± 0.00	1.39 ± 0.01	0.44 ± 0.00

GAE: Gallic acid equivalents.
CE: Catechin equivalents.
TE: Trolox equivalents.

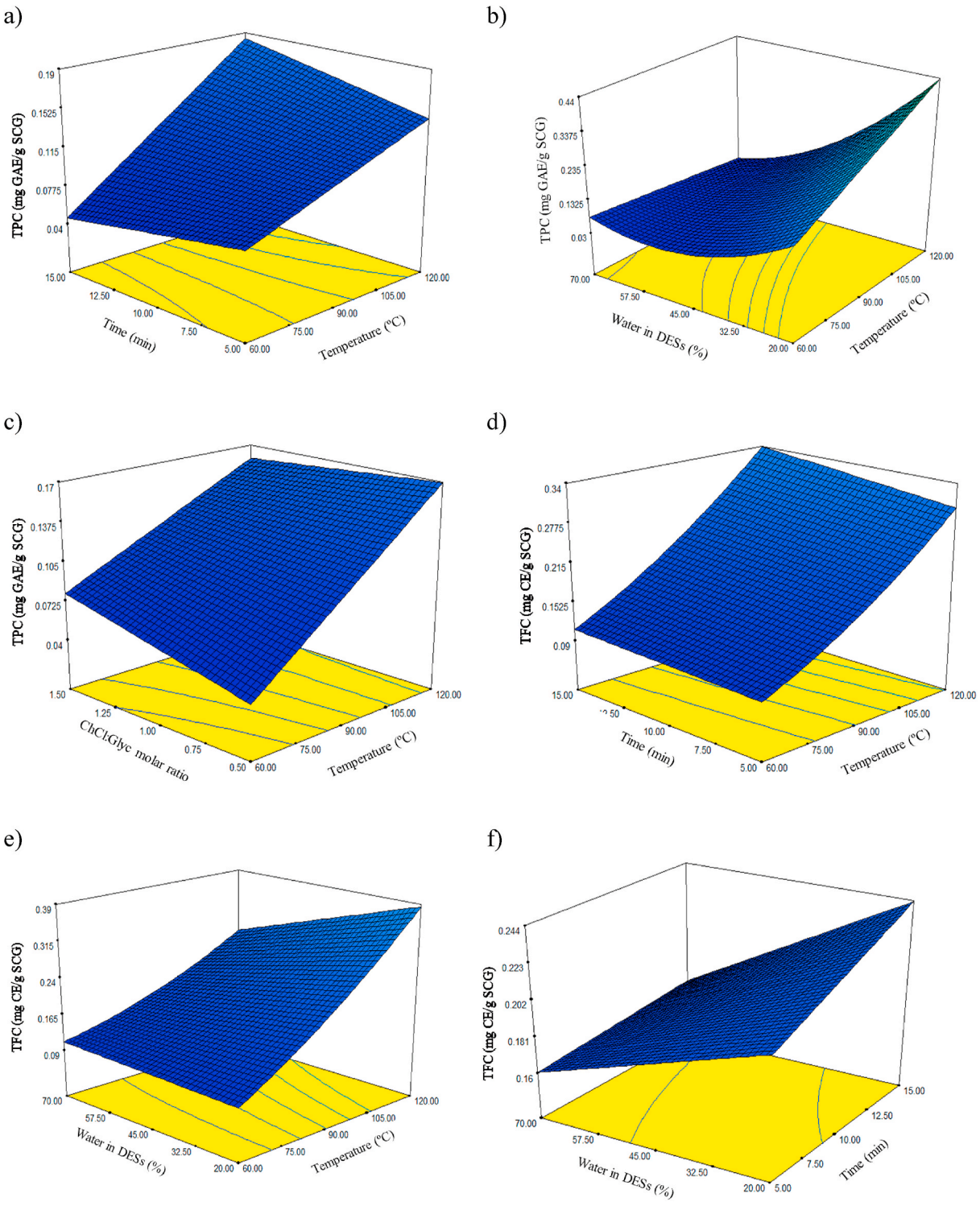

Fig. 1. Response surface for total phenolic content (TPC) (a,b,c) and total flavonoid content (TFC) (d,e,f) as a function of (a,d) temperature and extraction time at 45% water in NADES and 1 ChCl:Glyc molar ratio (b,e) temperature and percentage of water in NADES at 10 min and 1 ChCl:Glyc molar ratio (c) temperature and ChCl:Glyc molar ratio at 10 min and 45% water in NADES (f) extraction time and percentage of water in NADES at 90 °C and 1 ChCl:Glyc molar ratio.

sponses, the values of R^2 and adjusted R^2 (Eq. (1) and (2)), as well as the confidence levels (95%, $p < 0.05$), show good agreement between experimental and predicted data.

As can be observed in Eqs. (1) and (2), and Table 2, in both TPC and TFC responses, the effect of temperature and time were positive, whereas a negative influence of the % of water in NADES was found. The effect of the ChCl:Glyc molar ratio in the TPC was slightly positive, while it was insignificant in the TFC. Moreover, it is worth noting that, unlike those achieved for the TPC response, where the effect of the % of water in the NADES was the highest, the factor with the highest influence in the TFC was the temperature, probably due to a higher thermosensitivity of the flavonoid compounds [49].

On the other hand, concerning the interactions between the different factors (Eq. (1) and (2)), a positive interaction between temperature and

time was achieved for both TPC and TFC responses. In this way, high values of both variables could lead to an increase in the TPC and TFC. This trend can also be observed in Fig. 1(a,d), which is a response surface plot for TPC (a) and TFC (d) as a function of temperature and extraction time at 45% water in NADES and 1 ChCl:Glyc molar ratio. However, the interaction found between temperature and % water in NADES was negative for both responses. Then, a reduction in the TPC and TFC responses could happen due to the combined effect of both variables, since low values of both could lead to high viscosity problems of the NADES [20], or because high values of both could originate the breakup of the hydrogen bonds between NADES components and thus the loss of the eutectic properties of the NADES [50]. Fig. 1(b,e) also describes this behavior, which shows the response surface for TPC (b) and TFC (e) as a function of temperature and % water in NADES at 10 min and 1 ChCl:Glyc molar ratio. Negative interactions between temperature and the ChCl:Glyc molar ratio (Eq. (1) and Fig. 1(c)), and between time and % water in NADES (Eq. (2) and Fig. 1(f)), were also appreciated for TPC and TFC, respectively. Therefore, a reduction in the TPC response could also happen due to the combined effect of temperature and the ChCl:Glyc molar ratio because of the impossibility of the NADES extraction operating at high values of both factors, due to stability problems of the NADES in terms of the mixture remaining in the liquid phase for prolonged periods, or to the use of only one component of NADES (for example, glycerol) when low values of both factors are used [14]. Likewise, the TFC response could also be reduced due to the combined effect of time and % water in NADES due to the same reasons described above for temperature-% water in the NADES interaction. Finally, it is worth mentioning that, for TPC, the negative interaction between temperature and % water in the NADES was the highest appreciated among the interactions, while the most important interaction between factors, found for the TFC, was the negative interaction between time and % water in NADES.

With regard to the individual phenolic compounds (4-hydroxybenzoic acid, vanillic acid, vanillin, coumaric acid, ferulic acid and caffeine) identified for each experimental run, these are displayed in Fig. 2 (expressed as %, referred to the sum of individual phenolic contents). In general, as can be observed, individual phenolic compounds were identified in the following order (concerning their abundance): coumaric acid > vanillin > ferulic acid > 4-hydroxybenzoic acid > vanillic acid > caffein, coumaric acid (ranging between 24 and 39%) thus being the most abundant in all runs. Coumaric acid has been reported as one of the most abundant phenolic compounds identified in SCG extracts [4,7]. This compound, which is important for its hypoglycemic, hepatoprotective, antiviral, antibacterial, and anticarcinogenic properties, could be used as a potential agent in the pharmaceutical, cosmetic and food industries [4].

3.1.2. Antioxidant capacity

In order to assess the influence of microwave assisted NADES extraction conditions on the antioxidant activity of SCG extracts, three methods of antioxidant capacity determination were carried out: DPPH, ABTS and FRAP.

As can be seen in Table 2, the antioxidant capacity in SCG extracts ranged from 0.14 to 0.57, 0.78 to 3.15 and 0.24–1.58 mg TE g^{-1} SCG (in DPPH, ABTS and FRAP tests, respectively). Thus, as can be appreciated, the lowest values of antioxidant capacities were achieved for the lowest extraction temperature used in this work (30 °C, run 22) in the three tests used. Around the central point (90 °C, 10 min, 45% water in NADES and 1 ChCl:Glyc molar ratio), average values of 0.31, 1.58 and 0.53 mg TE g^{-1} SCG (in DPPH, ABTS and FRAP tests, respectively) were determined. According to Silva et al. [51], the presence of antioxidant activity in SCG is associated to different compounds contained in this raw material, such as phenolic compounds, lipids and terpenes.

Second-order polynomial equations were used to adjust these three responses (DPPH, ABTS and FRAP) (Eq. (3), (4) and (5)):

$$DPPH = 0.35 + 0.12\,T - 0.045\,W - 0.080\,M - 0.039\,TW - 0.018\,WM \quad (R^2 = 0.9547;\ R^2adjust = 0.9428) \tag{3}$$

$$ABTS = 1.64 + 0.64\,T + 0.050\,t - 0.22\,W - 0.082\,M - 0.32\,TW + 0.079\,T^2 + 0.082\,M^2 \quad (R^2 = 0.9782;\ R^2adjust = 0.9706) \tag{4}$$

$$FRAP = 0.54 + 0.31\,T + 0.042\,t - 0.20\,W + 0.040\,Tt - 0.21\,TW + 0.10\,T^2 + 0.042\,t^2 \quad (R^2 = 0.9780;\ R^2adjust = 0.9703) \tag{5}$$

where T is the temperature (°C), t is the time (min), W is the percentage of water in NADES (%) and M is the ChCl:Glyc molar ratio. As the values of R^2 and adjusted R^2 (Eq. (3), (4) and (5)), show, as well as the confidence level (95%, $p < 0.05$), a good agreement between experimental

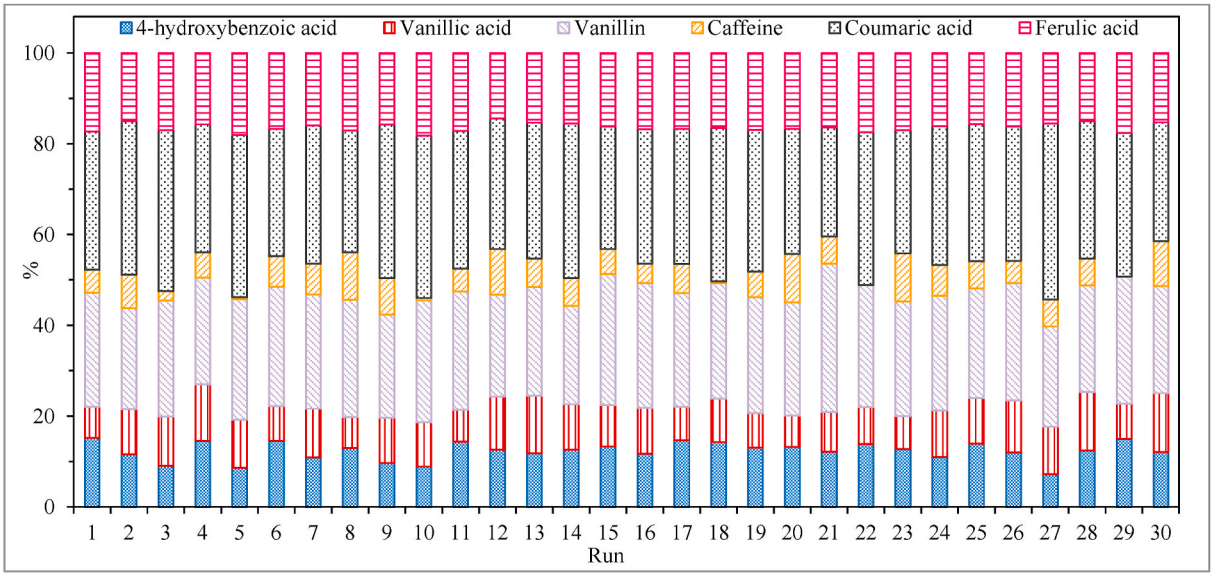

Fig. 2. Percentages (referred to sum of individual phenolic content) of the different individual phenolic compounds (4-hydroxybenzoic acid, vanillic acid, vanillin, coumaric acid, ferulic acid and caffeine) identified in the liquid extracts resulting from microwave assisted NADES extraction of SCG.

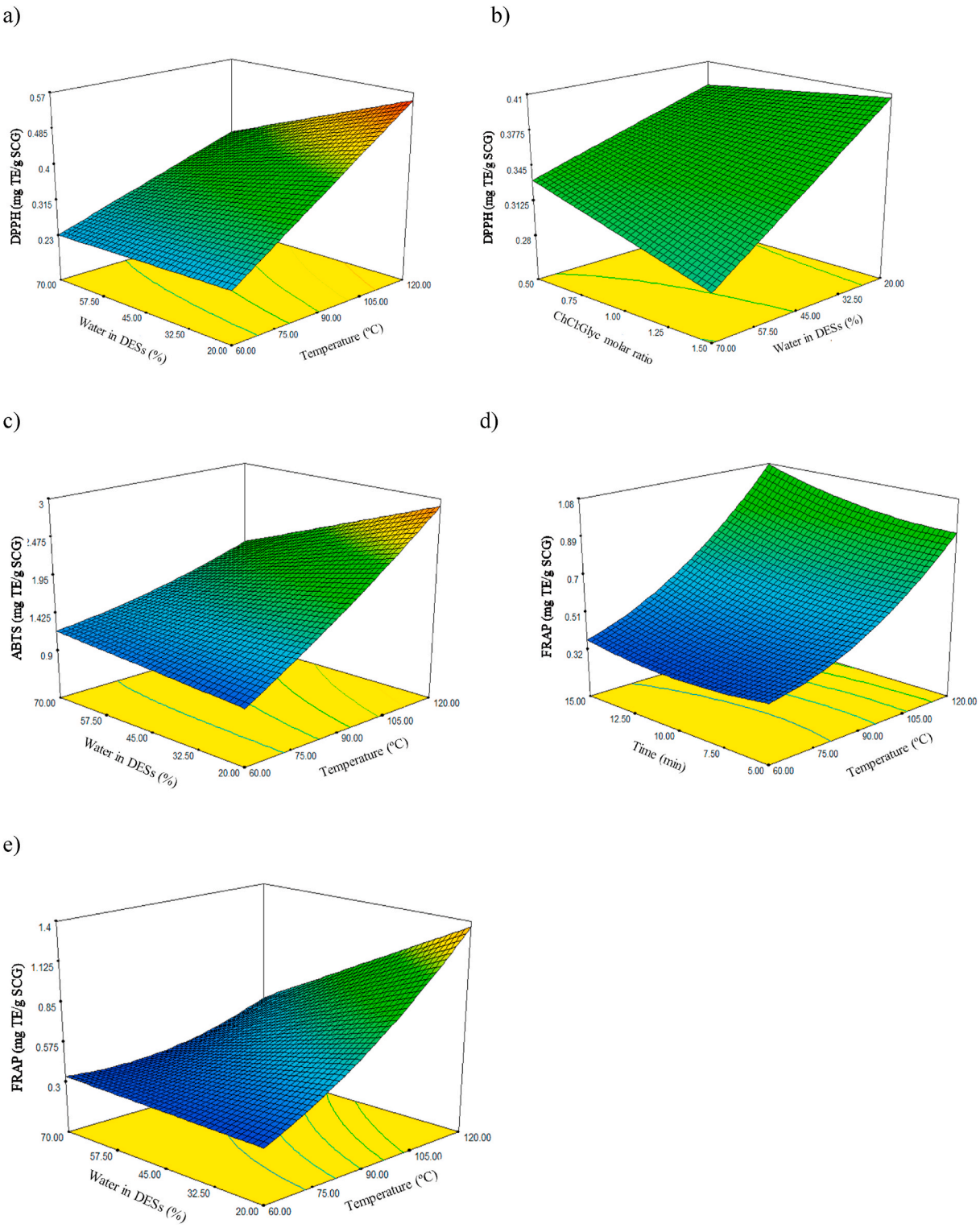

Fig. 3. Response surface for (a) DPPH as a function of temperature and percentage of water in NADES at 10 min and 1 ChCl:Glyc molar ratio (b) DPPH as a function of percentage of water in NADES and ChCl:Glyc molar ratio at 90 °C and 10 min (c) ABTS as a function of temperature and percentage of water in NADES at 10 min and 1 ChCl:Glyc molar ratio (d) FRAP as a function of temperature and extraction time at 45% water in NADES and 1 ChCl:Glyc molar ratio (e) FRAP as a function of temperature and percentage of water in NADES at 10 min and 1 ChCl:Glyc molar ratio.

and predicted data occurred for the three responses.

As can be appreciated in Table 2 and Eqs. (3)–(5), a positive influence of the temperature factor was found for the three responses, as well as a negative influence of % water in NADES. This same behavior was also observed for the TPC and TFC. Moreover, temperature was the most important variable evaluated in the DPPH, ABTS and FRAP responses, as happened for the TFC (Eq. (2)). Yoo et al. [52] also observed a significant positive effect of temperature on antioxidant activity, measured by both DPPH and FRAP assays in the ultrasound-assisted NADES extraction from SCG. Regarding the time factor, this variable positively

affected the ABTS and FRAP responses, whereas it was not significant for the DPPH response. Considering the ChCl:Glyc molar ratio, although no significant effect of this factor was found for the FRAP response, it negatively affected the DPPH and ABTS responses.

On the other hand, concerning the interactions between different factors, as happened for the TPC and TFC, a negative interaction between temperature and % water in NADES occurred for the DPPH, ABTS and FRAP responses (Eqs. (3)–(5) and Fig. 3(a,c,e)) due to the same reasons described above for TPC, this being the most important interaction. Moreover, in the case of the DPPH response (Eq. (3)), a slight negative interaction between % water in NADES and the ChCl:Glyc molar ratio was found, which is also shown in Fig. 3(b). Then, the combined effect of these variables could lead to a reduction in the DPPH, as low values of both variables could lead to high viscosity problems of the NADES and the use of a NADES mainly formed by only one component (for example, glycerol), or because high values of both variables could originate the breakup of the hydrogen bonds between the NADES components and then the loss of the eutectic properties of the NADES, as well as stability problems of the NADES in terms of the mixture remaining in the liquid phase for prolonged periods [50]. What is more, a slight positive interaction between temperature and time factors can be also appreciated for the FRAP response (Eq. (5) and Fig. 3 (d)), as was also found for TPC and TFC.

3.2. Optimization of operating conditions for microwave assisted NADES extraction

An optimization of microwave assisted NADES extraction from SCG was carried out by simultaneously maximizing the five responses studied in this work (TPC, TFC and antioxidant capacity by DPPH, ABTS and FRAP essays), since the antioxidant compounds content is closely related to their bioactive properties. In this way, the optimal experimental conditions found by the model were: 120 °C, 15 min, 20% water in NADES and 0.53 ChCl:Glyc molar ratio. Under these extraction conditions, the values predicted by the model for the five responses studied were: TPC, 0.46 mg GAE g^{-1} SCG; TFC, 0.41 mg CE g^{-1} SCG; DPPH, 0.54 mg TE g^{-1} SCG; ABTS, 3.10 mg TE g^{-1} SCG; and FRAP, 1.49 mg TE g^{-1} SCG.

A confirmatory experimental run was done under optimal extraction conditions to validate the model. As a result, the following experimental values were achieved for the five responses studied: TPC, 0.48 mg GAE g^{-1} SCG; TFC, 0.44 mg CE g^{-1} SCG; DPPH, 0.55 mg TE g^{-1} SCG; ABTS, 3.17 mg TE g^{-1} SCG; and FRAP, 1.52 mg TE g^{-1} SCG. Then, as can be observed, a good adjustment (deviations < 7%) between the predicted and experimental values was achieved. Different individual phenolic compounds were identified in the optimal liquid extract, being expressed as % (referred to the sum of individual phenolic contents): 4-hydroxybenzoic acid, 7.8; vanillic acid, 9.1; vanillin, 19.6; caffeine, 12.4; coumaric acid, 36.7; and ferulic acid, 14.3; coumaric acid thus being the most abundant.

In order to compare the efficiency of the NADES extraction process, a microwave assisted extraction with a conventional organic solvent (ethanol-water 25:75, v/v) was carried out, the following values being achieved for the five responses evaluated in this study: TPC, 0.30 mg GAE g^{-1} SCG; TFC, 0.23 mg CE g^{-1} SCG; DPPH, 0.42 mg TE g^{-1} SCG; ABTS, 1.83 mg TE g^{-1} SCG; and FRAP, 0.67 mg TE g^{-1} SCG. From these results, it was concluded that NADES exhibits a greater extraction capacity of phenolic and antioxidant compounds in comparison with conventional organic solvents. Ivanović et al. [11] also demonstrated a better efficiency of NADES by comparing it with conventional solvents, such as methanol, in the extraction of phenolic compounds from *Lippia citriodora* (lemon verbena).

The results obtained in this work, considering the DPPH response, which is one of the most commonly used methods to determine antioxidant capacity, agree with those achieved (0.05–0.93 mg g^{-1}) by Ramón-Gonçalves et al. [7], who extracted (at 60 °C and 25% ethanol for 15 min) valuable polyphenols using spent coffee grounds from different coffee varieties. No different TFC and FRAP values (0.75 mg CE g^{-1} SCG and 2.68 mg TE g^{-1} SCG, respectively) were attained in the extraction with 50% methanol (at 60–65 °C and 10 mL methanol g^{-1} raw material for 90 min) of bioactive compounds from SCG [12]. Ballesteros et al. [10] also reported a TFC of 1.87 mg CE g^{-1} SCG in the extraction by autohydrolysis (at 200 °C and 15 mL water g^{-1} SCG for 50 min) of antioxidant phenolic compounds from SCG. Similar results (TPC, 1.36 mg GAE g^{-1} SCG; DPPH, 0.64 mg TE g^{-1} SCG; ABTS, 2.02 mg TE g^{-1} SCG) were achieved by Bravo et al. [53] in the extraction of phenolic and antioxidant compounds from Arabica SCG (mocha coffeemaker), using petroleum ether (1:11, w/v) for 3 h at 60 °C in a Soxhlet extraction system, followed by water extraction at 90 °C for 6 min. In this context, it is worth mentioning that, even though organic solvents have been reported in the literature as good solvents for the extraction of bioactive compounds, due to their high toxicity, their application in the food and pharmaceutical fields are very limited [10]. Nevertheless, the NADES used in this work have a very low toxicity, so the bioactive compounds extracted can be used in food and life sciences industries [21].

Table 3
Carbohydrates and inhibitor compound concentrations in slurry enzymatic hydrolysate before and after detoxification.

Carbohydrates (g L^{-1})	Detoxification Method		
	None	Activated charcoal (5% w/v)	Activated charcoal (10% w/v)
Glucose	22.3 ± 0.3	20.6 ± 0.2	17.2 ± 0.2
Mannose	19.2 ± 0.1	17.2 ± 0.2	14.0 ± 0.1
Inhibitor compounds (g L^{-1})			
Formic acid	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Acetic acid	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
HMF	0.3 ± 0.0	n.d.	n.d.
Furfural	0.1 ± 0.0	n.d.	n.d.
Total phenols	0.3 ± 0.0	n.d.	n.d.

HMF: 5-hydroxymethylfurfural.
n.d.: not detected.

On the other hand, an extracted solid was obtained under optimal extraction conditions, its composition being the following: cellulose, 16.4 ± 0.3%; hemicellulose, 28.4 ± 0.3%; and lignin, 49.1 ± 0.4%. Then, by comparing with the composition of raw SCG, the optimal extracted solid was slightly enriched in cellulose and hemicellulose, but mainly in lignin, achieving high recoveries of glucose and hemicellulosic sugars in extracted solid (95.5 and 97.5%, respectively). Therefore, these results show that the residual extracted solid obtained under optimal extraction conditions had not disturbed its structural carbohydrates, and this needs to be valorized in the context of a biorefinery in order to guarantee the profitability of the microwave assisted NADES extraction process of bioactive compounds from SCG. In this way, it could be used as substrate in a process of acetone-butanol-ethanol (ABE) fermentation for biobutanol production.

3.3. Butanol production from extracted SCG

In order to break the lignocellulosic structure of residual extracted SCG obtained under optimal NADES extraction conditions, a microwave pretreatment assisted by dilute sulfuric acid was carried out. In this way, the solubilization of hemicellulosic sugars is obtained, maintaining the cellulose in the pretreated solid fraction. Next, the resulting whole slurry (without solid-liquid separation) was enzymatically hydrolyzed using Cellic CTec2 (a cellulolytic complex) and Pectinex Ultra SP-L (a

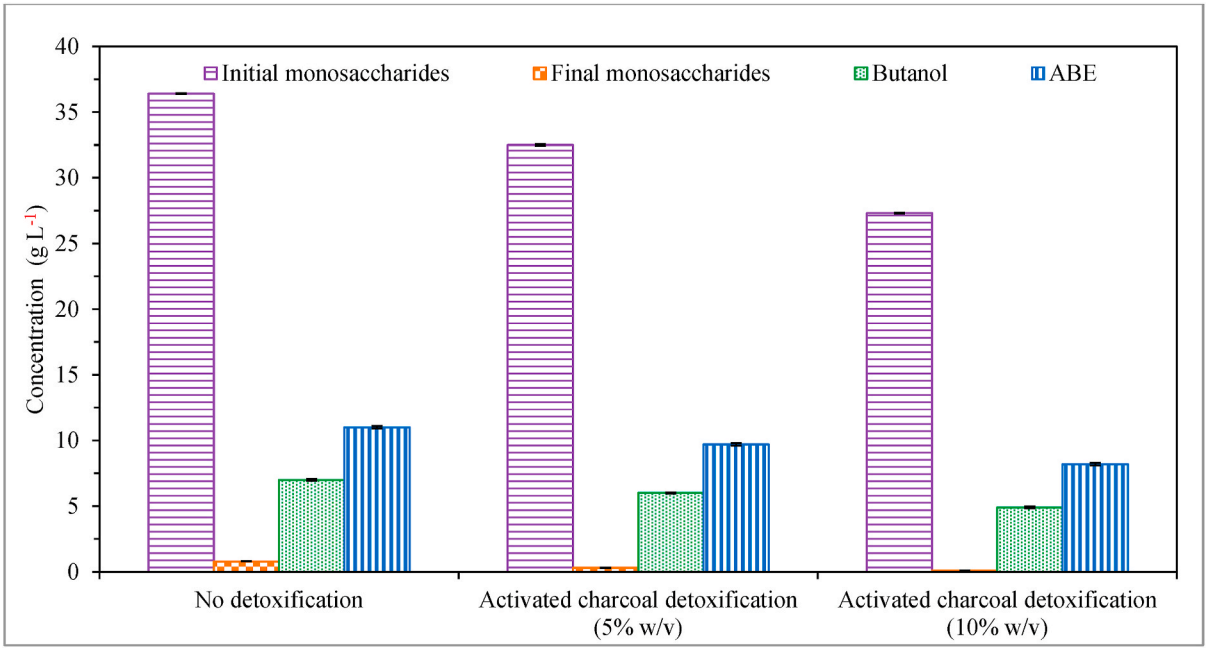

Fig. 4. ABE fermentation of slurry enzymatic hydrolysate before and after detoxification by activated charcoal.

Table 4
ABE fermentation of the slurry enzymatic hydrolysate before and after detoxification by activated charcoal. Sugar uptake (%), final acetic and butyric acid concentration (g L^{-1}), butanol and ABE yields ($Y_{BUT/sugars}$, $Y_{ABE/sugars}$ expressed as g g^{-1} sugars consumed), and butanol and ABE productivities (P_{BUT}, P_{ABE} expressed as g $L^{-1}{\cdot}h^{-1}$) at the time of maximum production of butanol and ABE (48 h).

	Slurry enzymatic hydrolysate		
	No detoxification	Activated charcoal detoxification (5% w/v)	Activated charcoal detoxification (10% w/v)
Sugar uptake (%)	97.9	99.1	99.5
Final products (g L^{-1})			
$C_{Acetic\ acid}$	0.3 ± 0.0	0.3 ± 0.0	0.0 ± 0.0
$C_{Butyric\ acid}$	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0
Yields (g g^{-1})			
$Y_{BUT/sugars}$	0.20	0.19	0.18
$Y_{ABE/sugars}$	0.31	0.30	0.30
Productivities (g $L^{-1}{\cdot}h^{-1}$)			
P_{BUT}	0.147	0.126	0.102
P_{ABE}	0.229	0.203	0.170

commercial β-galactosidase). The use of the acid whole slurry is considered essential, since it allows both cellulosic and hemicellulosic sugars to be present in a single hydrolysate, which can easily be fermented in a single reactor.

It is worth mentioning that, unlike other lignocellulosic residues, SCG contains an important content in galactans and mannans [54]. So, in order to recover the totality of the carbohydrates contained in SCG, besides the typical cellulolytic complex used, it is also necessary to use more specific enzymes, such as β-galactosidases and mannosidases. In this context, different enzymes have been reported in the literature as suitable for breaking up galactans and mannans, highlighting for instance the Pectinex Ultra SP-L and Saphera 2600L enzymes [55,56]. Both enzymes were tested in this work, and the Pectinex Ultra SP-L enzyme turned out to be much more efficient, considering mainly the breakup of the mannans groups contained in the acid pretreated SCG (data not shown).

The composition of the enzymatic hydrolysate attained is shown in Table 3. So, as can be seen, the recuperation of the total sugars (glucose and mannose) contained in the raw SCG was 72%. Moreover, in order to reduce the potential inhibitor compounds content, the enzymatic hydrolysate was detoxified with activated charcoal (at 5 and 10% w/v), its effect on the reduction of inhibitor compounds and resulting loss of sugars being evaluated (Table 3). The activated charcoal treatment is considered as one of the more economical and effective detoxification methods [57]. The detoxification allowed the total elimination of HMF, furfural and total phenols, while it was not effective for the reduction of formic and acetic acids. High furan and phenolic compound reductions by activated charcoal detoxification were also reported from hydrolysates of the sweet sorghum bagasse [58] and corn pericarp [59].

Therefore, an initial fermentation broth with a total sugar concentration as high as 36.4 g L^{-1} (without detoxification), and 32.5 and 27.3 g L^{-1} (after activated charcoal detoxification at 5 and 10% w/v, respectively) were attained (Fig. 4), which were subjected to ABE fermentation with *C. beijerinckii*. In this way, as can be seen in Table 4, the most sugars were consumed by *C. beijerinckii* at 48 h fermentation for all cases (sugar uptake > 98%). Moreover, as can be appreciated in Fig. 4 and Table 4, the best fermentation results were obtained for non-detoxified slurry enzymatic hydrolysate, achieving butanol and ABE concentrations of 7.1 and 11.0 g L^{-1}, respectively, which corresponds to high butanol and ABE yields (0.20 and 0.31 g g^{-1} sugars consumed,

respectively) and productivities (0.147 and 0.229 g L^{-1} h^{-1}, respectively). It is worth highlighting that no detoxification was necessary to ferment the SCG slurry enzymatic hydrolysate, which may be due to its relatively low content of inhibitor compounds.

These results were favorable to those obtained in the fermentation with *C. acetobutylicum* (8.5 g L^{-1} ABE) of the non-detoxified whole slurry of green macroalgae *Enteromorpha intestinalis*, which was pretreated with sulfuric acid (121 °C, 60 min, 270 mM H_2SO_4) [60]. Lower butanol and ABE concentrations (4.43 and 6.69 g L^{-1}, respectively) were attained in the fermentation with *C. acetobutylicum* of sulfuric acid hydrolysates (121 °C, 15 min, 1.5% H_2SO_4) from sugarcane industry waste [61]. López-Linares et al. [62] also reported comparable butanol and ABE concentrations (8.2 and 11.8 g L^{-1}, respectively) and butanol and ABE yields (0.26 and 0.37 g g^{-1}) in the fermentation with *C. beijerinckii* of activated charcoal detoxified slurry enzymatic hydrolysate of brewer's spent grain, subjected to microwave assisted dilute sulfuric acid pretreatment (147 °C, 2 min, 1.26% H_2SO_4). Similar butanol and ABE concentrations (7.0 and 11.4 g L^{-1}, respectively), and a slightly higher butanol yield (0.27 g g^{-1}), were achieved by Hijosa-Valsero et al. [35] in the fermentation with *C. beijerinckii* of slurry enzymatic hydrolysate of coffee silverskin, which had previously been pretreated by autohydrolysis (170 °C, 20 min).

3.4. Overall process material balance

The material balance of the overall proposed process for antioxidant and ABE production with *C. beijerinckii* from SCG is shown in Fig. 5. SCG was extracted under microwave irradiation with NADES at optimal conditions (120 °C, 15 min, 20% water in NADES and 0.53 ChCl:Glyc molar ratio). The obtained extract contained: total phenolic content (TPC), 0.5 kg of gallic acid equivalents (GAE) t^{-1} SCG (dry matter) and total flavonoid content (TFC), 0.4 kg of catechin equivalents (CE) t^{-1} SCG (dry matter). The solid resulting from the extraction was pretreated with microwave pretreatment with sulfuric acid 1% (w/v) at 170 °C and 10% w/v solid-liquid ratio for 5 min, resulting in a slurry which was enzimatically hydrolized and fermented with *C. beijerinckii*. In this way, a total production of 81 kg butanol t^{-1} SCG (dry matter) and 126 ABE t^{-1} SCG (dry matter) was achieved. The integrated valorization process of SCG confirmed good recovery rates for antioxidant compounds as high-added-value products and a good yield for butanol as an excellent renewable biofuel.

4. Conclusions

A newfangled and green extraction process based on microwave-assisted NADES combined with for the recovery of antioxidant compounds from SCG is proposed. The optimal extraction conditions were found to be 120 °C, 15 min, 20% water in NADES and 0.53 ChCl:Glyc molar ratio, an extract being produced with 0.48 mg GAE g^{-1} SCG (TPC), mainly formed by coumaric acid; 0.44 mg CE g^{-1} SCG (TFC); and antioxidant activities: DPPH, 0.55 mg TE g^{-1} SCG; ABTS, 3.17 mg TE g^{-1} SCG; and FRAP, 1.52 mg TE g^{-1} SCG. Furthermore, the subsequent valorization of the residual extracted SCG led to 126 kg ABE t^{-1} SCG (81 kg butanol t^{-1} SCG) by ABE fermentation. Considering the efficiencies of the global process (0.48 mg GAE g^{-1} SCG and 81 kg butanol t^{-1} SCG) the profits of jointly selling both products could reach up to 0.36 € kg^{-1} SCG, showing the potential of this lignocellulosic residue for use in an integrated biorefinery approach.

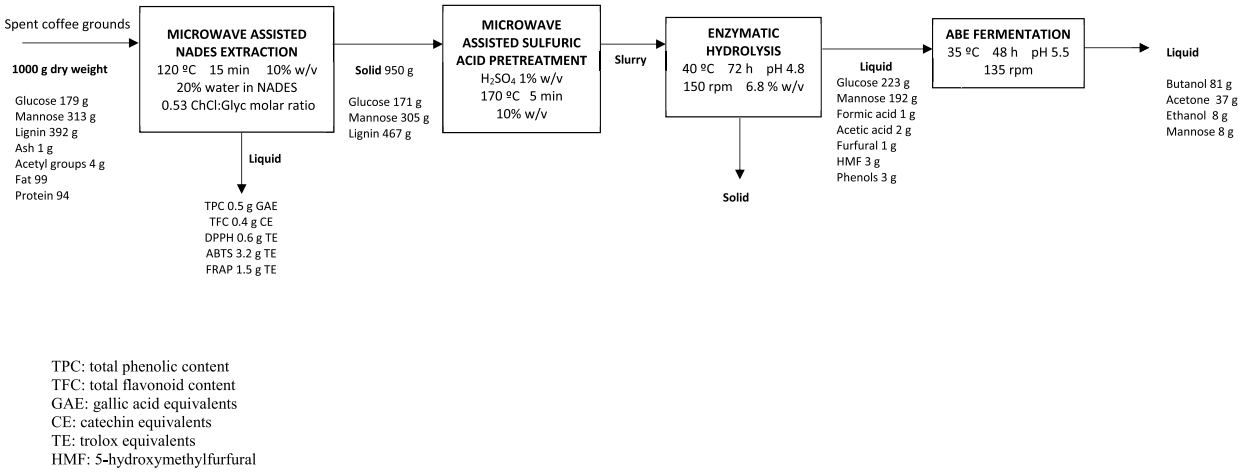

Fig. 5. Mass balance flow diagram of the overall process of antioxidants compounds extraction and biobutanol production from SCG by microwave assisted NADES extraction.
TPC: total phenolic content
TFC: total flavonoid content
GAE: gallic acid equivalents
CE: catechin equivalents
TE: trolox equivalents
HMF: 5-hydroxymethylfurfural.

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