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Pulsed electric field (PEF) as pre-treatment to improve the phenolic compounds recovery from brewers' spent grains

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Abstract

Brewers' spent grain (BSG) is the most abundant by-product obtained from beer production. However, it contains some bioactive compounds such as phenolic compounds, therefore, the valorization of BSG is important to recovery these compounds and reused them as functional ingredients in food industry. Therefore, in this work, pulsed electric field (PEF) has been used as extraction pre-treatment. PEF parameters such as electric field strength E (0.5, 1.5, 2.5 kV/cm), frequency (50, 100, 150 Hz) and total time of treatment (5, 10, 15 s) were optimized in order to maximize the content of flavan-3-ols, flavonoids, phenolic acid derivates and total free phenolic compounds. Optimal conditions to the maximum value of total free phenolic compounds were the following: 2.5 kV/cm, 50 Hz and 14.5 s. Concentrations of total free and bound phenolic compounds from BSG under these PEF optimum conditions were 2.7 and 1.7 times, respectively, higher than in case of the extraction without PEF pre-treatment, indicating an improvement in the phenolic recovery with the use of PEF as a pre-treatment in brewers spent grain samples.

Keywords: Pulsed Electric Field, free and bound phenolic compounds, brewers' spent grain, Box-Behnken design

1. Introduction

Brewers' spent grain (BSG) is the most abundant brewing by-product, corresponding to approximately 85% of total by-products generated. BSG may consist of the residues from malted barley, or those from malted barley and adjuncts (non-malt sources of fermentable sugars), such as wheat, rice, or maize added during mashing (Gupta et al., 2010).

Chemical composition of BSG varies according to barley variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process (Gupta et al., 2010); but in general, BSG is considered as a lignocellulosic rich in fiber and proteins and also contains appreciable amounts of lipids, carbohydrates, polyphenols and minerals (Mudura, Socaci, Dulf, & Tofan, 2015; Mussatto, 2014; Niemi et al., 2012). These compounds, when incorporated into human diets, may provide a number of benefits by lowering the risk of certain diseases including cancer, gastrointestinal disorders, diabetes, obesity and coronary heart disease (Mudura et al., 2015). Therefore, the valorization of BSG is important in order to recovery these high-value compounds that can be extracted, purified and reused as functional ingredients in food industry and in others industries (Hansen et al., 2004).

BSG consists predominantly of the husk, pericarp, and seed coat and is largely made up of cell walls (Mussatto, 2014). Barley provides a broad range of phenolic compounds that includes derivatives of benzoic and cinnamic acids, flavonoids, proanthocyanidins, tannins, and amino phenolic compounds, which are located mainly in the husk and hydroxycinnamic acids accumulate in the cell wall. Therefore, BSG is a potentially valuable source of these compounds (Guido & Moreira, 2017), which are an important source of antioxidants in cereals, and they are found in free and in the bound form. The majority of free phenolics in barley are flavanols, whereas the bound phenolics are mainly phenolic acids, which are ester-linked to cell wall polysaccharides (Gómez-Caravaca, Verardo, Berardinellic, Marconid, & Caboni, 2014; Guido & Moreira, 2017).

The re-emergence of nutraceuticals from agricultural by-products is achieved due to the existence of some conventional and emerging technologies, which allow both their recovery and also their reutilization inside foods (Galanakis, 2013). Five distinct recovery stages of high-added value

components from food waste are usually applied: macroscopic pre-treatment, macro- and micro-molecules separation, extraction, purification and nutraceuticals formation. Although, some steps are sometimes deleted or over-subscribe each other. Processing often advances from the macroscopic pre-treatment to the macro and micro molecular separation, after that, to the extraction of specific micro-molecules before the purification and finally to the encapsulation of the target ones. The objective of the macroscopic pre-treatment is the setting of the food waste matrix according to the water content, enzymatic activity and permeability of the bioresource tissues (Galanakis, 2012). Extraction technique represents the most important step in the recovery and isolation of phenolic compounds from brewers' spent grain. Many factors such as solvent composition, extraction temperature and solvent-to-solid ratio, may significantly influence the extraction efficiency, antioxidant activity and phenolic content. Therefore, it is necessary to optimize the extraction conditions to improve phenolic recovery (Carciochi, Sologubik, Fernández, Manrique, & D'Alessandro, 2018; Guido & Moreira, 2017). Solid–liquid extractions (SLE) are the most commonly used procedures prior to analysis of phenolics in BSG samples, due to their ease of use, efficiency, and wide applicability (McCarthy, O'Callaghan, Neugart, et al., 2013; Meneses, Martins, Teixeira, & Mussatto, 2013). Some studies have reported different extractions techniques for the recovery of phenolic compounds from brewers' spent grain such as ultrasound-assisted extraction (UAE) (Carciochi et al., 2018) and microwave assisted extraction (MAE) (Athanasios, Georgios, & Michael, 2007; Moreira, Morais, Barros, Delerue-Matos, & Guido, 2012). UAE and MAE have been considered as an alternative to SLE for the extraction of phenolic compounds from plants for various reasons: reduced extraction time, reduced solvent usage, and improved extraction yield (Guido & Moreira, 2017). Recently, pulsed electric field (PEF) has been used for the extraction in plants. The principle of PEF is to disintegrate the cell membrane structure for increasing extraction. When an electric field is applied to a living cell, an electric potential pass through the membrane of that cell. Based on the dipole nature of membrane molecules, electric potential separates molecules according to their charge in the cell membrane. After exceeding a critical value of approximately 1 V of transmembrane potential, repulsion occurs between the charge carrying molecules that form pores in weak areas of the membrane and causes a drastic increase of permeability. The effectiveness of PEF treatment strictly depends on the process parameters including

electric field strength, pulse shape, pulse width, number of pulses, pulse specific energy, and frequency (Heinz, Toepfl, & Knorr, 2003; Puértolas, Luengo, I.Alvarez, & Raso, 2012). PEF can increase mass transfer during extraction by drilling of the membrane structure of plant materials for enhancing extraction and decreasing extraction time. PEF has been applied to improve the release of intracellular compounds from plant tissue with the help of increasing cell membrane permeability (Azmir et al., 2013). PEF could be also applicable on plant materials as a pre-treatment process prior to conventional extraction to lower extraction time (Fincan & Dejmek, 2002; López, Puértolas, Condón, Raso, & Alvarez, 2009). Moreover, previous studies reported an increase in the phenolic content when PEF treatment was applied as the pre-treatment step in food samples such as grapes or grape pomace, onion, orange peel, sorghum flour and apple pomace (Esteve, 2015; Liu, Zeng, & Ngadi, 2018; Lohani & Muthukumarappan, 2016; Yang, Huang, Lyu, & Wang, 2016). In general PEF intensities ranging from 0.5 to 2 kV/cm are used for fresh materials whereas high dry matter containing materials require higher intensity e.g. 20 kV/cm (Boussetta, Soichi, Lanoisellé, & Vorobiev, 2014; Liu, Zeng, & Ngadi, 2018). A recent study, which applied PEF in Panax ginseng at electrical field strengths varying from 0.5 to 2.5 kV/cm, the pulse number of 500, the pulse frequency of 50 Hz, and the pulse width of 25 μs, showed a higher phenolic content in samples treated at 1.5 to 2.5 kV/cm than in the control one or the one treated at 0.5 kV/cm (Kim, Kwon, & Lee, 2019). For that, PEF could be applied to BSG samples as a pre-treatment process to conventional extraction to lower extraction effort.

One study has reported the effect of PEF treatment with an electric field strength of 2.8 kV/cm, frequency of 10 Hz and a total of 3000 pulses with a pulse width of 20 μs on the contents of bioactive constituents in dark and light BSG extracts as well as on their antioxidant, antimicrobial and immunomodulatory properties. Light BSG extracts pre-treated with PEF showed higher antimicrobial activity compared to the untreated extracts. Nevertheless, this study did not show significant differences on the total phenolic content, antioxidant activity and on the immunomodulatory activity in PEF treated extracts compared to untreated extracts for both the BSG samples (Kumari et al., 2019).

Therefore, this work was focused on the extraction by PEF treatment and identification and quantification, by HPLC-MS, of phenolic compounds from brewers spent grain. An experimental design response-surface Box-Behnken has been performed to optimize the extraction parameters of PEF: electric field strength, frequency and total time. In addition, in order to show the improvement on the efficiency of extraction by PEF as pre-treatment, a comparison on the content of free and bound phenolic compounds in PEF brewers spent grain extracts with those obtained without PEF treatment was carried out.

2. Material and methods

2.1. Chemicals

HPLC-grade acetonitrile, water, methanol, acetone, acetic acid, ethanol were purchased from Merck KGaA (Darmstadt, Germany). Ferulic acid, catechin and quercetin were from Sigma-Aldrich (St. Louis, MO).

2.2. Samples

Brewers' spent grain samples were obtained in a microbrewing plant after special beer production (Mastrobirraio, Cesena, Italy, 44°08′00″N 12°14′00″E).

2.3. Experimental design for Pulsed electric field extraction (PEF) in brewers' spent grain

The protocol of PEF pre-treatment was the following: 60 g of brewers spent grain with water ratio 1:1, was placed into a rectangular treatment chamber (5 x 5 x 5 cm) equipped with two stainless steel electrodes (5 x 5 cm) with a gap between them of 22 mm. The conductivity of the mix was of 463 mS/cm (measured by EC-Meter basic 30+, Crison). PEF treatments were applied by using pulse generator S-P7500 60A 8kV (Alintel srl., Bologna). The pulse width was fixed to 10 μs.

Box-Behnken design (BBD) was chosen for the optimization of PEF parameters since it is simpler and more efficient than other three-level factorial designs (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). The experimental design consisted of 15 experimental runs, with three levels (− 1, 0, 1) for each factor, and three center points. PEF parameters and values of the response variables in

each experiment appear in **Table 1.** Independent variables of PEF were the electric field strength - E (0.5, 1.5, 2.5 kV/cm), frequency (50,100, 150 Hz) and total time of treatment (5,10,15 s). Total time refers to the treatment time that is the number of pulses applied multiplied by the pulse width (or pulse duration) (Raso et al., 2016). Also, the total energy input of each experiment was calculated according to Raso et al. (2016) and it is reported in **Table 1.**

The response variables were fitted to a second-order polynomial model equation obtained by the response surface methodology (RSM) (**Eq.1**).

$$Y = \beta \quad \sum \beta \qquad \sum \beta \qquad \sum \sum$$

Where Y correspond with the response variables, which were the concentration of free phenolic compounds (Y_1), flavan-3-ols (Y_2), flavonoids (Y_3), phenolic acids derivates (Y_4) obtained from brewers' spent grain extracts by HPLC-MS, X_i and X_j are the independent factors affecting the response, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients of the model (intercept, linear, quadratic and interaction term).

The range of electric field strength was the same that the established by Liu, Zeng, and Ngadi (2018) for the extraction of phenolic compounds from onion. Moreover, the range of electric field strength and the minimum value of frequency chosen in this work was the same reported by (Kim et al., 2019) on raw gingesng samples and the pulse width was the same that the used in orange peel (Esteve, 2015).

The model building, experimental results, and designs were carried out using STATISTICA 7.0 (2002, StatSoft, Tulsa, OK). The results of quantification reported in this work are the averages of three repetitions (n=3).

2.4. Extraction of phenolic fractions of brewers' spent grain

After PEF treatment, extraction of free fraction was carried out according the protocol established by Hung and Morita (2008), with certain differences: 15 g from brewers' spent grain (2 g

of dry matter), previously submitted to PEF treatment was extracted by shaking twice with 30 mL of ethanol/water (4:1 v/v). The supernatants were collected and evaporated at 35 °C in a rotary evaporator, and finally the dried extract was reconstituted with 2 mL of methanol/ water (1:1 v/v). The extracts were stored at -18 ºC before the analysis.

In order to compare the effect of PEF treatment on the extraction of phenolic compounds, extraction of brewers spent grain without PEF treatment (Control) was carried out.

After establishing the PEF conditions the samples were extracted according to the previous methodology and the residue after phenolic extraction was submitted to alkaline hydrolysis as reported by Verardo et al. (Verardo, Gómez-Caravaca, Marconi, & Caboni, 2011) in order to recover the bound phenolic compounds.

2.5. Determination of phenolic compounds by HPLC- MS

Determination of free and bound phenolic compounds was carried out by using a liquid chromatography apparatus HP 1100 Series (Agilent Technologies, Palo Alto, CA, USA) equipped with a degasser, a binary pump delivery system and an automatic liquid sampler and coupled to single quadrupole mass spectrometer detector was used. Separation these phenolic compounds from brewers spent grains was carried out by using a C-18 column (Poroshell 120, SB-C18, 3.0×100 mm, 2.7 μm from Agilent Technologies, Palo Alto, CA, USA). The gradient elution was the same that the previously established by Gómez-Caravaca, Verardo, Berardinelli, Marconi, and Caboni 2014 using as a mobile phase A acidified water (1% acetic acid) and as mobile phase B acetonitrile. MS analysis was carried out using an electrospray ionization (ESI) interface in negative ionization mode at the following conditions: drying gas flow (N_2), 9.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 350°C; capillary voltage, 4000 V. The fragmentor and m/z range used for HPLC-ESI/MS analyses were 80 V and m/z 50-1000, respectively. Data were processed by the software MassHunter Workstation Qualitative Analysis Version B.07.00 (Agilent Technologies, Santa Clara, CA, USA).

3. Results and discussion

3.1. Characterization of phenolic compounds from brewers spent grain extracts by HPLC-MS

3.1.1. Analytical parameters of the method

Analytical validation of the method was performed considering linearity and sensitivity. In order to quantify phenolic compounds, four calibration curves were elaborated with the standards ferulic acid, catechin, quercetin and gallic acid. **Table S-1** lists the analytical parameters of the standards used containing linear range, calibration curve, determination coefficients, limit of determination (LOD) and limit of quantification (LOQ)..

Calibration curves were carried out by using the peak areas analyte standard against the concentration of the analyte for the analysis by HPLC. The external calibration of the standards was elaborated at different concentration levels from LOQ to 100 mg L^{-1}. All calibration curves revealed good linearity among different concentrations, and the determination coefficients were higher than 0.9984 in all cases. The method used for analysis showed LOD within the range 0.0040-0.0136 mg L^{-1} and the LOQ within 0.0134-0.0452 mg L^{-1}.

3.1.2. Identification of phenolic compounds

Free phenolic compounds in BSG extracts were analyzed by HPLC with MS detection. Free phenolic compounds were identified by rendering their mass spectra using the data reported in the literature and, when available, by co-elution with commercial standards.

A total of 13 free phenolic compounds were identified in beer by-products and they were previously identified in barley, millet, hop and brewers' spent grain extracts (Chandrasekara & Shahidi, 2011; Gómez-Caravaca et al., 2014; Magalhães et al., 2010) (**Table 2**). The peak 1 at 2.0 min with a m/z 451 corresponded with catechin-3-glucose and the peak 2 at 3.7 min presented the molecular ion at m/z 577 was identified as procyanidin B3, which was present in barley extracts (Gómez-Caravaca et al., 2014). The peak 3 at 4.1 min with a molecular ion at m/z 289 was identified as catechin and was present in barley extracts and brewers' spent grain (Gómez-Caravaca et al., 2014; Stefanello et al.,

2018). The peak 4 at 4.7 min at m/z 167 correspond with vanillic acid, which was identified in millet and brewers' spent grain extracts (Chandrasekara & Shahidi, 2011; Stefanello et al., 2018). The peak 5 at 4.5 min with a molecular ion at m/z 771 correspond with quercetin-3-hexosylrutinoside and was identified previously in hop extracts (Magalhães et al., 2010). The peak 6 at 5.2 min at m/z 121 was identified as p-hydroxybenzaldehyde and the peak 7 at 6.3 min at m/z 151 was identified as vanillin, both peaks were detected previously in millet extracts (Chandrasekara & Shahidi, 2011). The peak 8 at 6.4 min at m/z 593 was identified as prodelphinidin B3, which was detected in barley extracts (Gómez-Caravaca et al., 2014). The peak 9 at 6.8 min with a molecular ion at m/z 163 corresponded with p-coumaric acid, which was identified in brewers' spent grain and in barley (Moreira et al., 2012, 2013; Stefanello et al., 2018). The peak 10 at 7.5 min at m/z 371 was identified as hydroferuloyl-glucose according to the identification of this compound in barley samples (Gómez-Caravaca et al., 2014), the peak 11 at 7.6 min with a molecular ion at m/z 193 was identified as ferulic acid and it was identified previously in brewers' spent grain (Moreira et al., 2012, 2013; Stefanello et al., 2018). The peak 12 at 10.0 min with a molecular ion at m/z 385 was identified as sinapoyl hexose and was detected in barley samples (Gómez-Caravaca et al., 2014). The peak 13 at 15.0 min with a molecular ion at m/z 329 was identified as tricin, which was previously identified in millet and rice extracts (Chandrasekara & Shahidi, 2011).

3.1.3. Quantification of phenolic compounds

Free phenolic compounds were quantified through calibration curves of standards. Therefore, the calibration curve of ferulic acid was used to quantify vanillic acid, vanillin, p-coumaric acid, hydroferuloyl glucose and ferulic acid, the calibration curve of catechin was used to quantify catechin 3-glucose, procyanidin B3, catechin, prodelphinidin B3 and sinapoyl hexose, the calibration curve of gallic acid was used to quantify p-hydroxybenzaldehyde and the calibration curve of quercetin was used to quantify tricin. A total of 12 free phenolic compounds were quantified in brewers spent grain (**Table 3**). Quercetin-3-hexosylrutinoside was not quantified due to its value of concentration was less than the limit of quantification.

Tricin was the most concentrated flavonoid in brewers spent grain, which value varied from 27.936 µg g^{-1} d.w. in PEF-1 to 46.125 µg g^{-1} in PEF 2, whereas the most concentrated phenolic acid derivates was sinapoyl hexose, which ranged from 21.080 µg g^{-1} d.w. in PEF-1 to 36.108 µg g^{-1} d.w. in PEF-3.

Stefanello et al. (2018) quantified some phenolic compounds in brewers spent grain, in their study concentration of catechin was 68.4 µg g^{-1}, which was higher than the obtained in this work, whereas concentration of p-coumaric acid (8.4 µg g^{-1} d.w.) and ferulic acid (5.6 µg g^{-1} d.w.) were in the same order of magnitude than the obtained in this work. These differences in the concentration of phenolic compounds in brewers spent grain could be mainly due to barley variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process (Gupta et al., 2010). With respect to the study performed by Gómez-Caravaca, Verardo, Berardinelli, Marconi, and Caboni (2014) about the content of free phenolic compounds in barley samples, which is the main component in brewing by-products, the highest content of sinapoyl hexose obtained in the present work was 88-51-99.35 %, which was higher than the obtained in barley extracts (0.3-5.3 µg g^{-1} d.w.) and ferulic acid was in the same order than in the obtained barley extracts (1.4-7.3 µg g^{-1} d.w.), whereas the concentration of catechin-3-glucose (9.2-45 µg g^{-1} d.w.), procyanidin B3 (276.2-514.8 µg g^{-1}), catechin (68.8-350.6 µg g^{-1} d.w.) and prodelphinidin B3 (232-482.7 µg g^{-1} d.w.) in barley samples were higher than the obtained in the present work.

Content of total free phenolic compounds ranged from 68.664 µg g-1 d.w. in PEF-1 (0.5 kV/cm, 50 Hz and 10 seconds) to 96.842 µg g^{-1} d.w. in PEF-3 (0.5 kV/cm, 150 Hz and 10 seconds), content of flavan-3-ols ranged from 5.106 µg g^{-1} d.w. in PEF-6 (2.5 kV/cm, 100 Hz and 5 seconds) to 8.809 µg g-1 d.w. in PEF-2 (2.5 kV/cm, 50 Hz and 10 seconds). Concentration of flavonoids ranged from 33.853 µg g^{-1} d.w. in PEF-6 (2.5 kV/cm, 100 Hz and 5 seconds) to 54.933 µg g^{-1} d.w. in PEF-2 (2.5 kV/cm, 50 Hz and 10 seconds) and the content of phenolic acid derivates ranged from 34.296 µg g^{-1} d.w. in PEF-1 (0.5 kV/cm, 50 Hz and 10 seconds) to 56.916 µg g-1 d.w. in PEF-3 (0.5 kV/cm, 150 Hz and 10 seconds) (Table 1.).

Comparing the content of free phenolic compounds obtained in PEF extracts with the obtained in control samples it was possible to observe that concentration of flavan-3-ols was 55.8 % higher,

whereas, flavonoids content was 64.34 % higher than the obtained in control samples, content of phenolic acid derivates was 68.39 % higher in PEF treated sample and, finally, the total free phenolic content in PEF extract was 61.20 % higher than the obtained in control one. Therefore, these results have shown that the application of PEF treatment improves the phenolic extraction efficiency in brewers spent grains.

3.2. Fitting the model

The response surface methodology (RSM) was applied for the optimization of three PEF parameters to obtain the highest content of free phenolic compounds in brewers spent extracts. For that purpose, an experimental Box-Behnken design (BBD) was applied to evaluate the effects of electric field strength (0.5, 1.5 and 2.5 kV/cm) (X_1), frequency (50, 100 and 150 Hz) (X_2), and total time (5, 10 and 15 s) (X_3) on the response variable of free phenolic compounds, flavan-3-ols, flavonoids and phenolic acid derivates via HPLC- MS from brewers spent grain.

The data of the response variable were used to fit the model to a second order-polynomial equation by means of least squares method (LSM). Relied on Fisher test, the evaluation of the model was carried out according to the significance used in other works ($\alpha = 0.1$) (Díaz-de-cerio et al., 2017; Yan, Cao, & Zheng, 2017). Regression coefficients that describe free phenolic compounds, flavan-3-ols, flavonoids and phenolic acid derivates responses appear in the **Table 4**.. Most of the single factors, interactions between them and their cross-products reported a significant effect ($p < 0.1$) on the response variables, being the linear effect of electric field strength (X_1) the most influent, followed by the quadratic effect of electric field strenght (X_{11}).

The model was recalculated only with significant effects and the results of the analysis of variance (ANOVA) appears in the Table 5. Models presented a strong correlation between independent variables and response variables with coefficients of determination (R^2) between 0.9590 to 0.9999. In addition, the validity of the model was also verified by the p-value of the lack of fit as non-significant in all models ($p > 0.05$) and pure errors were also low. Therefore, models were accepted.

Three-dimensional response surface plots for the variables of free phenolic compounds, flavan-3-ols are presented in **Figure. 1**, while those for flavonoids and phenolic acid derivates are presented in **Figure. 2.** Electric field strength (X_1) has demonstrated the highest effect on the response variables.

In the **Figure 1.a** it can be observed the positive effect of electric field strength (X_1) and the positive effect of frequency (X_2), which had a higher effect than the negative effect of the quadratic term of frequency (X_{22}) in the response of free phenolic compounds, whereas the quadratic effect of electric field did not have a significant effect. In addition, the negative effect between electric field strength and frequency appears in the **Figure 1.a.** In the **Figure 1.b** it can be observed the positive effect between electric field strength and total time (X_{13}), which was higher than the quadratic effect of total time (X_{33}) and lower than the positive effect of electric field strength (X_1) and the negative effect of time (X_3). **Figure 1.c** shows the positive effect between total time and frequency, which had a lower effect than the positive effect of frequency (X_2), negative effect of total time (X_3) and the quadratic negative effect of time (X_{33}). Nevertheless, it had a higher effect than the quadratic effect of frequency (X_{22}). In the **Figure 1.d it** can be observed the positive effect of electric field strenght(X_1) in the response of flavan-3-ols, which was higher than the negative effect of the quadratic electric field strength (X_{11}) and the negative effect of frequency (X_2). The **Figure 1.e** shows the negative effect of cross product between electric field strength and total time (X_{13}), which had less influence than the linear (X_1) and quadratic effect (X_{11}) of electric field strength (X_1). The most influence on the response was attributed to the positive linear effect of electric field (X_1). In the **figure 1.f** appears the influence of total time and frequency on the content of flavan-3-ols, linear effect of frequency (X_2) has the most influence on the response following by the effect of the linear effect of total time (X_3).

In the **Figure 2.a** appears the positive effect of electric field strength (X_1), which has the most influence in the response of flavonoids. Negative cross effect between electric field strength and frequency (X_{12}) is lower than the positive linear effect of frequency (X_2) and higher than the negative quadratic effect of frequency (X_{22}). In the **Figure 2.b** it is possible to observe the positive effect of electric field strength and total time (X_{13}) that was lower than the positive linear effect of electric field strength (X_1) and negative linear effect of total time (X_3) and higher than the negative quadratic effect

of total time (X_{33}). In the **Figure 2.c** the positive effect between frequency and total time (X_{23}) in the response can be observed, which had a lower effect than the linear positive effect of frequency (X_2) and negative linear (X_3) and quadratic effect of total time (X_{33}). In the **Figure 2.d** it can be observed the negative influence between electric field strength and frequency (X_{12}). The **Figure 2.e** shows the positive effect of electric field strength (X_1) and total time (X_3) that had a higher influence on the response than the negative effect between the electric field strength and total time (X_{13}), which was higher than the quadratic negative effect of total time (X_{33}) and in the **Figure 2.f** it can be observed the positive effect between frequency and total time (X_{23}).

3.2.1. Optimization of PEF parameters

PEF factors were optimized in order to maximize the content for each family of free phenolic compounds and their total: flavan-3-ols, flavonoids, phenolic acid derivates and total free phenolic compounds. (Table 6.) Optimization of these factors was carried out by response surface plots of the combined effects of the factors.

Regarding the suggested model, a great value on free phenolic compounds could be obtained under the following optimized conditions: 2.5 kV/cm, 50 Hz and 14.5 seconds (energy input of 9.06 kJ/kg) to obtain a maximum value of 99 ± 2 μg g-1 d.w. This optimum extraction conditions are in concordance with a study by Kim et al. (2019), where the highest phenolic content was observed in ginseng samples following the application of PEF at frequency of 50 Hz and electric field strength higher than 0.5 kV/cm (1.5 and 2.5 kV/cm), showing that by increasing of electric field strength there is an increase of phenolic compounds extraction yield (Kim et al., 2019). Also other authors observed the similar effect of electric field strength on the polyphenols extraction yield, in particular, TPC of date palm fruit extract was of 64.20, 65.90 and 67.35 mg GAE/100 g for samples treated at 1, 2 and 3 kV/cm, respectively (Siddeeg et al., 2019). Also the frequency, which indicates the number of pulses applied by unit of time, is an important parameter to consider during the PEF application since it determines the amount of electrical energy delivered per unit of time on the treated product (Raso et al., 2016). In our study the lowest frequency and long time of the treatment (14.5 s) was more beneficial than short time and high frequency (e.g. treatment 4 with 2.5 kV/cm, 150 Hz and 10 s, and

18.75 kJ/kg) in the extraction of free phenolic compounds, indicating that, probably, energy input of 9.06 kJ/kg was sufficient for the electroporation of the majority of cells.

Optimal conditions to obtain the maximum value of families of phenolic compounds were the following: 2.5 kV/cm, 50 Hz and 15 second to obtain 10 ± 1 μg g^{-1} d.w. of flavan-3-ols, 2.5 kV/cm, 50 Hz and 15 seconds to obtain 59 ± 1 μg g^{-1} d.w. of flavonoids and 0.5 kV/cm, 150 Hz and 5 seconds to obtain 56.1 ± 0.3 μg g^{-1} d.w. of phenolic acid derivatives. These optimal conditions have been applied to obtain the experimental values of each responses and as reported in Table 5 any statistical difference was noticed between the predicted and obtained values.

The established PEF conditions that allowed the highest value of total free phenolic compounds were applied to obtain enriched phenolic extracts from BSGs and the phenolic content was compared with those obtained without the PEF pre-treatment. Moreover, the determination of bound phenolic compounds has been carried out at the optimum conditions established for free phenolic compound (2.5 KV/cm, 50 Hz and 14.5 s). These compounds were identified by HPLC-MS by rendering their mass spectra using the data reported in the literature and, when available, by co-elution with commercial standards (**Table 7**). The peak 1 at 4.2 min with a molecular ion at m/z 283 was identified as epicatechin, which was identified previously in buckwheat samples and in BSG (Chandrasekara & Shahidi, 2011; Stefanello et al., 2018). The peak 2 at 5.0 min with a molecular ion at m/z 179 was identified as caffeic acid, which was identified in barley, beer and BSG samples (Gómez-Caravaca et al., 2014; Pai et al., 2015; Stefanello et al., 2018). The peaks 3 and 4 at 6.9 min and 7.146 min respectively, at m/z 163 were identified as trans-p-coumaric acid and cis-p-coumaric acid, according to the identification of these compounds in barley samples (Gómez-Caravaca et al., 2014). The peaks 5 and 6 at 7.656 min and 7.972 min respectively, at m/z 193 were identified as tras ferulic acid and cis ferulic acid (Gómez-Caravaca et al., 2014). The peaks 7, 8, 9, 10, 12 and 13 at 9.2, 9.8, 11.2, 11.6, 12.5, 12.7 min respectively, with a molecular ion at m/z 385 were identified as sinapoyl-hexose isomers, which were identified previously in barley samples (Gómez-Caravaca et al., 2014). The peak 11 with a molecular ion at m/z 341 was identified as caffeoyl-hexose, which was identified in barley samples (Gómez-Caravaca et al., 2014).

Table 8 reports the content of free and bound phenolic compounds obtained from BSGs with and without PEF pre-treatment at optimal conditions.

The total content of free phenolic compounds, flavan-3-ols, sum of flavonoids and phenolic acid derivatives were 62.8 %, 61.5 %, 67.3 % and 67.3 % higher than the obtained in the control samples. Furthermore, the total bound content and flavan-3-ols were 39.6 % and 39.8 % higher than the obtained in the control samples (**Table 8.**). In addition, the total phenolic content (sum of free and bound) obtained in brewers' spent grains after the PEF treatment at optimum conditions (2.5 kV/cm, 50 Hz and 14.5 s) (640.46 μg g^{-1} d.w.) was 43.23 % higher than the obtained in the control sample (363.58 μg g^{-1} d.w.). This increase in the recovery of phenolic content with the application of PEF was in concordance with the study conducted by (Barba, Galanakis, Esteve, Frigola, & Vorobiev, 2015). They applied the PEF (13.3 kV/cm), and ultrasounds (USN) (400W; 24 kHz) treatments in blackberries in order to evaluate the effects of processing on protein, total phenolics and anthocyanins, showing that the phenolic content obtained following the PEF application (108.0 mg/100 g) was 57.2 % higher than the one obtained by ultrasounds (46.2 mg/100 g). Also, Kim et al. (2019) reported the highest phenolic content at 1.5 and 2.5 kV/cm (893.83 and 877.40 mg tannic acid equivalent/ 100 g), which were 8-10 % higher than the obtained in the control samples without PEF treatment (807.02 mg tannic acid equivalent/ 100g). Other study reported an increase on the phenolic content of 23 % in blueberries after PEF treatment (electric field strength 2.0 kV/cm, 100 pulses per s for 4 minutes, and pulse width 1 μs) (Jin, Yu, & Gurtler, 2017).. This substantial increase in the phenolic recovery after PEF treatment is due to the disintegration of the structure of cell cytomembrane and change its selective permeability properties, which caused an increased mass transfer through the cells (Liu, Esveld, Vincken, & Bruins, 2018). In fact, Kim et al. (2019) reported that the conductivity of ginseng samples increased with the application of PEF at 1.5 and 2.5 kV/cm, while no effect was observed when 0.5 kV/cm was applied in comparison to untreated samples. This increase in electrical conductivity values shows that PEF treatment at 1.5 and 2.5 kV/cm led to biological cell membrane disruption (Kim et al., 2019). Therefore, PEF can be used as a pre-treatment to increase the recovery of phenolic compounds in brewers spent grain.

The results of the total free and bound phenolic compounds content obtained in our study were lower than those obtained previously in barley samples (Gómez-Caravaca et al., 2014), this probably because part of the content of these compounds are extracted from brewers spent grain during the beer production, and some of them could have been degraded since high temperatures are used during the brewing processing (Gupta et al., 2010). Nevertheless, bound phenolic compounds are in the same order than the obtained in barley samples (Gómez-Caravaca et al. 2014), because these phenolic compounds are ester linked to the cell wall, for that reasons most of them are kept during the beer production (Gupta & De, 2017). Comparing the content of bound phenolic compounds of caffeic acid, p-coumaric acid and ferulic acid obtained in the present work, these were lower than the obtained previously in BSG (Ikram, Huan, Zhang, Wang, & Yin, 2017; McCarthy, O'Callaghan, Piggott, FitzGerald, & O'Brien, 2013), whereas, the content of free phenolic compounds of catechin and ferulic acid were higher than the obtained previously in BSG (Ikram et al., 2017). These differences could be because phenolic content of BSG varies according to barley variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process (Gupta et al., 2010).

According to the high content of phenolic compounds obtained from BSG with PEF treatment, these phenolic extracts could be beneficial as ingredients in food Industry because of the low cost and high nutritional value of BSG. For example, these extracts could be used to enriche bakery products such as bread, biscuits, cookies, muffins, cakes, waffles, pancakes, tortillas, snacks, doughnuts, brownies and pasta (Guo, Du, Zhang, Zhang, & Jin, 2014; Lynch, Steffen, & Arendt, 2016; Spinelli, Padalino, Costa, Del Nobile, & Conte, 2019).

4. Conclusions

The valorization of BSG is an important goal in order to recover the phenolic compounds that can be extracted, purified and reused as functional ingredients in food and cosmeceutical industry. Solid–liquid extractions are the most commonly used procedures to extract the phenolic compounds in BSG samples, due to their ease of use, efficiency, and wide applicability. However, to improve the phenolic recovery, pulsed electric field (PEF) have been used as extraction pre-treatment. PEF parameters were

optimized and this pre-treatment at electric field strength of 2.5 kV/cm, frequency of 50 Hz for 14.5 s was able to improve the total free and bound phenolics recovery of 2.7 and 1.7 times, respectively, compared to the control samples without PEF treatment, probably due to the increase of the permeability of the cell membrane, which facilitates the extraction of bioactive compounds. These promising results encourage further studies in order to check the extraction efficiency of PEF coupled to ultrasounds or microwave extraction technology and the possibility of the scale-up of the process.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https:// doi.org/xxxx

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure 1. Response surface plots showing combined effects of process variables for free phenolic compounds (a, b, c) and flavan-3-ols (d, e and f). a and d: Frequency with electric field strength, b and e: total time with electric field strength and c and f: total time with frequency

Figure 2. Response surface plots showing combined effects of process variables for flavonoids (a, b, c) and phenolic acid derivates (d, e and f). a and d: Frequency with electric field strength, b and e: total time with electric field strength and c and f: total time with frequency

Independent parameters					Dependent parameters			
Exp	X_1	X_2	X_3	Total energy input (kJ/kg)	Total free phenolic compounds	Flavan-3-ols	Flavonoids	Phenolic acid derivatives
1	0.5	50	10 (500)	0.25	68.7 ± 1.1	6.4 ±0.1	34.4 ±0.4	34.3 ±0.2
2	2.5	50	10 (500)	6.25	95.2 ±0.9	8.8 ±0.2	54.9 ±0.6	40.2 ±0.3
3	0.5	150	10 (1500)	0.75	96.8 ±0.5	6.0 ±0.1	39.9 ±0.3	56.9 ±0.4
4	2.5	150	10 (1500)	18.75	82.5 ±0.3	7.8 ±0.05	47.4 ±0.5	35.1 ±0.2
5	0.5	100	5 (500)	0.25	86.5 ±0.6	7.7 ±0.2	45.1 ±0.2	41.4 ±0.3
6	2.5	100	5 (500)	6.25	73.5 ±0.8	5.1 ±0.1	33.8 ±0.4	39.6 ±0.4
7	0.5	100	15 (1500)	0.75	82.9 ±0.7	7.1 ±0.3	41.9 ±0.3	41.0 ±0.6
8	2.5	100	15 (1500)	18.75	90.0 ±0.6	8.4 ±0.1	45.1 ±0.1	44.9 ±0.5
9	1.5	50	5 (250)	1.13	89.7 ±0.3	7.9 ±0.2	46.9 ±0.1	42.9 ±0.1
10	1.5	150	5 (750)	3.38	73.9 ±0.5	5.2 ±0.1	34.9 ±0.2	39.0 ±0.3
11	1.5	50	15 (750)	3.38	87.6 ±0.4	7.4 ±0.1	46.5 ±0.1	41.0 ±0.2
12	1.5	150	15 (2250)	10.13	83.4 ±0.4	6.1 ±0.2	43.9 ±0.2	39.5 ±0.3
13	1.5	100	10 (1000)	4.50	84.6 ±0.3	7.0 ±0.2	41.9 ±0.3	42.6 ±0.2
14	1.5	100	10 (1000)	4.50	84.9 ±0.5	6.6 ±0.2	42.3 ±0.3	42.6 ±0.3
15	1.5	100	10 (1000)	4.50	84.2 ±0.3	6.7 ±0.2	41.7 ±0.2	42.5 ±0.3

Table 1. Box-Behnken design with PEF parameters, values of total energy input in each experiment and dependent variables obtained (free phenolic compounds, flavan-3-ols, flavonoids and phenolic acid derivates) quantified by HPLC-MS in brewers' spent grain expressed by μg g^{-1} d.w..

X_1: E (kV/cm), X_2: Frequency (Hz), X_3: Total time (s) (pulses per second)

Peak	RT (min)	m/z $[M-H]^-$	Free phenolic compound
1	2.0	451.1	Catechin-3-glucose
2	3.7	577	Procyanidin B3
3	4.1	289	Catechin
4	4.7	167	Vanilllic acid
5	4.5	771	Quercetin-3-hexosylrutinoside
6	5.2	121	*p*-Hydroxybenzaldehyde
7	6.3	151	Vanillin
8	6.4	593	Prodelphinidin B3
9	6.8	163	*p*-coumaric acid
10	7.5	371	Hydroferuloyl glucose
11	7.7	193	Ferulic acid
12	9.8	385	Sinapoyl hexose
13	17.0	329	Tricin

Table 2. Table of identification of free phenolic compounds from brewers' spent grain extracts by HPLC-MS

	PEF-1	PEF-2	PEF-3	PEF-4	PEF-5	PEF-6	PEF-7	PEF-8	PEF-9	PEF-10	PEF-11	PEF-12	PEF-13	PEF-14	PEF-15
Catechin-3-glucose	0.39	0.57	0.20	0.44	0.45	0.23	0.25	0.39	0.42	0.10	0.49	0.22	0.43	0.39	0.36
Procyanidin B3	1.18	2.79	1.30	2.39	1.76	1.28	1.59	1.89	1.78	1.35	1.36	0.92	1.52	1.42	1.47
Catechin	2.74	3.37	2.60	3.18	3.08	2.24	3.0	3.61	3.06	2.21	3.24	2.97	3.11	2.97	2.95
Quercetin-3-hexosylrutinoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Vanillic acid	<LOQ	0.24	0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.19	<LOQ	<LOQ	<LOQ
***p*-Hydroxybenzaldehyde**	3.06	3.75	3.22	2.92	3.37	2.53	3.25	3.45	3.55	2.89	3.82	3.58	3.29	3.37	3.47
Vanillin	0.72	1.48	0.70	0.76	1.21	0.59	1.28	1.33	1.16	0.73	1.22	1.04	1.01	1.09	1.05
Prodelphinidin B3	2.12	2.08	1.92	1.81	2.43	1.35	2.34	2.51	2.61	1.58	2.34	1.96	1.99	1.80	1.87
***p*-coumaric acid**	<LOQ	3.03	3.09	2.57	3.94	1.94	3.51	3.64	3.10	2.10	3.20	3.10	2.92	2.42	2.55
Hydroferuloyl glucose	8.66	8.45	13.20	6.27	7.36	7.98	8.37	7.98	10.14	8.77	8.98	7.76	9.17	9.19	9.23
Ferulic acid	0.78	0.59	0.57	0.63	0.92	0.65	0.89	1.07	1.09	0.43	1.27	0.47	0.78	0.84	0.80
Sinapoyl hexose	21.08	22.70	36.11	21.95	24.62	25.96	23.73	27.42	23.77	24.11	22.58	23.36	25.46	25.70	25.43
Tricin	27.94	46.12	33.91	39.59	37.38	28.75	34.68	36.67	39.02	29.68	39.11	37.79	34.90	35.71	35.05

Table 3. Free phenolic compounds quantified in Brewers' spent grain (μg g^{-1} d.w.) in each PEF experiment by HPLC-MS

Regresion coefficients	Free phenolic compounds		Flavan-3-ols		Flavonoids		Phenolic acid derivates	
	Coefficients	p value	Coefficients	p value	Coefficients	p value	Coefficients	P value
β_0	12.9834*	0.000001	-0.49365*	0.000102	-4.39453*	0.000004	17.3779*	0.000000
Linear								
β_1	87.472*	0.006226	9.54454*	0.023825	50.55570*	0.000720	36.9159*	0.000078
β_2	0.9686*	0.017308	0.16624*	0.025020	1.00745*	0.004786	-0.0388*	0.000078
β_3	-0.2828*	0.001961	-0.01935*	0.032560	-1.00923*	0.002751	0.7264*	0.000973
Cross product								
β_{12}	-1.1005*	0.000256	-0.15341	0.362347	-0.97762*	0.001940	-0.1229*	0.000018
β_{13}	0.1770*	0.001052	-0.15821*	0.015798	0.81990*	0.001586	-0.6429*	0.000429
β_{23}	0.0116*	0.003173	0.00126	0.122936	0.00928*	0.003839	0.0023*	0.002616
Quadratic								
β_{11}	-20.1473	0.062937	-1.98423**	0.062937	-5.72219	0.183873	-14.4251	0.101027
β_{22}	-0.0024*	0.039608	-0.00083	0.859846	-0.00466*	0.006337	0.0023*	0.000843
β_{33}	-0.0698*	0.009345	-0.00560	0.386344	-0.03232*	0.032837	-0.0375*	0.001053

Table 4. Regression coefficients of the model

*Significant at $\alpha \leq 0.05$

**Significant at $\alpha \leq 0.1$

	Free phenolic compounds	Flavan-3-ols	Flavonoids	Phenolic acid derivates
R^2	0.9991	0.9590	0.9987	0.9999
p (Lack of fit)	0.1497	0.3220	0.3026	0.1010
Pure error	0.1067	0.0599	0.0832	0.0034

Table 5. Analysis of variance (ANOVA) of the model

Optimal conditions	Free phenolic compounds	Flavan-3-ols	Flavonoids	Phenolic acid derivates
E (KV/cm)	2.5	2.5	2.5	0.5
Frequency (Hz)	50	50	50	150
Total time (s)	14.5	15	15	5
Predicted (μg g^{-1} d.w.)	99 ± 2	10 ± 1	59 ± 1	56.1 ± 0.3
Obtained value (μg g^{-1} d.w.)	101 ± 2	10.1 ± 0.8	60 ± 2	55 ± 2
Significant differences	N.S.	N.S.	N.S.	N.S.

Table 6. Optimal conditions for PEF, N.S.: Not significant differences

Peak	RT	m/z $[M-H]^-$	Bound phenolic compounds
1	4.2	289	Epicatechin
2	5.0	179	Caffeic acid
3	6.8	163	trans-*p*-coumaric acid
4	7.1	163	cis-*p*-coumaric acid
5	7.7	193	trans ferulic acid
6	8.0	193	cis ferulic acid
7	9.2	385	Sinapoyl-hexose a
8	9.8	385	Sinapoyl-hexose b
9	11.2	385	Sinapoyl-hexose c
10	11.6	385	Sinapoyl-hexose d
11	12.7	341	Caffeoyl-hexose
12	12.5	385	Sinapoyl-hexose e
13	12.7	385	Sinapoyl-hexose f

Table 7. Table of identification of bound phenolic compounds from brewers' spent grain extracts by HPLC-MS

Free phenolic compounds	Control	PEF treated	Bound phenolic compounds	Control	PEF treated
Catechin-3-glucose	0.18 ±0.02	0.73 ±0.10	Epicatechin	3.34 ±0.12	5.55 ±0.24
Procyanidin B3	0.87 ±0.03	3.02 ±0.21	Caffeic acid	6.89 ±0.27	7.31 ±0.39
Catechin	1.34 ±0.01	3.96 ±0.34	trans-*p*-coumaric acid	76.74 ±1.02	141.49 ±2.57
Quercetin-3-hexosylrutinoside	n.d.	<LOQ	cis-*p*-coumaric acid	27.73 ±0.51	43.40 ±0.73
Vanillic acid	n.d.	0.30 ±0.17	trans ferulic acid	85.28 ±1.46	141.19 ±2.09
p-Hydroxybenzaldehyde	3.38 ±0.13	3.80 ±0.05	cis ferulic acid	21.42 ±0.67	33.14 ±0.52
Vanillin	0.58 ±0.02	1.50 ±0.22	Sinapoyl-hexose a	15.12 ±0.09	23.66 ±0.66
Prodelphinidin B3	1.49 ±0.06	2.40 ±0.02	Sinapoyl-hexose b	13.30 ±0.27	30.63 ±1.31
p-coumaric acid	1.21 ±0.10	3.30 ±0.05	Sinapoyl-hexose c	6.46 ±0.05	9.28 ±0.16
Hydroferuloyl glucose	3.15 ±0.08	8.60 ±0.12	Sinapoyl-hexose d	26.61 ±0.43	39.02 ±0.67
Ferulic acid	0.25 ±0.01	0.90 ±0.03	Caffeoyl-hexose	11.77 ±0.09	17.49 ±0.11
Sinapoyl hexose	9.43 ±0.21	23.00 ±0.73	Sinapoyl-hexose e	19.48 ±0.28	31.21 ±1.44
Tricin	15.70 ±0.19	49.76 ±1.20	Sinapoyl-hexose f	11.86 ±0.61	16.09 ±1.70
Sum	37.58 ±2.06	101 ± 2	***Total***	326 ±3.08	539.46 ±2.89
Sum flavan-3-ols	3.89 ±0.19	10.1 ± 0.8	***Flavan-3-ols***	3.34 ±0.22	5.55 ±0.38
Sum flavonoids	19.59 ±0.47	60 ± 2	***Flavonoids***	3.34 ±0.22	5.55 ±0.38
Phenolic acid derivatives	17.99 ±0.90	55 ± 2	***Phenolic acid derivatives***	322.66±2.49	533.91±3.06

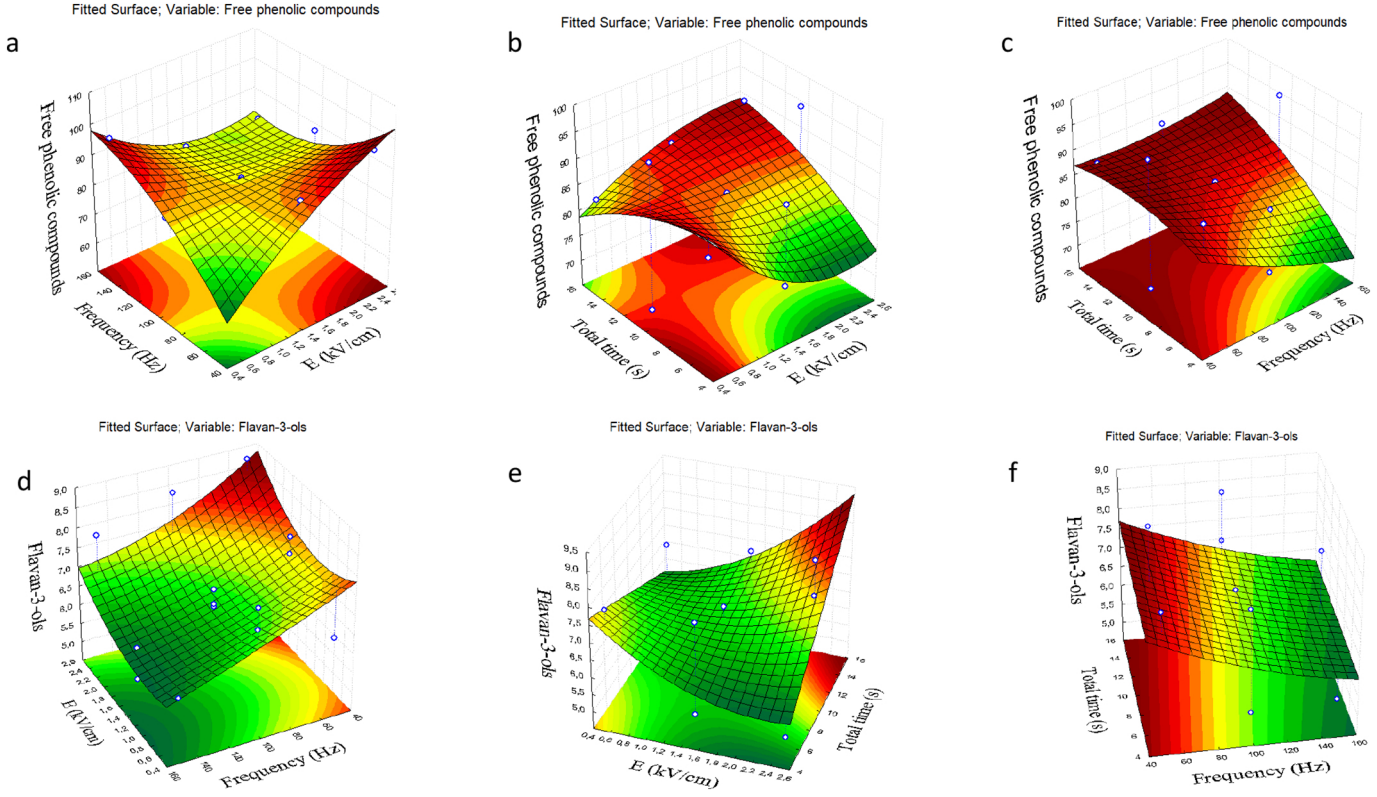
Table 8. Comparison of phenolic content (µg/g d.w.) (free and bound) with and without PEF treatment

Highlights

1. A pretreatment of pulsed electric fields in brewers' spent grain was optimized

2. Pulsed electric fields treated sample show higher amounts of free phenolic compounds

3. Bound phenolic compounds were more abundant in pulsed electric fields treated samples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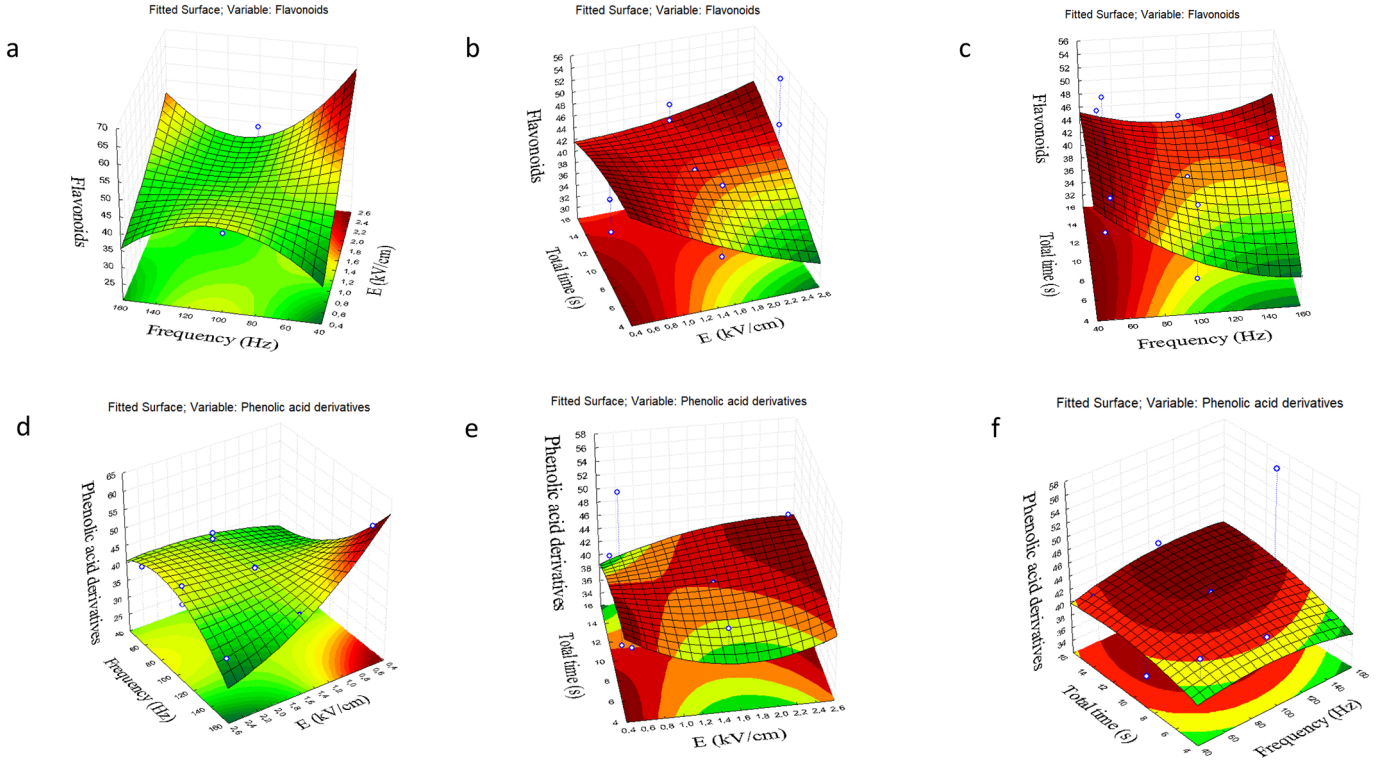
Free phenolic compounds
Frequency (Hz)
E (kV/cm)
b
Fitted Surface; Variable: Free phenolic compounds
Free phenolic compounds
Total time (s)
E (kV/cm)
c
Fitted Surface; Variable: Free phenolic compounds
Free phenolic compounds
Total time (s)
Frequency (Hz)
d
Fitted Surface; Variable: Flavan-3-ols
Flavan-3-ols
E (kV/cm)
Frequency (Hz)
e
Fitted Surface; Variable: Flavan-3-ols
Flavan-3-ols
E (kV/cm)
Total time (s)
f
Fitted Surface; Variable: Flavan-3-ols
Flavan-3-ols
Total time (s)
Frequency (Hz)

Figure 1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Fitted Surface; Variable: Flavonoids
Flavonoids
Frequency (Hz)
E (kV/cm)
b
Fitted Surface; Variable: Flavonoids
Flavonoids
Total time (s)
E (kV/cm)
c
Fitted Surface; Variable: Flavonoids
Flavonoids
Total time (s)
Frequency (Hz)
d
Fitted Surface; Variable: Phenolic acid derivatives
Phenolic acid derivatives
Frequency (Hz)
E (kV/cm)
e
Fitted Surface; Variable: Phenolic acid derivatives
Phenolic acid derivatives
Total time (s)
E (kV/cm)
f
Fitted Surface; Variable: Phenolic acid derivatives
Phenolic acid derivatives
Total time (s)
Frequency (Hz)

Figure 2