Pressurized GRAS solvents for the green extraction of phenolic compounds from *Hibiscus sabdariffa* calyces

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Abstract

An environmental friendly extraction procedure has been tested to extract phenolic compounds from *H. sabdariffa* calyces using pressurized GRAS solvents. A central composite rotatable design (CCRD) was performed to evaluate the influence of the main operational conditions: temperature (40 – 200 ºC) and solvent composition based on aqueous hidroalcoholic solutions (0 – 100 % ethanol). Phenolic composition of experimental extracts analyzed by HPLC-ESI-QTOF-MS showed that higher temperatures and greater ethanol percentages drove to solvents with lower dielectric constants, which resulted in extracts with major quantities of phenolic compounds. An exception was the extraction of cyanidin-3-sambubioside that could only be quantified in extracts performed at the lowest temperature (40 ºC) due to its thermal sensibility. In addition, a RSM was carried out with the aim to maximize the extraction of total phenolic content. To this end, the predicted optimal extraction conditions by RSM were 200 ºC and 100 % (v/v) of ethanol. Results showed that temperature and ethanol percentage had a significant influence on the extraction of total phenolic compounds (p value < 0.05). The mathematical model pointed out 200 ºC of temperature and 100 % of ethanol as the optimum conditions to perform the isolation of phenolic compounds by means of pressurized GRAS solvents.

Keywords: Pressurized GRAS solvents; *Hibiscus sabdariffa*; phenolic compounds; HPLC-ESI-QTOF-MS; response surface methodology;

1. Introduction

One of the main current challenges of the worldwide food industries is the achievement of food chain sustainability. Many strategies and policies are being developed to assure and balance the economic growth, sustain competitive advantage, resource sustainability and environmental protection as key drivers of success. Among them, the processing of food resources for alternative uses seems to be a good choice to create added-value products.

In this sense and due to the proven health-promoting activities, extraction of phytochemicals with bioactive properties from different plant matrix (Xiao & Bai, 2019) is pointed out as profitable practice to provide functional extracts to be exploited in food, nutraceutical, cosmetic or pharmaceutical sectors.

The edible flower *Hibiscus sabdariffa (Hs),* belonging to *Malvaceae* family, is a tropical plant commonly used in the preparation of herbal drinks, hot and cold beverages with antioxidant functions. Recently, an increasing body of studies has demonstrated its wide range of health claims and therapeutic applications in the prevention and treatment of chronic diseases such as diabetes mellitus, cancer, dyslipidemia and hypertension (Ali, Al Wabel, & Blunden, 2005; Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014; Kao et al., 2016; Micucci et al., 2015; Moyano et al., 2016; Rodríguez-Pérez, Segura-Carretero, & Contreras, 2019). Most of the beneficial properties are attributable to the presence of bioactive components in large quantities such as phenolic compounds: phenolic acids, anthocyanins and flavonoids (Borrás-Linares et al., 2015).

However, the extraction of the target compounds is not an easily accomplished task since they usually coexist with a multitude of other compounds. Therefore, their extraction must be efficiently but also environmentally benign. For that purpose, green

accelerated extraction techniques such as moderate electric field extraction (MEF), ohmic accelerated steam distillation (OASD), pulsed electric field (PEF), high pressure extraction (HPE) or pressurized liquid extraction (PLE) have emerged in the last years (Barba, Zhu, Koubaa, Sant' Ana, & Orlien, 2016; Gavahian, Yan-Hwa, & Sudhir, 2018; Gavahian & Farahnaky, 2018).

PLE has demonstrated its suitability for the extraction of bioactive compounds, particularly phenolic compounds, from plants (Erdogan, Ates, Durmaz, Yilmaz, & Seckin, 2011; Machado, Pereira, Barbero, & Martínez, 2017; Plaza & Turner, 2015). This technique employs elevated temperatures and pressures for the extraction of targeted compounds. Under these conditions, solvent remains in liquid state even at temperatures above the boiling point achieving a deeper penetration within the sample which turn into a rise in the extraction efficiency. Furthermore, the elevated temperature allows the sample to become more soluble attaining a higher diffusion rate (Janghel et al., 2015). The combination of both factors, temperature and pressure determines the dielectric constant dielectric constant (ε) of water is 78, but this value decreases to 21 at 300 ºC and 3335 psi of pressure being similar to that for ethanol (ε = 25) at 25°C (Carabias-Martínez, Rodríguez-Gonzalo, Revilla-Ruiz, & Hernández-Méndez, 2005). Consequently, modulating values of temperature and pressure, this technique enables custom efficient extractions by means of GRAS solvents (generally recognized as safe) such water or ethanol (Chemat, Vian, & Cravotto, 2012; Mustafa & Turner, 2011) to fulfil the requirements for a sustainable framework.

Therefore, the main aim of this work is to assess the feasibility of pressurized liquid extraction technique for the green isolation of bioactive compounds from *H. sabdariffa* with benign solvents. Extraction temperature and solvent composition based on water, ethanol and their mixtures were the main variables to be optimized, while the

extraction yield of phenolic compounds was chosen as response variable. For that purpose, a response-surface methodology (RSM) was performed to maximize the total phenolic compound content of extracts which were analyzed and characterized by HPLC-ESI-QTOF-MS.

2. **Material and methods**

2.1 Chemical and Reagents

Regards to extraction solvents, water was obtained by purification with a Milli-Q system from Millipore (Bedford, MA, USA), while ethanol was supplied by Fisher Scientific (Madrid, Spain). Otawa sand and cellulose filters inserted into extraction cells were obtained from Fisher Scientific (Madrid, Spain) and Dionex Corp. (Sunnyvale, CA, USA) respectively. Formic acid and acetonitrile for mobile phases were purchased from Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Madrid, Spain), respectively. Finally, the standards used for the quantification were acquired from Sigma-Aldrich, (Steinhemin, Germany): *p*-coumaric acid, gallic acid, citric acid, chlorogenic acid, myricetin, quercetin, quercetin-glycoside, rutin and apigenin (internal standard). All chemicals used during this research were of analytical HPLC-MS grade.

2.2 Plant material

All experiments were performed by using commercial dried calyces of *Hibiscus sabdariffa (Hs)* provided by Monteloeder Inc. (Elche, Alicante, Spain). The sample was grounded into a fine homogeneous powder with a Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany) equipped with 12-tooth rotor and ring sieve with aperture size of 1 mm. After that, the sample was stored at room temperature sheltered from sun-light until further experiments.

2.3 Pressurized Liquid Extraction (PLE)

Pressurized liquid extractions were carried out in a Dionex ASE 350 extractor (Dionex Corp., Sunnyvale, CA, USA), using ethanol and double-deionized water as GRAS solvents which were previously degassed.

The sample was placed in stainless steel cells of 34 mL prepared as follows: 5 g of sand at the bottom, 8 g of sample with 16 g of sand mixed homogeneously, and finally 5g of sand at the top of the cell. Moreover, disposable cellulose filters were placed into the cell´s inlet and outlet to prevent clogging in the metal frit of the extraction cell. Extractions were performed under the following conditions (Tripodo, Ibáñez, Cifuentes, Gilbert-López, & Fanali, 2018): static mode at a pressure of 1500 psi during 20 min of extraction time. After the extraction cycle, cells were flushed with solvent (60% of the cell volume), purged with nitrogen (100 s) and the resulting extracts were collected in 200 mL amber vials which were immediately cooled in ice. Afterwards, the extracts were centrifuged at 12.000 rpm for 15 min at 4 °C in a Sorvall ST 16 R centrifuge (Thermo Scientific, Leicestershire, UK). Then, the supernatants were evaporated using a Savant SC250EXP SpeedVac Concentrator (Thermo Scientific). The dried extracts were stored at −20°C and kept in darkness. Pior to HPLC analysis the dried extracts were reconstituted to a concentration of 4.5 mg mL^{-1} with methanol.

2.4 HPLC-ESI-QTOF-MS analysis

To determine the chemical profile of the extracts obtained at different conditions an analysis by HPLC-ESI-QTOF-MS was performed. The instrumentation used was an Agilent 1260 HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate Mass Q-TOF equipped with a Jet Stream dual ESI interface. Chromatographic separation was performed with a

reversed-phase C18 analytical column (Agilent Zorbax Eclipse Plus, 1.8 µm, 4.6×150 mm). The sample injection volume was 10 µl, whereas the column and auto-sampler compartments temperatures were set at 25 ºC and 4 ºC, respectively. The compounds were eluted with two polar mobile phases consisted of phase A (water-acetonitrile, 90:10 v/v plus 0.1 % of formic acid) and phase B (acetonitrile). Thus, the elution program was a multi-step linear gradient at a flow of 0.3 mL min^{-1}, beginning at 0 min with 5% of mobile phase B, followed by 20 % phase B at 34 min, at 45 min increasing until 95 % phase B, at 55 min back to 5 % of phase B. Finally, the initial conditions were maintained for 5 min (Diez-Echave et al., 2020).

The MS detection were performed in negative ionization mode with a mass range of 100-1700 m/z, the detection window was set to 100 ppm and data acquisition (2.5 Hz) was performed in centroid mode. The capillary voltage was set +4000V, nebulizer pressure 20 psi, fragmentor 130 V, nozzle voltage 500 V, skimmer 45 V and octopole 1 RF Vpp 750 V. Ultrahigh pure nitrogen was used as drying and nebulizer gas at temperatures of 325 and 350 ºC and flows of 10 and 12 L min^{-1}, respectively.

With the intention of recalibrate each single mass spectra acquired during the analysis providing accurate mass measurement typically better than 2 ppm, a continuous infusion of two reference masses were performed: trifluoroacetate anion (m/z 112.985587) and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921 (m/z 1033.988109).

The MS data were processed through the software Qualitative Analysis of MassHunter workstation version B.06.00 (Agilent Technologies, Palo Alto, CA, USA). Compound identifications were performed by comparison with available standards or by interpretation of the MS spectra obtained by the QTOF mass analyzer by the generation

of the candidate molecular formula with a mass accuracy limit of 5 ppm using the Smart Formula TM editor and considering their retention time (RT), mass spectra, isotopic distribution and the information available in literature. Quantification was carried out by eight calibration curves performed by these standards: p-coumaric acid, gallic acid, citric acid, chlorogenic acid, myricetin, quercetin, quercetin-3-glucoside and rutin. The linear range were set at seven concentration levels from the limit of quantification to 100 mg L^{-1} and analyzed in triplicate. In addition, apigenin was used as internal standard at a concentration of 20 mg L^{-1}. For those compounds with no commercially available standards, quantification was carried using calibration curves from other compounds with similar structure.

2.5 Statistical analysis and experimental design

Response-surface methodology (RSM) by central composite rotatable design (CCRD) was performed to determine the influence of independent variables, temperature and solvent composition (ethanol, water and their mixtures) on the extraction of the total phenolic compounds from *H. sabdariffa*. The experimental design covered the entire operational range of the independent variables that the device allows. The ranges tested were from 40 to 200 ºC for temperature factor and 100% water up to 100% ethanol.

A total of 10 experimental runs were carried out at different experimental condition provided by the experimental design: 4 to the full factorial design, 4 start points and 2 center points (Table 1). As is known, the center points ensure the reproducibility of this experimental design decreasing the number of experiments. In addition, runs were carried out randomly in order to minimize errors. The experimental data obtained was processed with the program Statgraphics Centurion software XVI

provided by Statpoint Technologies (Warrenton, VA, USA) in order to determine the optimum conditions to maximize the extraction of the total phenolic compounds.

In order to elucidate significant differences among the diverse families that comprised the phenolic composition of *H. sabdariffa* extracts obtained in each run, a one-way analysis of variance (ANOVA) at a 95% confidence level and a Student-Newman-Keuls (S-N-K) post-hoc test were applied. Statistical data treatment was performed using SPSS software: IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp., 2013).

3. Results and discussion

A comprehensive characterization of the phenolic composition of *H. sabdariffa* extracts obtained by PLE under the experimental conditions provided by the CCRD was carried out by HPLC-ESI-QTOF-MS. Supplementary information compiled the spectral data (peak number according to the elution order, retention time, theoretical m/z, error (ppm), score and molecular formula) as well as the bibliographic references previously reported, both used for the tentatively identification (Table S1).

Quantification was performed by means of eight calibration curves made with standards. Analytical parameters such as regression equation, linearity range and correlation coefficient of each curve are showed in Table 2. In addition, the sensitivity and validation of the proposed method were evaluated based on the limits of detection (LODs) whose signal / noise ratio was 3 and limit of quantification (LOQs) whose signal / noise ratio was 10 for each compound of the standard solutions. Moreover, the repeatability was deduced by relative standard deviations (RSDs) for multiple injections of the same sample and intermediate precision was determined based on intra- and inter-day variances. Thus, an *H. sabdariffa* extract was injected several times (n = 6) on the

same day (intraday precision) and 3 times on 2 consecutive days (interday precision, n = 12). The intraday repeatability of the proposed method for all analytes ranged from 0.81 to 7.87%, whereas the interday repeatability ranged from 1.03 to 9.60%.

A total of 35 compounds were tentatively identified and quantified in the *H. sabdariffa* extracts obtained by pressurized GRAS solvents under the experimental conditions provided by the CCRD. The identified phenolic compounds comprised different families: phenolic acids and their derivatives, and flavonoids such as flavonols, catechins, flavanones, anthocyanins and proanthocyanins. Other polar compounds joint organic acids were also identified. The total content of each family and the concentration of individual compound in all the extraction conditions tested are compiled in Table 3.

3.1. Characterization of the *H. sabdariffa* extracts obtained by CCRD

3.1.1. Phenolic acids and derivatives

A total of 15 compounds belonging to this family group were found in some of the extraction conditions, named as protocatechuic acid and its glucoside form, neochlorogenic acid, two isomers of chlorogenic acid, methylchlorogenate and three isomers of ethylchlorogenate, coumaroylquinic acid, two isomers of caffeoylshikimic acid, methyl digallate and sinapic acid. All of these compounds have been found in previous *Hs* researches (Borrás-Linares et al., 2015; Da-Costa-Rocha et al., 2014; Herranz-López et al., 2012; Zhen et al., 2016). Moreover, dihydroferulic acid-4-O-glucuronide was also characterized in the extracts whose occurrence was previously reported in other matrices such as in cranberry syrups (Contreras, Arráez-Román, Fernández-Gutiérrez, & Segura-Carretero, 2015).

However, not all the aforementioned compounds were detected in all the experimental extracts obtained. In runs 3 and 6 only the occurrence of neochlorogenic acid, chlorogenic acid isomer II and methyl digallate was quantified in small quantities. These extracts, runs 3 and 6, were those obtained with the lowest percentage of ethanol as solvent extraction (15 and 0 %).

The experimental conditions of run 3 were one of the lowest percentages of ethanol used as extraction solvent (15 %) and low temperature (63 ºC), meanwhile run 6 was performed exclusively by water at 120 ºC. Both conditions exhibited the highest dielectric constant values (ε_{run3} = 60.9 and ε_{run6} = 50.5) (Table 1), which resulted in extracts with the lowest phenolic acids content (4.58 ± 0.04 and 4.2 ± 0.2 mg $g_{extract}^{-1}$, respectively).

Contrary, those extractions performed by means of ethanol-water mixtures with the lowest dielectric constant values in combination with the highest temperatures (run 7: ε = 21.8, 176 ºC and run 9: ε = 26.8, 200 ºC) led to the highest extraction yields. This fact was in good agreement with the observations previously reported regarding polyphenols extraction from other plant matrices (Jovanović et al., 2017). From a qualitative point of view, the presence of phenolic acids such as protocatechuic acid and methyl chlorogenate isomer II only was above the limit of quantification in runs 7 and 9. Meanwhile other phenolic acids as protocatechuic acid glucoside and sinapic acid were solely quantified in run 9. The highest total concentration of phenolic acids was attained under the conditions of run (129 ± 7 mg $g_{extract}^{-1}$) which was significantly different from the following best extraction yield, run 7 (89 ± 4 mg $g_{extract}^{-1}$). However, it should be remarkable the quantitative differences found between both runs. In run 9 where a 50 % of ethanol and 200 ºC were employed, extracts were rich in chlorogenic

acid (43 ± 3 mg $g_{extract}^{-1}$), chlorogenic acid isomer II (19 ± 3 mg $g_{extract}^{-1}$), neochlorogenic acid (23 ± 1 mg $g_{extract}^{-1}$) and methyl digallate (12 ± 2 mg $g_{extract}^{-1}$). While in extracts obtained under the conditions of run 7, the major compounds detected were the ethylchlorogenate and its both isomers (10.8 ± 0.3; 19 ± 1; and 21.0 ± 0.7 mg $g_{extract}^{-1}$, respectively). This fact showed the feasibility of performing selective extractions by pressurized GRAS solvents modifying the solvent composition and the extraction temperature to obtain custom extracts.

3.1.2. Flavonoids

This family was divided in two groups: on one hand, flavonols, catechins and flavanones. On the other hand, anthocyanins and proanthocyanins were evaluated separately respect to the other flavonoids due to their different chemical structure and lower thermos-stability (Aurelio, Edgardo, & Navarro-Galindo, 2008) .

3.1.2.1. Flavonoids: Flavonols and catechins

Within this chemical group 7 compounds were identified: 6 flavonols (myricetin 3-arabinogalactoside, quercetin-sambubioside, quercetin-3-rutinoside, quercetin-3-glucoside, myricetin and quercetin) as well as one catechin, methylepigallocatechin. The presence of these flavonoids in *H. sabdariffa* calyces were in good agreement with other studies previously reported (Borrás-Linares et al., 2015; Da-Costa-Rocha et al., 2014; Herranz-López et al., 2012; Rodríguez-Medina et al., 2009).

Although the presence of all flavonoids aforementioned was confirmed, not all amounts extracted were quantifiable. Those runs performed with the lowest percentages of ethanol (0 - 15 %) drove to quantities of flavonoids below of quantification limit, runs 3, 6 and 8. Conversely, greater percentages of ethanol combined with the highest temperatures resulted in lower dielectric constant values (runs 7 and 9) which exerted a

positive effect on the extraction yield of flavonoids from *H. sabdariffa* calyces. Compared to bibliography, similar results were observed in a previous study in which the effect of ethanol percentage and temperature on total flavonoid content in *Momordica charantia* extracted by PLE was evaluated (Syahariza, Torkamani, Norziah, Ahmad, & Mahmood, 2017). In this work, total flavonoid content was measured using the Dowd method and expressed by g quercetin equivalents (QE) per 100 g of dried powder. The results showed that high ethanol percentage and temperature obtained the maximum amount of flavonoids (1.48 g QE per 100 g at 160 ºC and ethanol 80 %). In contrast, the minimum flavonoid yield was attained at 61 ºC and 20 % ethanol (0.07 g QE per100 g).

In term of quantification, run 9 performed with 50 – 50 % ethanol:water at 200 ºC and ε = 26.8 achieved the highest flavonol yield extracted (6.1 ± 0.2 mg $g_{extract}^{-1}$). Run 7 also exhibited excellent flavonoid extraction power working with ethanol 85 % at 176 º C and ε = 21.8 (5.4 ± 0.1 mg $g_{extract}^{-1}$). Despite being myricetin the main compound in both extracts, quantitative differences were observed. Run 7 exhibited major concentrations of quercetin, in run 9 were notable the significant higher quantities of myrectin 3-arabinogalactoside, quercetin sambubioside, quercetin-3-rutinoside and quercetin-3-glucoside.

3.1.2.2 Anthocyanins and proanthocyanins

Belonging to this phenolic family, cyanidin-3-sambubioside and prodelphinidin B3 were identified in some of the *H. sabdariffa* extracts. The presence of these compounds was in concordance to the previous characterization of *H. sabdariffa* composition described in other researches (Da-Costa-Rocha et al., 2014; Herranz-López et al., 2012).

Cyanidin-3-sambubioside was only present in amounts above the limit of quantification (0.33 ± 0.01 mg $g_{extract}^{-1}$) in that run obtained with the lowest temperature applied (run 1, 40 ºC). This finding pointed out the negative effect of temperature on the extraction of this compound. Indeed, the thermal kinetic degradation of anthocyanins observed in *H. sabdariffa* infusion previously studied would support this fact (Aurelio, Edgardo, & Navarro-Galindo, 2008). On the contrary, the opposite effect occured in the case of prodelphinidin B3, which was extracted in larger quantities exclusively in those runs performed at the highest temperatures, run 7 (176 ºC, 0.24 ± 0.05 mg $g_{extract}^{-1}$) and run 9 (200 ºC, 0.36 ± 0.01 mg $g_{extract}^{-1}$). Therefore, it was suggested that prodelphinidin B3 seems to be more temperature stable than cyanidin-3-sambubioside. This difference in thermal stability among both compounds could be related to chemical structure since proanthocyanidins, unlike anthocyanins, are composed by two, three, four or more flavan-3-ol molecules giving rise to a more stable conformation. Moreover, another influential feature on the extraction is that proanthocyanidins are mainly located in cell vacuoles while anthocyanins like cyanidin-3-sambubioside are located in the upper cellular layers (Bautista-Ortin et al., 2016).

3.1.3. Non phenolic compounds: Organic acids and other polar compounds

Despite not being phenolic compounds, organic acids resulted to be the most abundant family in all pressurized extracts of *H. sabdariffa*. For that reason, the chemical composition of organic acids should be mentioned although their data were not taken into account for the optimization of phenolic compounds extraction.

The organic acids family was comprised by gluconic acid ester with citric acid, quinic acid, hydroxicitric acid, hibiscus acid, hibiscus acid hydroxyethylester, hibiscus acid dimethylester and hibiscus acid hydroxyethyldimethylester. The chemical profile of

organic acids were in consonance with those previously reported in *H. sabdariffa* studies (Borrás-Linares et al., 2015; Herranz-López et al., 2012; Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall, 2011) with the exception of gluconic acid derivative, which was described in propolis plant (Bankova, Castro, & Marcucci, 2000).

The extraction of organic acids seemed to share the same tendency for the phenolic compounds. The lowest total concentration of organic acids was found in run 3 (33 ± 2 mg $g_{extract}^{-1}$), whose extract condition was 63 ºC and 15% of ethanol. Whereas the highest extraction values were detected in run 9 (774 ± 14 mg $g_{extract}^{-1}$) and run 7 (618 ± 20 mg $g_{extract}^{-1}$) carried out at 200 ºC with 50% of ethanol and 176 ºC with 85 % of ethanol, respectively. Results highlighted the increase of organic acids concentration in those pressurized extractions performed at high temperature and great percentages of ethanol. These results were in accordance to a previous study, in which the effect of temperature and solvent composition on organic acids content from radish sprouts was studied (Chlopicka & Dobrowolska-iwanek, 2014).

Again, the different extraction conditions in run 9 and run 7 drove to chemical differences among them. Equal portion of ethanol and water as solvent under high temperature (200 ºC) seemed to be selective conditions for the extraction of derivatives from hibiscus acids such as hibiscus acid dimethylester (90 ± 2 mg $g_{extract}^{-1}$) and hibiscus acid hydroxyethyldimethylester (480.0 ± 0.1 mg $g_{extract}^{-1}$). However, higher portions of ethanol under 176 ºC seemed to favor the extraction of hydroxycitric acid and hibiscus acid (261 ± 9 mg $g_{extract}^{-1}$ and 205 ± 1 mg $g_{extract}^{-1}$, respectively).

In addition other polar compounds were identified such as kinsenoside, benzopyran derivative, and two isomers of n-feruloyltyramine. These compounds have already been described in extracts from *H. sabdariffa* or in other plants (Alluis, Pérol,

Hajji, & Dangles, 2000; Borrás-Linares et al., 2015; Da-Costa-Rocha et al., 2014; Shen et al., 2012). The same extraction behavior was observed in comparison with the other families. Run 7 and 9 were the extracts with the greatest concentrations being kinsenoside the mayor component. The presence of benzopyran-4-one derivative and n-feruloyltyramine in run 7 was below to the limit of quantification. However, no significant differences were found in the total content of these polar compounds among both runs.

3.2. Optimization of total phenolic compounds extraction by RSM

The sum of the phenolic compounds for each run provided by the CCRD used as response variable are shown in Table 1. In order to maximize the extraction of phenolic compounds from *H. sabdariffa* calyces a response surface methodology (RSM) was applied to the chemical data (Figure 1). Table 4 summarizes the statistical parameters of the experimental design. According to the results, the model presented a great correlation coefficient (R^2 = 0.89) indicating a slight variance of data and a good prediction of the model to extract target compounds. Both independent variables, temperature and ethanol, had significant influence on the extraction of the total phenolic compounds (*p*-value < 0.05). Quadratic effect of temperature was also significant as well as the interaction between both parameters. However, the quadratic effect of the percentage of solvent was not significant. Hence, keeping only the significant parameters, the model equation provided by the method was (Eq.1):

$$\text{Phenolic compounds} = 109{,}836 - 1{,}96004A - 0{,}952867B + 0{,}00839877AA + 0{,}0107168AB \quad \text{(Eq. 1)}$$

In addition, the lack of fit was not significant (p-value > 0.05), which indicates that this model is suitable to the data observed at 95% confidence level. According to

the model, the optimized conditions to maximize the extraction of phenolic compounds by pressurized GRAS solvents were 200 ºC and 100 % of ethanol.

Due to the absence of previous studies about the use of pressurized GRAS solvents for the extraction of phenolic composition *H. sabdariffa*, the results have been compared with those reported in other plants whose pressurized liquid extraction conditions were optimized by RSM. In general, most of the previous studies also pointed out high temperatures to maximize the extraction of phenolic compounds. This fact could be explained because most of phenolic compounds present in plant matrixes are stored in vacuoles, and consequently, the use of high temperature in combination with alcoholic solutions, or other organic solvents, favor their extraction (Robbins, 2003). In this sense, the optimum temperature provided by the mathematic model to carry out the phenolic extraction of *Phyllantus amarus* by water pressurized was 192.4 ºC using the measure of total phenol index (Folin Ciocalteu assay) and gallic acid content as response variables (Sousa et al., 2016). In other work, conditions of PLE such as temperature and ethanol percentage for the extraction of phenolic compounds from apple pomace were also optimized by RSM. Considering antioxidant activity measured by DPPH, total phenol index measured by Folin-Ciocalteu assay and three polyphenol groups determined by HPL-DAD as response variables, the optimum conditions were 60 % of ethanol and 200 ºC (Wijngaard & Brunton, 2009). However, in both studies, spectrophotometric techniques were used, and although, these methods are widely used for quantification of phenolic compounds, they only provide a general estimation of these bioactive compounds. Furthermore, phenomena like degradations of original phenolic compounds (Sousa et al., 2016) or the generation of unwanted components (Wijngaard & Brunton, 2009) could act as interferences in the

determination. For this reason, it should be noted the importance of the individual quantification for each compound to carry out optimization processes.

4. Conclusions

For the first time, a green methodology based on the use of pressurized GRAS solvents has been optimized for the extraction of *H. sabdariffa* calyces. As it has demonstrated, the dielectric constant determined by extraction conditions such as temperature and solvent composition, is a strong factor that govern the extraction process. Results showed that the use of high temperatures combined to greater ethanol percentages drove to pressurized solvents with low dielectric constant enhancing the extraction of phenolic compounds. Furthermore, it was evidenced how the modification of both parameters could entail the customization of extracts by means of selective extractions. Using the sum of the individual phenolic compounds characterized by HPLC-ESI-QTOF-MS as response variable, the RSM pointed out the temperature of 200 ºC and ethanol 100 % as the best conditions to maximize the extraction of phenolic compounds. However, for some phenolic compounds as cyanidin-3 - sambubioside was only present at lower 40 °C. Therefore, the use of pressurized GRAS solvents seems to be good alternative to performed extractions of value-added compounds of interest for food, pharmacological or cosmeceutical industries.

Acknowledgements

This work was supported by the Research group AGR274 “Bioactive ingredients” from the Analytical Chemistry Department of the University of Granada. Also, the authors would like to thank to the Ministry of Science, Innovation and Universities (RTI2018-096724-B-C22). Finally the authors would like to thank the RED CYTED 118RT0543-ALSUB-CYTED.

Author Contributions:

SPM performed the statistical analysis and prepared the manuscript. IBL conducted the extraction experiment and the analysis by HPLC-ESI-QTOF-MS, realized the analytical data treatment and revised the manuscript. JLS performed the extraction experimental design, supported the experimental work and revised the data. MEA revised and edited the manuscript. DAR and ASC conceptualization, project administration and funding adquisition.

Conflicts of Interest:

The authors declare no conflict of interest.

Figure Captions

Figure 1. Estimated response surface for total phenolic compounds expressed in mg $g_{extract}^{-1}$

Tables

Table 1. Central composite rotatable design (CCRD) with the independent variables, dielectric constant values and total phenolic content as response variable.

Table 2. Analytical parameters of the proposed quantification method.

Table 3. Concentrations expressed in mg $g_{extract}^{-1}$ of phenolic compounds and other polar compounds detected in *H. sabdariffa* extracts obtained under conditions pointed by the central composite rotable design.

Table 4. Analysis of variance (ANOVA) of the regression model.

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Figure1

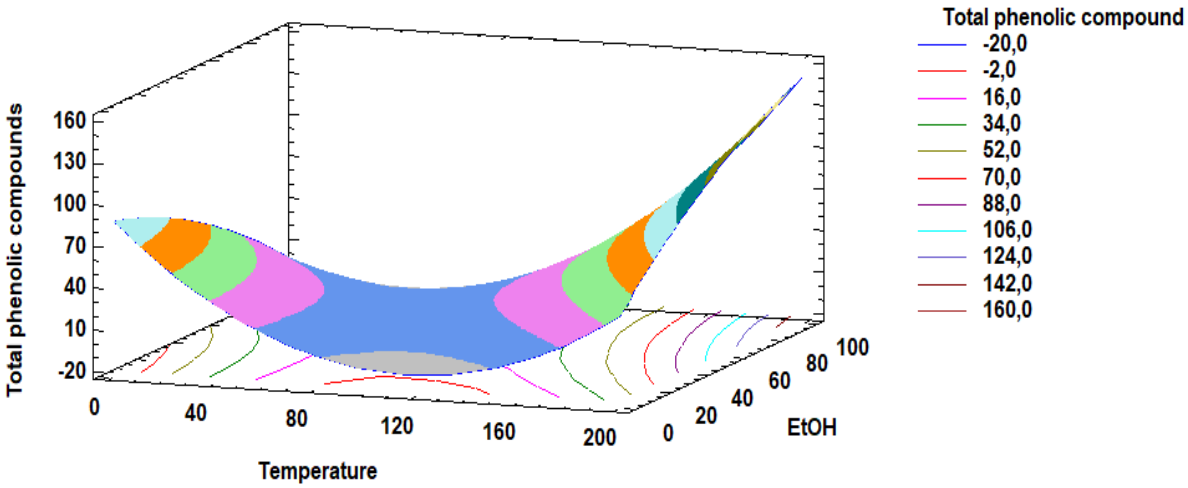

Fig 1. Estimated response-surface for total phenolic compounds expressed in mg $g^{-1}_{extract}$, temperature (ºC) and etanol (% EtOH)

Tables

Table 1. Central composite rotatable design (CCRD) with the independent variables, dielectric constant values, and total phenolic content as response variable

Run	Temperature (ºC)	Solvent Composition (% Ethanol)	Dielectric constant (Ɛ)	Total phenolic content (mg $g_{extract}^{-1}$)
1	40	50	49	$11.1^{b,c} \pm 0.2$
2	63	85	31.4	$8.6^{b} \pm 0.4$
3	63	15	60.9	$4.6^{a} \pm 0.1$
4	120	100	19	$14.1^{c} \pm 0.5$
5	120	50	34.7	$19^{d} \pm 1$
6	120	0	50.5	$4.1^{a} \pm 0.2$
7	176	85	21.8	$95^{e} \pm 5$
8	176	15	34.8	$10^{b} \pm 1$
9	200	50	26.8	$135^{f} \pm 14$
10	120	50	34.7	$21^{d} \pm 1$

Mean values superscripted by different letters indicate significantly different values ($p < 0.05$).

Table 2. Analytical parameters of the proposed quantification method

Standards	Linearity range (mg L^{-1})	LOD (mg L^{-1})	LOQ (mg L^{-1})	Calibration curves	R^2
p-Coumaric acid	LOQ-100	0.0006 ± 0.0002	0.0020 ± 0.0006	y = 0.0859 x + 0.1541	0.987
Gallic acid	LOQ-100	0.0028 ± 0.0010	0.009 ± 0.003	y = 0.0468 x + 0.1761	0.960
Citric acid	LOQ-100	0.005 ± 0.002	0.015 ± 0.005	y = 0.0408 x + 0.6539	0.995
Chlorogenic acid	LOQ-100	0.010 ± 0.002	0.033 ± 0.006	y = 0.0333x + 1.1761	0.983
Myricetin	LOQ-100	0.003 ± 0.001	0.010 ± 0.003	y = 0.1129x + 0.2238	0.980
Quercetin	LOQ-100	0.010 ± 0.003	0.03 ± 0.01	y = 0.0973x + 0.3613	0.951
Quercetin-3-glucoside	LOQ-100	0.0040 ± 0.0007	0.013 ± 0.002	y = 0.0262x + 2.1191	0.960
Rutin	LOQ-100	0.004 ± 0.001	0.012 ± 0.003	y = 0.0852x + 0.2182	0.963

LOD: limit of detection; LOQ: limit of quantification

Peak	Compound	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10
						Phenolic acids and derivatives					
6	Protocatechuic acid glucoside$^{\gamma}$	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	1.09^{b} ± 0.02	<LOQ^{a}
8	Neochlorogenic acid	2.9^{a,b} ± 0.06	1.6^{a} ± 0.1	2.33^{a,b} ± 0.09	1.6^{a} ± 0.1	3.1^{a,b} ± 0.3	2.3^{a,b} ± 0.1	5.2^{c} ± 0.3	1.8^{a} ± 0.1	23^{d} ± 1	3.8^{b,c} ± 0.4
10	Protocatechuic acid$^{\gamma}$	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	0.54^{b} ± 0.02	<LOQ^{a}	0.9^{c} ± 0.1	<LOQ^{a}
11	Chlorogenic acid$^{\phi}$	3.10^{a,b,c,d} ± 0.04	1.7^{a,b,c} ± 0.4	<LOQ^{a}	1.6^{a,b} ± 0.1	4.6^{d,e} ± 0.3	<LOQ^{a}	8.9^{f} ± 0.6	3.5^{c,d} ± 0.2	43^{g} ± 3	5.5^{e} ± 0.7
12	Chlorogenic acid isomer II	2.8^{a,b} ± 0.1	1.2^{a} ± 0.1	1.88^{a,b} ± 0.03	0.8^{a} ± 0.2	4.2^{b,c} ± 0.2	1.8^{a,b} ± 0.1	6^{c} ± 1	3.2^{a,b} ± 0.2	19^{d} ± 3	3.8^{b,c} ± 0.2
14	Methyl digallate$^{\gamma}$	1.07^{c} ± 0.01	0.04^{a} ± 0.02	0.376^{b} ± 0.007	0.45^{b} ± 0.06	1.8^{d} ± 0.2	0.06^{a} ± 0.05	1.1^{c} ± 0.1	<LOQ^{a}	12^{e} ± 2	1.9^{d} ± 0.1
16	Coumaroylquinic acid*	0.03^{a} ± 0.01	0.022^{a} ± 0.004	<LOQ^{a}	0.02^{a} ± 0.01	0.23^{a} ± 0.04	<LOQ^{a}	1.3^{b} ± 0.1	0.13^{a} ± 0.02	2.9^{c} ± 0.1	0.3^{a} ± 0.1
18	Methyl chlorogenate$^{\phi}$	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	0.11^{b} ± 0.07	<LOQ^{a}	0.58^{c} ± 0.04	<LOQ^{a}
19	Dihydroferulic acid-4-O-glucuronide$^{\phi}$	0.024^{a,b} ± 0.005	0.08^{a,b} ± 0.02	<LOQ^{a}	0.08^{a,b} ± 0.03	0.20^{b,c} ± 0.03	<LOQ^{a}	1.31^{d} ± 0.05	<LOQ^{a}	1.8^{e} ± 0.2	0.35^{c} ± 0.02
21	Ethylchlorogenate$^{\phi}$	<LOQ^{a}	0.88^{d} ± 0.01	<LOQ^{a}	0.86^{d} ± 0.07	0.57^{c} ± 0.06	<LOQ^{a}	10.8^{f} ± 0.3	0.02^{a} ± 0.01	2.2^{e} ± 0.1	0.32^{b} ± 0.01
22	Caffeoylshikimic acid*	0.47^{a,b} ± 0.03	0.73^{a,b} ± 0.02	<LOQ^{a}	1.28^{a,b} ± 0.06	1.9^{a,b} ± 0.1	<LOQ^{a}	12^{c} ± 1	0.97^{a,b} ± 0.06	11^{c} ± 2	2.6^{b} ± 0.2
23	Caffeoylshikimic acid isomer II*	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	0.46^{a,b} ± 0.04	<LOQ^{a}	2.0^{c} ± 0.5	0.62^{a,b} ± 0.07	4.4^{d} ± 0.9	0.23^{a,b} ± 0.03
26	Sinapic acid*	<LOQ^{a}	ND	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	<LOQ^{a}	ND	ND	0.436^{a} ± 0.001	<LOQ^{a}
27	Ethylchlorogenate isomer II$^{\phi}$	<LOQ^{a}	0.341^{a} ± 0.004	<LOQ^{a}	2.67^{b} ± 0.09	0.37^{a} ± 0.05	<LOQ^{a}	19^{c} ± 1	0.052^{a} ± 0.004	3.1^{b} ± 0.4	0.21^{a} ± 0.06
28	Ethylchlorogenate isomer III$^{\phi}$	<LOQ^{a}	0.67^{a} ± 0.06	<LOQ^{a}	3.4^{b} ± 0.3	0.52^{a} ± 0.08	<LOQ^{a}	21.0^{c} ± 0.7	0.42^{a} ± 0.04	3.9^{b} ± 0.9	0.85^{a} ± 0.07
	Total	**10.4^{b,c} ± 0.2**	**7.2^{a,b} ± 0.2**	**4.58^{a} ± 0.04**	**12.7^{c} ± 0.6**	**18^{d} ± 1**	**4.2^{a} ± 0.2**	**89^{e} ± 4**	**10^{b,c} ± 1**	**129^{f} ± 7**	**20^{d} ± 1**

	Flavonoids: Flavonols and catechins										
15	Myricetin 3-arabinogalactoside[ϙ]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	0.31[b] ± 0.06	<LOQ[a]
20	Quercetin sambubioside[ϙ]	0.20[b,c,d] ± 0.01	0.30[d] ± 0.04	<LOQ[a]	0.08[a,b,c] ± 0.04	0.07[a,b] ±0.05	<LOQ[a]	0.26[c,d] ± 0.07	<LOQ[a]	1.1[e] ± 0.1	0.36[d] ± 0.03
24	Quercetin-3-rutinoside[ϙ]	0.04[a] ± 0.02	0.25[b] ± 0.04	<LOQ[a]	0.42[b] ± 0.07	0.28[b] ± 0.01	<LOQ[a]	0.62[c] ± 0.06	<LOQ[a]	0.9[d] ± 0.1	0.28[b] ± 0.02
25	Quercetin-3-glucoside[ʊ]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	0.44[b] ± 0.07	<LOQ[a]	0.85[c] ± 0.04	<LOQ[a]
29	Methylepigallocatechin[γ]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	0.051[b] ± 0.006	<LOQ[a]	<LOQ[a]	<LOQ[a]
30	Myricetin[ſ]	0.059[a] ± 0.004	0.29[b] ± 0.01	<LOQ[a]	0.47[b,c] ± 0.06	0.50[c] ± 0.03	<LOQ[a]	2.7[e] ± 0.1	<LOQ[a]	2.41[d] ± 0.09	0.30[b] ± 0.01
35	Quercetin[ƛ]	0.028[a] ± 0.005	0.49[b] ± 0.02	<LOQ[a]	0.37[b] ± 0.08	0.5[b] ± 0.1	<LOQ[a]	1.332[d] ± 0.009	<LOQ[a]	0.48[b] ± 0.08	0.75[c] ± 0.03
	Total	**0.32[a] ± 0.01**	**1.33[b] ± 0.02**	**NQ[a]**	**1.3[b] ± 0.1**	**1.4[b] ± 0.1**	**NQ[a]**	**5.4[d] ± 0.1**	**NQ[a]**	**6.1[e] ± 0.2**	**2.1[c] ± 0.3**
	Flavonoids: Anthocyanins and proanthocyanins										
7	Cyanidin -3- sambubioside[ϙ]	0.33[b] ± 0.01	<LOQ[a]	<LOQ[a]	ND	<LOQ[a]	<LOQ[a]	<LOQ[a]	ND	<LOQ[a]	<LOQ[a]
33	Prodelphinidin B3[ϙ]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	<LOQ[a]	0.24[b] ± 0.05	<LOQ[a]	0.36[c] ± 0.01	<LOQ[a]
	Total	**0.33[c]± 0.01**	**NQ[a]**	**NQ[a]**	**NQ[a]**	**NQ[a]**	**NQ[a]**	**0.24[b] ± 0.05**	**NQ[a]**	**0.36[c] ± 0.01**	**NQ[a]**
	Organic acids										
1	Gluconic acid ester with citric acid[‡]	6.4[e] ± 0.5	6.4[e] ± 0.3	4.61[d] ± 0.06	2.9[b] ± 0.1	3.5[c] ± 0.3	3.6[c] ± 0.1	7.1[f] ± 0.5	1.7[a] ± 0.1	18.9[g] ± 0.9	3.8[c] ± 0.1
2	Quinic acid[‡]	<LOQ[a]	<LOQ[a]	<LOQ[a]	0.60[b] ± 0.07	0.31[a,b] ± 0.03	0.053[a] ± 0.007	5.4[c] ± 0.5	0.57[b] ± 0.03	13.1[d] ± 0.8	0.5[b] ± 0.1
3	Hydroxycitric acid[‡]	2.0[a] ± 0.7	4.4[a,b,c] ± 0.6	5.07[a,b,c] ± 0.08	4.0[a,b] ± 0.3	7.8[c] ± 0.4	4.9[a,b,c] ± 0.4	261[f] ± 9	75.9[e] ± 4	22[d] ± 3	6.5[b,c] ± 0.4
4	Hibiscus acid[‡]	20[b] ± 2	14.7[a] ± 0.7	21[b] ± 1	20[b] ± 1	20[b] ± 2	29.2[c] ± 0.8	205[f] ± 1	52[d] ± 6	131[e] ± 7	21[b] ± 1

5	Hibiscus acid hydroxyethylester‡	$5.1^{b} \pm 0.4$	$14.9^{c} \pm 0.2$	$<LOQ^{a}$	$26^{e} \pm 1$	$21.2^{d} \pm 0.5$	$<LOQ^{a}$	$28^{e} \pm 2$	$1.9^{a,b} \pm 0.5$	$90^{f} \pm 2$	$20^{d} \pm 1$
9	Hibiscus acid dimethylester‡	$10.0^{b} \pm 0.5$	$21^{c} \pm 2$	$3.14^{a} \pm 0.08$	$72^{f} \pm 7$	$60^{e} \pm 1$	$0.7^{a} \pm 0.04$	$44.3^{d} \pm 0.8$	$71^{f} \pm 3$	$480.0^{g} \pm 0.1$	$60.5^{e} \pm 0.9$
13	Hibiscus acid‡ hydroxyethyldimethylester‡	$<LOQ^{a}$	$1.4^{a,b} \pm 0.1$	$<LOQ^{a}$	$22.0^{d} \pm 0.2$	$4.63^{c} \pm 0.2$	$<LOQ^{a}$	$67^{e} \pm 1$	$0.85^{a,b} \pm 0.03$	$19^{d} \pm 1$	$3.5^{b,c} \pm 0.4$
	Total	**$43^{b} \pm 5$**	**$63^{c} \pm 3$**	**$33^{a} \pm 2$**	**$148^{e} \pm 11$**	**$117^{d} \pm 7$**	**$38^{a,b} \pm 1$**	**$618^{g} \pm 20$**	**$204^{f} \pm 18$**	**$774^{h} \pm 14$**	**$115^{d} \pm 3$**
	Other polar compounds										
17	Kinsenoside$^{\phi}$	$0.03^{a} \pm 0.01$	$4.5^{b} \pm 0.2$	$<LOQ^{a}$	$83^{f} \pm 1$	$21.6^{c} \pm 0.8$	$<LOQ^{a}$	$70^{e} \pm 5$	$1.67^{a,b} \pm 0.07$	$69^{d} \pm 4$	$22.3^{c} \pm 0.5$
31	4H-1-Benzopyran-4-one derivative*	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$0.28^{b} \pm 0.02$	$<LOQ^{a}$
32	N-feruloyltyramine$^{\gamma}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$<LOQ^{a}$	$0.20^{b} \pm 0.06$	$<LOQ^{a}$
34	N-feruloyltyramine isomer II$^{\gamma}$	$0.20^{b} \pm 0.02$	$0.40^{c,d} \pm 0.03$	$<LOQ^{a}$	$0.53^{d} \pm 0.04$	$0.32^{b,c} \pm 0.02$	$<LOQ^{a}$	$2.18^{f} \pm 0.02$	$<LOQ^{a}$	$2.4^{e} \pm 0.1$	$0.23^{b,c} \pm 0.02$
	Total	**$0.2^{a} \pm 0.1$**	**$4.9^{b} \pm 0.2$**	**NQ^{a}**	**$84^{e} \pm 2$**	**$23^{c} \pm 1$**	**NQ^{a}**	**$72^{d} \pm 6$**	**$1.67^{a,b} \pm 0.07$**	**$72^{d} \pm 4$**	**$22^{c} \pm 2$**

Table 3. Concentrations expressed in mg gextract-1 of phenolic compounds and other polar compounds detected in H. sabdariffa extracts obtained under conditions pointed by the central composite rotable design.

Number peak shows the elution order. ND: Compound not detected; NQ: Compound not quantified <LOQ: Concentration below of the limit of quantification. Quantification was performed using calibration curves of: *p-coumaric acid; $^{\gamma}$gallic acid; ‡citric acid; $^{\phi}$chlorogenic acid; $^{\int}$myricetin; $^{\lambda}$quercetin; $^{\upsilon}$quercetin-3-glucoside; $^{\partial}$rutin. Mean values superscripted by different letters indicate significantly different values between rows ($p < 0.05$).

Table 4. Analysis of variance (ANOVA) of the regression model.

Source	Total phenolic content				
	SS	**DF**	**MS**	**F-value**	**p**
A: Temperature	12248.9	1	12248.9	3488.47	**0.0108**[a]
B: % Ethanol	633.524	1	633.524	180.43	**0.0473**[a]
A^2:Temperature^2	2530.54	1	2530.54	720.69	**0.0237**[a]
AB: Temperature:% Ethanol	1796.49	1	1796.49	511.64	**0.0281**[a]
B^2: %Ethanol^2	260.509	1	260.509	74.19	0.0736
Lack of fit	1940.88	3	646.96	184.25	0.0534
Pure error	3.51125	1	3.51125		
Total	17799.2	9			
R^2	0.89076				
Adj. R^2	0.754209				

SS = sum of squares; DF= degrees of freedom; MS = mean square; R^2 = Quadratic correlation coefficient; [a] Significant ($p < 0.05$)

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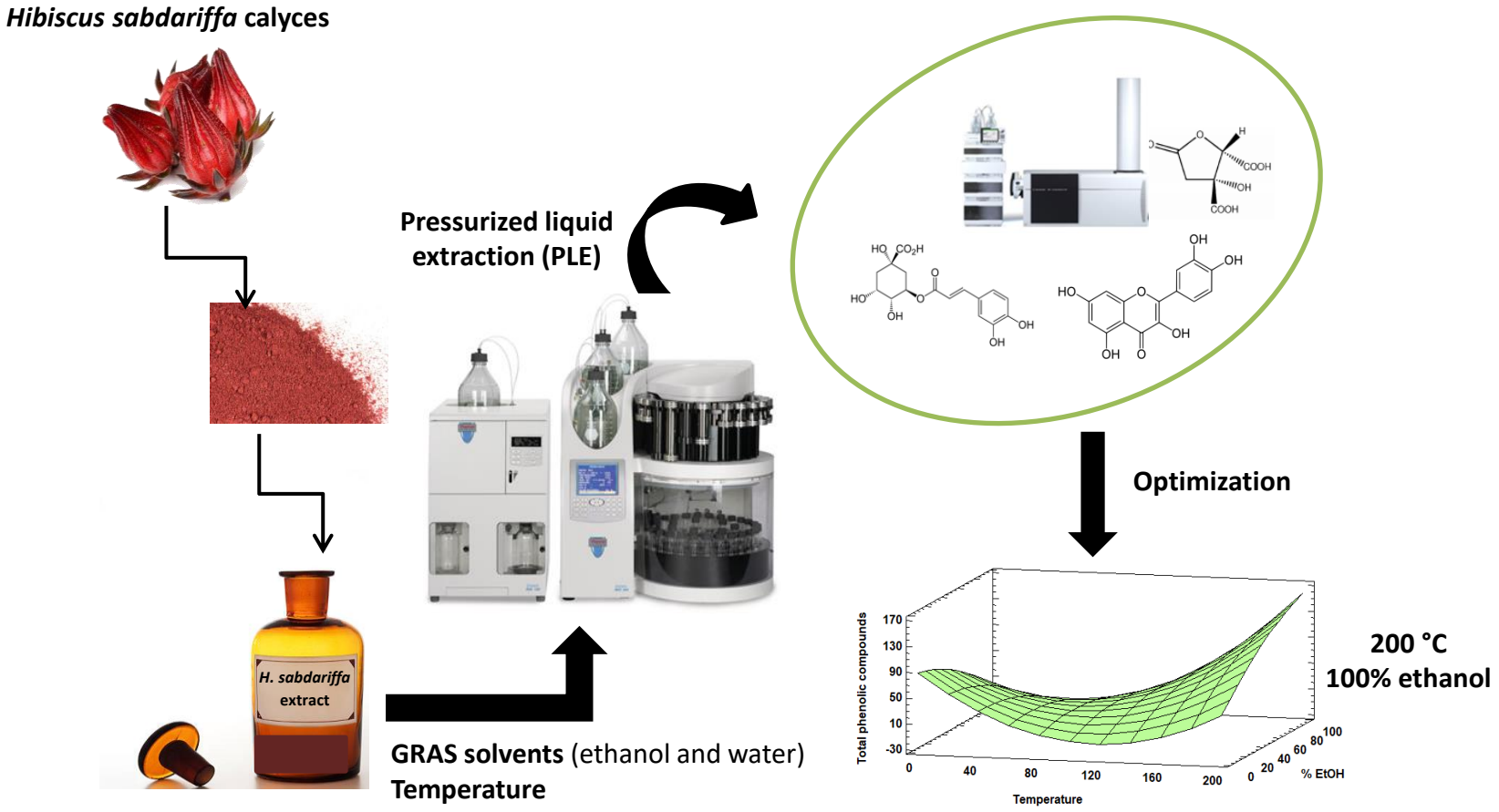
*Graphical Abstract



Credit Author Statement:

SPM performed the statistical analysis and prepared the manuscript. IBL conducted the extraction experiment and the analysis by HPLC-ESI-QTOF-MS, realized the analytical data treatment and revised the manuscript. JLS performed the extraction experimental design, supported the experimental work and revised the data. MEA revised and edited the manuscript. DAR and ASC conceptualization, project administration and funding adquisition.

*Conflict of Interest form

Conflict of interest statement

The authors declare that there are no conflicts of interest.