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A NOVEL SUSTAINABLE APPROACH FOR THE EXTRACTION OF VALUE-ADDED COMPOUNDS FROM *HIBISCUS SABDARIFFA* L. CALYCES BY NATURAL DEEP EUTECTIC SOLVENTS

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ABSTRACT

Hibiscus sabdariffa L. is widely acknowledged as an important source of value-added compounds as natural pigments and bioactive compounds whose isolation is of great interest in food industry. Due to the incessant demand of green extraction procedures, NADESs have emerged in the last years as new green solvents alternative to the conventional organic ones. The feasibility of NADESs for the extraction of value-added compounds of *H. sabdariffa* has been assessed in this work for the first time. An extraction technique based on microwave assisted extraction (MAE) and the use of NADESs was proposed testing different deep eutectic solvents based on choline chloride (ChCl). Among them, oxalic acid based NADES exhibited a great selectivity for anthocyanins in particular as well as higher extraction yields of bioactive compounds was also observed, even greater than those observed by methanol. The effect of extraction variables such as liquid-solid ratio, temperature and water percentage were studied by Box-Behnken design and a response surface methodology. The statistical program pointed out the liquid-solid ratio of 1:30 mL mg^{-1}, temperature of 75 ºC and a water percentage of 55 % as the conditions to maximize extraction within the experimental domain. Hence, NADES composed by oxalic acid and choline chloride resulted to be an effective green alternative tailor-made solvent to carry out selective extractions of value-added compounds from *H. sabdariffa.*

Keywords: natural deep eutectic solvents (NADES); *Hibiscus sabdariffa L.*; value-added compounds; green extraction; microwave-assisted extraction

1. Introduction

Current consumer demands require food industries to produce delightful, appealing, safer and healthier foodstuffs. In this sense, the awareness about the artificial colorants and the desire of promote health and well-being drive to the demand for food products formulated with natural ingredients as bio-based alternatives (Martins, Roriz, Morales, Barros, & Ferreira, 2016; Román, Sánchez-Siles, & Siegrist, 2017; Shahidi, 2009).

The global food natural colouring market has grown rapidly in the last years and it is expected to continue growing by 10 % to 15 % annually (Carle & Schweiggert, 2016) not only for being a key food sensory attribute but also for the interest in replacing artificially colorants by natural counterparts due to the adverse health effects (Ramesh & Muthuraman, 2018). On the other hand, due to the incessant interest of consuming food with health promoting activities, functional food market is soaring exponentially, so worldwide is entering the era of functional foods (Daliri, & Lee, 2015).

Edible flowers are a significant source of natural pigments endowed with colouring properties but also natural source of phytochemicals with bioactive activities to be used for functional food development (Jabeur, et al., 2017; Takahashi, Gonçalves Rezende, Fidelis Moura, Borges Dominguete, & Sande, 2020). Among them, the edible flower of *Hibiscus sabdariffa L.*, also known as roselle, is the most widely consumed genome between 300 different *Hibiscus sp.* species cultivated in tropical and subtropical areas of both hemispheres (Riaz & Chopra, 2018). Hibiscus flower is used and traded worldwide today as an important ingredient in teas, beverages, food and cosmetic products due to its exotic distinctive floral, berry-like flavour and its appealing reddish-purple colour properties (Bechoff et al., 2014; de Moura et al., 2019; Ismail, Ikram, & Nazri, 2008; Monteiro et al., 2017; Nguyen et al., 2018; Pinela et al., 2019)

Constituents of *H. sabdariffa* calyces responsible for the appealing coloration are anthocyanins such as delphinidin-3-sambubioside (hibiscin) and cyanidin-3-sambubioside (geossypicyanin) (Borrás-Linares et al., 2015a, 2015b; Galvão Maciel et al., 2018; Jabeur et al., 2017; Segura-Carretero et al., 2008; Sindi, Marshall, & Morgan, 2014). On the other hand, an increasing body of pharmacologic and clinical studies support the health and therapeutic claims of *H. sabdariffa* calyces due to the presence of phenolic compounds (Ali, Al Wabel,, & Blunden, 2005; Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014; Gomes Maganha et al., 2010).

In order to extract and recover value-added compounds from *H. sabdariffa* as natural pigments or bioactive compounds for their subsequent use as colorants and functional ingredients for the development of functional foods, it is necessary to conduct an efficient and sustainable extraction process. Traditionally, the extraction process was carried out by means of conventional extraction techniques (macerations, Soxhlet device…), which implied some disadvantages such as the use of organic solvents in large quantities, extended extraction time and low extraction recoveries among others (Borrás-Linares et al., 2015a; Salazar-González, Vergara-Balderas, Ortega-Regules, & Guerrero-Beltran, 2012; Segura-Carretero et al., 2008; Sindi et al., 2014). To overcome these limitations, alternative green extraction techniques such as microwave-assisted extraction (MAE) or supercritical fluid extraction (SFE) have been optimised for isolation of bioactive compounds from *H. sabdariffa* recently to be exploited in numerous applications in food industry (Pimentel-Moral et al., 2018; Pimentel-Moral et al., 2019; Yusoff & Leo, 2017).

In an attempt to find more effective and environmentally friendly extraction procedure in accordance with the main principles of green analytical chemistry (Gałuszka, Migaszewski, & Namieśnik, 2013), research interest has been triggered to greener extraction solvents. In this sense, the use of deep eutectic solvents (DESs) or natural deep eutectic solvents (NADESs), if

they are composed of metabolites naturally present in cells and organisms, has emerged in the last years (Cui et al., 2017; Dai, Van Spronsen, Witkamp, Verpoorte, & Choi, 2013; Smith, Abbott & Rydes, 2014). NADESs are simple, non-toxic, inexpensive and biodegradable solvents with good extraction properties. But, arguably, the most relevant advantage of NADESs is the endless opportunities of tailor-made solvents in a green media. This fact makes them an excellent choice to extract value-added compounds, not only increasing yields and preserving their biological effects and (Choi & Verpoorte, 2019; Murador, de Sousa Mesquita, Vannuchi, Braga, & de Rosso, 2019; Radošević et al., 2016; Zainal-Abidin, Hayyan, Hayyan, & Jayakumar, 2017) what will boost their further use food applications.

Therefore, the main aim of this study was the application of NADESs to the green extraction of value-added compounds of *H. sabdariffa* for the first time. For that purpose, a microwave-assisted extraction procedure was set up and the extraction abilities of eight tailor-made NADESs were tested in comparison with that provided by a conventional organic solvent, methanol. Once the best NADESs was selected, the effect of the main variables involved in the process (liquid-solid ratio, temperature and percentage of water of NADESs) were further analyzed by means of response surface methodology (RSM) .

2. Materials and methods

2.1. Plant material

Dried calyces of *H. sabdariffa* were generously provided by Monteloeder Inc. (Elche, Alicante, Spain). The sample was grounded and homogenized using an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany) equipped with 12-tooth rotor and ring sieve until a particular size of 2 mm. . A homogenized sample was stored at room temperature in darkness.

2.2. Chemical and reagents

Choline chloride (≥ 99.0%), lactic acid (≥ 98.0%), oxalic acid (≥ 99.0%), ethylene glycol (≥ 99.8%), 1,2-propanediol (≥ 99.5%), fructose (99.0%), maltose (≥ 99.0%), glucose (≥ 99.5%), and urea (≥ 99.5%) were purchase from Sigma-Aldrich (St. Louis, MO, USA). Standard compounds used for quantification process including chlorogenic acid, citric acid, gallic acid, p-coumaric acid, quercetin, quercetin-3-glucoside, rutin, myricetin and myricitrin were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin Ciocalteu's phenol reagent, gallic acid and Na_2CO_3 were supplied by Merck (Darmstadt, Germany). Additionally, LC-MS grade acetonitrile and HPLC-grade MeOH were purchased from Fisher Scientific (Madrid, Spain). Acetic acid (> 99.5%) was sourced from Fluka (Switzerland). Ultra-pure water (resistivity above 18.2 MΩ cm) used in this experiment was obtained from Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Preparation of NADESs

Eight different choline chloride based NADES were prepared (**Table 1**) by previously described method (Dai et al., 2013) in the proper molar ratios. In brief, choline chloride (ChCl) as hydrogen bond acceptor and different hydrogen bond donors (acids, polyols, sugars and urea) were heated at 80°C with constant stirring in a water bath until a clear, homogeneous liquid was formed. NADESs were store at room temperature.

2.4.Microwave-assisted extraction (MAE) procedure

Bioactive compounds from *H. sabdariffa* calyces were extracted using a microwave laboratory extractor (Anton Paar GmbH, Graz, Austria), which was equipped with a digital control system for the optimization of MAE parameters.

For initial screening of the prepared NADESs, 200 mg of powder sample were placed into an extractor vessel with 2 mL of each NADES solution (containing 25% water) to give liquid to solid ratio 1:10 mL g^{-1}. The mixture was subsequently microwave irradiated at 65 °C for 20

min to carry out the NADEs screening. All experiments were temperature-monitored and microwave power and pressure were fixed to 700 W and 18 bar, respectively. In parallel, a comparative experiment was also performed under the same experimental conditions using as reference a conventional organic solvent (methanol:water, 80:20, v/v).

After MAE, the extracts obtained were centrifuged (at 13.000 rpm for 15 min at 20 °C) and supernatants were filtered through a 0.2 μm regenerated cellulose (RC) filters. Clear samples were transferred into glass flask being diluted with ultra-pure water up to 10 mL and stored at -20°C for future analysis. All extractions were performed in duplicate.

2.5. Characterization of bioactive compounds from H. sabdariffa extract by HPLC-DAD-ESI-TOF-MS

HPLC analysis were performed using an Agilent 1200 series Rapid Resolution Liquid Chromatograph (Agilent Technologies, CA, USA) coupled to an orthogonal-accelerated micrOTOF mass spectrometer (Bruker Datonics, Bremen, Germany) and an electrospray interface (modelG1607A from Agilent Technologies, Palo Alto, CA, USA). The separation of bioactive compounds was achieved on a Zorbax Eclipse Plus C_{18} column (1.8 μm, 4.6 x 150 mm). The separation method was based on according to previous work with some modifications (Borrás-Linares, 2015a). Briefly, the mobile phases consisted of water:acetonitrile (90:10 v/v) with 0.1% formic acid (solvent A) and acetonitrile (solvent B). The gradient program was as follow: 0 min, 5 % (B), 8 min 22 % (B), 23 min 28 % (B), 27 min 95 % (B), 31 min 5 % (B) and finally a conditioning cycle of 5 min with the initial condition. Injection volume was set at 10 μL, oven temperature was fixed at 25 ºC and the flow rate was 0.5 mL min^{-1}. In order to ensure stable ionization conditions, the effluent from the HPLC was split before being introduced into the mass analyser. Detection was carried out considering a mass range of 50 – 1000 m/z. The ionization parameters were: capillary voltage,

4000V; drying gas temperature, 210 ºC; drying gas flow, 9.5 L min^{-1}; nebulizing gas pressure, 2.5 bar; and end plate offset, −500 V. External mass spectrometer calibration was carried out with a sodium formate cluster solution (5 mM sodium hydroxide and 0.1 % formic acid in water/2-propanol (1:1, v/v)) in quadratic plus high-precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) and all the spectra were calibrated before identification. The data were processed using the software Data Analysis 4.0 (Bruker Daltonik).

Identification was characterized 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 means of calibration curves of standards compounds (linearity range (μg mL^{-1}), R^2): gallic acid (0.75 – 130, 0.9997), chlorogenic acid (0.60 -120, 0.9999), citric acid (0.55 – 110, 0.9991), *p*-coumaric acid (0.55 – 110, 0.9999), quercetin (0.50 – 100, 0.9990), quercetin-3-glucoside (0.50 – 100, 0.9998), rutin (0.75 – 130, 0.9999, myricetin (0.70 – 140, 0.9999), myricitrin (0.30 – 60, 0.999) and cyaniding-3-sambubioside (0.39 – 100, 0.9954). Concentrations were expressed as milligrams of target compound per gram of dried plant material.

2.6. Statistical analysis and experimental design

For the selection of the appropriate NADES, statistical data treatment was performed using SPSS software: IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp., released 2013). 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 to determine the differences

amongst extraction yields of bioactive compounds with the NADESs tested and methanolic solution.

Once the appropriate NADES was selected, the MAE process was further optimized by using three-level, three-factor Box-Behnken design (BBD) and response surface methodology (RSM). The three main independent variables to optimized were temperature (X_1: 40 ºC, 80 ºC and 120 ºC), solvent to solid ratio (X_2: 1:10 mL mg^{-1}, 1:20 mL mg^{-1} and 1:30 mL mg^{-1}) and water percentage in NADESs (X3: 0 %, 35 % and 70 %). The range of the variables or factor was chosen based on the preliminary results and literature available (Alañón, Ivanović, Gómez-Caravaca, Arráez-Román, & Segura-Carretero, 2018; Craveiro et al., 2016; Dai et al., 2013;Ivanović, Alañón, Arráez-Román, & Segura-Carretero, 2018). The total phenolic content (TPC) and total anthocyanin content (TAC) were the response variables used to determine the optimum conditions of extracting phenolic compounds from *H. sabdariffa* calyces. A total fifteen experiments were carried out including three replications of the central point. The experimental data were fitted to a second-order polynomial model (equation 1) to obtain the regression equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{j=1}^{k} \beta_{ij} X_i X_j \quad (1)$$

where Y is the response variable, X_i and X_j are the independent variables and k is the number of tested variables (k = 3). The effect of each term in the model and its statistical significance for the response variable were analysed. Regression coefficients were regarded as significant when the level of significance was lower than 0.05, $p < 0.05$. Those regression coefficients not significant were excluded from the model and it was refitted by multiple linear regression. The new fitted model furnished the optimized conditions maximising the yield. For the graphical representation of the interactive effects of operational parameters on the extraction

yields, the three-dimensional (3D) profiles of multiple non-linear regression models were depicted using the fitted quadratic polynomial equation thus obtained. For the design of experiment, statistical data analysis and 3D graphs construction Design-expert software (Design Expert 11) was used.

2.7. Estimation of total phenolic content (TPC)

The analysis of total phenolic compounds was performed using the Folin-Ciocalteu reagent measuring the absorption at 760 nm (Slinkard, & Singleton, 1977). Briefly, 10 µL of properly diluted extracts were mixed with 600 µL of deionized water and 50 µL of Folin-Ciocalteu reagent. After 10 min, 150 µL of Na_2CO_3 solution 20% (w/v) and 190 µL of deionized water were added. The samples were allowed to stand for 2 h in a dark place at room temperature. After incubation, 200 µL of the samples were transferred into a 96-wells microplate and the absorbance was measured using BioTek spectrophotometer microplate reader (Winooski, Vermont, EEUU). The TPC were expressed as milligrams of gallic acid equivalents per gram of dry plant material. Each replicate of extraction were analysed in triplicate (n = 6).

2.8. Estimation of total monomeric anthocyanin content (TAC)

Total monomeric anthocyanin content (TAC) was determined by standard pH differential method (Lee, Durst, & Wrolstad, 2005). Extracts were diluted with two different buffers (pH=1 and pH=4.5). The absorbances measurements of the prepared solutions were adquired at two different wavelengths: 520 nm and 700 nm by a BioTek spectrophotometer microplate reader. TAC, expressed as mg of cyanidin-3-glucoside equivalents per litre (mg Cya-3-Glu L^{-1}), was calculated as follows (equation 2):

$$TAC = \frac{A\ x\ MW\ x\ DF\ x\ 10^3}{\varepsilon\ x\ 1} \quad (2)$$

where A = (A_{520nm} – A_{700nm}) pH 1.0 – (A_{520nm} – A_{700nm}) pH 4.5; MW (molecular weight) = 449.2 g mol^{-1} for cyanidin-3-glucoside; DF = dilution factor; 1 = path length in cm; Ɛ = 26,900 molar absorptivity coefficient, in L $mol^{-1}cm^{-1}$, for cyanidin-3-glucoside and 10^3 = conversion factor from g to mg. All measurements of each replicate extraction were performed in triplicate (n = 6).

3. Results and Discussion

3.1. Screening of NADES for the extraction of value-added compounds from calyces of H. sabdariffa

The analysis of *H. sabdariffa* extracts by means of HPLC-DAD-ESI-TOF-MS revealed the presence of 21 compounds which were identified by the interpretation of their MS spectra and the data from the literature and mass-spectra databases and quantified by calibration curves of standards. All spectral data as well as quantification aspects are compiled in Table 2. Chemical characterization of *H. sabdariffa* extracts isolated by NADESs was in good agreement with those previously reported in bibliography (Borrás-Linares et al., 2015a; 2015b; Da-Costa-Rocha et al., 2014; Pimentel-Mora et al., 2018; 2019).

Among the compounds detected (Table 3), anthocyanins are one of the groups of compounds found in *H. sabdariffa,* which has roused increasing interest in the last years. NADESs were able to extract delphinidin-3-sambubioside (hibiscin) and cyanidin-3-sambubioside (gossypicyanin), which are the dominant anthocyanins present in *H. sabdariffa* calyces and leaves (Da-Costa-Rocha et al., 2014). Among NADESs tested, those composed by organic acids (lactic and oxalic acid) exhibited higher extraction yields of both anthocyanins suggesting that their acid nature favour their extraction capacity of these target compounds. This fact was supported by findings reported in bibliography where the choice of

an appropriate NADES for anthocyanins extraction from diverse vegetal matrices (grape skins, *catharanthus roseus*, mulberry …), always lead to an organic acid component such as citric acid, lactic acid or oxalic acid (Bubalo, Curko, Tomasevic, Ganic, & Redovnikovic, 2106; Dai, Rozema, Verpoorte, & Choi, 2016; Guo et al., 2019; Jeong, et al., 2015). In our particular case of *H. sabdariffa*, NADES based on choline chloride and oxalic acid (ChCl-Ox) attained the highest value of the individual anthocyanins extracted (3.76 ± 0.03 mg g^{-1} and 3.60 ± 0.03 mg g^{-1} for delphinidin-3-sambubioside and cyaniding-3-sambubioside, respectively). Likewise, the highest value obtained in the TAC assay estimated by standard spectrophotometric method was 3.64 ± 0.01 $mg_{cyan\text{-}3\text{-}gluc}$ g^{-1}) . Oxalic acid is a dicarboxylic acid which makes it one of the strongest organic acid (pKa1: 1.25, pKa2: 4.27), meanwhile the acidic nature of lactic acid is weaker (pKa: 3.86). The different acidic feature among acids could explain the better extraction results of oxalic acid for anthocyanins depend on the pH supported by the fact that anthocyanins are prevalent in the flavylium cation form stable at pH < 2, while at a pHs > 7 is totally degraded (Panić, Gunjević, Cravotto, Radojčić Redovniković, 2019). Furthermore, it should be noted that ChCl-Ox proved to have higher extraction capabilities for anthocyanins than the use of methanolic solution (3.32 ± 0.09 mg g^{-1} and 1.73 ± 0.04 mg g^{-1} for delphinidin-3-sambubioside, and cyaniding-3-sambubioside respectively. For the extraction of *H. sabdariffa* anthocyanins, different solvents were used such as water or acidified water (Jabeur et al., 2017; Salazar-González, Vergara-Balderas, Ortega-Regules, & Guerrero-Beltrán, 2012;Sindi et al., 2014), ethanol and ethanol/ water mixtures (Salazar Gonzales et al., 2012; Jabeur et al., 2017) as well as methanol and acidified methanol (Sindi et al., 2014). Based on data reported by Sindi et al., 2014, acidified water and especially pure water at the boiling point lead to the highest extraction yields for the hibiscus anthocyanins (4.11±1.47 mg g^{-1} and 3.81±1.21 mg g^{-1} for delphinidin-3-sambubioside and cyanidin-3-sambubioside, respectively). These values were higher than those attained by

ChCl-Ox. However in other studies, microwave assisted extraction using water as solvent extracted lower amounts than ChCl-Ox (from 1.20 to 0.59 mg g^{-1} of delphinidin-3-sambubioside and values ranged between 0.36 and 0.16 mg g^{-1} for cyanidin-3-sambubioside) (Cassol, Rodrigues, & Zapata Noreña, 2019). These discrepancies are due to the different methodologies used as well as the chemical differences in the *H. sabdariffa* samples analysed (different cultivars, different locations of cultivation, different climatic conditions). Therefore, it is hard to compare the results of the present study with other published works.

Phenolic acids were also detected in *H. sabdariffa* extracts. Chlorogenic acid whose concentration range was from 4.64 ± 0.10 mg g^{-1} to 5.73 ± 0.00 mg g^{-1}, was the dominant phenolic acid found joint with its two isomers, cryptochlorogenic and neochlorogenic acid (Table 3). The presence of these compounds has already been reported (Borrás-Linares et al., 2015a; 2015b; Pimentel-Moral et al., 2018; 2019). The identification of other phenolic acids such as coumaroylquinic acid and 5-*O*-caffeoylshikimic acid was also confirmed. Although all NADESs tested were able to extract phenolic acids, those composed by sugar as fructose (ChCl-Fru), maltose (ChCl-Mal) and glucose (ChC-Glu) exhibited the lowest extraction values for the sum of phenolic acids, ranged from 14.57 ± 0.20 to 16.34 ± 0.07 mg g^{-1}. The use of urea and polyols like ethylene glycol and 1,2 propanediol provided higher extraction yields not finding significant differences with those attained by methanolic solution. This similar behaviour among NADEs formed by urea and polyols with methanol mixture was also observed for the extraction of phenolic compounds from olive leaves (Alañón et al., 2018). Again, lactic acid and especially oxalic acid lead to the highest extraction yields for the total phenolic acids recovery (19.12 ± 0.05 and 19.94 ± 0.60 mg g^{-1}, respectively). Comparing those results with the previously published data it can be concluded that proposed MAE-NADES methodology could represent a really promising tool for the quantitative extraction of phenolic acids from the hibiscus calyces. Cassol et al. reported a maximum contents of the

chlorogenic and neochlorogenic acid in the hibiscus flower of 2.58 mg g^{-1} and 1.71 mg g^{-1}, respectively, using exhaustive extraction with acidified methanol: water (80:20 v:v) as the extraction solvent (Cassol et al., 2019). Likewise, recently proposed supercritical fluid extraction (SFE) method, have showed a significantly lower recovery of the phenolic acids from hibiscus, with the total phenolic acids content of 10 mg g^{-1} (Pimentel-Moral, et al., 2019).

In addition, flavonoids and their glycosidic derivatives were also characterized. Quercetin, as well as its glucoside, rutinoside and sambubioside conjugates were detected in all extracts. Myricetin and kaempferol and their respective arabinogalactoside and sambubioside derivatives were other flavonoids identified. Regards to extraction of these flavonoids from *H. sabdariffa*, almost all of NADESs tested exhibited lower extraction power than hydro-methanolic solvent with the exception of oxalic acid (ChCl-Ox). The greatest amounts of myricetin, quercetin, kaempferol, myricetin-3-arabinogalactoside and kaempferol-3-*O*-sambubioside were attained when the extraction was performed by ChCl-Ox. Hence, the total flavonoid content extracted by the use of oxalic acid reached quantities of 4.57 ± 0.12 mg g^{-1}, which was significantly higher than those shown by methanolic solution. Comparing ~~those~~ result with the other *H. sabdariffa* extract, the levels of the flavonoids were significantly higher in the MAE-NADES than in other works (Pimentel-Moral et al., 2019).

Other polar compounds were detected, among them organic acids such as hibiscus acid and hydroxycitric acid, which comprised the most abundant fraction of *H. sabdariffa* extracts. Hibiscus acid was the predominant compound and its bioactive effects against diabetes and other glucose related diseases like metabolic syndrome have been recently demonstrated (Seung et al., 2018). In this particular case, none of the screened NADESs exhibited major extraction yields than methanol, which attained the highest content of hibiscus acid (31.70 ± 1.65 mg g^{-1}) and hydroxycitric acid (2.93 ± 0.07 mg g^{-1}). Among

NADESs, those composed by sugars and urea showed lower efficiencies in the extraction on these polar compounds such as organics acids. Contrary, NADES made with polyols and acids exhibited better capabilities to extract organic acids.

Therefore, based on the results, it could be pointed out that for the particular case the use of choline chloride and oxalic acid enhanced the extraction of bioactive compounds from *H. sabdariffa*, since due to its acidic nature favoured, mainly, the isolation of anthocyanins, phenolic acids and flavonoids reaching even major quantities than conventional solvent as hydro-methanolic solution. Comparing the results with those reported in bibliography, it can be concluded that the capacity of NADES to extract phenolic compounds varied significantly according to the structures and nature of the target compounds as well as the composition of each NADES. For example, the two secoiridoid derivatives most abundant and with major biological properties in olive oil were extracted in higher proportions with NADESs composed by polyols such as xylitol and 1,2-propanediol (García, Rodríguez-Juan, Rodríguez-Gutiérrez, Rios, & Fernández-Bolaños, 2016). Meanwhile, best recoveries of phenolic compounds from olive leaf were achieved with other polyol such as ethylene glycol (Alañón, et al., 2018). Urea-based NADES was reported as an excellent extraction solvent for the isolation of rutin from *Sophora japonica* (Zhao et al., 2015) while lactic acid-based NADES was the best solvent to extract bioactive compounds from *Lippia citriodora* (Ivanović et al., 2018). On the other hand, although the combination of choline chloride with sugars showed lower extraction efficiency for bioactive compounds of *H. sabdariffa* in this work and in *L. citriodora* (Ivanović et al., 2018), choline chloride and maltose resulted to be an excellent deep eutectic solvents for the extraction of polar and weak polar phenolics in *Cajanus cajan* leaves (Wei et al., 2015). Therefore, the deep eutectic mixture composed by oxalic acid and choline chloride was the most proper solvent to carry out the extraction of bioactive compounds from *H. sabdariffa* calyces.

3.2. MAE extraction conditions for value added compounds from calyces of H. sabdariffa: BBD experimental design.

After the natural deep eutectic solvent choice, the main parameters of the MAE microwave assisted extraction procedure were studied in order to achieve maximum extraction yields within the experimental domain. For this purpose, a Box-Behnken experimental design (BBD) based on a response surface methodology (RSM) was performed. The influence of three independent variables as liquid-solid ratio, temperature and water percentage of NADES was assessed by means of a three level factorial design. A total of 15 experiments, twelve factorial design points and three centre points for experimental error, were carried out in randomised run order (Table 4). Each experimental run were carried out by duplicate and the spectrophotometric measurement of TPC and TAC in triplicate (n = 6), were selected as response variables.

Table 5 summarizes statistical features related to the regression model and the analysis of variance. The quadratic correlation coefficients (R^2) of response variables were 0.994 and 0.985 for TPC and total anthocyanin content TAC respectively, while the lack of fit for both models was not significant. These parameters pointed out the good approximation of the statistic models proposed to the experimental conditions. The significance of regression was evaluated by the F-test. Values of the $F_{a,b}$ were calculated taken into account the degrees of freedom of the model (a) and the degree of freedom of the error (b). Values of $F_{9,2}$ were 91.23 and 36.87 for TPC and TAC respectively being both of them major than F-listed which was 19.385 . These values pointed out that the model is significant and therefore, the mathematical model is well fitted to the experimental data. Regards to the model proposed by TPC, the total phenolic content, the extraction seemed to be highly influence by liquid-solid ratio, water percentage of NADES and the interaction of temperature with water percentage ($p < 0.001$). Meanwhile, the quadratic effects for liquid-solid ratio and temperature were not significant (p

> 0.05), so experimental data can fit to a reduced model according to the following equation (3):

$$TPC = 28.34 + 1.78A + 3.96B + 3.81C + 0.39AB + 0.055AC - 2.61BC - 7.17C^2$$

For the particularly case of TAC, total anthocyanin content, the most influential parameters were the temperature, water percentage of NADES as well as its quadratic effect, and the interaction of temperature with the water percentage ($p < 0.001$). Solely the interaction of liquid-solid with the temperature was not significant ($p > 0.05$). Consequently, this parameter can be omitted in the proposed model (equation 4):

$$TAC = 3.28 + 0.26A - 0.41B + 0.47C + 0.36AC - 0.73BC + 0.056A^2 - 0.35B^2 - 0.69C^2 \quad (4)$$

For a visual interpretation of interaction between the independent variables, 3D plot of response surface were plotted (Figure 1). A higher liquid-solid ratio seemed to lead to greater extraction of TPC and TAC. The reason was probably that a larger concentration gradient of target compounds between matrix and solvent was obtained, which improved the extraction of phenolic compounds and anthocyanins from *H. sabdariffa* calyces. Increasing temperature favoured the extraction of TPC and TAC likely as consequence of the viscosity reduction of oxalic acid-based NADES, decrease in surface tension and enhancement of the target compounds diffusion. However, according to bibliography, the use of high temperatures with NADESs has some drawbacks due to their thermal stability (Craveiro et al., 2016; Haz, Strizincova, Majova, Skulcova, & Jablonsky, 2016). In addition, the increase in the temperature over 80ºC results in the remarkable increase in the rate of degradation of hibiscus anthocyanins (Galvâo Maciel_et al., 2018). The water percentage of oxalic acid-based NADES was a crucial parameter to take into account. In general, the main drawback of NADESs is the viscosity and mainly in those NADESs based on organic acids whose

viscosity is still significantly higher such as the ChCl-Ox. The addition of water to ChCl-Ox seemed to enhance the extraction of target compounds, however, excessive water content could break the hydrogen bonds between NADES components to the detriment of the eutectic character of solvent (Dai et al., 2013).

Based on the regression analysis, the three independent variables (liquid-solid ratio, temperature and water percentage) were maximized using the model equation provide by the statistical program to maximise the extraction of TPC and TAC of calices from *H. sabdariffa*. Results set the maximun extraction conditions within the experimental domain at liquid-solid ratio of 1:30 mg mL^{-1}, 75 ºC and 55 % of water. Extractions under these conditions were performed to carry out a comprehensive characterization of the extract (Table 6). Anthocyanin concentrations were 4.70 and 5.73 mg g^{-1} for delphinidin-3-sambubioside and cyaniding-3-sambubioside. These amounts were higher than those previously reported in bibliography using water as solvent extraction (1.20 – 0.59 and 0.36 – 0.16 mg g^{-1} for delphinidin- and cyaniding-3-sambubioside respectively) (Cassol et al., 2019) or even using acidified boiling water (4.11 mg g^{-1} for delphinidin-3-sambubioside and 3.81 mg g^{-1} for cyaniding-3-sambubioside) (Sindi et al., 2014). However, despite the greater amounts detected, these comparisons should be taken carefully since chemical differences can also be due to intrinsic variables of the *H. sabdariffa* samples. Other the other hand, higher content of phenolic acids and flavonoids were also detected with the methodology proposed by means of MAE-NADES in comparison with previous works (Cassol et al., 2019; Pimentel-Moral et al., 2019)

Therefore, the use of NADESs for the extraction of value-added compounds from *Hibiscus sabdariffa* has shown to be promising for the extraction of natural pigments or functional ingredient. Furthermore, other~~The main~~ advantage of the use of NADESs compared to conventional solvents as water and ethanol seems to be~~is~~ the increasing bioactive compounds stability (Benvenutti, Ferreira Zielinski, & Salvador Ferreira, 2019). Indeed, a

patent based on the use of eutectic solvent for efficient extraction from plant components, Naturex®, was deposited last year (U. S. Patent Nº. 0055904, 2018). On the other hand, new strategies are being developed for their recycling after the solute separation or solvent regeneration with the aim to cut off process costs (Benvenutti et al., 2019).

4. Conclusions

Results showed the extraction power of NADESs for the extraction of value-added compounds from c *H. sabdariffa* calyces. NADESs exhibited different extraction properties depending on their chemical components. Among them, oxalic acid-based NADES was the most promising solvent, especially for anthocyanin extraction, attaining higher extraction yields of value-added compounds from *H. sabdariffa* than the use of conventional organic solvents as methanol. The higher selectivity of oxalic-based NADES by anthocyanins and its acidic nature make it an excellent option to obtain stable extracts with great potential as natural pigments. Therefore, the optimise method of MAE using natural deep eutectic solvent such oxalic acid and choline chloride offers the possibility of design and customize extractions of bioactive compounds from *H. sabdariffa* which could be of interest for its further use in foodc industry. However, further studies addressing some issues such as stability, recovery yield, back-extraction, solvent regeneration and operational cost should be conducted to check the viability of the industrial application.

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Figure

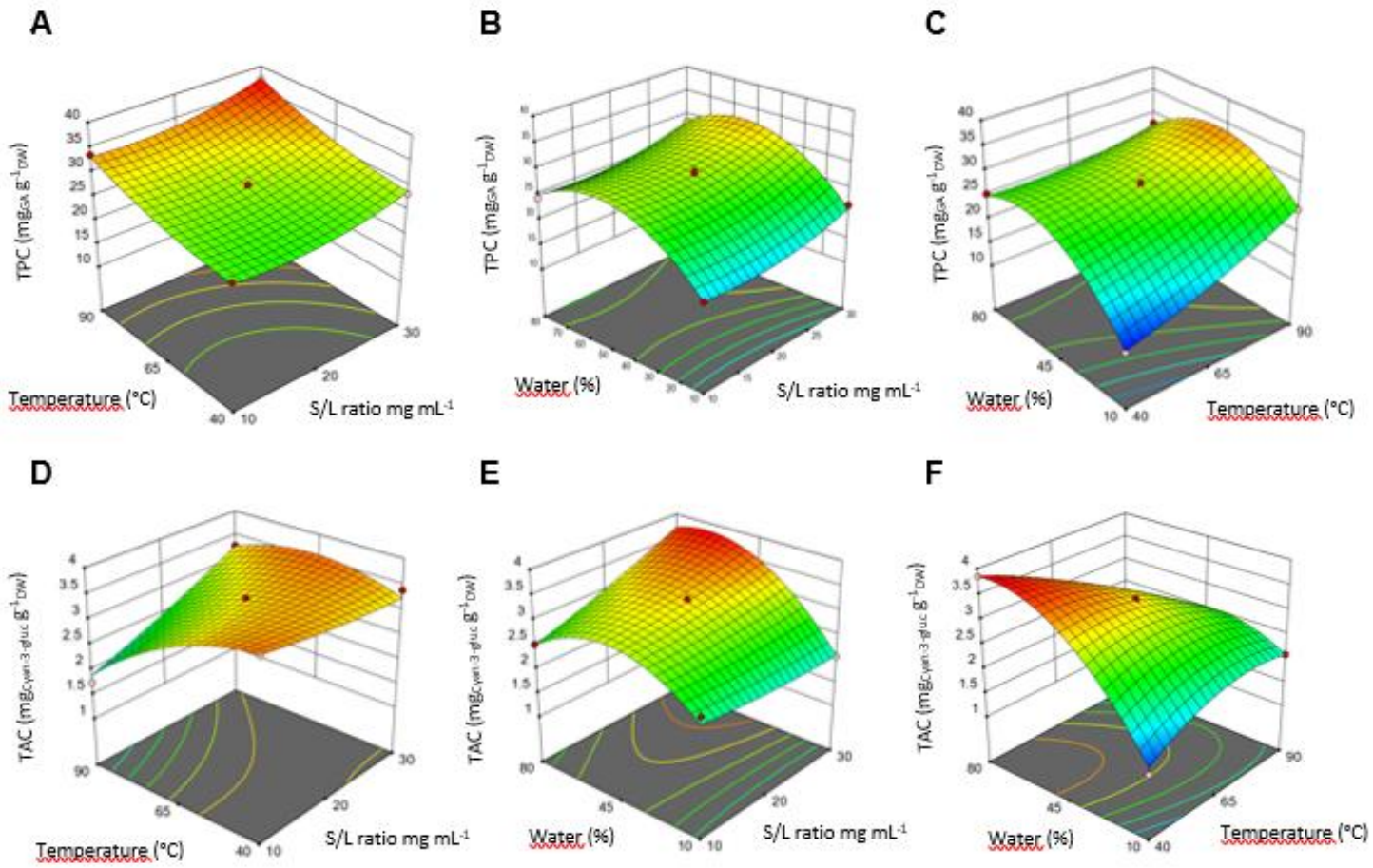

Figure 1. 3D response surface representations for interaction of three tested extraction variables on the total phenolic content, TPC (A-C) and total anthocyanin content, TPC (D-F).

Table

Table 1. Composition of natural deep eutectic solvents

Solvent abbreviation	NADESs composition			Molar ratio
	Component 1	Component 2	Component 3	
ChCl-La	Choline chloride	Lactic acid		1:2
ChCl-Ox	Choline chloride	Oxalic acid		1:1
ChCl-Eg	Choline chloride	Ethylene glycol		1:2
ChCl-1,2 Pro	Choline chloride	1,2-Propanediol		1:2
ChCl-Fru	Choline chloride	Fructose	Water	2:1:1
ChCl-Mal	Choline chloride	Maltose		3:1
ChCl-Glu	Choline chloride	Glucose	Water	2:1:1
ChCl-U	Choline chloride	Urea		1:2

Table 2. HPLC-ESI-TOF-MS data of the identified compounds in *H. sabdariffa* calyces extracted by NADESs

RT (min)	Proposed compound	Molecular formula	Measured m/z	Theoretical m/z	Error (ppm)
Anthocyanins					
3.9	Delphinidin-3-sambubioside[*]	$C_{26}H_{29}O_{16}$	595.1309	595.1305	-0.7
5.0	Cyanidin-3-sambubioside[*]	$C_{26}H_{29}O_{15}$	579.1342	579.1355	2.3
Phenolic acids					
4.2	Chlorogenic acid quinone[†]	$C_{13}H_{16}O_9$	315.0687	315.0722	3.5
5.1	Neochlorogenic acid[†]	$C_{16}H_{18}O_9$	353.0891	353.0878	-1.3
6.9	Chlorogenic acid[†]	$C_{16}H_{18}O_9$	353.0843	353.0878	3.5
7.2	Criptochlorogenic acid[†]	$C_{16}H_{18}O_9$	353.0885	353.0878	-2.1
10.4	Coumaroylquinic acid[†]	$C_{16}H_{18}O_8$	337.0916	337.0929	3.8
14.5	5-O-Caffeoylshikimic acid[đ]	$C_{16}H_{16}O_8$	335.0783	335.0772	-3.3
Flavonoids					
10.4	Myricetin-3-arabinogalactoside[ɤ]	$C_{26}H_{28}O_{17}$	611.1252	611.1254	0.4
14.2	Quercetin-3-sambubioside[‡]	$C_{26}H_{28}O_{16}$	595.1318	595.1305	-2.3
16.3	Quercetin-3-rutinoside[‡]	$C_{27}H_{30}O_{16}$	609.1476	609.1461	-2.5
18.0	Kaempferol-3-O-sambubioside[‡]	$C_{26}H_{28}O_{15}$	579.1347	579.1355	1.5
18.3	Quercetin-3-glucoside[ɧ]	$C_{21}H_{20}O_{12}$	463.0900	463.0882	-3.9
18.1	Methylepigallocatechin[ſ]	$C_{16}H_{16}O_7$	319.0823	319.0823	0.1
27.7	Myricetin[š]	$C_{15}H_{10}O_8$	317.0307	317.0303	-1.1
38.9	Quercetin[ſ]	$C_{15}H_{10}O_7$	301.0308	301.0354	4.6
42.5	Kaempferol[ſ]	$C_{15}H_{10}O_6$	285.0409	285.0405	-1.4
Other polar compounds					
2.94	Hydroxycitric acid[ϕ]	$C_6H_8O_8$	207.0150	207.0146	-1.7
3.1	Hibiscus acid[ϕ]	$C_6H_6O_7$	189.0078	189.0041	-3.7
8.1	Methyl digallate[•]	$C_{15}H_{12}O_9$	335.0397	335.0409	3.4
33.3	N-Feruloyltyramine[ϕ]	$C_{18}H_{19}NO_4$	312.1174	312.1241	6.7

Different superscripts for the identified compounds indicate the external standard used in the quantification process: [*]cyanidin-3-sambubioside; [†]chlorogenic acid; [đ]p-coumaric acid; [ɤ]myricitrin; [‡]rutin; [ɧ]quercetin-3-glucoside; [ſ]quercetin; [š]myricetin; [ϕ]citric acid; [•]gallic acid.

16 Table 3. Extraction yield (mg g^{-1}) of bioactive compounds from *H. Sabdariffa* isolated by different NADEs (25 % water w/w) in comparison with MeOH
17 (80:20). Extraction were performed by MAE at 65 ºC during 20 min and with a liquid-solid ration of of 10 mL g^{-1}.

Proposed compound	***MeOH***	***ChCl-La***	***ChCl-Ox***	***ChCl-Eg***	***ChCl-1,2 Pro***	***ChCl-Fru***	***ChCl-Mal***	***ChCl-Glu***	***ChCl-U***
Anthocyanins									
Delphinidin-3-sambubioside	3.32±0.09^{c}	3.35±0.07^{c}	3.76±0.03^{d}	2.70±0.05^{b}	3.13±0.11^{c}	2.75±0.11^{b}	2.63±0.07^{b}	2.85±0.11^{b}	1.26±0.06^{a}
Cyanidin-3-sambubioside	1.73±0.04^{f}	2.30±0.03^{g}	3.60±0.03^{h}	1.43±0.01^{c}	1.65±0.01^{e}	1.42±0.02^{c}	1.35±0.04^{b}	1.51±0.02^{d}	0.49±0.02^{a}
∑ anthocyanins	*5.05±0.13^{e}*	*5.65±0.04^{f}*	*7.36±0.06^{g}*	*4.12±0.04^{b,c}*	*4.79±0.12^{d}*	*4.17±0.13^{b,c}*	*3.98±0.11^{b}*	*4.36±0.13^{c}*	*1.74±0.08^{a}*
*TAC (spectrophotometry)	*3.29±0.16^{d,e}*	*3.10±0.04^{b,c,d,e}*	*3.44±0.01^{e}*	*2.94±0.00^{b,c,d}*	*3.15±0.12^{c,d,e}*	*2.66±0.20^{b,c,d}*	*2.52±0.08^{b,c}*	*1.92±0.30^{a}*	*2.76±0.11^{b,c,d}*
Phenolic acids									
Chlorogenic acid quinone	1.57±0.13^{a,b}	1.64±0.04^{a,b}	1.45±0.09^{a}	1.84±0.04^{b}	1.67±0.08^{a,b}	1.53±0.05^{a}	1.46±0.13^{a}	1.67±0.05^{a,b}	1.53±0.06^{a}
Neochlorogenic acid	3.73±0.09^{c}	3.52±0.00^{c}	4.21±0.02^{d}	3.72±0.00^{c}	3.47±0.02^{c}	3.11±0.06^{b}	2.75±0.05^{a}	3.14±0.02^{b}	3.84±0.33^{c}
Chlorogenic acid	6.08±0.33^{c}	5.72±0.00^{c}	5.73±0.03^{c}	5.93±0.06^{c}	5.81±0.12^{c}	5.22±0.13^{b}	4.64±0.10^{a}	5.13±0.06^{b}	5.70±0.08^{c}
Cryptochlorogenic acid	4.03±0.04^{b,c}	4.65±0.01^{c}	4.75±1.67^{d}	4.02±0.01^{b,c}	3.87±0.02^{a,b,c}	3.51±0.07^{a,b}	3.14±0.01^{a}	3.52±0.00^{a,b}	4.14±0.38^{b,c}
Coumaroylquinic acid	2.25±0.04^{c}	2.23±0.01^{c}	2.31±0.12^{c}	2.17±0.05^{c}	2.14±0.05^{c}	1.92±0.03^{b}	1.66±0.02^{a}	1.86±0.04^{b}	2.12±0.11^{c}
5-*O*-Caffeoylshikimic acid	0.74±0.01^{a}	1.35±0.02^{f}	0.94±0.02^{b}	1.10±0.02^{d}	1.14±0.01^{d}	1.01±0.02^{c}	0.92±0.03^{b}	1.02±0.01^{c}	1.20±0.05^{e}
∑ phenolic acids	*18.40±0.64^{c}*	*19.12±0.05^{c,d}*	*19.94±0.60^{d}*	*18.78±0.07^{c}*	*18.09±0.12^{c}*	*16.30±0.37^{b}*	*14.57±0.20^{a}*	*16.34±0.07^{b}*	*18.53±0.75^{c}*
Flavonoids									
Myricetin-3-arabinogalactoside	0.42±0.01^{a}	0.36±0.00^{a}	1.10±0.06^{c}	0.41±0.00^{a}	0.44±0.03^{a}	0.38±0.01^{a}	0.39±0.03^{a}	0.40±0.02^{a}	0.61±0.04^{b}
Quercetin-3-sambubioside	0.72±0.03^{f}	0.51±0.00^{c}	0.27±0.01^{a}	0.61±0.05^{d}	0.61±0.01^{d}	0.51±0.02^{c}	0.46±0.01^{b}	0.50±0.00^{c}	0.65±0.02^{e}
Quercetin-3-rutinoside	0.70±0.00^{f}	0.64±0.00^{e}	0.16±0.02^{a}	0.66±0.02^{e,f}	0.69±0.00^{f}	0.54±0.02^{c}	0.50±0.01^{b}	0.56±0.04^{c,d}	0.58±0.01^{d}
Kaempferol-3-*O*-sambubioside	0.05±0.00^{b}	0.03±0.00^{a,b}	0.11±0.01^{d}	0.04±0.00^{a,b}	0.02±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.04±0.00^{a,b}	0.06±0.00^{c}
Quercetin-3-glucoside	0.47±0.01^{e}	0.47±0.00^{e}	0.26±0.01^{a}	0.47±0.01^{e}	0.45±0.01^{d}	0.40±0.00^{c}	0.38±0.00^{b}	0.42±0.00^{c}	0.41±0.00^{c}
Methylepigallocatechin	0.11±0.01^{b,c}	0.11±0.01^{c,d}	0.08±0.00^{a}	0.11±0.00^{c,d}	0.13±0.00^{e}	0.11±0.00^{c,d}	0.10±0.00^{b}	0.10±0.00^{b,c}	0.12±0.01^{d}
Myricetin	0.61±0.01^{c,d}	0.67±0.01^{e}	0.89±0.03^{f}	0.61±0.01^{c,d}	0.63±0.00^{d}	0.54±0.01^{b}	0.52±0.00^{b}	0.57±0.01^{c}	0.41±0.02^{a}
Quercetin	0.75±0.01^{d}	0.77±0.01^{d}	1.42±0.09^{e}	0.72±0.01^{c,d}	0.82±0.01^{d}	0.64±0.00^{b,c}	0.61±0.01^{b}	0.62±0.01^{b}	0.45±0.04^{a}
Kaempferol	0.11±0.00^{a,b}	0.12±0.00^{b}	0.27±0.03^{c}	0.12±0.00^{a,b}	0.13±0.01^{b}	0.10±0.00^{a,b}	0.10±0.00^{a,b}	0.10±0.00^{a,b}	0.08±0.00^{a}
∑ flavonoids	*3.93±0.06^{d}*	*3.69±0.02^{c}*	*4.57±0.12^{e}*	*3.74±0.02^{c}*	*3.91±0.03^{d}*	*3.25±0.06^{b}*	*3.09±0.06^{a}*	*3.32±0.07^{b}*	*3.37±0.01^{b}*
†TPC (spectrophotometry)	*20.35±1.19^{b}*	*28.07±1.02^{c}*	*28.56±1.55^{c}*	*27.57±1.83^{c}*	*24.67±1.33^{c}*	*18.64±1.17^{b}*	*11.50±0.72^{a}*	*14.06±0.96^{a}*	*17.50±0.68^{b}*
Other polar compounds									
Hydroxycitric acid	2.93±0.07^{f}	1.94±0.00^{d}	2.21±0.06^{e}	0.62±0.07^{b}	0.24±0.03^{a}	1.10±0.15^{c}	0.51±0.09^{b}	0.74±0.09^{b}	0.58±0.02^{b}
Hibiscus acid	31.70±1.65^{d}	27.48±2.04^{c}	23.44±0.48^{a,b}	25.70±0.47^{b,c}	28.87±0.36^{c}	22.33±0.93^{a,b}	19.87±0.60^{a}	22.29±0.50^{a,b}	23.38±2.00^{a,b}
Methyl digallate	8.82±0.05^{c,d}	8.31±0.46^{c,d}	13.94±1.25^{e}	7.36±0.04^{b,c}	9.19±0.16^{d}	8.77±0.17^{c,d}	7.78±0.18^{c,d}	6.42±0.47^{b}	1.99±0.11^{a}
N-Feruloyltyramine	0.87±0.00^{c}	0.83±0.02^{c}	0.92±0.02^{d}	0.83±0.01^{c}	0.82±0.02^{c}	0.68±0.02^{b}	0.60±0.04^{a}	0.67±0.00^{b}	0.82±0.03^{c}
∑ other polar compounds	*44.32±1.77^{e}*	*38.56±2.52^{d}*	*40.51±0.73^{d}*	*34.52±0.37^{c}*	*39.11±0.57^{d}*	*32.88±1.27^{b,c}*	*28.76±0.72^{a}*	*30.12±0.88^{a,b}*	*26.76±2.06^{a}*

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Different superscripts for each compound in the same row denoted significant differences among solvents tested according to the Student–Newman–Keuls test at the 95 % confidence level ($p < 0.05$).

*Estimation of total anthocyanin content determined by standard spectrophotometric method expressed as mg equivalents of cyanindin-3-glucoside per gram of dried *H. Sabdariffa* calyces ($mg_{cyan\text{-}3\text{-}gluc}\ g^{-1}$)

†Estimation of total phenolic content determined by standard spectrophotometric method expressed ad mg equivalents of gallic acid per gram of dried *H. Sabdariffa* calyces ($mg_{ga}\ g^{-1}$)

ChCl-La (choline chloride:lactic acid; 1:2); ChCl-Ox (choline chloride:oxalic acid; 1:1); ChCl-Eg (choline chloride:ethylene glycol; 1:2); ChCl-1,2 Pro (choline chloride:1,2 propanediol; 1:2); ChCl-Fru (choline chloride:fructose: water; 2:1:1); ChCl-Mal (choline chloride:maltose; 3:1); ChCl-Glu (choline chloride:glucose: water; 2:1:1); ChCl-U (choline chloride:urea; 1:2)

Table 4. Box-Behnken design with coded and uncoded independent variables and experimental and predicted data for the response variables

Run	Experimental variables			Responses			
	A	B	C	TPC*		TAC†	
	Liquid-solid ratio (mL mg^{-1})	temperature (ºC)	water in NADES (%)	Experimental	Predicted	Experimental	Predicted
1	1:30 (+1)	90 (+1)	45 (0)	37.25 ± 0.05	37.55	3.27 ± 0.02	3.26
2	1:30 (+1)	40 (-1)	45 (0)	28.46 ± 2.10	28.84	3.39 ± 0.02	3.24
3	1:20 (0)	40 (-1)	80 (+1)	25.30 ± 0.21	24.88	3.85 ± 0.04	3.85
4	1:20 (0)	90 (+1)	80 (+1)	27.92 ± 0.81	27.58	1.70 ± 0.01	1.56
5	1:10 (-1)	65 (0)	10 (-1)	17.50 ± 2.79	17.46	2.43 ± 0.08	2.28
6	1:30 (+1)	65 (0)	80 (+1)	28.58 ± 0.72	28.63	3.57 ± 0.02	3.72
7	1:20 (0)	65 (0)	45 (0)	28.57± 0.85	28.34	3.33 ± 0.02	3.28
8	1:20 (0)	40 (-1)	10 (-1)	11.71 ± 0.28	12.05	1.32 ± 0.01	1.46
9	1:20 (0)	90 (+1)	10 (-1)	24.77 ± 1.25	25.19	2.09 ± 0.19	2.09
10	1:20 (0)	65 (0)	45 (0)	27.52 ± 0.39	28.34	3.23 ± 0.08	3.28
11	1:20 (0)	65 (0)	45 (0)	28.92 ± 0.72	28.34	3.28 ± 0.04	3.28
12	1:10 (-1)	40 (-1)	45 (0)	26.36 ± 0.73	26.07	3.56 ± 0.09	3.57
13	1:30 (+1)	65 (0)	10 (-1)	21.62 ± 2.58	20.91	2.06 ± 0.20	2.08
14	1:10 (-1)	90 (+1)	45 (0)	33.58 ± 0.51	33.20	1.74 ± 0.14	1.89
15	1:10 (-1)	65 (0)	80 (+1)	24.25 ± 0.96	24.96	2.51 ± 0.01	2.50

*Total phenolic content expressed as mg of gallic acid per gram (mg_{ga} g^{-1})

†Total anthocyanin content expressed as mg of cyanindin-3-glucoside per gram ($mg_{cyan\text{-}3\text{-}glu}$ g^{-1})

Table 5. Regression model and analysis of variance (ANOVA)

Regression Data								
	TPC				TAC			
	SS	DF	F-value	P-value	SS	DF	F-value	P-value
A	25.29	1	40.30	<0.001***	0.53	1	19.02	0.007**
B	125.58	1	200.10	0.001**	1.38	1	49.88	<0.001***
C	115.91	1	184.69	<0.001***	1.74	1	62.95	<0.001***
AA	0.62	1	0.98	0.366 ns	0.72	1	26.15	0.003**
BB	0.01	1	0.02	0.896 ns	0.51	1	18.51	0.008**
CC	27.23	1	43.39	0.001**	2.13	1	77.16	<0.001***
AB	12.20	1	19.44	0.007*	0.01	1	0.42	0.544 ns
AC	5.80	1	9.24	0.029*	0.44	1	16.02	0.010*
BC	189.81	1	302.44	<0.001***	1.78	1	64.33	<0.001***
Lack of fit	2.06	3	1.28	0.4673 ns	0.13	3	17.75	0.0538 ns
Pure error	1.08	2			0.005	2		

Analysis of variance (ANOVA)		
	TPC	**TAC**
R^2	0.994	0.985
Adj. R^2	0.983	0.958
Pred. R^2	0.932	0.770

A: liquid-solid ratio (mL mg^{-1}); B: temperature (°C); C: water in NADES (%)

SS: sum of squares; DF: degree of freedom; R^2: Quadratic correlation coefficient; Adj. R^2: Adjusted quadratic correlation coefficient; Pred. R^2: Predicted quadratic correlation coefficient.

Level of significance: *$p < 0.05$; **$p < 0.01$ and ***; $p < 0.001$; ns: not significant

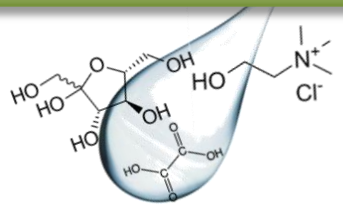
Table 6. Extraction yield (mg g^{-1}) of bioactive compounds from *H. Sabdariffa* isolated under experimental conditions provided by the statistical program as optimal conditions within the experimental domain.

Proposed compound	***Yield***
Delphinidin-3-sambubioside	4.70 ± 0.40
Cyanidin-3-sambubioside	5.73 ± 0.50
∑ *anthocyanins*	*10.43 ± 0.92*
Chlorogenic acid quinone	1.74 ± 0.15
Neochlorogenic acid	5.15 ± 0.35
Chlorogenic acid	6.96 ± 0.62
Cryptochlorogenic acid	5.77 ± 0.03
Coumaroylquinic acid	3.43 ± 0.06
5-*O*-Caffeoylshikimic acid	1.35 ± 0.07
∑ *phenolic acids*	*24.41 ± 0.32*
Myricetin-3-arabinogalactoside	1.21 ± 0.12
Quercetin-3-sambubioside	0.87 ± 0.03
Quercetin-3-rutinoside	0.21 ± 0.01
Kaempferol-3-*O*-sambubioside	0.15 ± 0.01
Quercetin-3-glucoside	0.55 ± 0.00
Methylepigallocatechin	0.25 ± 0.01
Myricetin	0.91 ± 0.01
Quercetin	1.45 ± 0.07
Kaempferol	0.33 ± 0.01
∑ *flavonoids*	*6.00 ± 0.09*
Hydroxycitric acid	2.42 ± 0.04
Hibiscus acid	24.84 ± 1.18
Methyl digallate	16.00 ± 0.31
N-Feruloyltyramine	0.98 ± 0.16
∑ *other polar compounds*	*44.25 ± 1.29*

*Graphical Abstract

Hydrogen bond acceptor (HBA)	Hydrogen bond donors (HBD)
Choline chloride	Lactic acid
	Oxalic acid
	Ethylene glycol
	1,2-propanediol
	Fructose
	Maltose
	Glucose
	Urea

NATURAL DEEP EUTECTIC SOLVENTS (NADES)

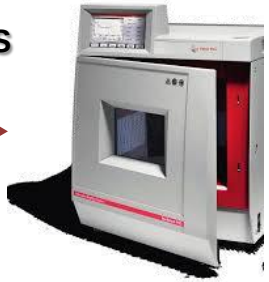


1 NADESs PREPARATION

Hibiscus sabdariffa L.

BIOACTIVE COMPOUNDS

Extraction



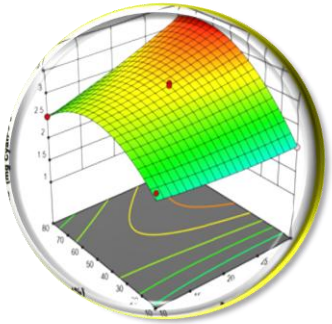
Microwave-assisted extraction (MAE)

Centrifugation

Dilution

2 EXTRACTION PROCEDURE

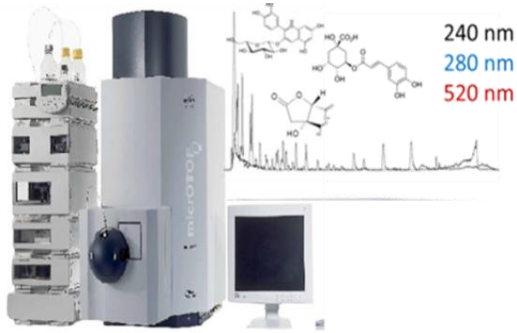
- ✓ **Total phenol content**
- ✓ **Total anthocyanin content**



✓ **Optimized extraction conditions:**
Solid-liquid ratio 1:30 mg/mL, temperature 75º C, 55 % water in NADES

Best NADES

ChCl-OXALIC ACID



HPLC-DAD-ESI-TOF-MS

3 INSTRUMENTAL ANALYSIS

***Credit Author Statement**

M. Elena Alañón: Conceptualization, methodology, writing, visualization, supervision, funding acquisition

Milena Ivanović: Investigation, validation, formal analysis

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